THE ANTIPROLIFERATIVE ROLE OF THE LIVER X RECEPTORS IN BREAST AND COLORECTAL CANCER

Lise-Lotte Vedin

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ABSTRACT

The liver X receptors (LXRα and LXRβ) are members of the nuclear receptor superfamily of ligand activated transcription factors and have functions as regulators of lipid and glucose metabolism, as well as inflammatory response. In recent years, several reports have demonstrated an important role of LXRs in the control of cell proliferation.

In **Paper I** we demonstrate that LXR activation with synthetic agonist GW3965 leads to a strong antiproliferative effect in four different human breast cancer cell lines. We show that LXR activation induces an arrest at the G1/S check point of the cell cycle with a hypophosphorylation of retinoblastoma protein and a downregulation of cell cycle modulators such as Skp2, cyclin A2 and cyclin D1. We further show that the antiproliferative function of LXRs is independent of lipid biosynthesis.

In **Paper II** we follow up the results in Paper I to elucidate more mechanisms of LXR activation in human breast cancer cell lines. Using microarray analysis, we find both cell line specific and common LXR target genes. The common responsive genes that were upregulated upon LXR activation are annotated to known metabolic functions of LXR, while the common downregulated genes mostly include those with function in cell cycle regulation and proliferation. Comparing the common downregulated gene set, with breast cancer tumour samples and patient data we find that patients with tumours expressing lower levels of these LXR target genes had better survival compared to patients with a higher expression of these genes. In addition, we identify the E2F family of transcription factors as mediators of the antiproliferative effect of LXR activation.

In **Paper III** we demonstrate that activation of LXRs with GW3965 decreases proliferation in human colorectal cell lines with a cell cycle arrest in the G1 to S phase transition. We demonstrate a decreased expression of cell cycle promoters such as Skp2, CDK1, CDK2, CDK4, cyclin E, cyclin B1 and c-myc, as well as hypophosphorylation of retinoblastoma protein. Moreover, we show that LXR deficient mice have an increased proliferation in the colonic crypt compared to wild type mice. Also, activation of LXRs with GW3965 reduces proliferation in the colonic crypt of wild type mice.

In **Paper IV** we demonstrate that activation of LXRs dampens the inflammatory response by downregulating pro-inflammatory mediators in two different mouse models of colitis. In addition, LXR deficient mice have a faster and more severe disease progression. We further demonstrate that expression of LXR regulated genes is suppressed in colon samples from patients with either Crohn’s disease or ulcerative colitis compared to healthy controls. Inflammatory bowel disease (IBD), including Crohn’s disease and ulcerative colitis, is associated to increased risk of developing colorectal cancer. The data in Paper IV suggests the potential for LXR mediated inhibition of inflammation during IBD, thus reducing the risk for developing colorectal cancer.
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ACAT2</td>
<td>Acetyl-Coenzyme A acetyltransferase 2</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl-Coenzyme A carboxylase</td>
</tr>
<tr>
<td>AF</td>
<td>Activation function</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>APOAI</td>
<td>Apolipoprotein AI</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
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<tr>
<td>BA</td>
<td>Bile acid</td>
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<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
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<td>BAX</td>
<td>Bcl-2–associated X protein</td>
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<tr>
<td>CAK</td>
<td>CDK-activating kinase</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CETP</td>
<td>Cholesterol ester transfer protein (plasma lipid transfer protein)</td>
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<tr>
<td>CKI</td>
<td>CDK inhibitor</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>Cyp51A1</td>
<td>Lanosterol 14α-demethylase</td>
</tr>
<tr>
<td>Cyp7A1</td>
<td>Cholesterol 7α-hydroxylase</td>
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<tr>
<td>DBD</td>
<td>DNA binding domain</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>ER</td>
<td>Oestrogen receptor</td>
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<tr>
<td>FAP</td>
<td>Familial adenomatous polyposis</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty acid synthase</td>
</tr>
<tr>
<td>FXR</td>
<td>Farnesoid X receptor</td>
</tr>
<tr>
<td>GCK</td>
<td>Glucokinase (hexokinase 4)</td>
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<tr>
<td>GLUT</td>
<td>Glucose transporter protein</td>
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<td>GR</td>
<td>Glucocorticoid receptor</td>
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<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
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<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HNPPC</td>
<td>Hereditary non-polyposis colorectal cancer</td>
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<tr>
<td>HRE</td>
<td>Hormone response element</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IDOL</td>
<td>Inducible degrader of the low-density lipoprotein receptor</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
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<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
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<td>LDLR</td>
<td>Low-density lipoprotein receptor</td>
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<td>LPL</td>
<td>Lipoprotein lipase</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LXR</td>
<td>Liver X receptor</td>
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<tr>
<td>LXRE</td>
<td>Liver X receptor response element</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Matrix metallopeptidase 9</td>
</tr>
<tr>
<td>NCoR</td>
<td>Nuclear receptor corepressor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>-------------</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor κ-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NPCL1</td>
<td>Niemann-Pick C1-like protein 1</td>
</tr>
<tr>
<td>NR</td>
<td>Nuclear receptor</td>
</tr>
<tr>
<td>NSAID</td>
<td>Nonsteroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PGC-1</td>
<td>Peroxisome proliferator-activated receptor γ coactivator-1α</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositide 3-kinase</td>
</tr>
<tr>
<td>PKLR</td>
<td>Pyruvate kinase, liver and red blood cell</td>
</tr>
<tr>
<td>PLTP</td>
<td>Phospholipid transfer protein</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PXR</td>
<td>Pregnane X receptor</td>
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<tr>
<td>RAR</td>
<td>Retinoic acid receptor</td>
</tr>
<tr>
<td>RB</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>RCT</td>
<td>Reverse cholesterol transport</td>
</tr>
<tr>
<td>ROR</td>
<td>Retinoic acid receptor-related orphan receptor</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>SCD1</td>
<td>Stearoyl-Coenzyme A desaturase 1</td>
</tr>
<tr>
<td>SCF</td>
<td>Skp, Cullin, F-box containing complex</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective oestrogen receptor modulator</td>
</tr>
<tr>
<td>Skp</td>
<td>S-phase kinase-associated protein</td>
</tr>
<tr>
<td>SMRT</td>
<td>Silencing mediator for retinoic acid and thyroid hormone receptor</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element binding protein</td>
</tr>
<tr>
<td>SULT1E1</td>
<td>Oestrogen sulfotransferase (EST)</td>
</tr>
<tr>
<td>SULT2A1</td>
<td>Hydroxysteroid sulfotransferase-2A1 (bile salt sulfotransferase)</td>
</tr>
<tr>
<td>SULT2B1</td>
<td>Hydroxysteroid sulfotransferase-2B1</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>TR</td>
<td>Thyroid hormone receptor</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>UCP1</td>
<td>Uncoupling protein 1</td>
</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin-proteasome system</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low-density lipoprotein</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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1 INTRODUCTION

1.1 NUCLEAR RECEPTORS AND THE LIVER X RECEPTORS

Liver X receptor α (LXRα) and LXRβ, belong to the nuclear receptor (NR) superfamily of ligand activated transcription factors. The NRs are attractive drug targets, thanks to their ability to control activity by small hydrophobic molecules and the wide range of processes they control. There are several good examples of NRs as drug targets; steroid hormones such as oestrogens and progestins have been used as contraceptives for decades targeting the oestrogen receptor (ER) and progesterone receptor (PR). ER has also been targeted in breast cancer treatment, using tamoxifen therapy. Dexamethasone activated the glucocorticoid receptor (GR) and is used for treatment of inflammatory diseases, while thiazolidinediones targets the peroxisome proliferator-activated receptor (PPAR) γ utilised in type II diabetes. [1, 2]

There are 48 NRs known in humans and 49 in mice, all are evolutionarily related and involved in both normal metabolic and developmental processes, as well as disease states such as cancer, inflammation, diabetes, obesity and atherosclerosis [3]. The first nuclear receptors to be cloned were the GR [4, 5] and ERα [6, 7]. Cloning and further studies of subsequent receptors presented a common structure for the NR superfamily consisting of five to six domains (A to F) with regions of high sequence homology [8, 9]. The variable N-terminal region, A/B, usually contains a transcriptional activation domain, or activation function 1 (AF-1), while the C region contains the DNA binding domain (DBD), including a highly conserved zinc finger motif. The DBD is responsible for binding to hormone response elements (HRE) in promoters of target genes. The D domain of NRs is poorly conserved and acts as a flexible hinge region between the DBD and the multifunctional E domain which contains both a ligand binding domain (LBD), the site for receptor dimerisation, and the activation function 2 (AF-2). Most NRs also contain a highly variable C-terminal F domain with a poorly understood function. [1, 2]

The two LXR isoforms were identified independently by several different groups during the mid 90’s. LXRα (official gene symbol: NR1H3) was first cloned by Apfel et al. (then called RLD-1) [10] and in the lab of David Mangelsdorf [11]. LXRβ (official gene symbol: NR1H2) was identified in the lab of Jan-Åke Gustafsson at Karolinska Institutet (named OR-1) [12], by Shinar et al. (NER) [13], Song et al. (UR) [14] and Seol et al. (RIP-15) [15]. LXRα and LXRβ are encoded by two separate genes, in humans, on chromosome 11p11.2 and 19q13.3, respectively, and the expression patterns differ between the isoforms. LXRβ is considered to be ubiquitously expressed, while LXRα expression seems to be more restricted, with the highest expression in the liver, but also in kidney, gall bladder, lung, testes, spleen, intestine, adipose tissue and skin [16, 17]. The two LXR isoforms share about 77% amino acid sequence identity in both the DBD and LBD.
1.1.1 Transcriptional regulation by NRs and LXR

NRs both positively and negatively regulate transcription through several distinct mechanisms, including ligand-dependent transactivation, ligand-independent repression and ligand-dependent transrepression, see Figure 1. Most of the NRs bind to DNA as homo- or heterodimers, but a subset of receptors binds to DNA as monomers. The latter is often an orphan receptor, or a receptor without known ligand, that can regulate transcription through changes in receptor expression or posttranscriptional modifications. The LXR form heterodimers with the retinoid X receptor (RXR) and the complex binds to LXR response elements (LXREs) which are characterised by direct repeats of the consensus sequence AGGTCA separated by four nucleotides (DR4) [11]. The permissive LXR-RXR dimer can be activated both by LXR and RXR ligands [11].

Figure 1. Different molecular mechanisms of LXR ligand activation and transrepression. (a) Classical transactivation where the unliganded LXR/RXR heterodimer is bound to DNA together with a corepressor complex. In response to ligand activation, the corepressor complex is exchanged for a coactivator complex and gene transcription can occur. (b) Alternative transactivation where methylated and repressed chromatin excludes unliganded LXR from binding. After demethylation and ligand activation, LXR/RXR heterodimer is recruited to the DNA, and subsequent recruitment of coactivator complex promotes transcription. (c) Transrepression where ligand activated LXR is post-translationally modified by the addition of a SUMO protein. The SUMOylated LXR docks to corepressor complex at the promoters of inflammatory response genes, inhibiting the degradation of the complex, and inhibits gene transcription. Adapted from [18] with permission from the publisher.

In the absence of ligand, LXRs are bound to LXREs on the DNA together with a corepressor complex, including the silencing mediator for retinoic acid and the thyroid hormone receptor (SMRT) and the nuclear receptor corepressor (NCoR) [19]. This corepressor complex acts as a strong repressor of target gene transcription, so called ligand-independent repression [20, 21]. In the classic mechanism of ligand-dependent transactivation, Figure 1a, ligand binds to the receptor heterodimer causing conformational changes, allowing for exchange of corepressor to a coactivator complex, and the initiation of transcription of target genes. An alternative ligand-
dependent activation pathway for the LXR has also been described, Figure 1b, where, in contrast to the previous pathway, unliganded LXR is not bound to the DNA [18].

