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New Insights of LXR Signalling In Inhibition of Cancer Proliferation and Inflammation

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Dedicated to

My Family

In Loving Memory of My Parents

Thesis defense

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ABSTRACT

Liver X receptors (LXRs) are ligand-activated transcription factors belonging to the nuclear receptor (NR) superfamily. Up-to-date, more than 1900 publications have established the role of LXRs in cholesterol homeostasis, lipogenesis, glucose metabolism, atherosclerosis, proliferation, and inflammation. In this thesis we have focused mainly on the role of LXR in proliferation and inflammation.

In Paper I we studied the role of LXR on the inhibition of proliferation in breast cancer cell lines through PI3K/AKT signalling pathway. Phosphorylation of several protein kinases in this pathway was reduced upon LXR activation, such as AKT and PI3K. Expression of both phosphatases PTEN and PHLPP, which directly regulate PI3K product (PIP3) and AKT respectively, was induced by LXR on transcriptional and protein levels. Furthermore, we showed that LXRβ was main executer of the anti-proliferative effect in human MCF7 breast cancer cell line.

In Paper II we continued the findings of paper I to explore more the role of LXR in inhibition of proliferation in human breast cancer cell line in relation to signalling pathway. In this study we investigated whether LXR regulates mTOR complex pathway in human breast carcinoma cell line. Thus, we identified that activated LXR inhibited proliferation of MCF7 cell via mTORC1 by affecting the phosphorylation of Raptor at Ser792 and mTOR at Ser2448, and its downstream target p70S6K and 4EBP1. Our data showed that there was no direct effect of LXR on the phosphorylation status of mTORC2. We further identified that LXR stimulation induced proliferation of MCF7 cells when Raptor was depleted, suggesting the crucial role of Raptor in LXR inhibition of cell proliferation.

In Paper III we investigated the impact of LXR agonists on triple negative human breast cancer using a patient-derived xenograft model. Primary tumors from patients were grafted into immune–compromised mice, where the tumour was allowed to expand. The primary tumour was then collected and used for subsequent xenografts, for generating a large mouse colony, all bearing tumors were shown that maintained the characteristics of the original tumour. We found that activation of LXR reduced progression of triple negative breast tumors *in vivo*. Moreover, we showed that LXR reduced phosphorylation of AKT at Ser473 residue, decreased expression of the proliferation marker Ki67, as well as reduction of both α-SMA (smooth muscle actin) and capillary density. The last two are angiogenic markers, thus suggesting a role of LXR in regulation of angiogenesis.

In Paper IV we demonstrated a protective role of LXR in inflammatory bowel disease (IBD). We used dextran sodium sulfate (DSS) and 2,4,6-trinitrobenzene sulfonic acid (TNBS) to induce colitis in mice. We observed that LXR deficient mice showed severe and fast disease progression with slower recovery as well as decreased survival rates. In addition, activation of LXR reduced the infiltration of immune cells and the expression of inflammatory cytokines, chemokines in the colon epithelium of mice. In patients with IBD, expression of both LXRα and LXRβ were significantly suppressed in inflamed colon compared with healthy controls.

LIST OF SCIENTIFIC PAPERS

I.   **Hassan, T. S.**, Paniccia, A., Russo, V., and Steffensen, K. R. LXR Inhibits Proliferation of Human Breast Cancer Cells through the PI3K-Akt Pathway. Nuclear Receptor Research, 2015. 2.

II.   **Hassan, T. S.**, Vedin, L. L., Steffensen, K. R., Parini, P., and Jakobsson, T., Liver X Receptor Mediates Inhibition of Estrogen Receptor Positive Breast Cancer Cells Proliferation through mTORP70S6K Pathway (Manuscript)

III.   Vedin, L. L.*, Kristian, A.*, **Hassan, T. S.,** Tenstad, E., Federico, G., Gythfelt, H.V., Juelle, S., Gröne, H. J., Engebåten, O., Mælandsm, G. M., and Steffensen, K. R., The effect of liver X receptors on breast cancer tumour progression in an orthotopic xenograft model (Manuscript)

IV.   Jakobsson, T., Vedin, L. L., **Hassan, T. S.**, Venteclef, N., Greco, D., D'Amato, M., Treuter, E., Gustafsson, J. A., and Steffensen, K. R., The oxysterol receptor LXRbeta protects against DSS- and TNBS-induced colitis in mice. Mucosal immunology, 2014; 7(6), p:1416-1428.

* Equally contributing authors.

CONTENTS

LIST OF ABBREVIATIONS

4EBP1	Initiation factor 4E-binding protein
ABC	ATP-binding cassette
ACAT2	1-1-Acyl-Coenzyme A: Cholesterol Acyltransferase 2
ACC	Acetyl-CoA carboxylase
AF-1	Activation function 1
AF-2	Activation function 2
AKT	Protein kinase B
AP-1	Activator protein-1
ApoE	Apolipoprotein E
APR	Acute phase response
AR	Androgen receptors
ATCC	American Type Culture Collections
BAD	B-cell lymphoma 2 associated death promotor
BRCA	Breast Cancer gene
CDK	Cyclin-dependent kinase
COS-2	Cyclooxygenase 2
CRISPR	Clustered regularly interspaced short palindromic repeats
CYP7A1	Cytochrome P450 cholesterol 7α hydroxylase
DAG	Diacylglycerol
DBD	Dendritic cells
DC	DNA Binding Domain
DR-4	Direct repeat-4
EC50	Half effective concentration
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
elF4E	Eukaryotic translation initiation factor 4F
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
ERα	Estrogen receptor alpha
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FASN	Fatty acid synthase
FBS	Fetal bovine serum
FOXO	Forkhead box O
FXR	Farnesoid X receptor
GSK-3	Glycogen synthase kinase 3
HDACs	Histone deacetylases
HDL	High-density lipoprotein
HER2	Human Epidermal Growth Factor Receptor 2
HP	Partial hepatectomy
IBD	Inflammatory bowel diseases
IGF-1	Insulin like growth factor

IHC	Immunohistochemistry
IL-6	Interleukin 6
IL-1β	Interleukin 1β
ILK	Integrin-linked kinase
iNOS	Inducible nitric oxide synthase
IRS1	Insulin receptor signalling 1
LBD	Ligand-Binding Domain
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat region
LXR	Liver X receptor
LXRE	LXR response element
MDM2	Mouse double minute 2 homolog
mLST8	Lethal with Serc13 protein 8
MMP-9	Matrix metallopeptidase 9
mSIN1	Mammalian stress activated protein kinase interacting protein
MST-1	Mammalian sterile 20-like kinase 1
mTORC	Mammalian target of rapamycin complex
NCoR	Nuclear receptor corepressor
NFκB	Nuclear factor kappa-B
NPC1L1	Niemann Pick C1-like protein
NR	Nuclear receptor
OR-1	Orphan receptor 1
PBD	PIP2 binding domain
PDGFR	Platelet derived growth factor receptor
PDK1	Phosphoinositide-dependent kinase 1
PEST	Penicillin/Streptomycin
PH	Pleckstrin Homology
PHLPP	Leucine-rich repeat protein phosphatase
PI3K	Phosphatidylinositide 3-kinase
PIP2	Phosphatidylinositol 4,5 diphosphate
PIP3	Phosphatidylinositol 3,4,5 triphosphate
PKCα	Protein kinase Cα
PLTP	Phospholipid transfer protein
PP2A	Protein phosphatase 2A
PP2C	Protein phosphatase 2C
PPAR	Peroxisome proliferator-activated receptor
PR	Progesterone receptor
PRAS40	Proline-rich AKT substrate 40 kDa
PROTOR-1	Protein observed with rictor-1
PTEN	Phosphatase and tension homologue 10
qPCR	Quantitative polymerase chain reaction
RAPTOR	Regulatory-associated protein on mTOR
RB	Retinoblastoma protein

RCT	Reverse cholesterol transport
RICTOR	Rapamycin insensitive companion of mTOR
RORs	Retinoic acid receptor-related orphan receptors
RTK	Receptor tyrosine kinase
PXR	Pregnane X receptor
RXR	Retinoid X receptor
SGK1	Serum-and glucocorticoid-regulated kinase-1
Shc	Src homology 2 domain containing protein
SKP2	S-phase kinase-associated protein 2
SMART	Silencing mediator for retinoic acid and thyroid hormone receptor
SOCS3	Suppressor of Cytokine signalling 3
SREBP	Sterol regulatory element binding protein
SULT2A1	Sulfotransferase enzyme 2 Androgen 1
SUMO	Small ubiquitin-related modifier
TH	Thyroid hormone
TZDS	Thiazolidinediones
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TNM system	Tumor, Node, Metastasis
UR	Ubiquitous receptor
USA	United State of America
VLDL	Very low-density lipoprotein
VSMC	Vascular smooth muscle cells
WHO	World Health Organization

1 INTRODUCTION

1.1 CANCER

Cancer is a worldwide-distributed disease. It is the second most frequent disease in USA, according to the American Cancer Society, which accounts 1 of every 4 deaths [2]. WHO, world health organization, has estimated 8.2 million cancer related deaths worldwide in 2012 [3]. In Sweden, 64 555 new cases of cancer were recorded for 2014 among these breast cancer was the most common among women, with 9730 new cases [4]. Lung, prostate, colorectal, stomach, and liver cancer are the most common types of cancer in men, while breast, colorectal, lung, uterine cervix, and stomach cancer are the most common types of cancer in women [5].

Cancer is a heterogeneous disease with abnormal and uncontrolled growth of cells that are able to spread to other parts of the tissues by a process called metastatization [6]. Hanahan and Wienberg have provided the principle concepts how cancer cells sustain their growth and escape from different anti-tumor mechanisms. This concept named the 'Hallmarks of Cancer', include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism, and evading immune destruction [6]. The reasons why some people develop cancer and others not are not well understood but there are many common risk factors that link to cancer development. Most of these factors are related to age, lifestyle habits, and the environment including: tobacco, alcohol, radiation, obesity, infectious agent, hormones, chronic inflammation, diet, immunosuppression, and cancer-causing substances [7, 8].

The process of cancer development occurs in various stages, which are mainly divided based on the TNM system. The TNM system normally stated as: **Tumor (T)** which refer to how large the primary tumor is and where it is located, while **Node (N)** observes the spreading of the tumor to the lymph node, and **Metastasis (M)** refers to the spreading of the tumor to other parts of the body. Based on the TNM staging system, most cancers have four stages:

- **Stage 0:** Cancer *in situ* (in place). The cancer has not spread to nearby tissues and still located in the place where it started.

- **Stage I:** The cancer has still not grown deeply to nearby tissues. This is called early stage cancers.

- **Stage II and III:** The tumors become larger and grow deeply into the nearby tissues and they may have spread to the lymph node, but not to other tissues and organs.

- **Stage IV:** The cancer has spread to other part of the body and this is called advanced cancer or metastasis.

Depending on the cancer stages and types, there are various strategies for cancer treatment. The most common cancer treatments are surgery, radiation, and chemotherapy but there are also other ways of cancer treatment that include targeted or biological therapies, hematopoietic stem cell transplantation, angiogenesis inhibitors, cryosurgery and photodynamic therapy. We have to take in consideration that every cancer treatment has potential risks and side effects and not all forms of treatments are suitable for all patients.

1.2 BREAST CANCER

Breast cancer is the most widespread malignancy among women and the most leading cause of cancer death worldwide. Of all cancer type diagnosed in women, breast cancer has the highest incidence rate of 25% and the mortality rate reaches 15% [9]. The risk of developing breast cancer increases with certain lifestyles (dietary habit, alcohol consumption and low number of children), age, obesity, high breast density, hormonal imbalance, exposure to toxins and ionizing radiation, and genetic factors. It has been estimated that inherited factors account approximately 5-10% of all cases of breast cancer [10]. Mutation in BRCA1 and BRCA2 tumor suppressor genes are among the most common genetic factors influencing breast cancer with a risk of 60-80% in those affected. Mutations in P53 and phosphatase and tension homologue 10 (PTEN) are among the rare significant hereditary factors for development of breast cancer [11, 12].

