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# Role of Low Density Lipoprotein Receptor as a Potential Suppressor of Growth and Survival of Colorectal Cancer Cells

Vaishali Basu  
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# **Role of Low Density Lipoprotein Receptor as a Potential Suppressor of Growth and Survival of Colorectal Cancer Cells**

By

Vaishali Basu

A Dissertation  
Submitted to the Faculty of Graduate Studies  
Through the Department of Biological Sciences  
in Partial Fulfillment of the Requirements for  
the Degree of Doctor of Philosophy  
at the University of Windsor

Windsor, Ontario, Canada  
2017

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February 7, 2017

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## Abstract

Colorectal cancers (CRC) express high level of 3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGCR) protein suggesting an increased requirement for endogenous cholesterol biosynthetic pathway by growing cancer cells. Intake of statins, pharmacological inhibitors of HMGCR has been reported to exert varying responses in reducing the risk of CRC in humans, suggesting the existence of tumours with statin-sensitive and statin-resistant phenotypes. Normally intracellular cholesterol homeostasis involves several proteins including HMGCR and the membrane bound low density lipoprotein receptor (LDLR) which allows uptake of plasma cholesterol and increase intracellular cholesterol level. Therefore, HMGCR activity within a cell is highly dependent on the level of LDLR. Whether LDLR is playing a role in CRC growth and cholesterol homeostasis remains poorly understood. In the first study, it was observed that experimentally induced colonic tumours, express lower LDLR and higher HMGCR, SREBP2 (Sterol Regulatory Element Binding Protein) and PCSK9 (Proprotein convertase subtilisin/kexin type 9) compared to colonic mucosa. This observation led to the hypothesis that a low LDLR phenotype favours tumour growth. To test this hypothesis, three human colorectal cancer cell lines (HCT 116, HT 29 and DLD 1) were selected. Through Western blot analysis and q-RT-PCR, it was established that all three cancer cell lines express lower levels of LDLR and higher levels of HMGCR, SREBP2 and PCSK9 compared to normal colonic epithelial cells, similar to solid tumours. DLD1 cells expressing the lowest LDLR protein exhibited the highest cell viability and proliferation amongst the three cancer cell lines. HCT 116 and HT 29 showed higher sensitivity to the growth inhibitory effect of cholesterol lowering drugs such as lovastatin and RO 48-8071 and were able to upregulate LDLR unlike DLD 1 cells. Ectopic overexpression of LDLR in the three CRC cell lines was associated with reduced cell viability, cell motility and migration and enhanced growth inhibition by lovastatin. Furthermore, ectopic over expression of LDLR induced tumour suppressive p38 and PTEN signaling and reduced activation of pro-survival signaling proteins such as ERK1/2 and AKT. The present dissertation alludes to a novel role of LDLR in CRC development. More importantly the findings support a tumour suppressive role

of elevated LDLR in CRC and support the contention that the drugs being used to inhibit HMGCR and/or increase LDLR, for the prevention of coronary artery diseases could also be effective in the prevention of CRC.

**Dedicated to**

**My family for their unconditional love and support**

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## List of Abbreviations and Nomenclature

ABCA1	ATP binding cassette subfamily A member 1
ABCG1	ATP Binding Cassette Subfamily G Member 1
ACAT	Acyl Coenzyme A Cholesterol Acyl transferase
ACL	Adenosine triphosphate citrate lyase
ADP	Adenosine diphosphate
AOM	Azoxymethane
APC	Adenomatous Polyposis Coli
APC/C	Anaphase promoting complex
APC <sup>MIN</sup>	APC = Adenomatous polyposis coli; MIN= multiple intestinal neoplasm
Apo	Apolipoprotein
ARH1	Autosomal recessive hypercholesterolemia 1
ATP	Adenosine triphosphate
BAD	Bcl-2-associated death promoter
bHLH-LZ	Basic helix loop helix- leucine zipper
BRAF	v-Raf murine sarcoma viral oncogene homolog B
BSA	Bovine serum albumin
CETP	Cholesteryl ester transfer protein
CIN	Chromosomal Instability
CoA	Coenzyme A
COPII	Coatomer II protein
CRC	Colorectal cancer
DMH	1,2-dimethylhydrazine
DR4	Direct repeat 4
EGFR	Epidermal growth factor receptor
ERK	Extracellular regulated kinases
FAP	Familial adenomatous polyposis
FASN	Fatty acid synthase



FH	Familial Hypercholesterolemia
FPP	Farnesyl pyrophosphate
5-FU	5-flourouracil
GEF	Guanine exchange factor
GGPP	Geranylgeranyl pyrophosphate
GLUT	Glucose transporter
GLS	Glutaminase
gp78	Glycoprotein 78
GSK-3 $\beta$	Glycogen synthase kinase-3 $\beta$
HDL	High density lipoprotein
HIF1 $\alpha$	Hypoxia inducing growth factor alpha
HMGCR	3-Hydroxy-3-Methylglutaryl-CoA Reductase
HNPCC	Hereditary Non polyposis Colorectal cancer
IDL	Intermediate density lipoprotein
IDOL	Inducible Degrader of LDLR
IGF-2	Insulin like growth factor-2
IL-1 $\beta$	Interleukin 1 beta
INSIG	Insulin induced gene
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
KRAS	Kirsten rat sarcoma viral oncogene homolog
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LOH	Loss of heterozygosity
LXR	Liver X receptor
MAPK	Mitogen activated protein kinase
MDM2	Mouse double minute 2 homolog
MLH	MutL homolog
MMP	Matrix metalloproteinase
MMR-	Mismatch repair deficient

MetS	Metabolic Syndrome
MSI	Microsatellite Instability
mTORC1	Mammalian target of rapamycin complex 1
NF- $\kappa$ B	Nuclear factor kappa B
NSAID	Non-steroidal anti-inflammatory drug
OSC	Oxidosqualene cyclase
PCSK9	Proprotein convertase subtilisin/kexin type 9
PI3K	Phosphoinositide 3-kinase
PIP <sub>3</sub>	Phosphatidylinositol 3,4,5-triphosphate
PMS	Postmeiotic Segregation Increased
PTEN	Phosphatase and tensin homolog
RONS	Reactive oxygen and nitrogen species
RO 48-8071	(4-Bromophenyl)-[2-fluoro-4-[6-[methyl(prop-2-enyl)amino]hexoxy]phenyl]methanone
RTK	Receptor tyrosine kinase
RXR	Retinoid X receptor
SAPK	Stress activated protein kinases
SCAP	SREBP cleavage activating protein
SREBP	Sterol regulatory element binding protein
STAT	Signal transducers and activators of transcription
TCF/LEF	T cell factor/Lymphocyte Enhancer family
TGF $\beta$	Transforming growth factor $\beta$
TNF- $\alpha$	Tumour necrosis factor alpha
TP53	Tumour protein 53
TSC1	Tuberous sclerosis 1
Ubc	Ubiquitin conjugatin
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau tumour suppressor protein
VLDL	Very low density lipoprotein

# **Chapter 1**

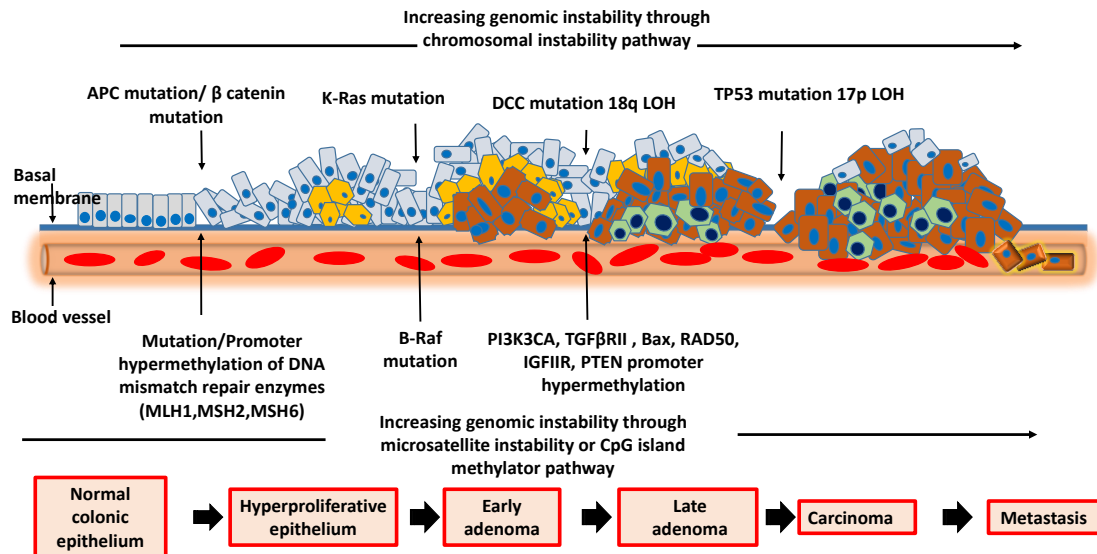
## **General Introduction**

## **1.1 Introduction**

Colorectal cancer (CRC), is one of the leading causes of cancer related deaths worldwide, alongside lung, prostate and breast cancer. In the Western world population, the average lifetime risk of a person developing colorectal cancer is approximately 5%, risk being marginally higher in males than females. 85% of the cases are sporadic caused by mutations due to environmental factors, diet and lifestyle [1]. Less than 10% of the cases are due to genetic predisposition in which a person inherits a single or several germline mutations in tumour suppressor gene or DNA mismatch repair (MMR) enzyme causing familial adenomatous polyposis (FAP) or called Hereditary Non polyposis Colorectal cancer (HNPCC) [2].

In 1990, Fearon and Vogelstein proposed that pathogenesis of CRC is a sequential, multistep process characterized by accumulation of mutations in tumour suppressor and oncogenes, leading to gross genomic instability[3]. The adenoma-carcinoma model (Figure 1.1) which shows the morphological transformation of hyper-proliferating colonic mucosa cells to metastatic carcinoma through a series of intermediate stages, still serves as a classic model to study solid tumour progression.

## Colorectal Cancer Pathogenesis



**Figure 1.1 Fearon and Vogelstein's model for the adenoma-carcinoma sequence of colorectal cancer development.** This model gave a schematic representation of stepwise morphological transformation of normal colonic epithelium to adenoma and metastatic carcinoma due to accumulation of mutations in key tumour suppressor and oncogenes, which is now known as the hallmark of chromosomal instability (CIN) pathway. Increasing genomic instability, which is the end point of all driver mutations, can also be achieved by alternate carcinogenesis pathway such as microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) pathway. MSI phenotype is characterized by defective DNA repair system and CIMP phenotype is characterized by hypermethylation of CpG island in promoter regions of tumour suppressor genes. This image was adapted from Toribara NW, 1995. *New England Journal of Medicine* [4].

## **1.2 Carcinogenesis pathways of CRC**

The prognosis of CRC and patients response to therapeutic treatment largely depends on the morphological subtype and underlying molecular carcinogenesis pathway. There are two main morphological subtypes that have been identified in CRC. One is the classical adenocarcinoma subtype which is characterized by tubular or tubulovillous adenomas that develop into carcinoma in later stages and the other one is the serrated neoplasia subtype identified by hyperplastic polyps or sessile, serrated adenomas [5]. The morphological transformation of preneoplastic lesions like adenomas or hyperplastic polyps to carcinoma is driven by three main carcinogenesis pathways- chromosomal instability pathway (CIN), microsatellite instability pathway (MSI) or the CpG Island Methylator Phenotype (CIMP) [6].

### **Chromosomal Instability Pathway (CIN)**

The CIN phenotype is the most common phenotype observed in 80-85% of sporadic CRC cases [7, 8]. CIN is characterized by accelerated genomic instability due amplification or deletion of whole or part of chromosomes resulting in karyotype abnormality or aneuploidy. This phenotype could result either due to defect in chromosomal segregation or DNA damage responses. Defective chromosomal segregation could arise due to abnormalities in spindle assembly checkpoints, anaphase promoting complex (APC/C) and other cell cycle proteins associated with chromosomal segregation[9]. Other mechanisms that contribute to karyotype abnormalities typical of CIN phenotype include dysfunction of the centrosome or telomere [10]. Amongst the DNA damage response proteins, tumour suppressor *p53* is the most frequently mutated genes in cancer and has been found to be inactivated in majority of CRC cases with CIN phenotypes [11]. Chromosomal aberrations cause increasing genomic instability, which results in accumulation of mutations in oncogenes and tumour suppressor genes. These mutations also known as driver mutations contribute to tumour progression by deregulating important growth signaling and apoptotic pathways.

One of the early events that marks the transition of preneoplastic lesion to proliferative early adenoma is deregulation of Wnt signaling pathway primarily through *APC*

(*Adenomatous Polyposis Coli*) mutation. *APC* is mutated in 60-80% sporadic adenomas and majority of the mutations are usually clustered in a specific region of the gene called ‘‘mutation cluster region’’[12]. Germline mutation of *APC* causes familial adenomatous polyposis (FAP). *APC* is a multi-functional protein that regulates cellular differentiation, migration, polarity, adhesion and apoptosis through Wingless/Wnt signaling pathway [13]. In the absence of Wnt glycoprotein signal, *APC* binds  $\beta$ -catenin into a ‘‘destruction complex’’ with axin and glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), directing it for phosphorylation and ubiquitination mediated proteasomal degradation. In an induced proliferating cell, binding of Wnt ligand to the membrane bound frizzled receptor, inactivates GSK-3 $\beta$ , thus stabilizing  $\beta$ -catenin in the cytoplasm.  $\beta$ -catenin binds to DNA binding proteins of T cell factor/Lymphocyte Enhancer family (TCF/LEF family) and facilitates transcription of several genes involved in cell proliferation, cell cycle regulators, membrane proteins, growth factors eg- *cMyc*, *cyclin D*, matrix metalloproteases (*MMPs*), *nuclear laminin* etc [14, 15]. Mutated *APC* disrupts  $\beta$ -catenin/ GSK-3 $\beta$  complex formation resulting in constitutive stabilization and activation of  $\beta$ -catenin that triggers transcription of several. Hypermethylation of the *APC* gene promoter has been observed in ~ 15-20 % of colorectal adenomas and carcinomas [16]. Deregulation of the Wnt signaling pathway is critical for early disruption of cellular homeostasis in the colonic crypt which is achieved either through genetic or epigenetic alteration of *APC* or through mutations in  *$\beta$ -catenin* and *GSK3  $\beta$*  [17].

Approximately 30-50% of colorectal tumours have Kirsten Rat Sarcoma Viral Oncogene Homolog or the *KRAS* mutation, which is often associated with resistance to anti-epidermal growth factor receptor (EGFR) therapy [18]. *KRAS* is a small molecular weight (~21 KDa) signaling protein that belongs to the G-protein superfamily. Growth factor binding to membrane bound growth receptors belonging to receptor tyrosine kinase (RTK) family such as EGFR activates guanine exchange factor (GEF) that catalyzes the conversion of inactive RAS-guanosine diphosphate (RAS-GDP) to active RAS-guanosine triphosphate (RAS-GTP). This triggers the activation of the downstream signaling pathways regulating cell growth, differentiation, apoptosis, cytoskeletal organization and vesicle trafficking [19]. Point mutation in codon 12 or codon 13 of exon 2, impairs the GTPase reaction that converts active GTP RAS to inactive GDP RAS, resulting in a

constitutively active RAS and amplification of its downstream signaling [20]. Of the several signaling pathways that RAS regulates, mitogen activated protein kinase (MAPK) and phosphoinositide -3 kinase/AKT (PI3K/AKT) pathways are the ones that are primarily responsible for cellular growth, survival, differentiation and apoptosis. Deregulation of MAPK and PI3K/AKT pathways due to mutations in growth hormone receptor, signaling molecules like RAS or BRAF is frequently observed in colorectal adenomas [21]. Mitogen activated protein kinases (MAPK) cascade is a key signaling pathway downstream of KRAS that regulates cell growth, proliferation, differentiation and apoptosis in a normal untransformed cell. This signaling cascade that belongs to the serine/threonine kinase superfamily, has three protein kinases that relay the signal downstream mainly through phosphorylation of their target proteins – MAPK kinase kinases (MAPKKK) that includes Raf protein kinases, MAPK kinase (MAPKK) also known as MEK  $\frac{1}{2}$  and lastly the MAPK. MAPK are the terminal effector serine threonine kinase that include the extracellular signal regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK) also known as stress activate protein kinases (SAPKs) and p38 kinases [22].

ERK1/2 pathway is generally activated by growth factors, whereas JNK or SAPK and p38 are activated in response to oxidative or nutrient stress, inflammation and DNA damage. MAPKKK or Raf is the first target in Raf/MEK/ERK pathway that is activated by GTP bound KRAS. Activation of Raf kinase initiates a phosphorylation cascade that results in downstream activation of MAPKK (MEK1/2) and MAPK (ERK1/2, JNK and/or p38). Activated ERK1/2 phosphorylates and activates a host of membrane bound, cytoplasmic and nuclear proteins that regulate cellular growth, proliferation and mitogenesis which makes this pathway an attractive target for chemotherapeutic drugs. In contrast to ERK, SAPK and p38 signaling pathways are less characterized in context of their role in chronic diseases including cancer. Majority of the p38 inhibitors are in phase I and phase II clinical trials for treatment of chronic inflammatory diseases. Now several *in vitro* studies are focusing on exploring the role of p38 and SAPK pathway in cancer development with an aim to target these pathways for restricting tumour growth.

Besides MAPK signaling, the other important growth signaling pathway that KRAS regulates, is the phosphatidylinositol- 3 kinase (PI3K)/AKT pathway. Genetic and epigenetic alterations leading to amplification of PI3K/AKT signaling is observed in



almost 10-12% of CRC cases and is often implicated in resistance to cancer therapies [23]. This pathway is activated in response to extracellular growth signals, cytokines, glucose and amino acids that lead to activation of PI3 kinase and its downstream effector AKT. Growth factor binding to receptor tyrosine kinases at the cell membrane (RTK) causes receptor dimerization that results in activation of PI3K. Activated PI3K phosphorylates Phosphatidylinositol 4,5- biphosphate (PIP<sub>2</sub>) to produce Phosphatidylinositol 3,4,5- triphosphate (PIP<sub>3</sub>) which is followed by recruitment of serine threonine kinases with PIP<sub>3</sub> binding domains, mainly phosphoinositide dependent kinase 1 (PDK1) and protein kinase B also known as AKT. Phosphorylation of AKT by PDK1 triggers a phosphorylation cascade that leads to activation of a plethora of cellular proteins involved in regulation of cell growth, survival, differentiation, proliferation and apoptosis. Some of the prominent genes activated by PI3K/AKT signaling include the mammalian target of rapamycin complex 1 (*mTORC1*), *BAD*, *caspase 9*, *FOXO*, *GSK3β*, *Mouse double minute 2 homolog (MDM2)* and *tuberous sclerosis 1 (TSC1)* [24]. Phosphatase and tensin homolog (PTEN) is a key molecule upstream of P3K/AKT which acts as a tumour suppressor by inhibiting cell growth and sensitizing cells to apoptosis. PTEN is a phosphatase that negatively regulates PI3K/AKT pathway by dephosphorylating PIP<sub>3</sub> thus blocking the activation of AKT and its downstream signaling [25]. Loss of PTEN activity due to genetic and/or epigenetic alterations underlines PI3K/AKT pathway dysregulation in several cancers including CRC [26, 27].

Another tumour suppressor gene *p53*, also known as the guardian of the genome, is a stress induced transcription factor that mediates cellular response to environmental stress, DNA damage, DNA replication or segregation and aberrant growth and proliferation signals. *p53* mutations are found in 30-50% of sporadic CRC cancers [11]. In humans, *p53* gene consisting of 11 exons and 10 introns is located on chromosome 17p. It encodes for a 393 amino acid long protein that has several functional domains including an N-terminal transactivation domain followed by a DNA binding domain, tetramerization domain and a C-terminal terminal regulatory domain [28]. In the absence of cellular stress, MDM2 (murine/human double minute 2), an E3 ubiquitin ligase regulates the ubiquitination and proteasomal degradation of *p53* thus keeping *p53* protein level low.

When the cell is under any internal or external stress, p53 is stabilized and its level is elevated dramatically due to self polyubiquitination and degradation of MDM2. Stabilized p53 regulates cellular response to stress through transcriptional activation of a host of genes involved in cell cycle arrest, DNA repair, apoptosis, autophagy and senescence. Some of the prominent p53 target genes are cell cycle regulators and mediators of intrinsic or extrinsic apoptotic pathways such as cyclin dependent kinase (CDK) inhibitor p21WAF1/CIP, pro- apoptotic Bcl-2 (B-cell lymphoma-2) family of proteins including BAX, Noxa and PUMA, death receptors such as Fas and DR-5 (Death receptor 5), DNA damage response proteins such as GADD45 (Growth arrest and DNA damage 45) [29]. In CRC, the majority of the mutations in p53 are located in exon 5-8 that includes the DNA binding domain that results in disruption of DNA binding and transactivation of the target gene.

The canonical transforming growth factor- $\beta$  (TGF- $\beta$ ) signaling pathway mediates growth inhibitory response in intestinal cells. This response is mediated by TGF- $\beta$  receptor, which upon binding with TGF- $\beta$  ligand, phosphorylates and activates SMAD proteins (SMAD 2 and SMAD 3). Receptor activated SMADs interact with the common mediator SMAD 4 which translocates into the nucleus and regulates the transcription of target genes. Deregulating of the TGF- $\beta$  signaling pathway due to somatic mutations in TGFBR1, TGFBR2 and SMADs has been observed in 10-15% of sporadic CRC cases [30].

### **Microsatellite Instability Pathway (MSI)**

The MSI phenotype also known as the mutator phenotype develops in CRC as a consequence of defective DNA mismatch repair (MMR) enzyme system. MSI phenotype is seen in 15-25% of CRC cases, majority of which are sporadic, acquired due to promoter hypermethylation of DNA mismatch repair enzymes and approximately 3% of the cases are due to germline mutations in MMR enzymes (Lynch syndrome) [31]. Colorectal tumours with MSI phenotype have unique clinicopathological features such as poor differentiation, mucinous or signet ring appearance, proximal location in the colon and are generally associated with a better prognosis compared to CIN phenotype [32]. Unlike CIN colorectal tumours, which are frequently aneuploid, MSI associated tumours rarely show karyotype abnormality. Genetic instability in MSI arises due to genetic and epigenetic

alterations in the simple sequence repeat (SSR) regions or microsatellite regions of the genome. Microsatellites or short tandem repeats (STRs) also known as simple sequence repeats of di, tri, tetra nucleotides are distributed throughout the genome. The bulk of microsatellite sequences are embedded in the non-coding sequences of the genome such as the intergenic regions or introns. However, it is the small proportion of microsatellites that are associated with coding DNA sequences, which due to susceptibility to DNA polymerase slippage can accumulate several mutations. These mutations can be a single base-base mismatch causing point mutation or insertion deletion loop causing frameshift mutation resulting in a truncated protein. The DNA mismatch repair enzyme system was first identified in bacteria and later found to have homologues in yeast and other eukaryotes including mammals. The human MMR enzyme consists of the mammalian *MutS* homologues (*MSH*) and the *MutL* homologues (*MLH*) such as *MLH1*, *MSH2*, *MSH3*, *MSH6*, *PMS1* and 2 [33]. Inactivation of these repair enzymes due to promoter hypermethylation leads to accumulation of mutations in genes, many of which are associated with tumourigenesis such as *B-Raf*, *Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA)*, *TGFβRII*, *Bax*, *RAD50*, *IGFIIR* and *PTEN* [33, 34].

### **CpG Island methylator phenotype (CIMP) pathway**

The CIMP phenotype is found in 30-40% CRC cases and is highly associated with the pathogenesis of sporadic MSI colonic tumours. DNA methylation is an important epigenetic regulatory mechanism in which DNA methyl transferases (DNMTs) transfer methyl groups to the cytosine base of CG dinucleotides also known as CpG (cytosine preceding guanine) islands. Approximately 70-80% of the CpG islands in the genome that lie in the non-promoter regions are methylated whereas the remaining CpG sequences lying closer to the promoter region remain unmethylated [35]. During cancer development, a genome wide hypomethylation and promoter hypermethylation of tumour suppressor, cell cycle inhibitors and DNA repair enzymes is frequently observed. Within CIMP phenotype, three sub groups have now been identified- CIMP1, CIMP2 and CIMP negative. CIMP1 colorectal tumours are highly associated with MSI phenotype and *BRAF* mutation whereas CIMP2 have a high incidence of *KRAS* mutations. CIMP negative subgroups showed a low

frequency of KRAS or BRAF mutations but were mostly *p53* negative. CIMP classification has kept evolving with time however they have certain unique clinicopathological features such high rate of KRAS or BRAF mutations, wild type *p53* and high incidence in proximal colon [36].

### **1.3 Biological heterogeneity within colorectal cancer**

CRC exhibits differences in incidence, pathogenesis, tumour histopathology and drug response outcome depending on the anatomical location of the tumours. In 1990, Bufill had proposed that the tumours appearing in the proximal region of the colon (right sided) are biologically distinct from those appearing in the distal region of the colon (left side) [37]. To begin with, they appeared histologically and morphologically different in appearance, the proximal tumours being mucinous and poorly differentiated in comparison to distal tumour. It was also observed that the proximal tumours had predominantly MSI phenotype whereas the distal tumours were mostly CIN phenotype [38]. Proximal tumours seem to have poorer prognosis and survival rates compared to distal tumours. These findings gave prominence to the idea that colorectal cancer is not a single entity and that colorectal tumours show intra-tumoural biological heterogeneity with distinct site specific pathogenesis. Anatomically, proximal or the right sided colon has an embryological origin in the midgut whereas distal colon develops from the hindgut region. The proximal colon has a richer blood supply from superior mesenteric artery whereas distal colon is connected to the inferior mesenteric artery. Proximal and distal colon are also exposed to different microenvironments including gut flora, nutrients, toxins and enzymes. Gene expression studies have revealed that proximal colon shows overexpression of several genes associated with inflammation and drug metabolism whereas distal colon has an overall high transcriptional activity of genes related to cell cycle and DNA metabolism [39]. Tumours appearing in the proximal region are generally slow growing, poorly differentiated and mucinous. Most of the proximal tumours exhibit an MSI or CIMP phenotype with high frequency of KRAS or BRAF mutation. The distal tumours are fast growing, well differentiated and in majority of the cases they show CIN phenotype. They also show karyotype abnormalities (aneuploidy) due to structural chromosomal aberrations such as deletion and amplification of large regions of the chromosome. The distal tumours also

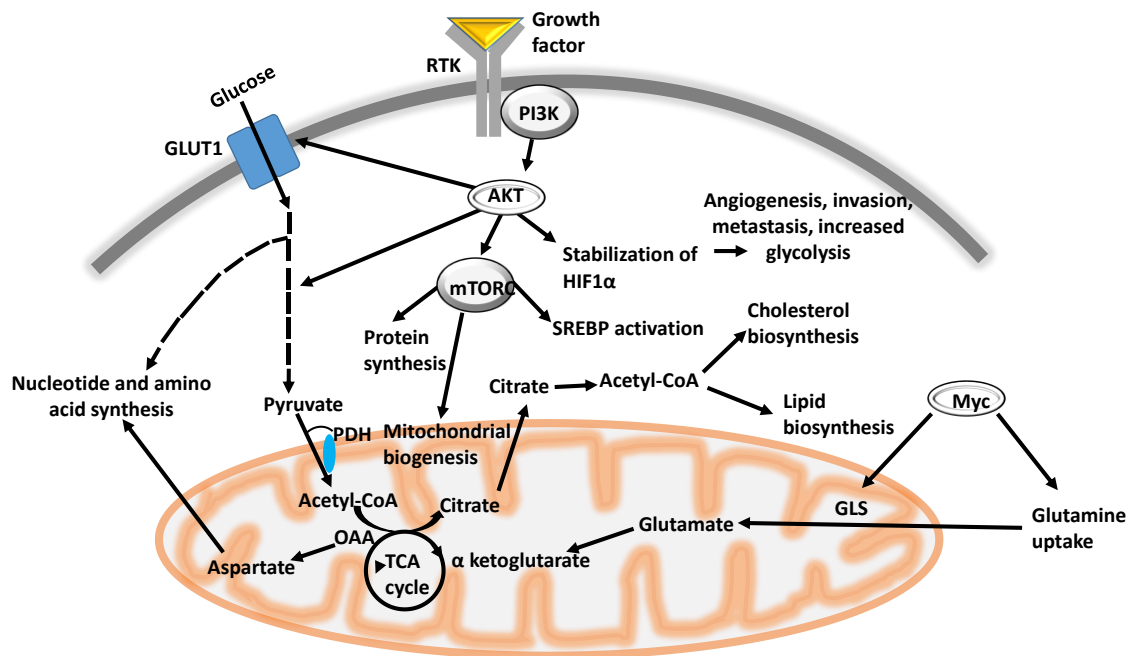
exhibit mutations in several tumour suppressors and oncogenes that contribute to genomic instability through deregulation of growth signaling and apoptotic pathways.

#### **1.4 Deregulation of metabolic pathways in colorectal cancer**

The first major breakthrough in understanding reprogramming of cancer metabolism came in 1920s, from the lab of Otto Warburg in Germany. He observed that tumour cells show a dramatic increase in the rate of aerobic glycolysis to “ferment” glucose to lactic acid [40]. This phenomenon known as the “Warburg effect” is one of the hallmarks of cancer. Earlier it was speculated that this abnormal behavior of cancer cells in which glucose intake is upregulated by almost 10 fold to produce far less adenosine triphosphate (ATP) molecules and lactic acid as a waste byproduct, was due to mitochondrial dysfunctioning. However, this idea faced criticism due to observations of normal mitochondrial functioning in cancer cells. A normal proliferating cell also shows an increase in aerobic glycolysis like a proliferating cancer cell, the difference being the regulation of the process, which is lost in case of cancer. Non-dividing, differentiated cell, under limited nutrient supply conditions, prefers the complete oxidation of glucose to maximize the production of ATP (36 molecules). A proliferating cell, on the hand, has altered preferences because it needs both energy as well as molecules for anabolism, which includes biosynthesis of lipids, proteins and nucleic acids. This is the reason it switches to aerobic glycolysis where pyruvate produced at the end of glycolysis instead of entering the TCA cycle and oxidative phosphorylation in the mitochondria, is converted to lactic acid in the cytosol. This is an “energy saving” mechanism for proliferating cells by which they compromise on energy and metabolize glucose aerobically through glycolysis to produce various intermediates required for biomass production. The entire process is regulated at various steps beginning at the level of upstream growth receptor signaling. However, in a proliferating cancer cell, genetic and/or epigenetic alterations in oncogenic growth signaling pathways and tumour suppressor genes allow the cancer cells to switch to aerobic glycolysis without any nutrient stimulus or growth signal induction. Glucose and glutamine are the main sources of carbon and nitrogen for cancer cells [41]. Glioblastoma cells, for instance, use 90% of glucose and 60% of glutamine as a source of carbon for acetyl CoA synthesis which is used for biosynthesis of fatty acids and lipids as well as

source of nitrogen for synthesis of non-essential amino acids for protein synthesis [42]. Overexpression of glucose transporter (GLUT) and several glycolytic enzymes such as hexokinase, pyruvate kinase, lactate dehydrogenase allows cancer cells to acquire this altered phenotype. Adaptation of cancer cells to reprogrammed cellular metabolism, requires assistance from the cellular growth machinery. PI3K/AKT signaling pathway which is often mutated, plays a major role in upregulating aerobic glycolysis through expression of glucose transporters (GLUT), hexokinase and phosphofructokinase [43]. PI3K/AKT pathway also directs glucose carbon flux towards biosynthetic pathways for lipid, cholesterol and isoprenoid synthesis through activation of SREBP pathway [44]. These pathways require acetyl-CoA, which is produced in the mitochondria from glucose, derived pyruvate. Since Acetyl-CoA cannot be transported to cytosol directly, it is first converted into citrate by another mitochondrial enzyme citrate synthase. Once citrate is in the cytoplasm, it is converted to Acetyl-CoA by ATP-citrate lyase (ACL). By phosphorylating and activating ACL, AKT ensures that mitochondrial citrate is diverted towards Acetyl-CoA production for feeding lipogenesis and sterol biosynthetic pathways. AKT also induces hypoxia inducing growth factor (HIF1 $\alpha$ ) that activates aerobic glycolysis in oxygen independent manner by upregulating the expression of glycolytic enzymes and glucose transporters. HIF1 $\alpha$  is the O<sub>2</sub> dependent subunit of the heterodimeric HIF1 protein and is frequently overexpressed in cancer. Overexpression of HIF1 and HIF2 is associated with poor prognosis in several cancers but the data is inconclusive for CRC due to small sample size in most of the studies [45, 46]. HIF1 $\alpha$  is subjected to O<sub>2</sub> dependent hydroxylation by von Hippel-Lindau tumour suppressor protein (VHL), which recruits an E3 ubiquitin ligase protein to the complex, targeting it for polyubiquitination and proteasomal degradation. Under hypoxia conditions, frequently observed in a tumour microenvironment, hydroxylation of HIF1 $\alpha$  by VHL is inhibited, resulting in its stabilization and dimerization with HIF-1 $\beta$ . This HIF1 complex then binds to the promoter of target genes and recruits coactivators to drive transcription of genes involved in cancer progression. Some of the key target genes activated by stabilization of HIF1 $\alpha$  that promote cancer progression are – angiopoietin 2, angiopoietin like 4, breast cancer resistant protein, endothelin1, fibronectin1, glucose transporter1, glucose phosphate isomerase, hexokinase1 and 2, lactate dehydrogenase (LDH) A, insulin like growth factor-2 (IGF-2), matrix

metalloproteinase (MMP) 2 and 14, vascular endothelial growth factor (VEGF), urokinase plasminogen activator receptor [47]. Downstream of the PI3K/AKT is the mammalian target of rapamycin (mTOR) signaling pathway, which enhances protein synthesis and also induced mitochondrial biogenesis [48]. mTOR driven activation of SREBP pathway for enhanced lipid and cholesterol synthesis is also part of metabolic reprogramming in cancer cells [49, 50]. Oncogenes such as *Myc* and *KRAS*, also assist in upregulating aerobic glycolysis in cancer cells. *Myc* transcriptionally activates splice variant of pyruvate kinase (*PKM2*) that directs the cell to upregulate aerobic glycolysis. *Myc* has also been shown to promote mitochondrial consumption of glutamine by upregulating the expression of *glutaminase (GLS)* [51]. Cancer cells with *cMyc* mutation are addicted to glutamine, which gives an opportunity to utilize *GLS* as therapeutic target. *KRAS* oncogene that is frequently mutated in colorectal cancers are also known to upregulate *GLUT1* expression thereby increasing glucose uptake in cancer cells. *In vitro* studies have shown that glucose deprivation in colorectal cancer cells with wild type *KRAS*, resulted in survival of few cells all of which had acquired *KRAS* mutation with concomitant upregulation of *GLUT1* [52]. Genetic alterations in metabolic enzymes like *PKM2*, isocitrate dehydrogenase (*IDH*), succinate-dehydrogenase assist in metabolic reprogramming of cancer cell by favouring aerobic glycolysis [49, 53]. Apart from heightened glucose uptake in cancer cells, glutamine metabolism is also key to cancer cell growth and proliferation. Glutamine metabolism is essential for tumour cells as a source of carbon, nitrogen, oxaloacetic acid (*OAA*) and *NADPH* for biomolecule synthesis [41, 54]. Additionally, glutamine is required for replenishing the TCA cycle intermediates to sustain the production of biomolecules in cancer cells, a process called anaplerosis [55, 56].



**Figure 1.2 Metabolic reprogramming in cancer cells.** A well-coordinated reprogramming of oncogenic signaling and metabolic pathways resulting in accelerated growth of cancer cells is considered to be one of the hallmarks of cancer. Increased glucose uptake and glycolysis (Warburg effect) is supported by hyperactive PI3K/AKT signaling through increased GLUT1 expression and activity. AKT also phosphorylates and activates ATP-citrate lyase (ACLY) that converts mitochondrial citrate to Acetyl-CoA required for lipid and cholesterol synthesis. AKT is also responsible for stabilization of HIF1 $\alpha$  subunit, which results in activation of genes required for glycolysis, angiogenesis, invasion and metastasis. mTORC1, located downstream of AKT is responsible for enhanced protein synthesis and mitochondrial biogenesis. It also mediates PI3K/AKT dependent upregulation of SREBPs for increased lipid and cholesterol synthesis. Figure adapted from Vito Iacobazzi\* and Vittoria Infantino, 2014. *Biol.Chem* [57].



## **1.5 Deregulation of lipid homeostasis in cancer**

Aberrant upregulation of lipid and cholesterol biosynthesis is frequently associated with several cancers.. In a normal cell, intracellular cholesterol homeostasis is maintained by a tight regulation of cholesterol biosynthesis, uptake and metabolism at both transcriptional and post-translational level. Cholesterol is synthesized in the cell from the precursor Acetyl-CoA through an enzymatic pathway called the mevalonate pathway [58]. The mevalonate pathway plays a crucial role in the normal growth and development of the cell by supplying cholesterol for membrane biogenesis, bile acid and steroid hormones synthesis. Cholesteryl esters derived from the mevalonate pathway are also utilized for the assembly of cholesterol and sphingomyelin rich microdomains in the plasma membrane known as lipid rafts that function as docking sites for several receptors required for growth and extrinsic apoptotic pathway signaling [59]. The non-sterol intermediates of mevalonate pathway such the isoprenoids are required for the post-translational modification of signaling proteins such as RAS, RHO, RAC and RAB. Studies in prostate cancer, hepatocellular carcinoma and acute myeloid leukemia have shown that an upregulation of cholesterol biosynthesis is associated with an aggressive tumour phenotype [60, 61]. Pharmacological inhibitors of mevalonate pathway enzymes such as statins have been shown to reduce cancer cell proliferation and viability in pre-clinical studies [62, 63].