NRs and LXRs may also negatively modulate transcription by ligand-dependent transrepression, Figure 1c, that does not require direct, sequence specific binding to DNA. This mechanism, where upon ligand binding LXRs inhibit transcription of target genes through protein-protein interactions with coregulatory proteins and transcription factors associated to the target site, has been shown to be important in response to inflammatory signals [22, 23]. In addition, LXRs have also been shown to be posttranslationally modified by phosphorylation [24-26], acetylation [27], SUMOylation [23] and O-linked β-N-acetylglucosamineylation [28] affecting the transcriptional activity of the receptors.

1.1.2 Ligands

NR ligands are small, hydrophobic, lipid-soluble, and vary greatly in characteristics. Endogenous NR ligands include various cholesterol derivatives, retinoids, steroid hormones, prostaglandins, benzoates and several fatty acids [1]. The ligand specificity is determined by the LBD, but some NRs are potentially promiscuous allowing molecules with the right stereochemistry to bind into the ligand binding pocket and mimic or block binding of the natural ligand. Environmental pollutants, including pesticides, and many plant and industrial chemicals have shown potential to disrupt the natural activity of NRs in this manner [29]. There are also a number of NRs referred to as orphan receptors lacking a known physiological ligand. Some of these NRs might still be ligand-dependent, whereas others have been found by structural studies to be “true” orphans without the ability to bind ligand and these receptors have a constitutively active AF-2 [1].

The first endogenous ligands for LXRs, identified by Janowski et al. [30], were naturally occurring oxysterols, or oxidised cholesterol derivatives, and the most potent agonists include 24(S),25-epoxycholesterol, 20(S)-, 22(R)-, 24(S)-, 25-, and 27-hydroxycholesterol [30, 31]. Theofilopoulos et al. recently also indentified cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) as an endogenous brain specific LXR ligand [32]. In addition, a wide range of various plant sterols and stanols have been shown to activate LXRs [33]. Among the synthetic ligands, GW3965 [34] and T0901317 [35] are the two most widely used. The latter has, however, shown potential to also activate farnesoid X receptor (FXR) [36], pregnane X receptor (PXR) [37] and retinoic acid receptor-related orphan receptors (RORs) [38]. Neither T0901317 nor GW3965 will discriminates between the two LXR isoforms. New synthetic agonists are being developed in order to selectively activate specific LXR pathways. LXR-623, activates LXRs without inducing hepatic lipogenesis [39]. Isoform specific agonists include the LXRB specific ligands; WYE-672, a partial and tissue-selective agonist [40], GW6340, intestine-specific agonist without lipogenic activity [41] and GSK9772, selective for LXR mediated transrepression of pro-inflammatory gene expression [42].
In addition, LXR antagonists have also been reported, both synthetic GSK2033 [43] and naturally occurring endogenous and plant ligands as reviewed by Viennois et al. [33].

### 1.1.3 LXR regulated pathways

#### 1.1.3.1 Cholesterol metabolism

Cholesterol is crucial for our survival, from being building blocks in cell membranes, involved in neural signalling and as precursor of vitamin D, bile acids and steroid hormones. The source of cholesterol is both **de novo** synthesis, primarily in the liver, as well as dietary intake. Cholesterol is transported in the body by a group of lipoproteins. High-density lipoprotein (HDL) transports cholesterol to the liver for excretion, while low-density lipoprotein (LDL) and very low density-lipoprotein (VLDL) transport cholesterol from the liver out to peripheral tissues when needed.

Reverse cholesterol transport (RCT) is a process where cholesterol is transported from peripheral tissues to the liver where it can be processed and excreted. When cholesterol is in excess, it can be bound to pre-HDL particles or apolipoprotein A1 (APOAI) with the help of sterol transporters of the ATP-binding cassette (ABC) family, and is subsequently transported to the liver via the plasma. In the liver, cholesterol is converted into bile acids (BAs) that can then be excreted via the bile and the intestine. The LXRs are involved in controlling RCT by regulating members of the ABC family. Activation of LXR leads to induction of ABCG1 and ABCA1, which in turn promotes cholesterol efflux to HDL [44-47]. LXR activation has also been shown to control macrophage cholesterol efflux through the joint regulation of apolipoprotein E (ApoE), ABCA1 and ABCG1 expression, promoting incorporation of cholesterol into HDL particles [48].

LXR activation also affects other members of the ABC family, specifically ABCG5 and ABCG8, that together with ABCA1 promote sterol excretion into the bile and faeces, and limiting cholesterol absorption from the intestine [41, 49]. Intestinal activation of LXR by synthetic agonist T091317 also decreases intestinal expression of Niemann-Pick C1-like protein 1 (NPC1L1), a vital factor for cholesterol absorption in the intestine [50]. NPC1L1 is responsible for absorbing free cholesterol from the intestinal lumen into the enterocytes, where it is esterified by acetyl-Coenzyme A acetyltransferase 2 (ACAT2) into cholesteryl esters. The cholesterol influx by NPC1L1 is opposed by the efflux of ABCG5 and ABCG8, pumping cholesterol back into the lumen. The importance of the intestine, and not only the liver, in RCT has been further highlighted by data from Brunham et al. on the role of intestinal ABCA1 to control plasma HDL levels [51, 52]. In addition, Lo Sasso et al. showed how intestinal specific, but not hepatic specific, LXRα activation stimulated RCT and decreased intestinal cholesterol absorption, by ABCG5/ABCG8 upregulation and NPC1L1 downregulation, increased HDL synthesis and reduced ACAT2 activity [53].
The LDL receptor (LDLR) is responsible for taking up LDL-cholesterol from the plasma and is tightly regulated by sterol regulatory element binding proteins (SREBPs). When cellular cholesterol levels are low, there is an enhanced processing of SREBPs to their mature forms, which leads to increased expression of LDLR [54]. The activated mature SREBPs will then promote both de novo synthesis and increased uptake of cholesterol. Activation of LXRα has been shown to negatively regulate this pathway by inducing expression of the inducible degrader of LDLR (IDOL, also known as Mir and Myl), an E3 ubiquitin ligase targeting LDLR for destruction [55]. In addition, activation of LXRα also reduces expression of two key enzymes in cholesterol synthesis; lanosterol 14α-demethylase (CYP51A1) and squalene synthase [56].

The importance of LXRs in cholesterol homeostasis was discovered by Peet and colleagues using LXRα deficient mice (LXRα−/−). The mice appeared normal and healthy under a normal low cholesterol diet, but when challenged with a diet high in cholesterol, the LXRα−/− mice failed to increase BA synthesis and excretion, leading to accumulation of cholesterol esters in their livers. The reason for this was a failure to induce gene expression of cholesterol 7α-hydroxylase (Cyp7A1), the rate-limiting enzyme in BA synthesis [57]. LXRβ−/− mice, however, maintain their resistance to high levels of dietary cholesterol, indicating that LXRα and LXRβ, although similar in many aspects, also have separate and unique functions [58, 59]. In contrast to rodents, LXR activation in primary human hepatocytes leads to suppression of Cyp7A1 [60, 61], demonstrating an important species difference upon LXR activation. Kotokorpi et al. further studied this interspecies difference through genome wide expression profiling of primary hepatocytes from humans and rats. They showed that GW3965 treatment in human primary hepatocytes, among other effects, leads to suppressed BA synthesis, reduced secretion of triglyceride (TG) bound to VLDL and increased lipid storage [62]. Wang et al. reported that lipid homeostasis was disturbed also in the brain from LXRαβ−/− mice compared to WT mice, with an accumulation of lipid droplets in the brain of LXR deficient mice [63].

The beneficial effects of LXR activation by reducing cholesterol levels and by anti-inflammatory signalling, discussed more below, suggests the LXRs as important targets for atherosclerosis prevention and treatment, and several studies have shown this to be true. Synthetic LXR agonists have been demonstrated in several animal models to have atheroprotective effects [53, 64-66] and also to reduce platelet aggregation [67].

Alterations in cholesterol homeostasis have also been connected to the initiation and development of Alzheimer’s disease and high cholesterol levels constitute a risk factor for this disease. Activation of LXRs increases cholesterol efflux and may lead to induced secretion of β-amyloid, thereby reducing the risk of amyloid deposition in the brain [68, 69]. The LXRs have also been linked to multiple sclerosis (MS) [70] and Parkinson’s disease [71], where both cholesterol homeostasis and the anti-inflammatory functions of LXRs might be of importance.
1.1.3.2 Lipid metabolism

Lipids (fatty acids) are synthesised in the liver from acetyl-CoA, and the esterification of fatty acids leads to the production of triglycerides; a process referred to as lipogenesis. In the liver, LXR activation induces *de novo* lipogenesis by induction of SREBP1c, acetyl CoA carboxylase (ACC), stearoyl-CoA desaturase 1 (SCD1) and fatty acid synthase (FAS), thereby increasing triglyceride (TG) content in the liver and circulating plasma [35]. TG-rich VLDLs secreted from the liver transport lipids to adipose and other peripheral tissues. LXR activation induces expression of lipid transfer proteins, such as cholesterol ester transfer protein (CETP) and phospholipid transfer protein (PLTP), as well as lipoprotein lipase (LPL), to help transport TGs to the adipose tissue [72-74].

The LXRs also appear to have functions within the adipose tissue; both LXRs are highly expressed in adipose tissue, and the expression of LXRα increases during adipocyte maturation [75, 76]. LXRs affect expression of genes involved in lipid metabolism in adipocytes, but do not seem to influence adipocyte differentiation [77, 78], however, this issue remains somewhat controversial. Stenson *et al.* showed that in white adipose tissue (WAT) in human and mouse, GW3965 activated LXR promotes lipolysis and fatty acid oxidation [79]. In brown adipose tissue (BAT), LXR stimulation decreases the expression of uncoupling protein 1 (UCP1), involved in thermogenesis and energy expenditure, and reduces energy expenditure [80]. Further, GW3965 treatment in ob/ob mice, a transgenic mouse model for obesity, altered the fat distribution by decreasing visceral fat and inversely increasing the subcutaneous fat depot, as well as changing the lipid composition with an overall change towards less lipotoxic lipids [81].

1.1.3.3 Glucose metabolism

Beyond the effect on lipid homeostasis, the LXRs also have other vital functions connected to glucose metabolism. After eating, circulating glucose levels will increase, thus promoting insulin release from the pancreas. Insulin stimulates glucose uptake from plasma primarily to muscle and adipose tissue. Insulin also suppresses gluconeogenesis in the liver and promotes conversion of glucose into glycogen for storage. The insulin-responsive glucose transporter GLUT4 facilitates the glucose uptake from plasma to muscle. Within the cell, glucose is converted into glucose-6-phosphate by hexokinase to be targeted for glycolysis and energy production. Other glucose transporters also play a role, including GLUT1 and GLUT2. GLUT1 has a high affinity for glucose, thus making it important in tissues with high energy demands, while GLUT2 operates as a bidirectional transporter with a low affinity for glucose and is expressed in kidney, intestine, liver and pancreas. When circulating glucose levels decrease, glucagon is released from the pancreas, stimulating breakdown of stored glycogen into glucose, gluconeogenesis, in the liver. In addition, glucagon also increases glucose production, gluconeogenesis, in the liver, kidney and intestine.
Activation of LXR promotes a lowering of circulating glucose levels by increasing the expression of transporters GLUT1 and GLUT4 [76, 82] and promotes glucose uptake in WAT and BAT [76, 83, 84]. In the liver, LXR activation suppresses expression of gluconeogenic enzymes. For example, it downregulates peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1α), phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase expression and it also induces the expression of glucokinase, which promotes use of hepatic glucose [78, 83]. The interspecies differences are also apparent in glucose homeostasis, as highlighted by Kotokorpi et al. who demonstrated that GW3965 reduces the expression of GLUT2, as well as glucokinase (GCK) and liver pyruvate kinase (PKLR), two key glycolytic kinases, in human, but not rat hepatocytes. This suggests a risk for high circulating glucose levels, hyperglycemia, in humans, but not rodents, after LXR activation in the liver [62].

