HUMAN 15-LIPOXYGENASE-1 AND TELOMERASE GENE EXPRESSION IN CANCER AND INFLAMMATION

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ABSTRACT

Human 15-lipoxygenase type 1 (15-LOX-1) is an enzyme that catalyzes the oxygenation of free or membrane-bond polyunsaturated fatty acids containing at least one bis-allylic methylene moiety. The enzyme has been proposed to be involved in various physiological and pathophysiological activities including inflammation, apoptosis, cell maturation and tumorigenesis. However, its expression and function has not been well studied yet. Telomerase, a ribonucleoprotein reverse transcriptase, synthesizes TTAGGG telomeric repeats which are essential for the stability and integrity of chromosomes and is involved in cell transformation and lymphocyte activation during the processes of tumorigenesis and immune response, respectively. In this thesis, the studies aimed at defining the transcriptional regulation of 15-LOX-1 and telomerase. We also delineated the effect of 15-LOX-1 upregulation in lung epithelial cells on chemokine production.

In paper I, we demonstrated that hyper- and hypomethylation of CpG islands in the 15-LOX-1 promoter region are intimately associated with the transcriptional repression and activation of the 15-LOX-1 gene, respectively. Inhibition of DNA methylation in the 15-LOX-1 negative Hodgkin lymphoma (HL) cell line L428 restores the inducibility of 15-LOX-1 by IL-4. Our results suggest that demethylation of the 15-LOX-1 promoter is a prerequisite for gene transactivation, which contributes to tissue- and cell-type-specific regulation of 15-LOX-1 expression. In paper II, we identified that three functional STAT6 binding sites are required for full activation of the 15-LOX-1 promoter in the 15-LOX-1 positive HL cell line L1236. We found that this region was occupied by STAT6 in L1236 cells but not in L428 cells. Furthermore, DNA hypomethylation and histone hyperacetylation were observed within the core promoter region of 15-LOX-1 only in L1236 cells. Our data indicate that STAT6 activation and chromatin remodeling by DNA demethylation and histone acetylation are crucial for transcriptional activation of 15-LOX-1 in cultured HL cells. In paper III, we ectopically expressed 15-LOX-1 in the human lung epithelial cell line A549. We found that over-expression of 15-LOX-1 in A549 cells leads to increased release of the chemokines Mip-1α, RANTES and IP-10, and thereby increases the recruitment of immature dendritic cells, mast cells and activated T cells. Our data suggest that an increased expression of 15-LOX-1 in lung epithelial cells is a pro-inflammatory event in the pathogenesis of asthma and other inflammatory lung disorders. In paper IV, we showed that SET and MYND domain-containing protein 3 (SMYD3) directly trans-activates the human telomerase reverse transcriptase (hTERT) gene. We found that SMYD3 occupies its binding motifs in the hTERT promoter and is required for maintenance of histone H3-K4 trimethylation in this region. Knocking down SMYD3 in some tumor cell lines abolished trimethylation of H3-K4, attenuated the occupancy by the transactivators c-Myc and Sp1, and led to diminished histone H3 acetylation in the hTERT promoter region, which was coupled with down-regulation of hTERT mRNA and telomerase activity. These results suggest that SMYD3-mediated trimethylation of H3-K4 functions as a licensing element for subsequent transcription factors binding to the hTERT promoter.
LIST OF PUBLICATIONS IN THE THESIS


* These authors contributed equally.
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<th>Description</th>
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<tr>
<td>15-LOX-1</td>
<td>15-lipoxygenase-1, 15-lipoxygenase type 1</td>
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<tr>
<td>13-HODE</td>
<td>13-hydroxoyctadecadienoic acid</td>
</tr>
<tr>
<td>15-HETE</td>
<td>15-hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>15-HPETE</td>
<td>15-hydroperoxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>5-LOX</td>
<td>5-lipoxygenase</td>
</tr>
<tr>
<td>azadC</td>
<td>5'-azadeoxycytidine</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
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<tr>
<td>CKR</td>
<td>Chemokine receptor</td>
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<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DiHETE</td>
<td>Dihydroxy eicosatetraenoic acid</td>
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<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>EX</td>
<td>Eoxin</td>
</tr>
<tr>
<td>GCN5</td>
<td>General control nonderepressible 5</td>
</tr>
<tr>
<td>H/RS</td>
<td>Hodgkin and Reed-Sternberg</td>
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<tr>
<td>H3-K4</td>
<td>Histone H3 lysine 4</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HL</td>
<td>Hodgkin lymphoma</td>
</tr>
<tr>
<td>HMT</td>
<td>Histone methyltransferase</td>
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<tr>
<td>hTERC</td>
<td>Human telomerase RNA component</td>
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<tr>
<td>hTERT</td>
<td>Human telomerase reverse transcriptase</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP-10</td>
<td>IFN-gamma-inducible protein 10</td>
</tr>
<tr>
<td>MBD</td>
<td>Methyl-CpG binding domain</td>
</tr>
<tr>
<td>Mip-1α</td>
<td>Macrophage inflammatory protein 1 alpha</td>
</tr>
<tr>
<td>MSP</td>
<td>Methylation-specific PCR</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>PCAF</td>
<td>p300/CBP-associated factor</td>
</tr>
<tr>
<td>PLA₂</td>
<td>Phospholipase A₂</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>PPAR</td>
<td>Peroxisome proliferator activated receptor</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon activation in normal T cells, expressed, and secreted protein</td>
</tr>
<tr>
<td>SMYD3</td>
<td>SET and MYND domain-containing protein 3</td>
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<tr>
<td>STAT6</td>
<td>Signal transducer and activator of transcription 6</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cells</td>
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1 INTRODUCTION

1.1 HODGKIN LYMPHOMA AND HODGKIN/REED-STERNBERG CELLS

Hodgkin lymphoma (HL) is one of the most common malignant disorders among young adults in the Western world and originates from lymphoid tissues. The disease is named after Thomas Hodgkin, who described it in 1832. The symptoms include fever, lymph node enlargement, and weight loss. In later stages the nodes become rubbery and the spleen and liver become enlarged. Approximately 200 new cases of HL are diagnosed in Sweden each year and the incidence of HL has increased among adolescents and young adults in the Nordic countries over the last decades.

The Hodgkin/Reed-Sternberg cells (H/RS), which are considered as the hallmark of HL, are named after Dorothy Reed and Carl Sternberg. The origin of H/RS cells has been debated for many decades and a number of recent studies strongly support that the large majority of tumors of classical HL and lymphocyte predominant (LP) subtypes are monoclonal and originate from germinal centre (GC) B cells. Based on single cell analysis, it appears that H/RS cells in classical HL are derived from crippled GC B cells, and H/RS cells in LP HL are derived from antigen-selected GC B cells.

1.2 15-LIPOXYGENASE-1 (15-LOX-1)

1.2.1 Identification, structure and biochemistry

15-LOX-1 catalyzes the oxygenation of free or membrane-bond polyunsaturated fatty acids containing at least one bis-allylic methylene moiety. Fluorescent in situ hybridization (FISH) indicated localization of a human lipoxygenase gene on chromosome 17p13.3, close to the tumor suppressor gene p53. Some 15-LOX-1 metabolites exhibit bioactivities and are involved in signal transduction in physiological or pathophysiological immune response.