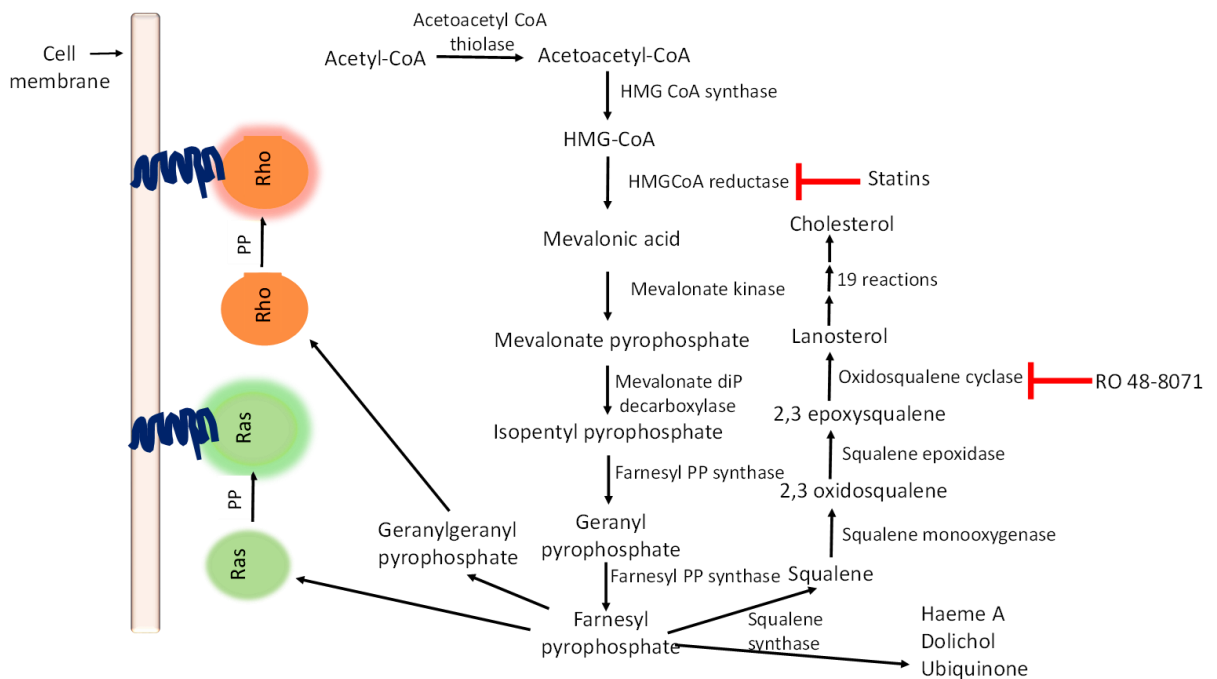
The other mechanism through which cells are able to derive cholesterol is the low-density lipoprotein receptor (LDLR) mediated endocytosis pathway. Both LDLR and 3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGCR), the rate-limiting enzyme of cholesterol biosynthesis pathway are under transcriptional regulation of sterol regulatory element binding protein (SREBP) family of transcription factors. Elevated accumulation of nuclear SREBP (mature form) resulting in upregulation of lipogenesis and cholesterol biosynthesis has been observed in prostate cancer with hyperactive PI3K/AKT/mTORC1 signaling [64]. Loss of sterol mediated feedback regulation of HMGCR and LDLR has been reported in several cancers including prostate cancer, colorectal cancer, breast cancer, liver cancer, lung cancer and leukemia [65-69]. Previous studies have shown that upregulated LDL-cholesterol uptake is correlated to increase in cyclooxygenase (COX-2) activity and prostaglandin (PGE-2) levels [70]. Elevated influx of essential fatty acids

through LDLR increases synthesis of prostaglandins (PGE<sub>2</sub>) that play an important role in cancer cell growth, proliferation and metastasis. The pro-inflammatory cytokines and chemokines such as tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) and C-reactive proteins secreted by the tumour cells and their microenvironment have been implicated in disruption of sterol mediated regulation of HMGCR and LDLR gene expression [71, 72]. Downregulation of PCSK9 and concomitant increase in LDLR protein level has been observed in several cancers including but not limited to hepatocellular carcinoma and melanoma [73, 74]. In a recent study in breast cancer cells, it was demonstrated that the mutant oncogenic form of *p53* can disrupt mammary acinar morphology in 3-dimensional culture conditions by upregulating the mevalonate pathway through SREBP activation [75].

## **1.6 Cholesterol biosynthesis pathway**

Cholesterol is required by the cell for membrane biogenesis, synthesis of steroid hormones, bile acids and for mediating intracellular signaling through lipid raft. Cholesterol is derived by the cell either through endogenous synthesis of cholesterol through the mevalonate pathway or import of serum cholesterol through membrane bound low density lipoprotein receptor (LDLR). Cholesterol is synthesized from Acetyl-CoA in three stages. The first stage is conversion of Acetyl-CoA into activated isoprene unit called isopentyl pyrophosphate (IPP) through a series of enzymatically catalyzed reaction [76]. The second step is condensation of six isoprene units to form squalene and the third stage is cyclisation of squalene to form cholesterol. Cholesterol biosynthesis starts with condensation of Acetyl-CoA and Acetoacetyl-CoA to form 3-Hydroxy-3-methylglutaryl coenzyme A (HMGCA). The first committed step in cholesterol biosynthesis pathway is the conversion HMGCA to mevalonate by 3-Hydroxy-3-methylglutaryl coenzyme-A reductase (HMGCR) enzyme. HMGCR, an approximately 98 KiloDalton (KDa), endoplasmic reticulum (ER) and peroxisomal glycoprotein, is the rate limiting enzyme of this pathway. It has a sterol sensing NH<sub>2</sub>-terminal transmembrane domain spanning the ER membrane and COOH-terminal catalytic domain facing the cytosol. HMGCR has been under intense scrutiny for therapeutic targeting of cholesterol biosynthesis to treat

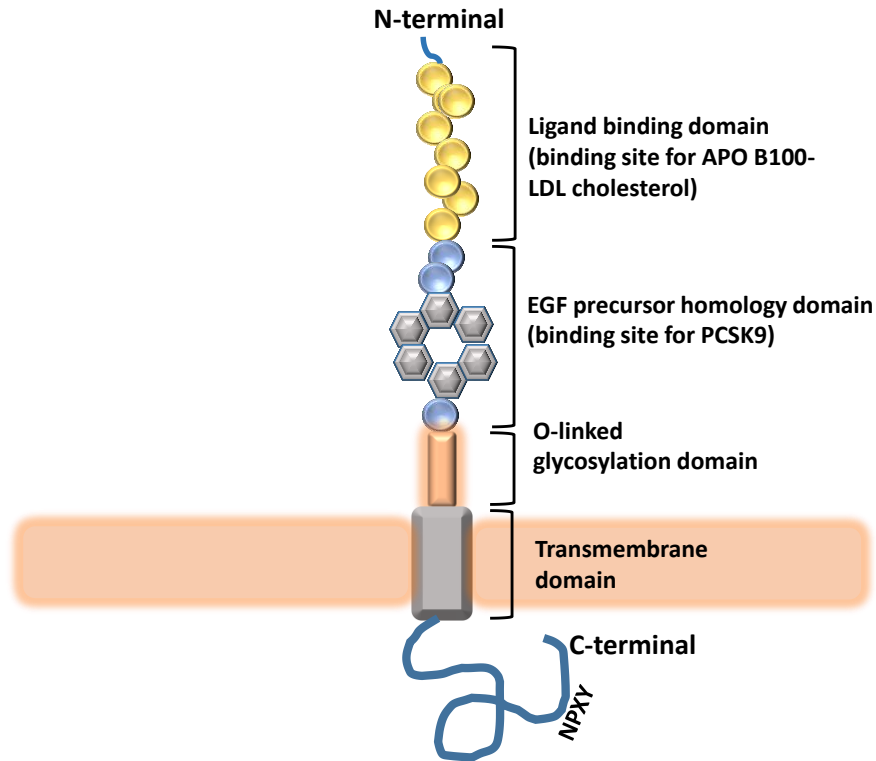
hypercholesterolemia. Statins are a class of pharmacological inhibitors of HMGCR that bind to the active site of HMGCR and inhibit its enzymatic activity by reversible competitive inhibition. Mevalonate is converted to isopentyl pyrophosphate, which is the structural unit of isoprenoids like farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). FPP and GGPP are required for membrane anchorage and activation of signaling molecules like RAS, RHO, RAC etc. In the next set of reactions, an ER enzyme called squalene synthase catalyzes synthesis of a 30 carbon isoprenoid called squalene from FPP. In the final stages of cholesterol biosynthesis, squalene is first cyclized to form squalene epoxide, which forms the nucleus of the tetracyclic steroid structure of cholesterol. Squalene epoxide is then converted into lanosterol by oxidosqualene cyclase (OSC). Inhibitors of OSC such as RO 48-8071 are being studied in pre-clinical models as an alternative to statins for therapeutic targeting of cholesterol biosynthesis. Lanosterol is converted into cholesterol through a series of enzymatic reactions that include several oxidation, reductions and demethylation reactions. Some of the prominent enzymes functional in the final stages of cholesterol biosynthesis are 7-dehydrocholesterol reductase, which catalyzes the synthesis of the immediate precursor of cholesterol called 7-dehydrocholesterol. Newly synthesized cholesterol can be acylated by Acyl-CoA Cholesterol-acyl transferase (ACAT) to form cholesteryl esters or oxidized to form oxysterols such as 25-dehydrocholesterol or used for biosynthesis of bile acids and steroid hormones. While cholesteryl esters are stored in lipid droplets as cellular store for cholesterol, oxysterol can translocate freely in the aqueous cytosolic environment thus functioning as potent signaling molecules.



**Figure 1.3 Mevalonate pathway of de novo cholesterol biosynthesis.** Intracellular cholesterol is synthesized by a series of enzymatic reactions in a pathway called the mevalonate pathway. This pathway is critical for cellular functioning since it provides the cell with essential biomolecules that include sterols and non-sterol products. Cholesterol, the end product of this pathway is required for cell membrane biogenesis, steroid and bile acid synthesis as well as assembly of lipid rafts. Non-sterol bioactive intermediates such isoprenoids are required for membrane anchorage and activation of signaling proteins like RAS and RHO. Other important non-sterol intermediates of the pathway include haeme A, dolichol and ubiquinone, which are required for posttranslational modification of several cellular proteins. Portions of this figure have been adapted from Valerie Leduc et al., 2010. *Trends in Molecular Medicine* [77].

## 1.7 Low Density Lipoprotein Receptor pathway

Investigation into the pathogenesis of the genetic disorder Familial Hypercholesterolemia (FH) by Goldstein and Brown in 1980s led to the discovery of low-density lipoprotein receptor (LDLR). FH is an autosomal dominant disease that is characterized by abnormally high levels of plasma cholesterol which was later found to be a result of homozygous mutation in LDLR [78]. LDLR receptor is approximately 840 amino acids long 160 KDa transmembrane glycoprotein that is primarily involved in clearance of plasma LDL cholesterol. It has five functional domains: Extracellular ligand binding domain that binds to apolipoprotein B 100 (apoB100) or apolipoprotein E (apoE) of very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL) and low density lipoprotein (LDL) cholesterol complex followed by epidermal growth factor (EGF) like domain, O-linked glycosylation domain, transmembrane domain and cytosolic domain [79]. LDLR can bind to all classes of lipoproteins with varying affinity, apoB100 (LDL) and apoE (VLDL) being the ones that have maximum affinity for LDLR. The dietary cholesterol absorbed in the gut is packaged along with triglycerides into chylomicron particles that are released in the blood stream. During their transport, triglycerides are hydrolyzed by lipases present on the surface of endothelial cells and are replaced by apolipoproteins such as apoE to form chylomicron remnants, which are taken up by hepatocytes. The other triglyceride rich lipoprotein is the very low-density lipoprotein or VLDL, which is synthesized in the liver and secreted in the blood stream to deliver cholesterol to extrahepatic tissues. Triglycerides in VLDL are hydrolyzed by endothelial cell lipases to form LDL, which are bound to apoE. LDL cholesterol binds to LDLR on the cell surface of hepatic or extra-hepatic tissues and is internalized by receptor-mediated endocytosis. In the low pH environment of the lysosome, the receptor unloads the protein-cholesteryl ester complex. Cholesteryl ester is hydrolyzed by lysosomal acid lipase to free cholesterol from protein complex. Free cholesterol is used for steroid and bile acid synthesis, incorporated in membranes or re-esterified by Acyl CoA Cholesterolacyl Transferase (ACAT) to form cholesteryl ester, which is stored in lipid droplets. Excess cholesterol is eliminated from the body by liver through bile acid secretion.



**Figure 1.4 Structure of low-density lipoprotein receptor.** Low-density lipoprotein receptor or LDLR is an 840 amino acids long membrane bound glycoprotein receptor which is primarily involved in uptake of blood cholesterol by receptor mediated endocytosis. Structurally, LDLR has 5 functional domains each having a distinct function. At the N-terminal lies the LDLR repeat domain consisting of seven homologous repeats which binds to apoAPOB100 and apoAPOE lipoproteins of LDL and VLDL cholesterol respectively. This is followed by the EGF repeat domain, which is also binding site for PCSK9 serine protease, which degrades LDLR. O-linked glycosylation domain is ~58 amino acids long and acts as a connecting link between the EGF repeat domain and transmembrane domain. The transmembrane domain is a 25 amino acids long membrane spanning domain rich in hydrophobic amino acids, which anchors LDLR to the plasma membrane. The cytosolic domain at the C-terminal contains a unique NPXY amino acid sequence, which interacts with ARH1 adapter protein on the inner side of the plasma membrane. This interaction is critical for sorting of the receptor in clathrin-coated pits and also for internalizing LDL cholesterol through receptor-mediated endocytosis. This figure has been adapted from Wasan K.M, 2008. *Nature Reviews Drug Discovery* [80].

## 1.8 Cholesterol efflux pathway

Over loading of cholesterol in the non-hepatic cells that cannot be metabolized into free cholesterol, for instance macrophage foam cells in the arterial wall, can increase the risk of cytotoxicity and cardiovascular diseases. Therefore it is imperative for the cells to have a system to efflux free cholesterol for transport to the liver where it can be metabolized. Most of this cholesterol efflux takes place through the ATP binding cassette receptors called the ABCA1 and ABCG2 through a process called ‘reverse cholesterol transport’. These receptors are transcribed by the liver x receptor (LXR) family of transcription factors [81]. Peripheral tissues release excess cholesterol into lipid poor apoA1 containing nascent high density lipoprotein (HDL) particles through ABCA1/ABCG1 and scavenger receptor SR-B1. This cholesterol rich HDL particle delivers cholesterol to the liver either directly through SR-B1 or indirectly through cholesteryl ester transfer protein (CETP) which converts cholesterol rich HDL into triglyceride rich LDL and VLDL. LDL particles interact with LDLR and enter the hepatocytes where they are metabolized and secreted as bile acids. This process called reverse cholesterol transport is critical in eliminating excess cholesterol from the body, especially from macrophage foam cells and atherosclerotic plaques thus reducing the risk of cardiovascular diseases.

## 1.9 Transcriptional regulation of cholesterol homeostasis

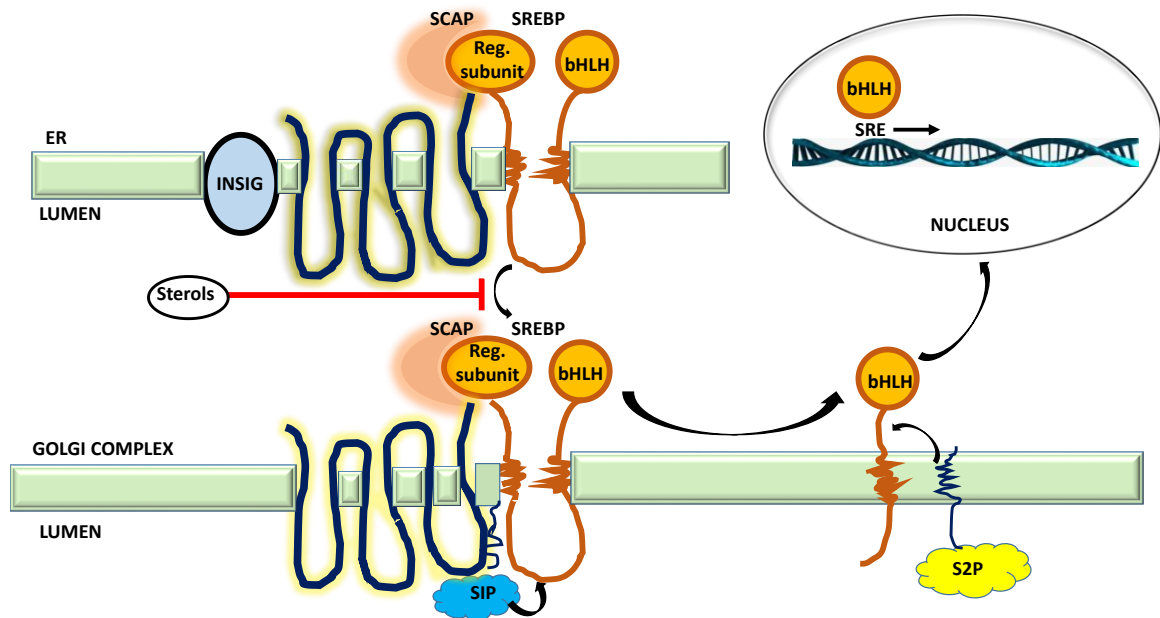
Cholesterol biosynthesis and uptake is under transcriptional regulation of a subclass of the basic helix loop helix leucine zipper (bHLH-LZ) family of transcription factors known as the sterol regulatory element binding proteins (SREBPs). The basic structure of SREBPs consists of an N-terminal DNA transactivation domain (~ 480 amino acids), a hydrophobic transmembrane domain (~ 80 amino acids) and a 590 amino acids C-terminal regulatory domain [82]. There are three isoforms of SREBPs- SREBP1a, SREBP1b and SREBP2. While *SREBP1a* and *SREBP1c* are transcribed from different promoters on the same gene, *SREBP2* is encoded by a separate gene on a different chromosome. SREBP1a and SREBP2 are responsible for transcription of genes involved in cholesterol biosynthesis and uptake whereas SREBP1c is associated with transcription of lipogenesis genes. Unlike

other bHLH-LZ transcription, which recognize E-box sequence in the target promoters, SREBPs recognize a unique 10 base pair long sequence called the sterol regulatory element (SRE) in the enhancer region of target promoter. In the case of the LDLR gene, SREBP2 binds to 5'-ATCACCCAC-3' called SRE1 [83]. The full length precursor form of the SREBP is anchored to the endoplasmic reticulum (ER) membrane in a complex with the SREBP cleavage activating protein (SCAP) and the Insulin induced gene (INSIG) protein. Excess of cholesterol or oxysterol derivatives of cholesterol such as 25-hydroxycholesterol in the ER membrane binds to sterol sensing domain of INSIG which blocks the loading of SREBP-SCAP complex to ER transport vesicles. When sterol is depleted in the ER membrane, INSIG undergoes a conformational change and releases the SCAP-SREBP complex which gets loaded in the coatamer II protein (COPII) vesicles and is carried to the Golgi complex. In the Golgi complex two proteases, site proteases I and II, cleave the transactivation N-terminal domain of the SREBPs. This cleaved, mature form of SREBP which is approximately 68 KDa, then translocates to the nucleus where it binds to the SRE in the enhancer region of genes like *HMGCR*, *LDLR*, *PCSK9* and *INSIG* and upregulates their transcription. *HMGCR* shows basal level of transcription in the presence of sterols, to meet the demand for non-sterol products of the mevalonate pathway, especially the isoprenoids which are critical for functioning of signaling proteins like RAS and Rho.

The other class of transcription factors that regulate cholesterol homeostasis in the cells are the liver X receptors or LXRs. They regulate the transcription of a host of cholesterol pathway related genes such as ATP binding cassette subfamily A member 1 (ABCA1) cholesterol efflux transporter, inducible degrader of LDLR (IDOL) and apoE [81]. The transcriptional activity of LXRs require binding of physiological ligands like oxysterols to its sterol sensing domain which triggers their hetero-dimerization with retinoid X receptor (RXR). LXR-RXR heterodimer binds to the direct repeat 4 (DR4) sterol regulatory element in the enhancer region of its target genes.

Alternate splicing of genes like *HMGCR*, *LDLR*, *HMGCS*, *MVK*, *PCSK9*, *apoE* is another mechanism of transcriptional regulation of cholesterol pathway related genes [84]. An *HMGCR* splice variant with an exon 13 deletion is associated with reduced statin response and enzyme activity [85]. *LDLR* splice variants with deleted exon 4 and 12 are associated with reduced expression and uptake of LDL cholesterol [86].

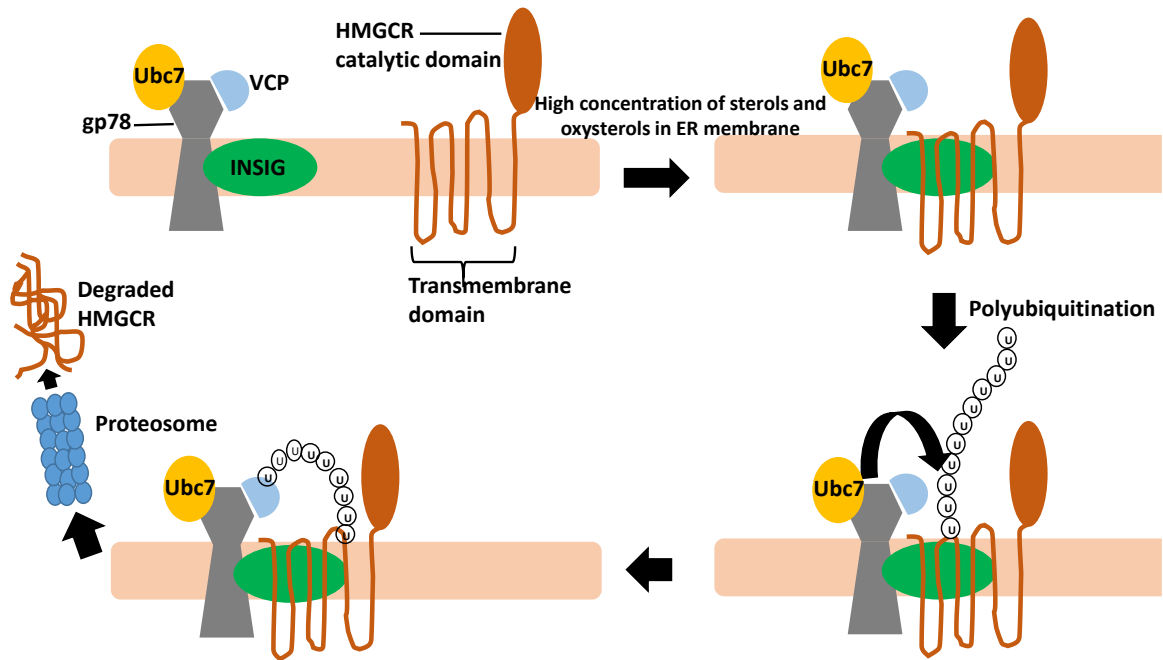




**Figure 1.5 Proteolytic cascade of SREBP activation.** Sterol regulatory binding element proteins (SREBP) are ER membrane bound leucine zipper family of transcription factors that regulate lipid and cholesterol homeostasis. In their inactive state, SREBPs are bound to ER membrane along with SREBP cleavage activating protein (SCAP) and Insulin induced gene (INSIG) protein. When sterol levels are low in ER membrane, SREBP translocates to the Golgi complex where it is cleaved by site proteases 1 and 2, resulting in release of the mature, transcriptionally active form. This mature form of the transcription factor translocates to the nucleus and binds to the sterol regulatory element (SRE) in the promoter region of lipid and cholesterol pathway genes to drive their transcription. This figure has been adapted from Russel A DeBose-Boyd, 2008. *Cell Research* [87].

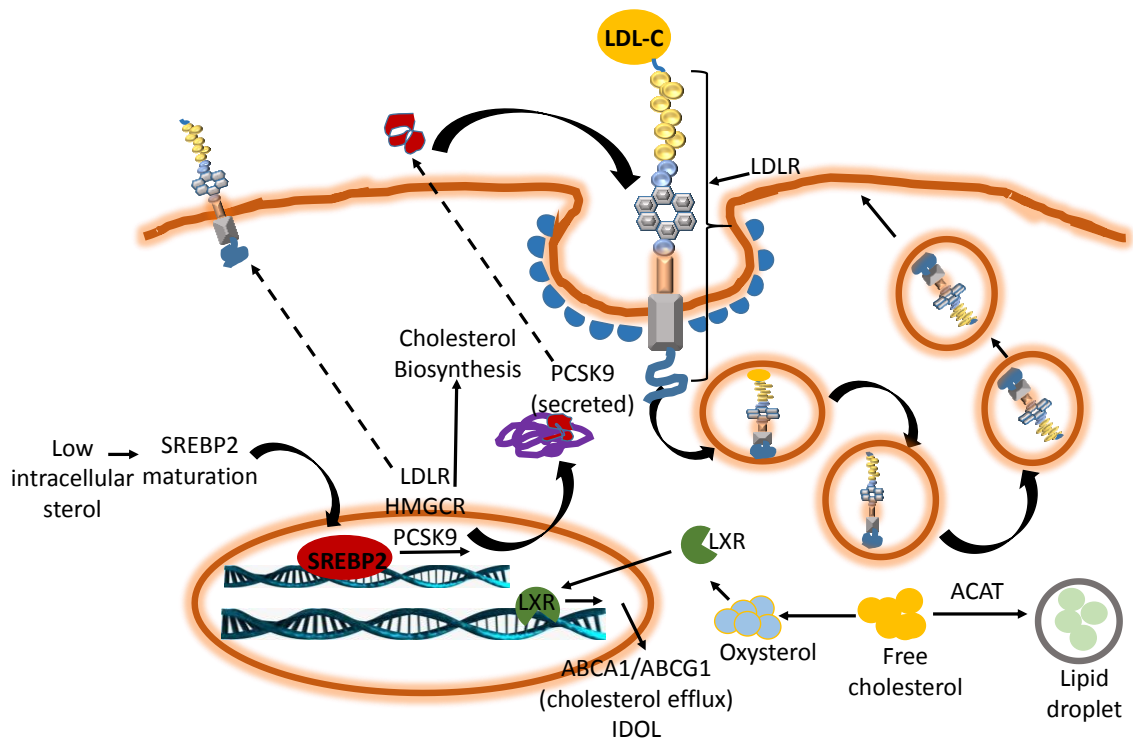
### **1.10 Post translational regulation of HMGCR and LDLR**

Accumulation of sterols in the ER membrane, triggers degradation of the HMGCR enzyme by ubiquitination and proteasomal degradation. This entire process is a complex mechanism that involves interaction of HMGCR transmembrane domain embedded in the ER membrane with sterol derivatives like oxysterols, isoprenoids and INSIG [88]. When sterol levels are high, INSIG binds to the ER membrane bound HMGCR and recruits gp78 E3 ubiquitin ligase and E2 Ubc7 ligase. This results in a conformational change in the cytoplasmic domain of HMGCR which gets exposed to gp78 and Ubc7 mediated polyubiquitination. An ER associated ATPase called valosin containing protein (VCP) regulates the extraction of polyubiquitinated HMGCR from ER and its subsequent degradation in the proteasome.



**Figure 1.6 Endoplasmic reticulum associated degradation of HMGCR.** Saturation of ER membrane with 25-hydroxycholesterol, lanosterol etc. results in a conformational change in insulin induced gene1 (INSIG) ER resident protein. INSIG binds to HMGCR transmembrane domain along with gp78 that is ER membrane bound E3 ubiquitin ligase. HMGCR-INSIG-gp78 protein scaffold at the ER membrane is bound by E2 Ubc7 and VCP ATPase which is required for extraction of ubiquitinated HMGCR from ER membrane. HMGCR is poly-ubiquitinated by combined action of gp76 and Ubc7, followed by extraction from ER membrane by VCP and proteasomal degradation. Portions of this figure have been adapted from Russel A DeBose-Boyd, 2008. *Cell Research* [87].

The LDLR protein turnover is regulated by Proprotein convertase subtilisin like Kexin type 9 (PCSK9) serine protease and an E3 ubiquitin ligase called the Inducible degrader of LDLR (IDOL). PCSK9 is primarily expressed in the liver in an inactive precursor form that contains an N terminal signal peptide, followed by a prodomain, subtilisin like catalytic domain and the C terminal domain [89]. Autocatalysis of the inactive precursor, releases the prodomain from the catalytic domain however it remains bound to mask the catalytic domain and direct it for extra cellular secretion. Once secreted out of the cell, the exposed catalytic domain binds to the extra cellular EGF like repeat domain of LDLR in a calcium dependent manner, resulting in its uptake and lysosomal degradation. IDOL or Induced degradation of LDLR is an E3 ubiquitin ligase transcribed by the liver X promoter (LXR) which targets LDLR for ubiquitination and proteasomal degradation [90]. Along with statins, PCSK9 inhibitors are being clinically tested as therapeutic targets for cardiovascular disorders [91].



**Figure 1.7 Regulation of the LDLR pathway.** Cellular cholesterol is imported into the cell through membrane bound receptors called the low-density lipoprotein receptor (LDLR) by receptor mediated endocytosis. In the low pH environment of the lysosome, free cholesterol is released and LDLR is recycled back to the cell membrane. LDLR gene is under transcriptional regulation of SREBP2 transcription factor, which also transcribes PCSK9. PCSK9 is a secretory serine protease, which binds to the extracellular EGF domain of LDLR and targets it for lysosomal degradation. Another protein that is responsible for LDLR turnover is an E3 ubiquitin ligase called Inducible degrader of LDLR (IDOL), which is transcribed by LXR transcriptional factor. This figure has been modified from *LDLR and Familial Hypercholesterolemia, Genetic 677*. UW-Madison and Moore KJ, 2010. *Trends in Endocrinology and Medicine*.

### 1.11 Regulation of SREBP and its role in cancer signaling

Despite of significant overlap in the pathways regulated by the three isoforms of SREBPS, they show distinct tissue distribution and response to regulatory cues. The regulation of SREBP expression occurs at both the transcriptional and post-transcriptional level. At the post transcriptional level, the SREBP activity is regulated by intracellular cholesterol level as well as a cross talk between SCAP and INSIG proteins as described in the earlier section (Figure 1.5). Out of the three isoforms of SREBPs, SREBP1a is expressed constitutively at a low level in the liver as well as in other mammalian tissues. *SREBP1c* and *SREBP2* share a common feed forward transcriptional regulation mediated by the SREs in the enhancer regions of the promoters of both the genes. Other factors that are known to regulate SREBP1c expression are the liver X activated receptors, insulin and glucagon. Insulin not only induces SREBP1c promoter through LXR but also regulates ER to Golgi transport of precursor SREBP1c and its nuclear accumulation through mTOR activation [94]. ER retention protein INSIG, which binds SREBP to ER membrane in a complex with SCAP, is negatively regulated by insulin. This implies that when insulin signaling is low, INSIG is active which results in reduced nuclear accumulation of SREBP1c. Studies in the past have shown that insulin like growth factor 1 (IGF-1) upregulates SREBP1 mRNA and protein expression with a concomitant increase in its target gene expression [95]. Similarly, platelet derived growth factor increase ER to Golgi complex translocation of SREBP thus increasing its nuclear accumulation [96]. AKT plays an important role in regulation of SREBP expression and activity at many levels. In prostate cancer, high expression levels of SREBPs and their downstream effector genes has been positively correlated with progression to an androgen independence aggressive tumour phenotype. In a xenograft model of human prostate cancer cells, upregulation of SREBPs has been shown to increase AKT phosphorylation and lipid raft growth signaling [98]. Studies have shown that a feed forward regulation between AKT signaling and lipogenesis promotes cell proliferation in advanced stages of cancer [99] . A similar bidirectional relationship between AKT and SREBPs has been observed in ovarian cancer, where hyperactive AKT signaling induces SREBP activity upregulating lipogenesis, which in turn feeds AKT signaling to support cell growth and proliferation [100]. Studies in prostate

cancer cell lines have shown that SREBP-1 besides regulating lipogenesis, also induces expression of androgen receptor resulting in enhanced growth and survival of PCa cells [101]. SREBP-1 also promotes prostate cancer cell proliferation by inducing AKT signaling and increasing oxidative stress through induction of Nox5 and generation of reactive oxygen and nitrogen species [98]. In glioblastoma cases, studies have shown that rapamycin resistant phenotype have an EGFR and AKT dependent pro-survival pathway that promotes glioblastoma cell growth and survival through SREBP mediated lipogenesis pathway [102]. Whereas most of the evidence of deregulation of the SREBP pathway and its role in modulating cancer growth signaling come from studies on prostate cancer, ovarian cancer and glioblastoma, there is limited work done in CRC.

### **1.12 Cholesterol reducing drugs and cancer**

In a tumour cell, multiple signaling pathways can deregulate cholesterol homeostasis to support their growth and proliferation and also modulate the tumour microenvironment to favour cancer cell survival. Upregulation of the mevalonate pathway is critical for cholesterol accumulation as well as for effective growth signaling in a tumour cell. For this reason, the mevalonate pathway serves as an ideal therapeutic target for cancer prevention. One of the most prominent therapeutic agents that reduce intra cellular cholesterol biosynthesis by inhibition of HMGCR, are the statins. They have traditionally been used to lower serum cholesterol in patients suffering from hypercholesterolemia or atherosclerosis. Several case control and epidemiological studies have shown that long-term use of statins lowers the risk of cancer associated mortality [103-105]. There are evidence from *in vitro* studies in preclinical models that have also established the role of statins as a negative regulator of cancer cell growth and viability. However, the protective role of statins has been a matter of intense debate due to many contradicting evidence from clinical and epidemiological studies that challenge the efficacy of statins to retard cell growth and proliferation. Since statins target HMGCR located upstream of the mevalonate pathway, they also reduce the production of bioactive isoprenoids which are required for posttranslational modification of signaling molecules. This limitation of statins was overcome with the advent of other cholesterol reducing drugs such as PCSK9 inhibitors and RO-48-8071. PCSK9 inhibitors block the protease activity of PCSK9, concomitantly

increasing LDLR protein which accelerates uptake of serum cholesterol. RO 48-8071 inhibits the enzymatic activity of oxidosqualene cyclase (OSC), which catalyzes the conversion of squalene epoxide to lanosterol thus blocking cholesterol biosynthesis without affecting the synthesis of isoprenoids.

### **Statins as chemotherapeutic drugs**

Statins, are a class of cholesterol reducing drugs that bind to the catalytic domain of HMGCR and block its enzymatic activity by competitive inhibition. Compactin, a secondary metabolite of the fungus, *Penicillium citrinum*, was the first statin to be discovered by the Japanese biochemist Akira Endo in the year 1973. A few years later, in 1978 Merck discovered lovastatin (earlier called mevinolin) from the secondary metabolite of the fungus *Aspergillus terreus*. After several clinical trials, lovastatin finally got approval from Food and Drug Administration (FDA) and was commercially released by the name of Mevacor in 1987 [106, 107]. Structurally, statins have three main domains-one is the structural analogue that competitively binds to the active site of HMGCR enzyme, the other is a covalently attached ring structure which helps in binding of the analogue to the enzyme and third is the side chain that determines the solubility and absorption of the drug. Most of the statins are lipophilic and are easily absorbed by hepatic and extra hepatic tissues whereas some like Fluvastatin, which are hydrophilic need carrier mediated absorption. Prodrug, or the inactive form of statins have a closed lactone ring which upon oxidation by microsomal enzyme cytochrome P450, turn into an active open ring acidic form that binds to the catalytic domain of HMGCR [108, 109]. The reversible competitive inhibition of HMGCR by statins has several direct and indirect effects on cellular metabolism. The direct effect of statin inhibition is decrease in intracellular cholesterol level as well as downstream secondary metabolites of mevalonate pathway such as isoprenoids (FPP, GGPP), dolichol, ubiquinone etc. The lowering of intracellular cholesterol upregulates LDLR expression which effectively reduces serum cholesterol level in patients suffering from hypercholesterolemia. In addition to this, statins also reduce the synthesis of isoprenoids required for membrane anchorage of signaling proteins like RAS, Rho, RAC which effects cell growth and proliferation. There are reports that have shown statins to inhibit epidermal growth factor induced tumour cell invasion by blocking



the prenylation of RhoA. [110, 111]. Other protective roles of statin that are independent of HMGCR, include synchronizing tumour cells for G1-S phase cell cycle arrest. Since S phase cells are most resistant to radiotherapy, arresting tumour cells in G1-S phase sensitizes them to radiotherapy. There are reports of statins increasing the expression of p21 and p27 cyclin dependent kinase inhibitors in prostate cancer cells, causing cell cycle arrest in G1-S phase [112]. On its own, statins have shown several anti-tumourigenic characteristics that have encouraged researchers to test them along with other known anticancer drugs in pre-clinical models. For instance, in human colon cancer cell lines, lovastatin in combination with cisplatin and 5-Flourouracil, have been shown to increase apoptosis [113]. Similarly, in animal models, statins have shown anti-tumourigenic effect in combination with doxorubicin, cisplatin, TNF $\alpha$  and non-steroidal anti-inflammatory drugs like Sulindac [113, 114].

Studies with evidence on the protective role of statins in patients suffering from CRC are limited and inconsistent. This may be partially due to colonic tumour heterogeneity associated with tumour location and molecular subtype or due to analytical limitations. For instance, some of these observational studies that analyzed statin effect pre and post CRC diagnosis, did not take into account several unmeasured variables such as medication history that included long term use of hypotensive drugs, non-steroidal anti-inflammatory drugs (NSAIDs), hypoglycemic agents, molecular profile of the tumour, diet and lifestyle, that could have impacted the final outcome [105, 115]. In another population based study which examined the association of statin use and colorectal cancer risk, it was observed that statin efficacy was associated with KRAS mutation status in colonic tumours [116]. Statins are known to modulate KRAS signaling through isoprenoids that are required for posttranslational prenylation of signaling molecules like RAS and RHO. KRAS mutation, observed in ~40% of CRC cases, results in constitutively active growth signaling independent of EGFR. In these KRAS mutated tumours both anti EGFR and statin therapy were found to be ineffective. Hence the conclusion drawn from this study was that the effectiveness of statin in reducing cancer specific mortality seems to be restricted to colorectal tumour with wild type *KRAS*. In yet another case control study of CRC, a single nucleotide polymorphism in *HMGCR* gene was identified which modulates serum cholesterol levels and CRC risk [117]. In the same study, mRNA expression of full length

*HMGCR* and alternately spliced isoform was studied in lymphocyte cell lines derived from patients on simvastatin medication. *HMGCR v1*, alternately spliced isoform of *HMGCR* has an exon 13 deletion, which encodes for the statin binding domain. Not surprisingly, cells enriched with *HMGCRv1* were more resistant to statin inhibition, which supported the idea that altered ratio of full length, and alternately spliced *HMGCRv1* mRNA is an important mechanism for differential sensitivity to statins in patients with CRC risk.