Effects of LXR in the pancreas have also been observed. Activation of LXR with synthetic agonist increases basal and glucose-induced insulin levels in a human pancreatic β-cell line [85] as well as in isolated human islets [86], thereby simulating high glucose levels. LXRβ seems to be the dominating isoform in this context, as demonstrated by the fact that LXRβ−/− mice show impaired glucose stimulated insulin secretion [87]. LXR activation in vivo has also been shown to decrease expression of gluconeogenic genes in models of diet-induced obesity and insulin resistance in wild-type (WT) and LXRβ−/− mice, but not in LXRα−/− mice [88], as well as in insulin resistant rats [89] supporting the role of LXRs in glucose tolerance and insulin sensitivity.

The close connection between the LXRs and important metabolic processes, has linked the receptors to metabolic disorders, for example diabetes. Activation of LXRs leads to improved insulin sensitivity and reduced blood glucose levels in animal models of type 2 diabetes [83, 89, 90] and induces insulin secretion by pancreatic islets [85]. LXRβ knockout animals on a high fat diet develop glucose intolerance due to impaired glucose-stimulated insulin secretion [87]. LXR activation in murine pancreatic islet β-cells inhibits proliferation and may induce apoptosis [91, 92]. Moreover, LXR agonists augment human islet function by stimulating insulin secretion [86]. Ketterer et al. have also reported a common variation within the LXRβ gene in subjects at increased risk for type 2 diabetes. The gene variation leads to impaired insulin secretion, which in turn might facilitate the development of the type 2 diabetes [93].

### 1.1.3.4 Inflammatory response and immunity

The LXRs have emerged as important regulators of inflammatory gene expression and innate immunity in many different cell types including skin, brain, T and B lymphocytes, monocytes, macrophages, pancreatic islet cells, muscle, bone, dendritic cells and liver (review in [18]). LXRs reduce the induction of classic inflammatory gene signalling such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2
(COX-2), matrix metallopeptidase-9 (MMP-9) and various chemokines in response to inflammatory signalling in the form of lipopolysaccharide (LPS), tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β) [94-96]. Arginase II, involved in nitric oxide inflammatory response, has also been shown to be an anti-inflammatory target for the LXRs [97].

In addition, LXRs have been shown to be essential for macrophage survival and elimination of invading bacteria in protective immune responses [98-100]. Activation with synthetic LXR agonists reduces inflammatory gene expression in macrophages [96]. In a contradictory study, LXR-deficient mice and macrophages were shown to be more resistant to *Leishmania chagasi*/*infatum* infection, suggesting an antimicrobial defence distinct from that in wild type mice and macrophages [101] and different from other infections where the LXRs seem to have a protective role.

Castrillo *et al.* have also shown a crosstalk between LXRs and Toll-like receptors (TLRs) in the response to viral and bacterial pathogens. Activation of TLRs with microbial ligands blocks the induction of LXR target genes in cultured macrophages and inhibits cholesterol efflux [102]. In addition, activation of LXRs ameliorated disease progression in a mouse model of lupus-like autoimmunity by helping apoptotic cell clearance, thereby maintaining immune tolerance [103].

In antigen presenting dendritic cells (DC), a crucial link between the innate and adaptive immune responses, there seems to be some divergent data. Some studies show that LXRs have an anti-inflammatory role, inhibiting the expression of chemokine receptor CCR7 on maturing DCs and their migration to lymph nodes [104]. It has also been demonstrated that they interfere with DC maturation and function, as well as DC-mediated T cell activation [105]. Others have revealed a pro-inflammatory role, where activation of LXRs increased expression of markers for DC maturation and promoted T cell proliferation; in addition, LXR positive DCs were found in reactive lymph nodes *in vivo* [106].

The role of LXRs as modulators of the immune response has linked the receptors to several disease states. As mentioned above, LXRs have been suggested to have a protective role against viral and bacterial pathogens, although this remains somewhat controversial and needs further exploring. Studies on LXRs in arthritis also reveal divergent results, showing both a protective role in rheumatoid arthritis [107, 108] and osteoarthritis [109, 110] after LXR activation by downregulation of pro-inflammatory mediators, as well as exacerbated progression of rheumatoid arthritis by promoting inflammation and cartilage destruction after treatment with synthetic agonists [111, 112].

Fowler *et al.* demonstrated the anti-inflammatory activity of LXRs in both irritant and allergic contact models of dermatitis, suggesting that LXR activators could provide a new class of therapeutic agents for the treatment of cutaneous inflammatory disorders.
Moreover, topical treatment with LXR agonist modulates epidermal proliferation, differentiation and permeability barrier function, together with the anti-inflammatory activity providing a role for LXR in both cutaneous inflammatory disorders and skin disorders [114]. Recently, evidence has also emerged that LXRs are involved in melanin production, something that is of importance in pigmentation conditions such as vitiligo [115, 116]. LXRs have also been suggested to be involved in decreasing cutaneous inflammation associated with the ageing of the skin induced by chronic UV radiation [117].

1.2 CANCER

Cancer is a worldwide problem with 12.7 million new cases and 7.6 million deaths reported for 2008, with lung, breast, colorectal and stomach cancer being the most common [118]. As the world’s population continues to grow and live longer lives, the incidence of cancer is thought to increase to a total of 22.2 million cases by the year 2030 [119].

A massive amount of research has been conducted to further understand how and why cancers develop, but many questions remain. The process of cancer development appears to be stepwise, see Figure 2, starting with increased proliferation of normal cells and ending with a metastatic tumour with the ability to spread throughout the body. The stepwise process demands time and accumulation of mutations and changes. The concept of cancer is a collection of many different forms of cancer, each with their own characteristics, but some features in common. Over ten years ago, Hanahan and Weinberg, first coined the concept “hallmarks of cancer” in their classic review published in Cell [120]. They described six capabilities acquired during the development of a tumour in an attempt to better explain the characteristics of disease development and progression. The six original hallmarks were: 1) sustaining proliferative signalling, 2) evasion of apoptosis, 3) sustained angiogenesis, 4) enabling replicative immortality, 5) tissue invasion and metastasis and 6) insensitivity to growth suppressors. In the updated version [121], Hanahan and Weinberg also included two additional hallmarks; avoiding immune destruction and deregulating cellular energetic, and two enabling characteristics; genome instability and mutation, and tumour promoting inflammation. These characteristics make it possible for the cancer cell to acquire the necessary capabilities and promote tumour progression. The hallmarks as a concept are a useful way to organise the necessary characteristics to be obtained and possessed by cells at some point in order to generate cancer. However, cancer as a system is evolving, dynamic and heterogeneous, so the complexity grows which explains the difficulties of finding efficient ways to treat cancer.
Figure 2. A model of cancer progression; from a normal cell to a metastatic tumour. Tumour progression starts, here illustrated by epithelium cells of the colon, with normal cells that acquire mutations (often in tumour suppressor genes) that lead to excessive proliferation, hyperplasia, and finally dysplasia. With the help of further mutations, inhibition of tumour suppressors and activation of oncogenes, cells will continue to grow and survive, until a metastatic tumour is formed, with the ability to invade surrounding tissues and spread (metastasise) to the whole body.
The cause of cancer is a widely researched area today and many correlations have been made from the earliest data with ionising irradiation and carcinogenic chemicals. One link that is of interest is the increased risk for some cancers in individuals with excess bodyweight, an increasing problem in the world today. The 2007 report from the World Cancer Research Fund highlighted the evidence for an association between body fatness and increased risk for oesophageal adenocarcinoma and cancers of the colorectum, pancreas, breast (after menopause), endometrium and kidney [122]. Clinical and epidemiological studies have also linked cancer to chronic medical conditions, such as metabolic syndrome and obesity, inflammatory diseases and autoimmune conditions [123-125].

As mentioned above, inflammation has been proposed to be included in the “hallmarks of cancer”, suggested as the seventh hallmark [123, 126]. It has been shown that chronic inflammatory conditions predispose to various forms of cancer, including gastric, lung, colorectal and liver cancer [127-130]. In addition, several clinical studies have also shown that long-term treatment with a low dose of non-steroidal anti-inflammatory drugs (NSAIDs) might reduce incidence and mortality of some cancers [131, 132]. There is also a suggested inflammatory component in the tumour microenvironment that promotes tumour proliferation and survival. The inflammatory microenvironment has also been suggested to contribute to inducing genetic instability, an important step in tumour progression [126]. In short, the immune and inflammatory mechanisms seem to be connected to all stages of tumour development; helping tumour initiation, with increased mutations, genomic instability and epigenetic modification of the DNA mismatch repair system; survival of tumour cells, by activation of tissue repair systems and inducing proliferation as well as stimulating angiogenesis; and in final promotion of metastatic tumours [133]. However, the inflammatory response also has initial beneficial effects, killing off developing tumour cells. It is when the tumour escapes this defence that the protumorigenic effects of inflammation dominates.

1.2.1 Breast cancer

Breast cancer is by far the most commonly diagnosed form of cancer in Swedish women, corresponding to 30.3 percent of diagnosed cancers in 2011, or over 8000 individuals, of which only 45 were male [134]. Globally, an estimated 1.38 million new breast cancer cases were diagnosed in 2008 [118]. There are some established factors connected with a higher incidence; high age, having given birth to few children, early menarche, late menopause, high age at first full-term pregnancy, obesity, alcohol and a hereditary component with family history of breast and ovarian cancer. Inherited mutations in the tumour suppressor genes BRAC1 and BRAC2 are much discussed, and account for 80 percent of inherited breast cancers, or 5-10 percent of all breast cancer cases [135].

Breast cancer is complex and can be considered as a heterogeneous group of different diseases, all with different biological features and clinical outcomes. Before molecular
profiling, all classifications were made based on morphological observations. Now, classifications are based on histological type, tumour grade, lymph node status and presence of predictive markers such as ERα, PR and human epidermal growth factor receptor 2 (HER2). Nearly all breast cancers are carcinomas, most often originating from the mammary ducts, i.e. mammary ductal carcinomas, but also from the lobules that supply the milk to the ducts, so called lobular carcinomas. Only in rare cases the tumours arise from the connective tissue in the breast, producing breast cancer sarcomas. Gene expression profiling has not only contributed to a better understanding of the heterogeneity of breast cancer at a molecular level, but it has also lead to new classifications that today include five subgroups: luminal A, luminal B, basal-like, HER2-enriched and others. The classification is of importance when choosing appropriate therapy, the luminal A and luminal B subtypes express ERα and can therefore be targeted with endocrine therapy, while trastuzumab, a humanised monoclonal antibody against HER2, can be used for the HER2-enriched subgroup [136, 137]. Another commonly used term is triple-negative breast cancer. This is characterised by a lack of ERα, PR and HER2 expression, and is related to the basal-like subgroup; a majority of basal-like tumours are triple-negative. Patients with triple-negative tumour have a relatively poor outcome and cannot be targeted with either endocrine therapy, or HER2 directed therapies [138].

Early detection and diagnosis, followed by surgical removal of the tumour is still the most successful strategy for treatment. Surgical treatment is often combined with radiation or chemotherapy to increase survival rate and reduce the risk of metastases. Endocrine therapies, mentioned above, with selective oestrogen receptor modulators (SERM’s) (tamoxifen and raloxifene), progesterone-like drugs and aromatase inhibitors target ERα and/or PR and is are choices for luminal A and B subtypes, but not all cancers respond and the therapy may lead to endocrine resistance of the tumour. Monoclonal antibodies such as trastuzumab and bevacizumab selectively bind to HER2 and vascular endothelial growth factor (VEGF, important for angiogenesis), respectively, and may prevent tumour growth.