Human 15-LOX-1 is a 75 kDa protein and consists of a single polypeptide chain. The crystal structure of rabbit 12/15-lipoxygenase, which shares 81% amino acid identity...
to human 15-LOX-1\textsuperscript{10} has been solved\textsuperscript{11}. It consists of two domains: the large C-terminal domain is believed to be the catalytic domain and essential for enzymatic activity; the catalytic non-heme iron is buried deeply inside and shuttles between the active ferric and inactive ferrous forms during the catalytic cycles\textsuperscript{7}; the small N-terminal domain is involved in membrane binding\textsuperscript{12}.

1.2.2 Tissue specific expression of 15-LOX-1

In humans, expression of 15-LOX-1 is observed predominantly in eosinophils, airway epithelial cells, macrophages, dendritic cells and mast cells\textsuperscript{13,14}. The enzyme is also expressed in reticulocytes during the red blood cell development\textsuperscript{15}. 15-LOX-1 expression was reported in normal colorectal and pancreatic tissue while abolished in the corresponding tumor tissue\textsuperscript{16,17}. Several studies have shown that 15-LOX-1 is present at higher levels in prostate cancer tissue when compared with the normal surrounding tissue\textsuperscript{18}.

Peripheral blood monocytes do not have endogenous 15-LOX-1 expression in circulation but its induction can be achieved in monocytes and lung epithelial carcinoma cells A549 when treated by IL-4 or IL-13 \textit{in vitro}\textsuperscript{19,20}. But not all the cells with functional IL-4/13 receptor have inducible 15-LOX-1 expression\textsuperscript{21}, indicating additional mechanism(s) transcriptionally controlling tissue- and cell-type-specific expression of 15-LOX-1.

1.2.3 Regulation of expression

Since expression of 15-LOX-1 in cells could result in oxidative stress and membrane degradation, the expression and activity of the enzyme should be under serious control\textsuperscript{7,22}. Because of the physiological importance of 15-LOX-1 in multiple biological processes, the study of its expression regulation has developed into a major field in lipoxygenase research. In mammalian cells, lipoxygenases are regulated by transcriptional and post-transcriptional processes.
Transcriptional regulation

The 15-LOX-1 promoter is rich of GC, and absent of typical TATA or CAAT motifs. Transcription of the gene is initiated at one major site. IL-4 induces transcription of 15-LOX-1 in human airway epithelial cells A549 and monocytes. The 15-LOX-1 promoter also contains a binding site for the Ku auto antigen, which is involved in IL-13/-4-induced 15-LOX-1 expression in A549 cells. The promoter also exhibits putative binding sites for the transcription factors SP1, AP2, NF1 and GATA-1. In addition, the transcription of 15-LOX-1 in colorectal cancer cells can be inhibited by over-expressed transcriptional regulatory protein GATA-617.

IL-4/-13-induced 15-LOX-1 expression through the signal transducer and activator of transcription-6 (STAT6) cascade is the best studied transcriptional activation pathway of the enzyme. This transcription factor has been shown to be constitutively activated in primary H/RS cells as well as in HL cell lines, most likely due to autocrine secretion of IL-1324,25. STAT6 has also been suggested as a candidate gene implicated in the pathogenesis of asthma and prostate cancer, which diseases are associated with 15-LOX-1 deregulation26.

The binding of the IL-4/13 to their cognate receptors leads to the phosphorylation and activation of JANUS kinases, which are believed to be attached to the cytoplasmic tail of the receptor in the unactivated cells. The JANUS kinase then phosphorylates tyrosine residues on the IL-4 receptor which provides docking sites for STAT6. Once STAT6 has bound to the receptor, the transcription factor turns phosphorylated17. In addition, the phosphorylated transcription factor is acetylated by histone acetyltransferases CBP/p300. The phosphorylation and acetylation of STAT6 leads to dimer formation and translocation into the nucleus, where the active STAT6 dimers interact with the 15-LOX-1 promoter and activate gene transcription.

15-LOX-1 expression is also associated with the CpG island methylation status and the histone acetylation level in the promoter region. It was found that DNA hypermethylation is associated with silenced 15-LOX-1 transcription and that demethylation is required for 15-LOX-1 transactivation. However, it was recently reported that hypermethylation of the 15-LOX-1 promoter leads to upregulation of 15-LOX-1 expression and enzyme activity in prostate cancer cells. More confusingly, a very
recent study on colorectal cancer cells showed that 15-LOX-1 transcription can be silenced by DNA methyltransferase (DNMT)-1 independently of DNA methylation. It has been found that 15-LOX-1 expression could be induced by histone deacetylase (HDAC) inhibitors in A549 cells. Further studies showed a synergetic effect of DNA methyltransferase (DNMT) inhibition and HDAC inhibition on 15-LOX-1 expression.

Translational regulation

In young rabbit reticulocytes 12/15-lipoxygenase mRNA is accumulated in the cytoplasm but no protein is present. The repetitive sequence in the 3’-untranslated region of the 12/15-LOX mRNA, named differentiation control elements (DICE), is occupied by two regulatory proteins called hnRNP K and hnRNP E1 which inhibit 80s ribosome assembly on the 15-LOX-1 mRNA. During the late stage of red blood cell maturation, these two proteins are degraded and 15-LOX-1 protein is synthesized. A similar control mechanism could also happen in human cells. In some cases, 15-LOX-1 expression and activity are not detectable although 15-LOX-1 mRNA is present.

Metabolism of arachidonic acid

Arachidonic acid is found esterified to membrane phospholipids in mammalian cells. The release of arachidonic acid is principally initiated by the action of phospholipase A2 (PLA2), which cleaves arachidonic acid from membrane phospholipids. The liberated arachidonic acid can be metabolized to either prostaglandins, tromboxane A2 or leukotrienes, collectively called eicosanoids, depending on the cell type and physiological stimulus (Fig. 1). Cyclooxygenase (COX) catalyzes the first step in the metabolism of arachidonic acid to prostaglandins and thromboxane A2. Two forms of the enzyme exist, named COX-1 and COX-2. The key enzyme in leukotriene synthesis is 5-lipoxygenase (5-LOX). Only recently it was reported that 15-LOX-1 can catalyse the conversion of arachidonic acid to eoxins in human eosinophils and mast cells. Eoxins induce increased permeability of endothelial cell monolayer in vitro, indicating that eoxins can modulate and enhance vascular permeability, a hallmark of inflammation. The enzyme 15-LOX-1 catalyses the conversion of arachidonic acid to 15-hydroperoxy-eicosatetraenoic acid (15-HPETE) and the subsequent formation of
Eoxin (EX) A₄, also named 14,15-leukotriene A₄. This metabolite can be conjugated with glutathione, leading to the formation of EXC₄ which in turn can be further metabolised to EXD₄ and EXE₄ (Fig. 1). Reduction of 15-HPETE leads to the formation of 15-HETE, and in addition to be converted to 15-HETE and EXA₄, 15-HPETE can also (as well as EXA₄) be converted to various dihydroxy acids (Fig. 1)⁹. Linoleic acid is also readily converted to 13-hydroxy-octadecadienoic acid (13-HODE) via the 15-LOX-1 pathway⁹.