The breast tissue is mainly made up of two systems, glandular system called lobules that can produce milk and thin tubular system called ducts, which carry milk from glands to the nipple. Additionally, breast consists of fat, lymph nodes, blood vessels and connective tissues. The most common type of breast cancer is the mammary ductal carcinoma, but breast cancer also can originate from cells of the lobules, so called lobular carcinoma. In a rare situation breast cancer can a rise from connective tissue and produce breast cancer sarcoma. Also males can develop breast cancer, however it is very rare and principally only ductal carcinoma. Based on histological features, stage of the tumors, presence of different markers such as estrogen receptor alpha (ERα), progesterone receptor (PR) and human epidermal growth factor receptor 2(HER2); breast cancer can be classified into the following subgroups [13] Table 1:

Table 1: *Subgroups of breast cancers.*

Subtype	ERα	PR	HER2
Luminal, A	+	+	-
Luminal, B	+	+	+
HER2 overexpression	-	-	+
Basal-like	+	+	+

Identifying different types of breast cancer based on previous classification facilitate the way to diagnose and treat the patients. For example, triple positive breast cancer (ERα, PR and HER2 positive) can be targeted by using hormonal therapy against ERα, and PR as well as using humanized monoclonal antibody against HER2. The triple negative breast cancer (ERα, PR and HER2 negative) represents approximately 13% of breast cancer cases. Triple negative breast cancer is a basal-like carcinoma and among the most aggressive group and very challenging to treat [14].

Over the recent decades there have been an improvement in breast cancer treatment due to earlier detection, and availability of new therapeutic strategies. However breast cancer treatment is still challenging, since treatments are complex, affected by side effects, and expensive. Although surgery remains the first option and the most successful way of breast cancer treatment, there are also another ways with either adjuvant treatment (after surgery) or neoadjuvant treatment (before surgery). Chemotherapy, radiotherapy, biological therapy (targeted drug therapy), and endocrine therapy are among other options in breast cancer treatment [15-17].

1.3 LIVER X RECEPTOR (LXR)

Liver X receptors (LXRs) are ligand activated transcription factors that belong to the nuclear receptor (NR) superfamily. NRs are a large superfamily and regulate genes important to various physiological processes in the body such as metabolism, adult homeostasis, reproduction, embryonic development and cell growth. The natural ligands of NRs are endocrine hormones such as thyroid hormones and other small molecules like; fatty acids, cholesterol derivatives, retinoic acid, and prostaglandins [18]. Consequently, many NRs are targeted to develop drugs against different diseases such as cancer, diabetes, obesity and atherosclerosis [19]. For example, targeting androgen receptor (AR) and estrogen receptor (ER) for treatment of prostate cancer and breast cancer respectively, while thyroid hormone (TR) and peroxisome proliferator-activated
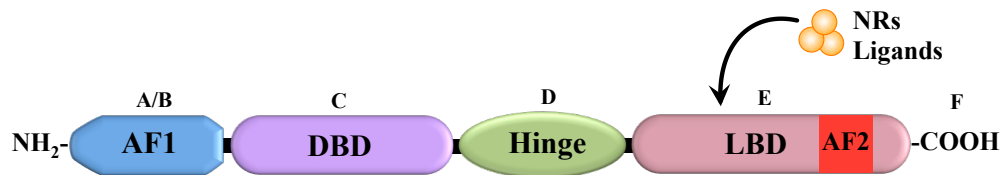
receptor (PPAR) ligands are used to treat both hypothyroidism, dyslipidemias, and diabetes respectively [18].

The NRs consist of 48 members in humans, 49 in mice, and 47 in rat [20]. The first classification of the NRs was based on functional and structural features and was subdivided into four subfamilies [21, 22] (Table 2). Later, NRs have also been subdivided into six superfamilies based on sequence homologies of the DNA binding domain (DBD) and the ligand-binding domain (LBD) [23-26]

NRs consist of five distinctive structural domains (A-F). The N-terminal regulatory domain (A/B domain) contains a ligand independent activation function 1(AF-1). The conserved DBD (C domain) consists of two zinc fingers that bind to the corresponding response element on the DNA. The hinge region (D domain) is a flexible region that links DBD to the LBD. The LBD (E domain) is responsible for ligand binding, receptor dimerization and ligand dependent interaction with various coregulators via activation function 2(AF-2). The AF-2 dependent interaction is often mediated via a NR-box (LxxLL motif) displayed on various coregulators [1]. Some NRs also have a highly variable C-terminal region (COOH, F domain) [21, 23] (Figure 1).

Table 2: *The nuclear receptor superfamily. The table shows the subdivision of NRs into four classes according to the functional and structural features.*

Steroid Receptors (Type I NRs)		Non steroid RXR heterodimers (Type II NRs)	
NR	**Ligands**	**NR**	**Ligands**
GR	Glucocorticoid	LXR	Oxysterols
MR	Mineralocorticoid	PPAR	Fatty acid
PR	Progesterone	FXR	Bile acid
AR	Testosterone	PXR	Xenobiotics
ER	Estrogen	TR (α and β)	Thyroid hormone
		RAR	Retinoic acid
		VDR	Vitamin D, bile acid
		CAR	Xenobiotics
		RXR	Retinoic acid
Dimeric Orphan Receptors (Type III NRs)		**Monomeric/Tethered Orphan Receptors (Type IV NRs)**	
COUP		NGFI-B	
HNF-4		SF-1	
TR (2 and 4)		Rev-erb	
TLL		ROR	
GCNF		ERR	

Figure 1: *Functional domains of nuclear receptors. AF1: Activation function 1; DBD: DNA binding domain; LBD: Ligand binding domain; AF2: Activation function 2.*

LXRs were initially classified as orphan nuclear receptors because at time of discovery no natural ligands were known. Latterly, identification of several physiological ligands has adopted these receptors. LXRs generally forms a permissive heterodimer with the retinoid X receptor (RXR) and were identified in 1994-1995 by screening rat and human liver cDNA library and firstly named ubiquitous receptor (UR), orphan receptor 1 (OR-1), RLD-1, NER and RIP-15 [27-32].

Up-to-date, more than 1900 publications have established the role of LXRs in cholesterol homeostasis, lipogenesis, glucose metabolism, atherosclerosis, diabetes, Alzheimer's disease, proliferation, and inflammation. Two isomers of LXRs have been identified in human, LXRα (NR1H3) encoded by a gene located on chromosome 11p11.2 and LXRβ (NR1H2) encoded by a gene located on chromosome 19q13.3. These two isomers share about 77% identity in their DBD and LBD, and are activated by the same ligands. Human LXRβ (460 amino acids) is ubiquitously expressed, whereas LXRα (447 amino acids) has a more restricted expression pattern predominantly in tissues with high metabolic rate including liver, kidney, adipose tissue, small intestine and macrophage [33].

1.3.1 Structural and Functional Organization of LXR

The LXRs accommodate a canonical NR structure [1, 30]. As mentioned previously, LXR function as a permissive heterodimer with RXR and binds to specific sequences on the DNA, called LXR response elements (LXREs). LXREs consist of two concensus 5´-AGGTCA-3´ sites separated by four nucleotides called direct repeat-4 motifs (DR-4). LXR/RXR heterodimer can be activated by either LXR ligand, RXR ligands or synergistically by ligands of both receptors [28, 30, 34].

In the absence of ligand, LXR is associated with corepressors such as nuclear receptor corepressor (NCoR), silencing mediator for retinoic acid and thyroid hormone receptor (SMART), and histone deacetylases (HDACs) at many regulatory regions (i.e. enhancer and promoters) and follows the conventional coregulator exchange mechanism. Upon ligand binding the corepressor complex dissociates and recruitment of coactivators results in activation of target genes

expression [35]. In addition to the classical coregulator exchange mechanism recent data also suggests an alternative mechanism, a strictly ligand dependent recruitment of LXR to target genes [36, 37]. A large number of coregulators have been identified that contribute to the NR function. Many of these proteins are component of multiple-protein complexes with associated enzymatic functions including nucleosome remodeling activity, histone acetyltransferase and histone methyltransferase activities, which directly or indirectly recruit core transcriptional machinery to the promoter region (Figure 2) [38]. In addition to the metabolic activities, LXR also display anti-inflammatory activities via a mechanism called transrepression [39, 40]. The mechanism of transrepression by LXR will be discussed in further detail below *(1.3.5)*.
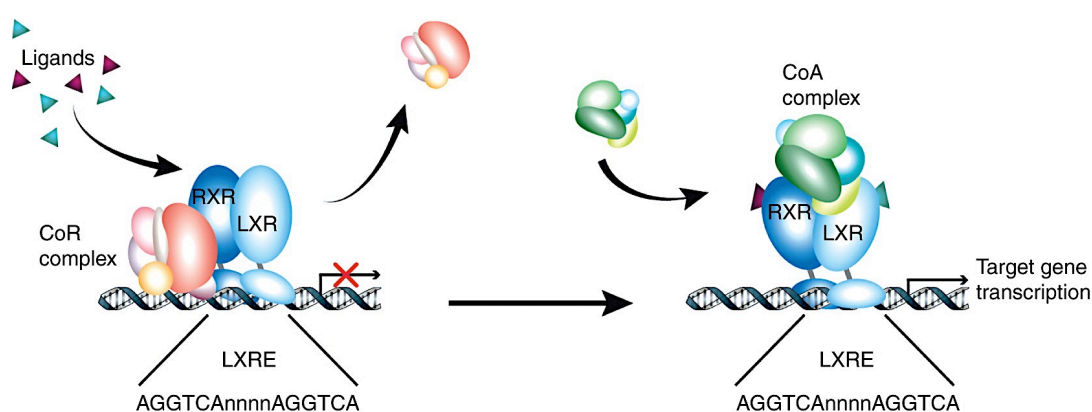
Figure 2. Model of classical transactivation pathway of LXR. *Unliganded LXR-RXR heterodimer is bound to the DNA via an LXRE together with a corepressor (CoR) complex, inhibiting target gene transcription. Ligand binding causes conformational changes promoting the release of the CoR complex and recruitment of coactivator (CoA), thus facilitating histone modification and chromatin remodeling, leading to the transactivation of target gene expression [34].*
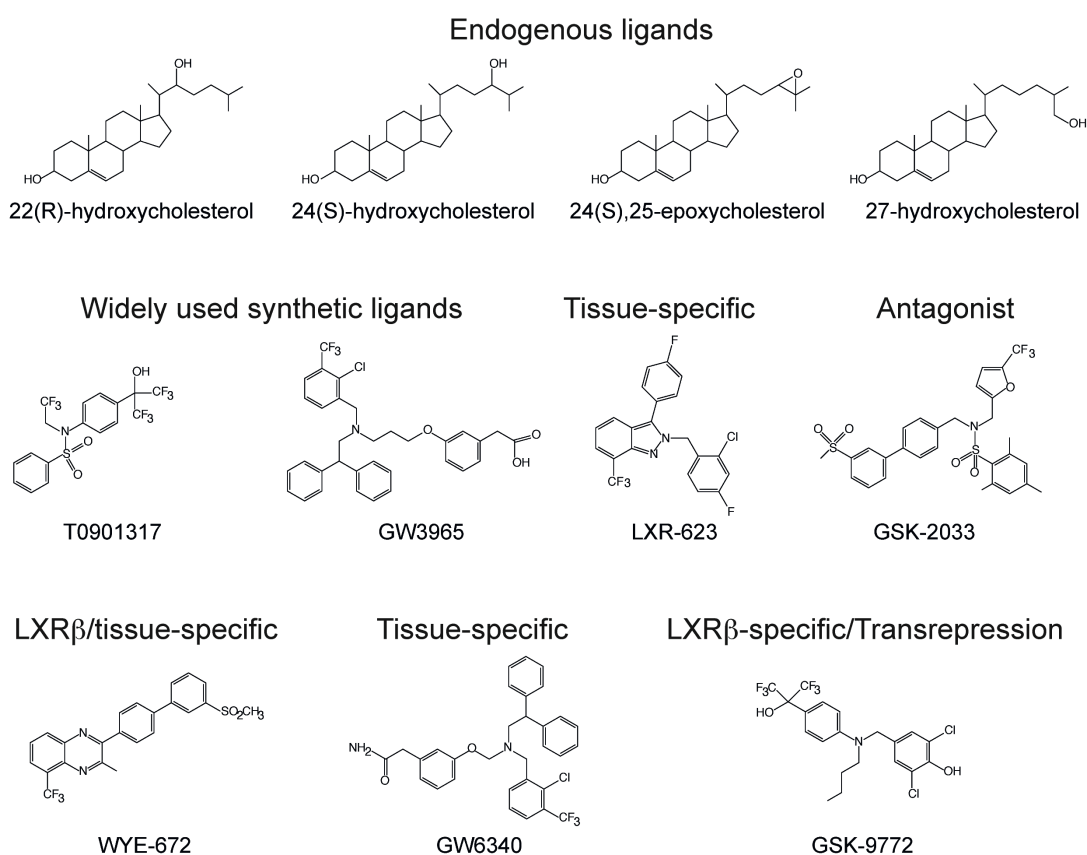
1.3.2 LXR ligands

The LXRs are activated by a specific group of mono-oxidized cholesterol derivatives (oxysterol). It is believed that cholesterol itself has no effect on LXRs, but their derivatives are the most potent naturally activators of LXRs including: 20 (S)-, 22 (R)-, 24 (S)-, 25-, 27-hydroxycholesterol, 24 (S), 25-epoxycholesterol (abundant in the liver). Importantly, these oxysterols were shown to bind and stimulate transcriptional activity of LXRs at a concentration within the physiological range [41]. Recently, it has been shown that LXRs can be activated by cholic acid (3α, 7α, 12α- trihydroxy-5β-cholan-24-oic acid) in the brain [42]. D-glucose, D-glucose-6-phosphate and phytosterols (plant sterols), were also reported to be LXR activators [43, 44]. In addition to the endogenous and natural ligands, various synthetic potent agonists can activate

LXRs. GW3965 [45] and T0901317 [46] are among the most commonly used ligands in experimental studies, displaying EC50 values in low nanomolar range for both LXRα and LXRβ [47]. Additionally to LXR activation, T0901317 has been reported to activate farnesoid X receptor (FXR) [48], pregnane X receptor (PXR) [49] and retinoic acid receptor-related orphan receptors (RORs) [50]. LXR heterodimerize with RXR and thus can also be activated by RXR ligands such as 9-cis retinoic acid [30]. There are other new synthetic agonists available that can activate LXRs pathway in specific tissues, known as tissue-selective agonists, such as: LXR-623, WYE-672 and GSK9772 [51-53].

