Epigenetic Regulation of Gene Transcription in Hematopoietic Tumors

MARINA TSHUIKINA WIKLANDER
Dissertation presented at Uppsala University to be publicly examined in Rudbeck Hall, Rudbeck Laboratory, Dag Hammarskjöld’s väg. 20 Uppsala, Friday, September 19, 2008 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

Epigenetic modifications were shown to play an essential role in tumorigenesis. Epigenetic mechanisms can alter transcription in several ways, through DNA methylation and/or through histone modification. DNA methylation at the TSS (transcriptional start site) has been implicated in tumor development and gene silencing. However, several examples of atypical methylation were shown. In Paper I we present the ICSBP/IRF8 gene that belongs to the IRF family and has characteristics of a tumor suppressor gene. The ICSBP/IRF8 is fully methylated in the promoter and TSS regions in U-937 and despite high expression of the gene. Presence of positive histone marks suggests that methylated DNA can be overridden by histone modification.

In Paper II a panel of 13 MM (multiple myeloma) cell lines and 9 primary patient tumors were analysed for methylation status of the ICSBP/IRF8 gene. In most cell lines (8/13) the gene was partially or fully methylated and partial methylation was also observed in 1/9 primary tumors. In vitro methylation analysis and treatment with 5-aza-2’deoxycytidine (DAC) proved that the ICSBP/IRF8 gene is silenced by methylation and may be associated with the malignant phenotype.

In Paper III and IV the NFκB signalling pathway was analysed and the role of ATRA and TNFα induction. In Paper III the data shows that activation of the NFκB pathway is essential in ATRA-induced terminal differentiation in the U-937 cell line and IkBα (S32A/S36A) inhibits ATRA-induced differentiation and G1 cell cycle arrest. This was accompanied by delayed down-regulation of several cyclins (A and E) and up-regulation of p21WAF1/CIP1 (CDKN1A) and p27KIP1 (CDKN1B).

TNFα alone did not induce expression of RA-induced genes analysed in Paper IV. However, ATRA in combination with TNFα showed enhanced activation of RA-induced genes. TNFα triggers demethylation of H3K9me3/H3K9me2 and H3K4me3 at RAR/ RXR target genes, which were not accompanied by changes in the level of H3K9-ac. This decrease in H3 methylation by TNFα may pave way for the later ATRA-induced gene transcription.

Keywords: IRF family, ICSBP/IRF8, epigenetics, ATRA, TNFα, histone modifications, methylation, transcription

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urn:nbn:se:uu:diva-9206 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-9206)
This world, after all our science and sciences, is still a miracle; wonderful, inscrutable, magical and more, to whosoever will think of it.

Thomas Carlyle
(1795-1881)

To my family.....
List of Papers

This thesis is based on the following papers referred to in the text by their roman numerals:

I  
Tshuikina M., Nilsson K. and Öberg F. “Positive histone marks are associated with active transcription from a methylated ICSBP/IRF8 gene” 2008 Gene 410, 259-267

II  
Tshuikina M., Jernberg-Wiklund H., Nilsson K. and Öberg F. ”Epigenetic silencing of the interferon regulatory factor ICSBP/IRF8 in human multiple myeloma.” Accepted

III  
Kårehed K., Tshuikina M., Nilsson K. and Öberg F. ”Activation of NFκB in all-trans retinoic acid induced differentiation, cell cycle arrest and p21 (CDKN1A/Waf1) expression in U-937 cells.” Submitted

IV  
Tshuikina Wiklander M., Kårehed K., Nilsson K. and Öberg F. ”TNFα induces a reduction of histone 3 lysine 9 trimethylation and dimethylation (H3K9me3 and H3K9me2) at all-trans retinoic acid target genes”. Manuscript