1.2.4 Physiological functions

Inflammation

15-LOX-1 products are involved in the immune response through different pathways. 15-LOX-1 expression is induced by the T helper (Th) 2 cytokines IL-4 and IL-13. 12/15-LOX knock-out mice showed decreased IL-12 and IL-23 production by
peritoneal macrophages in response to LPS challenge, indicating that the enzyme is involved in the reciprocal influence and coordinate regulation of type I and II immune responses. Initially this enzyme was considered as a pro-inflammatory factor: in human polymorphonuclear leukocytes, 13-HODE, a 15-LOX-1 metabolite from linoleic acid, induces chemotaxis at sub-micromolar concentrations; in asthmatic bronchitis elevated 15-HETE (a 15-LOX-1 metabolite from arachidonic acid) levels were found; the 15-LOX-1 metabolite 15-HPETE was reported to induce inflammation in rabbit skin; 15-LOX-1 expression and activity are found in human lung fibrosis. Later on, several lines of experimental evidences suggested that 15-LOX-1 could exert an anti-inflammatory effect on immune reactions: 15-HETE inhibits the activity of 5-LOX, attenuating leukotriene production by neutrophils; in addition, 15-LOX-1 can be involved in the formation of lipoxins which exhibit putative anti-inflammatory properties. Generally, the immune function of 15-LOX-1 is dependent on cell type and its microenvironment; conclusions from in vitro studies should be reviewed with caution.

**Cell differentiation and maturation**

Several lines of evidence suggested that 15-LOX-1 could be involved in the maturation of red blood cells through the degradation of mitochondrial membranes in late erythropoiesis. It was reported that 15-LOX-1 could integrate into the membranes of different organelles, allowing release of cell contents; inhibition of 15-LOX-1 leads to delayed organelle degradation in the reticulocytes. But this hypothesis is not supported by experiments in a transgenic animal model: even in 12/15-LOX knockout mice with induced anemia, defects in erythropoiesis were not observed.

**Cell proliferation and apoptosis**

Several in vitro and xenograft animal studies suggest that 15-LOX-1 expression inhibits proliferation and induce apoptosis. It was reported that ectopical expression of 15-LOX-1 or exogenous 13-HODE induced apoptosis and cell cycle arrest in colorectal cancer cells, nonsteroidal anti-inflammatory drugs can reestablish apoptosis in colonic, esophageal, and gastric cancer cells through restoring 15-LOX-1 expression, the ectopic expression of 15-LOX-1 inhibited osteosarcoma cell proliferation and promoted apoptosis in transformed 293 kidney cells. In contrast,
several studies suggest that 15-LOX-1 and 13-HODE play a pro-tumorigenic role in the development of prostate cancer by inducing cell growth and inhibiting apoptosis\textsuperscript{18,46}.

The underlying mechanisms through which 15-LOX-1 regulates apoptosis and cell proliferation have not been well studied yet. The possible mechanisms could be: (1) introduction of 15-LOX-1 can lead to oxidative stress\textsuperscript{47}; (2) 15-LOX-1 induces growth arrest through protein kinase-dependent pathways and phosphorylation of p53\textsuperscript{48}; (3) 15-LOX-1-derived oxidative metabolites of linoleic and arachidonic acid are natural ligands of peroxisome proliferator-activated receptors (PPARs) which can regulate cell proliferation and apoptosis\textsuperscript{43,49}; 15-LOX-1-derived oxidative metabolites of linoleic acid inhibit PPAR\textsubscript{γ} activity via MAP kinase phosphorylation\textsuperscript{50}.

### 1.2.5 15-LOX-1 and diseases

15-LOX-1 has been shown to be involved in the pathogenesis of various diseases, including cancer\textsuperscript{13}, asthma\textsuperscript{26}, orbital fibrosis\textsuperscript{51}, arteriosclerosis\textsuperscript{52} and nephritis\textsuperscript{53}.

### 15-LOX-1 and cancer

15-LOX-1 deregulation is implicated in several forms of cancer, including prostate cancer, colorectal cancer, breast cancer and pancreatic cancer\textsuperscript{54}. Its abnormal expression and effects on tumorigenesis seem to be tissue-dependent. The mechanisms by which 15-LOX-1 exerts its protumorigenic or antitumorigenic effects on cells have not been well studied yet, but may relate to various aspects of cancer development such as apoptosis, angiogenesis, signal transduction, oxidative stress and metastasis\textsuperscript{54}.

15-LOX-1 is expressed at high levels in prostate cancer tissue compared with the normal surrounding tissue. In prostate cancer cells, 15-LOX-1 could be induced by an oncogenic p53 mutant form\textsuperscript{55}. The enzyme could exert pro-tumorigenic effects by up-regulating the MAP kinase pathway and disrupting the balance of Bcl-family members across mitochondria membrane through its main metabolite 13-HODE\textsuperscript{18}. 15-LOX-1 also contributes to prostate cancer bone metastasis by increasing insulin-like growth factor-1 receptor (IGFR1) expression\textsuperscript{56}. Taken together, 15-LOX-1
expression and activity could exert protumorigenic effects during the development of prostate cancer. In contrast, a majority of studies demonstrated that in colorectal and pancreatic cancer, 15-LOX-1 expression is reduced or abolished in malignant tissues compared with normal surrounding tissues, and overexpression of the gene inhibits cancer cell growth\textsuperscript{16,39,57}.

**15-LOX-1 and chronic lung inflammatory diseases**

Asthma is a chronic inflammatory disease characterized by airway hyperreactivity, mucus hypersecretion and airflow obstruction. It was found that 15-LOX-1 protein is present at higher levels in bronchial epithelial cells of asthma and chronic bronchitis patients\textsuperscript{58}. A microarray study based on bronchial biopsies demonstrated that 15-LOX-1 has a higher expression level in asthma patients compared with healthy control subjects and could be reduced after treatment with inhaled corticosteroids\textsuperscript{59}.

The effect of 15-LOX-1 upregulation in the airway of asthma subjects has been controversial for a long time. Some studies suggest that 15-LOX-1 plays an anti-inflammatory role in the pathogenesis of asthma: inhalation of 15-HETE reduced airway responsiveness to histamine and methacholine\textsuperscript{60}; animal experiments showed that airways of 12/15-LOX-deficient mice had more pronounced inflammatory responses than those of control mice\textsuperscript{61}. In contrast, other evidence indicate that 15-LOX-1 is a pro-inflammatory enzyme in asthma pathogenesis: it was found that 15-HETE levels in bronchoalveolar lavage fluid (BAL) were significantly higher in severe asthmatics compared with normal controls\textsuperscript{61}; 15-LOX-1 expression is higher in the airway mucosa of smokers with chronic bronchitis than in healthy nonsmokers\textsuperscript{62}. It is important to notice that in some studies, very high nonphysiologic concentrations of 15-LOX-1 metabolites were used to obtain a measured response, and these conclusions should be viewed with caution.

### 1.3 Epigenetic Regulation of Gene Transcription

Epigenetics is the study of heritable changes in gene function that occur without a change in the sequence of the DNA. These changes of DNA environment are important for diverse biological processes including gene transcription, X-chromosome inactivation, DNA damage repair, telomere function and DNA
recombination. Epigenetic changes encompass modifications of DNA or its associated proteins, especially the histones. Major epigenetic mechanisms include DNA methylation, histone methylation, acetylation and phosphorylation. These epigenetic modifications interact with each other and control different biological processes together with the genetic code of the cells. The epigenetic changes can take place at different ranges, from a specific nuclear acid to a whole chromosome. Disruption of epigenetic regulation is involved in the pathogenesis of cancer and other diseases.