1.2.2 Colorectal cancer

Colon cancer is the third most common cancer in both men and women in Sweden, representing seven percent of reported cases in 2011. Colorectal cancer (CRC), where cancer of the rectum and anus also are included, represents ten percent of all reported cases, or over 6,300 individuals diagnosed [134]. Worldwide, colorectal cancer takes position as the third and second most common form of cancer in men and women respectively, with over 1.2 million cases and 608,000 deaths in 2008. Almost 60 percent of all cases reported occur in developed countries with Australia, New Zealand and Western Europe on the top [118].

Most patients with CRC are above 75 years, only about five percent are under 50 when diagnosed [134]. Besides age, a Western lifestyle is often strongly associated with an
increased risk of CRC. The incidence is, as mentioned above, higher in developed countries, but the risk is now also increasing in less developed countries that adopt a more Western lifestyle. Migration studies have shown that immigrants to high-incidence developed countries increase their risk to develop CRC compared to natives that remain in the low-incidence countries. The factors behind the increased risk are, however, debated. A diet rich in red meat, fat, carbohydrates and processed foods are often associated to an increased risk, while a diet high in fruit, vegetables and fibre seems to reduce the risk for CRC. Smoking, high consumption of alcohol, diabetes, obesity and lack of physical activity has also in some cases been connected to CRC. [139]

Patients with inflammatory bowel disease (IBD), such as ulcerative colitis and Crohn’s disease, account for about two-thirds of the incidence of CRC and the highest risks are found in patients with longer disease durations, extensive disease and in patients diagnosed before the age of 30 [140, 141]. Preventive colectomy is an option for these patients, but today, regular examinations with colonoscopy for early detection of cancer is preferred. There is also a hereditary component in some forms of CRC. The Lynch syndrome, also known as hereditary non-polyposis colorectal cancer (HNPPC), and familial adenomatous polyposis (FAP) colorectal cancer are the two most common hereditary CRCs, occurring in one in 300 and one in 7,000 people affected by CRC respectively [142].

The clinical model of CRC is often divided into four different stages, to better illustrate the stepwise and slow progress of the disease development, from normal epithelium to adenomatous polyps and invasive carcinomas. Tumours of stage I, which are limited to the wall of the colon, and those of stage II, which have begun to grow through the wall of the colon, are curable by surgical excision. If left untreated, the cancer will spread to the lymph nodes near the tumour, referred to as a stage III tumour. At this stage, adjuvant chemotherapy is added to surgical excision with a success rate up to 73%. Chemotherapy might also be given after surgery to reduce the risk for relapse. Stage IV CRC, where the tumour has grown into adjacent tissues or spread and formed metastases, often in the liver, is usually incurable [143]. New therapeutic methods, using for instance combinatorial approaches with anti-inflammatory agents, have been suggested in order to further increase patient survival. NSAID treatment, more specifically with the use of acetylsalicylic acid, seem not only to reduce the risk for CRC, but also, when used post-diagnosis, to reduce the number of distant metastasis and increase overall survival [144].

The molecular basis for the progression of CRC, through the different stages, is not fully understood, however certain factors have been suggested to be required to initiate and drive the progression of the tumour. Dysregulation of the Wnt signalling pathway, through inactivation of the adenomatous polyposis coli (APC) gene or activation of β-catenin, is considered to be the initiating event in CRC, leading to a constitutively active Wnt signalling pathway and induction of a number of cell proliferation and survival genes [145, 146]. It is germ line mutations of APC that give rise to FAP
syndrome, and a high risk for early onset CRC [147]. Tumour progression is then furthered by genomic instability, such as chromosomal instability, with an allelic imbalance at several chromosomal loci, chromosome amplification and translocation, or by defects in the DNA mismatch-repair system, as for patients with HNPCC. Several factors contribute to progression from adenomas into tumour. These include alterations of tumour suppressing pathways, such as transforming growth factor β (TGFβ), BCL2-associated X protein (BAX) and the p53 pathway, but also activation of oncogene pathways, such as RAS and BRAF, as well as phosphatidylinositide 3-kinase (PI3K) [143].

Figure 3. The structure of the colon (illustrated with a tissue section from mouse) including: **Muscularis externa**, an outer muscle layer responsible for the motility of the content in the lumen. **Submucosa**, a layer of connective tissue connecting the muscularis externa to the mucosa. The **mucosa**, or the inner layer of the colon, include a layer of **surface epithelial cells**, **lamina propria** (connective tissue) and an outer layer of muscle (**lamina muscularis mucosae**). In the colonic crypt, **stem cells** are found at the bottom, followed by a section with **transit cells** and finally a section with **differentiated cells**. The larger mucus secreting **Goblet cells** can also be seen in the colonic crypt.

The structure of the colon can be seen in Figure 3, illustrating how the colonic crypt is made up of different sections. A stem cell population, also known as crypt base columnar cells, resides at the bottom of the crypt. The stem cells produce progenitor cells, also called transit amplifying cells, which differentiate and migrate up the crypt to the crypt collar. At the top of the crypt, cells undergo apoptosis and are released into the lumen, a process referred to as cell shedding [148]. The whole process takes two to three days, making the intestinal epithelium the most rapidly regenerating tissue in the body. The various differentiated cell types of the crypt include enterocytes (or intestinal absorptive cells), mucus secreting Goblet cells, hormone-secreting enteroendocrine cells and Paneth cells. The latter are attributed a variety of roles, including secretion of antimicrobial products [149].

The stem cell population of the crypt is of special interest when discussing CRC, and is defined by two specific functional properties: self-renewal and multipotency. This
means that these cells have the ability to both continue dividing over a long time and to produce cells of any type specific to the colon. These two characteristics are also suspected to be of importance for cancer stem cells [150, 151]. In addition, the Wnt signalling pathway is crucial for both normal colon stem cell activity and CRC initiation. However, studies have indicated that the levels of active $\beta$-catenin differ between tumour cells in a population. To explain this, a model has emerged with a mixed tumour population of “normal” differentiated tumour cells and CRC stem cells. The CRC stem cells are thought to contain the tumourigenic capacity, with altered Wnt activity, self-renewal ability and the multipotency of stem cells [152].

One of the major risk factors for CRC is, as mentioned, IBD, and it is believed that the interaction between the colonic epithelial cells and macrophages, neutrophils and other inflammatory cells plays an important role in the tumour development. The inflammatory state leads to an overproduction of reactive oxygen and nitrogen species and an upregulation of several inflammatory genes and growth factors in the mucosa of the colon, resulting in DNA damages, as well as an increased epithelial cell turnover and further tumour progression. [153]

### 1.2.3 The cell cycle and cancer

The topic of the cell cycle is extensive and there is too much information on how cells manage division and proliferation to go into any depth in this thesis. This chapter is therefore focused on giving a brief summary of the concept of the cell cycle in eukaryotic cells. It will also connect the cell cycle to the uncontrolled cell proliferation seen in cancer in order to help discussions in later chapters.

The cell cycle has two main objectives. First, during the interphase period, the cell has to grow, duplicate its chromosomes and prepare for the second objective, mitosis, where the cell divides into two distinct cells, a pair of daughter cells. The cell cycle is often divided into four phases (see Figure 4); gap 1 ($G_1$) phase, synthesis or $S$ phase, gap 2 ($G_2$) phase and finally mitosis, or $M$ phase. In addition, the cell might leave the cell cycle and go into the quiescent gap 0 ($G_0$) phase, or the resting non-dividing phase. Specific checkpoints, or restriction points, exists for each phase to check if all necessary processes have been completed and the cell is ready to proceed to the next phase. The cell cycle might also be arrested due to external signals, such as in response to stress from DNA damage or lack of nutrients. Many players are involved in controlling these checkpoints, preventing DNA damages to be transferred into daughter cells as well as uninhibited proliferation of tumours [154].
Two groups of molecules central in the cell cycle are cyclins and cyclin-dependent kinases (CDKs). Together cyclins and CDKs form active complexes, with cyclins as the regulatory subunits and CDKs as the catalytic subunits, only active when bound to its specific cyclin. In $G_1$ phase, the entry and progression of the cell cycle is controlled by cyclin D1 and D2, bound to CDK4, CDK5 and CDK6. CDK2 needs to be bound to cyclin E in order for the cell to complete the $G_1$/S checkpoint and continue into S phase. The cyclin/CDK complexes phosphorylate retinoblastoma protein (RB) and inhibit the binding of RB to transcription factors needed for cell proliferation. In S phase, cyclin A bound to CDK2 or CDK1 will stimulates DNA duplication and regulates the start of the mitotic process. Another group of CDKs, namely CDK7 to CDK9 are also important during this phase. These CDKs have two distinct roles, first they phosphorylate and activate other CDKs (giving them the name CDK-activating kinases, or CAKs), and secondly they form a subunit of a transcriptional complex.
During the G₂ phase, cyclin B will binds to CDK1 (also called cdc2) and help the progression into mitosis [155].

Besides the CAKs mentioned, there are also other regulators of the CDKs, specifically two families of CDK inhibitors (CKIs). The INK4 (inhibitor of kinase 4) family, including p15, p16, p18 and p19, inhibit the binding of cyclin D to CDK4 and CDK6, whereas the KIP (kinase inhibitory protein) family, p21, p27 and p53, act by binding to CDK2. Other CDK modifiers include Cdc25 phosphatase and Wee1 kinase, the latter phosphorylates inhibitory sites on CDKs while the former removes these inhibitory phosphorylations [155, 156].

In addition, the cell cycle is controlled by the levels of the various cyclins, since the CDKs are only active when bound to their specific cyclin. The concentrations of the cyclins vary throughout the cell cycle, thereby controlling its progression. Cyclin E is expressed mid to late G₁ and peaks during the G₁/S transition, cyclin A is expressed during G₂ phase, and cyclin B is expressed from late S and G₂, but remains inactive until peak concentration is reached during late G₂ phase. The levels of D-type cyclins are, however, kept relatively constant throughout the cell cycle. [157]

The levels of cyclins and CKIs are controlled by the ubiquitin-mediated proteasome system (UPS) that ubiquitiates and targets the proteins for degradation. The UPS pathway consists of three steps. First the ubiquitin protein (Ub) is transferred to the ubiquitin-activating enzyme, E1, and is activated by hydrolysis of ATP, after which it is transferred to the ubiquitin-conjugating enzyme, E2. In the final step, the ubiquitin ligase, E3, covalently attaches the Ub to the target protein. Poly-ubiquitinated proteins are often targeted for degradation by the proteasome, but other types of ubiquitinylation events exist. There are many types of E3 ligases in humans, involved in many different biological processes. Two important E3 ligases concerning the cell cycle are the anaphase-promoting (APC) complex and the Skp1-Cullin-F-box protein (SCF) complex, responsible for the ubiquitinylation of cyclins and CKIs [158].

Deregulation of the control of the cell cycle is a fundamental aspect in tumour development, as discussed at the beginning of this chapter; therefore it is not surprising that many regulators of the cell cycle have been found to be mutated in various types of cancer. Changes include loss of CKIs and RB expression, as well as overexpression of cyclins and CDKs, thereby enabling cell cycle progression without the normal strict regulatory steps [156]. Many alterations in the APC and SCF complexes are also reported, linking UPS to tumour progression. An example is mutations in F-box and WD-40 domain protein 7 (FBW7), an SCF E3 ligase that is responsible for targeting several oncoproteins for degradation; including cyclin E, Myc, Jun, Notch1 and Notch 4 [158].