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## Abbreviations

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<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>APL</td>
<td>Acute promyelocytic leukemia</td>
</tr>
<tr>
<td>ATRA</td>
<td>All-trans retinoid acid</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>CLP</td>
<td>Common lymphoid progenitor</td>
</tr>
<tr>
<td>CML</td>
<td>Common myeloid progenitor</td>
</tr>
<tr>
<td>CoA</td>
<td>Co-activator</td>
</tr>
<tr>
<td>CoR</td>
<td>Co-repressor</td>
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<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyl-transferase</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>GMP</td>
<td>Granulocyte/macrophage progenitor</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HMT</td>
<td>Histone methyl-transferase</td>
</tr>
<tr>
<td>HSC</td>
<td>Haematopoietic stem cell</td>
</tr>
<tr>
<td>IAD</td>
<td>IRF associated domain</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>K</td>
<td>Lysine</td>
</tr>
<tr>
<td>MBD</td>
<td>Methyl CpG binding domain</td>
</tr>
<tr>
<td>MeCP1</td>
<td>Methyl CpG binding protein 1</td>
</tr>
<tr>
<td>MM</td>
<td>Multiple Myeloma</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>PML</td>
<td>Promyelocytic leukemia</td>
</tr>
<tr>
<td>POD</td>
<td>PML oncogenic domain</td>
</tr>
<tr>
<td>R</td>
<td>Arginines</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic acid receptor</td>
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<tr>
<td>RARE</td>
<td>Retinoic acid response element</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>S</td>
<td>Serine</td>
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<tr>
<td>T</td>
<td>Threonine</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
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<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>TRD</td>
<td>Transcriptional repression domain</td>
</tr>
<tr>
<td>TSS</td>
<td>Transcriptional start site</td>
</tr>
</tbody>
</table>
Introduction

Within the field of medical biology, significant research and clinical effort has been put into understanding and treating cancer. Subsequently, several papers, articles and books have been written on the topic of tumorigenesis or more simply put the origin and development of tumors. For a long time, cancer was believed to be solely a genetic disease. However, for the last decade the field of epigenetics was included in tumorigenesis due to the suggestion made by several researchers that epigenetics predisposes for cancer development.

The cancer research community is still wrestling with a proverbial question of what came first, the chicken or the egg? In tumorigenesis that question becomes: “What came first, genetic aberration or epigenetic modifications? There are several observations that support either standpoint, both the genetical and epigenetic theories on the origin of tumors. Familiar cancer, inherited from generation to generation, would support the genetic theory. It is also known that DNA methylation and chromatin modification lead to chromosomal instabilities, which would explain the chromosomal translocations and therefore support the epigenetic theory. Studies of twins also suggest epigenetic modifications to be a possible first hit for cancer development. It is also possible that epigenetics gives us predisposition for developing cancer or other diseases, and other factors like genetics and environment also contribute. Similar to the Knudsen’s two hit theory. Regardless, we cannot rule out either of them.

For many years, the role of genetic alterations in cancer development has been generally accepted. Point mutations, deletions, insertions or translocations—all added something to tumorigenesis. Several successful models were developed with time to introduce novel treatments for the patients, to overcome the drug resistance and to increase life expectancy at diagnosis.

For the last decade, the impact of epigenetics in tumor development has become more acknowledged and studied. Epigenetics concerns heritable states of the gene expression and does not affect the primary DNA sequence. Epigenetic changes influence gene expression via two mechanisms: DNA methylation and chromatin modifications. Chromatin modifications proved to be essential for the programming of gene expression and cell fate.
DNA methylation

Methylation of cytosine takes place at CpG dinucleotides by adding a methyl group at the C5 position of the pyrimidine ring. CpG’s are often clustered in CpG-rich regions and are then designated CpG-islands, often located at or nearby promoter regions, the first exon and untranslated regions [1].

A majority of the CpG islands in our genome are unmethylated, but there are examples of at least 4 types of genes normally silenced by methylation: imprinted genes, X-chromosome inactivation in women, germline-specific genes, and a number of tissue-specific genes [2].

