FUNCTIONAL CHARACTERIZATION OF 15-LIPOXYGENASE-1 EXPRESSION IN HUMAN COLORECTAL CARCINOMA CELL LINE HT-29

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FUNCTIONAL CHARACTERIZATION OF 15-LIPOXYGENASE-1 EXPRESSION IN HUMAN COLORECTAL CARCINOMA CELL LINE HT-29

submitted by **SEDA TUNÇAY** in partial fulfillment of the requirements for the degree of **Master of Science in Biology Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Zeki Kaya _____
Head of Department, **Biology, METU**

Assist. Prof. Dr. Sreeparna Banerjee _____
Supervisor, **Biology Dept., METU**

Examining Committee Members:

Prof. Dr. Mesude İşcan _____
Biology Dept., METU

Assist. Prof. Dr. Sreeparna Banerjee _____
Biology Dept., METU

Assist. Prof. Dr. Elif Erson _____
Biology Dept., METU

Assist. Prof. Dr. Çağdaş D. Son _____
Biology Dept., METU

Assist. Prof. Dr. Özlen Konu _____
Molecular Biology and Genetics Dept.,
Bilkent University

Date: _____

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Seda Tunçay

Signature :

ABSTRACT

FUNCTIONAL CHARACTERIZATION OF 15-LIPOXYGENASE-1 EXPRESSION IN HUMAN COLORECTAL CARCINOMA CELL LINE HT-29

Tunçay, Seda

M.Sc., Department of Biology

Supervisor: Assist. Prof. Dr. Sreeparna Banerjee

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Colorectal carcinoma (CRC) is often lethal when invasion and/or metastasis occur. 15-lipoxygenase-1 (15-LO-1), an enzyme of the inflammatory eicosanoid pathway, oxidatively metabolizes linoleic acid and its expression is repressed in CRC. In the present study, the hypothesis that the lack of 15-LO-1 expression in CRC cells may contribute to the tumorigenesis was investigated. Therefore 15-LO-1 was introduced to colon cancer cell line HT-29 that does not have detectable levels of the 15-LO-1. The HT-29 cells were transiently transfected with the eukaryotic expression vector pcDNA3.1-15-LO-1 and the effects of 15-LO-1 expression on the proliferation, apoptosis as wells as metastatic potential of the cells were investigated.

Cellular proliferation was analyzed by MTT assay and the apoptotic potential of 15-LO-1 was evaluated by acridine orange, floating cell ratio and caspase-3 assays as well as expression levels of the antiapoptotic protein XIAP. Cellular migration and invasion were investigated by Boyden chamber migration and Matrigel invasion assay.

The data indicates that 15-LO-1 expression significantly decreased cell proliferation and increased apoptosis. In addition, a significant reduction was observed in migratory and invasive capacity 15-LO-1 expression also significantly reduced the expression of metastasis associated 1 protein (MTA-1).

Taken together we propose that 15-LO-1 expression in CRC can inhibit colon cancer cell growth through induction of apoptotic cell death and may contribute to the inhibition of metastatic capacity *in vitro* which may be exploited for therapeutic purposes.

Key words: 15-lipoxygenase-1, colorectal carcinoma, metastasis, growth inhibition, apoptosis.

ÖZ

15-LİPOKSİJENAZ-1 İFADESİNİN HT-29 KOLOREKTAL KANSER HÜCRELERİNDE FONKSİYONEL KARAKTERİZASYONU

Tunçay, Seda

Yüksek Lisans, Biyoloji Bölümü

Tez Yöneticisi: Yrd.Doç.Dr. Sreeparna Banerjee

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Kolorektal kanser invazyon ve metaztaz aşamasına geldiğinde genellikle ölümle sonuçlanmaktadır. 15- lipoksijenaz–1 (15-LO–1) linoleik asiti okside etmekte olup enflamatuar eikosanoid yolağında görev alan enzimlerden bir tanesidir. 15-LO–1 ifadesinin kolorektal kanserde baskılandığı bulunmuştur. Bu çalışmada, 15-LO–1 ifadesi yokluğunun tümör oluşumuna katkıda bulunduğu hipotezi incelenmiştir. Bu yüzden 15-LO–1 geni, bu geni saptanabilir ölçülerde ifade edemeyen HT–29 kolon kanser hücrelerinde ifade ettirilmiştir. HT–29 hücreleri ökaryotik ifade pcDNA3.1–15-LO–1 vektörü ile geçici olarak transfekte edilmiştir. HT–29 hücrelerinde 15-LO–1 geninin ifadesinin doğrulanmasının ardından 15-LO–1 geninin bu hücrelerin çoğalması, apoptoz ve metastaz potansiyelinde yarattığı değişiklikler incelenmiştir.

15-LO–1 geninin hücre çoğalımı üzerindeki etkisine MTT analizi yöntemi ile apoptoz üzerindeki etkilerine akridine orange, floating cell ratio, kaspaz–3 tahlili ve XIAP proteini ifade seviyesindeki farklılığı saptanarak bakılmıştır. Hücre migrasyon ve invazyonu Boyden chamber ve Matrigel invazyon tahlilleriyle incelenmiştir.

Elde ettiğimiz bulgular, 15-LO–1 ifadesinin hücre çoğalmasını kayda değer ölçüde azalttığını apoptozu da yine kayda deger ölçüde artırdığını göstermiştir. Buna ek olarak, hücrelerin migrasyon ve invazyon edebilme kapasiteleri de önemli ölçüde azalmıştır. 15-LO–1 ifadesi, "metastasis associated 1" proteininin (MTA1) ekspresyonunu da önemli ölçüde azaltmıştır.

Elde ettiğimiz bulgular ışığında 15-LO–1 ekspresyonunun kolon kanserinde apoptozu indükleyerek hücre çoğalmasını durdurduğunu buna ek olarak metastaz kapasitesini azalttığını ve bu nedenle terapatik tedavi amacıyla kullanılabileceğini öne sürmekteyiz.

Anahtar kelimeler: 15-lipoksijenaz–1, kolorektal kanser, metastaz, çoğalmayı durdurma, apoptoz.

To My Family

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INTRODUCTION

1.1 Cancer

Cancer causes 7.1 million deaths annually (12.6% of the global total) and the number of new cases is expected to rise from 10 million to 15 million by 2020 according to the latest report of World Health Organization (WHO) (http://www.who.int/hpr/gs.fs.cancer.shtml). Lung, stomach, colorectal, liver and breast cancer lead to most cancer deaths each year. Table 1.1 shows the estimated incidence and deaths for 26 different kinds of cancer for men and women in the year 2002 (Parkin et al. 2005). Diet, accounting for about 30% of all cancers in Western countries and approximately up to 20% in developing countries is second only to tobacco as a preventable cause (http://www.who.int/dietphysicalactivity/publications/facts/cancer/en/).

A normal cell transforms into a tumor cell due to the interaction between a person's genetic factors and three categories of external agents: physical carcinogens (e.g. UV, ionizing radiation), chemical carcinogens (e.g. components of tobacco smoke, aflatoxin), biological carcinogens (e.g. certain viruses, bacteria or parasites).

The main goal of cancer research is to better understand the molecular pathways and mechanisms underlying the process of cancer growth, death and metastasis.

Over the last forty years our knowledge about the cancer etiology has been enriched substantially by the discovery of the mutations that produce oncogenes with dominant gain of function and tumor suppressor genes with recessive loss of function as well as epigenetic alterations.

Table 1. 1Worldwide incidence and mortality by sex and cancer type in 2002 (Parkin et al. 2005)**.**

	INCIDENCE						MORTALITY					
	MALES			FEMALES			MALES			FEMALES		
	Cases	ASR (World)	Cumulative risk (age 0–64)	Cases	ASR (World)	Cumulative risk (age 0–64)	Deaths	ASR (World)	Cumulative risk (age 0–64)	Deaths	ASR (World)	Cumulative risk (age 0–64)
Oral cavity	175,916	6.3	0.4	98,373	3.2	0.2	80,736	2.9	0.2	46,723	1.5	0.1
Nasopharynx	55,796	1.9	0.1	24,247	0.8	0.1	34,913	1.2	0.1	15,419	0.5	0.0
Other pharynx	106,219	3.8	0.3	24,077	0.8	0.1	67,964	2.5	0.2	16,029	0.5	0.0
Esophagus	315,394	11.5	0.6	146,723	4.7	0.3	261,162	9.6	0.5	124,730	3.9	0.2
Stomach	603,419	22	1.2	330,518	10.3	0.5	446,052	16.3	0.8	254,297	7.9	0.4
Colon/rectum	550,465	20.1	0.9	472,687	14.6	0.7	278,446	10.2	0.4	250,532	7.6	0.3
Liver	442,119	15.7	1.0	184,043	5.8	0.3	416,882	14.9	0.9	181,439	5.7	0.3
Pancreas	124,841	4.6	0.2	107,465	3.3	0.1	119,544	4.4	0.2	107,479	3.3	0.1
Larynx	139,230	5.1	0.3	20,011	0.6	0	78,629	2.9	0.2	11,327	0.4	0.0
Lung	965,241	35.5	1.7	386,891	12.1	0.6	848,132	31.2	1.4	330,786	10.3	0.5
Melanoma of skin	79,043	2.8	0.2	81,134	2.6	0.2	21,952	0.8	0.0	18,829	0.6	0.0
Kaposi sarcoma*												
Breast				1,151,298	37.4	2.6				410,712	13.2	0.9
Cervix uteri				493,243	16.2	1.3				273,505	9.0	0.7
Corpus uteri				198,783	6.5	0.4				50,327	1.6	0.1
Ovary				204,499	6.6	0.5				124,860	4.0	0.2
Prostate	679,023	25.3	0.8				221,002	8.2	0.1			
Testis	48,613	1.5	0.1				8,878	0.3	0.0			
Kidney	129,223	4.7	0.3	79,257	2.5	0.1	62,696	2.3	0.1	39,199	1.2	0.1
Bladder	273,858	10.1	0.4	82,699	2.5	0.1	108,310	4.0	0.1	36,699	1.1	0.0
Brain, nervous system	108,221	3.7	0.2	81,264	2.6	0.2	80,034	2.8	0.2	61,616	2.0	0.1
Thyroid	37,424	1.3	0.1	103,589	3.3	0.2	11,297	0.4	0.0	24,078	0.8	0.0
Non-Hodgkin lymphoma	175,123	6.1	0.3	125,448	3.9	0.2	98,865	3.5	0.2	72,955	2.3	0.1
Hodgkin disease	38,218	1.2	0.1	24,111	0.8	0.1	14,460	0.5	0.0	8,352	0.3	0.0
Multiple myeloma	46,512	1.7	0.1	39,192	1.2	0.1	32,696	1.2	0.1	29,839	0.9	0.0
Leukemia	171,037	5.9	0.3	129,485	4.1	0.2	125,142	4.3	0.2	97,364	3.1	0.2
All sites but skin	5,801,839	209.6	10.3	5,060,657	161.5	9.5	3,795,991	137.7	6.4	2,927,896	92.1	4.9

1.1.1 Colorectal cancer

Colorectal cancer (CRC) occurs in the tissues of the colon and rectum. Colorectal cancer is one of the leading causes of cancer related deaths throughout the world (McCormick, Kibbe and Morgan 2002). At the end of year 2009, 149,000 people are expected to be diagnosed with CRC only in the United States while 50,000 of them will die of CRC despite the increased screening and prevention efforts (Miller et al. 2008, Carter et al. 2009). However, CRC incidence and mortality are not uniformly distributed worldwide as there is at least a 25 fold variation in occurrence. North America, Australia/New Zealand, Western Europe and, Japan have the highest incidence while it is low in Africa and Asia (Parkin et al. 2005).

Histopathologically, it starts with hyper proliferation of the colon mucosa, and the formation of adenomas (monoclonal proliferation of non-malignant epithelial cells) with varying size, shape, villous architecture and dysplasia (Hamilton 1992). Those adenomas turn into adenocarcinoma and finally invasive colorectal carcinoma through various stages due to oncogene activations, loss of tumor suppressors or epigenetic changes. In CRC, inactivating mutations of both alleles of the tumor suppressor gene adenomatous polyposis coli (APC) on chromosome 5q; dysregulation of K-ras proto-oncogene due to a mutation are the earliest events. An alternative pathway for CRC is microsatellite instability (MSI) which is observed in almost all adenocarcinomas from patients with human non polyposis colorectal cancer (HNPCC) and 10-15% of sporadic colorectal cancers (Aaltonen et al. 1994, Gryfe et al. 1997, Leslie et al. 2002, Konishi et al. 1996). Chronic inflammation is considered as a major factor in the incidence of sporadic CRC (Atreya and Neurath 2008, Xie and Itzkowitz 2008).

The correlations between the risk of CRC and per capita consumption amounts of meat, fat and fiber is very significant (Armstrong and Doll 1975, McKeown-Eyssen

1994, Prentice and Sheppard 1990) reflecting the importance of diet in CRC. A diet low in vegetables, cereals; and rich in red meat increases the CRC risk.

CRC can be detected early and cured as a result of the development of sophisticated endoscopic or surgical techniques. However, mortality from CRC is still very high calling for better or complete understanding of the underlying mechanisms, in order to develop better chemoprevention strategies. CRC developing over the years and possibly decades (Gryfe et al. 1997), has the potential to be controlled during its preneoplastic stages physiologically or pharmacologically; thus we need to identify exact mechanisms whereby the progression of preneoplastic lesions can be stabilized, arrested or reversed. As a simple example, synthetic vitamin A analogs (retinoids) provide prevention for invasive epithelial cancers (Sporn 1976).

Evading apoptosis and capability of tissue invasion and metastasis are two very important events in CRC as well as in every other human cancer.

1.1.2 Apoptosis

Apoptosis (programmed cell death) may occur through both intrinsic and extrinsic pathways. The loss of a fully functional apoptotic program is considered to be one of the hallmarks of all types of malignant tumors.

1.1.2.1 Intrinsic pathway of apoptosis

A very important family of proteins involved in the intrinsic pathway of apoptosis are the Bcl-2 family proteins which can be subdivided as pro apoptotic (Bax, Bak, Bid, Bim) and anti apoptotic (Bcl-2, Bcl-XL, Bcl-W). In normal cells, the p53 tumor suppressor gene promotes apoptosis by upregulating the expression of pro

apoptotic Bax which in turn stimulates the mitochondria to release cytochrome c (Hanahan and Weinberg 2000). Once cytochrome c molecules are released, they form the apoptosome together with the Apaf-1 protein. Apoptosome converts latent protease procaspase-9 into active procaspase-9 which is a member of a family of cysteine aspartyl-specific proteases. Then active caspases-9 activates procaspase-3 into executioner caspase-3 which in turn activates a serious of executioner caspases (caspases-6 and caspases-7) that cleaves various death substrates creating apoptotic cell phenotype (Weinberg, 2007).

While cytochrome c in the cytosol activates caspases, Smac/DIABLO (released from mitochondria together with cytochrome c) inactivates Inhibitor of apoptosis proteins (IAPs). IAPs inhibit caspase action either through direct binding and inhibiting proteolytic activity of caspases or through marking the caspases for ubiquitylation and degradation (Weinberg, 2007).

1.1.2.2 Extrinsic pathway of apoptosis

Apoptosis can also be initiated outside of the cell through pro apoptotic cell surface receptors (death receptors). Tumor necrosis factor (TNF) family of proteins including TNF-α, TRAIL, and Fas Ligand (FasL) are the ligands of the death receptors. Once death receptors are activated due to ligand binding they associate with Fas-associated death domain protein (FADD) in the cytoplasm forming death inducing signaling complex (DISC) which in turn activates the initiator caspases-8 and less commonly caspases-10. The initiator caspases activates the executioner caspases-3,-6 and 7 thereby converging on the signaling pathway through which the intrinsic apoptotic program operates (Weinberg, 2007).

The convergence of apoptotic pathways are outlined in Figure 1.1.

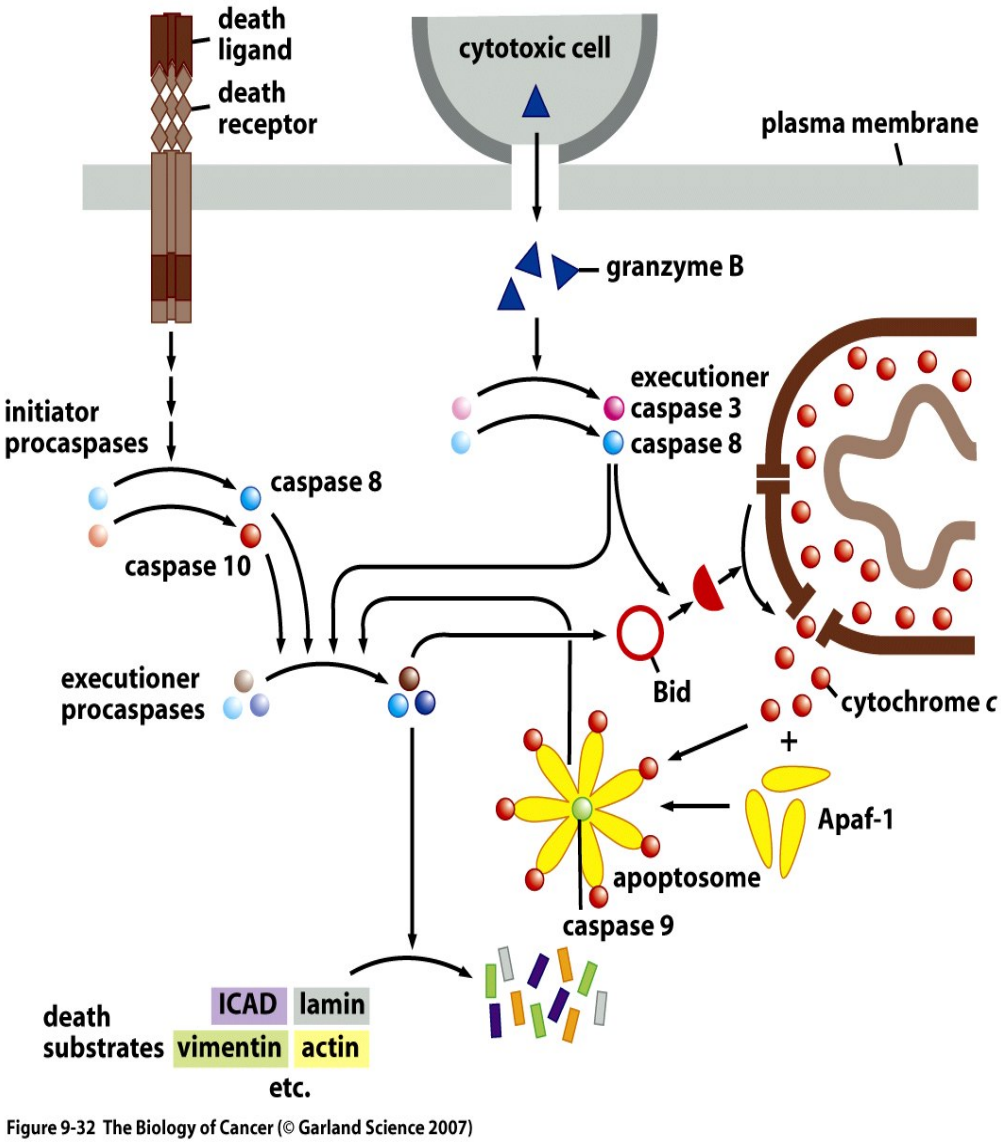

Figure 1.1 Convergence of intrinsic and extrinsic pathways of apoptosis (Weinberg, 2007).