### **RO 48-8071 as an alternate to statins**

RO 48-8071, chemically known as (4-Bromophenyl)-[2-fluoro-4-[6-[methyl(prop-2-enyl)amino] hexoxy]phenyl]methanone, is an inhibitor of oxidosqualene cyclase (OSC), a downstream mevalonate pathway enzyme that catalyses the conversion of 2,3 epoxysqualene to lanosterol [118]. Since OSC functions downstream of FPP and GGPP synthesis reactions in mevalonate pathway, blocking of this enzyme by any pharmacological inhibitor will block synthesis of sterols without affecting the synthesis of essential isoprenoids. Also, inhibition of OSC by RO 48-8071, does not upregulate *HMGCR* unlike statins, due to an indirect, negative feedback regulation of *HMGCR* [119]. RO 48-8071 has been reported to reduce cell viability and induce apoptosis in prostate cancer cells. Investigation into the mechanism of its anti tumourgenic role in prostate cancer cells showed that it reduced androgen receptor protein expression and in parallel increased the expression of anti proliferative estrogen receptor $\beta$  [120]. It also retarded the tumour growth in prostate cancer cells mice xenografts without any toxicity to normal cells. Similar results were seen in breast cancer cells, where RO was shown to reduce the expression of ER $\alpha$  and simultaneously increase the expression of antiproliferative ER $\beta$  to reduce breast cancer cell viability and induce apoptosis [121]. It concomitantly brought down the expression level of prosurvival BCL2 protein and increased the expression of cell cycle arrest protein p21 in a dose and time dependent manner. In breast cancer cells and even in xenograft models used to test the efficacy of the drug, RO proved to be more potent than statins in inducing apoptosis and containing tumour growth.

### **1.13 Pre-clinical models in colorectal cancer research**

Pre-clinical models using animals, cell lines, tumour xenotransplants and human biopsy specimens have become indispensable in understanding the complexities of tumour pathogenesis. Biological diversity of CRC makes it virtually impossible for a single animal model to recapitulate all the features of the human disease. There are three main criteria for selection of an animal model for CRC study. The first criterion is that the tumours should be restricted to the large intestine (colon). Secondly, the histological and morphological features of the lesions should resemble their human counterpart and lastly, the animal model should be able to recapitulate the basic mutation profile and alterations in cell signaling pathways.

#### **1,2 Dimethylhydrazine and Azoxymethane model**

The most commonly used model for study of sporadic CRC are 1,2-dimethylhydrazine (DMH) and azoxymethane (AOM) which are exclusively colon specific carcinogens [122]. Both these models are highly reproducible in murine models with different genetic backgrounds and faithfully recapitulate human CRC pathogenesis. The majority of the tumours produced by these carcinogens appear in the distal region of the colon and have a histology similar to the human adenomas and carcinomas [123]. DMH and its metabolite AOM both require metabolic activation by metabolizing enzymes of the liver such as cytochrome P450 to form active DNA alkylating compounds called methylazoxymethanol (MAM). The hydroxylated metabolite MAM is a stable compound with a half life of 12 hours which allows it travel from liver to the colon without getting degraded. In the colon, MAM is further metabolized to release methyl diazonium ions that can add methyl groups to the guanosine residues of macromolecules in the colon cells. Usually two intraperitoneal or subcutaneous injections of DMH (150mg/kg body weight) or AOM (15mg/kg body weight) given one week apart is sufficient to induce colonic tumours in rats or mice in 20-30 weeks. Tumour incidence and multiplicity can be manipulated by altering diet and genetic background, which makes this model useful to study the effect of chemopreventive drugs and also understand pathogenesis of CRC [122]. The synergistic effect of AOM (tumour inducing) and DSS (tumour promoting) in development of colonic tumours in the AOM/DSS colitis related murine CRC model

allows the investigation of the pathogenesis of inflammatory bowel disease (IBD) associated CRC. AOM/DSS induced colonic tumours generally appear in the distal region of the colon, which is where most of human sporadic tumours appear. These tumours rarely metastasize but they do show frequent mutation in *APC*,  *$\beta$ -catenin*, *KRAS* genes. They also show elevated levels of prostaglandin and nitric oxide synthesizing enzymes such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS).

Murine models have an advantage of other animal models in having extensive genetic information available which allows the development of recombinant inbred mouse, transgenics, knock out and knock in models for study of cancer pathogenesis. 6 week old male F344, wistar or Sprague Dawley rats are most commonly used to induce colonic tumours using DMH or AOM. The other animal model that has been widely used for colorectal cancer preclinical research is the *APC<sup>MIN</sup>* mouse model. This model is characterized by **m**ultiple **i**ntestinal **n**eoplasm (MIN) phenotype due to autosomal dominant mutation of the *APC* gene [125]. Heterozygous *APC<sup>MIN</sup>* carry a T to A transversion mutation in one of the alleles of *APC* that results in small lesions mostly in the small intestinal region [126]. For adenoma formation, loss of heterozygosity of *APC* gene is required. Because of close similarity to FAP, the *APC<sup>MIN</sup>* mouse model is used to study the etiology of CRC with mutated *APC* phenotype.

### **Cell culture based CRC models**

Cell lines derived from human tumours are widely used as preclinical model systems to study the pathogenesis of cancer. They allow mechanistic study of specific signaling pathways, chemotherapeutic drug screening as well as identification of novel targets and biomarkers. Despite their limitations of being largely monoclonal in nature and devoid of a tumour micro environment, their significance in micro examining pathogenesis of cancer cannot be undermined. The scope they provide, to genetically manipulate or delineate signalling pathways via specific treatments under controlled experimental conditions, is unquestionable.

The cancer cell line database is a repertoire of genetic profile of thousands of human cancer cells including CRC cells and it is ever expanding. The Cancer genome project of the Sanger institute and cancer cell line encyclopedia project as a joint collaboration

between the Novartis institute of Biomedical research and the Broad institute have substantial database containing genetic profiles of thousands of human cancer cells [127]. This database is particularly useful in research for finding new therapeutic targets in the wake of acquired resistance of tumour cells to pharmacological drugs.

CRC cell lines derived from primary tumour show heterogeneity in their growth characteristics, morphology, drug sensitivity, genetic and mutation profiles [128]. Mutation and gene expression data showed a close similarity between the cell lines and already published data on colorectal tumours, validating the use of cancer cell lines in pre-clinical research [129]

CRC cell lines have been classified into three classes based on their mutation profiles and altered genetic pathways. 1) MSI-H cell lines either have *MLH* hypermethylated in their promoter region or mutated DNA repair genes 2.) Hypermethylated mismatch repair deficient cell lines with defective DNA polymerase proof reading 3.) A CIN phenotype that show genomic instability due to chromosomal rearrangements in key oncogenes and tumour suppressors like *Wnt*, *MAPK*, *PI3K*, *p53*, *TGFβ* [129]. A fourth subclass of cell lines has the CIMP phenotype with epigenetic modifications in *BRAF*, *PTEN* etc. To a large extent, the molecular signature of CRC cell lines have shown a close resemblance to that of the primary tumours. Human colorectal derived cell lines also exhibit the biological heterogeneity observed at tissue level. For example- cell lines derived from same primary tumour like DLD 1 and HCT 15 show differences in their DNA copy number and mutation profiles, reflecting the inherent heterogeneity of the parent tumour tissue.

With a plethora of information centered around understanding the association of the cholesterol biosynthetic pathway with colorectal cancer tumorigenesis, there seems to be an increasing space for exploring the potential role of other molecules of cholesterol metabolism, LDLR in particular. The focus of my dissertation has been on elucidating the role of LDLR in modulating cholesterol drug response, growth and cell viability of colorectal cancer cells.

### **1.14 Evidence from pre-clinical studies**

Preclinical models have been extensively used to study the effect of statins on colorectal cancer cell growth, proliferation and development of colonic tumours. Some of the early evidence came from studies that showed that low dose of pravastatin was able to significantly reduce the tumour incidence in 1,2 dimethylhydrazine (DMH) induced colonic tumour model in mice by modulation of cholesterol biosynthesis pathway [130]. In another study, it was shown that a combination of low dose of atorvastatin, aspirin, a non-steroidal anti-inflammatory drug (NSAID) and celecoxib, a cyclooxygenase- 2 (COX-2) inhibitor can inhibit cell proliferation and induce apoptosis in AOM induced colonic tumours in F344 male rats [131]. In a recent study it was shown that simvastatin induces mitochondrial pathway mediated apoptosis in colorectal cancer cells by inhibiting geranylgeranylation of RhoGTPase and activating the JNK pathway [132]. A combination therapy including simvastatin and irinotecan, a chemotherapeutic drug used to treat metastatic colon cancer was reported to act synergistically in inhibiting colon cancer cell proliferation [133]. It was suggested that simvastatin mediated its anti-proliferation effects either by reducing the activity of ABCG1 receptors which confer irinotecan drug resistance or by reducing colorectal cancer stemness through bone morphogenetic pathway. In a study conducted by Clouston et.al, it was shown that dietary lipid modulate the tumour growth inhibitory effectors of lovastatin [134]. In the same study it was shown that all tumours regardless of dietary lipid or lovastatin treatment, exhibited high expression of HMGCR protein and low expression of LDLR protein compared to normal mucosa from tumour bearing rats. This study provided the first evidence that experimentally induced colonic tumours have heightened cholesterol biosynthetic pathway and this could be due to the fact the tumours had lower level of LDLR protein. The review of previous work done in pre-clinical models to study the role of statin in cancer prevention alludes to the possibility that statin can have pleiotropic effects on CRC development that goes beyond their lipid lowering activity. It also underlines the significance of HMGCR and the mevalonate pathway as attractive targets for chemotherapeutic drug intervention. The fact that HMGCR activity is regulated by coordinated feedback regulation between intracellular cholesterol and LDLR activity, raises the question about the role of LDLR in CRC development.

### **1.15 Specific Aims**

The main premise of this dissertation was to explore the role of the cholesterol biosynthetic pathway in particular the role of LDLR in CRC. We hypothesized that LDLR plays an important growth regulatory role in CRC development and that up-regulation of LDLR protein will negatively affect CRC growth. To test the hypothesis, the following aims were pursued :

1. To determine the protein expression levels of key molecules associated with cholesterol homeostasis in experimentally induced colonic tumour rat model (addressed in chapter 2).
2. To assess the suitability of human CRC cell lines as preclinical models to explore the role of LDLR in CRC development (addressed in chapter 3 ).
3. To determine if ectopic overexpression expression of LDLR affects the growth of CRC cells *in vitro* and to elucidate the underlying molecular pathways through which LDLR could be exerting its anti-tumourigenic effects (addressed in chapter 4).

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## **Chapter 2**

# **Evaluation of key proteins associated with cholesterol homeostasis in experimentally induced colonic tumour rat model**

## 2.1 Introduction

Cholesterol homeostasis is critical for the normal growth and development of a cell and its deregulation is frequently associated with pathogenesis of several chronic diseases including cancer. Besides regulating the fluidity of phospholipid bilayer plasma membrane, cholesterol is also an important structural component of sphingomyelin rich microdomains called lipid rafts, which are distributed throughout the plasma membrane [1, 2]. Additionally cholesterol is required as a precursor for steroid and bile acid synthesis. The cholesterol biosynthetic pathway also synthesizes several non-sterol bioactive intermediates called isoprenoids that are required for post translational activation of small G-proteins such as RAS, RHO and RAC which are located upstream of several growth signaling pathways [3].

In hepatocytes, endogenously synthesized cholesterol is incorporated into apolipoprotein B100 (apoB100) containing very low density lipoprotein (VLDL) particles and released into the blood stream to supply of cholesterol to peripheral tissues [4]. Alternately, it can be stored as cholesteryl esters or incorporated in lipid rafts. Cells can also acquire cholesterol by low density receptor mediated endocytosis which is either esterified by Acyl CoA Cholesterol-acyl Transferase (ACAT) enzymes and stored in lipid droplets or secreted from the cells as bile acids. Accumulation of excess cholesterol can be toxic to the cells, hence it is imperative for the cells to have a mechanism to control intracellular cholesterol levels, a process known as cholesterol homeostasis. Cholesterol homeostasis relies on an interplay between key proteins associated with cholesterol biosynthesis, uptake and metabolism. HMGCR, the rate limiting enzyme of the cholesterol biosynthesis pathway and LDLR, both are under transcriptional regulation of sterol sensitive transcription factors called sterol regulatory element binding protein (SREBPs) [5]. SREBPs have three structural isoforms of which SREBP2 is responsible for transcriptional regulation of cholesterol pathway associated genes such as *HMGCR*, *LDLR* and *Proprotein convertase subtilisin/kexin type 9 (PCSK9)*. SREBP1c regulates the transcription of lipogenic genes such as *fatty acid synthase (FASN)*. Cholesterol depletion in the endoplasmic reticulum (ER) membrane, promotes translocation of SREBP cleavage activating protein (SCAP) and precursor SREBP complex to the Golgi complex. At the

Golgi complex, the NH<sub>2</sub> terminal transactivation domain of SREBP2 is cleaved by proteolytic action of two site proteases (SP 1 and SP2) to release the mature, transcriptionally active form of SREBP2. This mature form of SREBP2 moves to the nucleus and binds to the sterol regulatory element (SRE) located in the enhancer region of LDLR and HMGCR thus upregulating cholesterol biosynthesis and uptake. Interestingly, SREBP2 also transcribes PCSK9 which negatively regulates LDLR expression by degrading LDLR through endosomal lysosomal pathway.

Deregulated cholesterol homeostasis resulting in abnormal accumulation of cholesteryl ester has been observed in several solid tumours such as prostate cancer, breast cancer, colorectal cancer (CRC), liver carcinoma, melanoma and glioblastoma [6-9]. Several cancer types, including but not limited to, breast, prostate and colon cancer express elevated levels of and HMGCR, fatty acid synthase (FASN) and other genes of cholesterol and lipogenic pathways [10]. Loss of sterol mediated negative feedback regulation of HMGCR and LDLR has been reported in different cancer types particularly in prostate cancer and CRC [11, 12]. Loss of feedback regulation of cholesterol homeostasis has been associated with resistance to cholesterol reducing drugs such as statins [13]. In case of CRC, apart from few studies that have reported the upregulation of cholesterol biosynthesis pathway, there is negligible information available to understand the role of cholesterol biosynthetic pathway in the development of CRC. There are few studies that have reported elevated expression of LDLR in CRC cells [12] whereas in some studies low expression of LDLR has been reported in colorectal cancer cells [14, 15] but the role that LDLR plays in tumourigenesis remains elusive.

The main objective of this study was to examine the steady state levels of HMGCR, LDLR, SREBPs and PCSK9 in azoxymethane (AOM) induced colonic tumours in Sprague Dawley rat model. We report that AOM induced colonic tumour rat model display deregulated cholesterol and lipid metabolism. All tumours examined exhibited elevated protein expression level of HMGCR and reduced protein expression level of LDLR. Moreover, the tumours showed significant upregulation of SREBPs and PCSK9 protein levels. These findings support the contention that deregulated cholesterologenic and lipogenic pathway may play an important role in colonic tumour initiation and progression in experimentally induced colonic tumour model. More importantly, in addition to elevated

level of HMGCR, a markedly reduced level of LDLR in tumours raises an important question about the role of LDLR in the sequential development of colonic tumours. This may represent a subset of colorectal tumour that requires low LDLR protein expression to support enhanced tumour growth. Whether or not this tumour phenotype is required for tumour initiation or progression is matter of further investigation. However, our study does open up a possibility of exploring a novel cancer prevention strategy targeted towards increasing the level of LDLR in colorectal tumours.

## **2.2 Materials and Methods**

### **Colon carcinogen**

Azoxymethane (AOM; Sigma Aldrich, St. Louis, MO, USA) was used as colon specific carcinogen. AOM was dissolved in 0.9% saline and subcutaneously injected into the animals once every week for two weeks at a concentration of 15mg/kg body weight.

### **Experimental animals and treatment**

Sprague Dawley weaning male rats (3-4 weeks old) were purchased from Charles River Laboratories (Saint-Constant, Quebec, Canada) and were housed in laboratory conditions with 12 hour light/12 hour dark cycle. Temperature and humidity were controlled at 22°C and 50%, respectively. Animals were acclimatized to the conditions for 1 week prior to treatment. During the entire course of the experiment, animals had free access to drinking water and food.

Animals were randomly categorized into two groups of 10 animals, each which served as experimental, and control respectively. Animals from experimental group were subcutaneously injected with azoxymethane (AOM), a colon specific carcinogen (15mg/kg body weight/week for 2 weeks) [16]. Animals injected with saline served as control. Animals were killed by CO<sub>2</sub> asphyxiation 30 weeks after the first AOM injection. Their colons were excised, flushed with cold PBS and slit open longitudinally from caecum to anus on a cold plate. The mucosal surface was examined for tumours and macroscopic lesions. The location and size of the tumours was recorded before snap freezing in liquid

nitrogen. All animal protocols were approved by the University of Windsor, Animal Care Committee according to the Canadian Council on Animal Care guidelines.

### **Tumour assessment and selection criteria for analysis**

The tumour assessment and selection parameters used, are previously described by Bird *et al* [16]. These included tumour incidence (percentage of total animals with tumours); tumour multiplicity (average number of tumours/tumour-bearing rat); average tumour size (mm<sub>2</sub>) per tumour-bearing rat; average tumour size/group (average size of all tumours in a group); and tumour burden (average of the total tumour area in each tumour-bearing rat). For the purpose of this study tumours ranging in size between 12 to 25 mm<sub>2</sub> or larger were used. Any visible tumours including micro adenomas of 1-2 mm<sub>2</sub> were removed. Therefore mucosal samples were devoid of any exophytic tumours. A majority of large tumours were located between 4-10 cm from the rectal end of the colon. For the sake of consistency and to avoid location specific heterogeneity observed in clinical samples [17], we used tumours which were located between 6-10 cm of the colon and were either adenomas or adenocarcinomas. The tumour incidence was 60% and each animals had 1-3 tumours in the selected colon region. Normal appearing surrounding mucosae from tumour bearing animals were used as control in the study.

### **Chemicals and antibodies**

The following antibodies were used for Western blot analysis: LDLR (sc-18823), HMGCR (sc-27578), SREBP1 (sc-366), SREBP2 (sc-5603),  $\beta$ -actin (sc-1616), secondary antibodies including horse radish peroxide (HRP) conjugate anti-mouse (sc-2005) and anti-rabbit (sc-2030) IgG were purchased from Santa Cruz Biotechnology. PCSK9 (NB300-959) antibody was purchased from Novus Biologicals.

Other chemicals used were: protease inhibitor cocktail (Roche/Sigma Aldrich), Bradford reagent (Bio-Rad Laboratories), Perkin Elmer Enhanced Chemiluminescence reagent (Thermo Fisher Scientific).

### **Sample preparation and Immunoblotting**

Frozen tissues were excised and weighed (approximately 50mg) before homogenization using Radioimmunoprecipitation assay (RIPA) buffer (see appendix 1). Whole tissue homogenate was centrifuged at 12000 x g for 20 minutes (4°C). Clear supernatant was collected and used for protein estimation by Bradford assay. Equal amount of protein was used for SDS-PAGE and transferred to PVDF membrane by electro-blotting using wet transfer method. Membranes were blocked in 5% skimmed milk for 1 hour at room temperature, followed by overnight incubation in primary antibodies prepared in 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS)-Tween (1% v/v) at 4°C. Membranes were washed three times in TBS-Tween (TBST) and then probed with HRP-conjugate secondary antibodies (1:10000) for 1 hour at room temperature. After washing three times in TBST, protein bands were visualized using Perkin Elmer ECL reagent and Fluor-Chem Western blotting imaging system. Protein bands were quantified using ImageJ software (Version 1.42q)

### **Statistical analysis**

All Western blot analysis are represented as mean  $\pm$  SEM (Standard error of mean). Statistical analysis were performed using Student t-test. P values were calculated using GraphPad Prism3.0 software (GraphPad software). Differences were considered significant for P values  $\leq$  0.05.

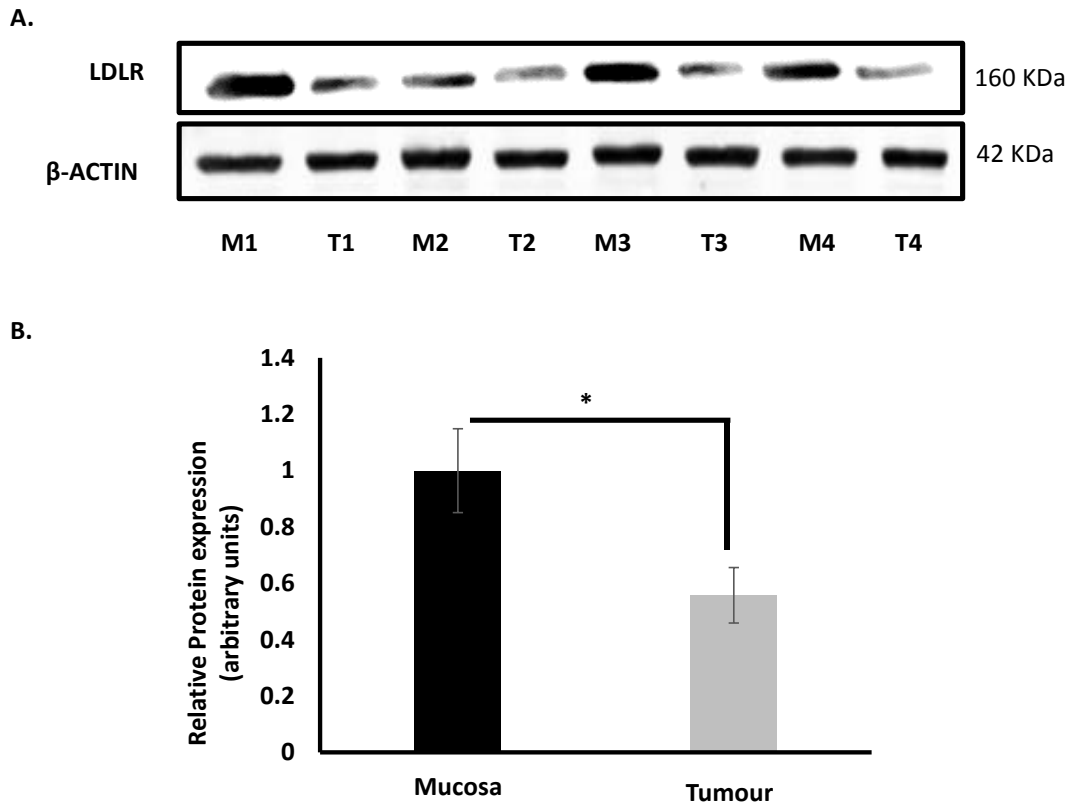
## **2.3 Results**

### **Colonic tumour show reduced level of LDLR protein expression and increased level of HMGCR protein expression**

To assess the protein expression level of LDLR in AOM induced colonic tumour model system, we collected tissue homogenate from colonic tumour and mucosa followed by protein quantification and Western blot analysis.  $\beta$ -actin was used as loading control. Results of Western blot analysis showed lower expression of LDLR protein in tumour tissue compared to normal mucosa tissue (Figure 2.1A). An average of LDLR protein

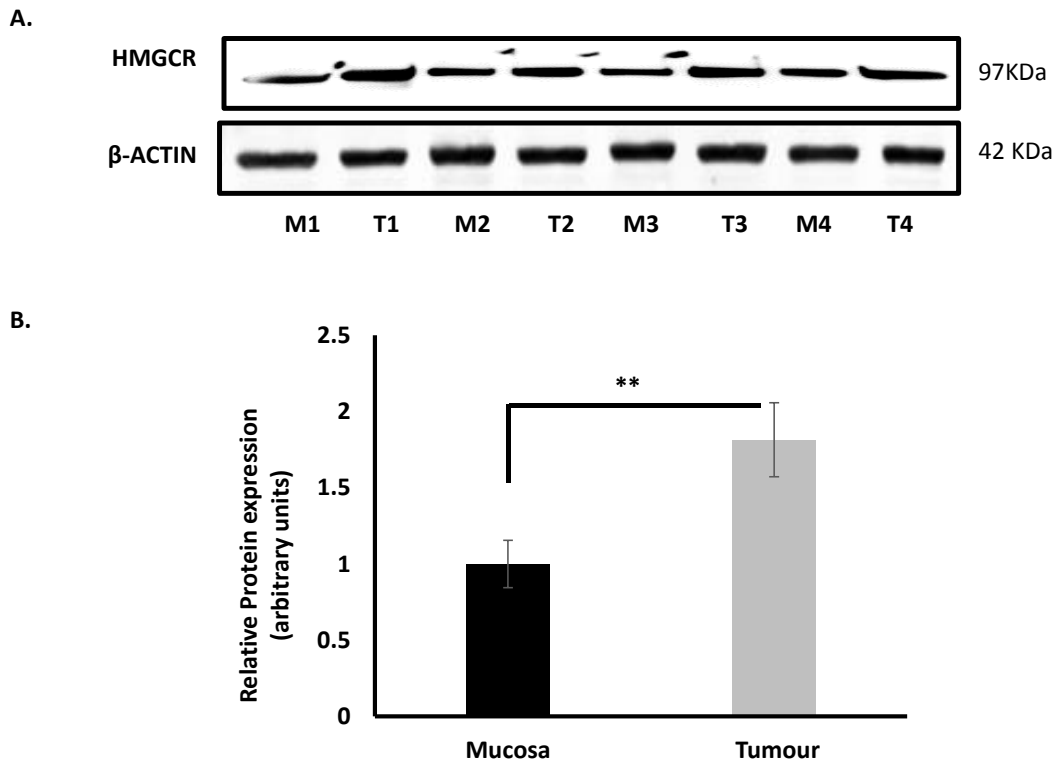


expression levels in colonic tumour and normal appearing mucosa from tumour bearing animals (n=10) showed significant downregulation of LDLR protein (~ 160 KDa) in tumour tissue samples compared to colonic mucosa (Figure 2.1B).



**Figure 2.1 LDLR protein expression is downregulated in colonic tumour.** Protein was extracted from colonic mucosa and tumour tissue from Sprague Dawley rats injected with azoxymethane (15mg/kg body weight) followed by Western blotting. (A) Representative image of Western blot showing LDLR protein expression in colonic mucosa and tumour tissue. (B) Quantification of LDLR protein expression in mucosa and tumour tissue (n=10) using ImageJ software.  $\beta$ -actin was used as internal loading control. Results are represented as mean  $\pm$  SEM (Standard error of mean), \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ . Statistical analyses were performed using student *t*-test. *P* values were calculated using Graph-Pad Prism 3.0 software. M1-M4=Mucosa protein samples, T1-T4=Tumour protein samples.

Several studies have shown an increase in HMGCR expression and upregulation of cholesterol biosynthesis pathway during the development of solid tumours [18, 19]. Consistent with the previous findings from clinical studies, we confirmed that HMGCR protein expression is upregulated in AOM induced colonic tumours compared to mucosa. Statistical analysis (n=10) showed significant upregulation of HMGCR protein in tumour tissue samples compared to normal mucosa (Figure 2.2). The band representing active form of the enzyme HMGCR (~ 97 KDa) was quantified and normalized using  $\beta$  actin as loading control. Heightened expression of HMGCR protein in tumours also confirms the loss of sterol dependent negative feedback regulation of cholesterol biosynthesis in experimentally induced tumour model.

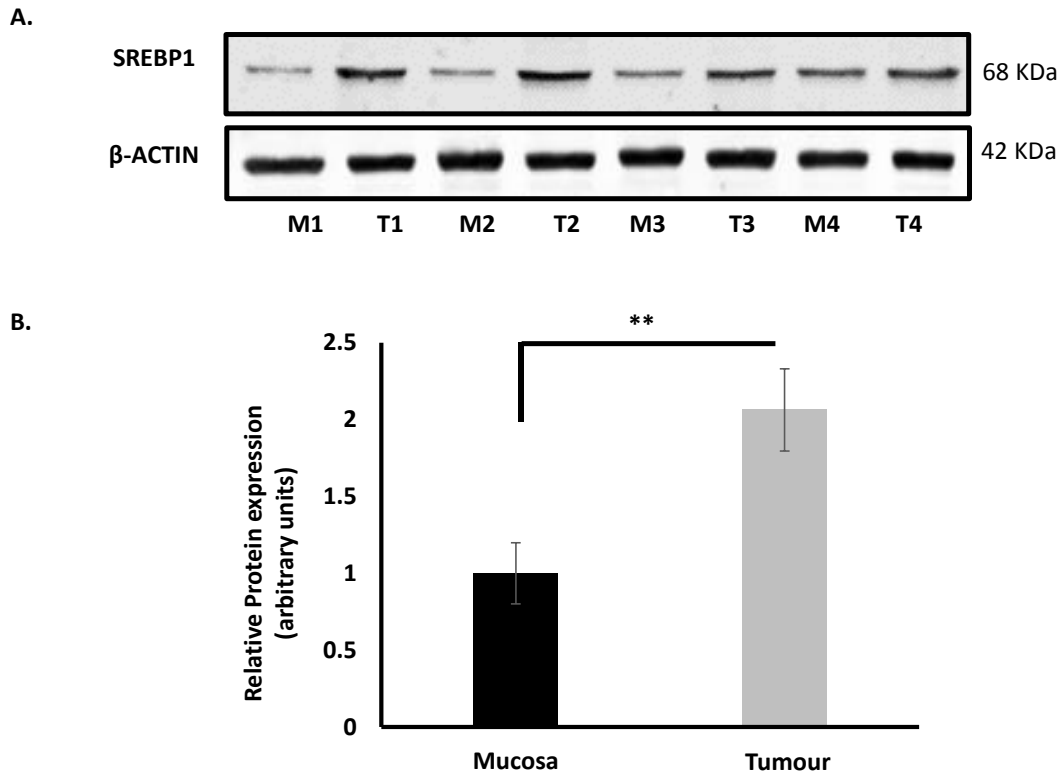


**Figure 2.2 HMGCR protein expression is upregulated in colonic tumour.** Protein was extracted from colonic mucosa and tumour tissue from Sprague Dawley rats injected with azoxymethane (15mg/kg body weight) followed by Western blotting. (A) Representative image of Western blot showing catalytically active form of HMGCR protein (~97KDa) expression in mucosa and tumour tissue. (B) Quantification of HMGCR protein expression in mucosa and tumour tissue ( $n=10$ ) using ImageJ software.  $\beta$ -actin was used as internal loading control. Results are represented as mean  $\pm$  SEM (Standard error of the mean), \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ . Statistical analyses were performed using student t-test.  $P$  values were calculated using Graph-Pad Prism 3.0 software. M1-M4=Mucosa protein samples, T1-T4=tumour protein samples.

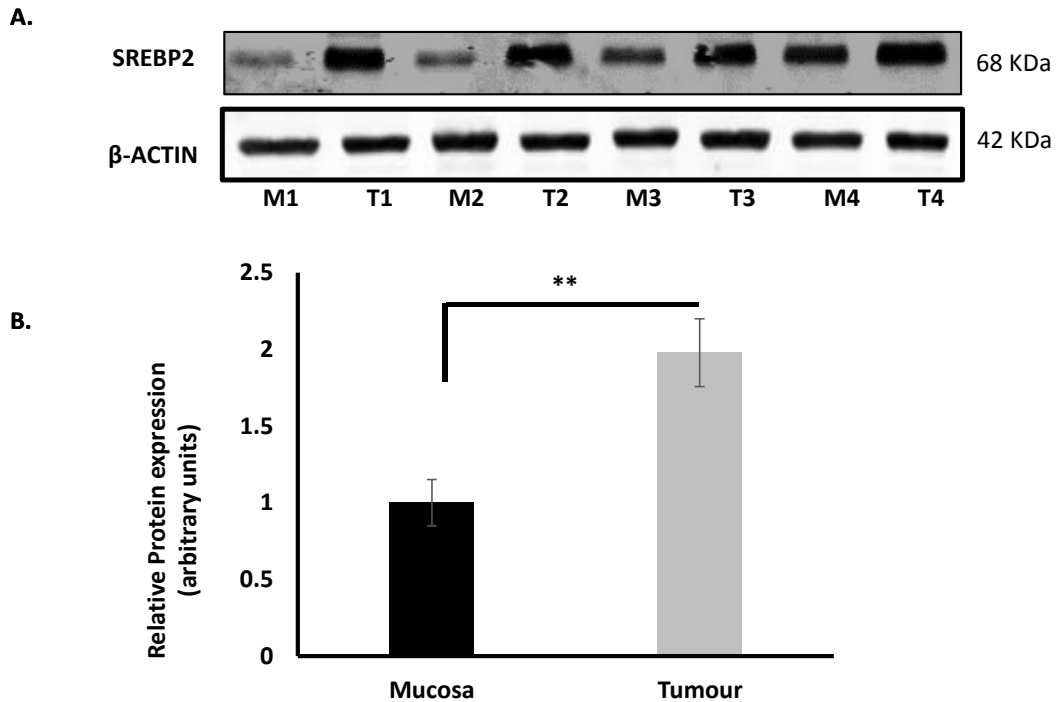
### **SREBPs and PCSK9 protein expression levels are upregulated in colonic tumours**

The sterol regulatory binding element proteins or the SREBPs are family of endoplasmic reticulum (ER) membrane bound transcription factors that regulate the expression of lipids and cholesterol pathway related genes. SREBPs have three isoforms – SREBP1a, SREBP1c and SREBP2. SREBP1a and SREBP2 are responsible for transcription of cholesterol associated genes including LDLR, HMGCR, PCSK9 whereas SREBP1c is solely dedicated to lipogenic pathway related genes. Previous studies have reported modulation of SREBP pathway and aberrant activation of its target genes in cancer [20]. Deregulation of cholesterol biosynthesis and lipogenesis in tumours has been shown to be mediated by aberrant activation of SREBPs through PI3K/AKT and mTORC signaling [21]. In a recent study in breast cancer cells, it was reported that mutant p53 interaction with SREBP2 upregulates cholesterol biosynthesis pathway which results in disruption of normal tissue architecture in 3-dimensional breast cancer cell model [22].

In our study we found that protein expression of transcriptionally active mature form of SREBPs (~ 68 KDa) are elevated in colonic tumour in comparison to normal mucosa (Figure 2.3A and 2.4A). High protein expression level of transcriptionally active SREBP1 and SREBP2 correlates with upregulated lipogenic and cholesterologenic pathways in colonic tumours.



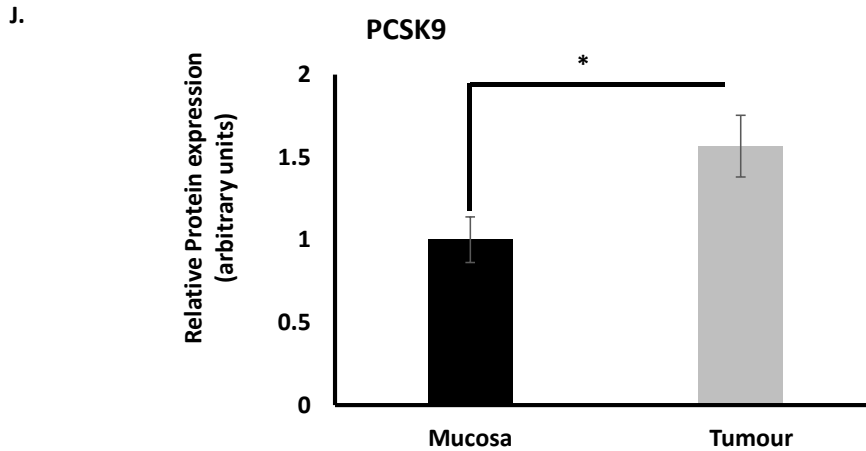
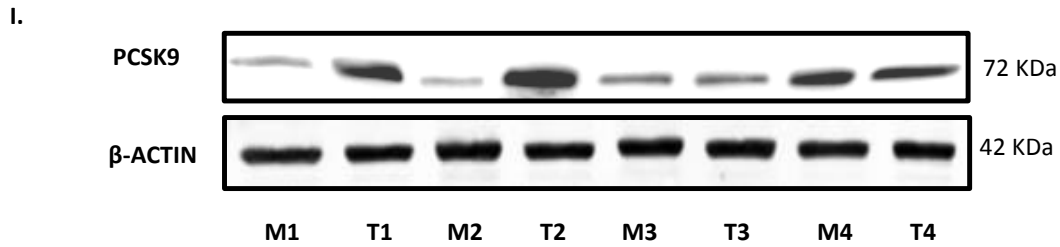
**Figure 2.3 SREBP1 protein expression is upregulated in colonic tumour.** Protein was extracted from colonic mucosa and tumour tissue from Sprague Dawley rats injected with azoxymethane (15mg/kg body weight) followed by Western blotting. (A) Representative image of Western blot showing SREBP1 (68 KDa mature form) protein expression in mucosa and tumour tissue (n=4). (B) Quantification of SREBP1 protein expression in mucosa and tumour tissue (n=10) using ImageJ software.  $\beta$ -actin was used as internal loading control. Results are represented as mean  $\pm$  SEM (Standard error of mean), \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ . Statistical analyses were performed using student t-test. P values were calculated using Graph-Pad Prism 3.0 software. M1-M4=Mucosa protein samples, T1-T4=tumour protein samples.



**Figure 2.4 SREBP2 protein expression is upregulated in colonic tumour.** Protein was extracted colonic mucosa and tumour tissue from Sprague Dawley rats injected with azoxymethane (15mg/kg body weight) followed by Western blotting. (A) Representative image of Western blot showing SREBP2 (mature form ~68 KDa) protein expression in mucosa and tumour tissue (n=4). (B) Quantification of SREBP2 protein expression in mucosa and tumour tissue (n=10) using ImageJ software.  $\beta$ -actin was used as internal loading control. Results are represented as mean  $\pm$  SEM (Standard error of mean), \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ . Statistical analyses were performed using student t-test. P values were calculated using Graph-Pad Prism 3.0 software. M1-M4=Mucosa protein samples, T1-T4=tumour protein samples.

We also analyzed the protein expression level of Proprotein convertase subtilisin kexin 9 (PCSK9) in colonic tumour in comparison to normal mucosa. PCSK9 is a serine protease that post transcriptionally regulates LDLR turn over in the cell by binding to its extra-cellular domain and targeting it for lysosomal degradation [23]. The subtilisin like catalytic domain (~ 72 KDa) is secreted by the cell after auto catalysis and proteolytic cleavage of the pro-protein. Gain of function mutation in PCSK9 catalytic domain results in increased serum cholesterol due to down regulation of LDLR [24]. Some of the previous studies have reported deregulation of PCSK9 expression in lung cancer, hepatocellular carcinoma, colorectal cancer, melanoma [25, 26]. Except for few studies that have reported a correlation of reduced PCSK9 protein expression with decrease in metastasis [25], not much is known about the role of PCSK9 in cancer progression. Western blot analysis for PCSK9 protein expression showed either elevated level of PCSK9 protein in tumour tissue or no significant change compared to mucosa tissue sample (Figure 2.5A). An average of PCSK9 protein levels in colonic tumour and normal appearing mucosa from tumour bearing animals (n=10) showed an overall upregulation of PCSK9 protein expression in colonic tumour.