There are two components in the process by which DNA methylation affects gene expression. The first one being DNA methyl transferases (DNMT1, DNMT3A, DNMT2 and DNMT3B) [3], that establish and maintain the DNA methylation status. The second one is defined by the group of methyl CpG binding proteins (MBD1, MBD2, MBD4, MeCP2 and Kaiso) [4-8].

Alterations of DNA methylation are associated with cancer. Cancer often leads to gene-specific hypermethylation and global hypomethylation. DNA hypermethylation leads to silencing of tumor-suppressor gene expression and increased tumor frequency. Global hypomethylation leads to chromosomal instability [9-11].

DNA methylation is usually associated with transcriptional repression. However, there are several exceptions where atypical methylation is associated with active genes. For example, estrogen-induced expression of Vitellogenin gene A1 and A2, which is shown to be expressed independent on methylation in Xenopus [12]. Most cases of atypical methylation were shown to be associated with methylation of the regions located far upstream of the transcriptional start site (TSS), like Interleukin-8 (IL-8) [13] and imprinted Igf2 genes [14], or an intronic or down stream region of the gene, like o2-Collagene gene [15] and Early Growth Response (EGR2) [16]. In all these cases the DNA methylation is suggested to inhibit the activity of the silencer
element, thereby increasing the promoter activity. The high levels of CpG methylation directly upstream from a region carrying histone H3K9-ac and H3K4me3 suggested prevention of spreading DNA methylation towards the promoter by active histone marks [17]. Direct evidence for transcriptionally active and densely methylated promoters is rare and involves displacement of MBD proteins by GATA-3 [18] or conversion of MBD2 proteins from repressor to an activator by interacting proteins like HTLV-1 Tax [19], or TACC3 and MBDin [6, 20]. In the HLA-DR gene the RFX protein has recently been shown to have the capacity to specifically bind the methylated promoter and active transcription [21]. The SIRT1 gene belongs to the class III of HDAC inhibitors and was shown to epigenetically silence tumor suppressor genes by binding to hypermethylated 5’ areas. Inhibition of SIRT1 would lead to reactivation of the genes without demethylation of the promoters [22]. Use of the LBH589 HDAC inhibitor showed re-expression of the estrogen receptor (ER) via reorganization of heterochromatin associated proteins also without loss of promoter hypermethylation [23]. The ICSBP/IRF8 gene is shown to be transcriptionally active in U-937 and account for high methylation over the TSS. However, enrichment of active histone marks (H3K4me3 and H3K9-ac) at TSS would explain accessibility of transcription factors (TFs) binding to TSS and activating the transcription [24]. These last three studies suggest that there is a combination of several mechanisms involved in gene silencing, making analysis of epigenetic silencing more complicated than it was believed to be. DNA methylation is an important mechanism in gene repression; however, hypermethylated promoters or regulatory elements can not be ultimately presented as silenced without investigation of the chromatin status.

DNA methyltransferase

DNA methyltransferases (DNMT’s) are the family of enzymes that catalyze the addition of a methyl group to the cytosine position 5 in the pyrimidine ring. The DNMT1, DNMT3a, DNMT3b, DNMT3L and DNMT2 proteins are all members of the DNMT family. DNMT1 is responsible for maintaining an already established DNA methylation pattern, where the function of DNMT3a and DNMT3b is to mediate establishment of new, de novo DNA methylation patterns [25, 26]. The DNA methyltransferase activity of DNMT2 is however weaker compared to other members of the family, but was also shown to have tRNA methyltransferase activity [3, 27]. Single nucleotide polymorphism (SNP) in DNMT3B gene was shown to be associated with increased risk for developing breast cancer [28]. Probably for cancer cells the expression of only DNMT1 is not enough for maintaining an abnormal hypermethylation status; a cooperation of both DNMT1 and DNMT3a and DNMT3b proteins is needed [29]. Reduced expression of
DNMT1 leads to global hypomethylation, which increases occurrence of genomic instabilities. It was shown that the amount of DNMT1 and DNMT3 in several solid and haematological tumors is higher than in normal tissue [30]. DICER involvement in siRNA/miRNA processing was recently discovered as a new mechanism for establishing and maintaining DNA methylation in human cancer [31]. This opens new insight into epigenetic silencing in tumorigenesis.