1.1.3 Metastasis

Metastasis is the ability of cancer cells to escape from the primary tumor mass and colonize at a new tissue or organ. This process leads to 90% of human deaths as a result of cancer (Sporn 1996, Hanahan and Weinberg 2000). Metastasis is governed by many gene products important for the detachment of neoplastic cells from the primary tumor, penetration into the blood and lymphatics, arrest at distant sites by adhesion to endothelial cells, extravasation, escape from the host antitumor responses an finally growth at metastatic sites (Nicolson 1988, Liotta, Steeg and Stetler-Stevenson 1991)

There is a dynamic interaction between cancer cells and the host stromal microenviroment which is mainly constituted of cellular elements such as fibroblast and non-cellular elements such as extra cellular matrix (ECM) (Tse and Kalluri 2007, Li, Fan and Houghton 2007, Gout and Huot 2008). The tumor stroma has an important role in the development of CRC with an increased number of fibroblasts, deposition of a new ECM rich in type-1 collagen and fibrin as well as enhanced capillary density (Kalluri and Zeisberg 2006)

The ECM is composed of five classes of macromolecules (collagens, laminins, fibronectins, proteoglycans and hyaluronans) and provides mechanical and physicochemical support for cells by binding to integrins at the cell surface (Gout and Huot 2008).

The basement membrane (BM) is a specialized sheet and a particular type of ECM with a significant role in cancer. It is constituted of ECM molecules such as Type IV collagen, laminins, Type VII collagen, and heparan sulfate proteoglycans (Gout and Huot 2008). The loss of BM components due to degradation by proteolytic enzymes and/or a lack of biosynthesis are correlated with tumour progression. For

example, Type VII collagen is lost early in the development of malignant melanoma (Kirkham et al. 1989), breast cancers (Wetzels et al. 1991) and prostate carcinomas (Nagle et al. 1995) Similarly, laminin-5 is commonly lost in colon carcinomas but not in pre-malignant tumours (Gout and Huot 2008).

Matrix metalloproteinases (MMPs) are the principal ECM-degrading enzymes involved in ECM turnover and they have a central role in cancer progression given that ECM degradation products can influence stroma– cancer cell interactions. It has been shown that metastatic colon cancer cells are able to induce the expression and/or secretion of MMP-2 and MMP-9 in stromal cells, either through a direct contact or via a paracrine regulation (Mook, Frederiks and Van Noorden 2004).

Cancer cell detachment from the primary site is one of the key initial events required for metastasis and mainly occurs due to the loss of E-cadherin- mediated cell–cell adhesion (Cavallaro and Christofori 2004).

This latter event results from mutations in the E-cadherin gene, proteolytic degradation of E-cadherin, IGF1-mediated internalization of E-cadherin and disruption of the function of E-cadherin involving β-catenin (Cavallaro and Christofori 2004)

After tumor cells detach from the primary neoplasm, they enter into the existing or newly formed blood or lymphatic vessels to disseminate which is called intravasation. In the case of colon cancer cells, they use the hepatic–portal circulatory system to enter the liver (Gout and Huot 2008).

The adhesive interactions between the cancer cells and the endothelial cells of the target organs determine the arrest of the circulatory cells in the capillaries and

initiates diapedesis of the cancer cells in the colonized organs that is known as extravasation (Nicolson 1988).

Integrins expressed on the cancer cell surface are activated by several chemokines and are very important in the process of extravasation as they provide adhesion of cancer cell to many ECM molecules including laminin and fibronectin (Joyce and Pollard 2009). Coinjection of tumor cells with fibronectin has shown to enhance cell adhesion and metastasis in experimental metastasis models (Liotta, Rao and Wewer 1986, Joyce and Pollard 2009).

1.2 Cancer related inflammation (CRI)

Inflammation occurs in response to tissue damage resulting from microbial pathogen infection, chemical irritation and/or wounding. Today it is well known that inflammation leads to various diseases like cancer, cardiovascular, pulmonary, neurological diseases as well as arthritis.
Functional links between inflammation, tissue injury and cancer date back to 1863, when Rudolf Virchow proposed that cancers arise from sites of chronic inflammation. Chronic inflammation and wound healing due to tissue injury go along with accumulation of inflammatory cells, cytokines, growth factors, oxidants and pro-inflammatory lipid mediators that provide the development and maintenance of an activated stroma and the proliferation of epithelial cells as well (Furstenberger et al. 2006).

Epidemiological studies have already shown that chronic inflammation is an important risk factor for certain cancer types as in the case of persistent hepatitis B, *Helicobacter pylori* infections, inflammatory bowel diseases, or chronic pancreatitis causing increased risk for liver, gastrointestinal, or pancreatic cancers, respectively.

Genetic alteration may occur in the proliferating cells under excessive or chronic inflammatory conditions; thus those cells may lead to autonomous tumors even after the inflammation has been terminated (Coussens and Werb 2002). A developing neoplasm produces various chemokines and cytokines attracting neutrophils, dendritic cells, eosinophils, mast cells and lymphocytes which are capable of producing more chemokines and cytokines as well as cytotoxic mediators including reactive oxygen species, serine and cysteine proteases, matrix metalloproteases (MMPs) and membrane perforating agents, as well as soluble mediators of cell killing, such as TNF-α, interleukins and interferons (Kuper, Adami and Trichopoulos 2000, Wahl and Kleinman 1998).

The "inflammation-cancer" link is not only due to an increased risk for some cancer types with chronic inflammation but also to the presence of inflammatory components in a tumor microenvironment of most neoplastic tissues together with those not related to inflammatory process (Colotta et al. 2009). Representative features of cancer related inflammation are: the infiltration of white blood cells, tumor associated macrophages (TAM), the presence of polypeptide messengers of inflammation (TNFα, IL-1,IL-6,chemokines such as CCL2 and CXCL8) and the occurrence of tissue remodeling and angiogenesis (Coussens and Werb 2002). Studies so far have elucidated the link between cancer and inflammation in two pathways, intrinsic and extrinsic which are outlined in Figure 1.2 (Mantovani et al. 2008).

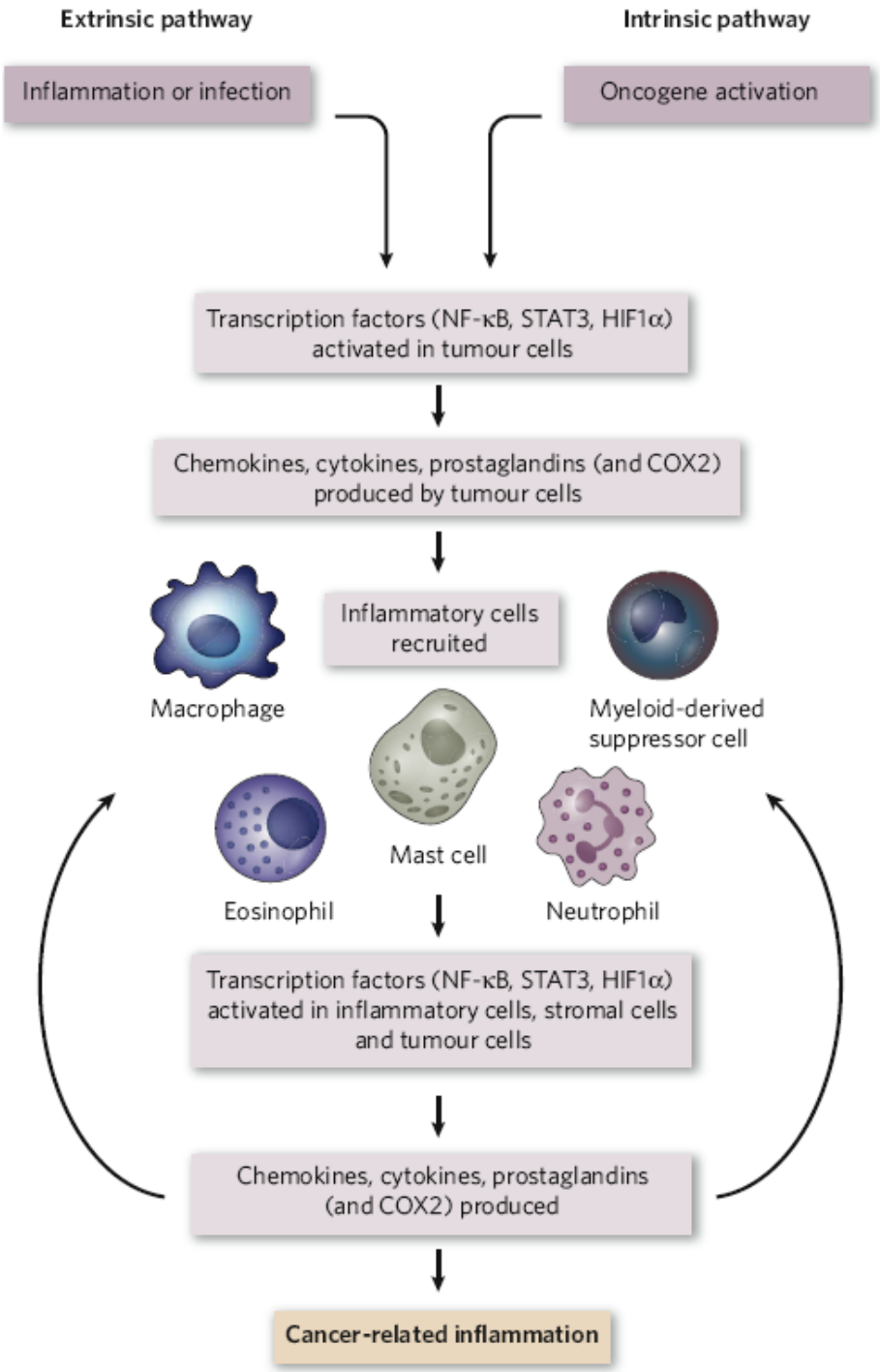

Figure 1.2 Pathways that connect inflammation and cancer (Mantovani et al. 2008)

1.2.1 Oxidative stress

Cancer cells and inflammatory cells produce free radicals, reactive oxygen species as wells as soluble mediators (such as metabolites of arachidonic acid, cytokines and chemokines) that further produce reactive oxygen species; which in turn recruit inflammatory cells. Therefore, a vicious circle occurs. The key substances linking inflammation to cancer via oxidative stress are prostaglandins and cytokines mainly produced by lipoxgenases and cyclooxygenases (Federico et al. 2007). The main steps linking inflammation to cancer via the oxidative stress pathway are summarized in Figure 1.3.

Increased nitric oxide (NO) production by inducible nitric oxide synthase (iNOS) due to inflammatory stimuli has been detected in many pathophysiological processes that cause the development and progression of colorectal cancer (Tamir and Tannenbaum 1996, Thomsen and Miles 1998).

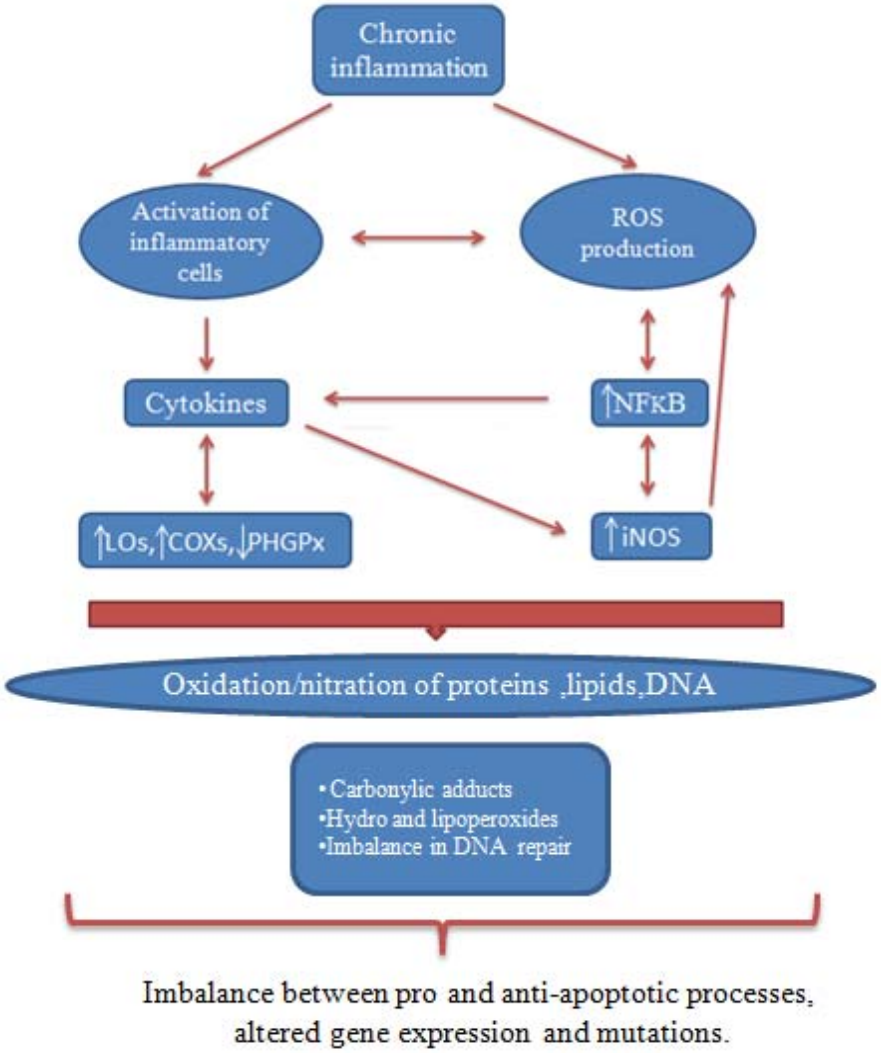

Figure 1.3 Main steps that link inflammation to cancer via oxidative pathway

1.2.2 NF-κB

NF-κB is an inducible transcription factor that regulates the numerous enzymes associated with inflammation, cellular apoptosis, growth, and adhesion as well as differentiation. Normally NF-κB is kept at the cytoplasm bound to IκB proteins which blocks its nuclear localization sequences (NLS). Upon various stimuli IκB is phosphorylated and dissociates from NF-κB and free NF-κB enters the nucleus and activates its target genes (Baeuerle and Baltimore 1996).

Being a central coordinator of innate immunity and inflammation, NF-κB has appeared as an important endogenous tumor promoter (Karin 2006). NF-κB has an important role in both tumor and potential tumor cells and in inflammatory cells. NF-κB operates downstream of the Toll-like receptor (TLR)-MyD88 signaling pathway as a response to microorganism or tissue damage and also in pathways activated by inflammatory cytokines such as TNF-α and IL-1β.

In tumor cells and epithelial cells which are predisposed to transformation by carcinogens and/or inflammatory cells, NF-κB activates the expression of genes that encode inflammatory cytokines, adhesion molecules, enzymes in the prostaglandin-synthesis pathway (e.g. Cyclooxygenase-2) and angiogenic factors (Mantovani et al. 2008). Besides, NF-κB can be activated due to cell-autonomous genetic changes like amplification, mutation and deletions in the DNA in tumor cells (Courtois and Gilmore 2006) .

Another study has shown that NF-κB promotes tumor formation by providing protection from apoptosis with activation of NF-κB dependent anti-apoptotic genes (A1/Bfl1, c-IAP1 and GADD45β) mainly due to TNF- α signaling as it is outlined in Figure 1.4 (Pikarsky et al. 2004).

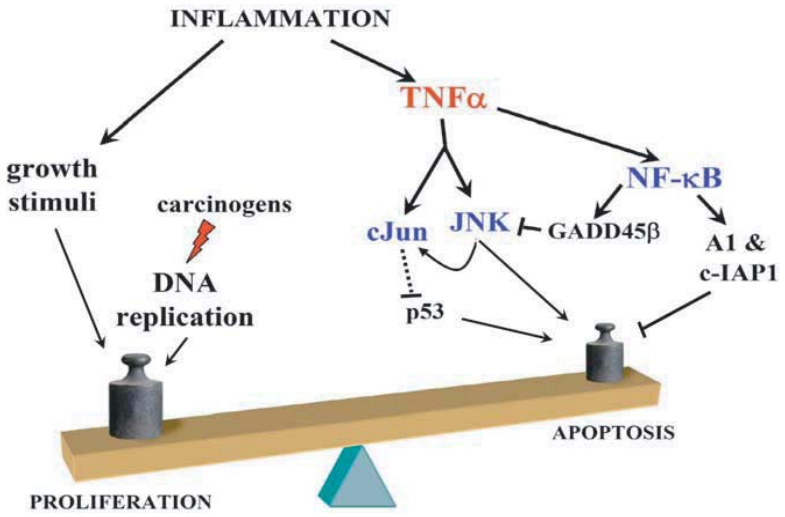

Figure 1.4 Inflammation induced NF-κB activation promotes tumor formation (Pikarsky et al. 2004).

Increased expression of NF-κB has an important role in the transition from colorectal adenoma with low-grade dysplasia to adenocarcinoma in the pathogenesis of colon cancer in patients (Yu et al. 2003).

1.2.3 PPARs

One of the members of the nuclear-receptor super family is the metabolite activated transcription factors that forms heterodimers with the retinoid X receptor (RXR). Most RXR heterodimers maintain active repression of target genes by binding to DNA together with histonedeacetylases (HDACs) and chromatin modifying factors (Glass and Rosenfeld 2000). One important group of these receptors is the Peroxisome Proliferator Activated Receptor PPAR family composed of PPAR-α, PPAR-δ (also known as PPAR-β) and PPAR-γ, an important family of receptors

which regulate metabolic and inflammatory signaling (Castrillo and Tontonoz 2004).

PPARs have been shown to inhibit inflammatory gene expression (Delerive et al. 1999). One important mechanism of inhibition is through direct interaction with NF-κB (Chung et al. 2000, Zingarelli et al. 2003). It has been proposed that the activation of PPAR-γ inhibits inflammatory gene expression by preventing the inflammation signal induced removal of co-repressor complex (Pascual et al. 2005); thereby inhibiting the NF-κB binding to inflammatory gene promoters and driving target gene expression.

PPARs act as nuclear receptors for metabolites of polyunsaturated fatty acids, arachidonic and linoleic acid whose metabolic products act as ligands for the transcriptional activity of PPAR gamma (Chawla et al. 2001).

1.3 Eicosanoid metabolism and cancer

Membrane phospholipids are acted upon by phospholipases which release polyunsaturated fatty acids such as arachidonic acid which are then enzymatically or non-enzymatically transformed to eicosanoids, forming lipid mediators of inflammation. The release of arachidonic acid (AA) and its subsequent oxygenation by lipoxygenases (LO) or cyclooxygenases (COX) is of major importance in cancer related inflammation.

While COXs converts AA to tromboxanes (A_2, B) and prostaglandins; LOs catalyze the conversion of AA to leukotrines (B4, C4,D4) and hydroperoxyeicosatetraenic acids (HpETE) and these inflammatory substances generated are involved in acute inflammatory response and tumorigenesis (Funk 2001, Furstenberger et al. 2006).

The LOs and COXs acting on arachidonic acids and the resulting inflammatory lipid metabolic products are shown in Figure 1.5

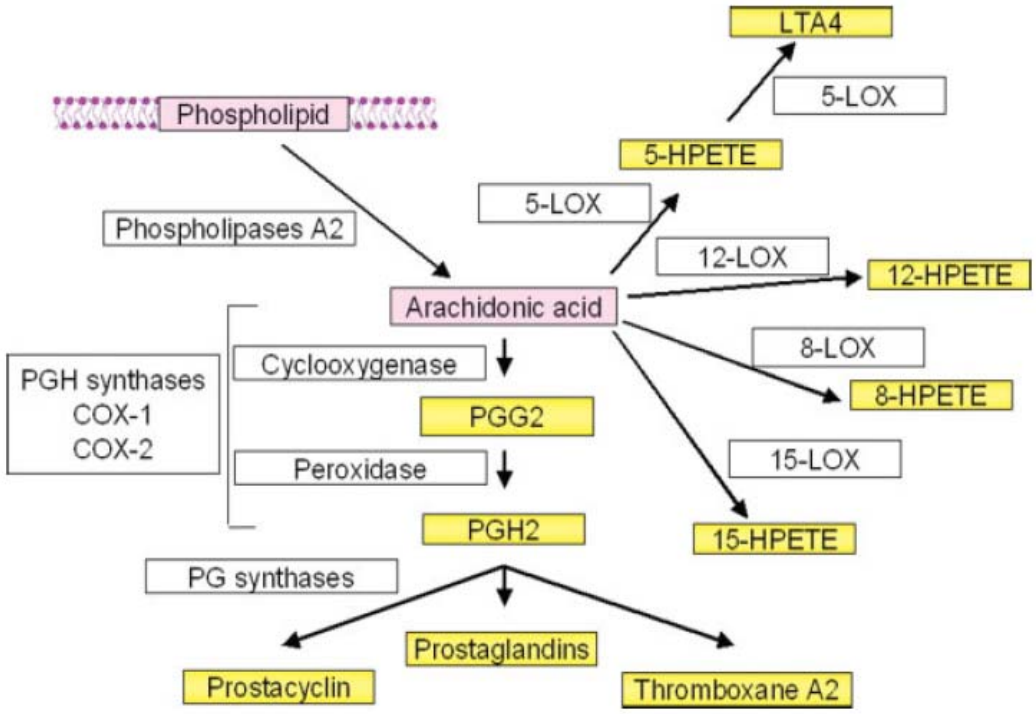

Figure 1.5 Overview of eicosanoid synthesis from arachidonic acid.
(Furstenberger et al. 2006)

PGH_2, the precursor prostaglandin generated by COX enzymes, can be converted to PGD_2, PGJ_2, PGE_2 and PGI_2 by different prostaglandin synthases which in turn function in different signaling pathways via specific G-protein coupled receptors (Funk 2001).