In contrast to LXR agonists, there are only few antagonists known to negatively regulate the transcriptional activity of LXR and inhibit the interaction of LXRs with coactivators. The natural antagonist geranylgeranyle pyrophosphate and GSK2033 (a synthetic antagonist) are reported to inhibit LXR activity [54, 55] (Figure 3).

Figure 3: *Structure of LXR ligands and antagonists. Chemical structures of LXR endogenous ligands, synthetic ligands, inhibitor and tissue specific agonists [33].*

LXRs activity is not only regulated by agonists or antagonists, but also by changes in receptor expression. A study has been demonstrated that LXRα but not LXRβ receptor expression is controlled by auto-regulatory mechanism. The auto-regulation of LXRα is limited to human cell types (macrophage, adipocytes, hepatocytes, skin fibroblasts and myocytes) [56].

1.3.3 LXR and cholesterol metabolism

Identification of oxysterols as a potent physiological ligand of LXRs pointed out the crucial role of this receptor in cholesterol metabolism. LXRs are involved in all aspects related to the cholesterol metabolism including regulation of reverse cholesterol transport (RCT), conversion of cholesterol to bile acid, and cholesterol absorption/excretion in the intestine [57]. RCT is regulated by LXRs through control of transcriptional regulation of genes involved in the efflux of cholesterol out of the cells, for instance ATP-binding cassette (ABC) including ABCA1 and ABCG1 [46, 58]. In the liver, ABCA1 induces cholesterol efflux to lipid poor apolipoprotein A1 (ApoA1). This process generates high-density lipoproteins (HDL) particles and initiates RCT, while ABCG1 transports cholesterol to phospholipid containing acceptors such as HDL [46, 59, 60].

ABCG5 and ABCG8 are two members of the ABC family responsible for the secretion of cholesterol from liver to bile. These two proteins also regulate cholesterol absorption by intestine. ABCG5/G8 forms an obligate heterodimer and are regulated by LXR [61]. Both cholesterol and phytosterols are absorbed by intestinal enterocytes via Niemann Pick C1-like protein (NPC1L1) [62]. Activation of LXR by synthetic ligand decreases intestinal expression of NPC1L1 and up regulates expression of ABCG5/G8. Cholesterol, but not phytosterols is esterified by 1-1-Acyl-Coenzyme A: Cholesterol Acyltransferase 2 (ACAT2) and incorporate into chylomicrons. Phytosterols remain unesterified and are transported back to the intestinal lumen by ABCG5/G8 transporters [63, 64]. Mutations in either monomers cause β-sitosterolemia, which is characterized by hyperabsorption and decrease in biliary excretion of phytosterols [65]. It has been shown that ABCA1 is an important factor in HDL biosynthesis in the intestine upon LXR activation [33, 66, 67]. Moreover, LXRα activation in mice stimulates RCT in intestine and inhibits the intestinal absorption through upregulation of ABCG5/G8 and downregulation of NPC1L1, which lead to increase in sterol secretion and regulation of cholesterol efflux [61, 68].

LXR not only influences cholesterol metabolism, but also regulates lipid homeostasis and inflammatory responses in the body, especially in macrophage. Activation of LXR by synthetic ligands is expected to have a great effect on the initiation and development of both atherosclerosis [69, 70] and Alzheimer's disease [71, 72]. Mechanistic studies have shown that upon LXR activation in

macrophage, the anti-atherosclerotic effects of LXR are largely attributed to the anti-inflammatory effects that are secondary to the effect on cholesterol metabolism [34, 73].

1.3.4 LXR and cancer proliferation

In the past few years, the antiproliferative effect of LXRs activated by either synthetic or natural ligands *in vitro* and xenograft have been observed in various types of cancer such as prostate [74-76], ovarian [77], breast [75, 78, 79], colon [80, 81] and glioblastoma carcinoma [82]. These studies not only suggest the effect of LXRs on cell proliferation, but also describe the potential mechanisms of action. LXRs inhibit proliferation by several mechanisms, for instance through regulation of cell cycle genes, regulation of signalling pathways, through regulation of metabolic genes (cholesterol metabolism), through induction of apoptosis, and/or through a potential mechanisms affecting hormone signalling.

The first report identifying the role of LXR in proliferation was in prostate cancer. Activation of LXR using synthetic ligand (TO901317) increased the protein levels of cyclin-dependent kinase (CDK) inhibitor (p27) but had no effect on the mRNA level. In contrast, the expression levels of S-phase kinase-associated protein 2(SKP2), a protein that targets p27 for degradation, was decreased upon ligand activation that led to inhibition of proliferation in prostate cancer cell line [75].

In ovarian cancer cells and in vascular smooth muscle cells (VSMC) treatment with LXR agonist also leads to increased expression of p27, as well as its family member p21, which significantly suppresses proliferation and induces apoptosis [77, 83]. Independently of the direct effects on the cell cycle, ovarian cancer cells proliferate in response to the accumulation of oxidized LDL cholesterol. LXR activation by the synthetic ligand TO901317 was shown to inhibit proliferation by increasing the cholesterol efflux and by suppressing the expression of pro-inflammatory cytokines [84]. In breast cancer cells the expression levels of SKP2 are decreased without any effect on p27 and p21 protein levels [78]. One study on ER-positive breast cancer cell lines treated with LXR agonist also showed decrease in the expression of E2F2, a transcription factor that regulates cell cycle progression [85]. Furthermore, in leukemic T-cells and lymphoblast, induction of LXR with TO901317 or GW3965 inhibit proliferation of the cells by preventing cell cycle progression from G1 phase to S-phase by alteration in the phosphorylation of Retinoblastoma protein (RB), a key regulator of entry to S-phase, without any effects on p27 [86]. In colon cancer and pancreatic cancer, SKP2 is downregulated upon LXR activation and consequently resulting in disruption of proliferation [87, 88].

Interestingly, activation of LXR in pancreatic cancer cells leads to increased expression of epidermal growth factor receptor (EGFR) suggesting a crosstalk between LXR and growth factor signalling pathways [88], as also shown in glioblastoma carcinoma [82]. In glioblastoma, which is one of the most common and lethal malignant brain tumors in adults, cells treated with GW3965 inhibit EGFR expression through upregulation of ABCA1, cholesterol efflux transporter, which regulates cholesterol homeostasis through suppression of PI3K/AKT signalling pathway and promotes cell death [82]. Intriguingly, a new role of non-transcriptional function of LXR has been addressed in colon cancer where LXRβ was shown to induce cell death by pyroptosis through induction of Caspase-1 [89].

As previously mentioned, LXR inhibit proliferation through several mechanisms, for example in prostate cancer suppression of proliferation is not just related to the cell cycle genes regulation but also through cholesterol depletion from the lipid rafts by induction of expression of ABCG1 that leads to an increase in cholesterol efflux that subsequently activates PI3K/AKT signalling pathway crucial for cell survival [76]. In another study, cholesterol homeostasis in prostate cancer has shown to be regulated by LXR target genes ABCA1 and ABCG1, which mediate cholesterol efflux in LNCaP prostate cancer cells and consequently lead to lipid deprivation in the cell membrane necessary for maintaining of cell growth [90].

The correlation between LXR, proliferation and cholesterol homeostasis has also been shown in mouse liver regeneration after partial hepatectomy (HP), in which part of the liver was removed in order to study the ability of liver to regenerate. In HP, the cholesterol level of the liver is increased in response to regeneration processes. At the same time, activation of LXR with GW3965 reduced the regeneration of liver thus indicating also in this model the importance of LXR in cell proliferation [91]. The antiproliferative effects of LXR in certain types of cancer cell lines is also related to the expression of lipogenic target genes SREBP1 and fatty acid synthase (FASN) [92]. In prostate cancer cell line, knockdown of FASN partially suppresses the anti-inflammatory effects of liganded LXR and increases expression of suppressor of cytokine signalling 3 gene (SOCS3), which lead to increase of cell death and decrease of proliferation[93], while in breast cancer the knockdown of SREBP1 shows no changes of the antiproliferative effect in response to LXR ligand [78].

Another mechanism of LXRs to inhibit proliferation is their effects on steroid hormone receptors. This mechanism seems to be specific to ER+ breast cancer and to prostate cancer associated with AR. The effect of LXR on hormone dependent proliferation in ovarian cancer has not been reported [34]. Androgens and androgen receptors signalling play an important role in the development of

both normal prostate growth and in diseases related to prostate, especially prostate cancer. One study has shown that LXR agonist TO901317, but not 22(R)-hydroxycholesterol and 24(S)-hydroxycholesterol acts as anti-androgenic agonist in human prostate cancer cells [94]. Furthermore another study has demonstrated that ligand treated LXRα overexpressed mice inhibits AR activity by increasing expression of sulfotransferase enzyme 2A1 (SULT2A1) and decreasing expression of steroid sulfate [95]. Similarly, LXRs regulate estrogen homeostasis in the liver by increasing expression of estrogen sulfotransferase (Est or SULT1E1), which consequently suppresses estrogen-dependent breast cancer cell proliferation in nude mice [96]. There are also evidence indicating that activated LXR suppresses breast cancer cell proliferation through inactivation of ER both on protein and transcriptional levels [78, 85].

1.3.5 LXRs regulate inflammatory responses and immunity

Recent studies have revealed that LXRs are important factors in innate and adaptive immunity and inflammatory response beside their known role in metabolism [57]. LXRs and other NRs, such as PPARs, upon ligand activation can inhibit the activities of other signal dependent transcription factors such as nuclear factor kappa-B (NFκB) and activator protein-1 (AP-1) through a mechanism called transrepression. This mechanism includes protein-protein interaction with the coregulator proteins and prevents the transcription of the target genes [97].

LXRs inhibit signal dependent activation of several inflammatory target genes by a transrepression mechanism (Figure 4). Upon ligand binding, LXR undergoes SUMOylation, which is a posttranslational modification, by conjugation of small ubiquitin-related modifier (SUMO 2 or 3) molecules to lysine residues at LXR in the LBD by E3 ligases. Subsequently, SUMOylated LXRs, upon docking, prevents the release of corepressors at several inflammatory response genes and inhibits the transcription [40, 98].