**Figure 2.5 PCSK9 protein expression is upregulated in colonic tumour.** Protein was extracted from colonic mucosa and tumour tissue from Sprague Dawley rats injected with azoxymethane (15mg/kg body weight) followed by Western blotting. (A) Representative image of Western blot showing PCSK9 (~72 KDa) protein expression in mucosa and tumour tissue (n=4). (B) Quantification of PCSK9 protein expression in mucosa and tumour tissue (n=10) using ImageJ software.  $\beta$ -actin was used as internal loading control. Results are represented as mean  $\pm$  SEM (Standard error of the mean), \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ . Statistical analyses were performed using student t-test. P values were calculated using Graph-Pad Prism 3.0 software. M1-M4=Mucosa protein samples, T1-T4=tumour protein samples.

## 2.4 Discussion

In a normal cell cholesterol homeostasis is a well-coordinated mechanism that involves several proteins interacting with each other to maintain intracellular cholesterol homeostasis. This is achieved through transcriptional and post-transcriptional regulation of cholesterol pathway related genes that are involved in biosynthesis, uptake and export of cholesterol. HMGCR, the rate limiting enzyme of mevalonate pathway and LDLR, the membrane bound glycoprotein receptor responsible for cellular uptake of cholesterol are under sterol mediated negative feedback regulation. HMGCR is the critical rate-limiting enzyme in the mevalonate pathway that catalyzes the conversion of HMGCoA to mevalonate. This pathway is also crucial for synthesis of several non-sterol isoprenoids that are required for posttranslational modification and activation of GTP-bound signaling proteins like Ras, Rho. Prenylation of these signaling molecules is crucial for downstream growth signaling pathways like the MAPK signaling pathway, PI3K/AKT/mTORC1 etc. Several studies have reported that *KRAS* mutation which is observed in 40% of CRC cases, results in constitutive activation of *KRAS* independent of isoprenylation [27]. Acquired resistance to anti-epidermal growth factor (EGFR) therapy has also been attributed to *KRAS* mutation [28]. *KRAS* mutation has also been associated with resistance to statin mediated reduction in cancer specific mortality in colorectal cancer case study [29]. The mevalonate pathway has interested cancer researchers worldwide because of the direct and indirect role it plays in tumourigenesis especially with growing evidence of statins and other cholesterol pathway inhibitors being able to induce apoptosis and cell cycle arrest in cancer cells [30-33] . LDLR has so far been in focus mostly for its role in uptake of cholesterol. It has been the target for drug therapy to reduce serum cholesterol to treat abnormal cholesterol level related diseases like atherosclerosis and hypertension. Both LDLR and HMGCR are under transcriptional regulation of SREBP2 transcription factors. Another key player in this pathway is the secretory protease called PCSK9, which is also under transcriptional regulation of SREBP2. PCSK9 binds to LDLR extracellularly and targets it for degradation. PCSK9 inhibitors have come up as a potent substitute for statins to reduce the levels of serum cholesterol by upregulation of LDLR. Deregulation of cholesterol homeostasis and loss of sterol dependent feedback regulation of LDLR and

HMGCR has been observed in several cancers like prostate cancer, CRC, glioblastoma, melanoma and breast cancer [9, 12, 34, 35]. In this study, for the first time we have assessed the protein expression of the key players that are involved in cholesterol homeostasis in AOM induced rat colon cancer model corroborating a link between these molecules and pathogenesis of colon cancer.

In order to answer the broader question about the role of LDLR in tumourigenesis, we wanted to first determine protein expression profile of LDLR and related molecules of cholesterol metabolic pathway in AOM induced colonic tumour rat model. Pre-clinical models like the Sprague Dawley rats which are well characterized to study the development of colonic tumours using like azoxymethane (AOM), provide an ideal model system for our study. By inducing tumour in the animals through carcinogen injection, we were able to detect tumours throughout the length of colon in 30 weeks. Tumour assessment along the length of colon showed considerable heterogeneity in tumour morphology, size and multiplicity. This heterogeneity may be attributed to the difference in the embryological origin as well as gene and protein expression pattern of proximal and distal tumour.

In our study, we observed that LDLR protein was significantly lower in tumour compared to normal mucosa. Our results are contradictory to some of the previous findings that have reported upregulation of cellular cholesterol levels in the tumour cells due to overexpression of LDLR and HMGCR protein. This suggests that colorectal cancer being a heterogeneous disease has a molecular subtype in which LDLR protein downregulation along with elevated levels of HMGCR might be playing an important role in early onset or late progression of the disease. The significance of the effect of low levels of LDLR protein in tumour is a question that we address through mechanistic study in colon cancer cell lines. Whether or not LDLR downregulation gives a survival advantage or drug resistant phenotype to the tumours are questions that are addressed in further studies. Compared to LDLR, there are far more compelling evidence on HMGCR that suggest that mevalonate pathway and related enzymes are upregulated in tumours. Our studies show that indeed this might be a more general phenomenon for tumours. We saw heightened expression of HMGCR protein in all tumour tissues compared to normal colonic mucosa. Increase in expression of HMGCR protein is directly related to increase in synthesis of cholesterol and non-sterol bioactive isoprenoids. Over expression of HMGCR ensures constitutive

activation of mevalonate pathway to meet the requirement of cholesterol for membrane biogenesis and assembly of lipid rafts for facilitating cellular growth signaling. Increase in HMGCR expression is directly linked to abnormal RAS activity that results in enhanced growth signaling in tumour cells [3]. Gene expression of both *LDLR* and *HMGCR* is dependent on SREBP2 transcription factor hence it was logical for us to analyze the expression of SREBPs in tumour. Several studies in the past have shown that regulation of lipid and cholesterol pathway by SREBPs is critical for growth and survival of cancer cells. Under hypoxic conditions of the tumour microenvironment, SREBPs expression is upregulated to increase lipid and cholesterol biosynthesis in brain tumour cells [36]. Depletion of SREBPs in glioblastoma cell lines, induced apoptosis due to ER stress and activation of unfolded protein response (UPR) pathway. Thus, SREBPs are critical for tumour growth and survival because of the role they play in coordinating lipid and protein biosynthetic pathways. In our study, we found elevated expression of transcriptionally active mature form of both SREBP1 and SREBP2 in tumours in comparison with normal mucosa. From these results, it is evident that the elevated expression of HMGCR and PCSK9 in tumour tissues can be attributed to upregulation of SREBP2. However, the downregulation of LDLR protein despite overexpression of SREBP2 suggests the involvement of another pathway in post translationally depleting LDLR protein levels in tumour. One of the proteins that is responsible for LDLR turn over in the cell, is PCSK9. C-terminal domain of PCSK9 binds to the extracellular domain of LDLR at the cell surface to form LDLR-PCSK9 complex that enters the endosomal pathway and is ultimately degraded in the lysosome. Hence LDLR protein abundance is inversely related to PCSK9 expression. In our study, we observed PCSK9 elevated in tumours when compared to mucosa. This suggests that PCSK9 might play a role in downregulation of LDLR protein in certain tumour phenotype. The significance of PCSK9 upregulation in colonic tumour, other than affecting LDLR protein levels, is yet to be understood. In some of the earlier reports, it was reported that PCSK9 deficiency reduces melanoma metastasis in liver by reducing serum cholesterol [25].

The overall objective of our study was to investigate the protein expression of key molecules of cholesterol metabolic pathway in AOM induced colon tumour rat model. Our results establish that in our selected model system, LDLR protein levels are significantly

low in tumour when compared to normal colonic mucosa. We also show that other key molecules of cholesterol pathway, such as HMGCR, SREBPs and PCSK9 have elevated protein expression in colonic tumour. Taken together, our results establish that experimentally induced colonic tumours have an altered lipid and cholesterol protein expression profile with reduced LDLR protein expression, which could be a tumour, acquired phenotype assisting the growth and survival of a subset of colorectal cancer.

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## **Chapter 3**

**Towards developing a cell culture based model to study  
the role of LDLR in CRC development**

### **3.1 Introduction:**

Most of our understanding of the pathogenesis of CRC comes from studies conducted in preclinical models of the disease [1, 2]. Human tumour derived CRC cell lines in particular have been instrumental in elucidating the complex signaling pathways that underline the process of human malignancy. Extensive genetic and epigenetic profiling of cancer cell lines has allowed researchers to genetically manipulate these cell lines by using techniques like homologous recombination, short hairpin RNA (shRNA) mediated gene silencing, gene knockdown and overexpression of specific genes using expression vectors[3, 4]. CRC lines are being widely used for drug screening, biomarker identification and mechanistic studies to delineate signaling pathways that drive tumourigenesis [5]. In comparison to an animal based model cancer cell lines are relatively inexpensive to maintain in the laboratory and are more amenable to genetic manipulation under controlled experimental conditions. Despite of limitations such as their monoclonal nature and the absence of tumour macro and micro-environmental interaction, cell lines have emerged as powerful tools to study the altered signaling pathways in cancer using a reductive approach.

Previous studies investigating the regulation of cholesterol biosynthesis in colonic adenocarcinoma cell lines have reported that colon cancer cells exhibit high endogenous cholesterol synthesis which is not inhibited by exogenous low density lipoprotein (LDL) treatment [6]. In the same study it was demonstrated that cholesterol biosynthesis could be reduced by oxysterol and mevinolin (inhibitor of 3-Hydroxy-3-Methylglutaryl-CoA Reductase) treatment but the cancer cells were unresponsive to LDL treatment. LDL binding and internalization studies showed that in these colon cancer cells the expression of LDLR was significantly downregulated. In another study, six human colonic adenocarcinoma cell lines showed no growth inhibition when cultured in media containing lipoprotein deficient serum. When treated with mevinolin, these colon cancer cells showed significant reduction in cell growth and proliferation which could not be rescued by exogenous LDL. An enzyme linked immunosorbent assay (ELISA) using human/bovine monoclonal antibody against LDLR was used to demonstrate that five out of the six colon cancer cells had a significantly low expression of LDLR [7]. In our previous study, we used azoxymethane (AOM) induced colonic tumour rat model to compare the basal protein

expression of key molecules of cholesterol metabolic pathway in colonic tumour with normal colonic mucosa. Our results showed that tumour tissue had lower levels of LDLR protein expression compared to mucosa along with elevated levels of HMGCR, SREBP1, SREBP2 and PCSK9 indicating that colonic tumour displayed a loss of sterol mediated feedback regulation of cholesterol biosynthesis pathway. To investigate the role of LDLR in CRC development, we selected three colorectal cancer cell lines. HCT 116, HT 29 and DLD 1 that broadly represented the main molecular subtypes of human CRC namely CIN and MSI phenotypes. While HCT 116 and DLD 1 have an MSI phenotype, HT 29 has a microsatellite stable (MSS) or CIN phenotype [3, 8, 9]. HCT 116 and DLD 1 have a defective DNA mismatch repair enzyme system with mutations in DNA repair enzyme *MLH132* and *MSH6* respectively [8, 9]. HT 29 shows karyotype abnormalities characteristic of CIN phenotype. It shows mutations in BRAF and p53 tumour suppressor gene [10]. Both HCT 116 and DLD 1 are near diploid and have mutated KRAS and PIK3CA genes. DLD 1 has mutated p53 while HCT 116 has a wild type p53 [11]. All three of these colon cancer cell lines are fast growing with a doubling time of 20-24 hours. HCT 116 cells were originally derived from primary tumour in ascending or proximal colon of a patient suffering from colorectal carcinoma Duke's D stage[3]. HT29 cells originated from primary tumour of a 44 year old Caucasian female suffering from colorectal adenocarcinoma, Duke's C stage. DLD1 cells were derived from primary tumour of a male with colorectal adenocarcinoma[9]. While HCT116 and DLD1 cells are well differentiated in culture, HT29 cells are poorly differentiated under standard growth conditions.

Statins are pharmacological inhibitors of HMGCR that are routinely used as lipid lowering drugs to treat hypercholesterolemia. There is compelling evidence from *in vitro* studies that have alluded to the possibility of statins exerting anti-tumourigenic effects on growth of cancer cells. By inhibiting HMGCR, statins block the synthesis of mevalonate and its downstream metabolites including farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), which are required for prenylation, and activation of G-protein signaling molecules like RAS and RHO. This is one of the several mechanisms through which statins are now known to negatively regulate cell growth, proliferation, migration and survival of cancer cells [12]. Statins have been shown to induce

G1/S cell cycle arrest, inhibit cell proliferation/viability and induce cellular autophagy in glioblastoma (GBM) and breast cancer cell lines. In prostate cancer cells, statins have been reported to induce apoptosis and inhibit cell invasion by blocking prenylation of signaling proteins such as RAS and RHO [13-15]. In a recent study, it was reported that in HCT 116 cells, simvastatin induces apoptosis by activation of p38 mitogen activated protein kinase (MAPK)-p53-survivin signaling pathway [16]. In another study, statins were shown to upregulate (Phosphatase and tensin homolog) PTEN activity through bone morphogenetic protein (BMP) signaling pathway that resulted in downregulation of PI3K/AKT/mTOR in HCT 116, RKO and HT 29 cells [13].

In this study, we set out to assess the suitability of CRC cell lines which could serve as a human CRC cancer model for investigation of the role of LDLR and cholesterol biosynthesis pathway in tumorigenesis. We began with determining the protein and mRNA expression of the key molecules associated with cholesterol homeostasis that were previously studied in animal model. Additionally, we analyzed the cell viability and proliferation rate of the CRC cells with and without lovastatin and RO 48-8071 (inhibitor of oxidosqualene cyclase enzyme of cholesterol biosynthesis pathway) treatment. We also assessed the ability of CRC cells to modulate mRNA and protein expression of cholesterol homeostasis associated genes in response to lovastatin and RO 48-8071. In this study we show that all the three colorectal cancer cells can serve as ideal pre-clinical models to study the role of LDLR in CRC development. Further, we confirm that lower LDLR expression is correlated with higher cancer cell viability, proliferation and resistance to cholesterol reducing drugs treatment.

### **3.2 Material and Methods**

#### **Cell culture**

Human CRC cell lines, HCT 116 (CCL-247; ATCC), HT 29 (HTB-38; ATCC) and DLD 1 (CCL-221; ATCC) were obtained from ATCC, USA. HCT 116 and HT 29 were maintained in McCoy's 5a Medium Modified (Sigma) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin streptomycin. DLD 1 cell line was maintained in RPMI-1640 (Sigma) supplemented with 10% FBS and 1% penicillin-streptomycin. Cells

were grown as monolayers at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Human normal colonic mucosa cell line (NCM-460), was kindly provided by Dr. Siyaram Pandey, Department of Chemistry/Biochemistry, University of Windsor. NCM 460 cells were maintained in RPMI-1640 media under standard culture conditions.

### **Antibodies and Reagents**

The following antibodies were purchased from Santa Cruz Biotechnology: LDLR (sc-18823;), HMGCR (sc-27578) ), SREBP1 (sc-366); , SREBP2 (sc- 5603),  $\beta$  actin (sc-1616), horse radish peroxidase (HRP) conjugate secondary anti-mouse IgG (sc-2005) and anti- rabbit IgG (sc-2030). PCSK9 antibody (NB300-959) was purchased from Novus Biologicals. Other chemicals used were-lovastatin (Sigma Aldrich), RO 48 -8071 (Cayman Chemicals), high capacity c-DNA reverse transcription kit (Applied Biosystems), power SYBR green mastermix (Applied Biosystems), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Sigma Aldrich).

### **Sample preparation and immunoblotting**

Cells were plated in 6 well plates and cultured in their respective media. On reaching desired confluence, cells were washed with cold phosphate buffered saline (PBS) followed by lysis in Radioimmunoprecipitation assay (RIPA) buffer (see appendix1). Total cell lysate was centrifuged at 12000Xg for 10 minutes at 4°C. Supernatant was collected in sterile tubes and used for protein quantification by Bradford assay. Equal amount of protein was used for SDS-PAGE followed by electro blotting onto PVDF membrane using wet transfer method. Membranes were blocked in 5% skimmed milk for 1 hour at room temperature, followed by overnight incubation in primary antibodies prepared in 5% bovine serum albumin (BSA) TBS-Tween (1% v/v) at 4°C. Membranes were washed three times in TBS-Tween (TBST) and probed with secondary antibodies (1:10000) for 1 hour at room temperature. After washing three times in TBST, proteins were detected using Perkin Elmer Enhanced Chemiluminescence reagent and Fluor-Chem Western blotting imaging system. Protein bands were quantified using ImageJ software (Version 1.42q).

To analyze the time dependent effect of lovastatin and RO 48-8071 on protein expression level of LDLR, HMGCR, SREBP1, SREBP2 and PCSK9 in colorectal cancer

cells, cells were seeded in 6 well culture plates and allowed to grow for 24 hours. Next day cells were treated with 20 $\mu$ M lovastatin (Sigma Aldrich) for 8 hours, 16 hours, 24 hours and 48 hours. Untreated cells growing in their respective media at each time point served as control for the treated sample. At the end of each time point, protein was extracted, quantified and used for immunoblotting by the same method described above. For RO 48-8071 treatment, cells were treated for 24 hours before protein extraction and Western blot analysis.

### RNA isolation and Quantitative Real time PCR (Q-RT PCR)

Total RNA was extracted from control and treated cells using TRIzol reagent (Invitrogen) followed by spectrophotometric quantification. 250 ng of total RNA was used to synthesize cDNA in a 20 $\mu$ l reaction. 1 $\mu$ L of cDNA along with 1  $\mu$ L primer (final concentration 10mM) and 10  $\mu$ L of 2X SYBR green PCR master mix (ABI) was used in a 20  $\mu$ l reaction to perform quantitative Real time PCR in Applied Biosystems 7300 PCR system (ABI). The amplification was performed under the following conditions: 10 min at 95 $^{\circ}$ C for one cycle, 40 cycles of 95 $^{\circ}$ C for 15 sec and 60 $^{\circ}$ C for 60 sec. The relative levels of gene expression were quantified by using the comparative CT method of  $-\Delta\Delta C_t$  [17].  $\beta$ -actin was used as endogenous control for data normalization.

Gene	Primer Sequence
LDLR-F	tgctactggccaaggacat
LDLR-R	ctgggtggtcggtacagtg
HMGCR-F	gacctttctagagcgagtgcac
HMGCR-R	gctatattctcccttacttcatcctg
SREBP1-F	acaagattgtggagctcaagg
SREBP1-R	tgcgcaagacagcagattta
SREBP2-F	gtgcagacagtcgctacacc
SREBP2-R	aatctgaggctgaaccagga
PCSK9-F	gtaccgggcggatgaatac
PCSK9-R	tctgtatgctggtgtctaggaga
$\beta$ -Actin-F	ccaaccgagagaagatga
$\beta$ -Actin-R	ccagaggcgtaggggatag

**Table 3.1 Primer sequence**

### **Cell viability assay**

To analyze cell viability and proliferation rate, cells were seeded with a density of 2000 cells/well in 96 well plate (BD Biosciences, San Jose, CA) and cultured overnight to allow them to adhere. At the end of desired time point, cells were washed with PBS. 10  $\mu$ L of 5 mg/ml MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] solution was added to a final concentration of 0.5 mg/ml, followed by incubation for 4 hours. After 4 hours, MTT solution and media was removed and 100  $\mu$ L DMSO was added to each well to dissolve the formazan crystals. Absorbance was measured at 570 nm using microplate reader.

To study the effect of lovastatin and RO 48-8071 on cell viability and proliferation rate of colorectal cancer cells, cells were seeded at a density of 2000 cells/well and allowed to grow for 24 hour before treating them with 20 $\mu$ M lovastatin (Sigma) or 20 $\mu$ M RO 48-8071 for 24 hours. At the end of time point, MTT assay was performed as mentioned above.

### **Statistical analysis**

*In vitro* results are shown as mean  $\pm$  SEM (standard error of mean). Comparisons of datasets were performed using unpaired Student's t test (experimental group compared with control group) or ANOVA test to compare more than two experimental conditions. P values were calculated using GraphPad Prism 3.0 software (GraphPad software). Differences were considered significant for P values  $\leq$  0.05.

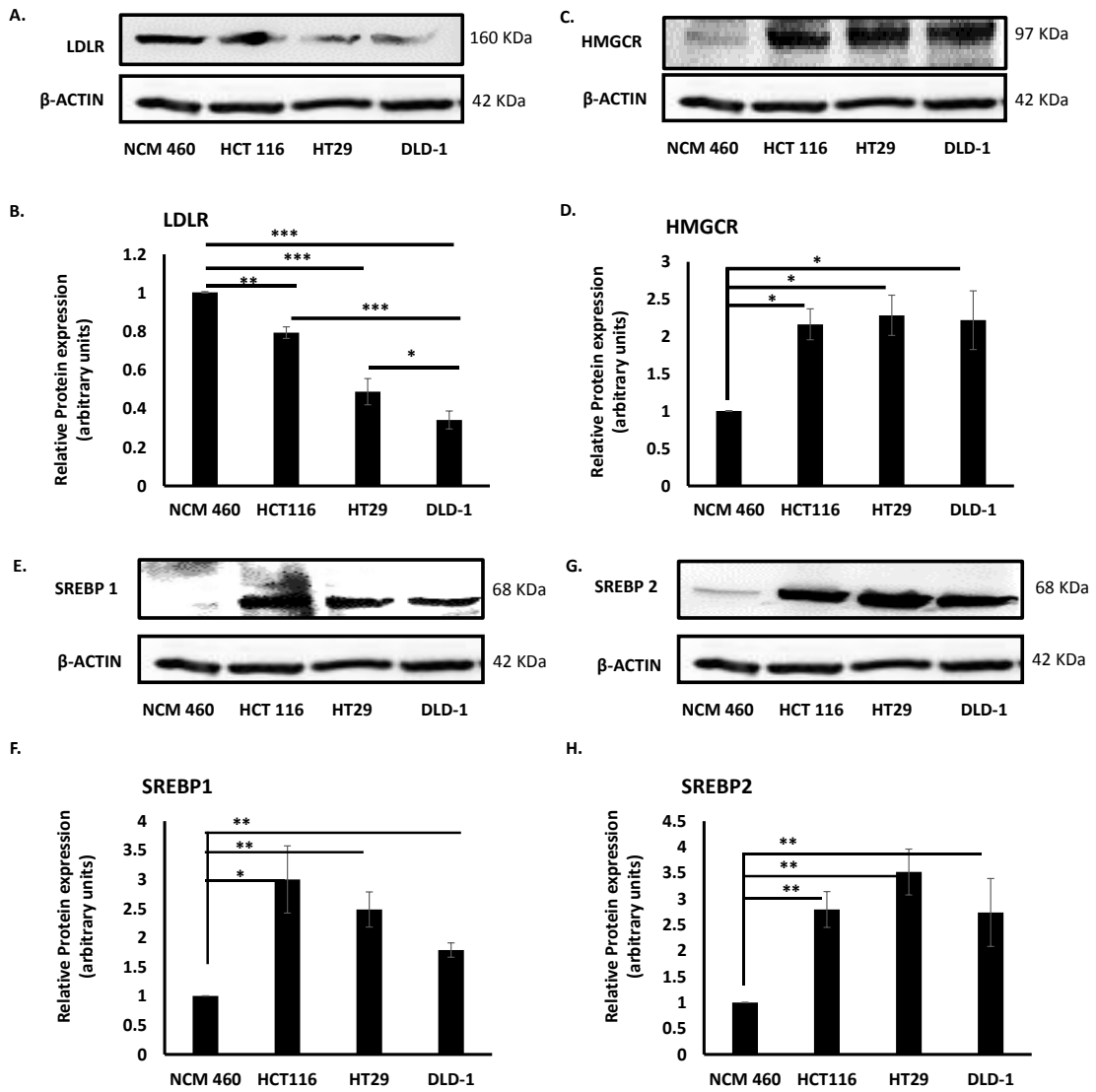
## **3.3 Result**

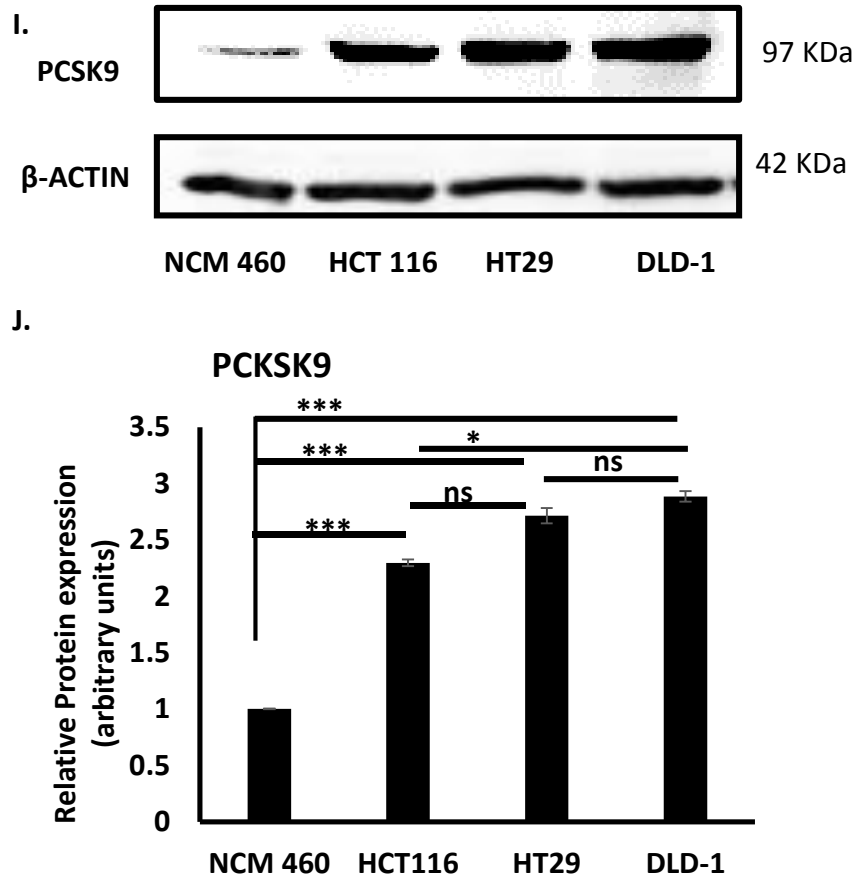
### **Human CRC cell lines mimic AOM induced colonic tumour rat model for protein expression levels of LDLR, HMGCR, SREBPs and PCSK9**

Our first objective was to establish cell culture based model that would recapitulate the protein expression of cholesterol homeostasis associated genes we had previously observed in animal model. To that effect, we compared the expression levels of LDLR, HMGCR, SREBPs and PCSK9 between colorectal cancer cells and untransformed colonic mucosa cells NCM 460. Our results showed significantly lower expression of LDLR

protein in HCT 116, HT 29 and DLD 1 when compared to NCM 460 (Figure 3.1A). We observed that all three colorectal cancer cell lines showed variability in downregulation of LDLR protein, with DLD 1 cells showing the lowest expression of LDLR amongst all three CRC cell lines. Consistent with our findings in animal model and previous reports on expression of HMGCR in tumour, we found that HMGCR protein expression level was significantly elevated in all three colorectal cancer cell lines in comparison with NCM 460 (Figure 3.1B). Protein expression of transcriptionally active, mature form (~68KDa) of SREBP1 and SREBP2 was also significantly higher in HCT 116, HT 29 and DLD 1 cells when compared to NCM 460 cells (Figure 3.1C, 3.1D). Furthermore, we observed that PCSK9 (~72kDa) protein level was increased in colorectal cancer cell lines compared to untransformed mucosa cells (Figure 3.1E). Taken together, our results for this study showed that our selected panel of human colorectal cancer cells, HCT 116, HT 29 and DLD 1 show significantly elevated expression of HMGCR, SREBP1, SREBP2 and PCSK9 in comparison to NCM 460 cells. Furthermore, LDLR protein expression is significantly reduced in all three colorectal cancer cell lines with DLD 1 showing the lowest expression of LDLR protein.



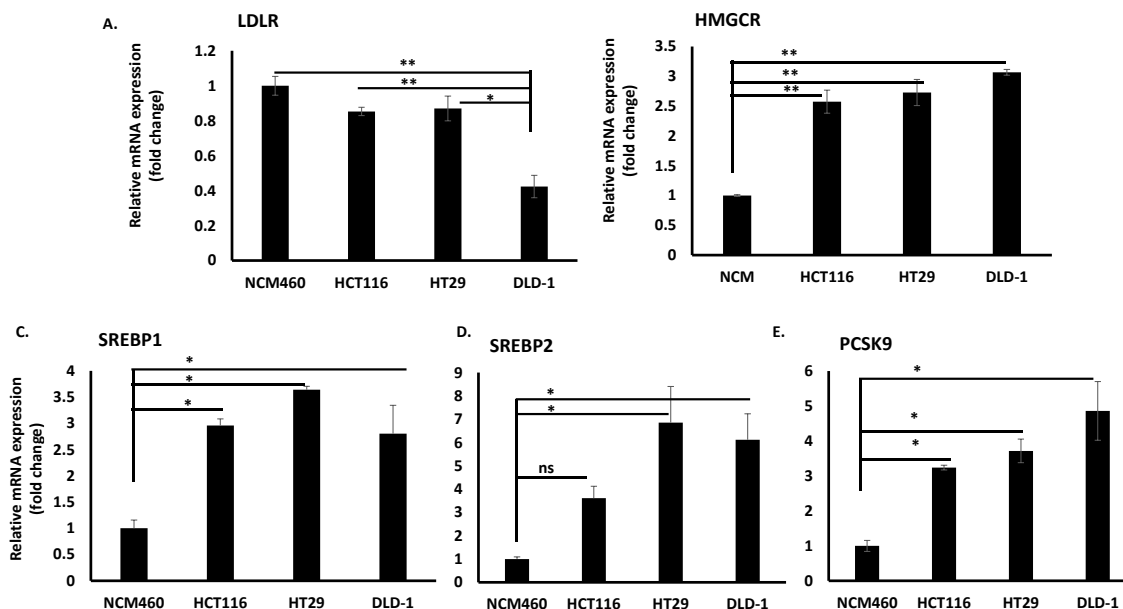




**Figure 3.1** CRC cells show differential protein expression of cholesterol homeostasis related genes in comparison with untransformed colonic mucosa cells. CRC cells (HCT 116, HT 29 and DLD 1) and untransformed colonic mucosa cells (NCM 460) were harvested for Western blot analysis. Representative image and quantification ( $n=3$ ) showing expression level of (A and B) LDLR, (C and D) HMGCR, (E and F) SREBP1 (G and H) SREBP2 (I and J) PCSK9 in HCT116, HT29 and DLD1, compared to NCM-460 cell line. Experiment was repeated three times. Results are represented as mean  $\pm$  SEM (Standard error of the mean), \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ . Statistical analyses were performed using one-way ANOVA. P values were calculated using Graph-Pad Prism 3.0 software.

### **Human CRC cell lines show differential mRNA expression of cholesterol homeostasis associated genes**

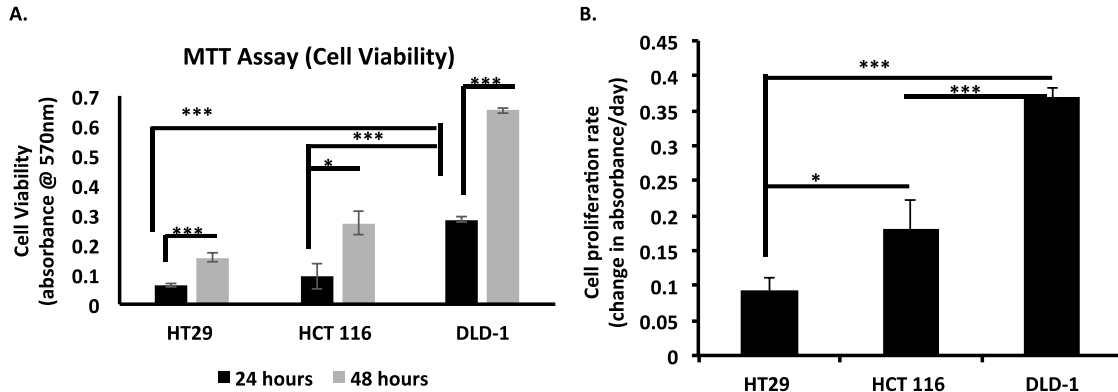
To analyze the relative mRNA expression of cholesterol metabolic pathway associated genes (LDLR, HMGCR, SREBP2 and PCSK9), quantitative real time PCR was performed. Results of q-RT PCR showed that LDLR mRNA level was significantly lower in colon cancer cells compared to NCM 460. Between the different colorectal cancer cell lines, DLD 1 cells showed lowest LDLR mRNA expression (Figure 3.2A). HMGCR mRNA expression was significantly elevated in all three colon cancer cell lines compared to NCM 460 (Figure 3.2B). All three CRC cells showed high mRNA expression for SREBPs (SREBP1 and SREBP2) (Figure. 3.2C, 3.2D). PCSK9 mRNA expression was elevated in all three cancer cell lines with DLD 1 showing the highest expression (Figure 3.2E). To summarize, we concluded that the transcripts levels of cholesterol homeostasis associated genes corroborated the protein expression data analyzed in the previous study.



**Figure 3.2 CRC cells show differential gene expression of cholesterol homeostasis related genes in comparison with normal colonic mucosa cells.** CRC cells (*HCT 116*, *HT 29* and *DLD 1*) and normal colonic mucosa cells (*NCM 460*) were cultured overnight in 6-well plate and used for total RNA extraction, followed by cDNA synthesis and quantitative real time PCR to analyze relative mRNA expression. (A) *LDLR* mRNA level was low in *HCT 116*, *HT 29* and *DLD 1* cells compared to *NCM 460*. *DLD 1* cells showed the lowest expression of *LDLR* mRNA. (B) *HMGCR*, (C) *SREBP1*, (D) *SREBP2* and (E) *PCSK9* mRNA expression were significantly higher in *HCT 116* *HT 29* and *DLD 1* cells compared to *NCM 460*. Experiment was repeated three times. Results are represented as mean  $\pm$  SEM (Standard error of the mean), \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ . Statistical analyses were performed using one-way ANOVA. *P* values were calculated using Graph-Pad Prism 3.0 software.

### **DLD 1 cells have greater cell viability and higher proliferation rate**

The mRNA and protein expression data for cholesterol homeostasis related genes suggested that colorectal cancer cells have an upregulated cholesterol biosynthesis pathway and low LDLR expression, which indicates that they have a deregulated cholesterol homeostasis. Next we measured the effect of downregulation of LDLR protein affects the growth and cell viability of the cancer cells. To this end, we determined cell viability using MTT assay. 2000 cells/well for each cell line, were seeded in replicates of six, in a 96 well plate and grown for 24 and 48 hours before performing MTT assay. After 24 hours, we observed that DLD 1 cells showed higher cell viability (Figure 3.3A) and proliferation rate (Figure 3.3B) compared to HCT 116 and HT 29 cells (Figure 3.3A and B). This supports our hypothesis that lower LDLR protein expression is associated with increased cancer cell survival and growth.



**Figure 3.3 DLD 1 cells show greater cell viability and higher cell proliferation rate.** CRC cells (HCT 116, HT 29 and DLD 1) were plated in 96 well plate at a cell density of 2000 cells/well in replicates of 6. After 24 and 48 hours MTT assay was performed. (A) Absorbance at 570nm showing cell viability after 24 hours and 48 hours for HCT 116, HT 29 and DLD 1. (B) Difference in cell proliferation rate of HCT 116, HT 29 and DLD 1 cells. DLD 1 cells showed highest cell proliferation and viability amongst the three CRC cells. Experiment was repeated three times. Results are represented as mean  $\pm$  SEM (Standard error of the mean), \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ . Statistical analyses were performed using (A) student t-test and (B) One-way ANOVA. P values were calculated using Graph-Pad Prism 3.0 software.