It has been widely reported that the modulation of AA metabolism by selective COX and LO enzyme inhibitors had anti-proliferative and pro-apoptotic effects on various human cancer cell lines including colon, breast, prostate, lung and malignant hematopoietic cell lines (Steele et al. 1999, Ghosh and Myers 1999, Kozubik et al. 1997). More recent studies also support direct functional evidence of the involvement of COX and LO in cancer development (Krishnamoorthy and Honn 2008, Cuendet and Pezzuto 2000).

1.3.1 COXs and cancer

COX has two isoforms COX-1 and COX-2, COX-1 is constitutively expressed and produces prostaglandins controlling normal physiological functions whereas COX-2 is transiently induced by pro-inflammatory stimuli, cytokines, growth factors and tumor promoters causing in increased rates of prostaglandin formation during tissue injury or repair (Furstenberger et al. 2006).

COX-2 promoter has two potential NF-κB binding sites and NF-κB is the major positive regulator of COX-2 expression upon inflammatory cytokines and various growth factors (Tanabe and Tohnai 2002).

There are many studies defining a causal role for COX in cancer development. Homologous disruption of genes coding for COX-1 and COX-2 was found to reduce polyp formation in the colon in MIN/+ mice by approximately 80% (Chulada et al. 2000). COX-2 gene null mutation was shown to decrease the number and size of the intestinal polyps in $APC^{\Delta 716}$ mice (Oshima et al. 1996). APC is a gatekeeper gene for normal colon epithelial cells which is found together with axin, β-catenin and glycogen synthase 3β in a complex. In the absence of growth signals, APC complex inhibits the release of β-catenin preventing the binding of β-

catenin to TCF-4 (T cell factor) which is a transactivator for growth (Vogelstein and Kinzler 2004). PGE_2 on the other hand, can directly regulate APC complex through interacting with axin which explains why COX over-expression is a key for colorectal tumorigenesis (Castellone et al. 2005).

The use of aspirin (Baron et al. 2003) and non-aspirin non-steroidal anti-inflammatory drugs (NSAIDs) is found to halve the risk of colon cancer and is an important factor for chemoprevention (Smalley et al. 1999). Part of the antitumor activity of NSAIDs stems from COX inhibition, particularly the COX-2 isoform. This gene is transcriptionally up-regulated by NF-κB and acts on both the malignant colonic epithelial cells and in the surrounding stroma to promote cancer growth. COX-2 cell dependent effects involves the inhibition of apoptosis as well as promotion of cell migration and invasion while the stromal effects involves angiogenesis and metastasis promotion (Gupta and Dubois 2001).

Additionally, over expression of COX-2 in basal epithelial cells of urinary bladder was found to induce transitional cell hyperplasia, dysplasia and transitional cell carcinomas (Klein et al. 2005). In another over expression study, in multiparous mice mammary glands, COX-2 was shown to generate mammary gland hyperplasia, dysplasia and the formation of metastatic cancer (Liu et al. 2001).

1.3.2 LOs and cancer

LOs comprise a family of nonheme iron dioxygenases that insert oxygen regio- and stereospecifically into polyunsaturated fatty acids forming 5-, 8-, 12-, 12R-, or 15-hydroxyeicosatrienoic acid (HETE) with arachidonic acid and 9- or 13-hydroxyoctadecadienoic acids (HODE) with linoleic acid (LA) as substrate (Brash 1999).

In Figure 1.6 three serial steps of LO reaction: hydrogen abstraction forming a carbon centered fatty acid radical; a radical rearrangement step and following oxygen insertion step forming an oxygen-centered fatty acid hydroperoxy radical.

With the exception of 5-LO all human LO genes including 15-LO-1 are aligned on the short arm of chromosome 17 within a few megabases to each other. As frequent gene duplications occur on the chromosome 17, multiplicity of the LO genes on the same chromosome may have stemmed from such duplications (Kuhn and O'Donnell 2006, Banerjee 2006).

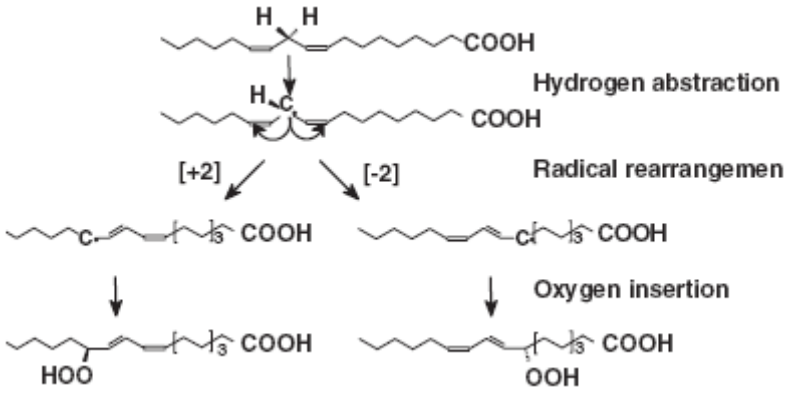

Figure 1.6 Lipoxygenase (LO) mediated oxygenation of a polyunsaturated fatty acid reaction (Liavonchanka and Feussner 2006).

LOs which can oxygenate arachidonic acid and linoleic acid, have an intriguing role in cancer development with its 6 different isoforms (Figure 1.7) with different profiles found in human tumor biopsies and experimentally induced animal tumor models (Cuendet and Pezzuto 2000, Shureiqi and Lippman 2001, Catalano and

Procopio 2005). LOs including 5-, 8- and 12-LO have procarcinogenic roles and work mainly within the arachidonic acid pathway while two LO isoenzymes, 15-LO-1 and -2 work either in the linoleic or the arachidonic acid pathway and have anticarcinogenic roles (Shureiqi and Lippman 2001).

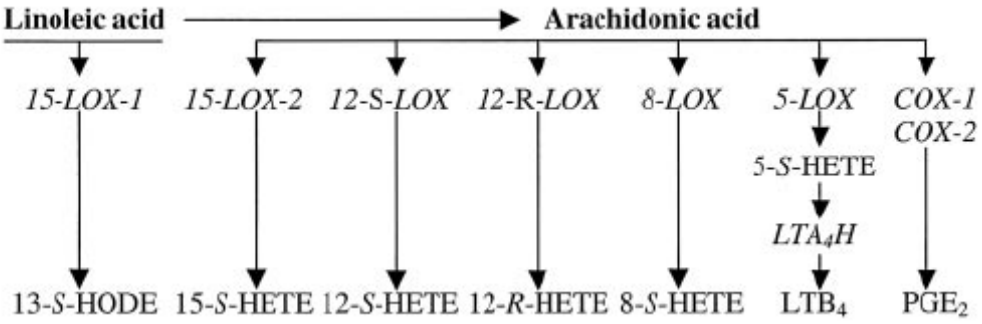

Figure 1.7 Polyunsaturated fatty acid metabolic pathway of arachidonic acid and linoleic acid through different LOs and COXs.

1.4 Metabolism of arachidonic acid by procarcinogenic LOs

Most of the LO isoforms functioning in the arachidonic acid pathway and their metabolic products have shown to provoke tumorigenesis which are: 5-LO and its products 5-S-HETE and LTB_4; 8-LO and 8-*S*-HETE; 12-*S*-LO and 12-*S*-HETE; and 12-*R*-LO and 12-*R*-HETE.

5-LO is found to be up-regulated in adenomatous colon polyps and cancer compared with normal colonic mucosa and inhibition of the enzyme prevents colon cancer cell proliferation both *in vitro* and *in vivo* (Melstrom et al. 2008). 5-LO

expression was found to be increased in intraepithelial neoplasia (early noninvasive precursor lesions for pancreatic adenocarcinomas) (Hennig et al. 2005). Immunohistochemistry studies showed that 5-LO is over expressed in esophageal adenocarcinogenesis in rat models and humans while; chemoprevention studies proved that selective 5-LO inhibitors reduce esophageal carcinogenesis incidence in a dose-dependent manner (Chen et al. 2004). Besides, 5-LO was found to be over expressed as along with COX-2 during oral carcinogenesis in hamsters and humans; and combined 5-LO and COX-2 selective inhibitor therapy had an additive protective effect on the incidence of squamous cell carcinoma (Li et al. 2005). A 5-LO selective inhibitor (A-79175) was shown to inhibit chemically induced lung cancer in mice (Rioux and Castonguay 1998).

12-S-LO (platelet type) deficient mice were found to have significantly reduced development of skin carcinoma (Virmani et al. 2001). However, in human prostate cancer, 12-S-LO expression level was found to correlate with advanced stage and poor differentiation (Jiang, Douglas-Jones and Mansel 2003).

Although there is limited information about 12-R-LO, its metabolic product 12-R-HETE and its link to tumorigenesis; it was reported that 12-R-HETE promotes colon cancer cell proliferation *in vitro* (Bortuzzo et al. 1996).

Both 5- and 12-LO inhibitors were shown to suppress growth of human pancreatic xenografts in mice (Tong et al. 2002). Simultaneous inhibition of cancer cells pretreated with cigarette smoke extract was achieved by the use of 5-LO and COX-2 selective inhibitors through down regulation of LTB4 and PGE2 respectively (Ye et al. 2005).

8-S-HETE, a high affinity ligand for PPARα (Forman, Chen and Evans 1997), was found to be elevated substantially in early stages of mouse skin tumorigenesis and

8-LO up-regulation in mice was found to promote skin carcinogenesis (Burger et al. 1999, Muga et al. 2000). On the other hand, 8-S-LO in mice has 78% homology in amino acid sequence with the anticarcinogenic human 15-LO-2 (Brash et al. 1999) and 8-LO induced growth inhibition in premalignant cells is probably through a common signaling pathway (Schweiger, Furstenberger and Krieg 2007).

1.5 Metabolism of arachidonic and linoleic acid by anticarcinogenic LOs

Two isoenzymes of 15-LO; 15-LO-1 and -2 have been shown to have important anticarcinogenic effects through the metabolism of AA or LA. While AA is oxygenated through multiple metabolic pathways, the enzymatic oxygenation of LA oxidation is solely limited to 15-LO-1, producing 13-(S)-HODE as a metabolic product (Daret, Blin and Larrue 1989, Baer, Costello and Green 1991).

1.5.1 15-LO-2

15-LO-2 catalyzes the conversion of arachidonic acid into 15-(S)-HETE and is differentially expressed in normal human tissues (not in colon but in cornea, prostate, lung) (Shureiqi and Lippman 2001). 15-LO-2 expression is found to be lowered in human prostate carcinomas as well as high grade prostatic intraepithelial neoplasia (Shappell et al. 1999, Jack et al. 2000).

1.5.2 15-LO-1

15-LO-1 enzyme preferentially catalyzes the conversion of LA to 13(S)-HODE (Nagy et al. 1998, Zuo et al. 2006). 15-LO-1 has a controversial role in cancer; it has been unambiguously shown to have a pro-tumorigenic role in prostate cancer

and preliminary reports in colorectal cancer also assigned a pro-carcinogenic role to 15-LO-1 via activation of the mitogen activated protein kinase (MAPK) pathway (Kelavkar et al. 2001, Ikawa et al. 1999, Hsi, Wilson and Eling 2002). However, surprisingly, subsequent studies have indicated a tumor suppressive nature of the enzyme in colorectal, bladder and pancreatic cancers (Kelavkar et al. 2007, Hennig et al. 2007, Nixon et al. 2004).

1.6 15-LO-1 and colorectal cancer

15-LO-1 expression was shown to be reduced or lost in CRC by immunohistochemistry (Shureiqi et al. 1999, Yuri et al. 2007). Additionally, reduction of 15-LO-1 expression was significantly correlated with reduced overall survival with stage IV colorectal carcinoma patients (Heslin et al. 2005). Selective molecular targeting of 15-LO-1 expression was shown to be sufficient to inhibit tumorigenesis in mice (Wu et al. 2008).

The 15-LO-1 gene spans a region of 10.7 kilobases on chromosome 17p13.1. The gene has 14 exons, the sizes being 149, 202, 82, 123, 104, 161, 144, 210, 87, 170, 122, 101, 108 and 859 bps (Figure 1.8). The 15-LO-1 mRNA is 2702 bp (Banerjee 2006)

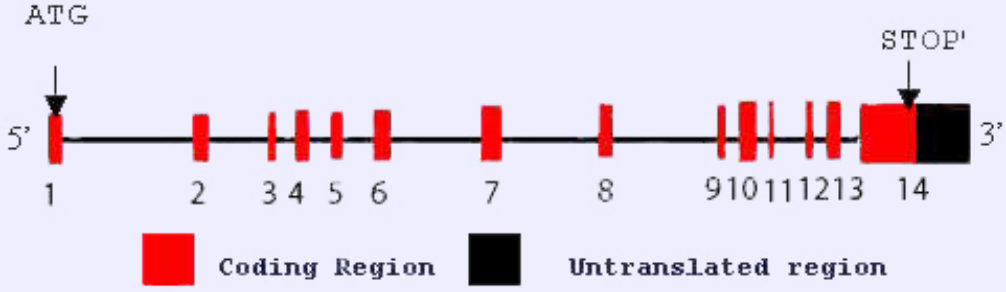

Figure 1.8 Diagram of the 15-LO-1 gene. Exons are represented by red boxes untranscribed sequences in black, with exon numbers on the bottom. The arrows show the ATG and the stop codons respectively (Banerjee 2006).

15-LO-1 protein is a 74.7 kDa, single polypeptide chain folded into a two- domain structure (Gillmor et al. 1997). The C-terminal domain is composed of 550 amino acids and has a helical structure. The domain contains the catalytic non-heme iron cluster that is buried inside the enzyme molecule. The small N terminal domain comprises 110 amino acids (consist of two four-stranded anti-parallel β-sheets) and has a role in membrane binding (Kuhn and O'Donnell 2006). The Polycystin/lipoxygenase/alpha-toxin (PLAT) domain in the 15-LO-1 protein provides it to access and catalysis of enzymatic lipid peroxidation in complex biological structures via direct dioxygenation of phospholipids and cholesterol esters of biomembranes and plasma lipoproteins (Banerjee 2006). The positively charged Arg403 of the human 15-LO-1 was shown to form a salt-bridge with the negatively charged carboxylic group of the fatty acid substrate while Phe415 of the human 15-LO-1 was suggested to interact double bonds of substrates via $\pi - \pi$ interactions (Gan et al. 1996).

T_H2 cytokines IL-4 and IL-13 have been shown to induce 15-LO-1 expression. Binding of IL-4 to its receptor activates many downstream protein kinases which in

turn phosphorylates transcription factors of the STAT family, particularly STAT-1, STAT-3, and STAT-6. Phosphorylated STAT proteins translocate to the nucleus, following acetylatylation by activated acetyl transferases (CBP/P300). In resting cells, STAT sensitive cis-regulatory sequences of 15-LO-1 promoter are blocked by histone proteins. However, IL4/13- activated histone acetyl transferases acetylate those histones thereby enables promoter binding of phosphorylated and acetylated STAT (Conrad et al. 1992, Heydeck et al. 1998, Brinckmann et al. 1996).

Promoter analysis of 15-LO indicated that expression of the gene is suppressed in tumors by several mechanisms. 15-LO-1 promoter methylation is one such mechanism (Liu et al. 2004). Methyltransferase inhibitors have been shown to upregulate gene expression (Hsi et al. 2005). STAT-6 inactivation has been also shown to reduce 15-LO-1 expression, while STAT-6 phosphorylation and acetylation enhance 15-LO-1 transcriptional activation (Shankaranarayanan et al. 2001). Another mechanism of suppression occurs via over expression of transcription factor GATA-6 (Shureiqi et al. 2007) and the most recent finding is the binding of the NuRD repression complex (Zuo et al. 2009a). The gene can also be transcriptionally reactivated by histone deacetylase inhibitors and non steroidal anti-inflammatory drugs to induce apoptosis (Shureiqi et al. 2000, Kamitani et al. 2001).

The forced expression of 15-LO-1 in a colorectal cancer cell line HCT-116, has resulted in reduced viability, probably by stoichiometrically activating the DNA-dependent protein kinase (DNA-PK) which in turn phosphorylates tumor suppressor gene p53 (Kim et al. 2005). It was recently shown that this effect is independent of the enzymatic activity of 15-LO-1, since a mutated form of 15-LO-1 that did not have any enzymatic activity was capable of phosphorylating DNA-PK (Zhu et al. 2008).

Normally, cells undergo apoptosis after finishing their life cycle which prevents the accumulation of damaged cells. Cancer cells, on the other hand, are found to escape apoptosis, which prevents the clearing of abnormal cells and allows them to form tumors. Therefore, the ability of cancer cells to escape apoptosis is thought to be important to cancer development. In colorectal cancer, escape from apoptosis has been linked to down-regulation of 15-LO-1, and its metabolic product 13-(S)-HODE has been found to restore apoptosis in colorectal cancer cells (Shureiqi et al. 2003). Forced expression of the enzyme in various colon cancer cell lines has shown a downregulation of anti-apoptotic proteins and activation of apoptotic pathways (Nixon et al. 2004, Zhu et al. 2008).

Numerous studies have shown that NSAID treatment, including selective COX-2 inhibitors, cause reduction of growth and proliferation in colorectal cancer cells, and interestingly, even with CRC cell lines with low levels of COX-2 expression (Boolbol et al. 1996, Kawamori et al. 1998, Steinbach et al. 2000). These effects were attributed, in part, to the upregulation of 15-LO-1 in response to NSAID treatment together with an increase in its metabolic product 13-S-HODE which induced apoptosis in colorectal cancer (Shureiqi et al. 2000, Heslin et al. 2005).

PPARδ/β expression is also known to promote colonic tumorigenesis through induction of COX-2 and EP4 (PGE2 receptor subtype) expression (Xu et al. 2006a, Xu, Han and Wu 2006b, Han et al. 2005); activation of extracellular kinases 1 and 2 (ERK1 and ERK2) (Daikoku et al. 2007); increased expression of vascular endothelial growth factor (VEGF) (Wang et al. 2006, Stephen et al. 2004, Fauconnet et al. 2002, Zuo et al. 2009b) as well as NF-κB activation and matrix metalloproteinase-9 secretion (Di-Poi et al. 2002). NSAIDs have shown to inhibit PPARδ/β (He et al. 1999). In subsequent studies, it was found that 13-(S)-HODE binds to and suppresses PPARδ/β activation and down regulates PPARδ/β. This

molecular mechanism also explains how 15-LO-1 and 13-S-HODE induce apoptosis in response to NSAIDs (Shureiqi et al. 2003).