Tontonoz and colleagues were the first to demonstrate the link between the anti-inflammatory actions of LXR and the cholesterol metabolism. In response to inflammatory signals mediated by bacterial lipopolysaccharide (LPS), tumor necrosis factor α (TNFα), and interleukin 1β (IL-1β) in macrophage, activation of LXRs inhibit expression of inflammatory genes such as interleukin 6 (IL-6), inducible nitric oxide synthase (iNOS), matrix metallopeptidase 9 (MMP-9), and cyclooxygenase 2 (COS-2). Additionally, upon ligand binding, the expressions of LXRs target genes (ABCA1 and ABCG1) are increased in macrophage stimulated with inflammatory signals, connecting the innate immunity and cholesterol metabolism regulated by LXRs [73, 99].
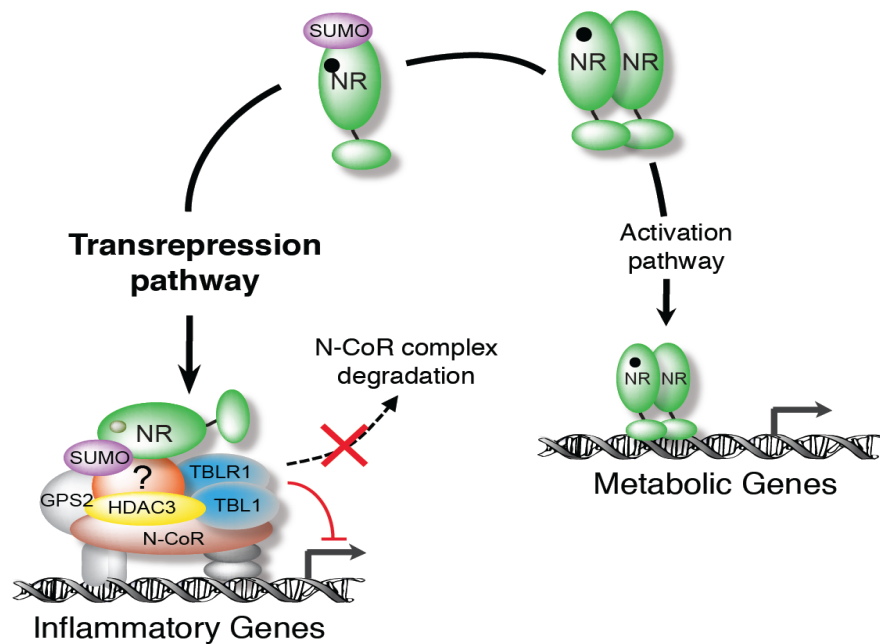
Figure 4. *Anti-inflammatory transrepression by NRs* [100].

Protecting macrophage from apoptosis by LXRs was another finding that showed the anti-inflammatory action of LXRs. LXRα-deficient mice are susceptible to bacterial infection subsequently accelerates macrophage cell death. Activation of LXR by synthetic ligands induces expression of several antiapoptotic factors, inhibits other pro-apoptotic factors, and reduces this phenomenon [101].

In addition to anti-inflammatory activities, LXRs also display immune regulatory functions. LXRs activated with GW3965 and TO901317 inhibit LPS induced neutrophil infiltration in the lung airway and reduce cytokine production in primary human and rodent alveolar macrophages [102, 103]. Several *in vivo* studies have demonstrated the role of LXRs in immune response triggered by pathogens in the host. For example, in mice infected with *Klebsiella pneumonia*, the mortality rate is increased when LXR is activated with TO901317 [103], while LXRα-deficient mice infected with *Mycobacterium tuberculosis* show increased susceptibility to infection and reduced neutrophil response compared with wild type mice [104]. On the other hand, LXR deficient mice exhibit more resistance to infection with the protozoan parasite *Leishmania chagasi/infantum* [105].

It appears that activation of LXRs inhibits the inflammatory response in ulcerative colitis, which is a type of inflammatory bowel diseases (IBD), and even in colon cancer [106]. The molecular mechanisms behind the pathogenesis of IBD are not well understood, but evidently it causes deregulation of the inflammatory response in small and large intestine. Besides the known

components of the inflammatory response, few factors have been reported to contribute in the initiation and progression of IBD including environmental factors, the luminal flora and genetic predisposition [107]. Moreover, studies have shown that increased permeability of the epithelial layer to some extent might contribute to the progress of IBD [108, 109]. We have recently reported that both LXRα and LXRβ have anti-inflammatory roles in suppression of various inflammatory mediators in the colon [106].

Important immunomodulatory properties of LXRs have been described in various cell types including monocytes, macrophage, T and B cells, skin, dendritic cells, brain, muscle, liver, bone, and pancreas [33, 110]. Interestingly, the role of LXRs in antigen presenting dendritic cells (DC) and T cells provides evidence that link LXR to both adaptive and innate immune response. One study suggests that LXR inhibit the ability of DC to activate the adaptive immune response via T-cells through reduced expression of inflammatory genes associated to maturation and migration of DC in the lymph nodes [111]. But, other studies suggest that LXRs induce expression of other pro-inflammatory markers that supports maturation and DCs function and induce T-cell proliferation [112]. Therefore, the above findings reveal divergent data that correlate LXRs with DCs.

LXRs, especially LXRβ, show anti-inflammatory response in the liver, where they inhibit acute phase response (APR) [98]. APR is a systemic change that associated with the release of acute phase proteins in response to inflammatory stimuli [113]. The anti-inflammatory responses of LXRs are further reported in various immune related tissues and diseases including: skin diseases, arthritis, and neurodegenerative diseases such as multiple sclerosis, Parkinson's disease and Alzheimer's disease [72, 114-118]. Also, it has been shown that LXRs decrease graft versus-host inflammatory reactions and suppresses the chronic damage allograft in rat kidney [119].

1.4 PI3K/AKT/MTOR SIGNALLING PATHWAY

1.4.1 Normal PI3K/AKT/mTOR signalling cascade

Receptor tyrosine kinases (RTKs) are cell surface receptors that are activated by binding of growth factors such as epidermal growth factor (EGF), insulin like growth factor (IGF-1), cytokines, and integrin [120]. They in turn activate phosphoinositide 3-kinases (PI3Ks). PI3K is involved in various cellular processes such as cell proliferation, growth, motility, survival and angiogensis [121, 122] (Figure 5).
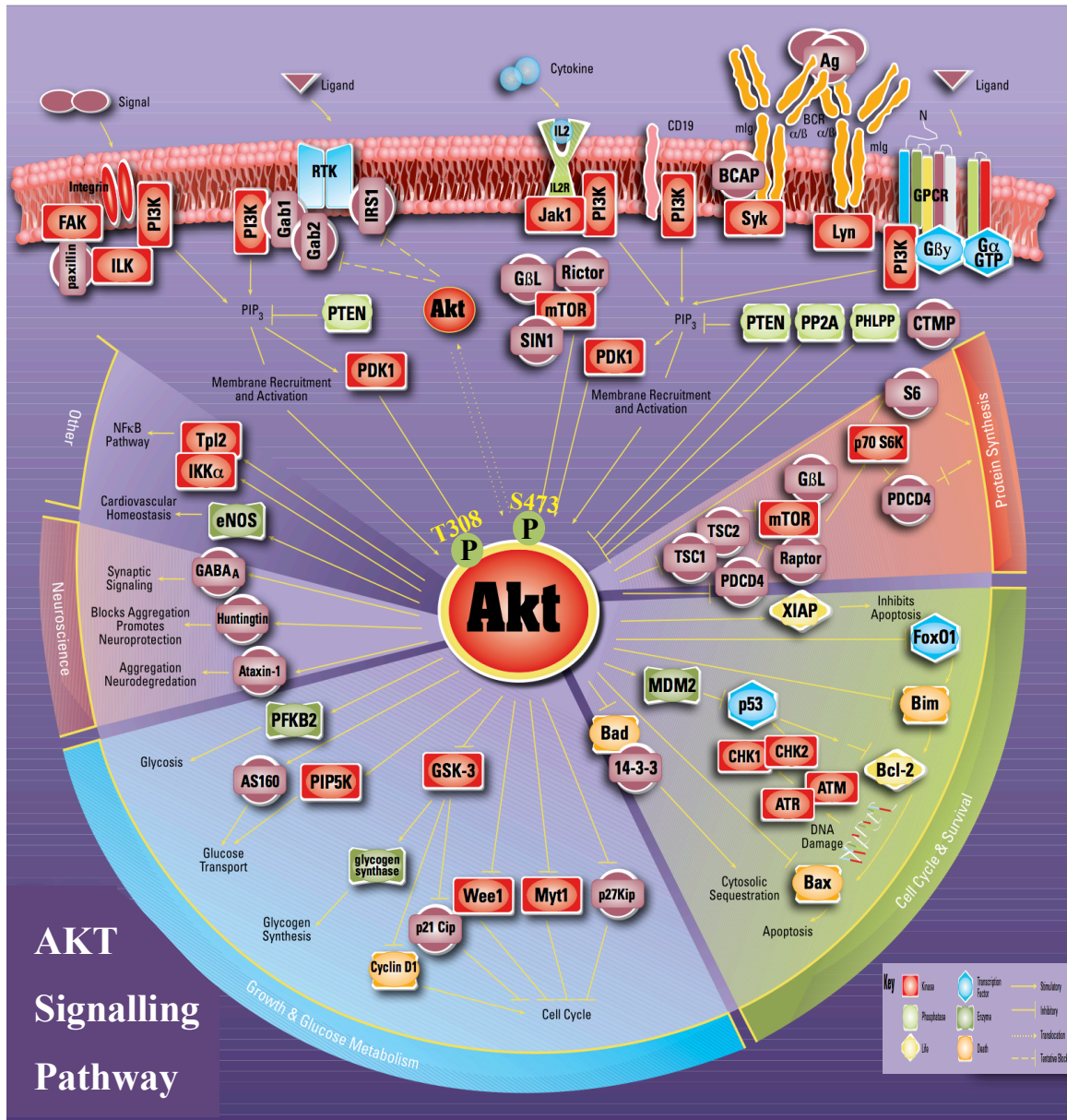
Figure 5: *Schematic representation of PI3K/AKT/mTOR signalling pathway. Illustration reproduced by the courtesy of ROCKLAND antibodies and assays (www.rockland-inc.com) with slight modification.*

PI3Ks is a family of kinases that consists of several classes and subclasses divided according to their structure, tissue distribution, substrate affinity activation and function [123]. The class IA is the most abundant and well studied in term of signal transduction. PI3Ks also play a central role in the pathogenesis of cancer. PI3Ks are heterodimeric lipid kinases composed by two-separated regulatory (p85) and catalytic (p110) subunit. The regulatory subunits include p85α, p85β, and p85γ that are encoded by *PIK3R1, PIK3R2*, and *PIK3R3* genes, respectively. The catalytic subunits are p110α, p110β, p110γ, and p110δ that are encoded by *PIK3CA, PIK3CB, PIK3CG*, and *PIK3CD* genes, respectively [124-126]. The regulatory subunits bind directly to RTK, which mediate its translocation to the plasma membrane where the PI3K is activated. The

regulatory subunits also protect the catalytic subunit from enzymatic degradation [127]. Upon activation, PI3K phosphorylates phosphatidylinositol 4,5 diphosphate (PIP$_2$) and yields the biologically active second messenger phosphatidylinositol 3,4,5 triphosphate (PIP$_3$). PIP$_3$ changes its cellular localization and undergoes conformational changes, leading to the recruitment of 3′-phosphoinositide-dependent kinase 1(PDK1) and protein kinase B (PKB/AKT) to the cell membrane. Afterwards, activated PDK1 phosphorylates AKT, which in turn leads to the phosphorylation and activation of the downstream signalling pathways [128]. PI3K is negatively regulated by dephosphorylation to its inactive lipid state (PIP$_2$) by the tumor suppressor protein phosphatase and tensin homologue deleted on chromosome 10 (PTEN) [129, 130].

AKT is a serine/threonine kinase and plays a key role in the regulation of various molecular functions such as cell cycle progression, apoptosis, transcription and translation [131]. There are three isoforms of AKT/PKB proteins encoded by three different genes: AKT1/PKBα, AKT2/PKBβ, and AKT3/PKBγ. They share around 80% identity in their structure and are similar to the members of AGC kinase family (cyclic AMP dependent-protein kinase, cyclic GMP dependent–protein kinase, and protein kinase C) [132-134]. AKT protein consists of the amino terminal pleckstrin homology (PH) domain, the central kinase domain (the site for substrate residues), and the hydrophobic carboxy terminal regulatory domain. The PH domain is one of the key component by which AKT gets activated by PI3K. The AKT isomers are activated by very similar mechanisms and are differently expressed in tissues. AKT1 is evenly distributed in all tissues whereas AKT2 is expressed in many metabolically active tissues such as liver, heart and skeletal muscle. AKT3 has a very restricted distribution; it is highly expressed in nervous tissues and testis [134, 135]. Recently, AKT3 has been detected in breast tissue [136].