Decreased protein expression of p27, a CKI from the KIP family, is frequently found in multiple human cancers and is often correlated to a poor prognosis. However, mutations of p27 are rare, and the decreased expression is thought to be connected with
an increased degradation especially by S-phase kinase-associated protein 2 (Skp2), identified as the SCF E3 ligase that targets p27 for ubiquitinylation and degradation. Skp2 also has other targets, including cyclin E, p57, p21 and the transcription factor E2F1. The importance and prognostic significance of overexpression of Skp2 in cancer, both with and without inverse correlations with p27 levels, has been seen in numerous studies, as reviewed by Hershko [160]. Skp2 is also believed to have a role in the regulation of the SCF complex itself, by being targeted by the APC complex for degradation [161]. A recent study also showed the importance of Skp2 in activating Akt kinases, helping tumour cells to use aerobic glycolysis in order to manage their increased energy needs, a process known as the Warburg effect. The study further showed that Skp2 deficiency could sensitise breast cancer tumours to specific treatment, showing a clinical value in targeting Skp2. [162]

RB is linked to cancer in several ways; the classic role of RB is as regulator of the G1/S checkpoint, as mentioned above. RB is inactivated by phosphorylation, leading to the release of bound members of the E2F family of transcription regulators, and the cell cycle may proceed. RB has also been appointed other functions, such as preservation of chromosomal stability, induction and maintenance of senescence, regulation of apoptosis, cellular differentiation, and angiogenesis, and inactivation of mutations in several members of the RB family have been found in a range of tumours [163].

Another key player in tumour progression is p53, found to be mutated or functionally inactive in most human cancers [164]. p53 has many functions in the cell, but in view of the cell cycle p53 activation, in response to DNA damage, actives CKI p21 and inhibits the formation of the cyclin E/CDK2 complex required for G1 to S phase progression [165]. Other processes that p53 affects include apoptosis, genomic instability, and inhibition of angiogenesis, all of which lead to tumour progression if p53 is mutated or inactivated.

1.3 THE LIVER X RECEPTORS AND CANCER

Publications linking LXRs to changes in proliferation and cancer have increased substantially during the last few years, showing that LXR agonists suppress proliferation in a wide range of cancer cell lines, including prostate, breast, colon, ovarian and leukaemia cells, and conversely, that LXR deficiency in mice will results in increased proliferation. Several mechanisms have been suggested behind the antiproliferative effects; direct regulation of the cell cycle, induction of apoptotic pathways, changes in cholesterol homeostasis and interrupted hormone-dependent proliferation.
1.3.1 LXRαs and effects on the cell cycle

Fukuchi et al. demonstrated that activation of LXRαs with synthetic agonist T0901317 reduces proliferation of LNCaP human prostate cancer cells both **in vitro** and in a xenograft model, as well as in other human prostate and breast cancer cell lines. Protein levels of cell cycle dependent inhibitor p27 were increased and Skp2, a SCF E3 ligase mediating degradation of p27 decreased [166]. Suppressed levels of Skp2 and increased levels of p27 were also seen in vascular smooth muscle cells (VSMC) treated with synthetic LXR agonists. T0901317 and GW3965 inhibited cell cycle progression from G₁ to S by altering the phosphorylation status of RB at the G₁/S checkpoint and this effect could be abolished by overexpression of Skp2 [66]. The antiproliferative effect of T0901317 was further shown in several human cell lines, including multiple prostate and breast cancer cell lines, as well as non-small lung cancer, osteoblastic, squamous carcinoma, cervical cancer, gastric cancer and hepatoma cells. In contrast, HEK293, transformed human embryonic kidney cells, and Wi38, human diploid fibroblast cells, seem to be more resistant to T0901317 [167, 168]. The T0901317 effect on PC3 prostate cancer cells seems to be unclear, with studies both showing reduced proliferation and no effect [166, 168, 169]. LXRαs have by Geyeregger et al. been shown to be active in leukemic T and B lymphocytes and activation with agonists T0901317 or GW3965 inhibited T cell proliferation and cell cycle progression with a G₁ to S phase arrest. Moreover, the LXR activation also promoted apoptosis in B cells isolated from patients with chronic lymphoblastic leukaemia (CLL) [170].

Data from pancreatic cells seem to be somewhat conflicting. Although Wente et al. could not see any changes in proliferation after T0901317 treatment of insulin secreting MIN6 cells, a mouse pancreatic islet β-cell line, they reported an antiproliferative effect when activating both LXRα and RXRα in these cells. The dual LXR/RXR activation increased expression of both tumour suppressor Smad3 and caspase activity in MIN6 cells and isolated rat pancreatic islets, suggesting increased apoptosis, but no change in Skp2 level was observed [171]. Short thereafter, another group reported that LXRα activation alone promoted lipid accumulation, lipotoxicity and increased β-cell apoptosis in INS-1, a rat insulinoma cell line, and isolated rat pancreatic islets [92]. In addition, Meng et al. demonstrated that LXRα activation with T0901317 in MIN6 and the hamster islet β-cell line HIT-T15 inhibited cell proliferation through a G₁ to S cell cycle arrest by down-regulating Skp2 mRNA expression and upregulating p27 protein levels. The importance of p27 was further highlighted when siRNA knockdown of p27 partially reversed the antiproliferative effect seen in MIN6 cells [91]. The same group further showed that LXRα activation with T0901317 results in lipid accumulation and lipotoxicity in pancreatic β-cells, via induction of SREBP1c [172], supporting the initial study.

In a recent study, Lo Sasso and colleagues demonstrated that activation of either LXRα or LXRβ with GW3965 resulted in G₁/G₀ cell cycle arrest and increased apoptosis in human CRC cell lines. Skp2 and cyclin D1 mRNA and protein levels were decreased, while p27 protein levels increased. Activation of LXRα was also shown to slow down
the growth and induce apoptosis of xenograft tumours in mice [173]. Besides the studies already mentioned, reduced Skp2 expression after LXR activation with T0901317 has also been seen in human non-small lung cancer, hepatoma and cervical cancer cell lines [169]. The effects on p27 protein levels are somewhat inconclusive. Some studies have shown an accumulation of p27 protein, often accompanied by Skp2 downregulation, while others observe the antiproliferative effect of LXRs without changes in p27 levels [170, 174]. In addition, Pascual-Garcia et al. showed that the antiproliferative effect still remained in p27 deficient macrophages. Instead they focused instead on other changes in the cell cycle, namely reduced protein expression of cyclin D1 and cyclin B1, as well as CDK2 and CDK4 after LXR activation. Although cyclin B1 is involved in later stages of the cell cycle, the effect of LXR activation appears to influence G1 to S progression, suggesting that the effects on cyclin B1 are secondary [175]. Other effects on cyclins and CDKs have also been reported, such as on cyclin B [170, 175, 176], cyclin D1 [66, 173-175], cyclin A [66, 168, 174], cyclin E [176, 177], CDK1 [176], CDK2 [175, 176] and CDK4 [175], demonstrating the clear influence of LXRs on the cell cycle.

The antiproliferative effect of LXR activation has also been suggested to be linked to a key mediator in Wnt signalling, β-catenin, in colon cancer cell line HCT116 [178]. The Wnt signalling pathway controls cell to cell communication needed for appropriate cell proliferation and differentiation during development and healing. Treatment with T0901317 suppressed β-catenin transactivation activity and downregulated mRNA expression of some β-catenin target genes, Myc, Bmp4 and MMP7 [178]. Dysregulation of Wnt signalling, leading to accumulation of β-catenin, has also been reported in human CRC [145, 146], and other NRs, particularly androgen receptor (AR), have been shown to modulate the Wnt/β-catenin pathway [179].

### 1.3.2 LXRs and antiproliferative effects of cholesterol homeostasis

The initial studies by Fukuchi et al. and others also showed that LXR target gene ABCA1 expression was connected to reduced proliferation of LNCaP cells. While ABCA1 knockdown increased the rate of proliferation, activation of LXRs, and thus increased ABCA1 expression, resulted in reduced proliferation [166, 180]. The mechanism behind this effect was suggested to involve cholesterol homeostasis. The activation of LXRs stimulates cholesterol efflux, which deprives the cancer cell membranes of lipids essential for their growth and thereby prevents further growth. Transino et al. showed that LXRβ, and not LXRα, is the major regulator of both ABCA1 and ABCG1 mRNA expression levels in prostate cancer cell lines [181], while Pommier et al. showed that T0901317 treatment leads to upregulation of ABCG1 only, in LNCaP cells in vitro and in vivo. The latter study further demonstrated that the increased cholesterol efflux, due to LXR activation, resulted in downregulation of the AKT survival pathway and induced apoptosis in LNCaP cells [182]. An in vivo study from the same lab using LXR deficient mice, showed that LXRs also regulate
proliferation in epithelial cells from the dorsal prostate by modulating cholesterol homeostasis and the AKT survival pathway [183], supporting the initial findings.

Lo Sasso and colleagues further highlighted the connection between cholesterol homeostasis, cell proliferation and LXR by using a mouse model of partial hepatectomy (PH) [184]. In PH, part of the liver is removed in order to study the ability of the liver to regenerate. The cholesterol content in the liver increased after the procedure to meet the increasing demands for regeneration and, at the same time, a suppression of pathways involving LXR could be seen due to lower levels of oxysterols. Activating LXR with GW3965 reduced the proliferation of the hepatocytes, thereby reducing the rate of liver regeneration. Scoles et al. showed cholesterol related proliferation also in ovarian cancer cells, where oxidised LDL cholesterol stimulated ovarian cancer cells to proliferate and LXR activation with T0901317 increased cholesterol efflux, reduced expression of proinflammatory cytokines and inhibited proliferation [185]. LDL cholesterol is also needed for glioblastoma proliferation, one of the most common forms of malignant primary brain tumours, as shown by Guo and colleagues [186]. Treatment with GW3965 resulted in dose-dependent inhibition of proliferation and induction of tumour cell death due to lower levels of available cholesterol in vitro and in an in vivo xenograft model. Both increased cholesterol efflux by overexpression of ABCA1 and reduced cholesterol influx was demonstrated as mechanism behind the antitumour effects seen.

A recent study also demonstrated a clear link between LXR and CRC. Intestinal specific LXR activation was shown to reduce intestinal tumour number and size in two different in vivo mouse CRC models, one chemically inducible and one genetic [173]. By comparing data from three different in vivo tumour models, the group further demonstrated that intestinal specific LXRα activation induced antitumour effects through increased cholesterol efflux.

1.3.3 LXR and hormone dependent proliferation

It is widely known that certain hormones and hormone regulated NRs are vital for normal cell growth, as well as aberrant growth in some types of cancer. The most well known examples are probably the connection between ER and breast cancer, or AR and prostate cancer. It appears that there is a cross-talk between LXR and other NRs, possibly through competition for coactivators [187, 188], that link LXR to hormone dependent cell growth.

Androgens are important both for normal prostate cell growth and development, as well as during prostate cancer. Chuu et al. showed that LXR agonist T0901317 delayed progression from androgen-dependent to androgen-independent tumours [189]. T0901317 might act as an AR antagonist in prostate cancer cell lines competing with androgen for binding to AR [190]. T0901317 also represses AR activity by upregulating sulfotransferase enzyme SULT2A1 and downregulating steroid sulfatase.
The first reaction deactivates androgens and the second one reverses this process. These changes result in lowered levels of circulating androgens in vivo, inhibited androgen-dependent prostate regeneration and decreased prostate cancer cell growth [191].

In studies on LXR deficient mice, Kim et al. demonstrated that both LXRα and LXRβ are expressed in the mouse ventral prostate, one of the four lobes of the murine prostate. LXRα−/− mice, and also to a lesser extent LXRαβ−/− mice, developed symptoms of benign prostatic hyperplasia of the ventral prostate [192]; this was supported by later studies showing that LXRα is the dominant isoform in AR regulation [193]. A study by Dufour et al. showed that LXRs also regulate proliferation in epithelial cells from the dorsal prostate, a lobe more similar to the human peripheral zone where most prostate cancers originate [183].

The cross-talk between LXRs and AR was further investigated by Krycer et al. as a mechanism in cholesterol homeostasis and prostate cancer [194]. They showed that AR antagonises LXR, potentially via competition for coactivators. Consequently, AR activation leads to LXR downregulation and reduced cholesterol efflux. High cholesterol levels are observed in both the ageing prostate and prostate cancer, and in addition epidemiological studies have associated high circulating cholesterol levels with an increased risk for prostate cancer, while cholesterol-lowering drugs reduced the risk [195].