PPARγ is well studied in terms of its anti-inflammatory, anti-proliferative proapoptotic effects in epithelial cells (Carter et al. 2009) and the expression of PPARγ in colorectal cancer has shown to be associated with a good prognosis (Ogino et al. 2009). PPARγ ligands (both natural and synthetic) down regulate COX-2 and cyclin D1 thereby inhibiting colon cancer cell proliferation and hepatic metastasis (Takano et al. 2008). Besides, phosphorylation of PPARγ leads to NF-κB inhibition via physical interaction with the p65 subunit of NF-κB and thereby activating apoptotic pathways. (Chen et al. 2003). The 15-LO-1 metabolic product, 13-S-HODE, is a ligand and potentiates the transcriptional activity of PPARγ. (Nagy et al. 1998, Bull et al. 2003, Nixon et al. 2003, Huang et al. 1999). LA in the presence of 15-LO-1 with PPARγ was shown to inhibit the iNOS promoter, (Huang et al. 1999); thereby suppressing the formation of reactive oxygen species. 15-LO-1 expression was also shown to activate PPARγ through downregulation of PPARβ/δ (Zuo et al. 2006).

Patients with inflammatory bowel disease (IBD) have an increased risk of developing CRC (Coussens and Werb 2002). Activation of PPARγ by its ligands, including 13-S-HODE, have been shown to ameliorate IBD by reducing the mucosal damage and preventing or downregulating the inflammatory response (Su et al. 1999, Desreumaux et al. 2001, Naito et al. 2001, Takagi et al. 2002, Bassaganya-Riera et al. 2004).

1.7 Scope of the study

Colorectal cancer (CRC) is one of the most common cancers worldwide and a prevalent cause of morbidity and mortality. Cancer cells and inflammatory cells produce free radicals, reactive oxygen species as wells as soluble mediators such as metabolites of arachidonic acid, cytokines and chemokines that further produce reactive oxygen species; which in turn recruit inflammatory cells.

Arachidonic acid is metabolized via two principal pathways: the cyclooxygenase (COX) and the lipoxygenase (LOX) pathways. Inflammatory molecules formed from these pathways exert profound effects that may exacerbate the development and progression of colon and other cancers.

While COXs and most of the LOs enhance tumorigenesis in colon and other cancers; based on supporting previous studies we have proposed that 15-LO-1 has an anticarcinogenic role. However, very little is known about the effect of the enzyme on cancer metastasis. To that extent, we wanted to investigate the biological effect of 15-LO-1 on the metastatic potential of CRC.

In this study we have transiently expressed 15-LO-1 in the CRC cell line HT-29 which does not express 15-LO-1 in detectable levels. We have investigated the effects of transient 15-LO-1 expression on cellular proliferation, apoptosis, metastatic potential (adhesion to the extracellular matrix, migration and invasion) in the HT-29 cell line. Additionally, we have conducted some preliminary experiments on the effect of 15-LO-1 expression on the tumor promoting transcription factor NF-κB.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

HT-29 human colon cancer cell line was purchased from ŞAP Enstitüsü (Ankara, Turkey).

Phenol red free RPMI 1640 medium, fetal bovine serum (FBS) (tested for mycoplasma), penicillin/streptomycin, trypsin-EDTA solution (0, 25% Trypsin&EDTA), L-glutamine and Optimem were purchased from Biochrom (Berlin, Germany). The transfection reagent FuGENE HD was purchased from Roche (Mannheim, Germany).

The 15-LO-1 cDNA cloned into a pcDNA3.1 vector was obtained from Dr Uddhav Kelavkar.

Plasmid DNA was isolated with a plasmid DNA isolation kit and RNA was isolated with RNeasy Mini_kit obtained from Qiagen (Hilden, Germany). cDNA synthesis kit and DNase1 enzyme were purchased from Fermentas (Lithuania). 15-LO-1 and MTA-1 primers were obtained from Iontek (Istanbul, Turkey).

For the detection of the enzymatic activity of 15-LO-1, the 13(S)-HODE enzyme immunoassay kit was purchased from Assay Design Inc (Ann Arbor, MI, USA).

For western blots, nitrocellulose membrane was purchased from Bio-Rad (Hercules, CA, USA). The 15-LO-1 antibody was obtained from Cayman Chemical (Ann Arbor, MI, USA). XIAP and MTA-1 antibodies were obtained from Santa Cruz (CA, USA). Coomassie Plus protein assay reagent and chemiluminescence kit ECL Plus was obtained from Pierce (Rockford, IL, USA).

Cellular proliferation was detected with the Vybrant MTT assay kit (Invitrogen Carlsbad, CA, USA). Acridine orange was purchased from Sigma (Taufkirchen, Germany). Caspase-3 kit was purchased from Biovision (Mountain View, CA, USA).

Fibronectin (50μg/ml) was purchased from Biological Industries (Israel). Boyden chambers containing membranes with 8μm pores were obtained from Corning (NY, USA). Matrigel was purchased from BD Biosciences (San Jose, CA, USA).

2.2 Methods

2.2.1 Cell culture

HT-29 cells were grown in phenol red free complete RPMI-1640 culture medium containing 10% fetal bovine serum (FBS) and 2 mM L-glutamine in a humidified atmosphere containing 5% CO_2 at 37˚C. Cell culture media were supplemented with 1% penicillin/streptomycin.

2.2.2 Transfections

2.2.2.1 Plasmid isolation and measurement for transfections

Escherichia coli Top10 strain carrying pcDNA3.1-15-LO-1 or pcDNA3.1 empty vector was grown in LB ampicillin broth at 37˚C with shaking O/N. Plasmid isolation was carried out with a Qiagen Plasmid isolation kit according to manufacturer's instructions.

Plasmid DNA concentration and its purity was measured by using spectrophotometer. Plasmid DNA was diluted in 1:20 ratio with molecular biology grade water and the absorbances were recorded at 260 and 280 nm in a quartz cuvette. The concentration of plasmid DNA in the solution was determined by measuring the absorbance of the solution.

Absorbance μg/ml = 50 × Measured OD_{260} × dilution factor

The ratio of OD260/OD280 was calculated to determine the purity. Clean DNA solutions have a ratio of 1.8.

2.2.2.2 Transient transfections

HT-29 cells were transiently transfected with 5 μg pcDNA3.1-15-LO-1 vector in order to study the biological effects of 15-LO-1 expression on colorectal cancer. Control cells included the cells transfected with the empty pcDNA3.1 vector and mock transfected cells as well as parental untransfected cells. The cells were seeded in 6 wells plates the day before the experiment at a confluency of 80% (2×10^6 cells).

Before the transfections, RPMI-1640 complete culture medium was changed with antibiotic free RPMI-1640 medium containing 2 mM L-glutamine, 1% FBS. FuGENE HD was used as a transfection reagent in 3:1 ratio of FuGENE to plasmid. The various transfection mixes were prepared in serum free and Optimem medium. (Table 2.1). Transfection conditions were the same for each experiment and transfection period was either 24 or 48 hours.

Table 2.1 Transfection mixture for 2×10^6 cells in 6 well plates

Transfection mixture	**pcDNA3.1 -15-LO-1**	**pcDNA3.1- (EV)**	**Mock**
DNA (5µg)	5µg	5µg	----
FuGENE 3:1(µl)	15	15	15
Optimem (µl)	Complete to 100 µl	Complete to 100 µl	Complete to 100 µl

The ingredients were added into a sterile eppendorf tube in the order of Optimem, DNA and FuGENE HD. FuGENE HD was added last avoiding contact anywhere, directly into the Optimem DNA mix. Mixtures was left at RT for 15 min and added accordingly by swirling into the cell media.

2.3 15-LO-1 mRNA expression analysis

2.3.1 RNA isolation and measurement

Total cellular RNA was extracted from transiently transfected cells after 48 hours by using the Qiagen RNeasy Minikit according to manufacturer's guidelines. RNA isolates were kept at -80˚ till cDNA synthesis and an aliquot was separated for concentration measurement. RNA concentration and its purity were measured by using spectrophotometer. RNA was diluted in 1:20 ratio with molecular biology grade water and the absorbances were recorded at 260 and 280 nm in a quartz cuvette (Agilent). The concentration of RNA in the solution was determined by measuring the absorbance of the solution.

Absorbance µg/ml = 40 × Measured OD_{260} × dilution factor

The ratio of OD_{260}/OD_{280} was calculated to determine the purity.

2.3.2 DNase-1 treatment

In order to remove any genomic DNA, DNase-1 treatment for the isolated RNA was carried out with Fermentas DNase-1 treatment kit. Briefly 1 µg RNA, 1 µl 10X Reaction Buffer with $MgCl_2$, 1µl DNase-1 was completed to 9 µl in a RNase-free tube and incubated at 37 ˚C for 30 minutes. Next, 1µl 25 mM EDTA was added into the reaction mixture and incubated at 65°C for 10 minutes (RNA hydrolyzes during heating in the absence of chelating agent). The prepared RNA was used as a template for reverse transcriptase.

2.3.3 15-LO-1 RT-PCR

First strand cDNA synthesis was carried out with Fermentas cDNA synthesis kit from total RNA (1µg) using oligo dT primers according to manufacturer's guidelines**.** Amplification of cDNA was carried out in a 30 µl reaction mixture containing synthesized cDNA as template, 5 µM 15-LO-1 gene specific primers (5'-GAGTTGACTTTGAGGTTTCGC-3' and 5'-GCCCGTCTGTCTTATAGTGG-3'); Taq polymerase buffer, of 2mM dNTPs, 1mM $MgCl_2$ and PCR grade water. For positive control, pc.DNA.3.1-15-LO-1 was also used as a template. Since RNA absorbance at OD_{260} may not be sufficiently accurate for quantification; we also PCR amplified GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) by using GAPDH specific gene primers (5'- CAAAGTTGTCATGGATGACC-3' and 5'-GGTGAAGGTCGGAGTCAACG-3') using same reaction mixture conditions for 15-LO-1 PCR. Negative control reaction mixture, without template cDNA was included for both PCR reactions.

15-LO-1 PCR thermal cycling conditions used consisted of an initial denaturation at 94˚C for 3 minutes followed by 28 cycles of 94˚C for 40 seconds, 62˚C for 30 seconds, 72˚C for 30 seconds and final extension at 72˚C for 10 minutes.

GAPDH PCR thermal cycling conditions used consisted of an initial denaturation at 94˚C for 3 minutes followed by 23 cycles of 94˚C for 40 seconds, 60˚C for 30 seconds, 72˚C for 30 seconds and final extension at 72˚C for 10 minutes.

10 µl of the final PCR products of both 15-LO-1 and GAPDH PCR were electrophoresed on a 2% agarose gel at 100V and photographed under UV light.

2.4 15-LO-1 protein expression analysis

2.4.1 Protein isolation and quantification

Proteins of HT-29 cells were isolated after transfection for 24 h, 48 h and 72 h by using M-PER protein isolation kit (Pierce, Rockford, IL, USA) containing protease inhibitors (Roche, Mannheim, Germany) according to the manufacturer's guidelines. The protein content was measured using the modified Bradford Assay using a Coomassie Plus protein assay reagent with 1:5 dilutions in molecular biology grade water. Protein concentrations were calculated according to the standard curve given in appendix A.

2.4.2 15-LO-1 Western blot analysis

Whole-cell extracts (50µg) and prestained Pageruler protein ladder (Fermentas) were separated in a 10% polyacrylamide gel and transferred onto a nitrocellulose membrane at 4˚C for 2 hours. The membrane was blocked in 10% skim milk and probed with 15-LO-1 antibody (1:1000 dilution) followed by a horseradish peroxidase-conjugated rabbit anti-sheep (1:2000 dilution) secondary antibody. After final washing steps with PBS-T, the excess buffer on the membrane was removed and the bands were visualized by using an enhanced chemiluminescence kit (Pierce) according to the manufacturer's instructions. Briefly, 1.5 ml of solution A was mixed with 1.5 ml of solution B of the chemiluminescence kit and applied onto the surface of membrane, left for 1 minute after which the membrane was dried and wrapped with stretch film. The image was taken by a Kodak X-ray processor.

Equal protein loading was confirmed by probing the same membrane for GAPDH. The membrane was stripped after 15-LO-1 Western blot analysis by using a

stripping buffer (100 mM β-meOH, 2% SDS, 62.5 mM Tris-HCl pH: 6.8) Membrane was stripped at 65˚C for 15 minutes with shaking, and then it was blocked in 10% skim milk and probed with a GAPDH polyclonal antibody (1:2000 dilution) followed by a horseradish peroxidase-conjugated goat anti-rabbit (1:3300 dilution) secondary antibody. After final washing steps with PBS-T the bands were visualized by enhanced chemiluminescence kit as described above.

2.5 15-LO-1 activity measurement

The metabolic product of 15-LO-1 oxygenation of linoleic acid, 13(S)-HODE was measured with EIA kit according to manufacturer's instructions. This kit specifically measures 13(S)-HODE with less than 2% sensitivity for the racemic 13(R)-HODE**.** The latter is usually formed in large amounts during the non enzymatic oxidation of linoleic acid. Briefly, 15-LO-1 expressing and control cells were harvested and lysed at 48 hours post-transfection. The lysates were acidified with 0.2 N HCl. 13(S)-HODE was extracted with water-saturated ethyl acetate. 13(S)-HODE, being a lipid molecule, remained in the organic phase and was collected through 3 sequential centrifugation steps. The samples were dried under a general stream of nitrogen and dissolved in ethanol before applying into 96 well plates coated with an antibody to 13(S)-HODE.

The level of 13-(S)-HODE was measured colorimetrically at 405 nm in a microplate absorbance reader (Bio-Rad, Hercules, CA, USA). The 13(S)-HODE concentrations were determined from a standard curve generated with standard 13-S-HODE (given in appendix B) and expressed as ng per mg of crude protein.

2.6 Cellular proliferation assay

2.6.1 MTT assay

The effects of 15-LO-1 expression on cell proliferation was measured by using the Vybrant MTT colorimetric assay kit (Invitrogen) according to manufacturer's instructions which is a simple method for determination of the viable cell number using a microplate absorbance reader.

The water soluble MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) is converted to an insoluble formazan by mitochondrial reductase in the mitochondria of viable cell (Figure 2.1). The formazan is then solubilized by adding SDS solution and the concentration is determined by optical density at 570 nm.

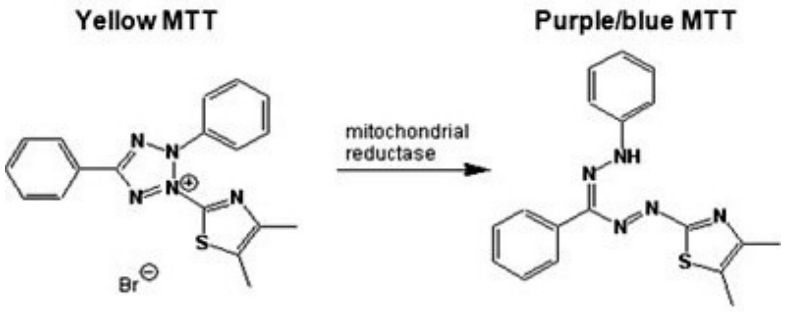

Figure 2.1 The reduction of MTT to formazan.

Briefly, parental HT-29 cells (15,000 cells/ well) were seeded in 96 well plates and transfected 24 hours later with the pcDNA3.1-15-LO-1 or pcDNA3.1 empty vector

using FuGENE HD. Mock transfected and parental HT-29 cells were used as controls. The transfection mixture (5µl) was prepared as in Table 2.1 and was added into each well accordingly.

The cells were allowed to grow for 48 hours after which the transfection media was changed with 100 µl RPMI-1640 complete media and 10 µl of MTT labeling reagent was added. Following incubation with MTT labeling reagent for 4 hours, 100 µl SDS-HCL solution prepared by dissolving 1 g of SDS in 10 ml 0.01 M HCl solution was added to the wells and incubated for 18 hours. The medium with MTT reagent was used as a blank. The absorbance of each well was read at 570nm in a Bio-Rad 680 microplate reader. Experiment was performed with 8 replicates.

2.7 Apoptosis assays

2.7.1 Acridine orange staining

Acridine orange staining assay is an apoptosis assay that provides examination of morphological changes of cells and evaluates apoptosis quantitatively. This assay is based on the difference in the emission of fluorescence when acridine orange (AO) interacts with normal and apoptotic cells.

Acridine orange is a metachromatic dye which differentially stains double-stranded (ds) and single-stranded (ss) nucleic acids. As AO intercalates into dsDNA it emits green fluorescence while it emits red fluorescence when intercalates into ssDNA or RNA upon excitation at 480-490 nm. On the other hand, apoptotic cells have condensed DNA (early event in apoptosis) which is more prone to DNA denaturation than normal chromatin. Condensation of chromatin in apoptotic bodies is a hallmark of apoptosis but not necrosis. Thus, if RNA is removed from cell

suspension by pre-incubation with RNase A and DNA is denatured *in situ* by exposure to HCl shortly before the acridine orange staining apoptotic cells having larger fraction of denaturated DNA display an intense red fluorescence and a low green fluorescence with respect to non-apoptotic cells.

In order to elucidate the apoptotic potential of cells in the 15-LO-1 background, parental HT-29 cells were transiently transfected with pcDNA3.1-15-LO-1 or pcDNA3.1 empty vector. Other controls included mock transfected and parental HT-29 cells.

After 48 hours of transfection, the transfected cells as well as control cells were collected by trypsinization, washed in PBS and centrifuged at 200 x *g* for 5 min and resuspended in 1 ml PBS. Next, cells were fixed in 9 ml 1% paraformaldehyde/PBS solution and kept on ice for 15 min. The fixed cells were centrifuged at 200 x *g* for 5 min and then resuspended in 5 ml PBS, which was followed by another centrifugation at 200 x *g* for 5 min. The cells were then resuspended in 1 ml PBS and transferred to 9 ml 70% ethanol (v/v) and stored for at least 4_h at 4°C. Afterwards, the cell suspensions were centrifuged at 200 x *g* for 5 min, resuspended in 1 ml PBS and 0.2 ml of DNase free- RNase A solution was added and incubated at 37˚C for 30 min. After incubation another centrifugation step at 200 x *g* for 5 min was performed and cells were resuspended in 0.2 ml PBS. Following this, 0.5 ml 0.1 M HCl was added into the cell suspensions at RT and after waiting for 30 to 45 seconds, 2 mg of AO was dissolved in 2ml of sterile dH_2O and diluted 1:100 with buffer solution containing 150 ml dH_2O, 9.92 ml 0.1 M citric acid, 5.46 ml 0.2 M Dibasic sodium phosphate and 1.7 g of NaCl.

Finally, the cell suspension was pipetted onto a glass slide and visualized under a Leica fluorescence microscope with a blue-green filter at 490 nm. The apoptotic cells were defined as cells showing cytoplasmic and nuclear shrinkage and

chromatin condensation or fragmentation. At least 500 cells were counted and the number of apoptotic cells was determined. The experiment was carried out in 4 replicates.

2.7.2 Floating cell ratio and Dead/ Total cell ratio experiments

A floating cell ratio experiment was performed to confirm the apoptotic effect of 15-LO-1 expression in HT-29 cells. Therefore, HT-29 cells were transiently transfected with pcDNA3.1-15-LO-1 or pcDNA3.1 vector. Other controls included mock transfected and parental HT-29 cells.

After 48 hours of transfection, the cell medium which contains floating (dead) cells was collected into a falcon tube, centrifuged for 3 min at 100 x *g*. The supernatant was discarded and the cells were resuspended in 1 ml PBS. A drop of the cell suspension was applied on a hemocytometer and the countings was recorded for each sample.

In the next step attached cells were trypsinized; collected in falcon tubes and centrifugated 3 minutes at 100 x *g*. The supernatant was discarded and cells were resuspended in 1 ml PBS (serum proteins stained with trypan blue dye produce misleading results, therefore, determinants were made in PBS or serum-free solution). Then, 40 µl trypan blue and 40 µl cell suspensions were mixed and mixture was allowed to wait approximately 3 min at RT. This step was carried out one by one for each sample since the cells should be counted within 3 - 5 minutes of mixing with trypan blue (longer incubation periods would lead to cell death). A drop of the trypan blue/ cell suspension mixture was applied to the hemocytometer. The stained and unstained cells were counted separately. Results were calculated as fold difference by using the control untransfected cell numbers as the reference.