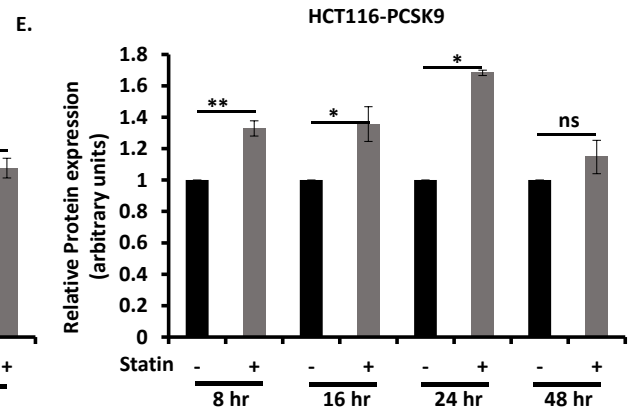
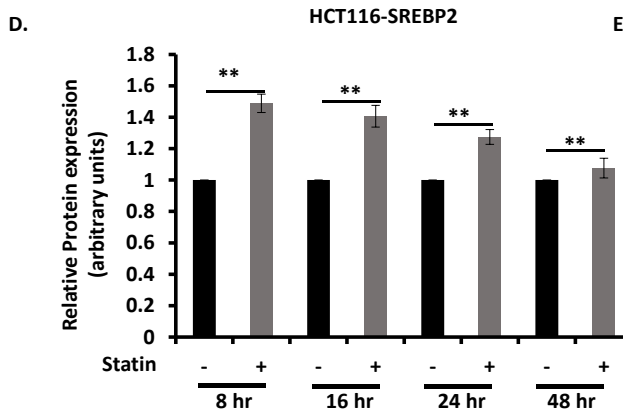
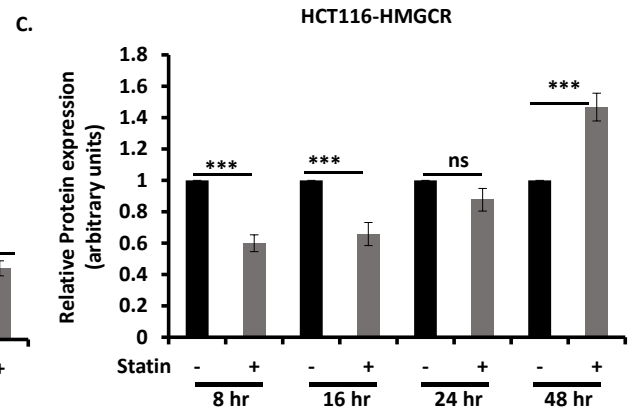
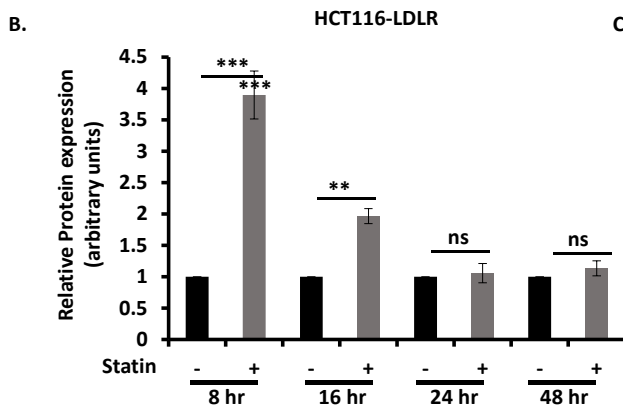
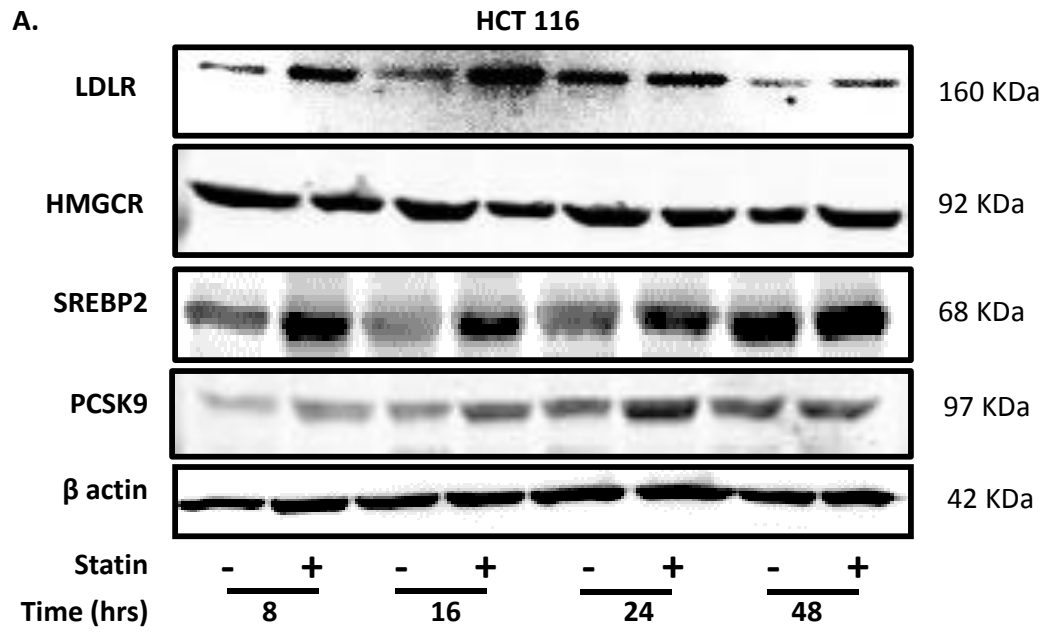
### **Lovastatin treatment is able to induce LDLR protein upregulation in HCT 116 and HT 29 but not in DLD 1 cells**

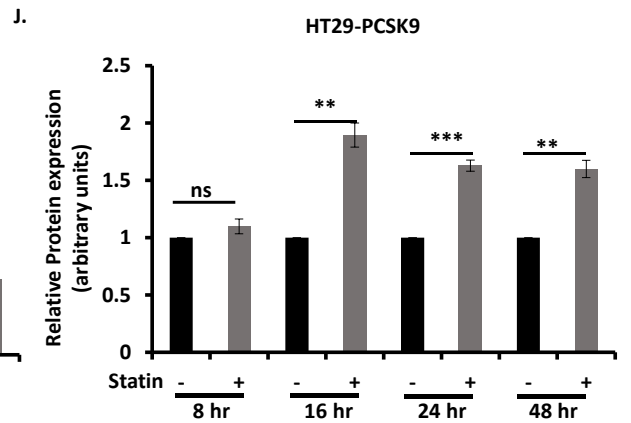
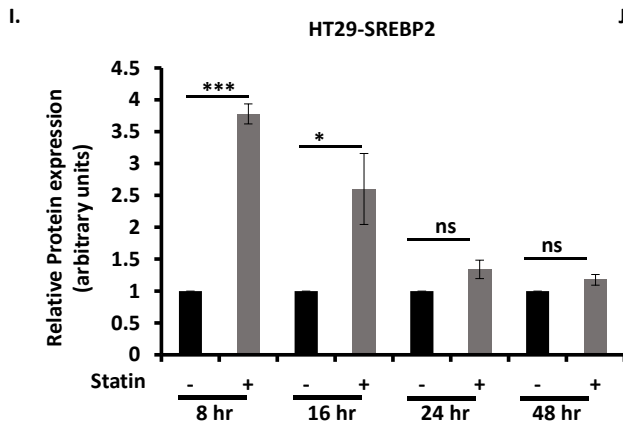
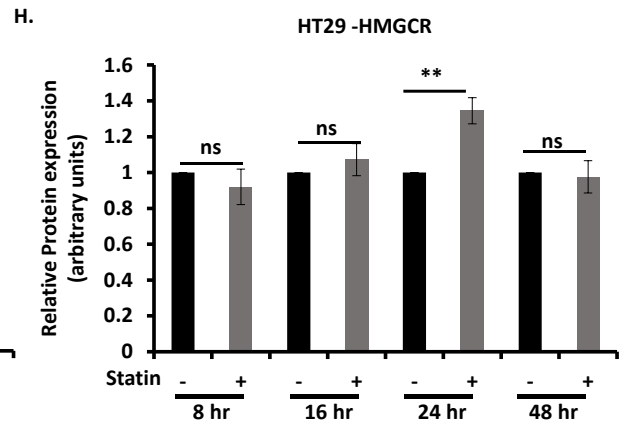
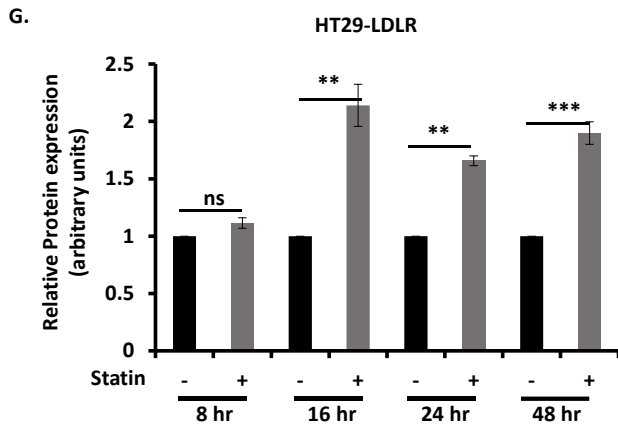
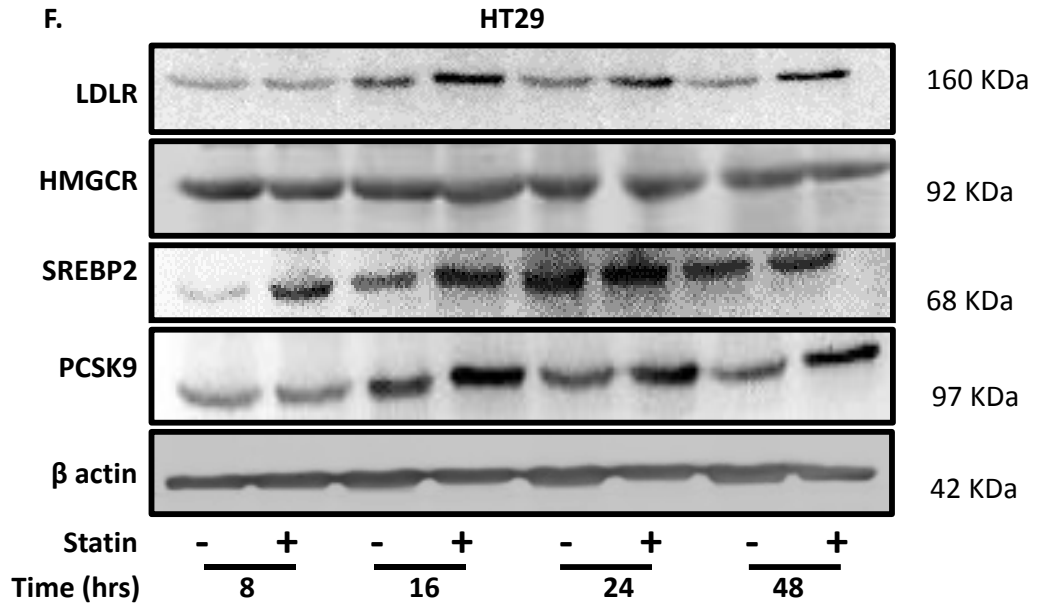
To further characterize the CRC cell lines, we studied their response to cholesterol reducing drugs such as lovastatin. Statins binds to HMGCR, the rate-limiting enzyme of cholesterol biosynthetic pathway, blocking the synthesis of cholesterol and several non-sterol intermediates such as isoprenoids. The downstream effect of this inhibition is upregulation of LDLR expression, which reduces serum cholesterol levels in patients suffering from hypercholesterolemia or atherosclerosis. Studies in prostate cancer cells such as PC-3 and DU145, have shown that these cancer cells do not respond to statins by upregulating LDLR protein expression [18, 19]. This is due to defective sterol dependent negative feedback regulation of LDLR and HMGCR. To study the effect of statins, we cultured colorectal cancer cells in 6 well plates overnight in their respective media followed by treatment with or without 20  $\mu$ M lovastatin for 8 hours, 16 hours, 24 hours and 48 hours. At the end of each time point, protein was extracted to analyze the response of colorectal cancer cells to lovastatin followed by Immuno-blotting. All three colorectal cancer cell lines showed differential response to lovastatin treatment. HCT116 showed an early response to lovastatin treatment by upregulating LDLR protein expression at 8 and 16 hours (Figure 3.4B) with a concomitant decrease in HMGCR at the same points (Figure 3.4C). However, after 48 hours of lovastatin treatment HCT 116 cells significantly upregulated HMGCR protein level. We looked at the protein expression of transcriptionally active form of SREBP2, which regulates the transcription of cholesterol pathway associated genes and found that it was significantly elevated in lovastatin treated samples at all time points (Figure 3.4D). We also observed an elevation in PCSK9 protein expression in HCT 116 cells treated with lovastatin (Figure 3.4E). (Figure 3.4C). The response of HT 29 cells to lovastatin treatment was similar to HCT 116. We observed an increase in LDLR expression between 16 and 48 hours (Figure 3.4G). Lovastatin did not seem to have an effect on HMGCR protein expression though we did see an increase in protein level after 24 hours (Figure 3.4H). SREBP2 and PCSK9 protein levels were also significantly elevated after lovastatin treatment in HT 29 cells (Figure 3.4 I and Figure 3.4J).

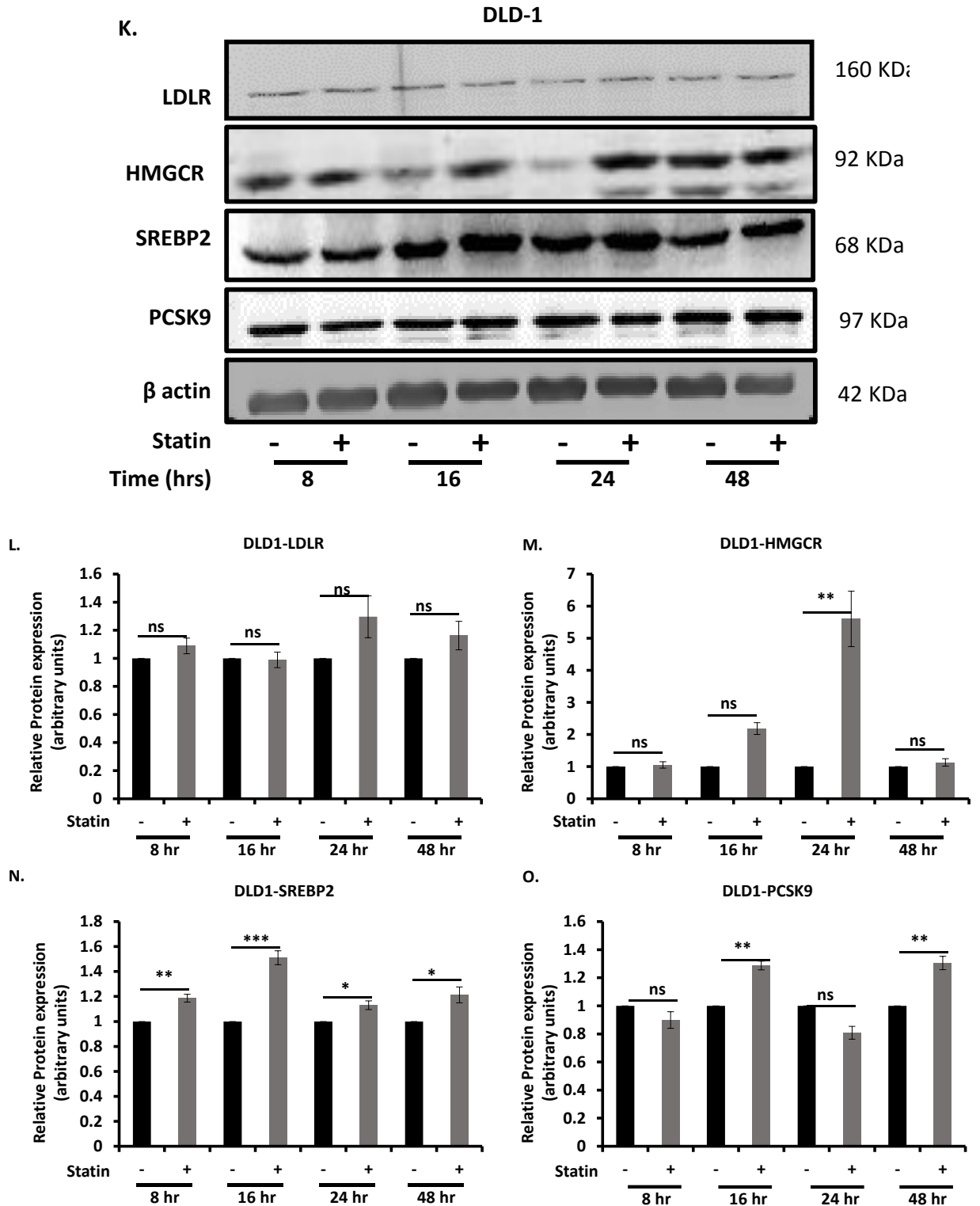
In DLD 1 cells, we did not observe any change in LDLR protein expression in response to lovastatin as observed in HCT 116 and HT 29 cells (Figure 3.4 L). Around 24 hours, we observed a significant elevation in HMGCR protein expression in lovastatin treated DLD 1 cells compared to untreated control (Figure 3.4M). SREBP 2 showed an early elevation in protein level at 8 hours in response to statin followed by a steady decline at later time points (Figure 3.4N). Lovastatin treatment was able to induce PCSK9 protein expression between 16 and 48 hours (Figure 3.4O).

In summary, all three colorectal cancer cell lines showed variable response to lovastatin treatment with respect to increase in protein expression of cholesterol metabolic pathway associated genes. HCT 116 and HT 29 cells responded to lovastatin treatment by upregulating LDLR in time dependent manner. However, lovastatin was unable to induce LDLR protein expression in DLD1 cells which exhibited the lowest level of LDLR protein amongst the three cancer cell lines as shown in the previous study.









**Figure 3.4** Lovastatin treatment increases LDLR protein expression in HCT 116 and HT 29 cells but not in DLD 1 cells. CRC (HCT 116, HT 29, DLD 1) were plated in 6 well tissue culture plates and grown in their respective media for 24 hours before treating them

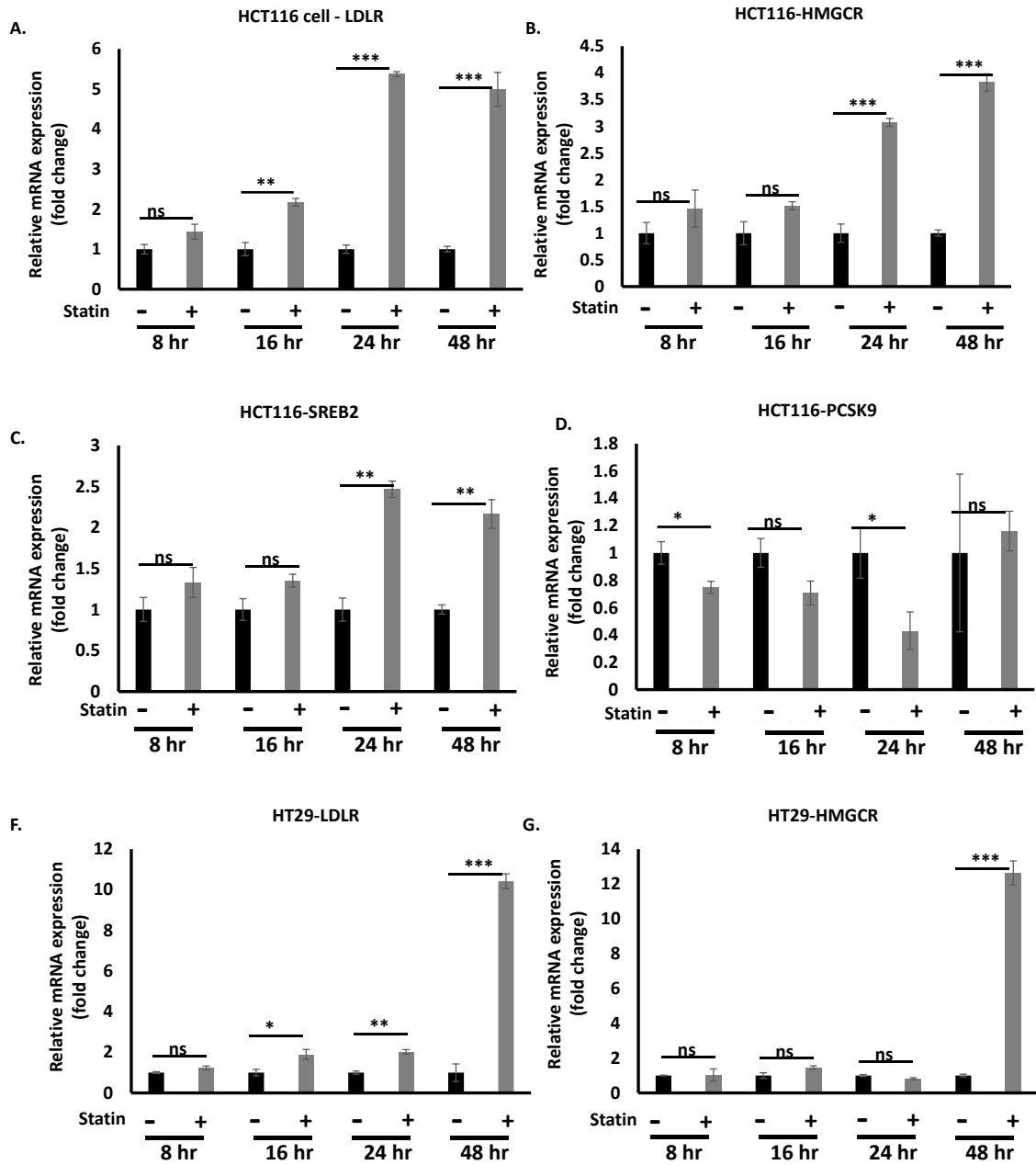
*with lovastatin (20 $\mu$ M final concentration) for 8 hours, 16 hours 24 hours and 48 hours under standard culture conditions. Untreated control for each time point had cells growing in cell specific media supplemented with 10% FBS and 1% penicillin-streptomycin. At the end of each time point, cells from treated group and control group were used to extract whole cell lysate. Equal amount of protein was loaded onto SDS PAGE followed by Western blot analyses. Representative image and quantification (n=3) showing expression levels of LDLR, HMGCR, SREBP2 and PCSK9 in (A-E) HCT116, (F-J) HT29 and (K-O) DLD1. Experiment was repeated three times. Results are represented as mean  $\pm$  SEM (Standard error of the mean), \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ . Statistical analyses were performed using student t-test. P values were calculated using Graph-Pad Prism 3.0 software.*

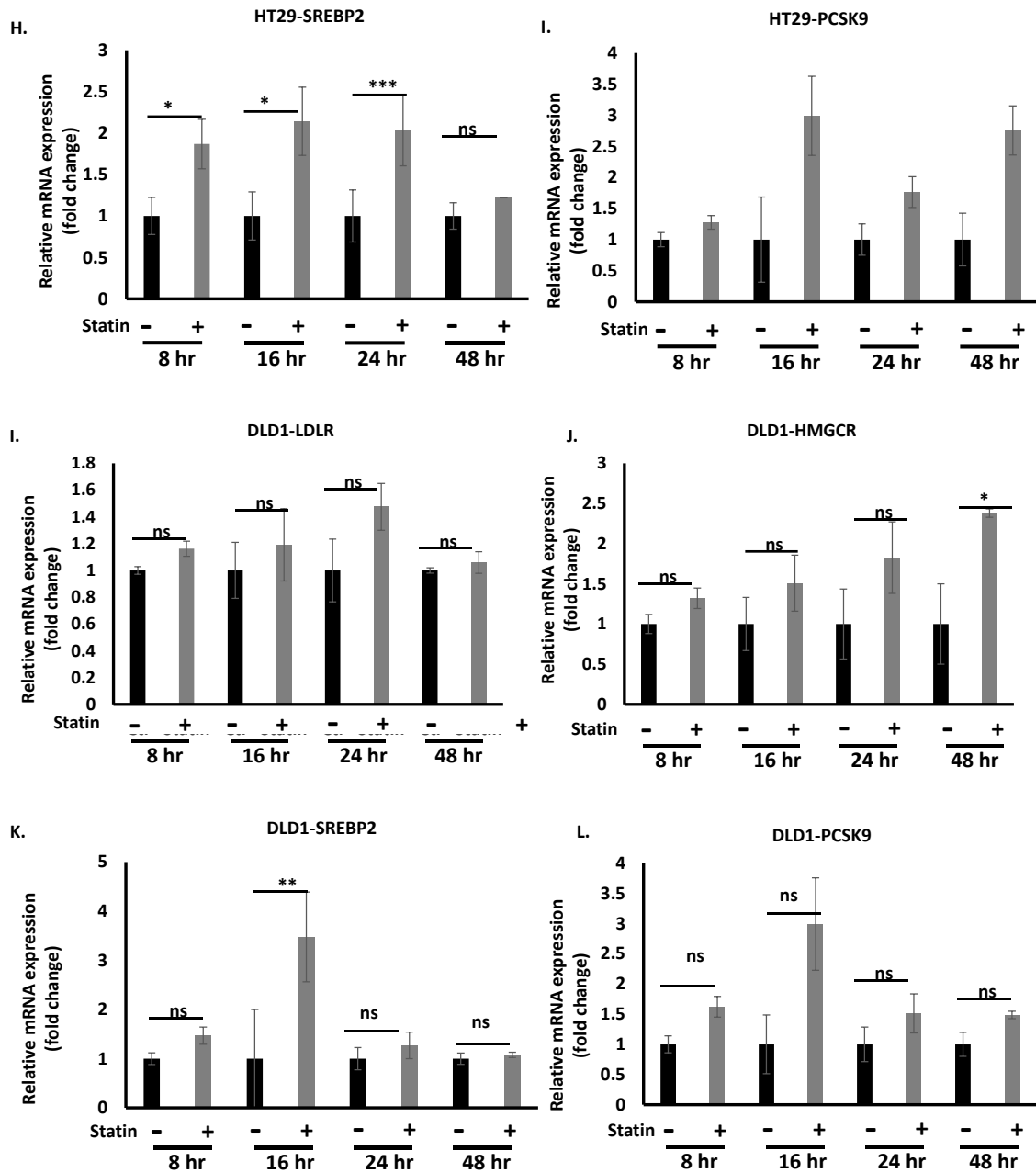
### **DLD1 cells do not upregulate LDLR mRNA in response to lovastatin treatment unlike HCT 116 and HT 29 cells**

To determine whether the time-dependent response of the colon cancer cell lines to lovastatin can be seen at mRNA level, we extracted total RNA from control and lovastatin treated cells and performed quantitative real time PCR (q-RT PCR). Analysis of q-RT PCR results showed that HCT 116 cells responded to statin treatment by significantly upregulating LDLR mRNA expression at almost all time points (Figure 3.5A). HMGCR mRNA expression was elevated around 24 and 48 hours in lovastatin treated HCT 116 cells (Figure 3.5B). There was an elevation in SREBP2 transcript level however, the results were only significant between 24 and 48 hours (Figure 3.5C). In contrast, upregulation of PCSK9 transcript level was not significant at any time point (Figure 3.5D). A similar trend was observed in HT 29 cells which showed a consistent increase in LDLR mRNA levels in response to lovastatin (Figure 3.5E), however HMGCR mRNA was significantly upregulated only at 48 hours (Figure 3.5F). We also noted that lovastatin is able to induce SREBP2 (Figure 3.5G) and PCSK9 (Figure 3.5H) mRNA expression between 16-48 hours.

Unlike HCT 116 and HT 29 cells, DLD 1 cells did not show any significant increase in LDLR mRNA expression in response to lovastatin treatment at any given time point. (Figure 3.5I). Lovastatin did induce HMGCR mRNA expression after 48 hours (Figure 3.5J). In DLD 1 cells, we saw a significant elevation in SREBP2 transcript level only after 16 hours of lovastatin treatment (Figure 3.5K). There was no significant difference in PCSK9 transcript level between untreated control and lovastatin treated DLD 1 cells (Figure 3.5L).

The results of this study demonstrate that HCT116 and HT29 respond to lovastatin by upregulating LDLR mRNA and protein expression, whereas, DLD 1 cell are resistant to lovastatin treatment, suggesting a disconnect in LDLR-HMGCR feedback regulation.





**Figure 3.5** mRNA expression levels of LDLR, HMGCR, SREBP2 and PCSK9 are upregulated in HCT 116 and HT 29 cells in response to lovastatin but not in DLD 1 cells. Colorectal cancer cells (HCT 116, HT 29 and DLD 1) treated with or without lovastatin (20 $\mu$ M) for 8, 16, 24 and 48 hours, were harvested for total RNA and q-RT PCR analysis. Bar graph shows relative mRNA expression of LDLR, HMGCR, SREBP2 and PCSK9 in (A-D) HCT 116 cells, (E-H) HT 29 cells and (I-L) DLD 1 cells. Data was normalized using  $\beta$  actin as endogenous control. All experiments were repeated three

*times. Results are represented as mean  $\pm$  SEM (Standard error of the mean), \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ . Statistical analyses were performed using student t-test between treated and untreated samples. P values were calculated using Graph-Pad Prism 3.0 software.*



## **DLD 1 cells do not upregulate LDLR mRNA and protein expression in response to RO 48-8071 unlike HCT 116 and HT 29**

Our previous results showed that DLD1 cells fail to respond to lovastatin treatment by upregulating LDLR expression even though they do upregulate HMGCR at a later time point. Resistance of DLD 1 cells to lovastatin treatment could be attributed to higher expression of HMGCR v1 splice variant which lacks exon 13 known to contain the statin binding domain [20, 21]. Another reason for DLD 1 cells not upregulating LDLR expression in response to lovastatin could be a loss of LDLR feedback regulation, which has earlier been observed in some prostate cancer cells [19]. To analyze if DLD 1 cells exhibit a loss of sterol mediated negative feedback regulation of LDLR, we selected another cholesterol reducing drug, which inhibits the enzymatic activity of a mevalonate pathway enzyme called oxidosqualene cyclase (OSC). OSC catalyzes the conversion of 2, 3- squalene epoxide to lanosterol, reducing the level of intracellular cholesterol without depleting the supply of isoprenoid intermediates [22].

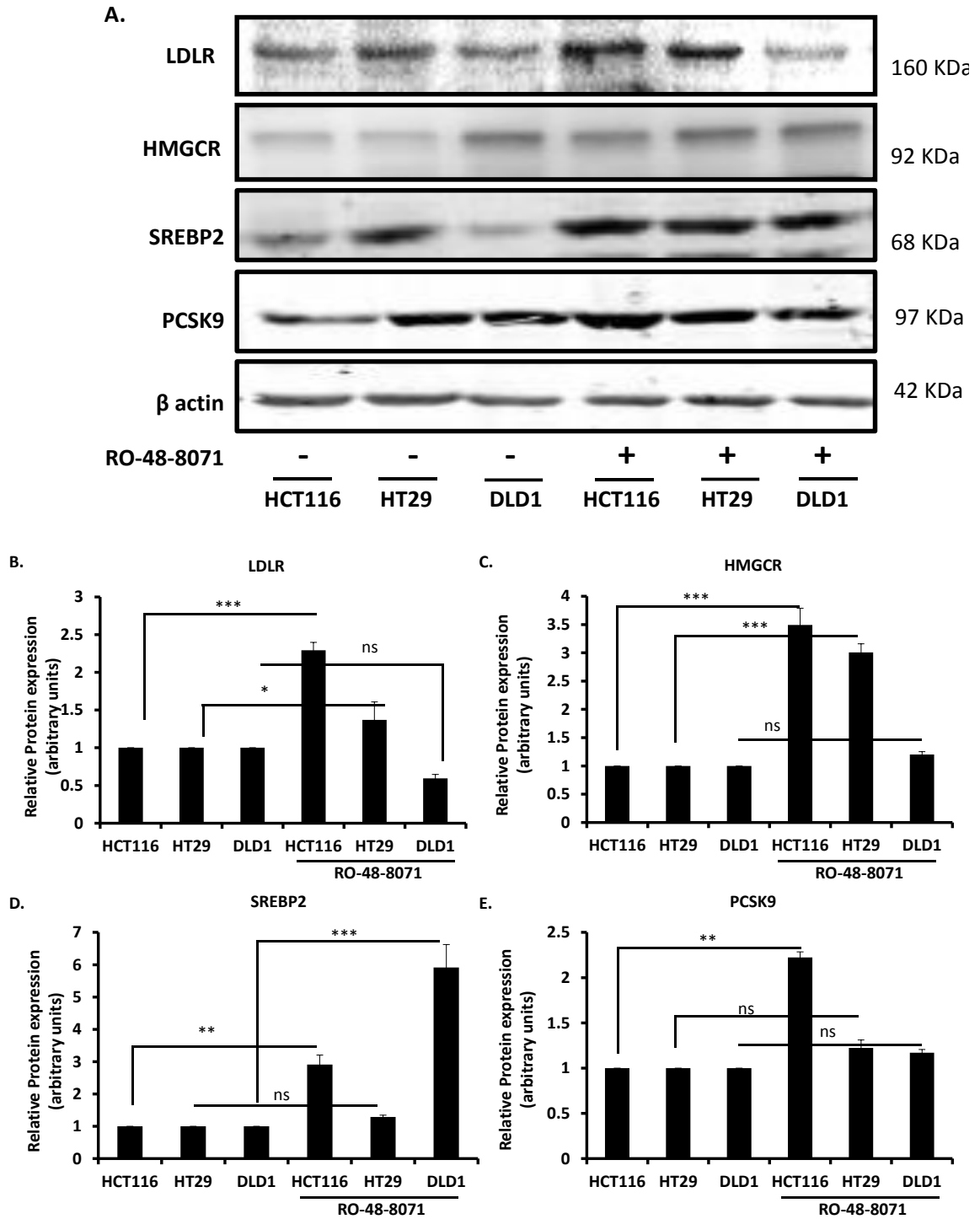
For the purpose of this study, we seeded HCT 116, HT 29 and DLD 1 cells in 6 well plates and cultured them for 24 hours in their respective media before treating them with 20  $\mu$ M of RO 48-8071 for 24 hours. At the end of each time point, total RNA and protein was extracted for mRNA and protein expression study.

Results of immunoblotting (Figure 3.6A), showed that HCT 116 upregulated the protein expression of LDLR (Figure 3.6B), HMGCR (Figure 3.6C), SREBP2 (Figure 3.6D) and PCSK9 (Figure 3.6E) in response to 24 hours of RO 48-8071 treatment. HT 29 cells showed a significant increase in LDLR (Figure 3.6B) and HMGCR (Figure 3.6C) protein levels after RO 48-8071 treatment. Unexpectedly there was no significant change in SREBP2 (Figure 3.6D) and PCSK9 (Figure 3.6E) protein levels. DLD 1 cells again failed to upregulate LDLR (Figure 3.6B) and HMGCR (Figure 3.6C) protein levels in response to RO 48-8071 even though there was a significant elevation in protein level of mature form of SREBP2 (Figure 3.6D). PCSK9 (Figure 3.6E) protein expression remained unaltered by RO 48-8071 treatment.

At the mRNA level, LDLR expression was significantly increased in HCT 116 and HT 29 cells but not in DLD 1 cell after RO 48-8071 treatment (Figure 3.7A). HMGCR

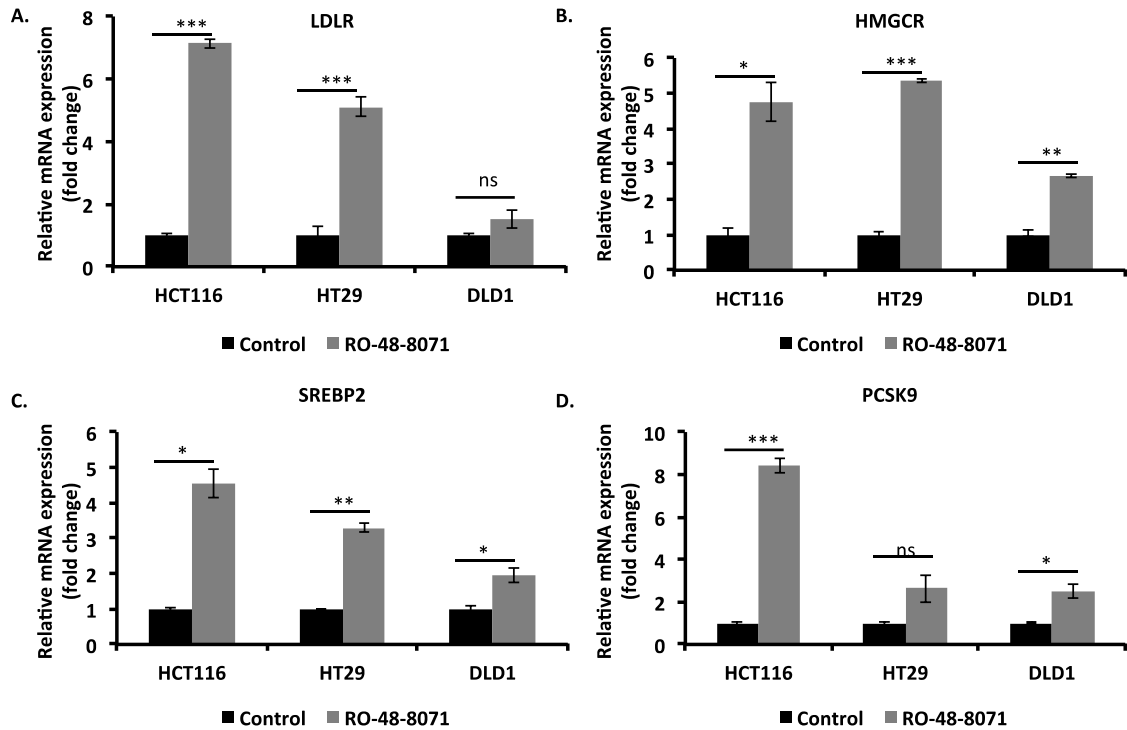
(Figure 3.7B) and SREBP2 (Figure 3.7C) mRNA level was significantly upregulated after 24 hours of RO 48-8071 treatment in all three colorectal cancer cell lines with highest increase in HCT 116 and HT 29 cells. PCSK9 (Figure 3.7D) mRNA expression was increased in HCT 116 cells and DLD 1, whereas HT29 showed increase in PCSK9 transcript level but it was statistically insignificant.

Taken together, we conclude that lovastatin and RO 48-8071 induce similar response in HCT 116, HT 29 and DLD 1 cells with regards to altering the expression of key molecules of cholesterol metabolic pathway. HCT 116 and HT 29 colorectal cancer cell lines respond to lovastatin and RO 48-8071 by upregulating mRNA and protein expression of cholesterol biosynthetic and uptake genes, however DLD 1 cells show a resistant phenotype by not inducing LDLR expression in response to lovastatin and RO 48-8071.



**Figure 3.6** DLD 1 cells do not upregulate LDLR protein expression in response to RO 48-8071. CRC cells were plated in 6 well culture plates and cultured overnight before treatment with 20μM of RO 48-8071 for 24 hours. At the end of time point, cells were harvested for protein extraction and Western blot analyses. (A) Representative image

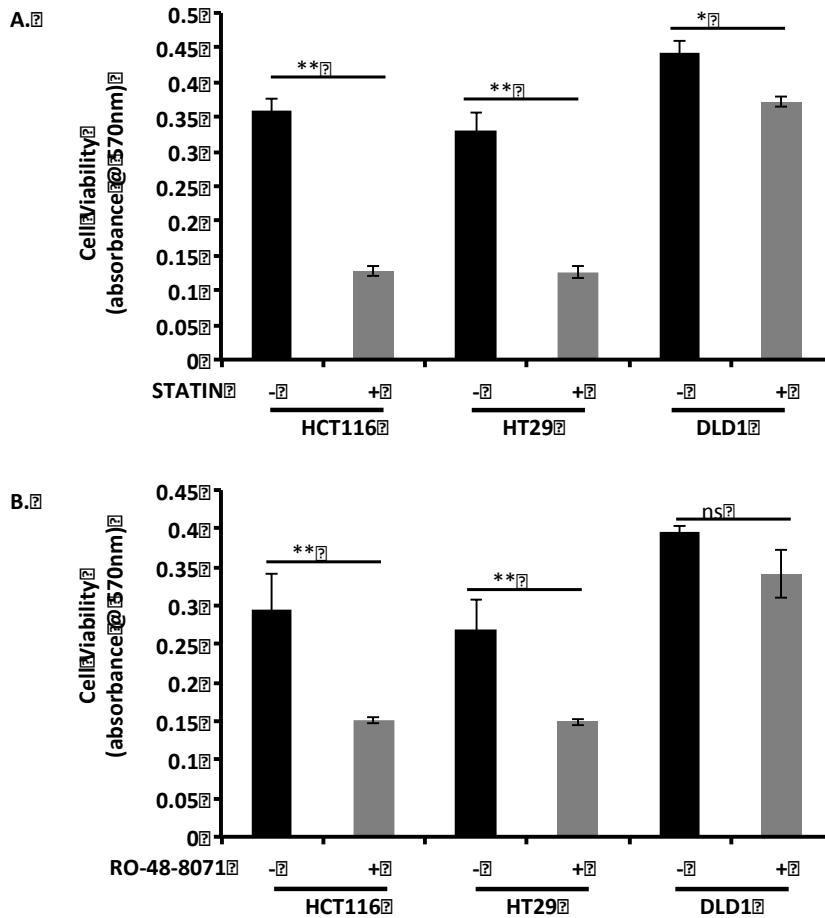
*showing protein expression of LDLR, HMGCR, SREBP2 and PCSK9 in RO 48-8071 treated and untreated HCT 116, HT 29 and DLD 1 cells. Bar graphs showing quantification (n=3) of (B) LDLR, (C) HMGCR, (D) SREBP2 and (E) PCSK9 protein expression after 24 hours of RO 48-8071 treatment in HCT 116, HT 29 and DLD 1 cells. Results are represented as mean  $\pm$  SEM (Standard error of the mean), \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ . Statistical analyses were performed using unpaired student t-test between treated and untreated samples. P values were calculated using Graph-Pad Prism 3.0 software.*



**Figure 3.7** DLD 1 cells do not upregulate LDLR mRNA expression in response to RO 48-8071. CRC cells were plated in 6 well plates and cultured overnight followed by treatment with 20 $\mu$ M of RO 48-8071 for 24 hours. At the end of time point, total RNA was extracted from the cells and used for cDNA synthesis and quantitative real time PCR (q-RT PCR). Bar graph shows (A) LDLR, (B) HMGCR, (C) SREBP2 and (D) PCSK9 in HCT 116, HT 29 and DLD 1 in response to RO 48-8071 treatment. All experiments were repeated three times. Results are represented as mean  $\pm$  SEM (Standard error of the mean), \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ . Statistical analyses were performed using unpaired student t-test between treated and untreated samples. P values were calculated using Graph-Pad Prism 3.0 software.