Krycer et al. and others have also shown cross-talk between LXR and other NRs besides AR, such as ER, PR and GR, something that has been supported by both studies on LXR deficient mice and treatment with LXR ligands. Gabbi et al. showed that LXRβ−/− female mice, but neither LXRα−/− nor LXRαβ−/− female mice, or LXRβ−/− male mice develop inflammation and cancerous changes in the gallbladder in an oestrogen-dependent manner [196]. Activation of LXR has also been shown to control oestrogen homeostasis regulating hepatic oestrogen sulfotransferase (EST or SULT1E1), an enzyme responsible for the sulfurylation of oestrogen and other steroids [197]. The study additionally showed that LXR activation inhibited oestrogen-dependent breast cancer cell growth in a mouse xenograft model.

In studies on breast cancer cell lines and tissue biopsies, Vigushin et al. showed that LXRα mRNA is expressed in both normal human breast tissue and primary breast carcinomas, but to a lower extent in the latter samples. However, the LXRα expression in the tumour samples could not be correlated to ER or PR status, nor to patient age or tumour size. The group further described that LXR activation inhibited proliferation of both ERα positive and negative breast cancer cell lines [198], something that has also been shown by several other groups [166, 167].
1.3.4 LXR\textsuperscript{s} and cancer immunology

Avoiding the immune surveillance is one of the hallmarks of cancer and an important characteristic of tumour cells and the advance of tumour growth and metastasis \cite{133}. Growing tumours release immunogenic material from dead or dying tumour cells, thereby engaging tumour-associated DCs to collect this debris. The DCs thereafter migrate to tumour-draining lymph nodes and activate naive T and B cells, thus starting an immune response against the tumour. Villablanca \textit{et al.} reported a new role for LXR\textsuperscript{s} in cancer biology, namely to prevent this antitumour response from the immune system. Tumours can produce oxysterol LXR agonists that can inhibit the expression of CCR7 on the DCs. CCR7 is a chemokine receptor critical for the migration of DCs to the tumour-draining lymph nodes. The inhibition of CCR7 expression by LXR activation consequently dampens the immune response. Treatment with sulfotransferase 2B1b (SULT2B1b), an enzyme that sulfurylates cholesterol, decreased the availability of LXR ligands and increased the antitumour immune responses \cite{104}.
2 AIMS OF THE THESIS

The major aim of this thesis is to investigate the role of LXRs in breast and colorectal cancer and the underlying mechanisms. Specific aims are to investigate:

- the molecular mechanisms behind the antiproliferative effect of LXRs and in human breast and colorectal cancer cell lines (Papers I, II and III)

- the correlation between LXR-responsive genes in human breast cancer cells and gene profiles associated with the survival of breast cancer patients (Paper II)

- the role of LXRs in proliferation of colonic epithelial cells in vivo in mice (Paper III)

- the anti-inflammatory role of LXRs in the colon using DSS and TNBS induced colitis in mice (Paper IV)
3 METHODOLOGICAL CONSIDERATIONS

Materials and methods are described in each paper included in this thesis, but this chapter aims to highlight specific aspects about some of the methods used. Only a general discussion is included here, more details are described below for each paper.

3.1 CELL LINES

Several different immortalised cell lines from various tissues have been used in this thesis. The cell lines represent an excellent *in vitro* model system, being easy to grow, manage and manipulate, creating a reliable pool of samples with a high degree of homogeneity. Even though the use of cell lines can be considered as standard laboratory practice, there are caveats in using these model systems, in particular when trying to translate results from *in vitro* cell line experiments to *in vivo* situations.

An illustration of one of the problems using cell lines, and the variation in data between laboratories, can be seen in the classic experiment from Osborne and colleagues from the 1980’s [199] where they compared one of the most widely used human breast cancer cell lines, MCF-7, from four different laboratories. The data showed that the MCF-7 lines differed in amounts of ER and PR, important markers in breast cancer, as well as having different growth rates, response to oestrogen treatment, tumorigenicity in mice and chromosomal alterations. It was even found that one of the lines was derived from an entirely different patient.

A review in Breast Cancer Research [200] highlights many important concerns using cell lines, with the focus on breast cancer cell lines. Issues being raised include “false” cell lines or cell lines contaminated with HeLa cells [201] and that many of the most frequently used breast cancer lines used are derived from tumour metastases, and not from primary tumours, thereby representing late-stage disease. The clonogenicity of the cell lines with continuous culturing also leads to a phenotypic and genotypic drift from the original cell population.

One additional major disadvantage in using cell lines is the lack of interaction between the tumour cell and the environment. The microenvironment of a tumour cell *in vivo* will has a strong influence on growth and survival of the tumour, which is lacking *in vitro*. Xenograft mouse models are an option to simulate a more natural environment, but it means going from the convenience and controllability of *in vitro* studies.

The intention has been to address limitations using cell lines throughout the work in this thesis. Several different cell lines have, when possible, been used as model systems for breast and colorectal cancer. Limits have also been put to how many passages cells were allowed to undergo before a new stock of cells has to be used, in order to maintain the homogeneity of the culture. Other model systems, such as primary cultures are a
good alternative, but the ease and convenience of the cell lines make them hard to replace.

3.2 PROLIFERATION ASSAYS

Several different types of proliferation assays have been performed throughout this thesis, all with specific advantages and limitations.

The simplest method used in this thesis is viable cell count using a vital cell stain, trypan blue, that selectively stains dead or dying cells with disrupted cell membranes. Both stained and non-stained cells are counted in a haemocytometer and the percentage of viable cells may be calculated. To see effects on proliferation with this method, a time course needs to be set up and changes in absolute numbers of viable cells examined. This is a cheap and easy, but somewhat time consuming, method where the biggest drawback is that it only gives changes in absolute cell numbers, and no information about the cell cycle status of the cells.

By using a flow cytometry based proliferation assay with a DNA stain, such as propidium iodide (PI), it is possible to get a snapshot of the cell population and determine which cell cycle phases the cells reside in. It also gives an indication if the treatment in question might be toxic to the cells. Additional stainings can be added to examine apoptosis, viability and other characteristics of the cells.

5-bromo-2'-deoxyuridine (BrdU) incorporation assays have been used in this thesis both in vitro and in vivo. It is based on the fact that BrdU is a structural analog to thymidine, allowing it to be incorporated in newly synthesised DNA. The amount of incorporation of BrdU thus corresponds to the amount of cells going through S phase. We have measured BrdU incorporation both by immunohistochemistry (IHC) on tissue sections and by flow cytometry together with PI staining on fixed cells. Using BrdU and PI staining together makes it easier to identify changes in the cell population going through S phase.

In addition to BrdU incorporation, tissue sections were also, using IHC, stained for proliferation marker Ki67. Ki67 is strictly associated with cell proliferation and the protein is expressed during all active cell cycle phases, but not in resting cells in G0 [202].

3.3 MESSENGER RNA AND PROTEIN ANALYSIS

Quantitative polymerase chain reaction (qPCR, real-time PCR) was used to measure mRNA expression levels. It is a widely used, specific and sensitive method, but nonetheless limitations exist. Good housekeeping, or reference, genes are required to normalise data, as well as well designed and gene specific primers. All primers used in
this thesis have been carefully tested with dissociation curve analysis in order to detect unspecific binding and primer dimers, and changes in housekeeping genes due to treatments etc. have been monitored to avoid false results. qPCR analysis needs to be complemented with analysis of protein levels and activity.

Protein levels have been measured in this thesis using Western blot, where the two major problems are antibody specificity and determination of signal intensity. The first problem is hard to address, new antibodies should be tested against negative or positive controls if possible, and antibody dilutions and incubation times optimised. There are several different methods for quantifying Western blot results, both by analysing band density from photographic films (see below) and using newer, possibly more accurate, detection methods utilising CCD cameras and digital imaging systems, or by using fluorescent secondary antibodies.

Within this thesis, enhanced chemiluminescent horse radish peroxidase (HRP)-linked secondary antibodies were used with detection detection by photographic film. For quantification, films were scanned and band density analysed using ImageJ 1.43u software (Wayne Rasband, National Institutes of Health, USA) according to online protocol from Luke Mille [203] and normalised against a loading control. Even though care was taken to avoid problems such as saturated films, this is still a significant problem when using photographic film, and needs to be considered when evaluating the results.

Another way to measure protein levels included in this thesis is enzyme-linked immunosorbent assay (ELISA), used in Paper IV. This method still uses antibody technology to recognise target protein, but the detection is connected to intensity of transmitted light by spectrophotometry. The ELISA is usually performed in plate format, making it possible to measure a larger number of samples simultaneously.
4 RESULTS

4.1 PAPER I: THE OXSTEROL RECEPTOR LXR INHIBITS PROLIFERATION OF HUMAN BREAST CANCER CELLS

LXR has been shown to be antiproliferative in several cell types and in Paper I, the aim was to investigate the role that LXRs might have in human breast cancer cell lines. The study was done using four different cell lines; MCF-7 and T47D, both of a luminal subtype expressing ER$\alpha$ and PR but not HER2, MDA-MB-231, a basal triple negative cell line, and SKBR3, a luminal HER2-enriched cell line lacking ER$\alpha$ and PR expression [204]. All four cell lines showed expression of both LXR$\alpha$ and LXR$\beta$, and activation with synthetic LXR agonist induced classical LXR target genes, demonstrating functional LXR pathways.

We further showed, using a flow cytometry bromodeoxyuridine (BrdU) incorporation assay, that synthetic LXR agonists, both GW3965 and T0901315, had an antiproliferative effect on all four cell lines. The cells appear to accumulate in G1 and G0 cell cycle phases, indicating that LXR activation leads to an arrest at the G1 to S cell cycle phase transition. The effect was boosted by reducing the initial rate of cell proliferation, through the use of growth media with reduced serum concentration (2-5% serum compared to 10% (data not shown)).

Interestingly, the antiproliferative effect was most pronounced in the ER$\alpha$ positive cell lines, MCF-7 and T47D, which lead us to investigate the role of ER$\alpha$ further. Using charcoal stripped media, thus removing endogenous hormones and growth factors, we had a model system where we could control the growth of oestradiol responsive MCF-7 cells. By adding oestradiol in increasing concentrations, proliferation increased in a dose-dependent manner. However, when co-treating the cells with GW3965, the oestradiol-induced proliferation was significantly reduced. In addition, following addition of a synthetic ER$\alpha$ antagonist to block ER-dependent proliferation, the antiproliferative effect of the LXR agonist in MCF-7 cells was eliminated. Also, investigation of the gene expression profiles of MCF-7 cells after T0901317 and GW3965 treatment, showed that ER$\alpha$ was reduced both at mRNA and protein levels. Together, all this data indicates that LXR induced cell arrest involves an ER$\alpha$ dependent pathway.

Even though the antiproliferative effect of LXR was most pronounced in ER$\alpha$ positive cells, both GW3965 and T091317 caused to a significant reduction of proliferation also in the two remaining, ER$\alpha$ negative cell lines. We therefore concluded that the antiproliferative effect of LXR activation must engage several pathways. One possible factor was the role of LXRs as regulators of lipid homeostasis. We tested this hypothesis using siRNA technology, knocking down SREBP1c, a key target of LXRs responsible for regulating genes required for de novo lipogenesis. Reduced levels of
SREBP1c lead to reduced rate of proliferation of MCF-7 cells, in agreement with the link between lipid biosynthesis and proliferation. However, there was no changes in the antiproliferative effect of LXR activation following knock down of SREBP1c, indicating that SREBP1c activation and resulting lipogenesis were not involved in LXR effects on proliferation of MCF-7 cells.

By further investigating the effects of LXR activation on MCF-7 cellular proliferation, we found alterations of expression of genes involved in cell cycle regulation. Treatment with GW3965 or T0901317 reduced expression of mRNA and protein levels of Skp2, though no increase of p27 protein levels was seen. Cyclin D1 and A2 levels were also decreased upon LXR activation, whereas the levels and activity of tumour suppressor protein p53 protein were increased. RB protein levels remained the same after LXR activation, but became hypophosphorylated, a state of RB that inhibits the cell cycle transition from G1 to S phase.