The floating cell and Dead/Total cell ratios were calculated according to the formulae:

Floating cell ratio = number of floating cells/ the number of attached cells

Dead/Total cell ratios = number of floating cells + number of dead cells/ the total number cells.

2.7.3 XIAP expression Western blot analysis

Whole cell extracts (50 µl) and prestained Pageruler ladder (Fermentas) were separated in a 10% polyacrylamide gel and transferred onto a nitrocellulose membrane at 4˚C, 100V for 2 h. The membrane was blocked in 10% skim milk and probed with a polyclonal XIAP antibody (1:500 dilution) followed by a horseradish peroxidase-conjugated goat anti-rabbit (1:3300 dilution) secondary antibody. The bands were visualized by using an enhanced chemiluminescence kit as described above.

Equal protein loading was confirmed with a Western blot for GAPDH. The membrane was stripped after probing for XIAP expression by using a stripping buffer. The membrane was stripped at 65˚C for 15 minutes with shaking, and then it was blocked in 10% skim milk and probed with a GAPDH polyclonal antibody (1:2000 dilution) followed by a horseradish peroxidase-conjugated goat anti-rabbit (1:3300 dilution) secondary antibody. After the final washing steps with PBS-T buffer, the bands were visualized by enhanced chemiluminescence kit as explained previously.

2.7.4 Caspase-3 activity assay

Caspase-3 activity assay was carried out by using Biovision caspase-3 kit according to the manufacturer's protocol. Caspases recognize the sequence DEVD and the Caspase-3/CPP32 Colorimetric Assay Kit are based on spectrophotometric detection of the chromophore ρ-nitroanilide (ρNA) preceding cleavage from the labeled substrate DEVD- ρNA. The quantification of the assay is done by reading absorbances at 400 or 405 nm.

HT-29 cells were transiently transfected with pcDNA3.1-15-LO-1 or pcDNA3.1 vector**.** Other controls included mock transfected and parental HT-29 cells. After 48 hours of transfection, cells were counted by a hemocytometer and resuspended in 50 µl chilled Cell Lysis Buffer (CLB) and incubated on ice for 10 minutes. After incubation on ice, cell suspensions were centrifugated at 10000 x *g* for 1 min and the supernatant (cytosolic extract) was transferred to a fresh tube. An aliquot was taken for protein concentration measurement and the remaining extract was kept at -80˚C until use. The protein concentrations were determined by modified Bradford Assay using the Coomassie plus protein assay reagent standard curve (appendix A).

In the assay, 100-µg protein lysates from each sample were mixed with 50 µl chilled Cell Lysis Buffer in fresh eppendorfs tubes. 50 µl of the 2X Reaction Buffer (containing 10 mM DDT) was added to each sample. Then 5 µl 4 mM DEVD- ρNA substrate (200 µM final concentrations) was added to the tubes and mixture was incubated for 2 hours at 37˚C. Finally the samples were diluted in Dilution Buffer (100 µl sample in 900 µl) and the absorbances were read by using a spectrophotometer at 405 nm.

2.8 Quantitation of activated NF-κB

The NF-κB colorimetric assay is a non radioactive DNA binding assay that quantitates the amount of NF-κB subunits present in the nuclear extract of the sample by the use of 96 well ELISA plates that are coated with DNA binding NF-κB consensus sequence. The DNA bound NF-κB subunit p50 is selectively recognized by the primary antibody (p50 and the dimeric p105 specific), which, in turn, is detected by the secondary antibody-alkaline phosphatase conjugate. The Optical Density (OD) is measured by a colorimetric plate reader after addition of TMB substrate. The linear correlation (r2 typically 0.95) between the optical densities and the amount of NF-κB, allows precise and quantitative measurement of the transcription factor in biological samples using a standard curve generated according to manufacturer's instructions.

HT-29 cells were transfected for 48 hours and then cytosolic and nuclear proteins were isolated separately by NE-PER Nuclear and Cytoplsamic Extraction buffer (Pierce) which enables stepwise separation and preparation of cytoplasmic and nuclear extracts from mammalian cultured cells, according to manufacturer's instructions. Then protein concentrations were determined and proteins were kept -80˚C until use.

200 µg/µL of both nuclear (activated NF-κB) and cytoplasmic (total NF-κB) proteins were diluted accordingly with NF-κB colorimetric assay dilution buffer and added into 96-well ELISA plates that are coated with DNA binding NF-κB consensus sequence. Assay was carried out according to manufacturer's instructions.

OD values of standards and samples were recorded at 450 nm in a microplate reader. A standard curve was generated by plotting standard concentrations (pg/mL)

versus x-axis in pg/mL) OD values. We determined the corresponding concentrations (pg NF-κB/mL) using the standard curve and divided the NF-κB value obtained in pg/mL by the protein content of the sample extract in mg/mL, taking the dilution factors into account to quantitate the amount (pg) of NFκB/ mg protein in the sample extract.

2.9 Cell adhesion assay

In order to investigate the effect of 15-LO-1 on the ability of HT-29 cells to metastasize, HT-29 cells were transfected with pcDNA3.1-15-LO-1 and pcDNA3.1 vector and also mock transfected for 48 hours.

96 well plates were coated with 75 µl of fibronectin at a concentration of 50 µg/ml and left at 37˚C in a CO_2 incubator for 1 hour. Some wells were left uncoated for negative controls. Then, the 96 well plates were washed twice with washing buffer (0. 1% BSA in RPMI-1640 medium). The plate was blocked with 1% blocking buffer (1%BSA in RPMI-1640) by adding 100µl of blocking buffer into the wells previously coated with fibronectin and incubated at 37˚C in a CO_2 incubator for 1 h. After incubation, the plate was washed again with washing buffer and chilled on ice. The transfected HT-29 cells were washed with PBS, trypsinized and counted with a hemocytometer after stopping trypsin action. 400,000 cells were resuspended in 1 ml RPMI-1640 medium for each group (15-LO-1 transfected, empty vector transfected and mock transfected) and 100 µl of this was added per well so that each well contained 40,000 cells. Uncoated empty wells were also plated with 40,000 cells per well as negative controls. Additionally, a second 96 well plate coated with fibronectin was also seeded with 40,000 cells as a total cell control. Both plates were left at 37˚C in a CO_2 incubator for 2 h. At the end of the incubation, the first

96-well plate (containing both fibronectin coated and uncoated wells) was washed twice with PBS and inverted on a filter paper gently to remove non-adherent cells.

Finally, 10 µl of MTT was added into the wells and incubated for 4 h, followed by solubilization of the formazan crystals with the addition of 100µl of 1 mM SDS into each well. The absorbance value for each well was recorded in Bio-Rad 680 microplate reader at 570 nm. The experiment was carried out with 9 replicates. Adhesion values for each sample was calculated by dividing the obtained absorbance value in fibronectin coated wells by uncoated, total cell wells corresponding to each particular sample. The ratios were multiplied by 100 and data was interpreted as % values.

2.10 Boyden chamber cell migration and invasion assays

In order to elucidate the effects of 15-LO-1 expression in migration and invasion cell adhesion assay was carried out.

2.10.1 Migration assay

We investigated whether there is a difference in the migration ability of HT-29 cells expressing 15-LO-1 by a Boyden chamber cell migration assay. Firstly, HT-29 cells were transfected either pcDNA3.1-15-LO-1 or pcDNA3.1- EV for 24 hours (longer transfection period may lead to loss of expression since the invasion assay itself required another 48 hours). Trans-well cell migration assays were done in a two-chamber 24 well migration assay plate which is composed of upper and lower chambers.

In the 24th hour of transfection cells were harvested by the use of Trypsin/EDTA and washed three times in RPMI-1640 media containing 1% FBS. Next, the cells were counted in a hemocytometer and resuspended in RPMI-1640 media containing 1% FBS. 100 µl of the cell suspension containing 50,000 cells was applied onto the 8 µm Transwell filters (upper chamber). In order to stimulate migration of the cells through the 8 µm pored membranes, 600 µl RPMI-1640 complete media containing 5µg/ml fibronectin (a chemoattractant) was added to the bottom of the well. Therefore the migratory cells passed through the pores of the membrane and migrated onto the lower surface of the filter. The cells were left at 37˚C in a CO_2 incubator for 48 hours. At the end of incubation period transwells were removed from the 24-well plates and the unmigrated cells and media on the upper portion of the Transwell membrane filters was swabbed of by the use of sterile cotton swabs. The swabbing repeated at least 2 times with fresh swabs.

Subsequently, the Transwells were fixed in 100% methanol for 10 minutes, and then stained with Giemsa solution for 2 minutes at RT. Finally, the Transwells were washed in sterile distilled water to remove excess stain and left to air dry inside the laminar flow hood. After the membrane filters were dry, they were cut out and mounted onto a glass slide with a drop of oil. The total number of cells that had migrated in each transwell filter was then counted at 20x magnification under a Leica light microscope. The experiment was performed with 5 replicates.

2.10.2 Invasion assay

One of the hallmarks of metastatic phenotype is invasion. To study the effect of 15-LO-1 expression on metastatic potential of HT-29 cells, the Boyden chamber invasion assay was carried out which mimics the *in vivo* metastasis process.

Invasive capacities of cancer cells can be determined by establishing a barrier of extracellular matrix (ECM) through which the cells are expected to invade. Matrigel is a basement membrane matrix preparation and composed of mostly laminins and collagen IV isolated from the Englebreth-Holm-Swarm mouse sarcoma and used as an ECM barrier in these assays. Invasion of tumor cells into Matrigel has been used to characterize involvement of ECM receptors and matrix degrading enzymes which play roles in tumor progression.

Firstly, HT-29 cells were transfected either pcDNA3.1-15-LO-1 or pcDNA3.1 (EV) for 24 hours (longer transfection period may lead to loss of expression since the invasion assay itself requires another 48 hours). At the 20th hour of the transfection, Matrigel (thawed overnight at 4˚C) was diluted in 1:5 ratio (400 µl matrigel was mixed with 1600 µl serum free cold RPMI-1640 media) and applied into upper chamber of the 24-well transwell plate Figure 2.2 and kept at 37˚C incubator for 4 hours.

Matrigel solidifies very quickly, thus matrigel preparation steps were carried out on ice with all materials used were cold. At the 24th hour of transfection, cells were harvested by the use of Trypsin/EDTA and washed three times in RPMI-1640 media containing 1% FBS. Next, cells were counted in a hemocytometer and resuspended in RPMI-1640 media containing 1% FBS. Subsequently the gelled matrigel prepared before was gently washed with warmed serum free culture media and 100 µl of the cell suspension containing 100,000 cells was applied onto the matrigel. In order to stimulate invasion through matrigel coated membranes, 600 µl RPMI-1640 complete media containing 5µg/ml fibronectin (chemoattractant) is added to the bottom of the well.

The invasive cells invade through the matrix barrier, the pores of the membrane and finally migrate onto the lower surface of the filter. The Boyden chamber was incubated at 37˚C in a CO_2 incubator for 48 h.

At the end of the incubation, the Transwells were removed from the 24-well plates and cells and Matrigel were scrapped off from the upper portion of the Transwell membrane filters by the use of sterile cotton swabs. The swabbing repeated at least three times. By doing so, the entire surface of the filter was swabbed and all of the cells that have not invaded were removed. Subsequently, the Transwells were fixed in 100% methanol for 10 min, and then stained with Giemsa solution for 2 min at RT. Finally, the Transwells were washed in sterile distilled water to remove excess stain and left to air dry inside the laminar flow hood. After the transwell membranes were dry; they were cut out and mounted on a glass slide with a drop of oil. The total number of cells that had migrated in each transwell filter was then counted at 20x magnification under a Leica light microscope. The experiment was performed with 5 replicates.

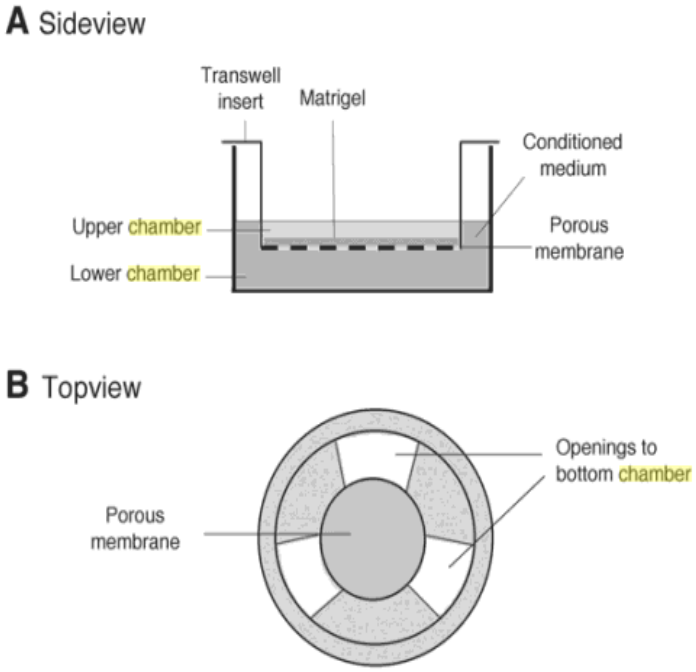

Figure 2.2 A. Side view of a Transwell, lower and upper chambers B. Top view of upper chamber (Shaw 2009)

2.11 Expression level analysis of MTA-1 in HT-29 cell line

To examine the pathway through which 15-LO-1 could have effect on metastasis, we performed MTA-1 expression level analysis by RT-PCR and western blot analysis.

2.11.1 MTA-1 RT-PCR

HT-29 cells were transfected either with pcDNA3.1-15-LO-1 or pcDNA3.1-EV for 48 hours. RNA isolation, measurement, cDNA synthesis were carried out as

explained previously. The cDNAs from RNA isolated from both 15-LO-1 expressing and control cells were amplified in a duplex (RT)-PCR using MTA-1 gene specific primers (5'-TAGGGGGACCCCAAGAATAC-3 and 5'-GTG GAAGACCACCGACAGAT-3') and GAPDH primers (5'- CAAAGTTGTCATG GATGACC-3' and 5'-GGTGAAGGTCGGAGTCAACG-3'). The amplification was carried in a 30 µl reaction mixture containing 2 µl of cDNA as template, 5 µM MTA-1 gene specific primers, 5 µM GAPDH gene specific primers, 3 µl 10x Taq polymerase buffer, Taq polymerase, 2mM dNTPs, 1mM $MgCl_2$ and PCR grade water. A negative control reaction mixture, without the template DNA was included for PCR reaction.

MTA-1 PCR thermal cycling conditions used consisted of an initial denaturation at 94˚C for 3 minutes followed by 26 cycles of 94˚C for 30 seconds, 55˚C for 30 seconds, 72˚C for 1 minute and final extension at 72˚C for 7 minutes.

2.11.2 MTA-1 Western blot

We wanted to investigate the expression levels of MTA-1 found by Western blot analysis, therefore we transiently transfected HT-29 cells either with pcDNA3.1-15-LO-1 or pcDNA3.1-EV for 48 hours. Parental HT-29 cells were also used as controls. Protein isolation was carried out with M-PER isolation kit according to manufacturer's guidelines. 50µg whole-cell extracts and Pageruler ladder were separated in a 10% polyacrylamide gel and transferred onto a nitrocellulose membrane at 4˚C for 2 hours. The membrane was blocked in 5 % skim milk and probed with MTA-1 monoclonal antibody (1:150 dilution) followed by a horseradish peroxidase-conjugated goat anti-mouse (1:2000 dilution) secondary antibody. The bands were visualized by using an enhanced chemiluminescence kit as explained previously.

Equal protein loading was confirmed with GAPDH Western blot. The membrane was stripped after MTA-1 western blot analysis by using a stripping buffer. The membrane was stripped at 65˚C for 15 minutes with shaking, blocked in 10% skim milk and probed with GAPDH monoclonal antibody (1:2000 dilution) followed by a horseradish peroxidase-conjugated goat anti-rabbit (1:3300 dilution) secondary antibody. After final washing steps with PBS-T the bands were visualized by enhanced chemiluminescence kit as explained previously.

2.12 Statistical analyses

Data analysis and graphing was performed using the GraphPad Prism 5 software package. Statistical analysis between experimental results was based on Mann Whitney U-test. Significant difference was statistically considered at the level of $P<0.05$.

Densitometric analyses of Western blots were carried out with image processing programme Image J.

CHAPTER THREE

RESULTS

3.1 Expression and enzymatic activity of 15-LO-1 in HT-29 cells

15-LO-1 has been shown to be transcriptionally silenced in colorectal cancer (Shureiqi et al. 1999, Yuri et al. 2007). Therefore, to study the biological effects of 15-LO-1 in colorectal cancer, we generated transient transfectants of the HT-29 cell line using a pcDNA3.1-15-LO-1 vector as described in Materials and Methods. As controls, HT-29 cells were transfected with the empty pcDNA3.1 or mock transfected with the transfection reagent FuGENE HD. Parental HT-29 cells were also used as controls.

The expression of 15-LO-1 was confirmed by semi-quantitative reverse transcriptase RT-PCR (Figure 3.1). Results were normalized by PCR of the housekeeping gene GAPDH. RT-PCR carried out with cDNA from pcDNA3.1-15-LO-1 transfected cells showed high level of 15-LO-1 transcript, while pcDNA3.1 (empty vector), mock transfected HT-29 cells and parental HT-29 cells showed the presence of very low levels of 15-LO-1 mRNA transcripts.

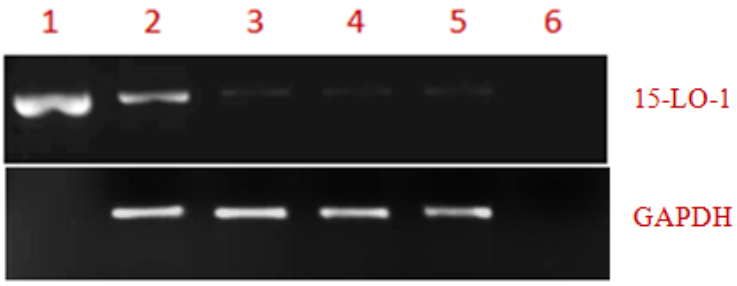

Figure 3.1 RT-PCR analysis of the 15-LO-1 transcript (product size: 952 bp). Lane 1: 15-LO-1 vector (positive control); lane 2: pcDNA3.1-15-LO-1 transfected cells 15-LO-1; lane 3: empty vector (pcDNA3.1) transfected cells; lane 4: mock transfected cells; lane 5: parental HT-29 cells; lane 6: negative control.

However, when the protein expression was analyzed by Western blot as described in the Materials and Methods, we did not detect any protein expression with the empty vector transfected or parental (untransfected) HT-29 cells but high level of expression with pcDNA3.1-15-LO-1 transfected cells for 24 and 48 h of as shown in Figure 3.2. Equal protein loading was confirmed by the levels of GAPDH.

The 15-LO-1 protein expression at 72 h of post transfection was below detectable levels. The doubling time of HT-29 cells is 30 hours. Therefore at 72 h post transfection cells should have lost the pcDNA3.1-15-LO-1 vector as they had gone through two rounds of cell division which explains the very low levels of the 15-LO-1 protein expression.