When AKT is recruited to the plasma membrane, it is partially activated through phosphorylation by PDK1 at threonine 308 (Thr308) in the activation loop of the central kinase domain. Moreover, another phosphorylation at serine 473 (Ser473) in the hydrophobic domain is necessary to get AKT fully activated. Phosphorylation of AKT at Ser473 is performed by various kinases including: mammalian target of rapamycin 2 (mTORC2), integrin-linked kinase (ILK) and by AKT itself due to auto-phosphorylation [137]. Partial activation of AKT at Thr308 is enough to activate other AKT downstream targets, such as the mammalian target of rapamycin 1 (mTORC1) [138-142]. Two tumor suppressor proteins, the PH domain and the leucine-rich repeat protein phosphatase (PHLPP) and the protein phosphatase 2A (PP2A) negatively regulate AKT at Ser473 and Thr308 residues respectively, due to direct dephosphorylation. When AKT is activated, it translocates to different areas in the cytoplasm or nucleus to

mediate various phosphorylations on the key substrate and promotes different cellular effects [143-145].

AKT phosphorylates many substrates based on the consensus motif containing the amino acid sequence RXRXXS/T [132]. Some of these substrates are cell cycle-related proteins such as glycogen synthase kinase 3 (GSK-3), p21, and p27, whereas others are apoptotic proteins such as mouse double minute 2 homolog (MDM2), forkhead box O3 (FOXO3), Caspase and B-cell lymphoma 2-associated death promoter (BAD). AKT also regulates proteins important for angiogenesis and inflammatory signalling such as eNOS and NFκB. Activation of AKT is necessary in many processes related to the cell integrity, glycolysis, and tumorigenesis [146]. Another major downstream substrate of AKT is mTORC1 that is crucial in protein synthesis, autophagy and cell survival [126].

mTOR is a serine/threonine kinase, which belongs to PI3K related kinase family. mTOR is 289 kDa protein that forms two distinct multi-protein complexes: mTORC1 and mTORC2. The two mTOR complexes are regulated differently and they show different sensitivities to rapamycin. mTORC1 functions as downstream target of AKT, while mTORC2 is known to be upstream regulator of AKT activation [147-149]. mTOR signalling is commonly regulated by oncogenes and tumor suppressor proteins such as Ras, Raf, and PTEN [150].

mTORC1, the rapamycin sensitive complex, consists of at least five subunits: mTOR, (catalytic subunit of the complex); Raptor (regulatory-associated protein on mTOR); mLST8 or GβL (lethal with Serc13 protein 8); PRAS40 (proline-rich AKT substrate 40 kDa); and Deptor (DEP domain-containing mTOR interacting protein). Raptor positively regulates mTORC1 activity and directs the complex toward the substrates whereas PRAS40 and Deptor are known as negative regulator of mTORC1 [151, 152]. AKT activates mTORC1 at Ser2448 residues indirectly by Rheb-GTP activation and through PRAS40 inhibition. Rheb-GTP is induced through inhibition of TSC1/2 complex mediated by AKT [153, 154]. When activated, mTORC1 phosphorylates two downstream effectors that play a role in the regulation of protein synthesis and translation, namely p70 ribosomal protein S6 kinase (p70S6K1 or S6K1) and the eukaryotic translation initiation factor 4E-binding protein (4EBP1) [147].

S6K1 is the main downstream effector of mTORC1. It can also be activated independently on mTORC1 by other signalling pathways such as PDK1 and MAPK but at different phosphorylation sites. Phosphorylation of S6K1 at Thr389 in the C-terminal hydrophobic domain by mTORC1 is the main target in ribosomal protein synthesis [155, 156]. S6K by a negative feedback mechanism phosphorylates and inhibits insulin receptor signalling 1(IRS1) [157] and Rictor (mTORC2 component), leading to the subsequent inhibition of mTORC2 [158].

4EBP1 is another well-characterized downstream target of mTORC1, which inhibits initiation of protein translation by binding and inactivating the eukaryotic translation initiation factor 4F (elF4E). When elF4E is released, the transcriptional level on mRNA of 7-methylguanine caps is increased. The inhibition of mTORC1 by rapamycin also causes dephosphorylation of 4EBP1 and prevents protein translation [131, 159].

mTORC2, the rapamycin insensitive complex, consists of at least six proteins subunits: mTOR, rapamycin insensitive companion of mTOR (Rictor), mammalian stress activated protein kinase interacting protein (mSIN1), protein observed with rictor-1 (Protor-1), mLST8, and Deptor. Little information is available about mTORC2 structure, components, upstream signal integration and downstream targets. But it has been shown that knockout of mTORC2 in mice causes early lethality in the embryo [155, 160]. There is no specific inhibitor available for mTORC2 so far, and many researchers are trying to identify them. Rictor is the core protein for the mTORC2 catalytic activity and recruits the complex toward the substrate as Raptor does in mTORC1. mSIN1, which binds to Rictor, is an important mTORC2 subunit because it helps in stabilizing the complex and is responsible to direct the complex toward AKT. Protor-1 binds with Rictor and its function is unknown. mLS8 is necessary for complex stability [154].

mTORC2 can be activated by the same growth factors and cytokines that activate PI3K but by unknown mechanisms [161]. Another evidence suggests a possible link between the two complexes that may lead to activation and phosphorylation of mTORC2 at Ser2481 residues [162]. mTORC2 regulates mTORC1 activity through AKT-TSC1/2 pathway, and in turn mTORC1 controls mTORC2 activity through docking with proteins that could be synthesized by their downstream target S6K1 [141, 155, 158]. Activated mTORC2, beside AKT phosphorylation at Ser473 residue, also phosphorylates and activates other downstream targets, such as protein kinase Cα (PKCα) and the serum-and glucocorticoid-regulated kinase-1 (SGK1) [163, 164].

PKCα is a member of serine/threonine protein kinases and is activated by mTORC2, even if the exact mechanism is unknown. However, studies have shown that mTORC2 activation results in the stabilization of PKCα protein by direct PKCα phosphorylation at Ser657 residue. Like P70S6K, PKCα can also be phosphorylated by PDK1. Additionally, PKCα is activated by calcium and the second messenger diacylglycerol (DAG) [165].

SGK1 is another target of mTORC2 activated by growth factors and regulated by mTORC2 that lead to SGK1 phosphorylation at Ser422 residues, which is involved in growth and sodium transport through plasma membrane [152, 166].

1.4.2 PTEN and PHLPP

The tumor suppressor protein PTEN is a well-characterized lipid phosphatase that regulates various cellular processes including survival, proliferation, energy metabolism and cellular architecture by inhibition of PI3K/AKT/mTOR pathway [129]. The structure of PTEN includes several features not observed in other protein phosphatases. It consists of 403 amino acids and it is structured into five functional domains: PIP2 binding domain (PBD), phosphatase domain (is the site of PIP2 binding capacity), C2 domain, carboxy terminal tail, and PDZ domain. Phosphatase and C2 domain are required for PTEN activity [167, 168]. Some reports suggest the importance of the carboxy terminal region of PTEN for the regulation of its binding capacity and its membrane recruitment [168, 169].

PTEN has a double phosphatase activity on both lipids and proteins. The main lipid substrate is PIP_3 [170], while protein phosphatase activity regulates cell migration by dephosphorylation of focal adhesion kinase (FAK), Src homology 2 domain containing protein (Shc), platelet derived growth factor receptor (PDGFR) and PTEN itself [171]. PTEN converts PIP_3 to PIP_2 through removing the phosphatase group at 3-position of the inositol sugar ring of PIP_3 [172]. PTEN can regulate protein activity at post-translational and transcriptional level by protein-protein interaction and/or by mediating translocation to different cell organelles. At the transcriptional level, PTEN has been shown to positively regulate EGFR, PPARγ, and p53 [171, 173-175]. At the post-translational level, PTEN regulates proteins by different mechanisms: phosphorylation, acetylation, and oxidation, all of them leading to PTEN inactivation. PTEN remains phosphorylated and inactive in most of the normal cell types; it becomes activated when dephosphorylated. The phosphorylated PTEN remains in the cytoplasm, but the activated dephosphorylated form it is recruited to the plasma membrane to access its substrate [176, 177].

Another tumor suppressor protein that regulate PI3K/AKT/mTOR pathway is PHLPP. PHLPP is a novel serine/threonine phosphatase that belongs to protein phosphatase 2C family (PP2C) [178]. PHLPP directly dephosphorylates AKT and PKC on their hydrophobic motif resulting in the inhibition of the kinase activity, triggering apoptosis and suppressing tumor growth [179-181]. The two isomers of PHLPP, PHLPP1 and PHLPP2 are shown to dephosphorylate both AKT2 and AKT1 at Ser473 respectively. The dephosphorylation function of AKT by PHLPP is mainly depending on the PDZ binding domain [143]. Both isomers are present in the cytoplasm, nucleus and plasma membrane. They have similar structure consisting of various domains: the amino terminal PH domain, leucine-rich repeat region (LRR), PP2C phosphatase domain, and a C-terminal PDZ-binding motif [182]. Moreover, PHILPP dephosphorylates PKC resulting in the destabilization of the protein, thus leading to its degradation [181, 183].

Further studies have shown that PHLPP directly dephosphorylates S6K1, the downstream target of mTORC1 as part of a negative feedback loop and deletion of PHLPP results in the activation of S6K1 [184, 185]. Another substrate of PHLPP is mammalian sterile 20-like kinase 1(MST-1). PHLPP directly dephosphorylates MST-1 at Thr387 residue that is required to regulate MAPK pathway leading to activation of the downstream signalling effectors, namely p38 and JNK, and subsequently to stimulation of apoptosis [186].

1.4.3 *Alteration of PI3K/AKT/mTOR pathway in cancer*

The PI3K/AKT/mTOR pathway is a prototypic survival pathway that is constitutively activated in many types of cancer such as colon, pancreas, ovarian, breast and glioblastomas [82]. Alteration of signals from upstream membrane RTK or signals generated directly by PI3K/AKT/mTOR pathway may lead to uncontrolled cellular proliferation, tumour progression, metastasis and resistance to drug treatment [120, 187, 188]. Mechanisms of activation include mutation (germline and somatic mutation), overexpression, amplification, hyper-activation, methylation, and aberrant splicing [131, 189]. It is well known that hyper-activation of RTK results in several types of cancers. These can be caused by either overexpression of the growth factor ligands, such as insulin like growth factor-1/2 (IGF-1/2) and epidermal growth factor (EGF) or by amplification/overexpression of the receptor themselves such as IGFR and EGFR, which lead to activation of PI3K/AKT/mTOR signalling. These events have been implicated in tumor progression and invasion [190, 191]

Amplification of PI3K gene is the major alteration in the signal transduction in tumors, and this is confirmed in ovarian and cervical cancer [192, 193]. Also mutations of *PIK3A* gene are frequently occurring in several cancers, such as breast, colon and ovarian cancer. *PIK3A* mutations lead to PIP_3 accumulation and hyper-phosphorylation of AKT and its downstream targets. This results in several phenotype changes, including uncontrolled cell proliferation, reduced apoptosis, and malignant transformation [194]. PI3Ks also regulate integrins related to cell motility in normal and cancerous status. In breast cancer PI3K is induced upon integrin ligation, promoting formation of lamellae and invasion [195].

Loss of PTEN due to either mutations, gene deletion or promoter methylation leads to constitutively hyperactivation of all PI3K downstream effectors such as AKT, PDK1 and mTOR, as can be observed in many primary and metastatic human cancers [196, 197].

AKT deregulation does not only determine the increase in its protein level, but also leads to constitutively increase in its activity. Constitutively active AKT can

be seen in various types of cancers, including breast cancer, and is related to bad prognostic outcome [198]. A mutated AKT1 can be seen in gastric carcinoma, while AKT2 gene amplification is demonstrated in breast, ovarian, pancreas and stomach cancer [199]. Activation of AKT3 is found in estrogen receptor-deficient breast cancer, triple negative breast cancer, and androgen-insensitive prostate cancer [136, 200].

Alterations in the downstream substrates of AKT have been shown to correlate with relapse, metastasis, poor prognosis, and relevant mortality. TSC1/2, which regulates mTOR, undergoes hypermethylation and leads to activation of mTOR in several kind of cancers [201]. Hyperactivation of mTOR itself is common in many types of cancer. mTOR is activated by a mutation that cause cell cycle progression and resistance to amino acid depletion [202]. The other two effectors of mTOR, namely S6K1 and 4EBP1, are thought to be altered and activated in different kind of cancers. Their activation is associated with aggressiveness of the cancer phenotype, malignant transformation, and/or progression [196, 203].

Reduced expression of PHLPP1 or PHLPP2 has been associated with a wide spectrum of cancer diseases including prostate cancer [204], breast cancer [205], glioblastoma cancer [206], colon cancer [207] and melanoma [208]. Loss of PHLPP at both mRNA and protein levels has been associated with progression in cell proliferation, whereas overexpression of PHLPP is largely responsible for the inhibition of cell growth [207].