4.2 PAPER II: LIVER X RECEPTOR LIGANDS DISRUPT BREAST CANCER CELL PROLIFERATION THROUGH AN E2F-MEDIATED MECHANISM

The aim of Paper II was to further investigate the effects of LXR activation on breast cancer cell lines, as observed in Paper I, by microarray analysis of gene expression. In addition, we also wanted to compare any differentially expressed genes in the cell lines to disease parameters and survival outcome from cancer patient databases.

We performed microarray analysis of gene expression in the four cell lines from Paper I; MCF-7 and T47D, both expressing ERα, and ERα negative cell lines SK-BR-3 and MDA-MB-231, treated with synthetic LXR agonist GW3965 or vehicle under the same conditions as in Paper I. Differential expression after GW3965 treatment could be seen for a total of 2800 genes. MCF-7 cells showed the highest number of changed genes, 2021, while 462 genes were differentially expressed in T47D cells. ERα transcripts were reduced in both cell lines after LXR activation, consistent with what we saw in Paper I. In ERα negative SK-BR-3 cells 603 genes where changed after agonist treatment, whereas 926 genes were altered in the triple negative MDA-MB-231 cells. In total, 83 genes were differentially regulated in all four cell lines after treatment with GW3965; off these 23 were upregulated and 60 downregulated.

We continued by performing gene ontology (GO) analysis on the 83 induced genes that were common between the four cell lines, in order to find which biological processes were involved in the common response to LXR activation. Among the 23 upregulated genes, GO terms for known LXR functions, such as lipid and cholesterol transport, and metabolism, were enriched. In contrast, and perhaps more interesting, the downregulated gene set, was enriched for GO terms involved in cell cycle regulation, DNA replication and other processes associated with cell proliferation.
In order to find known transcription factor binding site motifs, we used the downregulated gene set and performed a transcription factor binding site analysis of the promoter regions of the selected genes. Only one sequence motif enriched in this gene set was found, namely the E2F family of transcription factors, enriched in 15 of the 60 genes. The downregulation of the majority of these 15 genes after GW3965 treatment was confirmed with qPCR analysis in all four cell lines. The E2F family includes nine members, all involved in cell cycle regulation, and by going back to the 60 commonly downregulated genes, E2F2 was found to be responsive to LXR agonist treatment. E2F2, together with two other E2F members, E2F1 and E2F3a, are cell cycle promoters expressed in late G1 phase of the cell cycle and interact with G1/S checkpoint regulator RB.

The reduced expression of this new cell cycle target for LXR activation, E2F2, was confirmed at the mRNA level with the most pronounced regulation in the ERα positive cell lines, but also, to a lesser extent, in MDA-MB-231 cells. To investigate the role of E2F2 in breast cancer cell lines, E2F2 was knocked down, using siRNA technology, in MCF-7, T47D and MDA-MB-231 cells. The knockdown lead to reduced cell proliferation in MCF-7 and T47D cells, compared to control cells transfected with unspecific siRNA, as measured by absolute number of viable cells. However, no antiproliferative effect of the siRNA knockdown of E2F2 could be seen in the ERα negative MDA-MB-231 cells. These cells showed clear reduction of E2F2 mRNA expression, but no significant changes in E2F2 protein levels after 48h possibly explaining the unchanged proliferation rate. In addition, the differences between the ERα positive and negative cell lines were further highlighted when examining E2F target gene expression in MCF-7 and MDA-MB-231 cells. 13 out of the 15 examined genes were downregulated in MCF-7 cells, while only 5 out of 15 were changed in MDA-MB-231 cells. Of the other members of the E2F family, E2F7 was downregulated in MCF-7 and T47D cells, while in MDA-MB-231 cells E2F5 was upregulated after GW3965 treatment.

To determine the clinical relevance of the 83 genes found to be differentially regulated in all four breast cancer cell lines, the expression of these genes was examined in expression data from 258 breast cancer patients from Uppsala, Sweden, previously described by Miller et al. [205]. Hierarchical clustering of breast cancer patients based on the expression profiles of the 60 genes commonly down-regulated after LXR activation showed a strong association of these genes with patient survival. Particularly, a better outcome could be seen in patients with lower expression levels of the down-regulated genes, corresponding to the effect of GW3965 treatment, compared to the group with higher expression of these genes.
4.3 PAPER III: THE OXSTEROL RECEPTORS LXRα AND LXRβ
SUPPRESS PROLIFERATION IN THE COLON

Having observed that LXR activation is antiproliferative in various cell types, shown by us and others, we aimed with this study to examine the antiproliferative role of LXRs in colon cancer cells. We further wanted to make use of LXR deficient mice, produced previously in the lab [58], to determine the \textit{in vivo} role of LXRs in the colon.

Two different human colorectal carcinoma cell lines, Colo205 and HCT116, were used in this study. Both expressed LXRα and LXRβ, although LXRα mRNA expression was significantly higher in both cell lines and Colo205 had overall higher expression of both isoforms. Colo205 and HCT116 also showed functional LXR pathways, with induction of classical LXR target genes upon activation with synthetic LXR agonist GW3965.

LXR activation with GW3965 lead to a dose dependent reduction of proliferation, measured both by flow cytometry and absolute cell number, in Colo205 and HCT116 cells. In agreement with our previous data from the breast cancer cell lines, LXR activation seemed to lead to an arrest of the cells in G1 and G0 phases, with no changes of cell viability, checked by trypan blue staining. The antiproliferative effect could be further boosted by decreasing the serum concentration in the cell growth media, also something we discovered when studying breast cancer cell lines (see Paper I). In the subsequent experiments, we therefore continued to use 1% serum in the treatment media.

To investigate the role of the two different LXR isoforms, a lentiviral shRNA system was used in the Colo205 cells to generate monoclonal cell lines with knockdown of either LXRα (shLXRα) or LXRβ (shLXRβ). A nonspecific shRNA was used as control. By knocking down any of the isoforms, the amount of cells in G1 and G0 phases decreased, showing that both LXRα and LXRβ control cell proliferation in Colo205 cells. However, the effect was more pronounced, and could be seen at an earlier time point, in shLXRβ cells. We continued by treating the cells with GW3965, and once again, the antiproliferative effect of LXR activation could be seen in both knockdown cell types, but the effect was reduced in shLXRβ cells compared to shLXRα and control cells.

To determine the mechanism behind the effect of LXR activation, expression levels of several cell cycle regulators were determined. We observed altered expression of key players in the G1 to S phase transition; CDK4, cyclin E and CDK2 were downregulated at mRNA and/or protein levels, CKI p15 was upregulated and RB protein showed both a reduction of total protein level and a hypophosphorylated status, all consistent with an arrest at the G1/S checkpoint. However, no reduction could be seen on levels of cyclin D1 and CDK4 protein levels. Other changes observed include downregulation of cyclin B1 and CDK1, involved in G2 to M phase of the cell cycle, as well as, as seen in Paper I, a downregulation of Skp2 at both protein and mRNA levels. When examining the
LXR knockdown cells, LXRβ again appeared to have a more pronounced antiproliferative role than LXRα, since shLXRβ cells had a reduced suppression at mRNA level of the cell cycle genes mentioned above. This was especially clear when checking Skp2 protein levels, where the GW3965 induced reduction was abolished in shLXRβ cells. In addition, the shLXRβ cells had a higher basal protein level of Skp2 compared to shLXRα and control cells.

Continuing in vivo, we examined the colon of wild type (WT), LXRα−/−, LXRβ−/− and LXRαβ−/− mice. No differences in colon structure could be seen between the different groups. We continued by investigating the proliferation of the epithelial cells in the crypt of the colon by using immunohistochemistry and staining for proliferation marker Ki67, as well as first pulsing the mice with BrdU and then staining for BrdU positive cells. We found, using both methods, that LXRαβ−/− mice had a significantly higher number of proliferating cells in the crypts of the colon compared to WT controls. No differences could be seen in crypt length between the groups, instead the proliferating zone seemed to extend higher up in the crypt of LXRαβ−/− mice. No significant changes were seen in basal proliferation in LXRα−/− or LXRβ−/− compared to WT animals.

To further confirm the antiproliferative role of LXRs in the colon, WT animals were treated for 13 days with GW3965. Compared to controls treated with vehicle, GW3965 treated animals showed a significant reduction of proliferating cells in the colonic crypts. Short term treatment, for 3 days, did not show any significant changes in proliferation, although there was a trend toward reduced proliferation in WT and LXRα−/− mice, but not in LXRβ−/− mice. Short term GW3965 treatment induced mRNA expression of classical LXR target genes in WT mice. This induction was reduced in LXRα−/− mice and gone in LXRβ−/− mice. No changes could be seen in Skp2 mRNA expression after GW3965 treatment, however interestingly, LXRβ−/− mice had increased basal level of Skp2 compared to WT mice, consistent with what we found in shLXRβ Colo205 cells.

4.4 PAPER IV: THE OXYSTEROL RECEPTORS, LXRα AND LXRβ, PROTECT AGAINST DSS- AND TNBS-INDUCED COLITIS IN MICE

Given the fact that inflammatory bowel disease (IBD), including Crohn’s disease and ulcerative colitis, is closely connected to CRC, and the anti-inflammatory role of LXRs have emerged during recent years, it is interesting to investigate the role of LXRs in the colon during IBD, and more specifically colitis.

In order to study the role of LXRs in colitis in vivo, we used two different mouse models to induce colitis in wild type (WT) and LXR deficient (LXRα−/−, LXRβ−/− and LXRαβ−/−) mice. The first model took advantage of the toxicity of dextran sulphate sodium polymers (DSS) on gut epithelial cells. DSS added to the drinking water induces acute colitis characterized by bloody diarrhoea, ulcerations and inflammatory
infiltration of the colon, first showed in mice by Okayasu et al. [206]. In the second complementary model we used intrarectal administration of inflammatory agent 2,4,6-trinitrobenzenesulfonic acid, TNBS [207, 208]. To monitor the progress of inflammation certain clinical symptoms were observed throughout the study, such as body weight, and appearance of diarrhoea and rectal bleeding.

Using the DSS model for colitis, we investigated both the effect of LXR activation with synthetic LXR agonist GW3965 and the effect on inflammation in LXR deficient mice compared to WT controls. WT mice were pre-treated with GW3965 or vehicle by gavage for four days, thereafter DSS was added to the drinking water to both WT and LXR KO mice for seven to nine days. At day nine, the WT mice on DSS treatment had lost 13% of their body weight, and a slight, but significant, protective effect of the GW3965 treatment could be seen. The LXR deficient mice 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 at day seven.

The more severe phenotype after DSS treatment seen in LXRαβ−/− and LXRβ−/− mice, compared to WT and LXRα−/− mice, was further supported by additional clinical data. These mice showed an earlier onset as well as increased scoring, done by manual inspection, of both diarrhoea and rectal bleeding. In addition, all animals showed reduced colon length after DSS treatment, and this effect was more pronounced in LXRαβ−/− and LXRβ−/− mice. Using the TNBS model, we could further support the more severe effect of DSS treatment in LXRαβ−/− and LXRβ−/− mice compared to WT mice; LXRα−/− mice showed an intermediate phenotype.

Histopathological examination of the colon from all groups showed that DSS treatment induced severe ulceration, disruption of crypt structure and hyperplasia. Even though these effects were apparent in all groups, they were more pronounced in LXRαβ−/− and LXRβ−/− mice, consistent with previous observations. DSS treatment also caused an increased accumulation of neutrophile granulocytes and macrophages in the colon, as examined with immunohistochemistry. The infiltration of macrophages was also increased in LXRαβ−/− mice under basal conditions, compared to WT controls.