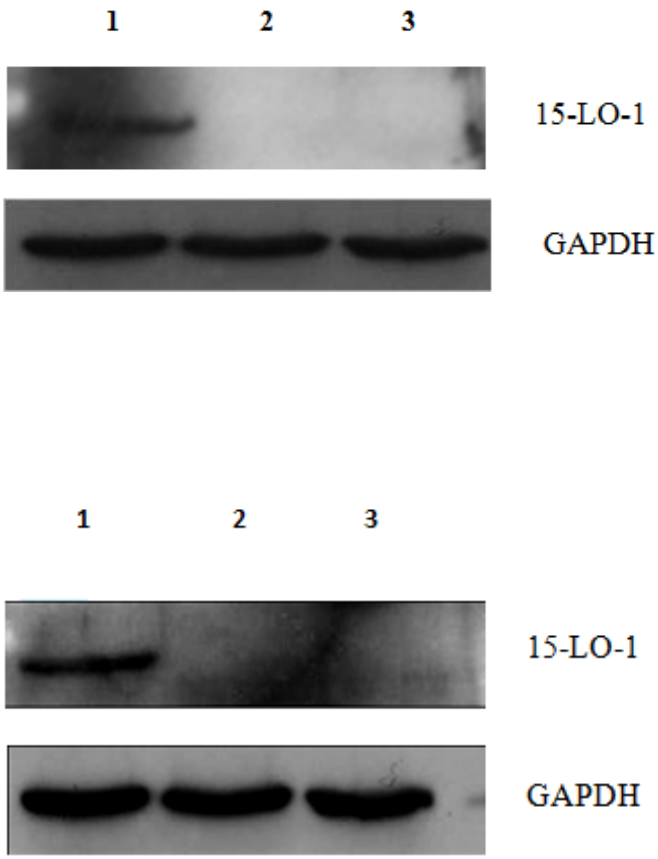

Figure 3.2 Western blot analysis of 15-LO-1 protein (75 kDa) expression. (A) 24 h post transfection (B) 48 h post transfection Lane 1: 15-LO-1 transfected cells; lane 2: empty vector transfected cells and lane 3: untransfected parental cells (for both A and B).

After confirming the presence of the protein, the enzymatic activity of 15-LO-1 was detected with an ELISA kit for 13(S)-HODE as described in the Materials and Methods. The amount of 13(S)-HODE in HT-29 cells expressing 15-LO-1 was significantly higher; that is 108.4 ng/mg of protein compared to 26.7 ng/mg protein in empty vector transfected cells (***P ≤0.0001). The results of enzymatic activity measurement are given in Figure 3.3.

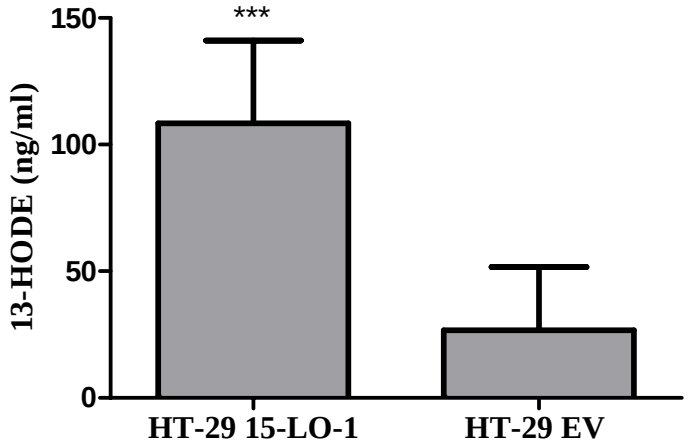

Figure 3.3 The enzymatic activity of 15-LO-1 transfected and empty vector transfected cells (***P <0.0001). Error bars represent the SD of five independent experiments.

3.2 15-LO-1 expression reduced cell proliferation *in vitro*

Self sufficiency in growth signals, insensitivity to growth inhibitory signals and limitless replicative potential are three hallmarks of cancer (Hanahan and Weinberg 2000). Therefore, firstly we investigated if 15-LO-1 expression had an effect on cell proliferation.

The effects of 15-LO-1 expression on cell proliferation were measured by using the Vybrant MTT colorimetric assay kit according to manufacturer's instructions as explained in the Materials and Methods.

HT-29 cells transiently transfected with the 15-LO-1 expression vector in 96 well plates for 48 hours proliferated significantly more slowly (***P ≤ 0, 0001) when compared to empty pcDNA3.1 transfected, mock transfected and parental HT-29 cells (HT-29_UT). The results are shown in Figure 3.4.

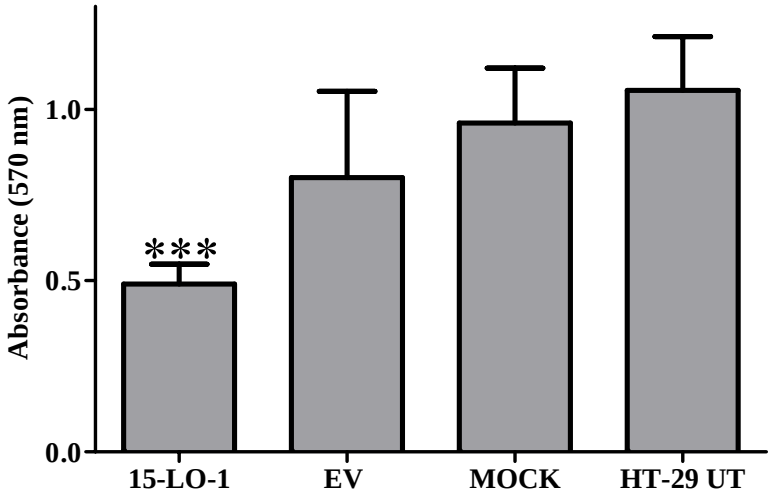

Figure 3.4 MTT assay of 15-LO-1 transfected, empty vector transfected, mock transfected and untransfected (parental) HT-29 cells (****P* < 0.0001 for all comparisons). Error bars represent the SD of three independent experiments.

3.3 15-LO-1 expression increased apoptosis *in vitro*

The ability of cancer cells to escape from apoptosis is a common requirement for tumorigenesis (Hanahan and Weinberg 2000). Therefore, we evaluated if 15-LO-1

enzyme can provoke apoptosis in HT-29 cells. In each particular apoptosis assay, we found out that 15-LO-1 expression induced cells to go to apoptosis.

3.3.1 Acridine Orange staining

An apoptotic cell has special morphological features such as cell shrinkage, chromatin condensation, margination and apoptotic body formation. A qualitative analysis of apoptotic body formation and nuclear changes was carried out using the acridine orange assay in HT-29 cells transfected with a 15-LO-1 expression vector for 48h as described in the Materials and Methods. When the 15-LO-1 expressing and control cells were fixed, stained with acridine orange and viewed under a fluorescent microscope, the apoptotic cells, with their denatured DNA displayed an intense red fluorescence whereas non-apoptotic cells appeared green. The results (Figure 3.5) indicated that 15-LO-1 expression significantly induced apoptosis in HT-29 cell line compared with empty vector, mock transfected or untransfected parental cells ($P \leq 0.05$). HT-29 cells transiently transfected with 15-LO-1 vector have greater number of apoptotic cells compared to empty vector (EV), mock transfected cells or parental cells. Fluorescent microscope pictures from 15-LO-1 transfected and control cells are given in Figure 3.6, Figure 3.7, Figure 3.8 and Figure 3.9.

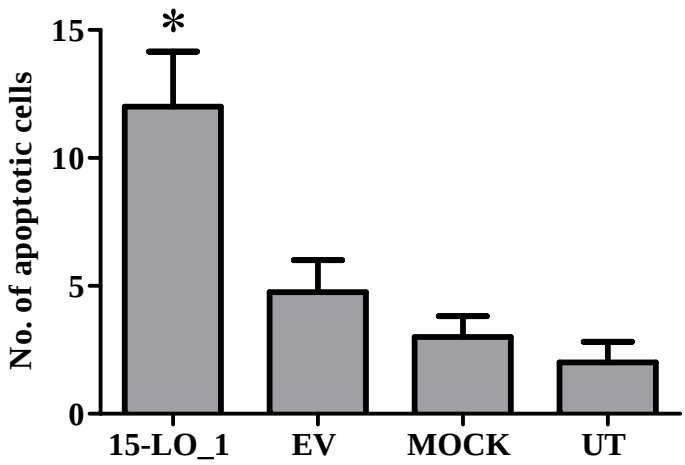

Figure 3.5 Number of apoptotic cells in 15-LO-1 transfected, empty vector transfected, mock transfected and untransfected (parental) HT-29 cells (*$P < 0.05$ for all comparisons)

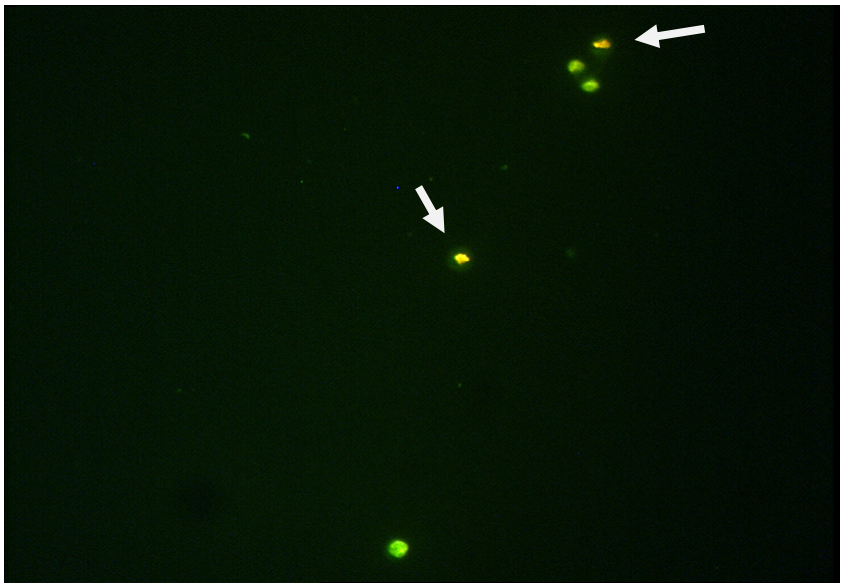

Figure 3.6 pcDNA3.1-15-LO-1 transfected HT-29 cells. The arrows indicate apoptotic cells.

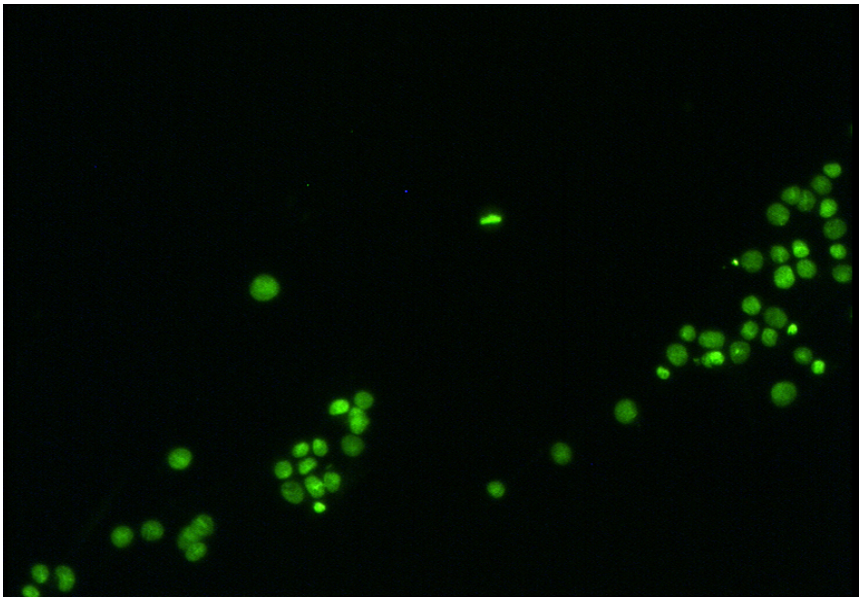

Figure 3.7 pcDNA3.1 (EV) transfected HT-29 cells

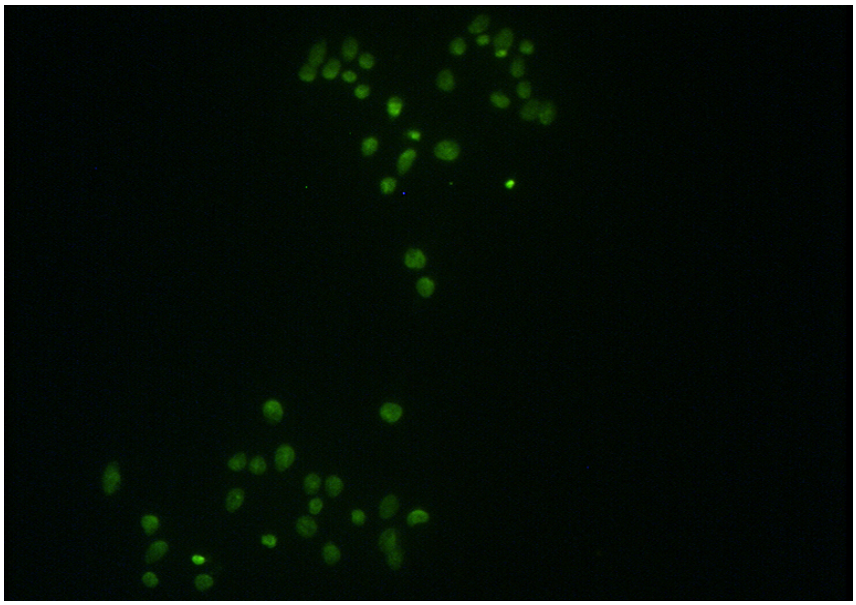

Figure 3.8 Mock transfected HT-29 cells

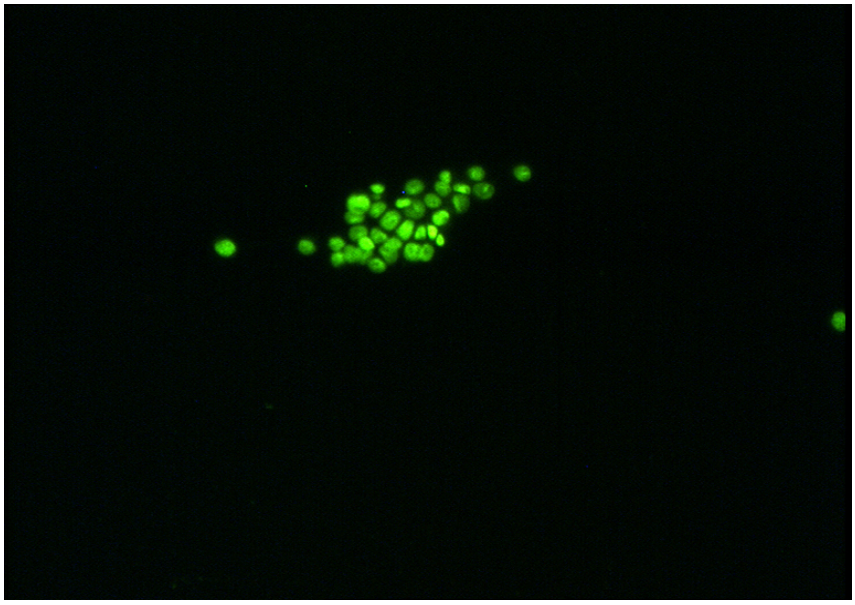

Figure 3.9 Parental HT-29 cells.

3.3.2 Floating cell ratio and Dead/ Total cell ratio

We further confirmed the induction of apoptosis in 15-LO-1 expressing cells quantitatively by determining the floating cell ratio and dead/ total cell ratio experiments quantitatively. pcDNA3.1-15-LO-1 transfected cells showed a significantly (***$P < 0.0001$) higher floating cell ratio (***$P < 0.0001$) (Figure 3.10) and a higher dead/total cell ratio (Figure 3.11) compared to empty vector, mock transfected or untransfected parental cells. Results are given as fold difference.

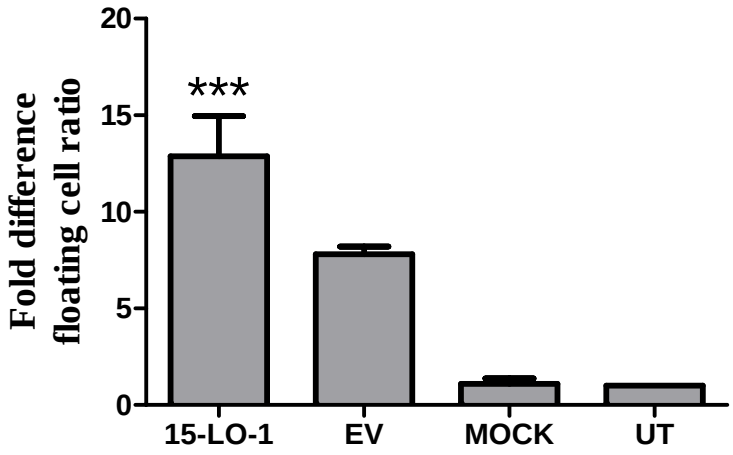

Figure 3.10 Floating cell ratio of 15-LO-1 transfected, empty vector transfected, mock transfected and parental HT-29 cells (***$P < 0.0001$ for all comparisons). Error bars represent SD of three independent experiments.

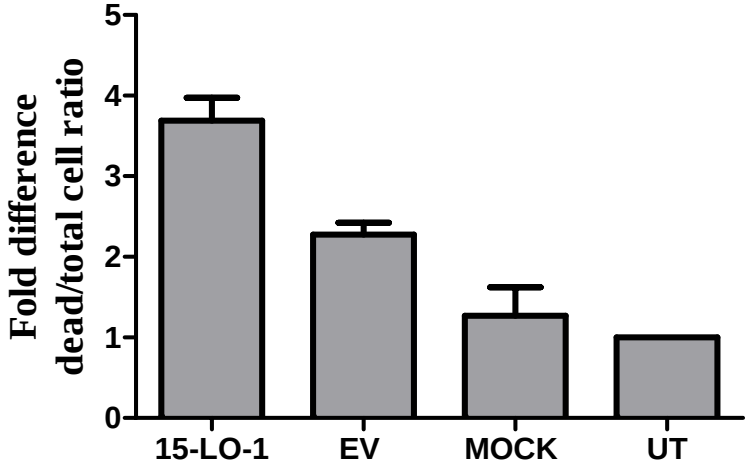

Figure 3.11 Dead/ Total cell ratio of 15-LO-1, empty vector, mock transfected and untransfected cells. Error bars represent SD of three independent experiments.

3.3.3 XIAP expression levels

X- Linked Inhibitor of Apoptosis Protein (XIAP) is an apoptosis inhibitor protein. To understand the pathway by which 15-LO-1 expression induced apoptosis in HT-29 cell line as assessed by acridine orange staining and floating cell or dead/total cell ratio experiments, we examined the XIAP expression levels by Western blot analysis. We have observed that the 15-LO-1 expressing cells reduced expression of XIAP compared to the control cells. The results are given in Figure 3.12. Equal protein loading was confirmed with a GAPDH Western blot of the same membrane.

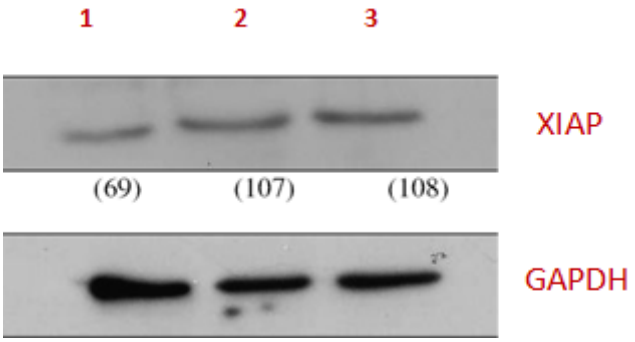

Figure 3.12 Western blot analysis of XIAP protein (55 kDa) expression. 15-LO-1 transfected cells (lane 1), empty vector transfected (lane 2) and parental HT-29 cells (lane 3). The numbers in parenthesis indicate densitometric values after normalization to GAPDH (37 kDa).

3.3.4 Caspase-3 activity assay

Diverse groups of molecules are involved in the apoptosis pathway. One set of mediators implicated in apoptosis belong to the asparate-specific cysteinyl proteases or caspases which are a family of cysteine proteases functioning in the initiation and execution of apoptosis. A member of this family, caspase-3 (CPP32) has been determined as being a key mediator of apoptosis in mammalian cells. In addition, XIAP can directly inhibit the activity of caspase-3 along with caspases-7 and -9 (Dubrez-Daloz, Dupoux and Cartier 2008) and we have showed that XIAP levels are reduced in 15-LO-1 background. Therefore, we examined the effect of 15-LO-1 expression on the caspase-3 activity. We observed that 15-LO-1 expression significantly increased caspase-3 activity (Figure 3.13) in HT-29 cells compared to control cells (*$P \leq 0.05$ for all comparisons).