1.3.1 CpG island methylation

DNA methylation is a biochemical modification of DNA in which a methyl group (CH3-) is enzymatically added to the 5-carbon of cytosine within 5'-CpG-3' dinucleotides (Fig. 2). The reaction is catalyzed by members of the DNA DNMT family, which contain DNMT1 (responsible for DNA methylation maintenance during cell division, development and cancer), DNMT3a, and DNMT3b (responsible for de novo methylation during early development). Around 10-15% of the CpG dinucleotides in mammals show up in CpG islands. The CpG islands, regions with at least hundred base pairs and rich of cytosine-guanosine dinucleotides (CpGs), are localized in the promoter regions of around 60% of genes in humans and are normally maintained in the non-methylated form. It is well established that the CpG island methylation status of gene promoters correlates with gene transcription levels. In most of cases, CpG island hyper-methylation is associated with silenced gene expression; active gene transcription is associated with a less methylated promoter.

Two mechanisms could be involved in DNA methylation-mediated gene transcriptional regulation: (1) In rare cases, the methyl group which is covalently bound to cytosine could prevent the interaction between transcription factor/s and the binding motif at the promoter region; (2) More commonly, methylated cytosine can
serve as a binding platform for specific proteins containing a specific binding domain, the so-called methyl-CpG binding domain (MBD). Some of these proteins are a part of chromatin-modifying complexes which comprise histone modification enzyme/s and could regulate accessibility of transcription activators to promoters\(^{63,68}\).

The 15-LOX-1 promoter region is GC-rich and apparently belongs to the CpG islands\(^8\). It was reported that demethylation of the 15-LOX-1 promoter is required for transcriptional activation of the 15-LOX-1 gene in diverse human primary cells and cancer cell lines and that the HDAC inhibitor 5'-azadecytidine (azadC) induces 15-LOX-1 expression in human colorectal cancer cells\(^{21,71}\). Paradoxically, it was found that hyper-methylation of the 15-LOX-1 promoter is associated with the upregulation of 15-LOX-1 expression and enzyme activity in prostate cancer cells and tumor tissues\(^{28}\).

### 1.3.2 Histone modifications

Histones are the basic scaffolding proteins that organize genomic DNA into chromatin in eukaryotic cells. The nucleosome is the basic unit of this organization and comprise 147 base pairs of DNA superhelically wrapped around a histone octamer composed of two copies each of the core histones H2A, H2B, H3 and H4\(^{72}\). Each histone has a 20–40 residue segment that extends from the surface of the nucleosome and can undergo a plethora of post-translational modifications including acetylation, phosphorylation, methylation, ubiquitination and sumoylation\(^{73}\). These ‘tails’ play an essential role in controlling the folding of nucleosomal arrays into higher-order structures by different patterns of modification and is believed to closely correlate with gene transcriptional activity\(^{73}\). Distinct chemical modifications at different amino acids in histone tails can generate synergistic or antagonistic interaction affinities for proteins interacting with chromatin and in turn regulate gene expression. The combination of histone modifications may work as a marking system that is read by regulatory machinery. This is the hypothesis of the so-called ‘histone code’\(^{74}\).

Among the diverse histone covalent modifications, lysine acetylation and methylation have been under intensive investigation and are highly associated with transcription activity.
**Histone acetylation**

The histone’s N-terminal lysines undergo reversible acetylation/deacetylation switches which are achieved through the action of histone acetyltransferases (HATs) and HDACs respectively\(^{72}\). Acetylated histones are usually associated with active promoter activity which is permissive for transcription activators to interact with binding motifs; in contrast, hypo-acetylated histones correlate with a silenced promoter and repressed gene transcription. Several experimental studies showed that around 2% of all human genes including 15-LOX-1 and human telomerase reverse transcriptase (hTERT) are subject of transcriptional regulation by histone acetylation in different primary and transformed cells\(^{21,27,75-77}\).

Several mechanisms are considered to be involved in the regulation of gene transcription by histone acetylation/deacetylation switch. A covalent modification by an acetyl group could neutralize the positive charge of histone tails, reduce the affinity between adjacent histones and affect interaction between lysine residue and negatively charged DNA. Acetylation also renders the histone less prone to form hydrogen bonds, inhibits histone oligomerization and consequently leads to looser chromatin architecture. In addition, conjunction of an acetyl group to a lysine residue could form a new specific docking site for proteins. For example, bromodomains of chromatin remodeling proteins general control nonderepressible 5 (GCN5) and p300/CBP-associated factor (PCAF) are able to recognize acetyl lysine residues of histones\(^{78,79}\).

**Histone methylation**

Lysine is the key substrate residue in histone methylation; arginine could also be methylated as well. The methylation of a single lysine residue can occur one, two or three times (mono-, di- or trimethylation), which leads to different biological outcomes (Fig. 3). Histone methylation could have various effects on gene transcription, depending on the precise residues and levels of methylation\(^{80}\). Generally, Histone 3 lysine 4 (H3-K4), H3-K36, and H3-K39 hyper-methylation are associated with an active transcription status; H3-K9 and H3-K27 hyper-methylation has a repressive effect on gene expression\(^{80,81}\).
The histone methylation status of a specific residue is an outcome of a dynamic balance between corresponding histone methyltransferases (HMTs) and histone demethylases. The dynamic regulation is also affected by the mutual interaction with other forms of chromatin modifications.

Unlike acetyl groups, methyl groups are not electrically charged. Adding methyl groups could therefore not affect chromatin structure by directly neutralizing the positive charge of the histones. Histone methylation could exert its transcriptional regulation function by recruiting multiple effector proteins. For example, the chromodomain of the chromatin remodeling protein Chd1 binds to methylated H3-K4, recruiting the yeast SAGA (Spt-Ada-Gcn5 acetyltransferase) complex which contains the H3 acetyltransferase GCN5.

Among the diverse HMSs, SET and MYND domain-containing protein 3 (SMYD3) contains a SET domain and has histone H3-K4 di- or tri-methyltransferase activity. SMYD3 is also a transcription factor that specifically interacts with its binding motif in the target genes. Endogenous expression of SMYD3 is undetectable or very weak in most normal human tissues whereas significant up-regulation was observed in the great majority of investigated colorectal carcinoma, hepatocellular carcinoma, and breast cancer specimens. SMYD3 is considered as an oncogene that exerts its carcinogenic function through up-regulation of its pro-tumorigenic target genes by methylating histone H3-K4.
1.4 ASTHMA

Asthma is a major public health problem. The prevalence of asthma in 1990s was about 5-9% in western European countries and has considerably increased over the past five decades\textsuperscript{87}. The disease has been defined as a chronic inflammatory disorder of the airways and is characterized by infiltration of inflammatory cells and the clinical development of wheezing, shortness of breath, chest tightness, and cough\textsuperscript{87,88}.

Allergic asthma is featured by intermittent airway obstruction which could cause difficulty in breathing. This is mediated by hyperresponsive bronchial smooth muscle, secreted airway glycoproteins and inflammatory debris\textsuperscript{89}. Asthma belongs to the so-called ‘Th2-type’ immune diseases which means that cytokines secreted by Th2 cells, including IL-4, IL-5, IL-9 and IL-13, predominantly contribute to the pathogenesis of the immune disease\textsuperscript{90}.