### **DLD 1 cells show minimum reduction of cell viability in response to lovastatin and RO 48-8071**

Previously we had observed that DLD 1 cells which showed the lowest expression of LDLR protein amongst the three colorectal cancer cells was associated with maximum cell proliferation and viability. We further observed that DLD 1 did not respond to lovastatin and RO 48-8071 treatment by upregulating LDLR expression unlike HCT 116 and HT 29 suggesting that they are resistant to cholesterol reducing drugs. Therefore, it was of interest to us to determine if the colorectal cancer cells, DLD 1 in particular are able to maintain their cell viability after treatment with lovastatin and RO 48-8071. Cells were plated at a density of 2000cells/well in replicates of six, in 96 well plates and allowed to adhere overnight before treating with 20 $\mu$ M of lovastatin and RO 48-8071 for 24 hours. At the end of time point, MTT assay was performed. Our results showed that following lovastatin treatment, there was a significant reduction in HCT 116 and HT 29 cell viability whereas DLD 1 showed no change in cell viability. Similar observation was made for RO 48-8071 treatment in which DLD 1 cells maintained cell viability after 24 hours of RO 48-8071 treatment.



**Figure 3.8 DLD 1 cells show minimum reduction of cell viability in response to lovastatin and RO 48-8071.** CRC cells (HCT116, HT29 and DLD1) were plated in 96 well plate at a cell density of 2000 cells/well in replicates of 6 and cultured overnight. Cells were treated with 20 $\mu$ M of lovastatin and RO 48-8071 for 24 hours followed by MTT assay. (A) Absorbance at 570nm showing cell viability after 24 hours of lovastatin treatment in HCT116, HT29 and DLD1. (B) Absorbance at 570nm showing cell viability after 24 hours of RO 48-8071 treatment in HCT116, HT29 and DLD1. Experiment was repeated three times. Results are represented as mean  $\pm$  SEM (Standard error of the mean), \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ . Statistical analyses were performed using student t-test. P values were calculated using Graph-Pad Prism 3.0 software.

### **3.4 Discussion**

Our previous work in animal model was suggestive of a dysregulation in cholesterol homeostasis in colonic tumours, which was characterized by elevated protein expression of cholesterol, and lipid biosynthesis pathway associated genes such as HMGCR, SREBP1 and SREBP2. Additionally we had observed that colonic tumours have a significant low expression of LDLR protein and elevated PCSK9 protein expression which could be the factor responsible for high turnover of LDLR in tumour cells. The conclusion that we had drawn from these results was that elevated cholesterol synthesis and less dependence of LDL cholesterol is a colonic tumour phenotype that is selected and clonally expanded during CRC development. In order to explore the role of LDLR in CRC tumourigenesis, it was of importance to us to select a model system that would allow us to perform the mechanistic studies with relevance to human disease. Human tumour derived cell culture based models have become instrumental as biomedical research tools in elucidating signaling pathways, drugs screening and discovering novel biomarkers.

For the purpose of our study, we selected three human colorectal cancer cell lines, HCT 116, HT 29 and DLD 1, each with distinct tumour origin, growth characteristics and mutation profile. These CRC cell lines are genotype authenticated and broadly represent the main molecular subtypes of CRC. To begin with, we determined the expression of HMGCR, LDLR, SREBP2 and PCSK9 at both protein and mRNA level, in all three cancer cell lines in comparison with untransformed colonic epithelial cell line NCM 460. Our results showed that all three cancer cell lines had high mRNA and protein expression of HMGCR, SREBP2 and PCSK9 with a concordant low expression of LDLR compared to the normal colonic cell line. While all three cancer cell lines varied in their expression of LDLR with respect to untransformed mucosa cell, it was interesting to note that DLD 1 had the lowest expression of LDLR at both mRNA and protein level. Low expression of LDLR despite of elevated protein expression of transcriptionally active form of SREBP2 suggests that LDLR could have be negatively regulated at post translational level, either through PCSK9 or IDOL [23]. PCSK9 is a secretory serine protease with binds to extracellular domain of LDLR and degrades it through endosomal-lysosomal pathway whereas IDOL is an E3 ubiquitin ligase that targets LDLR for ubiquitination and



proteasomal degradation [24]. Previous studies conducted in female C57BL/6 mice showed that inflammation induced by lipopolysaccharides (LPS), Zymosan A or turpentine oil upregulated PCSK9 expression through SREBP2 pathway activation which resulted in a significant increase in PCSK9 mediated degradation of LDLR [25]. It is now well established that inflammation plays a crucial role in the etiology of cancer. Colonic tumour and their microenvironment have been shown to secrete pro-inflammatory cytokines and chemokines like tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1  $\beta$  (IL-1 $\beta$ ), IL-6 etc. in an autocrine fashion by positive feedforward regulation that promotes tumour growth [26, 27]. Hence, the possibility of inflammation playing a role in PCSK9 mediated downregulation of LDLR in colon cancer cells cannot be ruled out. It was interesting to note that colonic tumours had high expression of SREBP2 which regulates transcription of all three cholesterol pathway associated genes such as LDLR, HMGCR and PCSK9. However, our results showed that high SREBP2 expression correlated with upregulated HMGCR and PCSK9 mRNA expression but could not explain the low expression of LDLR mRNA suggesting that increased SREBP activation does not necessarily underline the changes in LDLR expression. It could also mean that LDLR expression is negatively regulated at both mRNA and protein level, probably through microRNAs and/or PCSK9. MicroRNAs are small endogenous RNAs that negatively regulate gene expression by targeting mRNA for degradation. In a recent study, microRNA 185 was shown to target 3' untranslated region (UTR) of LDLR resulting in its rapid degradation [28].

Our cancer cell line data corroborated with the finding in animal models indicating that low LDLR expression could be playing a role in CRC development. LDLR has mainly been implicated in its role of serum cholesterol uptake but there is very little known about its role in tumorigenesis. In prostate cancer cells, loss of LDLR feedback regulation has been reported in PC-3 cells and DU145 due to defect in SREBP2 regulation of LDLR transcription [19, 29]. Our results from cell viability assay showed that DLD 1 cells have highest cell viability and proliferation rate followed by HCT 116 and HT 29. Coincidentally, DLD 1 had also shown the lowest expression of LDLR mRNA and protein amongst all three cancer cell lines which suggests that low LDLR expression along with elevated cholesterol biosynthesis could favour a highly proliferative phenotype in colorectal cancer. Considering that all three cancer cell lines have a unique genetic background, mutation

profile and carcinogenesis pathway, one can argue that it would affect the way these cells grow, proliferate and respond to various chemotherapeutic drugs. For instance, both HCT 116 and DLD 1 cells have an MSI phenotype with mutations in *KRAS* and *PIK3CA* oncogenes whereas HT 29 has CIN phenotype with *BRAF* mutation. *KRAS* and *PIK3CA* mutation has frequently been associated with increase in pro-survival and growth signaling through the AKT/mTOR and/or the MAPK signaling pathway which could be a reason for greater cell viability and proliferation rate in HCT 116 and DLD 1 cells compared to HT 29 cells [30, 31]. Previous reports on the efficacy of cholesterol reducing drugs such as statins, PCSK9 inhibitors and RO 48-8071 have shown promising results in reducing cancer cell viability and proliferation *in vitro* [32-35]. Pharmacological inhibition of key effectors of cholesterol metabolism such as HMGCR, squalene synthase and liver X receptor (LXR), have shown reduction in tumour growth in xenograft models [36-38]. It is generally accepted that cholesterol lowering drugs such as statin will reduce tumour growth by reducing endogenous production of cholesterol and other important metabolites such as isoprenoids required for cell growth signaling. Lovastatin binds to HMGCR by reversible competitive inhibition which blocks the production of mevalonate and its downstream metabolites including cholesterol and isoprenoids [12]. RO 48-8071 inhibits cholesterol biosynthesis in the cell by targeting oxidosqualene cyclase (OSC) which converts 2,3-squalene epoxide into lanosterol downstream of mevalonate pathway [22]. These two drugs allowed us to analyze the effect of cholesterol biosynthesis inhibition with and without depletion of mevalonate and isoprenoids on cancer cell growth and viability. Based on review of previous work done with statins in *in vitro* and our own optimization, 20 $\mu$ M of lovastatin and 20  $\mu$ M of RO 48-8071 were selected to treat the colorectal cancer cells [35, 39].

Our results for lovastatin treatment showed a time dependent elevation in LDLR protein expression level in HCT 116 and HT 29 but not in DLD 1. Other proteins of cholesterol metabolism pathway such as HMGCR, SREBPs and PCSK9 were elevated in all three colorectal cancer cell lines at different time points. While HCT 116 and HT 29 responded to lovastatin by increasing the expression of LDLR, DLD 1 cells seem to lack LDLR feedback regulation. Elevated protein levels of HMGCR suggests that the cholesterol biosynthetic pathway could be resistant to sterol dependent negative feedback

regulation in these colorectal cancer cells. Earlier studies have also reported lack of cholesterol mediated feedback regulation of HMGCR [40]. This implies that sterol regulatory element 1 (SRE 1) which is shared by promoters of LDLR and HMGCR gene could play an important role in loss of sterol mediated regulation of LDLR. However, our results showed an upregulation of SREBP2, HMGCR and PCSK9 expression levels in all cancer cell lines which suggests that LDLR protein and mRNA downregulation in DLD1 cells could be independent of SREBP activation.

We observed that the response of the cancer cells to RO 48-8071 was similar to what we had observed in case of lovastatin. RO 48-8071 was able to upregulate cholesterol biosynthesis and uptake related genes significantly in HCT 116, HT29. DLD 1 cells again failed to upregulate LDLR mRNA and protein levels in DLD 1 cells though we did see an increase in expression of HMGCR, SREBP2 and PCSK9 mRNA level and protein level after 24 hours. Our results from lovastatin and RO 48-8071 treatment suggest that HCT 116 and HT 29 have a functional sterol mediated feedback regulation of LDLR and HMGCR, however DLD 1 cells an alteration in sterol mediated regulation of LDLR expression. Interestingly, DLD1 had the lowest expression of LDLR protein and highest cell viability compared to the other two cancer cell lines which suggests that the loss of LDLR feedback regulation resulting in low LDLR levels may be correlated to increased cell survival of DLD1 cells. It also suggests that DLD 1 cells may be more dependent on endogenous biosynthesis of cholesterol than cellular uptake for its growth and additionally LDLR may have an alternate role to play in growth of DLD 1 cells. Results of cell viability assay demonstrated that DLD 1 cells showed minimum reduction of cell viability after 24 hours of lovastatin and RO 48-8071 treatment. HCT 116 and HT 29 showed significant loss in cell viability after treatment with lovastatin and RO 48-8071. Hence, we concluded that in both HCT 116 and HT 29, statin was able to reduce cell viability possibly by lowering intracellular cholesterol isoprenoids synthesis required growth and signaling as discussed earlier, though there are reports of statins showing growth inhibitory effects independent of HMGCR inhibition [41, 42]. Furthermore, the unresponsiveness of DLD 1 cells to lovastatin and RO 48-8071 treatment suggest that these cells have a loss of LDLR feedback regulation since they were unable to upregulate LDLR expression in response to cholesterol reducing drug treatment. Statin resistance in DLD 1 cells could also be

attributed to elevated expression of HMGCR v1 splice variant which shows a deletion of exon 13 that contains the statin binding domain [43]. However, the fact that DLD 1 cells showed an upregulation of SREBP2 in response to lovastatin and RO 48-8071 indicates that HMGCR may not be playing a role in statin resistance in DLD 1 cells. In the present study we demonstrated that the three colorectal cancer cell lines show deregulated cholesterol metabolic pathway most importantly a phenotype, which favoured a lower level of LDLR protein. However, each cell line showed different sensitivity to inhibitors of endogenous cholesterol biosynthetic pathway. Importantly, DLD 1 cells with lowest LDLR expression showed least response to statin and RO-48-8071 treatment. Therefore it was concluded that all three colorectal cell lines could serve as model systems to explore a role of LDLR in the growth and survival of developing colonic tumours harboring different genetic aberrations. Additionally, they could serve as model systems to test the efficacy of cholesterol reducing drugs in retarding tumour growth and survival.

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## **Chapter 4**

### **Ectopic overexpression of LDLR negatively regulates colorectal cancer cell growth and viability**

## 4.1 Introduction

The low density lipoprotein (LDL) gene family consists of seven structurally related cell surface receptors that are involved in receptor mediated endocytosis and lysosomal delivery of a diverse spectrum of extracellular ligands [1]. Members of this evolutionary conserved gene family were initially thought to be mere cargo receptors involved in lipid metabolism. However, recent studies have shown that they play several important biological functions that are much more diverse than initially thought. These include regulating calcium homeostasis, protease uptake, regulating Wnt signaling and intracellular signaling during embryonic brain development [2, 3]. LDL receptor (LDLR) is the prototype of this gene family and also the first member to be identified [4]. The role of LDLR in regulating cholesterol homeostasis through clearance of LDL cholesterol from the blood circulation has been extensively researched. The pioneers in this field are Goldstein and Brown who were working to unravel the genetics behind the pathogenesis of an inherited autosomal dominant diseases called familial hypercholesterolemia (FH) [5, 6]. Patients suffering from this disorder had abnormally high levels of blood cholesterol which was later found to be due to a genetic defect in LDLR. The extracellular ligand binding domain of LDLR binds to apolipoprotein B of the cholesterol rich LDL particle. This LDL particle receptor complex formed at the clathrin coated pits on the cell surface is internalized through the endosomal lysosomal pathway. In the low pH environment of the lysosomes, LDLR releases the free cholesterol, which is either esterified and stored in lipid droplets or metabolized into bile acids. Ligand free LDLR is recycled back to the plasma membrane to repeat the process. The intracellular cholesterol level is regulated by sterol dependent transcriptional and post translational regulation of cholesterol biosynthesis and uptake genes. At the transcriptional level, a family of basic helix loop helix leucine zipper (bHLH-LZ) transcription factors called the sterol element regulatory binding proteins (SREBPs) regulate both LDLR and HMGCR, the rate limiting enzyme of mevalonate pathway [7]. Interestingly, SREBP also regulates transcription of PCSK9 which regulates LDLR turnover in the cell by degrading it through the endosomal lysosomal pathway. Post translationally, LDLR can also be also subjected to an E3 ubiquitin ligase called inducible degrader of LDLR (IDOL) mediated ubiquitination and

proteasomal degradation [8]. HMGCR protein turnover is regulated by oxysterol and/or isoprenoid mediated degradation by endoplasmic reticulum associated E3 ubiquitin ligase gp78 [9]. Role of LDLR as a membrane receptor responsible for uptake and delivery of cholesterol and essential fatty acids has been extensively studied whereas limited attention has been paid to its role in tumorigenesis.

Alteration in sterol mediated feedback regulation of LDLR and HMGCR has been observed in several cancers, mainly prostate, colorectal and ovarian cancer [10-12]. Previous studies have shown that prostate cancer cells PC-3 and DU145 lack sterol dependent regulation of SREBP2 mediated LDLR expression [13-15]. The loss of cholesterol mediated feedback regulation of LDLR and HMGCR has been observed in other cancers such as hepatocellular carcinoma, glioblastoma, acute myeloid leukemia and melanoma [15-19]. Most of these studies have focused on abnormal cholesteryl ester accumulation in cancer cells through upregulation of cholesterol biosynthetic and LDLR pathway. Whether genetic or epigenetic alteration of LDLR expression can be correlated to an enhanced colonic tumour cell growth, invasion and/or metastasis through modulation of key signaling pathways, remains elusive.

Our previous data in CRC cell lines showed downregulated LDLR protein phenotype in cancer cells compared to normal colonic mucosa cells corroborating the findings in experimentally induced colonic tumours. We selected all the three CRC cell lines with varying LDLR protein expression to test the hypothesis that LDLR protein downregulation imparts a favourable growth and survival environment for the colonic tumours. It is now well known that tumours alter their growth signaling and metabolism to favour rapid cell proliferation, growth and survival. Recent studies have brought into focus the role of SREBPs in mediating the upregulation of lipogenesis and cholesterol biosynthesis in cancer through Phosphatidylinositol-3 kinase (PI3K)/AKT and cMyc regulated glucose and glutamine pathway [20-22]. There are evidence that hyperactive EGFR and AKT dependent rapamycin insensitive pathway increases cell survival of glioblastoma through SREBP1 mediated enhanced lipogenesis [23]. Alteration of the MAPK signaling pathway has been characterized as one of the most prominent genetic markers in CRC that regulates early clonal expansion of transformed cells and also modulates invasion and metastasis in later stages [24-26]. *KRAS* and *EGFR* mutation

observed in 30-40% of CRC cases are directly correlated with amplification of cell signaling through RAF/MEK/extracellular signal related kinases (ERK1/2) resulting in enhanced cell proliferation and growth [27]. The three terminal kinases of the MAPK signaling cascade namely, ERK, stress activated protein kinases (SAPK) also known as C-Jun N-terminal kinases and p38 MAPK have all been shown to play diverse roles in cancer progression as well as tumour growth inhibition [28, 29]. Previous studies have reported that ERK signaling pathway is involved in SREBP2 mediated upregulation of LDLR expression in human hepatoma cells [30, 31]. Stress activated protein kinases or SAPK/c-Jun N-terminal kinase signaling pathway has been implicated in NF- $\kappa$ B inhibition mediated apoptosis in HCT 116 cells [32]. Both ERK and SAPK pathways have also been shown to have a cross talk with other signaling pathways such as the signal transducer and activator of transcription (STAT) pathway that regulates gene expression of pro and anti-apoptotic genes resulting in enhanced cancer cell growth, invasion and migration [33]. SAPK signaling pathway can have a context dependent role in either promoting or inhibiting tumour growth. Another important stress induced pathway in the MAPK cascade, is the p38 MAPK signaling pathway which has shown apoptosis inducing and growth inhibitory effects in several cancers including CRC [34, 35]. Pharmacological growth inhibitors such as selenite, anti-angiogenic agent 5,6-Dimethylxanthenone-4-acetic acid (DMXAA) and anti-proliferation agent  $\beta$ -elemene exert their growth inhibitory effect through p38 signaling pathway [36-38]. Correlation of cholesterol biosynthetic pathway with altered growth signaling through deregulated PI3K/AKT and MAPK pathway is well established, however there is limited information on the involvement of the LDLR pathway. Hyperactivation of the PI3K/AKT growth signaling is often due to deletion or mutation of Phosphatase and TENsin homologue deleted on chromosome 10 (PTEN) tumour suppressor gene which a negative regulator of P13K/AKT signaling pathway [39]. PTEN is a phosphatase that antagonizes the function of oncogenic PI3K by dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate (PIP3). This results in deactivation of AKT and its downstream growth signaling. PTEN phosphatase activity is regulated by several post translational modifications including acetylation, oxidation, ubiquitination and phosphorylation [40]. Many phosphorylation sites have been identified on PTEN that are subject to phosphorylation by cellular kinases such as casein kinase,

glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) resulting in inactivation of its phosphatase activity. An increase in phosphorylation at serine 380 and threonine 382/383 has been observed in many cancers [41, 42]. Loss of PTEN activity due to genetic and epigenetic alterations has been correlated with increased cholesterol accumulation through PI3K/AKT signaling mediated SREBP2 activation [43, 44].

The main premise of this study was to determine if ectopic over expression of LDLR in colorectal cancer cell lines can modulate their growth, cell viability and cell motility. We also analyzed the underlying molecular mechanisms through LDLR could exert its anti-tumourigenic effect in colorectal cancer cells by looking at the induction of the three main downstream effector kinases of MAPK signaling pathway such as ERK, SAPK and p38 signaling. Additionally, we studied the AKT/PTEN pathway to determine if elevated LDLR protein levels can regulate AKT signaling through PTEN, which is critical for cancer cell growth as noted in earlier studies.

## **4.2 Materials and Methods**

### **Cell culture**

Human CRC cell lines, HCT 116 (CCL-247; ATCC), HT 29 (HTB-38; ATCC) and DLD 1 (CCL-221; ATCC) were obtained from ATCC, USA . HCT 116 and HT 29 were maintained in McCoy's 5a Medium Modified (Sigma) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin streptomycin. DLD 1 cell line was maintained in RPMI-1640 (Sigma) supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were grown as monolayers at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

### **Construction of LDLR expression vector**

Human LDLR gene was amplified by polymerase chain reaction (PCR) from pJP1520 retroviral expression vector (DNASU) containing cytomegalovirus (CMV) promoter and ampicillin resistance gene. PCR primers used were as follows: Forward primer:GATAAGCTTGGGCCCTGGGGCTGG,Reverseprimer:AAAGCGGCCGCCTACGCCACGTCATCC with Hind111 and Not1 sites for cloning. The PCR product was cloned into a linearized p3XFLAG-CMV-7.1 Expression Vector (Sigma) with Hind111

and Not1 overhangs. Positive clones selected by restriction enzyme digestion were sent for sequencing to confirm LDLR open reading frame using JPO113 sequencing primer (GCGGTTTTGGCAGTACATCAATGGGCG). After DNA sequence confirmation, LDLR p3XFLAG-CMV-7.1 was used for overexpression of LDLR in colorectal cancer cells HCT 116, HT 29 and DLD 1.

### **DNA transfection**

Cells were plated in 10 cm tissue culture plates and cultured in their respective media till they reached desired (~80-90%) confluency. The plasmid p3XFLAG-CMV-7.1 LDLR was transfected using Xtreme GENE HP DNA Transfection Reagent (Roche Applied Science, Mannheim, Germany). Briefly, 5ug of DNA was diluted in 200ul of serum free media containing 15ul of DNA transfection reagent. After gently mixing the contents, the transfection mixture was incubated at room temperature for 20 minutes. Transfection mixture was then added dropwise to the cells followed by gentle swirling before incubating them at 37°C for 24 hours. The next day media was removed and fresh media containing 1% penicillin-streptomycin was added. LDLR overexpression was confirmed by Western blotting.

### **Antibodies and Reagents**

The following antibodies were purchased from Santa Cruz Biotechnology-: LDLR (sc-18823), HMGCR (sc-27578), SREBP1 (sc-366), SREBP2 (sc-5603),  $\beta$  actin (sc-1616), secondary antibodies including horse radish peroxidase (HRP) conjugate anti-mouse IgG (sc-2005) and anti-rabbit IgG (sc-2030,). PCSK9 (NB300-959) antibody was purchased from Novus Biologicals. Other chemicals used were- lovastatin (Sigma Aldrich), RO 48-8071(Cayman Chemicals), high capacity c-DNA reverse transcription kit (Applied Biosystems), power SYBR green mastermix (Applied Biosystems), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Sigma Aldrich)

### **Sample preparation and immunoblotting**

CRC cells growing in their respective media with or without treatment were washed with cold phosphate buffer saline (PBS) followed by lysis in Radioimmunoprecipitation

assay (RIPA) lysis buffer (See appendix 1). Total cell lysate was centrifuged at 12000 x g for 10 minutes at 4°C. Supernatant was collected in sterile tubes and was used to quantify protein by Bradford assay. Equal amount of protein was used for SDS-PAGE followed by electro-blotting onto PVDF membrane (PVDF; Bio-Rad, Hercules, CA, USA), using wet transfer method. Membranes were blocked in 5% skimmed milk for 1 hour at room temperature, followed by overnight incubation in primary antibodies prepared in 5% bovine serum albumin (BSA) TBS-Tween (1% v/v) at 4°C. Membranes were washed three times in TBST and probed with HRP conjugated secondary antibodies (1:10000) for 1 hour at room temperature. After washing three times in TBST, proteins were detected using Perkin Elmer Enhanced Chemiluminescence reagent and FluorChem Western blotting imaging system. Protein bands were quantified using ImageJ software (Version 1.42q)

### **RNA isolation and Quantitative Real time PCR (Q-RT PCR)**

RNA was isolated from p3XFLAG-CMV-7.1 or p3XFLAG-CMV-7.1 LDLR expressing HCT 116, HT 29 and DLD 1 cells using TRIzol reagent (Invitrogen) followed by spectrophotometric quantification. 250ng of total RNA was used for first strand cDNA synthesis in a 20 µl reaction using ABI High Capacity cDNA Reverse Transcription kit using the given cycling conditions (25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 seconds). 1µL of cDNA along with 1 µL primer (final concentration 10mM) and 10 µL of 2X SYBR green PCR master mix (ABI) was used in a 20 µl reaction to perform quantitative Real time PCR in Applied Biosystems 7300 PCR system (ABI). Following PCR cycling conditions was used: 10 min at 95 °C for one cycle, 40 cycles of 95 °C for 15 sec and 60 °C for 60 sec. The relative levels of mRNA expression were quantified by using the comparative CT method of  $-\Delta\Delta C_t$  [45].

### **Cell viability assay**

To assess the growth of CRC cells in an LDLR overexpression background compared to empty vector transfected control cells, cell viability assay was performed. Briefly, the cells were seeded in 6-well plates and cultured overnight before transfecting them with p3XFLAG-CMV-7.1 empty vector or p3XFLAG-CMV-7.1 LDLR. After confirming the overexpression of LDLR by Western blotting using anti-Flag antibody,

transfected cells were re-plated in 96 well tissue culture plates. At the end of desired time point, 10  $\mu$ l of MTT solution (5 mg/ml) was added into each well for a final concentration of 0.5mg/ml, followed by incubation for 4 h at 37°C. After 4 hours, media was replaced with 100  $\mu$ l of dimethyl sulfoxide (DMSO) in each well to dissolve the formazan crystals. Absorbance was measured at 570 nm using microplate reader.

### **Wound healing scratch assay**

Cellular motility was measured by *in vitro* scratch-wound healing assay. Equal number of HCT 116, HT 29 and DLD 1 expressing p3XFLAG-CMV-7.1 or p3XFLAG-CMV-7.1 LDLR, were seeded in six-well plates (replicates of three) and incubated until they were 90-95% confluent. The monolayer of cells was scratched with a sterile pipette tip (2-10ul) followed by washing twice with PBS to remove cell debris. Images were captured immediately after wounding using an inverted microscope with 4X objective (Olympus). The cells were then incubated in complete media for 16 and 32 hours. At the end of each time point, wound closure was monitored under the microscope. The percentage wound closure between the wounded edges was analyzed using the ImageJ 1.42 program.

### **Statistical analysis**

*In vitro* results are shown as mean $\pm$  SEM (standard error of mean). Comparisons of datasets were performed using unpaired Student's t test (experimental group compared with control group) or ANOVA test to compare more than two experimental conditions. P values were calculated using GraphPad Prism 3.0 software (GraphPad software). Differences were considered significant for P values  $\leq$  0.05.

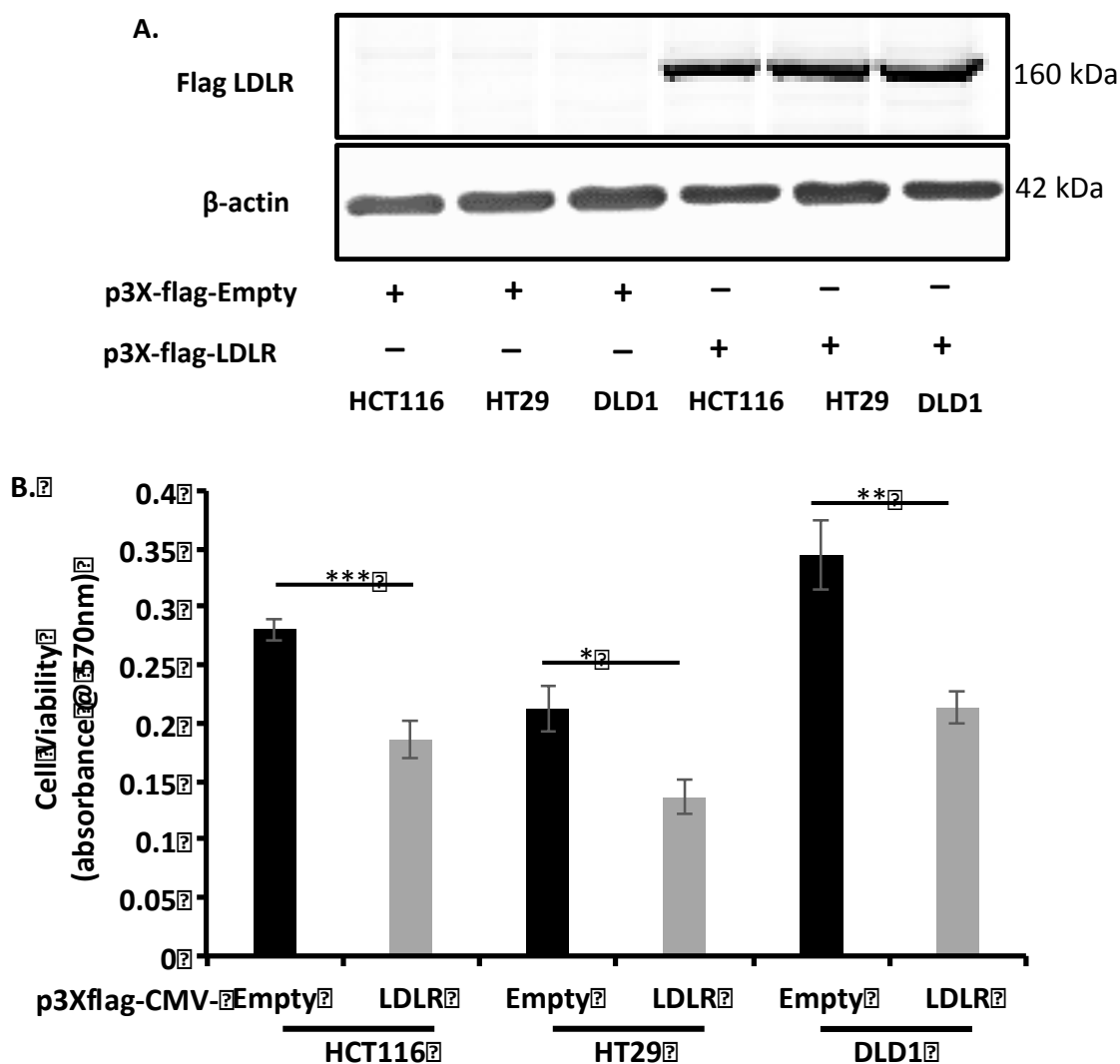
## **4.3 Results**

### **LDLR overexpression reduced cell viability of colorectal cancer cells**

The deregulation of lipogenesis and cholesterol metabolism is a hallmark of tumourigenesis and has been under intense scrutiny for development of pharmacological inhibitors of the involved pathways. Several effectors of cholesterol metabolism such as



HMGCR and SREBP 1 and 2 have been reported to have increased expression that increases cancer cell growth, proliferation and migration [16, 46, 47]. In our previous studies, we found that colorectal cancer cells showed a significantly low expression of LDLR compared to untransformed colonic cells. We also reported that DLD 1 cells with the lowest LDLR protein expression amongst the three colorectal cancer cell lines, had the highest cell viability and proliferation rate. To investigate if ectopic overexpression of LDLR in the colorectal cancer cells can modulate cancer cell viability, we transfected HCT 116, HT 29 and DLD 1 cells with LDLR overexpression vectors or empty vector (control). After confirming the overexpression of LDLR in transfected cell by Western blot analysis using anti-Flag antibody (Figure 4.1A), we followed up with MTT assay to analyze cell viability. Our result showed that ectopic overexpression of LDLR significantly reduced cell viability in HCT 116, HT 29 and DLD1 colorectal cancer cells when compared to the empty vector transfected control cells. (Figure 4.1B). This implies that LDLR could be involved in negative regulation of cancer cell growth and survival in a subset of CRC cases.

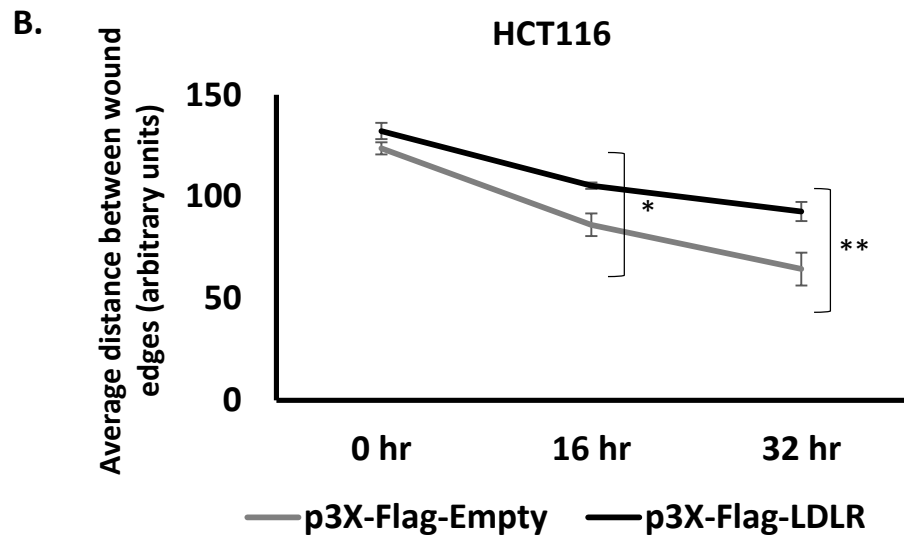
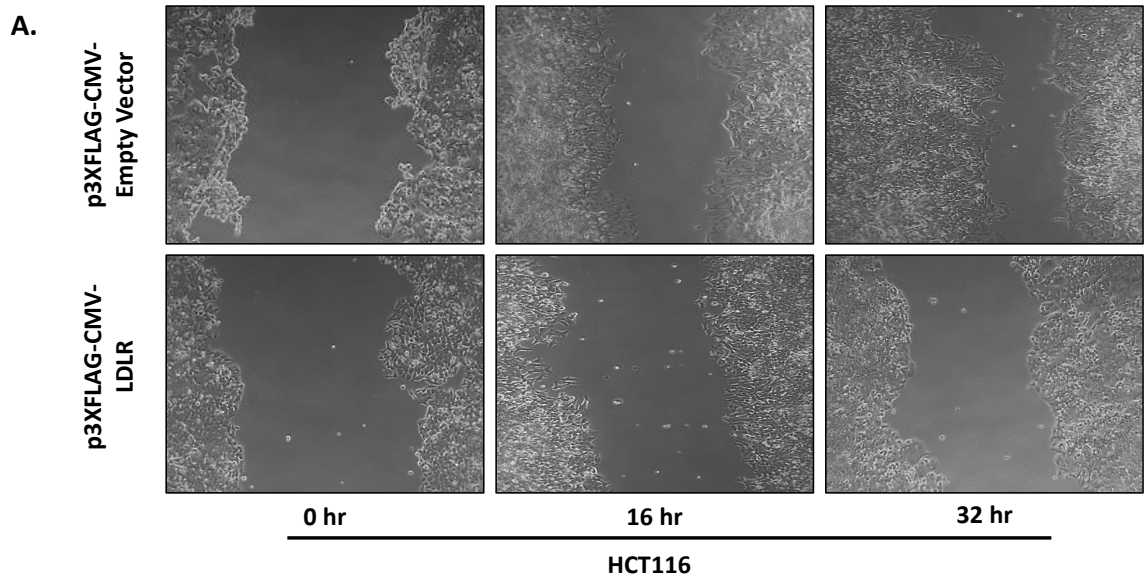


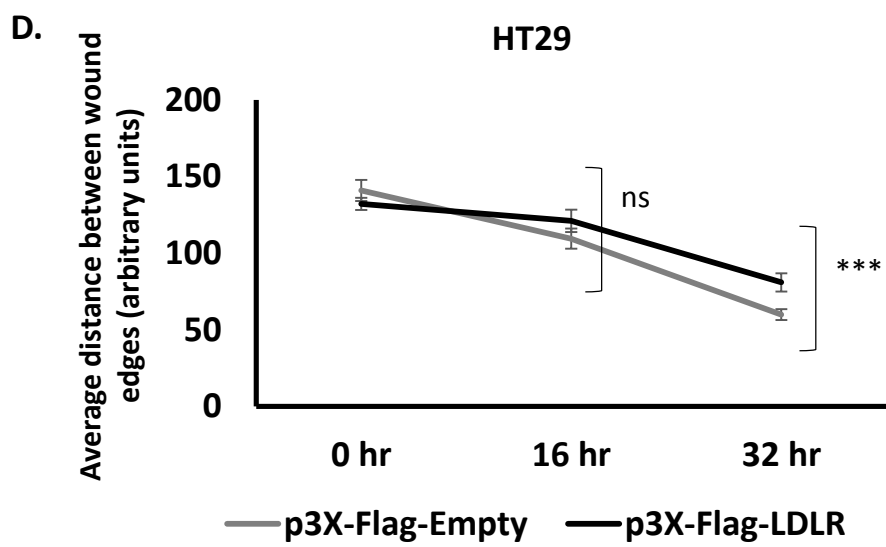
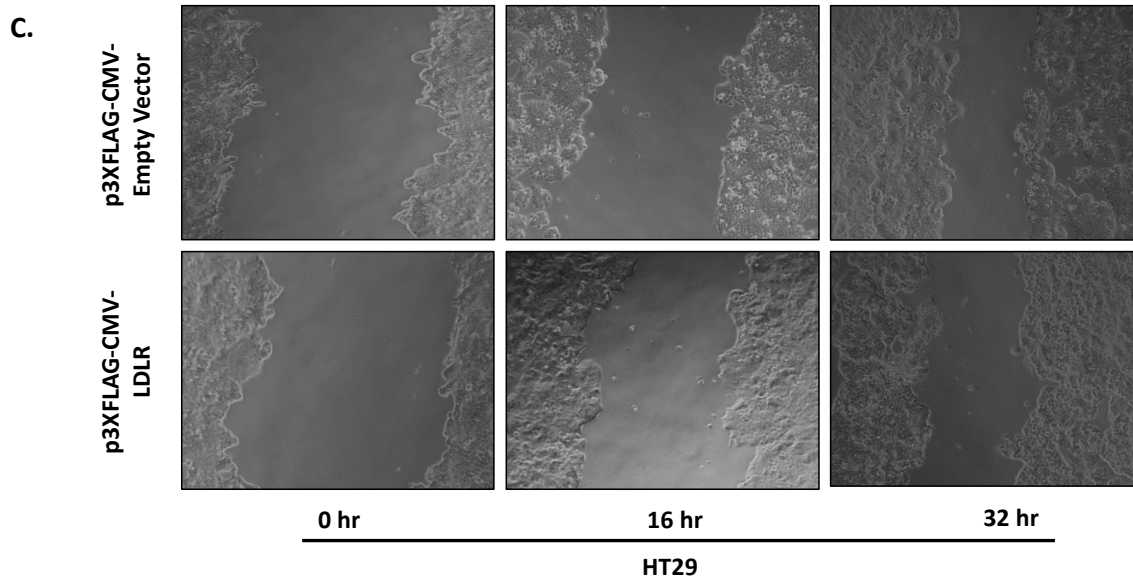
**Figure 4.1 Overexpression of LDLR reduces CRC cell viability.** CRC cells (HCT 116, HT 29 and DLD 1) were transfected with p3XFLAG-CMV-7.1-Empty vector (control) or p3XFLAG-CMV-7.1-LDLR. After confirming overexpression of LDLR by Western blotting using anti-Flag antibody, cells were re-plated in 96 well tissue culture plate and MTT assay was performed to analyze the cell viability. (A) Western blot showing over-expression of LDLR in HCT 116, HT 29 and DLD 1 cells using anti-flag antibody. (B) Change in cell proliferation rate of HCT 116, HT 29 and DLD 1 cells expressing p3XFLAG-CMV-7.1-Empty vector (control) or p3XFLAG-CMV-7.1-LDLR. Experiment was repeated three times. Results are represented as mean  $\pm$  SEM, \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ .

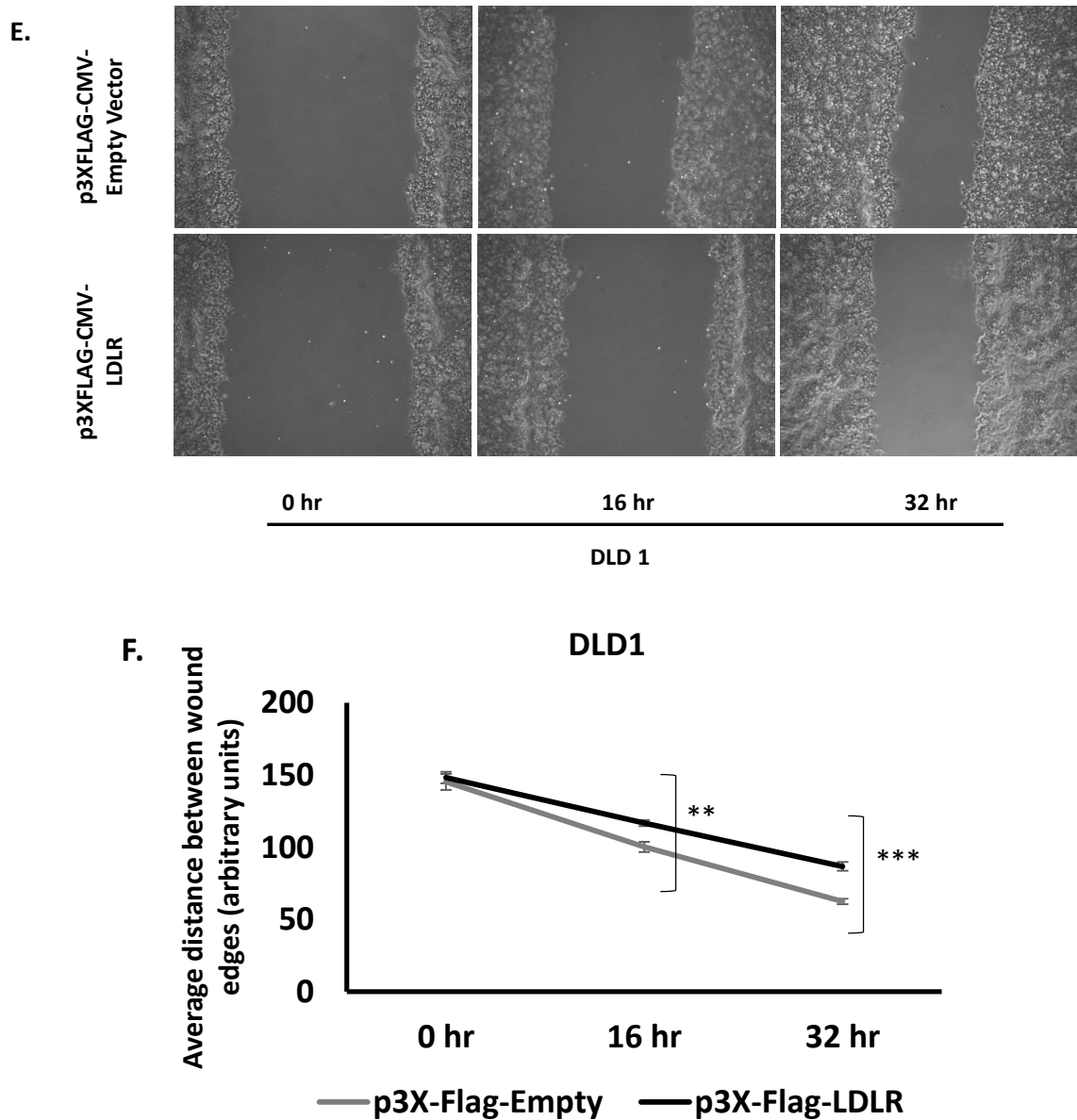
*Statistical analysis was performed using student t-test. P values were calculated using Graph-Pad Prism 3.0 software.*

### **Overexpression of LDLR reduces cell motility and migration of CRC cells**

Tumour invasion and metastasis is a complex, multi-stage process that requires increase in cell motility through cytoskeletal remodeling. Cancer cells show high degree of plasticity in the mechanisms through which they achieve increased cell motility that would support their invasive behavior for metastatic growth. A complex network of regulatory mechanism ensures appropriate actin polymerization and its response to external or internal stimuli [48]. Alteration of this regulatory mechanism and over expression of pro-migratory actins promote cancer cell invasion. This is achieved through modulation of several growth signaling and metabolic pathways. For instance, cytoskeletal rearrangement mediated by altered activity of Rho guanosine triphosphatases (Rho GTPase), Rac and PI3K has been well documented in cancer [49, 50] . Aberrant lipid, glucose and glutamine metabolism has been positively correlated with increased cancer cell migration and invasion [51-53]. For the purpose of our study, we wanted to assess if increase in LDLR expression can modulate cancer cell migration and motility. HCT 116, HT 29 and DLD 1 cells expressing empty vector (control) or LDLR expression vector were used for wound healing scratch assay. Our results showed that in all three colorectal cancer cell lines, ectopic overexpression of LDLR significantly reduced the cell migration from wounded edges to scratched cell free area as analyzed by the average distance measured between the wounded edges at the end of 16 hours and 32 hours (Figure 4.2). Overall, our results suggest that ectopically overexpressed LDLR negatively regulates cancer cell motility and migration.







**Figure 4.2 Ectopic expression of LDLR reduces colorectal cancer cell motility and migration.** Colorectal cancer cells (*HCT 116*, *HT 29* and *DLD 1*) were transfected with *p3XFLAG-CMV-7.1-Empty vector* (control) or *p3XFLAG-CMV-7.1-LDLR*. After confirming *LDLR* overexpression by Western blotting, *in-vitro* scratch wound healing assay was performed. Representative images showing wound closure after 16 and 32 hours in (A.) *HCT 116* (C) *HT 29* and (E.) *DLD 1* cells transfected with *p3XFLAG-CMV-7.1-Empty vector* (control) or *p3XFLAG-CMV-7.1-LDLR*. The wound closure was quantified

*at 16 hours and 32 hours post wounding by measuring the average distance between wounded edges using ImageJ software in (B.) HCT 116 (D.) HT 29 and (F). DLD 1. Experiment was repeated three times. Results are represented as mean  $\pm$  SEM (standard error of the mean), \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ . Statistical analysis was performed using student t-test. P values were calculated using Graph-Pad Prism 3.0 software.*



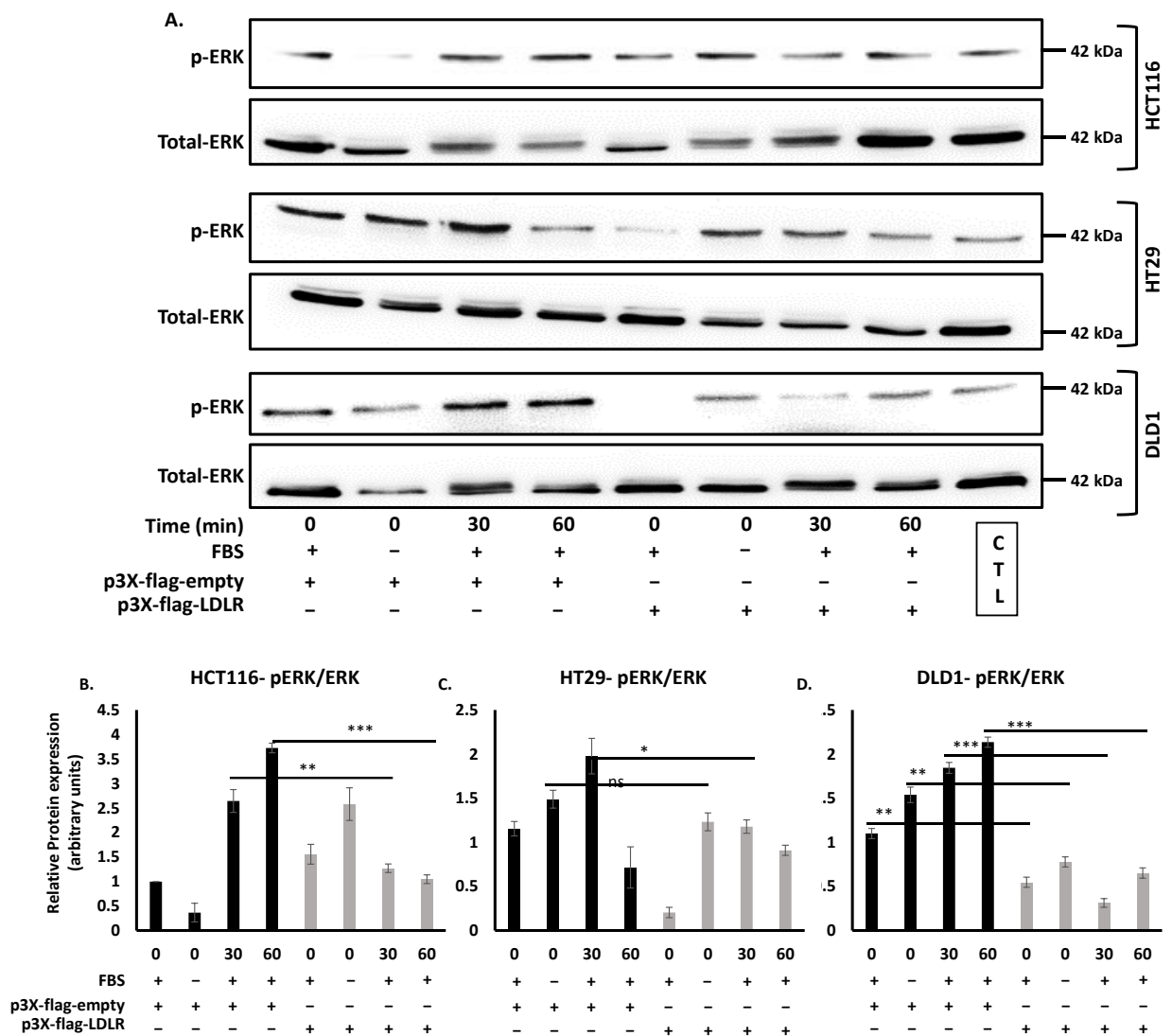
### **Overexpression of LDLR retards cell growth and cell proliferation of CRC cells through modulation of the MAPK signaling cascade.**

The mitogen activated protein kinases (MAPK) signaling pathways relay extracellular signals to regulate the expression of several cytoplasmic and nuclear proteins through a phosphorylation cascade. The three effector MAPK proteins including ERK, JNK (SAPK1) and p38 (SAPK2) show aberrant expression in several cancers [29]. Altered MAPK signaling has been associated with enhanced tumour cell growth, proliferation, invasion and metastasis. Tumour initiation in CRC which is frequently driven by *KRAS* or *BRAF* mutation, show upregulated ERK1/2 signaling [54]. Besides increasing tumour cell proliferation and growth, the ERK signaling has also been shown to promote epithelial to mesenchymal transition, migration and invasion [55]. To gain insight into the molecular pathways through which LDLR could be exerting its anti-tumourigenic effects in CRC cells, we analyzed the induction of MAPK signaling pathway in cancer cells overexpressing LDLR.

Briefly, p3XFLAG-CMV-7.1 expressing control cells and p3XFLAG-CMV-7.1 LDLR expressing HCT 116, HT 29 and DLD 1 cells were plated in four, 6 well plates. Except for one plate which served as control to determine the basal expression of MAPK signaling proteins, all other plates containing cancer cells with (transfected with p3XFLAG-CMV-7.1-LDLR) or without LDLR overexpression (transfected with p3XFLAG-CMV-7.1-Empty vector control) were serum starved for 4 hours. After 4 hours, serum was added back to the media for 30 and 60 minutes. Cells were harvested for protein extraction and Western blot analysis to detect induction of ERK, p38 and c-Jun N-terminal kinase (JNK) or SAPK signaling pathway. For the analysis of MAPK signal pathway Western blot results, we normalized activated phosphorylated MAPK protein (p-ERK, p-p38, p-SAPK) with total protein (t-ERK, t-p38, t-SAPK). The ratio of phosphorylated MAPK protein to total protein was indicative of the induction of the signaling pathway.

In HCT 116, induction of ERK signaling was significantly reduced after 30 and 60 minutes of serum replenishment in LDLR overexpressing cells compared to the control cells (Figure 4.3B). HT 29 cells overexpressing LDLR had significantly low basal level of ERK signal induction in comparison with control cells and serum starvation was found to

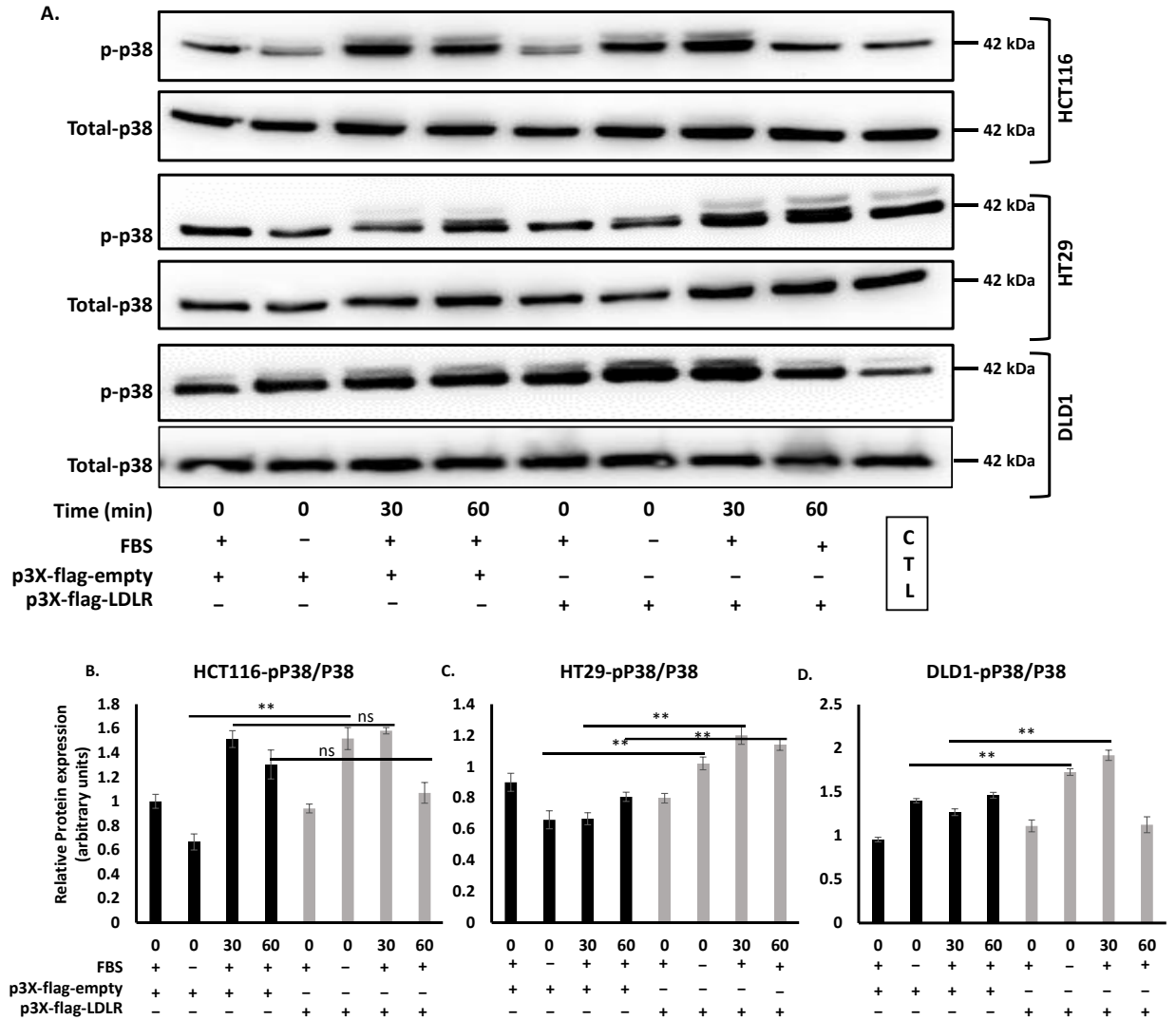
have no effect on ERK signaling. We did observe an induction in ERK signaling after serum replenishment for 30 and 60 minutes, but it was significantly less in LDLR overexpressing HT 29 cells compared to the empty vector transfected control cells (Figure 4.3 C). DLD 1 cells with ectopic expression of LDLR exhibited an overall decrease in induction of ERK signaling at basal level as well as after serum starvation. Serum replenishment for 30 and 60 minutes resulted in significantly less induction of ERK signaling in DLD 1 cells with ectopic overexpression of LDLR compared to mock transfected control cells. To summarize, overexpression of LDLR in CRC cells reduced the induction of ERK1/2 signaling suggesting that LDLR can negatively regulate tumour growth, survival and migration by reducing the activation of pro-survival ERK signaling pathway.



**Figure 4.3. LDLR over-expression reduces ERK activation in CRC cells.** *p3XFLAG-CMV-7.1 empty vector (control) and p3XFLAG-CMV-7.1 LDLR expressing HCT 116, HT 29 and DLD 1 cells were used to analyze the induction of ERK signaling in control versus LDLR overexpressing cancer cells by serum starving the cancer cells for 4 hours followed by serum replenishment for 30 and 60 minutes. Relative expression of phospho-ERK was normalized with total-ERK expression. Representative image (A) and quantification (n=3) showing ratio of expression levels of phospho-ERK and total-ERK (p-ERK/total-ERK) in (B) HCT116, (C) HT29 and (D) DLD1. Experiment was repeated three times. Results are represented as mean  $\pm$  SEM (standard error of mean), \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ .*

*Statistical analysis was performed using student t-test. P values were calculated using Graph-Pad Prism 3.0 software.*

To determine if LDLR mediated suppression of cancer cell growth and migration involves p38 signaling pathway, we looked at the induction of p38 under serum starved conditions in empty vector transfected (control) and LDLR overexpressing colorectal cancer cells. Results for Western blot analysis showed that 4 hours of serum starvation induced p38 signaling significantly higher in LDLR overexpressing HCT 116 cells when compared to the control cells (Figure 4.4 B). However, there was no significant difference observed in induction of p38 after 30 and 60 minutes of serum replenishment. In HT 29 cells with ectopic LDLR overexpression, we observed a significantly higher induction of p38 signaling after 4 hours of serum starvation when compared to control cells (Figure 4.4C). After 30 minutes of serum treatment, HT 29 overexpressing LDLR had a higher induction of p38 signaling compared to control cells. DLD 1 cells showed no significant difference in basal level induction of p38 signaling between LDLR overexpressing and control cells (Figure 4.4D). In in DLD 1 cells overexpressing LDLR, we observed that p38 signaling was significantly induced after serum starvation. Even after 30 minutes of serum replenishment, p38 was induced significantly higher in LDLR overexpressing DLD 1 cells compared to mock transfected control cells. Taken together, these results suggest that the anti-tumourigenic effect of LDLR in colorectal cancer cells could be mediated by induction of p38 MAPK signaling pathway.

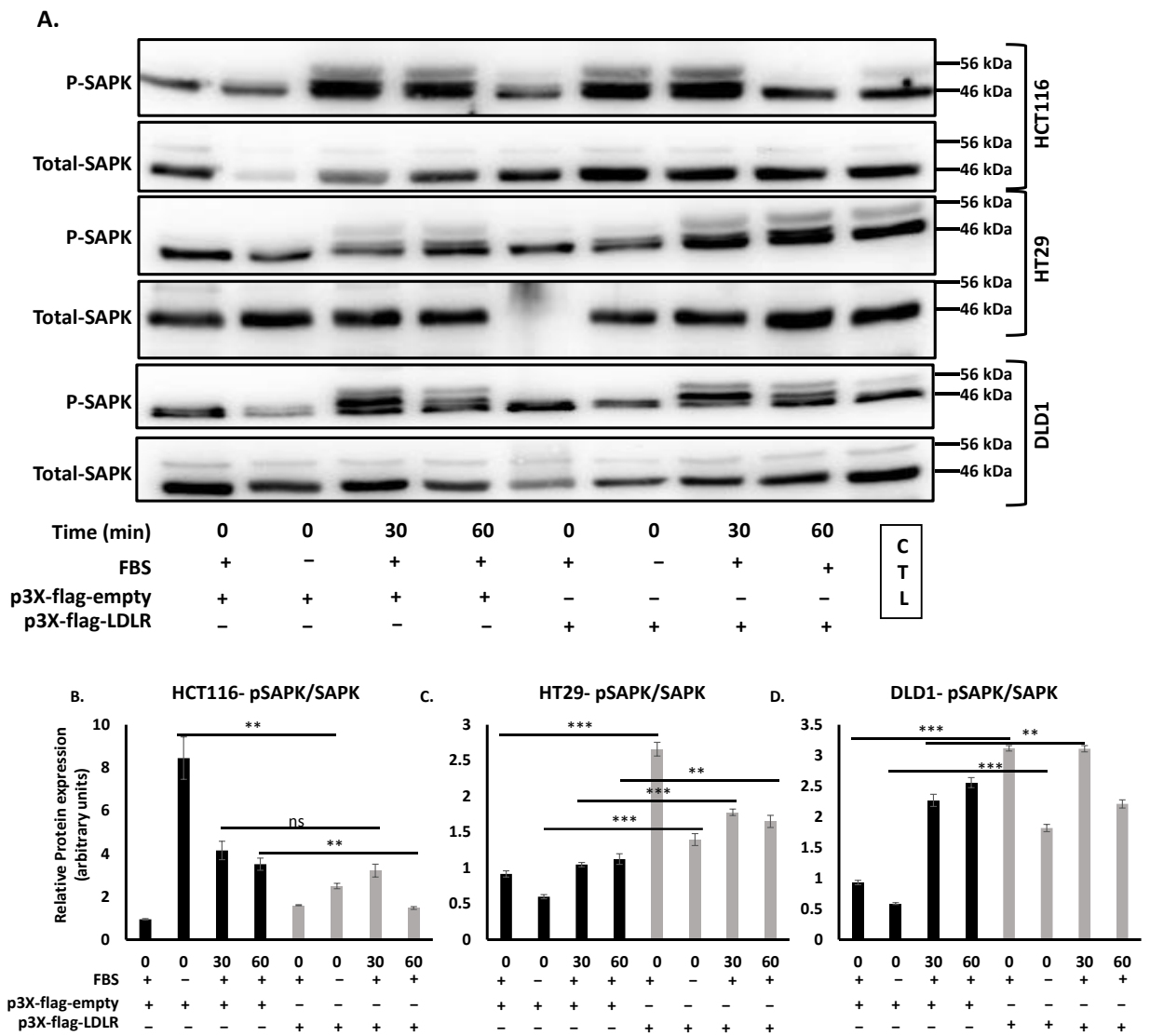


**Figure 4.4. LDLR overexpression induces p38 MAPK signaling in colorectal cancer cells.** *p3XFLAG-CMV-7.1* empty vector (control) and *p3XFLAG-CMV-7.1* LDLR expressing HCT 116, HT 29 and DLD 1 cells were used to analyze the induction of p38 signaling in control versus LDLR overexpressing cells by serum starving the cancer cells for 4 hours followed by serum replenishment for 30 and 60 minutes. Relative expression of phospho-p38 was normalized with total- p38 expression. Representative image (A) and quantification ( $n=3$ ) showing ratio of expression levels of phospho-p38 and total-p38 ( $p\text{-p38}/t\text{-p38}$ ) in (B) HCT 116, (C) HT 29 and (D) DLD 1. Experiment was repeated three times. Results are represented as mean  $\pm$  SEM (standard error of mean),  $*p \leq 0.05$ ,  $**p \leq$

*0.01, \*\*\* $p \leq 0.001$ . Statistical analysis was performed using student t-test. P values were calculated using Graph-Pad Prism 3.0 software.*

In case of c-Jun N-terminal kinase (JNK) or SAPK signaling, we observed that HCT 116 cells with ectopic overexpression of LDLR had an overall less induction of SAPK compared to control cells (Figure 4.5B). There was a significant induction of SAPK after 4 hours of serum starvation in control HCT 116 cells, however in LDLR overexpressing cells we did not observe much of an induction of SAPK. Interestingly, in HT 29 and DLD 1 cells, we saw the opposite trend in which ectopic expression of LDLR, increased SAPK induction when compared to control cells. HT 29 cells overexpressing LDLR had a significant induction of SAPK signaling at all time points in comparison to control cells (Figure 4.5C). Similarly, DLD 1 had a significantly high induction of SAPK at basal level as well as after 4 hours of serum starvation under LDLR overexpression conditions compared to control. After 30 and 60 minutes of serum replenishment, DLD 1 overexpressing LDLR had a higher induction of SAPK than control cells, though the difference was not significant at 60 minutes time point (Figure 4.5D). To summarize, our results suggest that LDLR is a negative regulator of tumour proliferation, growth and survival and the underlying mechanisms include modulation of MAPK signaling pathway.





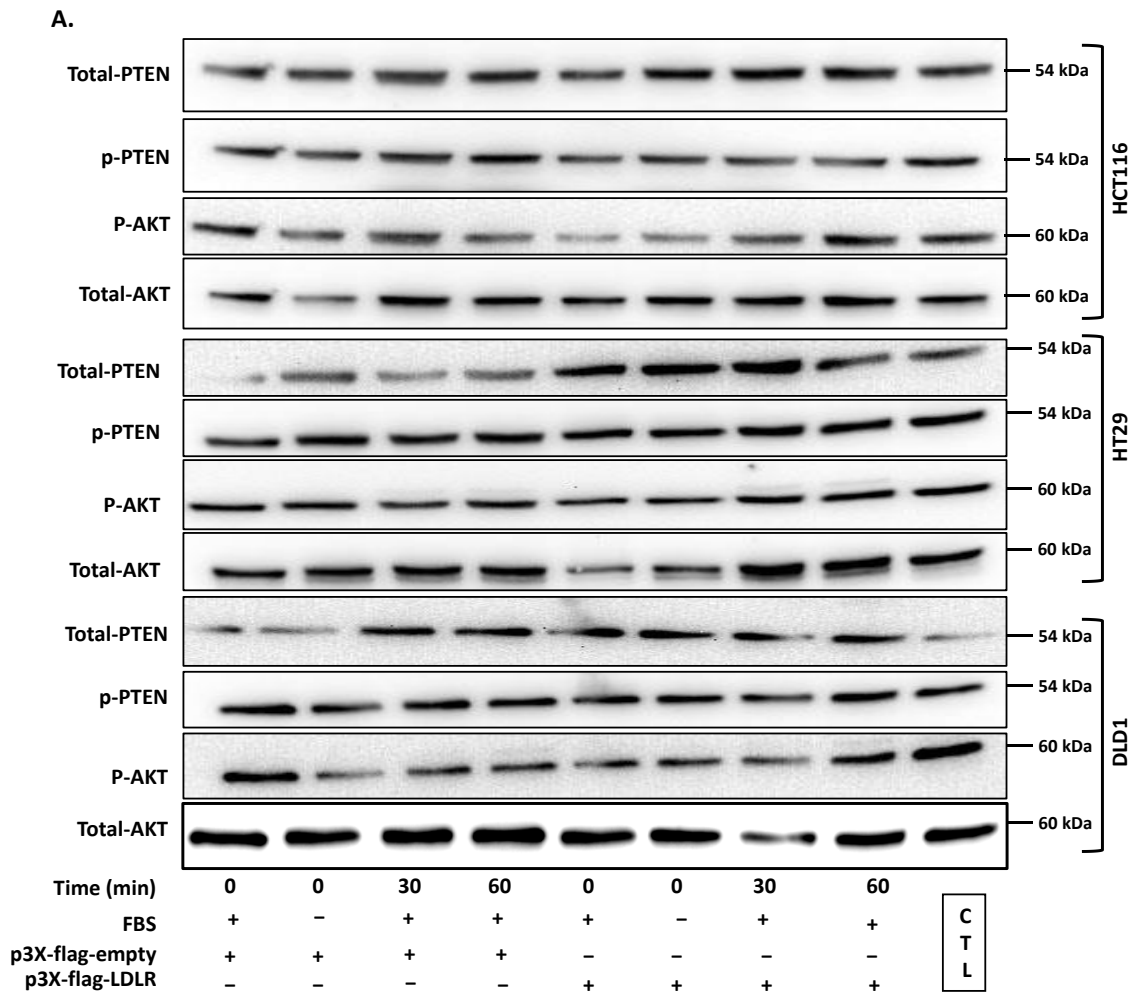
**Figure 4.5** LDLR over expression differentially regulates SAPK signaling in colorectal cancer cells. p3XFLAG-CMV-7.1 empty vector (control) and p3XFLAG-CMV-7.1 LDLR expressing HCT 116, HT 29 and DLD 1 cells were used to analyze SAPK signaling in control versus LDLR overexpressing cells by serum starving the cancer cells for 4 hours followed by serum replenishment for 30 and 60 minutes. Relative expression of phospho-SAPK was normalized with total-SAPK expression. Representative image (A) and quantification ( $n=3$ ) showing ratio of expression levels of phospho-SAPK and total-SAPK

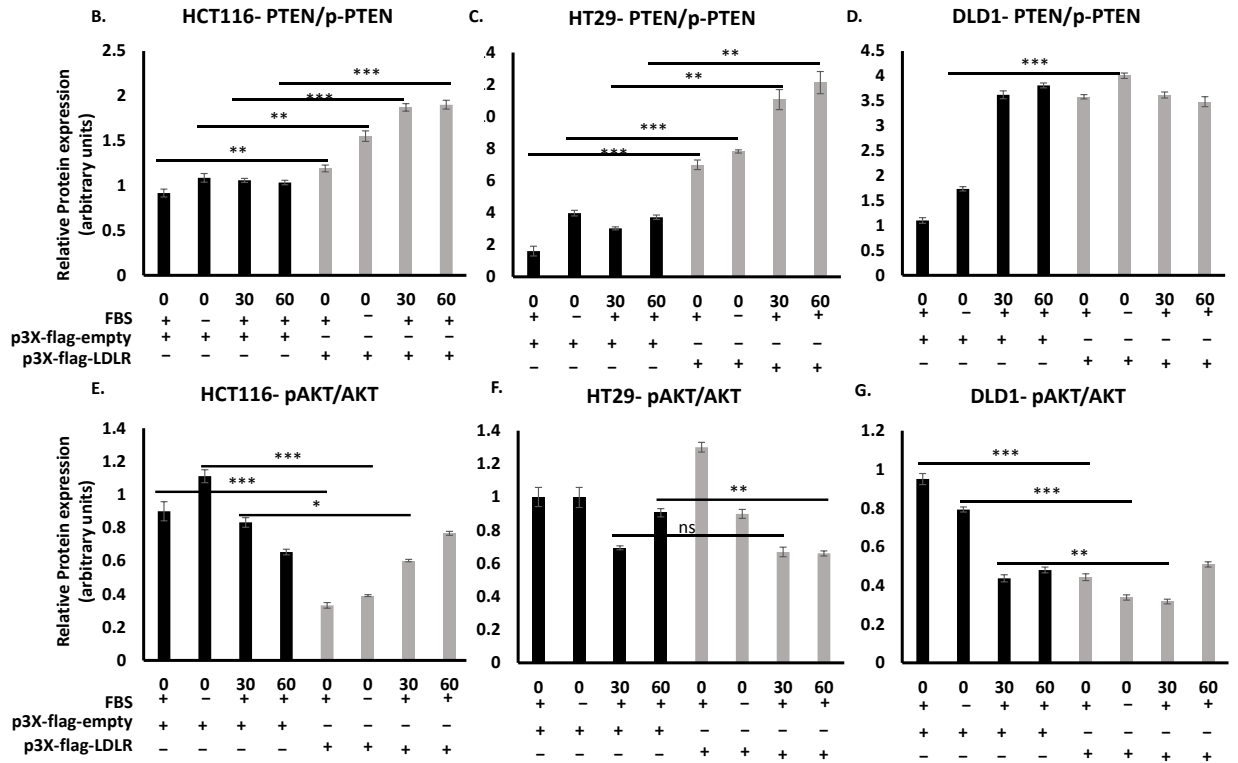
*(p-SAPK/total-SAPK) in (B) HCT 116, (C) HT 29 and (D) DLD 1. Experiment was repeated three times. Results are represented as mean  $\pm$  SEM (standard error of mean), \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ . Statistical analysis was performed using student t-test. P values were calculated using Graph-Pad Prism 3.0 software.*

### **Overexpression of LDLR suppresses AKT activation by inducing PTEN signaling**

The role of AKT also known as protein kinase B as the central node of cellular signaling network connecting various components of the growth and survival machinery, has been extensively researched. In context of CRC, AKT has been implicated with SREBP mediated increase in lipogenesis and tumour growth [56]. Pharmacological inhibitors of AKT have shown to reduce SREBP2 protein levels and its downstream transcriptional activity [57]. These results were complemented with the findings that showed insulin growth factor- 1 (IGF-) mediated activation of AKT resulted in direct activation of SREBP 2 and upregulation of its downstream targets LDLR and HMGCR. Activation of AKT by upstream kinase PI3K, mediates several pro-survival pathways that play important role in development and progression of cancer. In this study, we aimed to explore the effect of ectopic expression of LDLR on induction of AKT signaling. We also analyzed PTEN activity by determining the expression of total active PTEN and phosphorylated inactive PTEN in LDLR overexpressing colorectal cancer cells and compared that to control cells transfected with empty vector. AKT induction was analyzed as ratio of phospho-AKT to total AKT, same as MAPK signaling. For analysis of PTEN signal induction, the ratio of total PTEN to phosphorylated PTEN was interpreted as induction of PTEN activity. Analysis of Western blot results showed that HCT 116 cells over expressing LDLR have a significantly higher PTEN induction, i.e higher expression of total PTEN compared to phosphorylated inactive PTEN, at basal level compared to control cells (Figure 4.6B). Additionally, they also showed higher PTEN induction after 4 hours of serum starvation. Significantly high level of active total PTEN was observed after 30 and 60 minutes of serum replenishment. Interestingly, we observed lower AKT induction in HCT 116 cells with ectopically over expressed LDLR. There was significantly less induction of AKT signaling at basal level and after 4 hours of serum starvation in HCT 116 overexpressing LDLR (Figure 4.6 E) compared to control cells. Even after 30 minutes of serum starvation, we observed that AKT was induced less under LDLR overexpression conditions. HT 29 cells had high induction of PTEN in LDLR overexpressing cells (Figure 4.6F). Contrary to HCT 116 cells, AKT induction at basal level was significantly higher in HT 29 cells with ectopic expression of LDLR. We could only observe a significant decrease in AKT

induction after 60 minutes of serum replenishment in HT 29 cells overexpressing LDLR compared to control cells. DLD 1 cells had higher PTEN induction at basal level under LDLR overexpression conditions though it was not statistically significant (Figure 46 G). After 4 hours of serum starvation, there was a much higher induction of PTEN activity in DLD 1 cells overexpressing LDLR compared to control. However, 30 and 60 minutes of serum replenishment, LDLR overexpression did not seem to have an effect on induction of PTEN since it was found to be the same as control cells. These results provide another line of evidence suggesting that elevated expression of LDLR protein could suppress the proliferation and growth of tumours by PTEN mediated suppression of AKT signaling pathway.