Nowadays, many researchers are focusing their attention on the study of signalling pathways in order to find out possible new therapeutic targets. Targeting of PI3K/AKT/mTOR signalling cascade with different anticancer drugs may lead to decrease of the progression and diffusion of different kind of cancers. Nevertheless, the complexity of this pathway, the crosstalk with other alternative cascades, and the presence of feedback loops have created some difficulties to identify the specific targets for the different type of cancers. However, during the last two decades intensive studies are trying to find out novel therapeutic strategies of targeting PI3K/AKT/mTOR pathway in cancer patients.

1.4.4 LXR and PI3K/AKT/mTOR pathway

The PI3K/AKT/mTOR is frequently activated in human cancers [126]. As mentioned earlier, mechanisms of activation of this pathway includes loss of tumor suppressor PTEN function, amplification/mutation of PI3K or AKT, activation of growth factor receptors and/or exposure to carcinogens [79]. Activation of LXR by synthetic ligands such as T0901317 can repress growth of human prostatic cell lines *in vitro* and *in vivo* [76]. This event resulted in the induction of apoptosis after disruption of lipid rafts due to AKT dephosphorylation [209]. It has been shown that LXR potentially induces cell death in glioblastoma, both *in vitro* and *in vivo* by decreasing LDLR protein expression and increasing ABCA1-dependent cholesterol efflux transporter via PI3K/AKT/mTOR signalling [210]. It has further been shown that activated LXR significantly reduces the survival rate in breast cancer due to inactivation of the PI3K/AKT/mTOR pathway induced by IGF1 [79]. Recent evidences suggest that mTORC1 is also involved in LXR-mediated transcription of lipogenic gene including induction of SREBP1c, FAS and acetyl-CoA carboxylase (ACC) in liver from mice subjected to a high fat diet [211, 212]. There are very few mechanistic studies on how LXRs regulate PI3K/AKT/mTOR pathway, especially in cancer.

2 AIM OF THE STUDY

2.1 GENERAL AIM

The general aims of this study was to investigate the role of LXR in breast cancer proliferation in relation to the major signalling pathways and to investigate the role of LXR in the inhibition of inflammation in colon.

2.2 SPECIFIC AIMS

- To point out the role of PI3K/AKT signalling pathway in the antiproliferative effect of LXR signalling in breast cancer cells. **(Paper I)**

- To investigate whether LXR pathway and mTOR complex interact in human breast cell carcinoma. **(Paper II)**

- To study the impact of LXR on triple negative human breast cancer using a patient derived xenograft model. **(Paper III)**

- To characterize the role of LXRs in colon of mice using a DSS- and TNBS-induced colitis model. **(Paper IV)**

3 METHODOLOGY

3.1 CELL LINES

Different cell lines were used in this thesis. MCF7 and MDA-MB-231 (human breast cancer cell lines), HuH7 (human liver cancer cell line), SW480 and Colo205 (human colorectal adenocarcinoma). The cell lines were purchased from American Type Culture Collections (ATCC). Cells were grown in different growth media and supplemented with different concentrations of fetal bovine serum (FBS) and Penicillin/Streptomycin antibiotics (PEST) and were kept in 5% CO_2 humidified atmosphere at 37°C.

3.2 LABORATORY ANIMALS AND TUMOR GRAFTING

3.2.1 Animal experiments

C57B1/6J female mice were used in paper IV. The experimental protocols were approved by the Stockholm south ethical committee-Huddinge, according to the guidelines of the Swedish National Board of Laboratory Animals at Karolinska Institutet, (permit numbers S97-09, S98-11, S148-11, S67-13). Dextran sodium sulfate (DSS) and 2,4,6-trinitrobenzene sulfonic acid (TNBS) were used to induce colitis in mice [213]. Furthermore, LXR synthetic agonist (GW3965) was used in order to elucidate the molecular mechanism underlying the anti-inflammatory action of LXR.

3.2.2 Patient derived xenograft model (PDX)

Immunocompromised SCID mice were used in paper III and kept under pathogen-free conditions, at constant temperature (21.5 ± 0.5 °C) and humidity ($55 \pm 5\%$), 20 air-changes/hr and a 12 h light/dark cycle. Patient-derived triple negative breast cancer tumors with a size of 1-2mm^3 were grafted into small pockets created in mammary fat pads of the mice. After expanding, the tumors were collected and used for subsequent xenografts to generate a large mouse colony (Figure 6). In order to study the role of LXR in tumor progressions, mice were treated with varying concentration of GW3965. After the end point of the experiments, the tumor tissues were collected for subsequent analysis. All procedures and experiments involving animals were approved by The National Animal Research Authority in Norway (permit number 5806), and were conducted according to the European Convention for the Protection of Vertebrates used for Scientific Purposes.
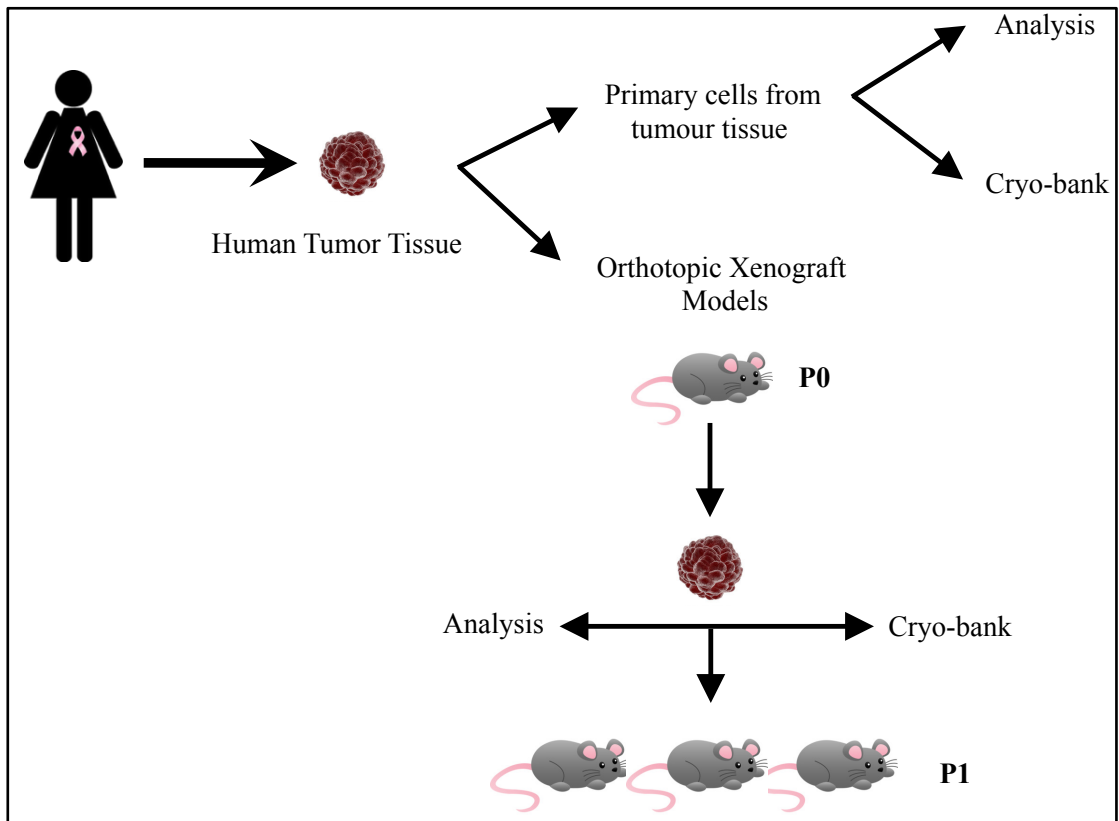
Figure 6: *Workflow of the xenograft model.*

3.3 ANTIBODY BASED TECHNIQUES

3.3.1 *Western blotting*

Western blotting is a widely used semi-quantitative technique in cell and molecular biology. Western blotting was first introduced by Towbin and colleagues in 1979 [214]. The principle of this technique is to separate proteins in a mixture depending on the size. In this thesis we have focused on phosphorylated proteins. In brief, we homogenized the cells or tissue in lysis buffer made fresh and containing a cocktail of protease inhibitors and phosphatase inhibitors. As soon as lysis occurs, proteolysis, dephosphorylation and denaturation begin. These events can be tremendously slowed down if samples are kept on ice or at 4°C at all times and appropriate inhibitors are added fresh to the lysis buffer. The details of the method regarding the using of the buffers, gels, antibodies, and detection systems are described in each paper of this thesis.

3.3.2 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA (also called Enzyme Immunoassay EIA) is a plate-based technique used to measure the concentrations of a specific substance in solution such as peptides, proteins, hormones and antibodies. The principle of ELISA is based on the use of an enzyme linked to an antibody or antigen as a marker for the detection of a specific protein. It is very simple, quick and sensitive method. However ELISA has some disadvantages. False positive are often reported due to nonspecific reactions with samples or cross reactivity. We have used monoclonal antibodies, in order to reduce the risk for cross reactivity (paper II and paper IV).

3.3.3 Immunohistochemistry (IHC)

We have used immunohistochemistry method in paper III and paper IV. IHC is a very efficient and commonly used laboratory technique based on detection of antigens (proteins) in cells of the tissue sections by binding with specific antibodies tagged with visible marker. IHC is very important technique for the identification and visualization of the distribution and localization of various cellular components especially for cancer. The samples can be viewed either by light or fluorescence microscopy.

3.3.4 Isolation of immune cells and flow cytometry

Flow cytometry is a laser-based technique used in analytical cell biology to count and profile cells in a heterogeneous mixture containing live cells. Flow cytometry technology is applied in cell counting, cell sorting, biomarker detection and protein engineering. In this thesis we have used fluorescence-activated cell sorting (FACS) analysis to study different populations of immune cells from lamina propria of the mice colon. FACS is a derivative of flow cytometry and is used to sort the cells in a heterogeneous mixture into different population by using antibodies tagged with a fluorescence dyes.

The isolation of lamina propria immune cells from the colon in this study was performed as previously described by Weigmann and colleague [215] . Briefly, the colon was removed from the mice and opened longitudinally and dissected into 1 cm pieces. After several washings and digestion steps, the isolated immune cells were incubated with different antibodies labeled with fluorescence dye and analyzed by flow cytometry using a FACS calibur (BD Bioscience).

3.4 QUANTITATIVE POLYMERASE CHAIN REACTION

SYBR Green based quantitative Polymerase Chain Reaction (qPCR) was used to measure mRNA expression level in a real time. It also called real time PCR because the amplification of DNA with PCR is monitored in real time. For qPCR, total RNA from cells or tissues was extracted and reverse transcribed into cDNA and analyzed using gene-specific primers. The specificity of the primers must be thoroughly tested by melting curve analysis in order to get a single PCR product. Endogenous control genes were used to normalize the data. Relative gene expression quantifications were identified based on the comparative threshold cycle method normalized with housekeeping genes.

3.5 CELL PROLIFERATION AND VIABILITY ASSAY

Various types of assays are available to identify proliferations and viability cells. The simplest method to examine cell viability is by monitoring the number of cells overtime and counting the number of dividing cells *in vitro*. Utilizing this concept, we used Countess™ automated cell counter from Life Technologies with trypan blue dye, which selectively stain dead cells. By counting both stained and non-stained cells we can identify the percentage and absolute numbers of viable cells, to investigation the cell response after treatment.

4 RESULTS AND DISCUSSIONS

4.1 PAPER I

LXR Inhibits Proliferation of Human Breast Cancer Cells Through The PI3K/AKT Pathway

It has been demonstrated in many studies that LXRs activation inhibit proliferation of tumor growth in different types of cancers including breast, colon, prostate, glioblastoma and ovarian cancer [74, 76-78, 82]. The anti-proliferative functions of LXRs in various models were through various mechanisms. Our previous studies in breast and colon cancers have shown that LXRs inhibit proliferation through regulation of genes controlling cell cycle such as p27, SKP2 and RB [78, 81]. In this study, we showed that activation of LXRs with specific synthetic ligands GW3965 reduced the proliferation of breast (MCF7) and colon (SW480) cancer cell lines. This reduction was associated with the inhibition of the PI3K/AKT signalling activity by decreasing phosphorylation of various proteins of this pathway such as AKT on Ser473 residue. It is well known that PI3K/AKT pathway is prototypic surviving pathway that controls various cell physiology such as proliferation, apoptosis, protein synthesis and cell survival, and this pathway is activated in various kinds of cancer [216]. In the previous studies, it has been shown that loss of tumor suppressor proteins PTEN and PHLPP on both mRNA and protein levels are associated with progression in various cell processes including cell survival and proliferation. On the other hand, overexpression of PTEN and PHLPP seems to be largely responsible for inhibition of cell growth due to the activation of PI3K/AKT signalling [129, 207]. Interestingly, in this study we found that LXRs decreased the level of PIP3 and increased the expression of both PTEN and PHLPP.