The Th2 dependent allergic reaction could be mediated by immunoglobulin E (IgE) which is secreted by B cells activated by IL-4 or IL-13\textsuperscript{91}. The interaction between IgE and its receptors present on effector cells such as eosinophils, mast cells and basophils leads to cellular activation, degranulation and release of diverse inflammatory molecules including leukotrienes and other lipid mediators of inflammation. These lipid inflammatory mediators play very important roles in airway hyperresponsiveness, eosinophilia and airway hypersecretion through a variety of G-protein-coupled receptors\textsuperscript{92}. In addition, IL-4 and IL-13 could directly elicit airway hyperreactivity through IL-4 receptors present on airway smooth muscle and epithelium cells. Activated IL-4 receptors could exert their inflammatory effects by inducing Janus kinases and STAT6 cascades and activating downstream gene expression\textsuperscript{93}.

1.5 CHEMOKINES AND CELL MIGRATION

Chemokines represent a large family of 8–15 kDa, predominantly secreted, proteins with essential roles in immune responses. They exert their function through a cluster of receptors which belong to the G-protein coupled receptor family\textsuperscript{94}. According to the number and spacing of the first two N-terminal cysteines in a peptide sequence, chemokines are classified into four groups: the main -CC- and -CXC- groups and the less described -C- and -CX3C- groups\textsuperscript{95}. Chemokine receptors (CKR) are G-protein–
coupled receptors and also divided into four groups according to the spectrum of chemokines they associate with\textsuperscript{96}. Most CKR recognize several chemokines but some are relatively monogamous\textsuperscript{97}.

Chemokines mediate a wide array of effects on the behavior of cells expressing their receptors, including chemotaxis, morphology change, proliferation, adhesion and release of cell components\textsuperscript{96,98-100}. The most important effect brought about by all chemokines involves the migration of cells toward areas with higher concentrations of the chemokine. Chemokines could be immobilized on negatively charged glycosaminoglycans of cells or extracellular matrix surfaces and thus direct the migration of cells along surfaces\textsuperscript{101}.

\subsection*{1.5.1 Mip-1α}

Mip-1α (Macrophage inflammatory protein-1, CCL3) belongs to the C-C chemokine family and exerts its function through the receptors CCR1, CCR3 or CCR5\textsuperscript{102}. Mip-1α is associated with an acute inflammatory state in the recruitment and activation of polymorphonuclear leukocytes and is involved in cell adhesion and migration\textsuperscript{103,104}. Receptors for Mip-1α are expressed in a majority of inflammatory cells including T-cells, dendritic cells (DC), eosinophils, macrophages and mast cells. It has been well established that Mip-1α induces chemotaxis and transendothelial migration of T-cells, dendritic cells and neutrophils\textsuperscript{104}. Mip-1α also plays an important role in mast cell degranulation, which is highly involved in the pathogenesis of asthma\textsuperscript{8}.

\subsection*{1.5.2 RANTES}

RANTES (Regulated on activation, normally T-cell expressed and secreted) is one of several C-C cytokines with genes clustered on the long-arm of chromosome 17. It is involved in Th1 and Th2 immune responses through CCR1/3/4/5 expressed by leukocytes\textsuperscript{94}. RANTES production, which is generated mainly by CD8 T cells, epithelial cells, fibroblasts and platelets, is a particular feature of inflammation. It was reported that RANTES plays a critical role in recruitment, activation and eventually degranulation of eosinophils which are highly involved in asthma\textsuperscript{105}. Several lines of evidence suggest that RANTES also contributes to the infiltration of monocytes, mast
cells, T-cells and dendritic cells in inflamed tissues of immune diseases, including arteriosclerosis, arthritis, asthma, atopic dermatitis and nephritis\textsuperscript{106-108}.

\subsection*{1.5.3 IP-10}

IP-10 (IFN-gamma-inducible protein 10, CXCL10) is a secreted C-X-C chemokine of 10 kDa that was first identified as an early response gene induced after IFN-\(\gamma\) treatment in a variety of cells\textsuperscript{109,110}. It was found that the IP-10 receptor CXCR3 is the most abundantly expressed chemokine receptor on mast cells and smooth muscle cells in the airway of asthma patients\textsuperscript{111}. Human lung mast cell migration can be induced by airway smooth muscle cells predominantly via activation of CXCR3. IP-10 was expressed preferentially by asthmatic airway smooth muscle cells in bronchial biopsies and \textit{in vitro} cultured cells of asthma patients compared with those from healthy controls\textsuperscript{111}. These studies indicate that inhibition of the CXCL10/CXCR3 axis could provide a novel approach for targeted therapy of asthma\textsuperscript{111}. IP-10 can also induce T-cell migration and plays important roles in diverse host defense immune reactions\textsuperscript{112,113}.

\subsection*{1.6 TELOMERE AND TELOMERASE}

\subsubsection*{1.6.1 Telomere}

The human telomere is a TTAGGG repetitive DNA array with associated proteins (e.g. TRF1, TRF2 and hPOT1) that cap the end of chromosomes\textsuperscript{114}. It has been suggested that the telomere ends in a large loop where the TTAGGG 3’ overhang invades into the duplex telomeric DNA, displaces one strand and hybridizes to the complementary strand, thereby forming a structure named t-loop. The telomere protects the end of the chromosome from damage, erosion or fusion\textsuperscript{114-116}. Unprotected DNA ends could be regarded as double-stranded breaks that lead to DNA damage responses including cell cycle arrest and senescence\textsuperscript{117}.

\subsubsection*{1.6.2 Identification, expression and function}

Human telomerase is a reverse transcriptase complex which consists of two essential subunits: a 127 kDa protein named human telomerase reverse transcriptase (hTERT) and a 451 nucleotide RNA subunit named human telomerase RNA component
In most human somatic cells without enough telomerase activity, telomere gets eroded as cell grows due to incomplete DNA replication by DNA-dependent DNA polymerases (the so called ‘end-replication problem) and the digestion by nucleic enzymes. Telomerase exerts its telomere elongation function by hTERT with reverse transcriptase activity using hTERC as template. Telomerase is also suggested as a cap of the telomere 3’ overhang to keep the structure of the telomere without the requirement of reverse transcriptase activity.

In most human somatic cells, the expression and activity of telomerase are very low or undetectable; in reproductive cells, tumor cells or immortalized cell lines, telomerase is frequently upregulated, endowing these cells with the ability of endless replication. The relative specific expression and critical functions of telomerase in tumor cells make it as a promising target for cancer therapy.

### 1.6.3 Regulation of telomerase activity

In most human cells, hTERC is widely present; hTERT is the rate-limiting factor for assembly of the functional telomerase complex. hTERT expression is mainly regulated at the level of transcription. It could also be regulated at the post-transcription level, such as protein phosphorylation, differential splicing or sub-cellular localization.

The hTERT promoter has no TATA or CAAT boxes but is highly GC rich and the transcription start sites lies at 60 to 120 bp upstream of the translational start site. The hTERT promoter contains several binding sites for transcription factors including two E-boxes and several SP1 binding sites between them. Intensive studies during the last decade showed that hTERT expression is subject to multiple levels of control by different transcription factors in different cell types. hTERT transcription is also subject to epigenetic regulation. Histone acetylation and methylation pattern is highly related to hTERT promoter activity. However, the CpG island methylation is not responsible for repressing hTERT expression in most telomerase-negative cells.
2 MATERIALS AND METHODS

2.1 MATERIALS

The cell lines used were the following: Human lung epithelial carcinoma cell line A549; mixed cellularity HL cell line L1236; nodular sclerosis HL cell line L428; Burkitt’s lymphoma cell line BL41; histocytic lymphoma cell line U937; epidermoid cancer cell line A431; cervical carcinoma cell line HeLa; osteosarcoma cell line Saos2; colorectal carcinoma cell line HCT116; hepatocellular carcinoma cell line Hep3B; human normal foreskin fibroblasts BJ; and lung fetal fibroblasts LF1.