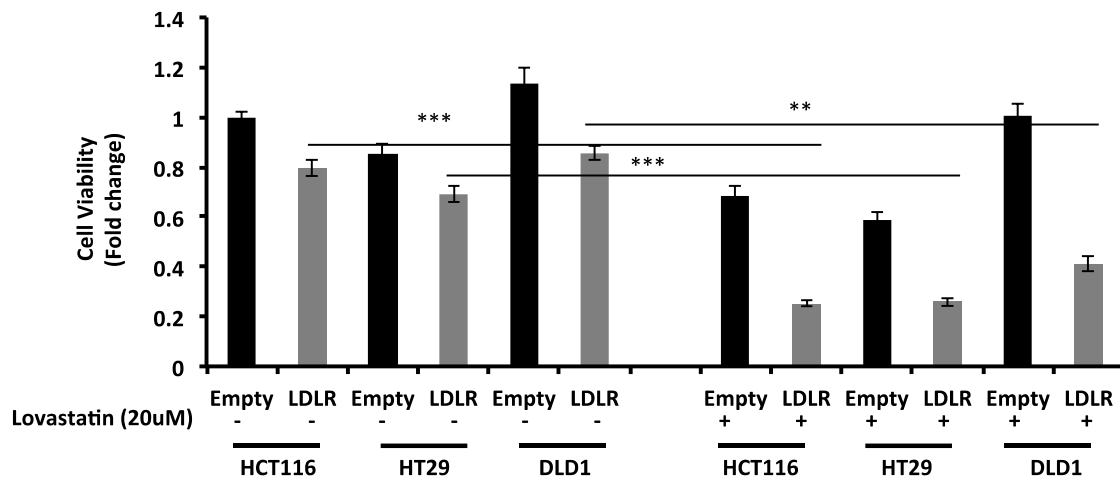




**Figure 4.6. LDLR over-expression suppresses AKT induction through PTEN signaling in CRC cells.** *p3XFLAG-CMV-7.1 empty vector (control) and p3XFLAG-CMV-7.1 LDLR expressing HCT 116, HT 29 and DLD 1 cells were used to analyze PTEN and AKT induction by serum starving the cancer cells for 4 hours followed by serum replenishment for 30 and 60 minutes. Relative expression of PTEN was normalized with phospho-PTEN expression. Relative expression of phospho-AKT was normalized with total AKT expression. Representative image (A) and quantification (n=3) showing ratio of expression levels of t-PTEN/p-PTEN and p-AKT/t-AKT in (B, E) HCT 116, (C, F) HT 29 and (D, G) DLD 1 respectively. Experiment was repeated three times. Results are represented as mean  $\pm$  SEM (standard error of mean), \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ . Statistical analysis was performed using student t-test. P values were calculated using Graph-Pad Prism 3.0 software*

### **Overexpression of LDLR enhances the growth inhibitory effect of lovastatin**

As we have discussed in our earlier sections, statins have showed inconsistency in their efficacy to retard tumour growth in clinical and epidemiological studies. Results in preclinical models have been more convincing in suggesting that statins have an anti-proliferative effect and that they can induce cell cycle arrest and apoptotic response in cancer [58-60]. KRAS mutation, altered ratio between full length HMGCR and HMGCR v1 splice variant which shows a deletion of statin binding domain containing exon 13, are some of the mechanisms associated with differential host sensitivity to statins [61]. In our initial finding (chapter3), we reported that all three colorectal cancer cells selected for the study had significantly low levels of LDLR protein compared to untransformed colonic mucosa cells, with DLD 1 showing the maximum downregulation. Next, we observed that DLD 1 cells showed the least sensitivity to statins amongst the three colorectal cancer cell lines, with respect to upregulation of LDLR, though they did increase HMGCR protein level at a later time point. DLD 1 cells were also able to maintain their cell viability and proliferation rate in the presence of statins, compared to HCT 116 and HT 29 cells which showed a significant decrease in cell viability on statin treatment. These results suggested that low LDLR protein in DLD 1 cells was protecting the cells from statin mediated anti-growth and survival effects. We, therefore sought to examine the role of LDLR in modulating the response of colorectal cancer cells to cholesterol reducing drug like lovastatin. To that effect, we measured cell viability in LDLR over expressing cell lines treated with or without 20 $\mu$ M of lovastatin for 24 hours by MTT assay. Our results showed that LDLR overexpression enhanced the growth inhibitory effect of lovastatin by significantly reducing cell viability in all three colorectal cancer cell lines compared to control cells transfected with empty vector (Figure 4.7).



**Figure 4.7 Overexpression of LDLR enhances the growth inhibitory effect of statin.** Colorectal cancer cells (HCT 116, HT 29 and DLD 1) were transfected with p3XFLAG-CMV-7.1-Empty vector (control) or p3XFLAG-CMV-7.1-LDLR. 24 hours post transfection, cells were trypsinized and re-plated in 96 well plates (2000 cells/well) followed by treatment with or without lovastatin (20uM) for 24 hours. At the end of time point, MTT assay was performed. Bar graphs represent fold change in cell viability of HCT 116, HT 29 and DLD 1 cells expressing p3XFLAG-CMV-7.1-Empty vector (control) or p3XFLAG-CMV-7.1-LDLR treated with or without lovastatin for 24 hours. Experiment was repeated three times. Results are represented as mean  $\pm$  SEM, \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ . Statistical analyses were performed using student t-test. P values were calculated using Graph-Pad Prism 3.0 software.



#### 4.4 Discussion

The low density lipoprotein receptor or LDLR has traditionally been implicated in clearance of blood LDL cholesterol to maintain intracellular and extracellular cholesterol homeostasis[1]. Besides its role as a cargo receptor delivering cholesterol and essential fatty acids into the cell, not much is known about its role in etiology of chronic diseases like cancer. LDLR, being an indispensable component of cellular cholesterol homeostasis, is under stringent sterol mediated negative feedback regulation by a family of transcriptional factors called sterol regulatory element binding proteins or SREBPs [62]. SREBPs have three isomers, SREBP1a, SREBP2 and SREBP1c which despite of having a significance sequence overlap, have distinct role to play in lipid and cholesterol metabolic pathways. In humans, SREBP1c primarily regulates fatty acid metabolism whereas SREBP1a and SREBP2 on account of their almost identical N-terminal transcriptional domain regulate cholesterol metabolism [63]. LDLR protein turnover is regulated mainly by the activity of PCSK9 serine protease that degrades LDLR by endosomal lysosomal pathway or by IDOL which degrades LDLR in the cell by polyubiquitination and proteasomal degradation pathway. The other component of cholesterol metabolism which has got a lot more attention for therapeutic intervention as a part of cancer therapy, is the cholesterol biosynthesis or mevalonate pathway. Pharmacological inhibition of key enzymes of this pathway such as HMGCR, squalene synthase, oxidosqualene cyclase and farnesyl pyrophosphate transferase have shown promising *in vitro* results that underline the significance of cholesterol biosynthesis pathway in initiation and progression of cancer. Statins in particular, have been a subject of intensive research for its potential anti-cancerous effects. Several *in vitro* studies have supported the idea that statins activate anti-proliferation and pro-apoptotic markers in cancer cells. By inhibiting HMGCR enzymatic activity, statins reduce the flux of mevalonate and downstream metabolites of the cholesterol biosynthesis pathway, most importantly isoprenoids and cholesterol, thus reducing cancer cell proliferation and growth[64]. Clinical data has not always complimented the *in vitro* results from statin study and there are few cases where contradictory claims have been made [65]. However, there is an increasing body of evidence corroborating the statin studies done in cancer cell lines. Interestingly, in a

number of case control population based studies, host specific differential sensitivity to statins have been reported which was associated with the KRAS mutation status, ratio of full length HMGCR to alternately spliced HMGCR v1 variant, over expression of multiple drug resistance associated proteins such as P-glycoproteins, breast cancer resistance protein (BCRP/ABCG2) or elevated PCSK9 levels [66]. High expression of mevalonate pathway associated genes has also been associated with statin resistance in cancer cell lines and clinical biopsies[67]. Cancer cells, owing to their increased requirement of cholesterol, upregulate expression of several enzymes of cholesterol biosynthesis pathway, including HMGCR, the rate limiting enzyme. Loss of sterol mediated negative feedback regulation of HMGCR is one of the several mechanisms through which cancer cells increase endogenous cholesterol synthesis. This is achieved through alteration of LDLR gene expression, loss of ABCA1/ABCG1, LXR mutation, loss of function mutation in ER membrane associated E3 ligase gp78 and upregulated growth factor or steroid hormone receptor through hyperactive PI3K/AKT or ERK1/2 signaling [68-70]. Recently it was reported that mutant p53 upregulates mevalonate pathway in breast cancer cells by associating with sterol gene promoters through SREBPs [71]. Collectively, these evidence emphasize the significance of cholesterol biosynthesis and its regulation in initiation and growth of cancer. LDLR mediated cholesterol uptake pathway on the other hand, has not attracted much of an attention for its role in tumorigenesis. In several cancers mainly prostate cancer, colorectal cancer and ovarian cancer aberrant expression of LDLR and loss of its sterol mediated feedback regulation has been reported [13, 15, 72]. In a recent study it was shown that statins reduce cell proliferation in androgen independent prostate cancer cells PC-3 which show a loss of LDLR feedback regulation [73]. In accordance with previous studies, we observed elevated expression of HMGCR, SREBP1, SREBP2 and PCSK9 in azoxymethane induced colonic tumour rat model indicating a deregulation of lipogenic and cholesterol metabolism (chapter 2). Furthermore, we observed significantly low expression of LDLR protein in colonic tumour compared to normal mucosa. Based on our *in vivo* observations, we hypothesized that low LDLR protein expression is associated with increased tumour growth and survival. To test our hypothesis, we established a cell culture based model system consisting of three human colorectal cancer cell lines , HCT 116, HT 29 and DLD 1 cells broadly representing the genetic landscape of human CRC

(Chapter 3). All three colorectal cancer cell lines showed significantly low expression of LDLR protein compared to untransformed colonic mucosa cells along with high expression of HMGCR, SREBPs and PCSK9. Interestingly, DLD 1 cells with the least expression of LDLR had the highest cell viability and proliferation rate. Lovastatin treatment was unable to induce LDLR mRNA and protein expression in DLD 1 cells unlike HCT 116 and HT 29 cells. Additionally, they showed resistance to lovastatin mediated reduction of cell viability in comparison to HCT 116 and HT 29 cells. We made similar observations with RO 48-8071, another cholesterol reducing drug that targets oxidosqualene cyclase (OSC), the downstream enzyme of mevalonate pathway. These findings supported the idea that LDLR protein expression in colorectal cancer cells is correlated with their growth and proliferation as well as their response to cholesterol reducing drugs like statins and RO 48-8071. To fully understand the role of LDLR in cancer progression and drug response and to explore the molecular pathways through which it mediates its anti-proliferative effects, we ectopically overexpressed LDLR in the cancer cells using a CMV driven expression vector. The first observation we made was a significant reduction of cell viability in colorectal cancer cells with LDLR overexpression. This was contrary to what one would expect because of the role LDLR is known to play in a cell which is uptake of cholesterol. However, our results indicate that LDLR could be interacting with cell survival and proliferation pathways to negatively regulate cancer cell growth. A plausible scenario is that in these cells, LDLR is primarily involved in regulation of cancer cell growth instead of cholesterol uptake which makes the mevalonate pathway indispensable as well as a target for therapeutic intervention. In order to explore the role of LDLR in cancer progression, we assessed cancer cell motility and migration rate in LDLR overexpressing colorectal cancer cells and compared that to control cells transfected with empty vector by performing time course wound healing scratch assay. Our results showed that ectopic overexpression of LDLR significantly reduced migration of cells from the wounded edges to cell free scratched area compared to control cells. HT 29 had the slowest migration rate amongst the three cancer cells in both control and LDLR overexpressing group. High cell motility and migration ability is a phenotype acquired by cancer cells to support invasion and metastasis. This requires cytoskeletal rearrangement involving altered expression and activity of several genes associated with actin polymerization and its regulation including

Rho GTPase, Rac, PI3K/AKT [74, 75]. Our results suggest that ectopically expressed LDLR could be interacting with some of these pathways to negatively regulate the expression of pro-migratory actins resulting in reduction of cancer cell motility and migration rate. Since wound healing scratch assay is a semi quantitative assay this result could be complimented by trans-well cell migration assay for further validation. In order to elucidate the underlying signaling pathways through which LDLR exerts its putative tumour suppressor functions, we studied the MAPK signaling pathway. In a genome wide association study, MAPK signaling pathway was identified as one of the most strongly associated genetic markers for colorectal cancer [26, 76]. This pathway relays extracellular cues such as growth hormones, cytokines, inflammation or stress to the intracellular transcriptional machinery which responds by altering gene expression resulting in altered cellular growth, proliferation, differentiation, apoptosis etc. The three main downstream effector kinases of the MAPK signaling cascade, are the extra cellular response kinases (ERK1/2), c-Jun N-terminal kinases or stress activated protein kinases1 (JNK or SAPK1) and stress activated protein kinases 2 (SAPK 2 or p38) [28]. While ERK1/2 is generally associated with mitogenic responses, SAPK1 (JNK) and SAPK2 (p38) are associated with cellular stress and inflammatory responses. Early genetic alterations in CRC such as KRAS and BRAF mutations, result in constitutive activation of ERK1/2 signaling which increases proliferation of tumour cells. ERK1/2 signaling has also been implicated in later stages of CRC tumourigenesis such as invasion and metastasis [77, 78]. Downregulation of p38 $\alpha$  and inhibition of p38 MAPK activity is associated with increased colonic tumour and lung metastasis [34, 36]. Recent studies in colorectal cancer cells have shown that selenite induces apoptosis in colon cancer cells and xenograft tumours by activating p38 MAPK mediated inhibition of cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) and Bcl2 pro-survival pathways [36]. Another stress activated kinase, c-Jun N-terminal kinase (SAPK) is known to have a context dependent function depending on the cell type, external or internal cues and growth phase. There are studies that have showed that SAPK exerts its anti-tumourigenic effects by inducing apoptotic response through inhibition of NF- $\kappa$ B signaling [79]. SAPK or the JNK signaling pathway has also been shown to regulate the multi drug resistance (MDR) mechanism of ABCG2 transporter protein in colon cancer cells such that the inhibition of JNK activity reverses

the MDR effect of ABCG1 [80]. For the purpose of our study, we serum starved control cells and LDLR over expressing cells for 4 hours followed by serum replenishment for 30 minutes and 60 minutes to analyze the effect of LDLR over expression on induction of MAPK signaling pathways. Phosphorylated MAPK protein was normalized with total protein to show the induction of the protein. Our results showed that LDLR overexpression significantly reduced the induction of ERK signaling after serum replenishment in HCT 116 and HT 29 cells overexpressing LDLR compared to control cells. LDLR overexpressing DLD 1 cells had an overall low induction of ERK signaling at all time points in comparison with control cells. This suggests that in these colorectal cancer cells LDLR could be interacting with ERK signaling to reduce cancer cell growth and proliferation. In case of p38 signaling which has shown to have an anti-tumourigenic effect on the growth of several cancer cells, we observed that LDLR overexpression significantly induced p38 signaling after 4 hours of serum starvation in HCT 116 with ectopic LDLR overexpression compared to control cells though we did not observe the same trend after 30 and 60 minutes of serum replenishment. HT 29 and DLD 1 cells showed a higher induction of p38 at all time points in LDLR overexpressing cells compared to control cells. Given the role p38 as a tumour suppressor as discussed earlier, our results suggest that LDLR could be functioning in association with p38 MAPK signaling to inhibit tumour cell growth and proliferation. Analysis of the effect of LDLR overexpression on SAPK signaling could be interpreted in more than one way since it showed differential regulation in the three colorectal cancer cell lines. LDLR overexpression reduced induction of SAPK Jun signaling after serum replenishment in HCT 116 cells. In HT 29 and DLD 1 cell, we saw an opposite trend where LDLR overexpressing cells showed a higher induction of SAPK signaling at basal levels as well as after of serum replenishment for 30 and 60 minutes compared to control cells. Our results suggest that SAPK or JNK pathway is differentially regulated by LDLR in colorectal cancer cells, however the physiological relevance of this pathway and interaction with LDLR in modulation of cancer cell growth in unclear. The other possibility could be that LDLR exerts its anti-tumourigenic effect independent of SAPK Jun signaling pathway. Taken together, we established that LDLR interacts with the components of MAPK signaling pathway to regulate the growth and survival of colorectal cancer. The other growth signaling pathway, which was of interest

to us, was the AKT pathway that is one of the most frequently altered signaling pathways in cancer. We also analyzed the effect of LDLR overexpression on PTEN induction, which is the negative regulator of AKT signaling. AKT is downstream of PI3K and is activated by phosphorylation. In order to determine the level of AKT induction, we normalized phospho-AKT with total AKT. In case of PTEN, which is inactivated by phosphorylation, we normalized total PTEN with phospho-PTEN to analyze its induction. Our results showed that all three colorectal cancer cells over expressing LDLR had high basal induction of PTEN after serum replenishment compared to control cells. This was complimented by inhibition of AKT signaling as indicated by low p-AKT/t-AKT expression suggesting that LDLR overexpression decreases in AKT signal induction in HCT 116, HT 29 and DLD 1. Overall, our results showed that LDLR over expression suppresses AKT signal induction through PTEN activation in colorectal cancer cells, implying that LDLR modulate tumour growth by regulating PTEN-AKT signaling.

In conclusion, our results confirmed that ectopic over expression of LDLR in colon cancer cell lines negatively regulates cancer cell viability and motility, underlining its anti-tumourigenic properties and putative tumour suppressor role in cancer. Overexpression of LDLR was also shown to enhance the anti-proliferative and growth inhibitory effect of lovastatin resulting in greater reduction of cell viability. We further established that overexpressed LDLR negatively regulates the growth and survival of these cancer cells by modulating the MAPK, AKT and PTEN signaling. These results suggest that upregulating LDLR expression can retard tumour growth and motility in a subset of colonic tumours, which are exclusively dependent on mevalonate pathway for their survival and growth. It appears that these colonic tumours, which do not seem to require LDL cholesterol for their growth represent an aggressive phenotype that is selected and clonally expanded with increasing downregulation of LDLR and a concordant elevation of cholesterol biosynthesis owing to constitutive high expression of HMGCR and SREBP2. Besides imparting a growth and survival advantage to the colonic tumours, low LDLR expression is also correlated with resistance to cholesterol reducing drug treatment, as seen in case of DLD 1. Based on the current findings, we propose that upregulation of LDLR using PCSK9 inhibitors in conjunction with statins could give promising results in retarding growth and proliferation of cancer cells in a subset of CRC tumours.

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**Chapter 5**  
**General Discussion**

Altered lipid and cholesterol biosynthesis has increasingly been recognized as a signature of several cancers types. A growing body of evidence from clinical and epidemiological studies has alluded to the possibility that accumulation of cholesteryl esters is associated with a more aggressive tumour phenotype [1, 2]. In pre-clinical models, the cholesterol biosynthesis pathway inhibitors such as statins have shown promising results in reducing tumour growth and cancer cell proliferation, underlining the significance of cholesterol biosynthesis in tumourigenesis [3-7]. In a study conducted by Clouston *et.al* it was shown that dietary lipid modulate the tumour growth inhibitory effectors of lovastatin [20]. In the same study it was shown that all tumours regardless of dietary lipid or lovastatin treatment, exhibit high expression of HMGCR protein and low level of LDLR protein compared to normal mucosa from tumour bearing rats. Besides few isolated reports on deregulation of LDLR expression in cancer, mainly in prostate and colorectal cancer cells, the correlation of LDLR with cancer has not been explored in great detail [8-10]. Based on the observations made by Clouston and some of the earlier studies reporting downregulation of LDLR in colonic tumour, we hypothesized that low LDLR expression represents an aggressive CRC phenotype with enhanced growth and survival. To test our hypothesis, we first assessed the expression of key proteins of cholesterol homeostasis pathway in experimentally induced colonic tumour rat model. Our results showed that colonic tumours exhibited high protein expression of HMGCR, SREBP1, SREBP2 and PCSK9 along with a significant downregulation of LDLR protein (refer to Chapter 2) in comparison to normal appearing mucosa from tumour bearing rats. The increase in HMGCR, SREBP1 and SREBP2 protein expression implied that tumours preferred to upregulate the lipid and cholesterol biosynthesis pathway for their growth. Furthermore, low expression of LDLR protein along with elevated protein levels of PCSK9 in colonic tumour suggested that downregulation of LDLR possibly was mediated through the PCSK9 lysosomal degradation pathway, a phenotype preferred for sustaining growth. The question of whether or not the low LDLR expression in colonic tumours was correlated with a more aggressive phenotype characterized by enhanced growth, cell proliferation or the resistance to chemotherapeutic interventions, particularly cholesterol reducing drugs, were important questions that were addressed in the present study.

In order to examine the role of LDLR in colon carcinogenesis and its relevance to human carcinogenic process, it was important to determine if one or more CRC cell lines also exhibit low LDLR status and hence could serve as preclinical models. We selected three colorectal cancer cell lines that allowed us to study the role of LDLR in CRC tumorigenesis and the underlying mechanisms, from a human disease perspective. The three human colorectal cancer cell lines we selected for our study (HCT 116, HT 29 and DLD 1) broadly represented the main molecular subtypes and key mutations found in CRC. Our results indicated that all three colorectal cancer cell lines displayed differential expression of LDLR protein and mRNA at a level significantly lower than untransformed colonic mucosa cells (Discussed in chapter 3). Interestingly, HMGCR, SREBP1, SREBP2 and PCSK9 expression at both mRNA and protein levels, were elevated in all colorectal cancer cell lines. Thus our cell line data corroborated our *in vivo* data from experimentally induced colonic tumour supporting a model in which human colorectal cancer cells exhibit a tumour phenotype with low LDLR expression and an elevated cholesterol biosynthesis pathway. In the same study, we also found that DLD 1 cells had the lowest LDLR expression accompanied by the highest cell viability and proliferation rate amongst the three cancer cell lines. This lends further supports to our hypothesis that low LDLR expression gives a growth and survival advantage to colonic tumours. We also observed that unlike HCT 116 and HT 29 cells which upregulated LDLR expression in response to the cholesterol reducing drugs lovastatin and RO 48-8071, DLD 1 cells did not upregulate their LDLR expression. Furthermore the viability of DLD 1 cells is minimally affected by lovastatin or RO 48-8071 treatment, whereas both HCT 116 and HT 29 respond to these treatments with cell viability significantly reduced.

Taken together, these results suggested that the lowering of LDLR protein expression during colorectal cancer progression is not only associated with a more aggressive tumour phenotype characterized by enhanced growth but that it also may contribute to the resistance to cholesterol reducing therapeutic interventions. Thus it appears that during colorectal cancer progression, a group of hyper-proliferative transformed cells with low LDLR and heightened cholesterol biosynthesis (indicated by high HMGCR/SREBP2 expression) are selected and clonally expanded to form aggressive tumours that may or may not be sensitive to pharmacological inhibitors targeting



cholesterol biosynthesis pathway. Based on the responsiveness of three cancer cell lines to cholesterol lowering drugs and the levels of various cholesterol homeostasis associated proteins, we concluded that all three cell lines could serve as pre-clinical models to determine whether or not LDLR plays a role in colon carcinogenesis. We reasoned that all three cell lines representing tumour phenotypes with varying degree of deregulated cholesterol biosynthetic pathway could serve as ideal tools to study the role of LDLR in 1.) Regulating the growth of colon cancer cells 2.) Modulating the response of cancer cells to inhibitors of cholesterol biosynthesis pathway such as statins.

To get a better insight into the potential tumour suppressor role of LDLR in CRC and the underlying signaling pathways through which it exerts its anti-tumourigenic effect, we ectopically overexpressed LDLR in all three CRC cell lines using a CMV driven overexpression vector. After confirming the overexpression of LDLR through Western blot analysis, we assayed for the effect of LDLR overexpression on CRC cell viability. (refer to chapter 3, figure 3.1). Our results showed that ectopically overexpressed LDLR significantly reduced cell viability in all three colorectal cancer cells including DLD 1 cells. Additionally, overexpressed LDLR enhanced the growth inhibitory effect of lovastatin in all cancer cell lines (Figure 4.7). The negative effect of elevated LDLR protein expression on cancer cell viability suggests that LDLR primarily plays the role of a potential growth inhibitor or tumour suppressor and not just a receptor for cholesterol uptake in these cancer cells. Hence, one would expect that these cancer cells would be more dependent on the mevalonate pathway for their growth and survival making them vulnerable to therapeutic drugs that target cholesterol biosynthesis, as is the case we noted for HCT 116 and HT 29 cells. DLD 1 was an exception since despite being dependent on cholesterol biosynthesis for its growth; it was resistant to cholesterol biosynthesis inhibitors. Overall this result implies that a combination therapy targeting the cholesterol biosynthetic pathway along with LDLR upregulation through inhibiting PCSK9 inhibition and hence LDLR upregulation could be more effective. For example, in the case a tumour phenotype similar whose expression pattern in terms of PCSK9 and LDLR levels is similar to DLD 1 this may be an effective therapy in reducing tumour growth.

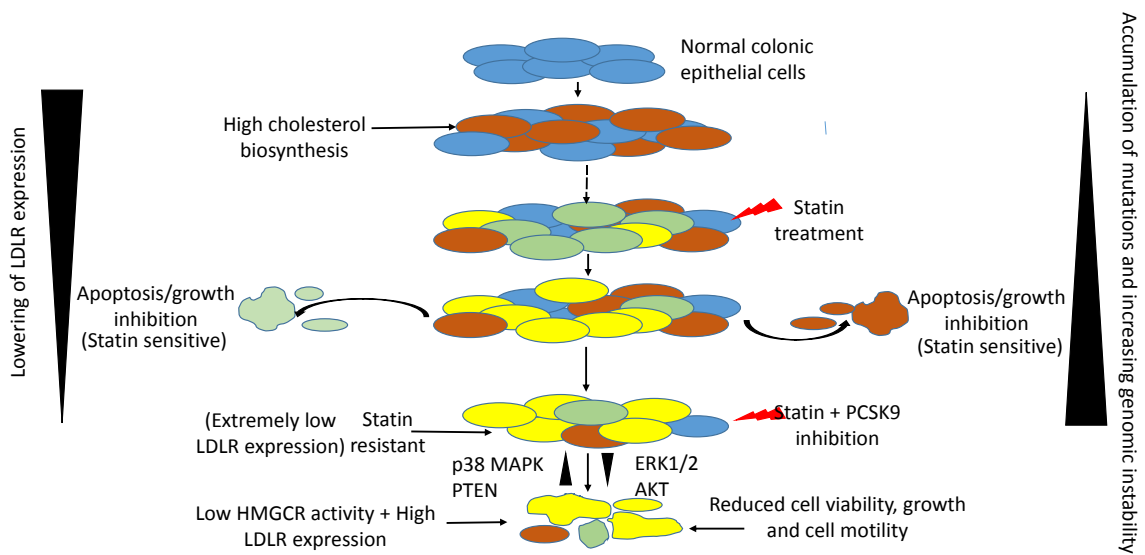
To explore the role of LDLR in CRC development, we assessed the cell motility and migration rate of colorectal cancer cells overexpressing LDLR and comparing that with

empty vector transfected control We observed that cancer cells with overexpressed LDLR had significantly reduced motility and migration rates as indicated by the distance travelled by the cancer cells from the wounded edges to the cell free scratched area over a period of 24 hours (Figure 4.2). Increased cell motility and migration ability is a phenotype acquired by cancer cells as they become more invasive. It is partially achieved by cytoskeletal remodeling through deregulation of actin polymerization which involves several signaling pathways such as PKB/AKT, Rho GTPase and Rac mediated pathways[11]. There is published evidence that supports a role for LDLR related proteins-1 (LRP-1) in promoting cancer invasiveness through modulation of the the ERK and JNK pathways [12, 13] but there is no evidence linking LDLR with cancer cell motility or invasiveness. This is the first study to report a correlation between LDLR and cancer cell motility. The exact mechanism through which LDLR could be regulating cell motility and migration in cancer needs to be investigated in future studies.

In order to investigate the molecular mechanisms through which LDLR could potentially modulate the growth and survival of cancer cells, we studied the two main growth signaling pathways that are deregulated in the majority of cancers including colorectal cancer, one is the MAPK signaling and the other is the AKT/PTEN signaling. We analyzed the induction of the three main effector kinase families of MAPK signaling pathway –ERK, p38 and SAPK in CRC cells with an ectopic overexpression of LDLR growing for 4 hours in serum depleted media followed by serum replenishment for 30 and 60 minutes. Similar treatment conditions were used to analyze the induction of AKT and PTEN signaling. Overall, our results established that LDLR overexpression decreases induction of pro-growth and proliferation signaling through lowering of the ERK and AKT induction. Ectopically overexpressed LDLR also activates anti-tumourigenic signaling through the induction of the p38 and PTEN signalling. Even though we observed differential regulation of SAPK in cancer cells with overexpressed LDLR, at this point in time it is not clear as to how and what role the LDLR- SAPK pathway interaction could play in tumourigenesis. Taken together, we demonstrated that ectopically overexpressed LDLR negatively regulates the growth and survival of colonic tumours by inducing p38 and PTEN signaling and reducing the induction of ERK MAPK/AKT signaling.

Through our work, we established that a subset of potentially aggressive colonic tumours exhibit low LDLR protein expression complimented with elevated expression of HMGCR and SREBP2. Heightened PCSK9 expression in colonic tumours alludes to the possibility of its involvement in high LDLR turn over in these cancer cells, though we cannot rule out another post-translational regulator of LDLR known as the Inducible Degradator of LDLR (IDOL). Since we observed significantly lower expression of LDLR mRNA in colorectal cancer cells compared to untransformed mucosa cells, it would be interesting to analyze the expression of micro-RNAs, such as the recently validated micro-RNA 185 that targets LDLR mRNA for degradation [14]. The role of inflammation in cancer is well appreciated as tumour cells and their microenvironment are known to secrete pro-inflammatory cytokines and chemokines such as tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukins and interferons [15]. Recently, it was reported that inflammatory stress induced by interleukin-1  $\beta$  (IL-1 $\beta$ ), TNF- $\alpha$ , IL-6 and lipopolysaccharides (LPS) resulted in statin resistance in human mesangial cells [16]. In the same study, it was shown that inflammatory stress increased HMGCR expression and enzymatic activity through upregulation of SCAP-SREBP2 pathway. Colonic tumours have been shown to express high levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 which are secreted in an autocrine fashion to maintain a state of low grade chronic inflammation that supports their growth [17]. Therefore, it is possible that inflammation could play a role in loss of sterol mediated negative feedback regulation of HMGCR resulting in upregulation of cholesterol biosynthesis pathway as well as statin resistance. Inflammation has also been reported to induce PCSK9 expression that results in accelerated degradation of LDLR and increased serum cholesterol levels [18]. Thus inflammation can have pleiotropic effects on cholesterol homeostasis primarily resulting in increased HMGCR expression, statin resistance and increased PCSK9 mediated degradation of LDLR. An increase in HMGCR expression and enzymatic activity could be the reason for further downregulation of LDLR to avoid cellular toxicity due to accumulation of free cholesterol. Upregulation of Acyl CoA cholesterol acyl transferase (ACAT) expression in cancer and altered compartmentalization, trafficking and storage of LDL cholesterol and endogenously derived cholesterol in cancer are other factors that could contribute to increased cholesterol biosynthesis and low LDL cholesterol uptake [9, 19].

To summarize, we show that LDLR plays the role of a potential tumour suppressor in colorectal cancer cells. It appears that during cancer progression, transformed cells with low LDLR expression and elevated cholesterol biosynthesis pathway are selected and clonally expanded to acquire a more aggressive tumour phenotype characterized with enhanced cell viability, motility and resistance to therapeutic drugs targeting the cholesterol biosynthesis pathway. In a clinical setting this means that low LDLR expression in CRC patients could serve as a prognostic marker for administration of a combination therapy that includes targeting of both cholesterol biosynthesis pathway through statins and LDLR through PCSK9 inhibitors. Additionally, a prolonged use of statins and PCSK9 inhibitors could reduce the risk of re-occurrence of adenomas in the colon after colonoscopy by targeting the seemingly dormant but potentially aggressive transformed colon cells that somehow escaped the treatment.



**Figure 5.1 Overall perspective of the role of LDLR in CRC development**

*Schematic diagram showing transformed colonic cells acquiring low LDLR protein expression phenotype as they accumulate mutations, which triggers their clonal selection and expansion within the population of tumour cells. These cells expressing high HMGCR and low LDLR protein rapidly proliferate and are resistant to chemotherapeutic intervention by statins. The combined effect of LDLR upregulation through PCSK9 inhibitions and statins reduces the cell viability, growth and migration of these cancer cells. This growth inhibitory effect of upregulated LDLR is due to suppression of AKT/ERK and induction of p38/PTEN signaling.*

### **Significance of the study and future directions**

In this dissertation, a series of experiments were carried out to test the hypothesis that LDLR plays an important role in CRC development and whether upregulation of LDLR will retard the growth of CRC. We demonstrated that LDLR overexpression negatively regulates CRC cell growth and motility by modulating the MAPK and AKT/PTEN pathway. Our work has expanded the information on the expression of key molecules of the cholesterol homeostasis pathway in both an experimentally induced colonic tumour rat model as well as a cell culture model. More importantly, this study has given us insight into the potential role of LDLR in CRC development as well as in the response to therapeutic interventions that target the cholesterol biosynthetic pathway. This study has also raised additional questions about the role of cholesterol biosynthetic pathway and LDLR in particular in CRC pathogenesis. It will therefore be of interest to investigate the stage of CRC development in which lowering of LDLR expression is critical for tumour growth. Another area of interest raised is in regard to the role that systemic inflammation plays in the development of CRC. It has been suggested that tumours which develop under a pro-inflammatory environment have a different genotype than those that are sporadic in nature. Future studies should focus on determining LDLR expression in these cells and furthermore whether they respond to cholesterol lowering drugs. Whereas the results presented in this dissertation can be considered quite convincing from a mechanistic point of view, they also raise a number of questions which should be pursued in the future. The importance of *in vitro* findings should be tested *in vivo*. To further explore the importance of a deregulated cholesterol biosynthetic pathway and LDLR, it will be important to carry out some of the studies in an *in vivo* models. The conditional overexpression or knockout of LDLR in colonic cells may help address questions about the role of LDLR in various stages of CRC development including metastasis. It will be important to determine as to when deregulation of the cholesterol biosynthetic pathway is important during the multistep process of colon carcinogenesis. Pre-clinical models should be used to understand the factors which lead to lowering of LDLR in tumours. There is limited or negligible information pertaining to the role of SREBPs and PCSK9 in the development of colonic tumours. CRC is a heterogeneous disease in which the genesis and biology of tumours appearing in the right side of the colon differ from those emerging in

left side of the colon. Therefore it is critical to determine whether or not there is a difference among these tumour types with respect to deregulation of the cholesterol biosynthetic pathway and sensitivity to HMGCR inhibitors. Furthermore, it will be important to target not just HMGCR but other molecules such as PCSK9 and SREBPs to determine their importance in CRC development.

The findings of the present dissertation present a novel avenue of investigation that is worth pursuing in human based clinical studies. An understanding of the biological heterogeneity in human CRCs depending on their location along the colonic axis, mutations spectra and their metabolic phenotypes must be considered, to advocate the role of personalized medicine in CRC prevention. There is a strong association between obesity, metabolic syndrome and diabetes with CRC as well as cardiovascular diseases. Therefore, it is important that oncologists assess the role of cholesterol lowering drugs in the prevention of cardiovascular disease as well as cancer.

The findings of the present study have significant translational value in actualizing the concept that many chronic diseases have common etiology and they share aberrant metabolic pathways. In this context, the lipogenic biosynthetic pathway and LDLR may play a central role in carcinogenesis and cardiovascular diseases.

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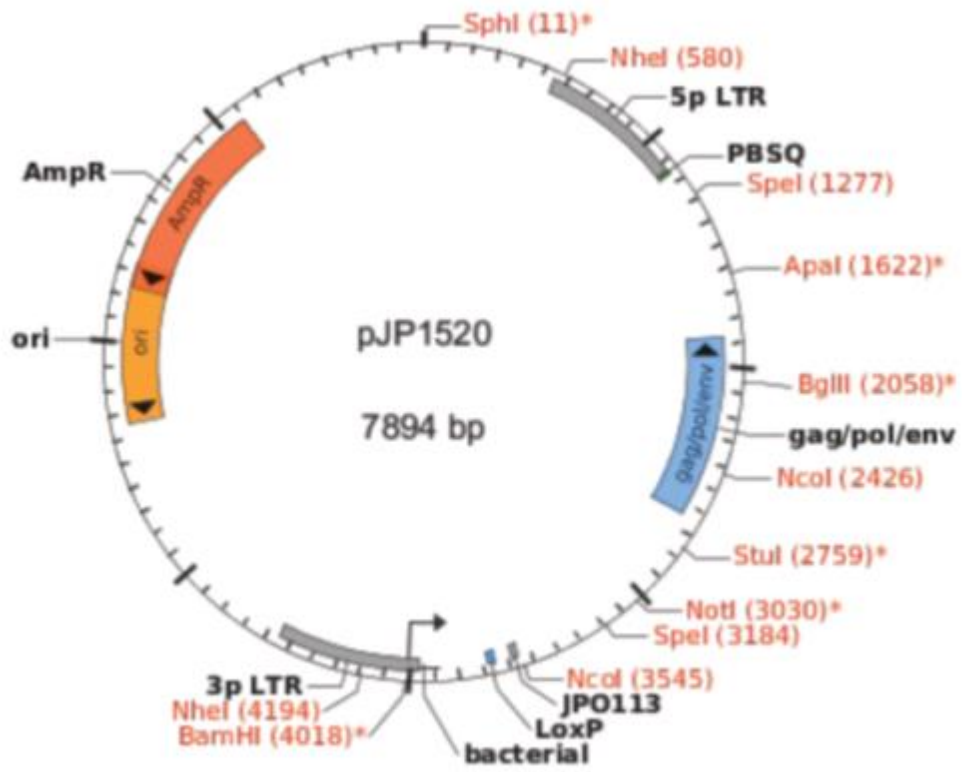
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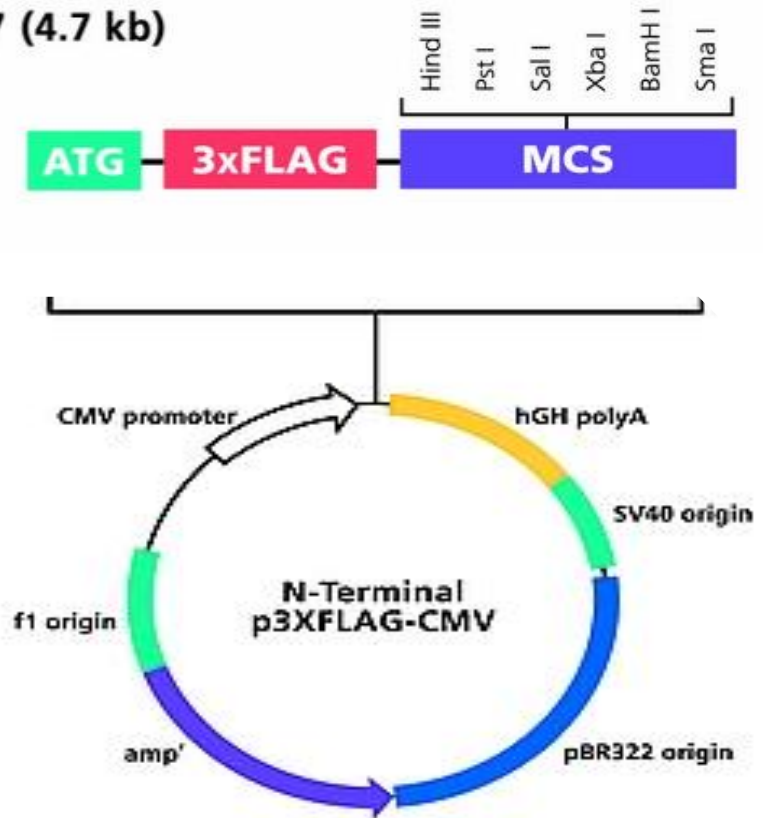
## **Appendices**

Appendix 1. Pjp1520 Retroviral expression vector (DNASU)



**Appendix 2. p3XFLAG-CMV-7 expression vector (Sigma)**

**p3xFLAG-CMV-7 (4.7 kb)**



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