Taken together we found that the LXRs-mediated anti-proliferative effects in breast cancer cell line were through different molecular mechanisms including: 1) induction of processes that inhibit degradation of PIP3, 2) suppression of processes that generate PIP3, and (3) suppression the phosphorylation of AKT by PHLPP (Figure 7). Furthermore, we found a big difference between our recent study in breast cancer (Paper I) and previous studies performed on prostate cancer and glioblastoma, mainly that the anti-proliferative effects of LXR seem to be mediated by PI3K/AKT signalling pathway. In prostate cancer cell line (LNCap), LXR reduced proliferation of the cells through disruption of lipid raft cell membrane by inducing cholesterol efflux via expressing of ABCG1 cholesterol transporter gene. The disruption of the lipid rafts led to decreased phosphorylation of AKT and inhibition of AKT survival signalling [76]. In

glioblastoma LXR potentially induces cell death both *in vitro* and *in vivo* by decreasing LDLR protein expression and increasing ABCA1-dependent cholesterol efflux transporter via PI3K/AKT signalling pathway [82, 217]. Altogether, our findings are consistent with the previous finding and suggest existing multiple mechanisms behind the LXRs effect on the proliferation correlated to PI3K/AKT pathway.

We next determined if the anti-proliferative effect was mediated by LXRα, LXRβ, or both and we took advantage of using small hairpin RNA (shRNA) and small interfering RNA (siRNA) techniques to knock down expression of LXRs. We found that LXRβ was the most potential inhibitor of the anti-proliferative effects of LXR. Regulation of PI3K/AKT pathway by LXR is giving us a potential strategy for the molecular therapy behind controlling breast cancer development. Beside identification of LXRβ as a potential LXR isomer modulates proliferation. Thus targeting approaches could be narrowed toward LXRβ specific agonist inhibitor.
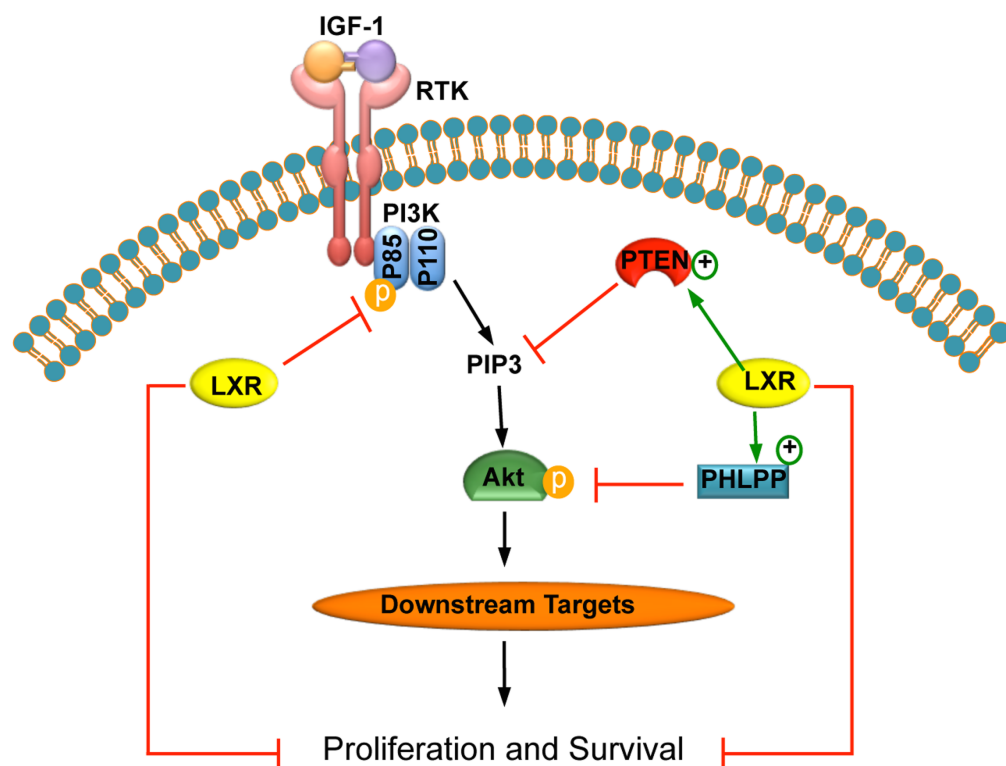
Figure 7: *Schematic presentation of the LXR-effects on the PI3/Akt intracellular signalling pathway.*

4.2 PAPER II

Liver X Receptor (LXR) Mediates Inhibition of Estrogen Receptor Positive Breast Cancer Cells (MCF7) Proliferation Through mTOR-P70S6K Pathway

This study is a follow up of the paper I, were we continued working on the anti-proliferative effect of LXR on signalling pathway in MCF7 breast cancer cell line after treatment with synthetic agonist GW3965. In this study, we investigated whether inhibition of phosphorylation of mTORC1/2 was mediated by LXR activation. We showed that Raptor phosphorylation at Ser792 residue (mTORC1 component) was increased upon LXR activation, subsequently reduced the phosphorylation of two main regulators of mRNA translation and ribosome biogenesis, p70S6K and 4E-BP1, which then led to prevention of protein synthesis and cellular growth [218]. Interestingly, this data confirms the previous finding suggesting that phosphorylation of Raptor on the S792 residue negatively regulates mTORC1 kinase activity [219].

mTORC2 is another main protein kinase related to PI3K signalling pathway. mTORC2 together with the presence of PI3K are necessary for proper phosphorylation of AKT at Ser473 residue, but its upstream activator and the molecular mechanism that regulates its functional interaction remain elusive [154, 161]. We showed that phosphorylation of Rictor, an essential subunit of mTORC2, at Thr1135 residue was decreased by LXR activation, while mTOR phosphorylation at Ser2481 was slightly reduced. We speculate that this effect of LXR on mTORC2 might be directly related to the reduction of mTORC1 and p70S6K activity. This based on previous findings that show that the mTORC1 downstream protein p70S6K positively regulates the phosphorylation of Rictor at T1135 residue upon growth factor stimulation. Nevertheless, Rictor phosphorylation at T1135 residue has no effect on mTORC2 assembly and kinase activity [158].

Our work showed that phosphorylation of AKT at Ser473 residue was decreased upon LXR activation. Accordingly, the mechanism behind dephosphorylation of AKT at Ser473 residue by LXR might not be due to decreased phosphorylation of Rictor and mTORC2, since the dephosphorylation of AKT at Ser473 residue following increased expression of PHLPPL (tumor suppressor protein) has been shown to occur in MCF7 cell upon LXR activation [79].

Another key finding of this study was that the transient knockdown of mTOR, Rictor and Raptor using siRNA significantly affected proliferation of MCF7 cells. We observed that the absence of mTOR blunted the inhibitory effect of LXR on cell proliferation. Finally, we showed that Raptor knockdown induced

phosphorylation of mTOR at Ser2448 residue and its downstream targets of mTORC1, p70S6K and 4E-BP1 in the presence or absence of an LXR agonist. Also, LXR stimulation induced proliferation of MCF7 cells when Raptor was depleted. Our findings suggest the critical role of Raptor for the LXR inhibition of cell proliferation. Taken together, we propose that LXR may play a role in the modulation of breast cancer cell proliferation through PI3K/AKT/mTOR/p70S6K pathway (Figure 8). Mainly, this alteration might be due to attenuation of mTORC1/mTORC2 subunits and their downstream targets phosphorylation.
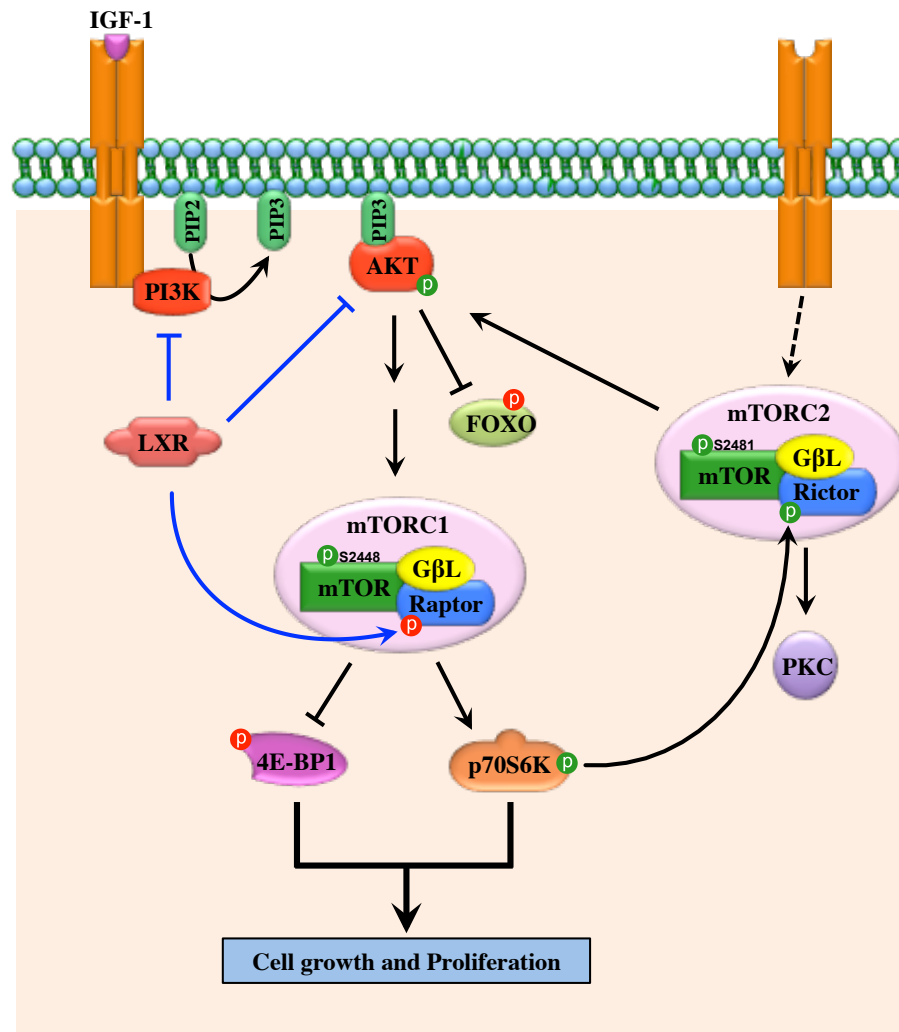
Figure 8: *A model depicting the relationship between LXR, AKT and mTOR complexes. mTOR forms complexes with other proteins, including Raptor (forming mTORC1) or Rictor (forming mTORC2). Growth factor (IGF-1) signals to the PI3K/AKT pathway that activates mTOR function. Then, activated mTORC1 phosphorylates 4E-BP1 and p70S6K. AKT is pivotal for mTOR signalling, it interplays as upstream activator of mTORC1 and downstream effector of mTORC2. LXR negatively regulates mTOR, which prevents activating mTORC1, by direct phosphorylation of Raptor and their downstream targets S6K1 and 4E-BP1. Growth factors also increase mTORC2 activity, albeit the exact signalling event is unknown. Another possible mechanism for LXR modulation of mTORC2 activity is due to the reduction of p70S6K activity that positively phosphorylates Rictor at Thr1135). Also, AKT phosphorylates FOXO1/FOXO3a in a manner independent of mTORC2, which are inhibited by LXR.*

4.3 PAPER III

The Effect of Liver X Receptors on Breast Cancer Tumour Progression In an Orthotopic Xenograft Model.

The aim of this study was to investigate the effect of LXR activation on breast cancer cells *in vivo*. In this study we used primary tumor from a patient with triple negative basal-like breast cancer. Using a so-called patient-derived xerograph model (PDX), cells from the primary tumor were grafted into immuno-compromised SCID mice and allowed to expand. We show that in mice treated with LXR agonist GW3965 there is a significant reduction of the tumor progression at the dose of 40mg/kg/day compared with control. We also show that the expression of the proliferative marker Ki67 was reduced, further strengthening the antiproliferative effect of LXR activation in this model. Our group has previously demonstrated that synthetic LXR ligands inhibit proliferation also in triple negative breast cancer cell lines [78, 85]. These findings support the importance of LXR in triple negative breast cancers, the most aggressive type of breast cancer with a very poor prognostic outcome.