Human primary monocytes and T lymphocytes were isolated from buffy-coated blood of healthy individuals. Mast cells derived from cord blood of healthy donors were generous gifts from Prof. Gunnar Nilsson at Karolinska Institutet (Sweden).

2.2 METHODS FOR STUDIES OF GENE EXPRESSION AND PROTEIN ACTIVITY

2.2.1 Quantitative determination of mRNA by real time PCR

Real time PCR is a technique to determine the abundance of mRNA transcripts. The experiment procedure starts with total RNA isolation, followed by cDNA synthesis by reverse transcription. Real time PCR was performed in an ABI 7700 sequence detector using Pre-made Gene Expression Assays primers and probes (Applied Biosystems, Foster City, CA). Levels of transcripts were expressed as the ratio versus human Beta-2 Microglobulin.

2.2.2 Western blot

Western blot was recruited to detect specific gene expression at the protein level. In our studies, protein extracts from whole cell lysates were separated electrophoretically on SDS polyacrylamid gels and subsequently transferred to nitrocellulose membranes. The membranes were probed with antibodies against the specific proteins followed by anti-mouse horseradish peroxidase–conjugated IgG and developed with the enhanced chemiluminescent method. The signal of β-actin was used as a loading control.
2.2.3 Analysis of 15-LOX-1 activity

HPLC and ELISA were employed to measure the catalytic activity of 15-LOX-1 based on the measurement of 15-LOX-1 metabolites from exogenous arachidonic acid. The details of 15-LOX-1 activity assay are given in Paper III.

2.2.4 Telomerase activity assay

Telomerase activity assay

Telomerase activity assay

TeloTAGGG Telomerase PCR ELISA Kit (Roche, Basel, Switzerland) based on telomeric repeat amplification protocol was used to determine telomerase activity in cell lysates. Briefly, a sample that contains telomerase will add these repetitive sequences to the 3'-end of the biotinylated synthetic P1-TS-primer. In a second step, these elongated products are amplified by PCR using the primers P1-TS and P2, generating PCR amplicons. An aliquot of the PCR is denatured and hybridized to a digoxigenin (DIG)-labeled, telomeric repeat-specific detection probe. The hybridization products are immobilized via the biotin-labeled primer to a streptavidin-coated microplate. The immobilized PCR product is detected with an anti-digoxigenin antibody conjugated to peroxidase. The probe is finally visualized by peroxidase, which metabolizes TMB to form a colored reaction product.

2.3 METHODS FOR STUDIES ON TRANSCRIPTIONAL REGULATION

2.3.1 Electrophoretic mobility shift assay (EMSA)

EMSA is a procedure used to determine the physical binding between a DNA fragment and protein in cells in vitro. In our study, EMSA was performed to investigate the association between the transcription factor STAT6 and its potential STAT6 binding motifs in the 15-LOX-1 promoter in nuclear extracts. Briefly, the probes were generated by P32 end-labelling of purified double stranded DNA oligonucleotides containing the potential STAT6 binding element. After incubation with nuclear extract of L1236 cells, the reaction mixture was electrophoretically separated on a polyacrylamide gel. To determine the specificity of the complex, a STAT6 antibody was added to the reaction mixture in a separate tube.
2.3.2 Chromatin immunoprecipitation (ChIP)

ChIP is a method for investigating the physical interaction of nuclear acids and protein in vivo. We used this procedure to determine the association between gene promoters and transcription factor/s or modified histones in living cells. Briefly, cells in culture were cross-linked by incubating them in 1% (v/v) formaldehyde-containing medium for 10 min at 37 °C and then sonicated to make soluble chromatin fragments between 200 and 1,000 bp. Antibodies against transcription factors or specifically modified histones were used to precipitate DNA fragments bound by their corresponding elements. The protein-DNA complex was collected with protein A or G sepharose beads, eluted, and reverse cross-linked. Following treatment with Protease K, the samples were extracted with phenol-chloroform and precipitated with ethanol. The recovered DNA was resuspended in TE buffer and used for PCR amplification.

2.3.3 Methylation-specific PCR (MSP)

MSP based on the bisulfite reaction is the most widely used method to determine the methylation status of genomic DNA. Sodium bisulfite can convert cytosine in genomic DNA into uracil through steps of sulfonation, hydrolytic deamination and subsequent desulfonation with alkali. However, methylated cytosine is protected from the bisulfite reaction because of the presence of the methyl group which blocks the sulfonation by bisulfite. Following bisulfite treatment, PCR is carried out using primers which could distinguish each modified strand of DNA, leading 5-methylcytosine substituted by a cytosine and unmethylated cytosine replaced by a thymine.

2.3.4 Analysis of promoter activity by luciferase activity assay

The luciferase reporter system provides a basis for the quantitative analysis of cis- or trans-acting elements that potentially regulate mammalian gene expression. The backbone of the pGL3 luciferase reporter vector contains a modified coding region for firefly luciferase which is driven by a fragment of the promoter to be studied. After the reporter vector is transfected into cultured cells, the intensity of luciferase reflects the transcription activity of the inserted promoter region. To monitor the transfection efficiency, the luciferase reporters were cotransfected with a Renilla reniformis
luciferase-containing plasmid, which gene is under control of the thymidine kinase promoter.

2.4 ASSAY OF CELL MIGRATION

To evaluate the chemoattractant properties of conditioned cell culture medium, chemotaxis assay using transwell microporous plates was carried out. Briefly, the upper and lower wells are separated by a gelatin-coated microporous filter membrane through which single cell could pass. Conditioned medium is loaded in the lower wells and cells to be tested are added to the upper wells. After two to four hours of incubation, the filters are washed with PBS, fixed in ice-cold methanol/acetone, and stained with Mayer’s hematoxylin and eosin. Cells remaining on the upper side of the filters are scraped off and cells at the lower side of the filters are counted by light microscopy.
3 AIMS OF THE STUDY

1. To delineate the mechanism of 15-LOX-1 gene expression in HL-derived and other cell lines (Paper I and II).

2. To study the role of 15-LOX-1 expression in lung epithelial cells in the pathogenesis of asthma and other inflammatory lung disorders (Paper III).