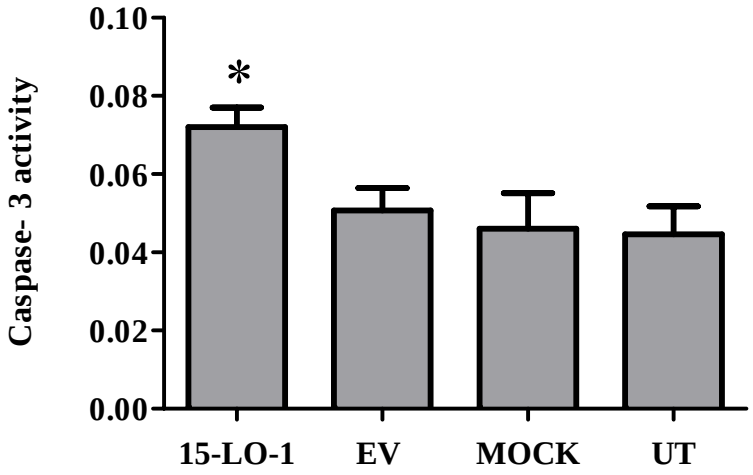

Figure 3.13 Caspase-3 activity assay of 15-LO-1, empty vector, mock transfected and parental HT-29 cells. (*$P < 0.05$ for all comparisons) Error bars represent SD of three independent experiments.

3.4 15-LO-1 expression reduced activated NF-κB quantitatively

The transcription factor NF-κB has been reported to be activated in response to a wide variety of stimuli such as cytokines, growth factors, physiological, physical and oxidative stress (Karin 2006). In response to these stimuli the inhibitory IκB is deegraded by phosphorylation and ubiquitination thereby releasing the NF-κB subunits p50 and p65 for nuclear translocation and transcriptional activity.

NF-κB induces the transcription of XIAP along with many other proteins that can inhibit apoptosis (Chu et al. 1997, Wang et al. 1998). On the other hand, XIAP has been shown to mediate activation of the upstream IKK kinase ultimately causing NF-κB activation (Jin et al. 2009). Since we observed a reduction in XIAP expression in the 15-LO-1 expressing cells, we wanted to see if there is also a reduction in the amount activated NF-κB. We found that pcDNA3.1-15-LO-1 transfected HT-29 cells have a significantly (***$P < 0.0001$) lower amounts of activated NF-κB in HT-29 cells compared to pcDNA3.1 (empty vector) and mock transfected HT-29 cells (Figure 3.14).

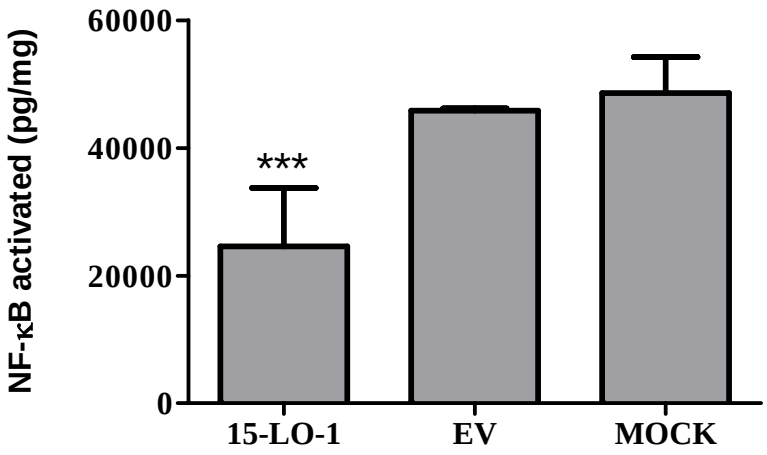

Figure 3.14 The bar diagrams represent activated NF-κB amounts (pg/mg) in 15-LO-1 transfected, empty vector transfected, mock transfected and parental HT-29 cells (***$P < 0.0001$ for all comparisons).

3.5 15-LO-1 expression reduced adhesion of HT-29 cells to the fibronectin

During tumor metastasis, cancer cell survival and adaptation to the new microenvironment is preceded by integrin-mediated cell adhesion to and migration on the ECM proteins such as fibronectin. We transfected HT-29 cells with pcDNA3.1-15-LO-1, pcDNA3.1-EV or mock for 24 hours and then cells were seeded in 96 well plates coated with 50µg/ml fibronectin and blocked with BSA as described in the Materials and Methods. The cells were allowed to attach for 2h following which the non adherent cells were gently removed and an MTT assay was carried out to quantify the cells that adhered to fibronectin at 570 nm.

Our data indicated that 15-LO-1 expression significantly reduced the ability of HT-29 cells to adhere to fibronectin (***$P<0.0001$) compared to control cells.

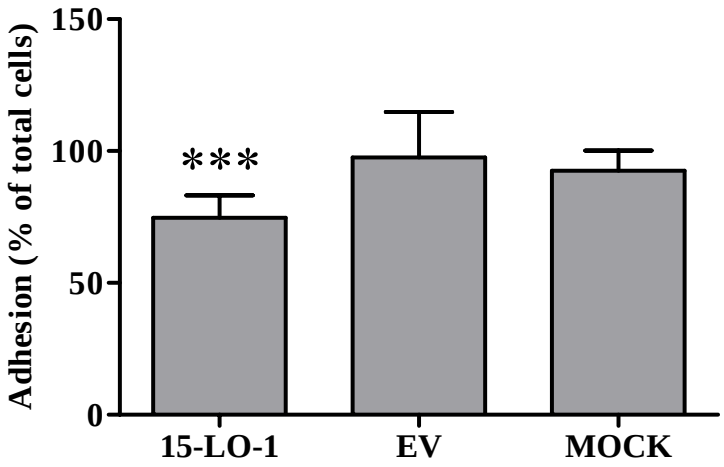

Figure 3.15 The bar diagrams represent 15-LO-1 transfected, empty vector transfected, mock transfected and parental HT-29 cells adhesion to fibronectin (***$P < 0.0001$ for all comparisons). Error bars represent SD from two independent experiments carried out in 9 replicates.

3.6 15-LO-1 expression reduced migration and invasion of HT-29 cells

Migration is the first event in tumor metastasis. Thus, we investigated whether there is a difference in the migration ability of HT-29 cells expressing 15-LO-1 background, by a Boyden chamber cell migration assay.

In order to demonstrate the effect of 15-LO-1 expression on the migration of HT-29 cells, an *in vitro* cell migration assay was performed based on the principle of the Boyden chamber assay. Cells transfected either pcDNA3.1-15-LO-1 or pcDNA3.1-EV for 24 hours were added to Transwell inserts containing membranes with 8µm pores in 1% serum. The bottom chambers contained 10% FBS and 5µg/ml

fibronectin (chemoattractant). After 48 hours, the membranes were fixed and stained and the number of cells which moved through the membrane was counted under a Leica light microscope (10 x objective).

HT-29 cells expressing 15-LO-1 exhibited a significant decrease in the number of cells that were able to migrate compared to empty vector transfected cells, as given in Figure 3.16 (**$P < 0.01$ for all comparisons). Thus, the forced expression of 15-LO-1 significantly inhibited the migration of HT-29 cells.

Membrane images for 15-LO-1 and empty vector transfected cells are given in Figure 3.17 and Figure 3.18 respectively.

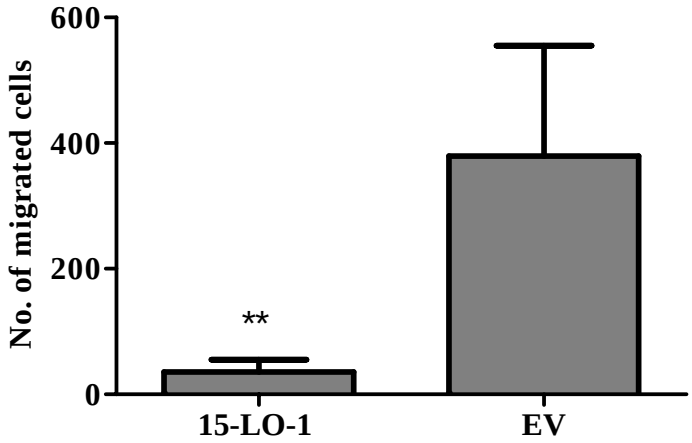

Figure 3.16 Transwell migration assay of 15-LO-1 transfected and empty vector transfected cells. The bar diagram represents the number of cells that could migrate through the Transwell in the experimental and control cells. (** $P < 0.01$). The experiment was carried out in 5 replicates.

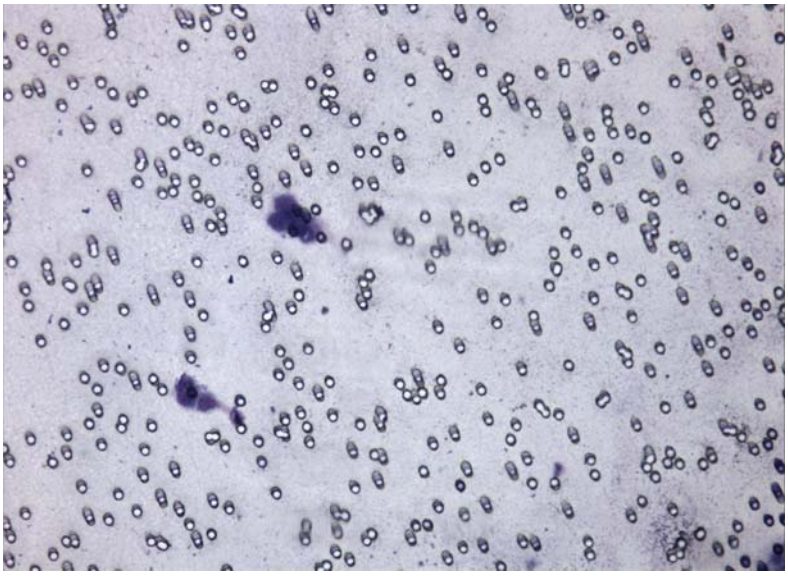

Figure 3.17 pcDNA3.1-15-LO-1 transfected HT-29 cells membrane image

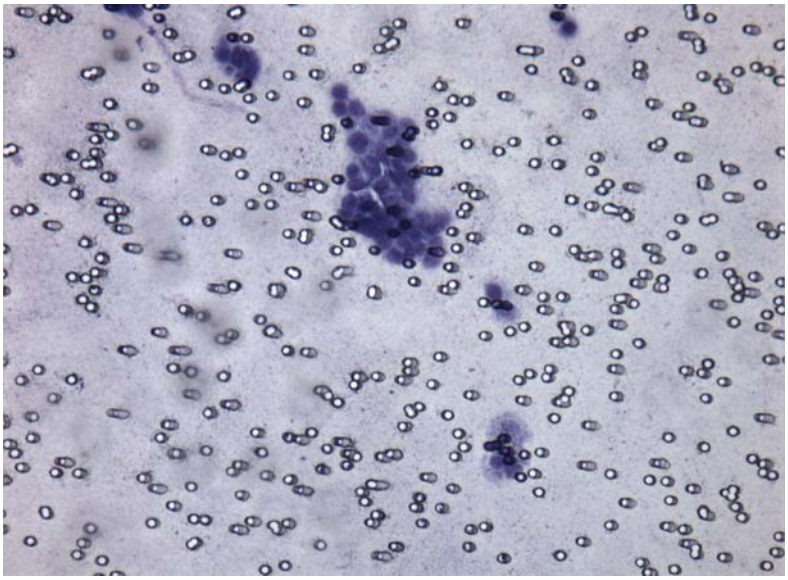

Figure 3.18 pcDNA3.1 (empty vector) transfected HT-29 cells membrane image.

3.7 Boyden chamber cell invasion assay

In order to demonstrate the effect of 15-LO-1 expression on the invasive capacity of HT-29 cells, the *in vitro* Matrigel invasion assay was performed based on the principle of the Boyden chamber assay. The Matrigel served as a reconstituted basement membrane *in vitro*. Cells in 1% serum were added to Transwell inserts containing membranes with 8μm pores coated with Matrigel. The bottom chambers contained 10% FBS and 5μg/ml fibronectin. After 48 hours, the membranes were fixed and stained and the number of cells which moved through the membrane was counted under a Leica light microscope (10x objective). HT-29 cells expressing 15-LO-1 exhibited a significant decrease in the number of cells that were able to invade across Matrigel-coated membranes compared to empty vector transfected cells. (

) (**P < 0.01 for all comparisons). Thus, the forced expression of 15-LO-1 significantly reduced the invasion capacity of HT-29 cells.

Membrane images for 15-LO-1 transfected and empty vector transfected cells are given in Figure 3.20 and Figure 3.21 respectively.

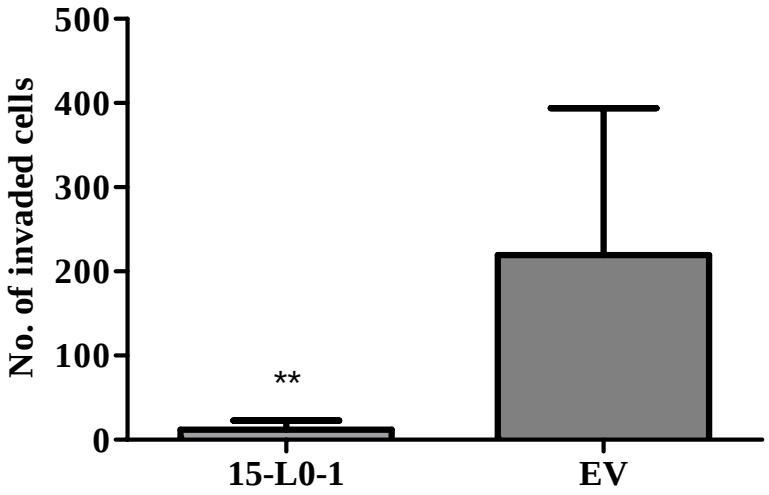

Figure 3.19 Transwell invasion assay of 15-LO-1 transfected and empty vector transfected cells. The bar diagram represents the number of cells that could invade through the reconstituted basement membrane Matrigel and Transwell in the experimental and control cells lines. (**P < 0.01 for all comparisons). The experiment was carried out in 5 replicates.

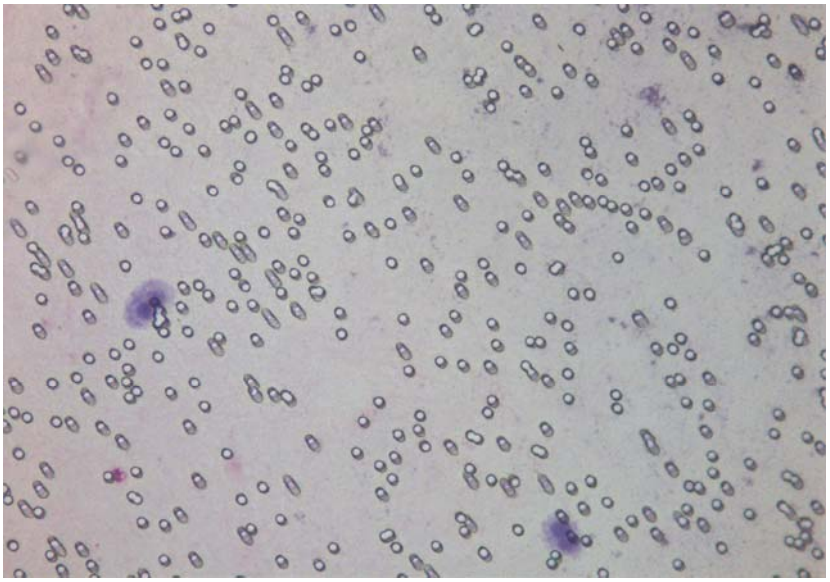

Figure 3.20 pcDNA3.1-15-LO-1 transfected HT-29 cells membrane image

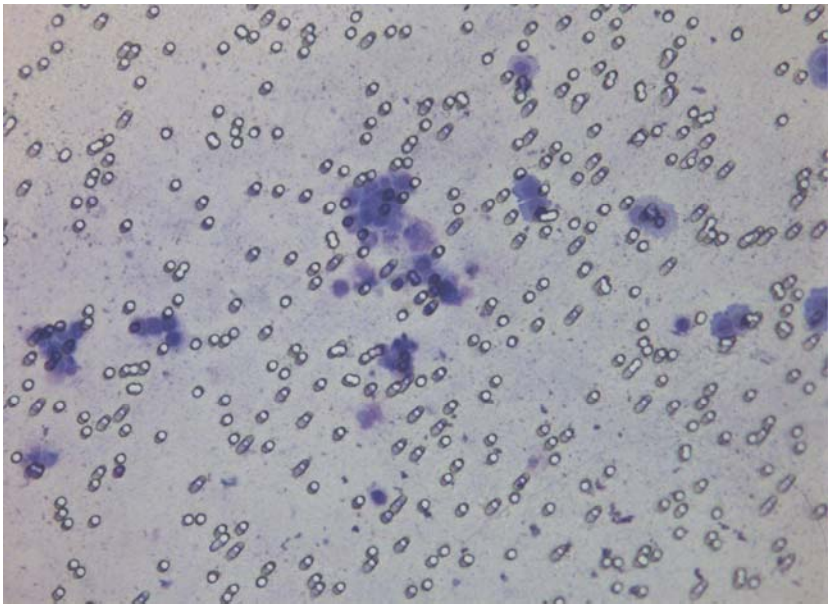

Figure 3.21 pcDNA3.1 (empty vector) transfected HT-29 cells membrane image

3.8 15-LO-1 expression reduced MTA-1 expression

We next examined the pathway through which 15-LO-1 could be inhibiting cellular motility and invasiveness. To that extent, we examined the expression of Metastasis associated protein-1 (MTA-1) by (RT)-PCR and western blot analysis. MTA-1 gene encodes a protein associated with increased invasiveness and anchorage independent growth (Mazumdar et al. 2001, Mahoney et al. 2002). The protein is a component of the nucleosome remodeling and histone deacetylase (NuRD) repression complex that is involved in the transcriptional repression of many different genes including 15-LO-1(Zuo et al. 2009a).

3.8.1 MTA-1 (RT)-PCR

Cells were transfected with pcDNA3.1-15-LO-1 or pcDNA3.1-EV for 48 hours. RNA isolation and cDNA synthesis was carried out as previously described in Materials and Methods. RT-PCR was performed with MTA-1 gene specific primers and GAPDH primers as duplex. The results showed an obvious decrease in MTA-1 gene transcription in 15-LO-1 expressing cells compared to empty vector transfected control cells while GAPDH expression levels are the same (Figure 3.22).

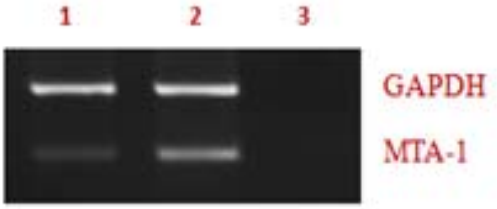

Figure 3.22 RT-PCR analysis of the MTA-1 transcript in HT-29 cells (product size: 244 bp). Lane 1: pcDNA 3.1-15-LO-1 transfected cells MTA-1 transcript. Lane 2: pcDNA 3.1 (empty vector) transfected cells MTA-1 transcript. Lane 3: Negative control.

3.8.2 MTA-1 western blot

Cells were transfected with pcDNA3.1-15-LO-1 or pcDNA3.1-EV for 48 hours. Protein isolation and western blot was carried out using a mouse monoclonal antibody as previously described in material methods. The data Figure 3.23indicates that both HCT-116 and HT-29 cells lines, transfected with 15-LO-1, had reduced protein levels of MTA-1 when compared to the empty vector expressing and parental cells. Equal protein loading was confirmed by GAPDH western blot.