Another key finding in this study is the anti-angiogenic properties of LXR activation. We can clearly see that the numbers of angiogenic markers (α-SMA positive staining and capillary density) are significantly decreased upon GW3965 treatment in tissue sections of the xenografts. Unfortunately, we have so far not found any effects of LXR activation on angiogenic genes on the transcriptional level. However, it has been previously shown that activation of LXRs reduces angiogenesis by restraining cholesterol-dependent vascular endothelial growth factor receptor-2 (VEGF-2) compartmentation and signalling. LXR agonists GW3965 and T0901317 reduced migration, tubulogenesis, and proliferation of human umbilical vein endothelial cells [220]. Furthermore, T0901317 reduced the growth of Lewis lung carcinoma grafts in mice by impairing angiogenesis [220]. Based on this work we need to further investigate the potential anti-angiogenic properties of LXR activation to elucidate if this is a direct effect due to LXR activation and if it can be targeted for new cancer therapies. We further continued to find the mechanism behind the reduced tumor growth by GW3965 treatment. We found that the phosphorylation status of some proteins in the PI3K/AKT/mTOR pathway was significantly reduced upon LXR activation with GW3965 compared to control group.

The PI3K/AKT/mTOR pathway plays an important role in regulating cell growth, proliferation, apoptosis, angiogenesis and protein synthesis [216]. Reduction of phosphorylation of AKT at Ser473, p70S6K at Thr389 and 4EBP1 at Thr70 by GW3965 further support our previous finding of the effect of LXR activation on the proliferation of human breast cancer cells *in vitro* [79].

4.4 PAPER IV

The Oxysterol Receptor LXRβ Protects Against DSS- and TNBS-Induced Colitis In Mice

In this work, we examined the role of LXRs in inflammatory bowel disease (IBD). IBD includes ulcerative colitis and Crohn's disease, which affects millions of people in the world. IBD is a major risk factor for colorectal cancer (CRC). In order to examine the role of LXR in colitis in mice, we used dextran sodium sulfate (DSS) and 2,4,6-trinitrobenzene sulfonic acid (TNBS) to induce colitis in mice with different genotype (wild type (WT), LXRα$^{-/-}$, LXRβ$^{-/-}$, LXRαβ$^{-/-}$). WT mice were pretreated with GW3965 or vehicle by gavage, thereafter DSS was added to the drinking water for all genotypes. At day nine, the WT mice treated with DSS had lost 13% of their body weight, and a small, but significant, protective effect of the GW3965 treatment was observed. The LXR deficient mice (LXRβ$^{-/-}$ and LXRαβ$^{-/-}$) had an earlier reduction of body weight, and at day seven the LXRαβ$^{-/-}$, LXRα$^{-/-}$ and LXRβ$^{-/-}$ mice had lost 15%, 2%, and 14% respectively, compared to a 5% reduction of body weight of WT mice.

We observed that activation of LXRs protected mice against colitis and reduced the inflammation, while loss of LXRs had an opposite effect. When colitis was induced in LXR-deficient mice (LXRβ$^{-/-}$ and LXRαβ$^{-/-}$), they showed a marked worsening of clinical symptoms including ulcers, diarrhea and rectal bleeding, and increased mortality in both DSS- and TNBS colitis models. Furthermore, we showed that loss of LXR led to increase recruitment of CD11b^{+} cells and consequently increased and prolonged release of inflammatory mediators in colon. Interestingly, mice treated with LXR agonist displayed faster recovery, increased survival, and suppressed expression of inflammatory mediators upon DSS-induced colitis.

To continue investigating on the role of LXRs as anti-inflammatory factor, we examined human colorectal adenocarcinoma cell line Colo205 and SW480 pretreated with GW3965 and stimulated with tumor necrosis factor (TNFα) and bacterial lipopolysaccharide (LPS) to induce an inflammatory response. We found that activated LXR suppressed the inflammatory response of both TNFα and LPS stimulation. Additionally, after depletion of LXRβ expression in SW480 using siRNA, we abolished the anti-inflammatory action of LXR. This result suggests that LXRβ is the dominant subtype responsible for repressing the inflammatory response in SW480 cells. Later, we used LPS to induce inflammatory response mediated by toll like receptor 4 (TLR4) in vivo. Mice pretreated with LXR ligand GW3965 and successively administrated with LPS intraperitonealy, showed stronger repression of inflammatory and

chemokine genes (Tnfα, Cxcl1/Kc, Cxcl2/mip2, Ccl2/Mcp1, and Il17a) in the colon from LXRα$^{-/-}$ mice compared to LXRαβ$^{-/-}$ and LXRβ$^{-/-}$ mice. These findings suggest that LXRβ has a protective role *in vivo* in colon. Our results also suggest that the effect of LXRs takes place on both colonic epithelial cells and on resident and infiltrating immune cells in colon. By suppressing both inflammatory cytokine and chemokines in immune cells and colonic epithelial cells, thus LXRs seem to affect the immunopathogeneis of IBD at multiple levels.

Finally in this paper, we studied the role of LXR in-patient with IBD and found that expression of both LXRα and LXRβ is suppressed in the inflamed colon (both Crohn's disease and ulcerative colitis) compared with normal healthy colon. Thus these data confirmed the recently reported paper concerning the role of nuclear receptor especially LXRβ in regulation of IBD [221].

5 CONCLUSIONS

The Liver X receptor is the main focus of the present thesis. The potential anti-proliferative and anti-inflammatory effects of LXRs and the underlying mechanisms of action were studied in breast cancer and inflammatory bowel disease, respectively. In regards to the aims, the main conclusions of this thesis can be summarized as follows:

1. Inactivation of the growth factor induced PI3K/AKT intracellular signalling pathway by GW3965 revealed an important role for LXRs as anti-proliferative factor. This observation suggetes that targeting of LXR may be a potential strategy to controll breast cancer development **(Paper I)**

2. Inhibition of breast cancer cell proliferation by LXRs takes place mainly through modulation of mTORC1 in the PI3K/AKT/mTOR signalling pathway. However, in order to identify the exact mechanism behind LXR regulation of mTORC1 and the downstream targets in breast cancer cell line remains to be investigated in the future. **(Paper II)**

3. We have further identified an additional role of LXRs in the regulation of breast tumor growth using patient-derived xenograft model. Upon LXR activation with GW3965, we found a reduced tumor progression in triple negative breast cancer xenografts. Further investigations are needed, but the anti-angiogenic role of LXR remains the most important finding of our study and certainly opens up to the use of different drugs in combination with an LXR agonist to target the most aggresive forms of breast cancer. **(Paper III)**

4. We provid evidence that LXRβ appears to have a protective role in DSS-induced colitis in mice. Moreover, mice treated with LXR agonist display faster recovery, increased survival, and suppressed expression of inflammatory mediators upon DSS-induced colitis **(Paper IV)**

6 FUTURE PERSPECTIVES

The work of this thesis highlighted the involvement of LXR in the regulation of PI3K/AKT/mTOR pathway. Our novel findings add a new player that might be considered in the future as important therapy target candidate to inhibit cancer proliferation and development, in particular for breast cancer. It has been shown that in other types of cancer, such as prostate cancer and glioblastoma, LXR inhibits proliferation through regulation of lipid metabolism and that is mediated by PI3K/AKT signalling pathway. In this study, we added new findings to this field, which are the involvement of protein phosphatases and mTOR with LXRs functions. Nevertheless, the exact mechanisms that characterize this action have not been fully elucidated and more mechanistic studies are needed.

To further study the antiangiogenic effects of LXR in PDX models we will use combination treatment of LXR agonist with the traditional cytostatic drugs used in cancer treatment. This could open a new window to investigate more about the therapeutic function of LXR specifically in triple negative breast cancer, but also in estrogen positive (ER+) breast cancer.

Preliminary results:

In ongoing study, we are trying to identify relation of LXR to the main proteins and phosphatases that regulate the above-mentioned PI3K/AKT/mTOR pathway. Now we are using CRISPR (clustered regularly interspaced short palindromic repeats) technology to facilitate efficient knockout of LXR genes in MCF7 breast cancer cell lines. By knockout of LXRs ($LXR\alpha\beta^{-/-}$, $LXR\alpha^{-/-}$, $LXR\beta^{-/-}$) and then study their interactions with the main effector proteins in PI3K/AKT/mTOR pathway, we can identify exactly how LXRs regulate this pathway. We obtained some preliminary data on $LXR\alpha\beta^{-/-}$ by using the CRISPR technique in MCF7 cell (Figure 9). The expression of LXR target genes ABCA1 and ABCG1 was predominantly reduced in LXR deficient cells (Figure 9, A and B). Furthermore, knockout of LXRs by CRISPR significantly reduced the antiproliferative effect of LXR in MCF7 breast cancer MCF7 cells (Figure 9, C and D). These data indicate the important role of LXR in the proliferation of cancer cells. Clonally expanded cells are currently analyzed using Sanger sequencing in order to verify biallelic knockout of LXR.
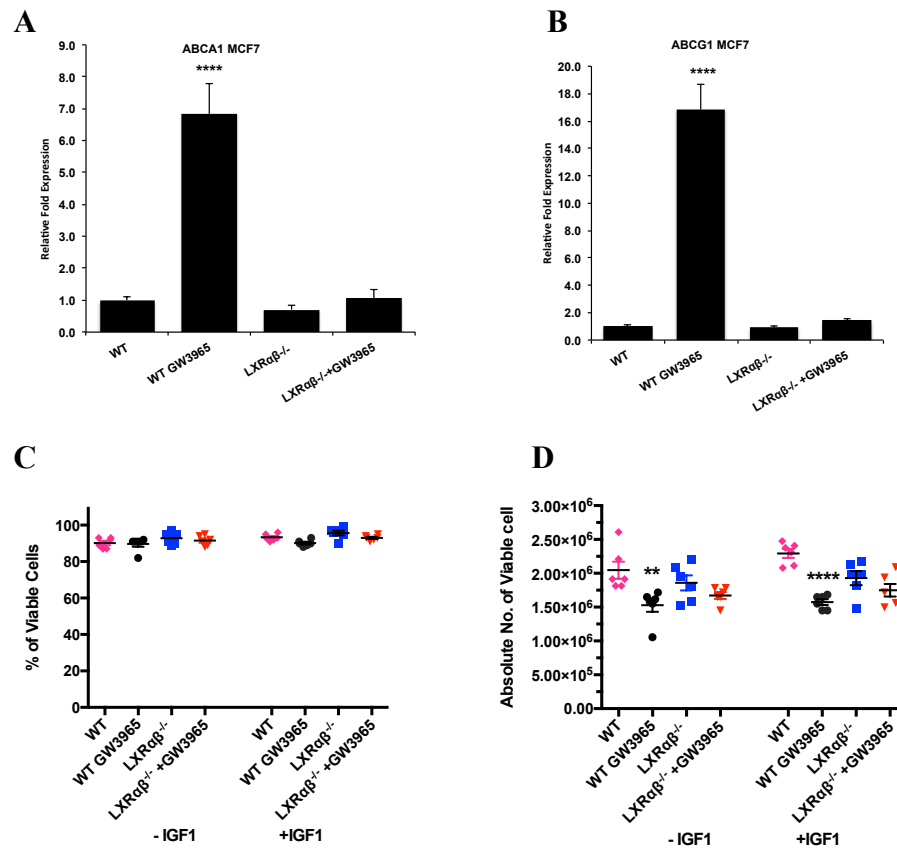
Figure 9: *Knockout results of LXRαβ⁻/⁻ in MCF7 cells using CRISPR technology. **(A and B)** Relative mRNA expression levels of LXR target genes ABCA1 and ABCG1. Wild type and LXRαβ⁻/⁻ cells cultured in 1% serum medium then treated with vehicle (DMSO) or 5μM GW3965 for 16hrs. The data is presented as mean ±SEM, ****P< 0.0001. **(C and D)** Wild type and LXRαβ⁻/⁻ cells treated with vehicle (DMSO) or 5μM GW3965 for 48hr. Cells were harvested after activation with 50ng/ml IGF-1 for 20 min, to perform a viability test, and the absolute number of viable cells was counted. The data is evaluated using ordinary one-way ANOVA (with Tukey test). The data is presented as means ± SEM, **p<0.01 and **** P< 0.0001.*

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