We continued investigating if LXRs might have a role in weight recovery after DSS induced colitis. The mice were given DSS in the drinking water until they reached approximately 10% weight reduction, thereafter the DSS was removed and the body weight continued to be monitored. WT mice, both treated with vehicle and GW3965, continued to lose weight for about three more days before recovering, while LXRαβ−/− mice continued to lose weight for about six days before beginning to recoup. The WT mice treated with GW3965 showed a clear trend to recover faster, a statistical significant change when compared to LXRαβ−/− mice. Due to ethical considerations, a cut off point was introduced at which point the mice were euthanised to prevent unnecessary suffering. When comparing the survival rate between the groups, it was
also evident that LXRαβ−/− mice recovered less efficiently while WT mice on GW treatment recovered faster. In addition, WT mice treated with GW3965 had significantly lower immune response compared to controls after DSS treatment, shown by lower mRNA expression of several known proinflammatory genes.

To further study the inflammatory effects of LXR activation, we used a human colorectal adenocarcinoma cell line, Colo205, pretreated with GW3965 and stimulated with TNFα to induce an inflammatory response. Interestingly, LXR activation suppressed the inflammatory response of TNFα stimulation, as judged from mRNA expression of several inflammatory markers. These results were confirmed in a second human colorectal cell line, SW480, using lipopolysaccharide (LPS) or TNFα to stimulate an inflammatory response. Using siRNA technology to knock down LXRβ in SW480 cells, we could show that the anti-inflammatory effect of LXR activation was abolished, suggesting LXRβ to be the most anti-inflammatory LXR isoform in these cells.

We then continued our studies by going back in vivo and inducing an inflammatory response in the mice by intraperitoneal injections of LPS. LPS, an endotoxin from gram-negative bacteria, induces a milder inflammatory response compared to DSS or TNBS, thus preventing unnecessary suffering of the animals. We found a stronger repression of inflammatory genes after GW3965 treatment in the colon from LXRα−/− mice compared to LXRαβ−/− and LXRβ−/− mice, suggesting LXRβ to have a more protective role than LXRα against LPS-induced inflammation. In addition, we found that LXRαβ−/− mice have a longer response to a single inflammatory stimulus by LPS compared to WT mice, with higher mRNA expression of several inflammatory mediators and a larger number of infiltrating CD11+ macrophages.

To connect our results back to inflammation and IBD in humans, we compared the mRNA expression of LXRα and LXRβ in inflamed colon from patients suffering from Crohn’s disease or ulcerative colitis with non-inflamed colon from matched healthy individuals. We found that the expression of both LXR isoforms was significantly suppressed in patients with IBD compared to healthy controls.
5 DISCUSSION

In **Paper I** we showed a strong antiproliferative effect in four different human breast cancer cell lines upon LXR activation with GW3965. In this paper we further demonstrated that the antiproliferative effect did not occur via changes in SREBP1c expression and lipogenesis. Instead we demonstrated changes in several cell cycle regulators. GW3965 activation of LXRs in MCF7 cells reduced mRNA and/or protein expression of Skp2, cyclin A2 and cyclin D1. However, in spite the clear changes of Skp2 levels, we could not see any indications of stabilisation and increased protein levels of the Skp2 target p27. Later studies, especially by Pascual-García *et al.* [175], also showed antiproliferative effects of LXR activation with changes in Skp2 levels without involving p27. The difference in p27 regulation might be due to the use of different synthetic agonists, GW3965 versus T0901317, different doses, or maybe cell lines.

We showed that GW3965 activation of LXR leads to an accumulation of cells in G₁ and G₀ phases of the cell cycle. In addition, we also observed a reduction of cyclin D1 levels and hypophosphorylation of RB. Together this suggests an arrest at the G1/S checkpoint of the cell cycle after LXR activation. This is in line with general antiproliferative effects of LXR activation now seen in a vast array of cell lines. Our results also correlate with what has been shown by others using T0901317 in human breast cancer cell lines [167, 180], as well as when activating LXR in a xenograft mouse model [197].

In this first paper, we also show other effects of GW3965 treatment in MCF7 cells. LXR activation leads to both increased protein levels and increased activity of tumour suppressor p53. In addition, ERα mRNA and protein levels were suppressed upon GW3965 treatment. The changes in ERα levels may be one mechanism behind the cell cycle arrest seen in ERα positive cells, but other pathways need to be considered to explain the effects seen in ERα negative cell lines. We saw no indications that the LXR activation increased apoptosis in these cells (data not shown), something that has been reported in MCF7 cells [209, 210]. The big difference between our study and the later study by El Roz *et al.* is that we used GW3965, while they used T0901317, the endogenous ligand 22(R)-HC or conjugated linoleic acids (CLA). These three latter LXR agonists have the potential to modulate other NRs as well. The difference might therefore be both ligand-specific and dose-dependent.

**Paper II** is a follow up study of Paper I, where we continued working with the same breast cancer cell lines treated with LXR agonist GW3965. Microarray analysis revealed both cell line specific and common LXR target genes. When comparing the common downregulated gene set, which was annotated to processes in cell cycle regulation and proliferation, with breast cancer tumour samples and patient data we found an interesting connection. Patients with tumours expressing lower levels of these LXR target genes had better survival compared to patients with a higher expression of
these genes. In association with disease outcome, the two clusters also varied in ERα and PR expression, as well as lymph node status and tumour grade. This suggests that LXR activation, leading to a downregulation of the genes in question, resulted in the same gene expression profile as patients with better disease outcome.

The second key finding in Paper II was the identification of the family of E2F transcription factors as mediators of the antiproliferative effect of LXR activation. This correlates well with the proposed G1 to S transition cell cycle arrest and hypophosphorylation of RB, which will sequester members of this family. The E2F family is very important for regulation of S phase gene expression, as reported by Blaschke et al., especially for E2F1 in VSMC [66]. They showed that LXR activation suppresses expression of MCM6, an E2F target gene [211], as well as that E2F1 overexpression in coronary artery smooth muscle cells (CASMCs) abrogated the LXR induced cell cycle arrest.

In our study, E2F2 was the only E2F family member that was differentially regulated in all four cell lines after LXR activation. However, there were also changes in some of the other E2Fs. Interestingly, both E2F1, as reported by Blaschke et al. [66], and E2F2, belong to the E2F activators that are maximally expressed in late G1 phase. The role of E2F2 and other E2F family members in the antiproliferative effects of LXR needs to be investigated further, as well as if E2F2 is regulated directly or indirectly by LXR activation.

Further highlighting the role of LXRs as regulators of cell proliferation, we showed in Paper III that activation of LXRs with GW3965 decreased proliferation in human colon cell lines. Interestingly, LXR deficient mice had increased expression of proliferation markers in the colonic crypts. Uno et al. previously showed that T0901317 inhibited proliferation in HCT116 human colon cancer cells by modulating the Wnt/β-catenin signalling pathway [178]. We could not see changes in β-catenin expression in the CRC cell lines after GW3965 treatment, nor in vivo when comparing the colon from WT and LXRαβ−/− mice. Our data therefore suggests that the antiproliferative LXR effects that we report are independent from β-catenin. However, additional experiments are needed to fully understand the effects on Wnt signalling.

Consistent with our findings, and with what has been reported by others, the LXR activation in the human colon cancer cell lines results in a cell cycle arrest in the G1 to S phase transition. We also demonstrated hypophosphorylation of RB, decreased expression of Skp2, as well as changes in other cell cycle regulators. Cell cycle promoters such as CDK1, CDK2, CDK4, cyclin E, cyclin B1 and c-myc were all downregulated, while PKI p15 expression was upregulated. Cyclin B1 and CDK1 are involved in later stages of the cell cycle, but these changes might be secondary and due to the cell cycle arrest.

Studying proliferation in colon cells also gave us the possibility to examine basal proliferation in vivo in WT and LXR deficient mice. We found that LXRαβ−/− mice had
an increased proliferation in the colonic crypt compared to WT mice. Also, activation of LXRα with GW3965 reduced proliferation in the colonic crypt of WT animals. Our results clearly show an in vivo antiproliferative effect of LXR activation in the colon. This has recently been supported by an extensive study from Lo Sasso et al. where they demonstrate that both GW3965 and LXRα specific activation reduced proliferation in human colon cancer cell lines [173]. In addition, they showed that intestinal specific LXRα activation in vivo in mice reduced tumour growth and induced apoptosis in a xenograft model, and resulted in fewer and smaller tumours in two different mouse cancer models. They further showed the importance of lipid and cholesterol metabolism as a factor in LXR-mediated antiproliferative events in CRC. Microarray experiments comparing tumours from the colon, ileum and xenografts from mice overexpressing LXRα with control tumours identified several LXR target genes annotated to metabolism that have previously been associated with the antiproliferative effect of LXRα.

With Paper IV we took a step away from direct regulation of proliferation by LXRα, and investigated one of the major risk factors behind CRC, namely inflammatory bowel disease, IBD. Using two different mouse models of colitis, DSS- and TNBS-induced colitis, we showed that activation of LXRα dampens the inflammatory response by downregulating pro-inflammatory mediators. LXR deficient mice had a faster and more severe disease progression, and recovered more slowly when the inflammatory agent was removed. This suggests that LXRα have a protective role in IBD.

We further demonstrated expression of LXR regulated genes is suppressed in colon samples from patients with either Crohn’s disease or ulcerative colitis compared to healthy colon, supporting the role of LXRα as anti-inflammatory mediators in the colon. Together, the data in Paper IV suggests the potential for LXR mediated inhibition of inflammation during IBD, reducing the risk for developing CRC.

In summary, our studies have strengthened the view of LXRα as modulators of cell proliferation. Activation of LXRα leads to a G1/G0 cell cycle arrest by regulation of key players in the cell cycle. Other reported mechanisms behind the antiproliferative effect of LXRα activation include regulation of cholesterol homeostasis, depriving the fast growing tumour cells vital nutrients, and induction of apoptosis.
6 CONCLUDING REMARKS

The antiproliferative effects of LXRs make them potential targets in cancer therapy, however, many questions still remain. LXRs, like many other NRs, are favourable drug targets since their activity is regulated by small molecular compounds that can relatively easily be delivered. The biggest caveat, so far, in using LXR ligands as therapeutic agents has been the increase of lipogenesis in the liver leading to hypertriglyceridemia and increasing the risk for cardiovascular disease. Finding new ligands in the form of subtype-, tissue- or pathway specific agonists, may be one solution. However, in mortal diseases, such as many cancers, the increased risks associated with induced hepatic lipogenesis could be considered acceptable, if it means a prolongation of life.

Many chronic inflammatory conditions are associated with a significant increased risk of developing cancer, as with CRC and IBD. On one side, using LXR agonists to treat these inflammatory diseases would also, indirectly, reduce the development of certain cancers. On the other side, activating LXRs could disrupt a proper immune response towards the developing tumour via DCs and their migration of lymph nodes and thereby providing an opportunity for the tumour to escape immune surveillance.

An additional point to consider is that much of the published data on LXR activation is performed using the synthetic LXR agonist T0901317. It has been shown that T0901317 can activate other NRs, specifically FXR [36], PXR [37] and RORs [38]. The LXR effect observed in studies where the T0901317 effect has not been shown to be abolished with LXR deficient systems, such as LXR deficient mice or siRNA in cell lines, remains unvalidated and should be confirmed using the GW3965 agonist.

This thesis has focused on the antiproliferative role of LXRs after activation with synthetic agonists. However, studies have also shown that endogenous LXR ligands [212], phytosterols ([213], also reviewed in [214] and [215]) and other ligands from plants [210, 213] may regulate proliferation and/or induce apoptosis in a variety of cell lines and cancer models, possibly via the LXRs. This connects the LXRs to dietary factors for increased or decreased cancer risk. For instance, there might be a link between a diet rich in phytosterols and phytostanols, protection against CRC and LXRs, giving these NRs a role in prevention of cancer and not only as a target for cancer therapy.
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