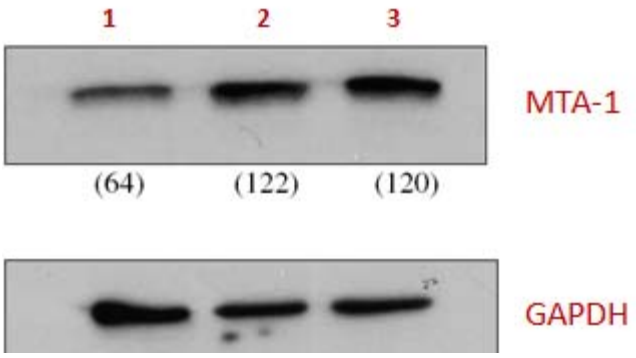

Figure 3.23 Western blot analysis of MTA-1 protein (80 kDa) expression. Lane 1: 15-LO-1 expressing cells MTA-1 protein, lane 2: empty vector expressing cells MTA-1 protein, lane 3: untransfected parental cells MTA-1 protein. The numbers in parenthesis indicate densitometric values after normalization to GAPDH protein (37 kDa).

CHAPTER 4

DISCUSSION

In this study we have described the effect of forced expression of 15-LO-1 on cellular characteristics of the colorectal cancer cell line HT-29, particularly on the ability of the protein to behave as a putative tumor suppressor. The gene was expressed transiently in HT-29 cells (Figure 3.1 and Figure 3.2). The protein was also enzymatically active as we could detect higher amounts of the enzymatic product of linoleic acid oxygenation, 13(S)-HODE (Figure 3.3).

Although much is known and confirmed about the ability of 15-LO-1 to suppress tumorigenesis, (Shureiqi and Lippman 2001, Liu et al. 2004, Nixon et al. 2004, Zhu et al. 2008, Hennig et al. 2007, Hsi et al. 2004, Philips et al. 2008, Shureiqi et al. 2003) the effect of this protein on metastasis needs further examination.

4.1 15-LO-1 expression reduces proliferation and induces apoptosis *in vitro*

Self sufficiency in growth signals, insensitivity to growth inhibitory signals and limitless replicative potential are three hallmarks of cancer (Hanahan and Weinberg 2000).

The first studies on 15-LO-1 metabolism of linoleic acid suggested a tumor promoter function for 13-(S)-HODE. The bioactive lipid has been shown to enhance the mitogenic response to epidermal growth factor (EGF) in BALB/c3T3 fibroblast

and Syrian hamster embryo (SHE) cells (Glasgow and Eling 1990, Glasgow et al. 1992). The levels of 13-S-HODE dehydrogenase enzyme, responsible for the conversion of 13-S-HODE to 13-oxoctadienoic acid, have been found to positively correlate with the degree of colonic cell differentiation, while human colonic cancer tissues have lower levels of 13-S-HODE dehydrogenase activity compared to normal tissues (Earles et al. 1991, Silverman et al. 1996). Therefore it was suggested that 13-S-HODE promotes colonic cell proliferation. However, there were no studies reported in the literature that explored a direct link between 15-LO-1, 13-S-HODE, and colorectal carcinoma.

The first evidence for antitumorigenic role of 15-LO-1 and 13-S-HODE had come in 1999. Shureiqi et al had shown that 15-LO-1 expression was low in tumors compared to normal tissue by immunohistochemistry and transformed colonic cell lines HT-29, RKO and SW620 had no 15-LO-1 expression. In addition, 13-S-HODE levels were reduced in human colonic tumors and 13-S-HODE treatment induced apoptosis in RKO cells in a dose dependent manner (Shureiqi et al. 1999). Exogenous addition of 13-S-HODE has also been shown to have antiproliferative effects in Caco-2 cells and 15-LO-1 transfected HCT-116 colorectal cancer cell lines have been shown to form reduced tumor sizes compared to empty vector transfected HCT-116 cells in tissue xenografts studies (Nixon et al. 2004). In addition, the role of 15-LO-1 in apoptosis has been shown in human esophageal epithelia by both *in vivo* and *in vitro* studies which suggests the idea that two common gastrointestinal cancers escape from apoptosis through down regulation 15-LO-1 expression and decreased 13-S-HODE production (Shureiqi et al. 2001). A suggested mechanism for antiproliferative effects of 15-LO-1 has been through p53 phosphorylation in human colorectal cancer cells (Kim et al. 2005).

We examined the effect of expression of the 15-LO-1 enzyme on cellular proliferation and apoptosis. Our data showed that the proliferation of 15-LO-1

expressing HT-29 cells was significantly slower when compared to the empty vector transfected cells and other controls (Figure 3.4). A similar reduction in proliferation after 72h-96h in HCT-116 cells transiently expressing 15-LO-1 via adenoviral delivery has been recently reported (Wu et al. 2008). Additionally, 15-LO-1 expression has been shown to decrease cellular proliferation in 15-LO-1 over expressing MiaPaCa2 and S2-O13 pancreatic cancer cell lines (Hennig et al. 2007).

We observed that increased apoptosis contributed towards this reduced growth rate of 15-LO-1 expressing cells, as seen from increased number of apoptotic cells by acridine orange staining (Figure 3.5); floating cell (Figure 3.10), dead to total cell ratios Figure 3.11; increased Caspase-3 activation (Figure 3.13) and decreased expression of the anti-apoptotic XIAP protein (Figure 3.12). Shureiqi et al. recently reported that 15-LO-1 expression decreases the expression of the anti-apoptotic proteins XIAP and Bcl-XL and increases Caspase-3 activity in HT-29 and HCT-116 colorectal cell lines (Wu et al. 2008). Since HT-29 colorectal cancer cell line is p53 mutated, our results therefore further confirm the findings of that study on the ability of 15-LO-1 to induce apoptosis through down regulation of antiapoptotic proteins, especially in CRC.

4.2 15-LO-1 expression decreases activated NF-κB levels

The transcription factor NF-κB is a key regulator of the inflammation inducing several inflammatory genes and behaves as an endogenous tumor promoter (Pikarsky et al. 2004, Karin 2006, Mantovani et al. 2008) NF-κB suppresses apoptosis through both intrinsic (mitochondrial pathway) and extrinsic pathways (through death receptors) (Beg et al. 1995, Doi et al. 1999). NF-κB implements its pro-survival activity through many anti-apoptotic proteins, comprising FLIP, Bcl-X_L, TNFR-associated factor 1 (TRAF1), TNFR-associated factor 2 (TRAF2),

cellular inhibitor apoptosis(c-IAP), X chromosome linked inhibitor of apoptosis (XIAP) (Karin and Lin 2002, Kucharczak et al. 2003).

XIAP together with some other IAP family proteins were elucidated as NF-κB induced transcripts, which contributes to the NF-κB mediated protection of some cells from TNF-α- induced apoptosis (Chu et al. 1997, Wang et al. 1998, Earnshaw, Martins and Kaufmann 1999). Moreover, XIAP has been shown to act as a cofactor in transforming growth factor (TGF-β) signaling by interacting with TGF-beta-activated kinase 1-binding protein 1 (TAB1) and thereby activating TGF-beta-activated kinase 1 (TAK1), leading to NFκB activation (Yamaguchi et al. 1999, Birkey Reffey et al. 2001, Sanna et al. 2002).

In our study we have shown that 15-LO-1 expressing HT-29 cells have reduced levels of activated NF-κB (Figure 3.14). We have also shown that XIAP expression is decreased in 15-LO1 expressing cells. Therefore, the low amount of activated NF-κB may be attributed to the lower expression of XIAP. This decrease in XIAP and activated NF-κB could be responsible for apoptotic behavior of 15-LO-1 expressing HT-29 cells.

4.3 15-LO-1 expression reduces adhesion, migration and invasion of colon cancer cells

Metastasis is a complex multistep process involving many factors and genes; which is one of the challenges for developing an effective therapy. As we take more steps in understanding the complex biology of metastasis, the development of new therapeutic strategies and genetic interventions will be possible. Two of the critical steps of metastasis are adhesion and migration of the primary tumor cell on the ECM at the distant site (Joyce and Pollard 2009).

We observed that 15-LO-1 expression significantly reduced the ability of HT-29 cells to adhere to fibronectin, a component of the extracellular matrix (Figure 3.15). A similar reduction in the binding of cancer cells to components of the extracellular matrix has been attributed to the metastasis suppressive properties of geftinib, (Toda et al. 2006) galectin-9, (Nobumoto et al. 2008) and 15- hydroxyprostaglandin dehydrogenase (Li et al. 2008) on colorectal cancer.

We then examined the ability of HT-29 cells to migrate through Transwell pores and invade through Matrigel, a reconstituted basement membrane. These systems are *in vitro* analyses of the invasive potential of malignant cells. Our data indicates that 15-LO-1 expression reduced the ability of HT-29 cells to migrate through the 8μm pores in the Transwell inserts (Figure 3.16). Additionally, when a layer of the Matrigel matrix was added to the insert membranes, the cells' ability to digest the Matrigel matrix occluding the membrane and migrating through the pores was significantly inhibited when they expressed 15-LO-1(Figure 3.19). Taken together our data provides, for the first time, compelling evidence supporting an inhibitory role for 15-LO-1 on the metastatic potential in colorectal cancer.

We then wanted to identify how 15-LO-1 modulates the migratory behavior of colon cancer cells. MTA-1, a component of the nucleosome remodeling and histone deacetylase (NuRD) repression complex, is involved in the transcriptional repression of many different genes including 15-LO-1, (Zuo et al. 2009a) estrogen receptor (Mazumdar et al. 2001) and BRCA1 (Molli et al. 2008). MTA-1 is regulated by the epidermal growth factor receptor family and is found to be over expressed in epithelial cancers including breast and colorectal carcinoma (Zuo et al. 2009a, Nicolson et al. 2003, Giannini and Cavallini 2005) and forced over expression of the protein is associated with increased invasiveness and anchorage independent growth (Mazumdar et al. 2001, Mahoney et al. 2002). We have observed a decreased expression of MTA-1 protein when 15-LO-1 is expressed in

HT-29 cell lines (Figure 3.23). We propose that the decrease in the invasive phenotype observed for the 15-LO-1 expressing cells may be due to the reduced expression of MTA-1. Zuo et al. (Zuo et al. 2009a) have shown a negative correlation between 15-LO-1 and MTA-1 expression in paired normal and cancerous colorectal mucosa. Based on this study and ours, we speculate the possible presence of a negative feedback loop between 15-LO-1 and MTA-1 expression. Forced expression of 15-LO-1 causes a loss of MTA-1 expression and results in a decrease in cellular motility and invasion. Over expression of MTA-1, commonly observed in malignant epithelial cells, causes a repression of 15-LO-1 expression and increases the migratory capacity of the cells.

4.4 Future perspectives

Further studies may be based on a complete understanding of the link between 15-LO-1 expression and the metastatic potential of colorectal cancer cells. Firstly, the relationship between MTA-1 and 15-LO-1 needs to be elucidated further. The NuRD complex has been shown to silence the expression of 15-LO-1 in colorectal cancer cell line (Zuo et al. 2009a). We have shown here that 15-LO-1 expression results in a suppression of MTA-1 expression. Therefore, the presence of interactions between these two proteins warrants further studies. Additionally, the loss of NF-κB activity in 15-LO-1 expressing cells needs to be further examined. This transcription factor is ubiquitous in several different inflammatory pathways and therefore the suppression of its transcriptional activity may be of therapeutic interest which should be further examined.

CHAPTER 5

CONCLUSION

The major findings of this study are as follows:

1. We have transiently expressed 15-LO-1 in the HT-29 colorectal cancer cell line by transfection of 15LO-1 mammalian expression vector. The expression was confirmed by RT-PCR and Western blot. The protein was enzymatically active, as indicated by the presence of significantly higher amount of the enzymatic reaction product 13(S)-HODE.
2. Expression of 15-LO-1 resulted in a significant loss of proliferation as shown by an MTT assay.
3. The loss of proliferation was accompanied by an increase in apoptosis. Several assays were conducted to demonstrate apoptosis in these cell lines:
    a. The floating cell and dead to total cell ratios also indicated significantly more cell death in the 15-LO-1 expressing cells when compared to the control cells.
    b. Acridine orange staining indicated the presence of significantly higher number of cells exhibiting apoptosis in the 15-LO-1 expressing HT-29 cells compared to control cells.
    c. The expression XIAP, an inhibitor of apoptosis, was lower in the 15-LO-1 expressing cell lines when compared to the control cells.
    d. The antiapoptotic XIAP gene promoter has consensus sequences for NF-κB binding and the gene has been shown to be regulated by NF-κB transcriptional activity (Chu et al. 1997). We propose that the

decrease in the expression of XIAP observed in this study could be due to a loss of NF-κB transcriptional activity, which we have shown by an NF-kB colorimetric assay. The assay showed that the NF-κB consensus DNA binding activity of the nuclear extracts of 15-LO-1 expressing HT-29 cells was significantly lower than that of the control cells.

e. The major function of XIAP is to inhibit the function of caspases involved in the process of apoptosis. We have therefore examined the activity of caspase-3 (involved in both the extrinsic and intrinsic pathways of apoptosis) in the presence of 15-LO-1 expression. The data indicates that the caspase-3 activity of HT-29 cells was significantly higher when 15-LO-1 was expressed. This indicates the inhibition of XIAP expression removed the inhibitory effect on caspase-3 thereby allowing it to activate the apoptotic pathway.

4. The expression of 15-LO-1 resulted in a loss of the metastatic potential of HT-29 cells. We have conducted a number of experiments to prove this hypothesis.
    a. Cells expressing 15-LO-1 lost their capacity for adhesion to the extracellular matrix, as shown by a reduced adhesion to fibronectin.
    b. 15-LO-1 expression caused the HT-29 cells to lose their invasive and migratory capacity through Transwell inserts in a Boyden Chamber assay
    c. The expression of MTA-1, a nuclear protein which is a member of the NuRD silencing complex, has been reported to enhance the invasive potential of cancer cells. We have shown here for the first time that 15-LO-1 expression causes a decrease in the expression of MTA-1, which may indicate the possible presence of a negative feedback loop regulating 15-LO-1 expression and the metastatic potential of colon cancer cells.

These properties of 15-LO-1 further emphasize the importance of this protein as a possible therapeutic option in colorectal carcinogenesis.

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APPENDIX A

STANDART CURVE FOR COOMASSIE PLUS REAGENT BRADFORD METHOD

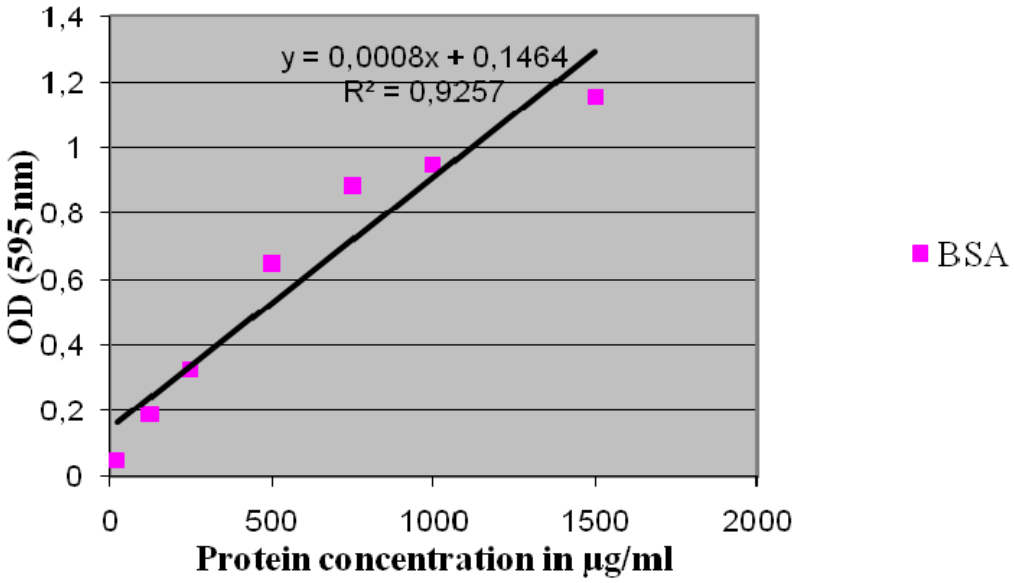

Figure 5.1 Protein concentration standard curve

APPENDIX B

13-S-HODE ENZYME ACTIVITY STANDARD CURVE

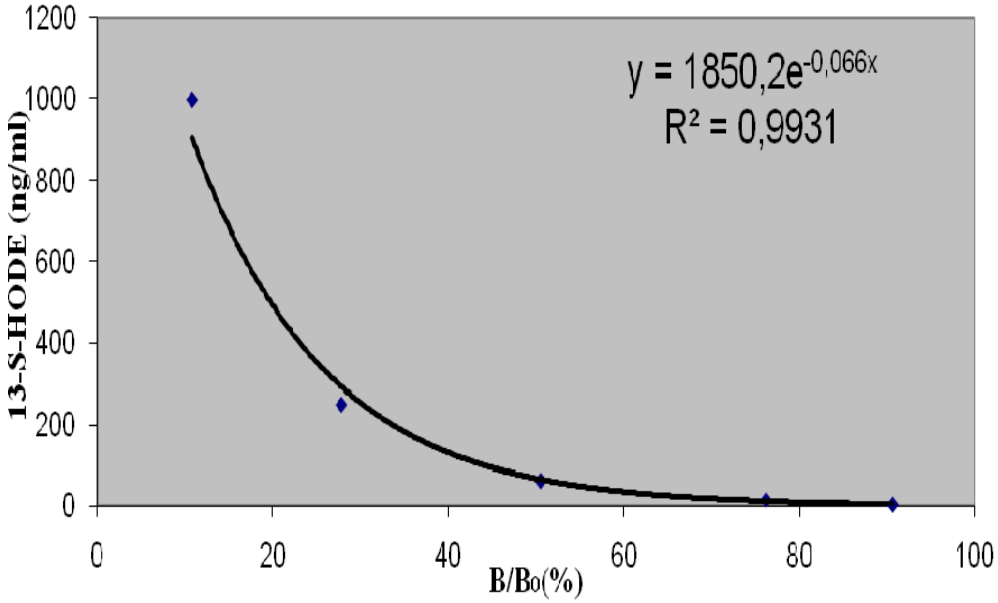

Figure 5.2 13-HODE enzyme activity standard curve

APPENDIX C

COLORIMETRIC NF-κB STANDARD CURVE

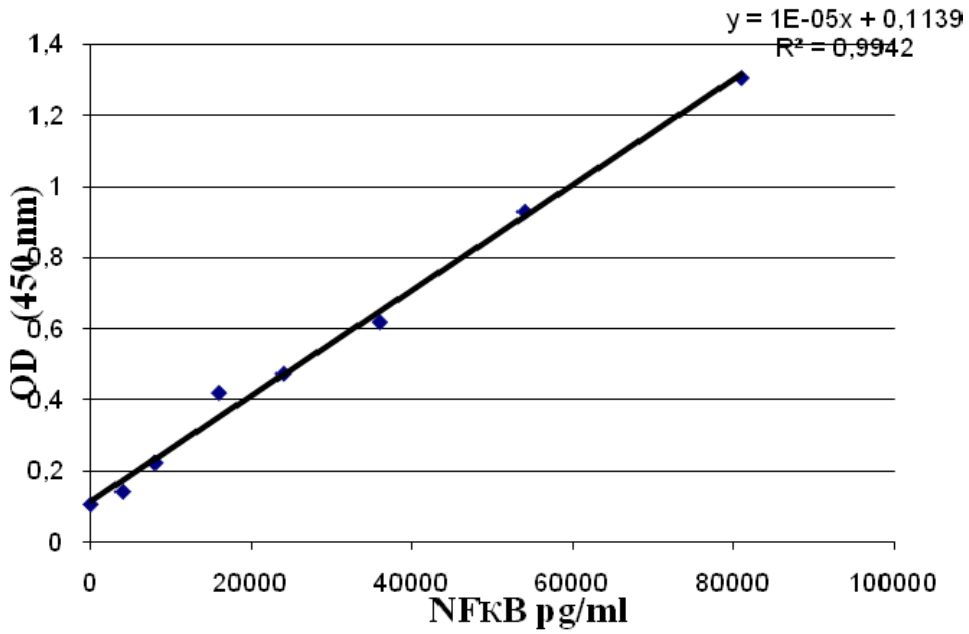

Figure 5.3 Activated NFκB standard curve