3. To evaluate the effect of SMYD3 expression in hTERT gene expression and telomerase activity (Paper IV).
4 RESULTS AND DISCUSSION

4.1 TRANSCRIPTIONAL REGULATION OF 15-LOX-1 EXPRESSION BY PROMOTER METHYLATION (PAPER I)

Because the 15-LOX-1 gene is highly regulated and specifically expressed in certain types of human cells, we attempted to investigate a potential relationship between DNA methylation and 15-LOX-1 expression. We examined 15-LOX-1 mRNA expression in a panel of human cell lines and normal human primary cells using RT-PCR. 15-LOX-1 mRNA was undetectable in all the studied cell lines including L428 HL cells, U937, and BL41 lymphoma cells, A549 lung epithelial cancer cells, and A431 epidermal cancer cells, normal human T lymphocytes and monocytes. A549 cells and monocytes could be induced to express 15-LOX-1 mRNA when exposed to IL-4 whereas the same treatment did not lead to transcriptional activation of the 15-LOX-1 gene in any of the other cells. Using MSP, we found hypomethylation of the 15-LOX-1 promoter in A549 cells and monocytes, in which IL-4 induces 15-LOX-1 expression. In striking contrast, the promoter was heavily methylated in the 15-LOX-1-incompetent cells. These results suggest a strong correlation of 15-LOX-1 gene silencing with a dense methylation of its promoter in both established human cell lines and primary cells.

To elucidate whether demethylation of the 15-LOX-1 promoter is required for the gene activation, 15-LOX-1-negative L428 cells were chosen because they express functional IL-4 receptors. We found that neither azadC nor IL-4 alone was capable of inducing significant levels of 15-LOX-1 mRNA. However, the combined treatment of L428 cells with azadC and IL-4 resulted in obvious 15-LOX-1 mRNA expression. Thus, inhibition of DNA methylation restores IL-4-inducibility of 15-LOX-1 mRNA, suggesting hypomethylation of the promoter as a prerequisite for the 15-LOX-1 gene activation, and that both the promoter demethylation and presence of appropriate transcription factors are required for transcriptional activation of the 15-LOX-1 gene in given types of human cells or tissues.

To investigate the correlation between 15-LOX-1 promoter methylation and its transcriptional activation, we synthesized an in vitro-methylated 15-LOX-1 promoter reporter construct that spans 1.5 kb upstream of the ATG and examined potential
effects of methylation on the promoter activities. We found that unmodified 15-LOX-1 promoter constructs exhibited threefold higher activity than its methylated counterpart. This finding demonstrates that DNA methylation leads to repression of the 15-LOX-1 promoter activity in cultured HL cells.

It has been shown that histone acetylation is involved in IL-13-mediated 15-LOX-1 induction\(^{27}\). Therefore, we investigated the effect of IL-4 on histone acetylation at the 15-LOX-1 promoter in the absence and presence of azadC. Histone H3 and H4 acetylation at the 15-LOX-1 promoter did not change when L428 cells were treated with either azadC or IL-4 alone. However, preexposure of the cells to azadC enabled IL-4 to increase H3 and H4 acetylation as determined by ChIP analysis. Substantial elevation of acetylated histones at the 15-LOX-1 promoter was well consistent with appearance of 15-LOX-1 mRNA in L428 cells treated with IL-4 and azadC. Our findings suggest that disruption of DNA methylation facilitates histone acetylation at the 15-LOX-1 promoter by TSA or IL-4 and in turn leads to significant 15-LOX-1 gene activation.

### 4.2 EPIGENETIC AND TRANSCRIPTIONAL CONTROL OF THE 15-LIPOXYGENASE-1 GENE IN A HODGKIN LYMPHOMA CELL LINE (PAPER II)

IL-4/13-STAT6 cascade, the best studied 15-LOX-1 activating pathway, has been shown to be constitutively activated in primary H/RS cells as well as in HL cell lines, most likely due to autocrine secretion of IL-13\(^{24}\). We recently found that 15-LOX-1 is over-expressed in a HL-derived cell line L1236, and in the primary H/RS cells of most classical HL tumors. In this study, we aimed at delineating the transcriptional regulation mechanisms leading to 15-LOX-1 gene expression in the HL cell line L1236 by analyzing both cis- and trans- acting elements.

We first investigated cis-acting elements of importance for the transcriptional regulation of 15-LOX-1 in L1236 cells. A combined approach of promoter deletion constructs cloned into reporter vectors and site-directed mutagenesis not only showed that all three putative STAT6 responsive elements within one kb of 5’ promoter sequence are of cooperative importance for 15-LOX-1 expression in these cells, but also that preserved STAT6 responsive elements are indispensable for promoter activity.
Physical interaction of STAT6 with the 15-LOX-1 promoter region in L1236 cells was confirmed in living cells as well as in vitro by ChIP analysis and EMSA, respectively. On the contrary, no STAT6 bound to the 15-LOX-1 promoter could be detected in L428 cells by ChIP analysis.

To address the different accessibilities of STAT6 to the 15-LOX-1 promoter in L1236 and L428 cells, its epigenetic environment in these cells was investigated. Using MSP, we found that L1236 cells exhibited hypomethylated CpG islands within the 15-LOX-1 promoter while this region in L428 cells is heavily methylated. Furthermore, we observed that the H3 acetylation at this region was very strong in L1236 cells compared with L428 cells. Collectively, these data suggest a correlation of 15-LOX-1 promoter CpG island methylation and histone acetylation status with STAT6 accessibility and 15-LOX-1 gene activation in cultured HL cells.

The biological consequences of 15-LOX-1 expression in H/RS cells are to date not clear, but ongoing investigations in our laboratory are focused on these issues. Except for the putative intrinsic activity in the tumor cells, as HL is characterized by inflammatory cells infiltration, effects of 15-LOX-1-derived metabolites on the surrounding tumor-associated cells are to be expected.

### 4.3 15-LIPOXYGENASE-1 INDUCES EXPRESSION AND RELEASE OF CHEMOKINES IN CULTURED HUMAN LUNG EPITHELIAL CELLS (PAPER III)

15-LOX-1 has been proposed to be involved in the pathogenesis of various disorders including inflammatory diseases. Asthma and chronic obstructive pulmonary diseases are associated with increased expression of 15-LOX-1 in bronchial epithelial cells, but the potential functions of 15-LOX-1 in airway epithelial cells have not been well clarified.

In order to evaluate the biological function of 15-LOX-1 in airway epithelial cells, we transiently transfected the 15-LOX-1-inducible lung epithelial cell line A549 with a vector expressing 15-LOX-1 cDNA or an empty control vector. Based on microarray analysis and real-time PCR at 24 hours post-transfection, we found that gene
expression of the chemokines Mip-1α, RANTES, IP-10 and I-TAC were up-regulated over three times by introducing 15-LOX-1.

Furthermore, we measured the concentration of chemokines in conditioned medium of cells ectopically expressing 15-LOX-1. A time course study using ELISA showed that after 2 days of transfection, cells expressing 15-LOX-1 produced higher amounts of Mip-1α than cells transfected with control vector. Multiplex luminex analysis demonstrated that conditioned medium of A549 cells expressing 15-LOX-1 had significantly higher levels of RANTES and IP-10 than that of cells transfected by empty vector. Our data indicate that 15-LOX-1 over-expression induces chemokine production in A549 cells.

Immature DC express receptors for inflammatory chemokines, including CC chemokine receptor 1 (CCR1), CCR2, and CCR5, which guide them to inflammatory sites where antigen sampling can take place. Since 15-LOX-1 over-expression results in up-regulation of Mip-1α, Mip1-1β and RANTES, we performed chemotaxis assays to determine if the ectopic expression of 15-LOX-1 in A549 cells could induce the migration of immature DC (iDC) in vitro. We found that iDC exhibited significant increased migration in response to the conditioned medium of A549 cells over-expressing 15-LOX-1 compared with medium of A549 cells transfected with the mock vector. The antibody neutralization experiments demonstrated that the enhanced iDC migration was highly dependent on the Mip-1α and RANTES induced by 15-LOX-1. These experiments show that over-expression of 15-LOX-1 in A549 cells increases iDC migration by inducing expression of Mip-1α and RANTES.

T cells play a critical role in immune responses and activated T cells have abundant expression of chemokine receptors, including CCR3 and CXCR3. Mast cells are also important players in the pathogenesis of asthma and other allergic diseases, and chemokines are involved in the accumulation of mast cells in inflamed tissues through a set of chemokine receptors including CXCR2, CCR3 and CCR5. Therefore, we performed similar chemotaxis and antibody neutralization experiments to test whether activated T cells and mast cells exhibited significant increased migration in response to the conditioned medium of A549 cells over-expressing 15-LOX-1. We observed that over-expression of 15-LOX-1 in A549 cells leads to increased recruitment of mast cells and activated T cells mainly via upregulated expression of RANTES and Mip-1α.
Collectively, our data suggest that expression of 15-LOX-1 in airway epithelial cells, by induction of chemokine synthesis and release, contributes to the accumulation of inflammatory cells seen in airway inflammatory conditions. This observation indicates that 15-LOX-1 plays a pro-inflammatory role in the airway epithelium and might constitute a therapeutic target in asthma and other inflammatory lung disorders.

4.4 THE TELOMERASE REVERSE TRANSCRIPTASE (HTERT) GENE IS A DIRECT TARGET OF THE HISTONE METHYLTRANSFERASE SMYD3 (PAPER IV)

SMYD3 is a histone H3-K4–specific dimethyltransferase and trimethyltransferase and plays an important role in oncogenesis. However, the mechanism underlying SMYD3-mediated oncogenesis is only incompletely understood and its target genes essential for transformation remain to be characterized further. Several lines of evidence have shown that activation of telomerase is a prerequisite for somatic cell immortalization and transformation and highly trimethylated H3-K4 was associated with the active hTERT expression in telomerase-proficient tumors. We sought to determine whether the hTERT gene is a transcriptional target of SMYD3.

To investigate the effect of SMYD3 on hTERT gene expression, we ectopically expressed SMYD3 in BJ, LF1 fibroblasts and the osteosarcoma cell line Saos2 which lack detectable telomerase activity and endogenous SMYD3 expression. Using RT-PCR, we found that introduction of SMYD3 into these cells induced hTERT mRNA expression. Our data demonstrated that SMYD3 positively regulates hTERT mRNA expression in human primary fibroblasts and cancer cells with a repressed hTERT gene. We further asked whether SMYD3 was required for constitutive hTERT mRNA expression in telomerase-proficient cancer cells. For this purpose, we knocked down SMYD3 expression with specific SMYD3 siRNAs in the human colorectal carcinoma cells HCT116, the HL cell line L1236 and the hepatocellular carcinoma Hep3B cell line. A significant reduction in hTERT mRNA and telomerase activity was seen following the inhibition of SMYD3 expression in these cells. Collectively, we found that the basic or constitutive hTERT expression and activity depends heavily on the presence of SMYD3 in the examined cells.
To determine whether SMYD3 up-regulates hTERT expression at the transcriptional level, we examined the effect of SMYD3 on the hTERT promoter activity. We identified five potential SMYD3 binding sites, CCCTCC, within the hTERT core promoter region. Cotransfection of the SMYD3 expression vector with a luciferase reporter driven by the hTERT core promoter sequence (hTERT-Luc p181) into Saos2 cells led to ~100% increase in reporter gene activity in a dose-dependent manner. Through substitution mutation of each SMYD3 binding site, we found that two of the potential SMYD3 binding sites were important for the transcriptional activity of the hTERT gene in all the three hTERT positive cancer lines we investigated.

To examine the ability of this region to interact directly with SMYD3 \textit{in vivo}, we transiently transfected Saos2 cells with the Flag-tagged SMYD3 expression vector and performed ChIP assay using an anti-Flag antibody. We found that Flag-tagged SMYD3 was bound to the hTERT promoter containing the SMYD3 binding motifs, indicating the direct association between SMYD3 and hTERT promoter.

Because SMYD3 induces transcriptional activation of its target genes by dimethylating or trimethylating H3-K4 in their promoter regions, we sought to determine whether the level of SMYD3 expression affected methylation patterns of H3-K4 at the hTERT promoter. We knocked down SMYD3 expression in HCT116 and L1236 cells using siRNA and then examined alterations in H3-K4 trimethylation in the hTERT promoter region using ChIP assay. We observed that H3-K4 trimethylation was abolished in the hTERT core promoter region when SMYD3 expression was inhibited, suggesting that SMYD3 is responsible for trimethylation of H3-K4 in the hTERT promoter region.

H3-K4 methylation alters chromatin folding that in turn contributes to increased accessibility of DNA to transcription factors and provides specific binding sites for certain proteins including histone acetyltransferases. We wanted to determine whether the abolished H3-K4 trimethylation affected the occupancy of the transcription factors c-Myc and Sp1, two essential \textit{trans}-activators for the hTERT gene, at the hTERT promoter in the SMYD3-silent cells. The ChIP assay showed that both c-Myc and Sp1 were present at the hTERT promoter in control HCT116 cells, whereas they were absent in the same cells with SMYD3 inhibition. Moreover, histone H3 acetylation at the hTERT promoter was detected in the control cells but not in the cells treated with SMYD3 siRNA.
A recent study showed that high H3-K4 methylation was one of the strict prerequisites for recognition of any target site by the c-Myc oncoprotein\textsuperscript{135}. Consistent with that observation, we found impaired occupancy of the transcription factors c-Myc and Sp1 on the hTERT promoter and diminished histone H3 acetylation following SMYD3 silencing and subsequent inhibition of H3-K4 trimethylation in the examined cancer cells. It is well established that the transcription factors c-Myc and Sp1 play a key role in activation of the hTERT gene transcription\textsuperscript{126}. Thus, by governing the access of positive transcription factors to and histone acetylation at the hTERT promoter, SMYD3-mediated H3-K4 methylation may function as a critical licensing element through which the \textit{trans}-activation of the hTERT gene is initiated.
5 CONCLUDING REMARKS

On the basis of our present findings, the following conclusions can be drawn:

1. Hyper- and hypomethylation of CpG islands in the 15-LOX-1 promoter region is intimately associated with the transcriptional repression and activation, respectively, of the 15-LOX-1 gene. Demethylation of the 15-LOX-1 promoter is a prerequisite for transactivation of the gene, which contributes to tissue- and cell-type-specific regulation of 15-LOX-1 expression (Paper I).

2. STAT6 activation and chromatin remodeling by DNA demethylation and histone acetylation are important for transcriptional activation of 15-LOX-1 in cultured HL cells (Paper II).

3. Over-expression of 15-LOX-1 in A549 cells leads to increased production of the chemokines Mip-1α, RANTES and IP-10, and thereby to increased recruitment of immature dendritic cells, mast cells and activated T cells. An increased expression of 15-LOX-1 in lung epithelial cells could be a pro-inflammatory event in the pathogenesis of asthma and other lung inflammatory diseases (Paper III).

4. SMYD3 induces hTERT transcription by directly binding to the hTERT promoter and affecting abundance of trimethylated H3-K4 associated with the hTERT chromatin. SMYD3-mediated H3-K4 methylation is required for inducible and constitutive hTERT expression (Paper IV).
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