Methyl CpG-binding proteins

Activation of transcription can be inhibited by DNA methylation through either direct or indirect mechanisms. Direct inhibition occurs when sequence-specific transcriptional activators are prevented from binding to DNA by methylation of cytosine within their target binding motif. The indirect mechanism is characterized by recruitment of methyl CpG binding proteins and their associated chromatin remodelling activity.

Two classes of proteins involved in indirect inhibition of gene expression have been identified: the methyl CpG binding protein 1 (MeCP1) and MeCP2.

MeCP2 consists of a single polypeptide that contains both a methyl CpG-binding domain (MBD) and a transcriptional repression domain (TRD) [8, 32]. It can bind to a single CpG and inhibit gene expression by recruiting histone deacetylases (HDAC) [33]. MeCP2 and other MBDs gain access to chromatin in order to target co-repressor or co-activator complexes that will further modify the chromatin structure. MeCP2 null mice are surprisingly viable and fertile, but they later develop neurological symptoms similar to the ones occurring in Rett Syndrome, a neurological disorder in females caused by mutation of MeCP2 [34].

MeCP1, in contrast to MeCP2, requires densely methylated DNA in order to inhibit gene expression. MeCP1 is detectable in somatic cells, but absent in ES cells as well as in germ cells [35]. MeCP1 is a complex containing histone deacetylases HDAC1, HDAC2 and RBAP48/46 [36, 37].

The screen for MBD-containing homologues revealed four additional methyl CpG-binding proteins to the MeCP2 group: MBD1, MBD2, MBD3, and MBD4. The MBD domains of MBD1, MBD2 and MBD3 are more similar to each other than to either MBD4 or MeCP2. The MBD domain of MBD4 is more similar to MeCP2. This analysis showed a striking similarity concerning the MBD, suggesting that all of them are evolutionarily related [38]. The MBD domain is essential for MeCP2 binding to chromosomes and re-
quired for specific binding of MBD1, MBD2, MBD3 and MBD4 to methylated DNA \textit{in vivo} and \textit{in vitro} [39]. Initially the MBD1 protein was believed to be a member of the MeCP1 repressor complex. However, later studies revealed that MBD1 does not belong to the MeCP1 complex. The MBD1 protein consists of the MBD, CxxC motifs and the transcriptional repressor domain (TRD) and represses transcription through co-operation of these domains in a deacetylase-dependent pathway. Binding of MBD1 protein through CxxC motifs can affect the gene expression from both unmethylated and hypomethylated promoters [4]. The MBD2 protein belongs to the MeCP1 repressor complex and can also be associated with HDAC2 and act as a repressor of transcription. However, MBD2 and TACC3 form a complex in vivo with histone acetyltransferase pCAF, showing that under certain circumstances MBD2 can act as an activator of the transcription [20]. There are two forms of MBD2; MBD2a and MBD2b and it has been reported that MBD2b has intrinsic demethylase activity [40]. The MBD3 protein is a member of the histone deacetylase complex with nucleosome remodelling activity designated the Mi-2/NuRD complex. MBD2 can form heterodimers with the MBD3 protein, bind to hemimethylated DNA and recruit deacetylases as well as DNMT1 [38]. MBD3 is a crucial protein in mammalian development. The MBD3 knock-out mice failed to develop to term [41]. It was shown that MBD3 inhibits gene expression in a similar way as MeCP2, MBD1 and MBD2 [42]. The MBD4 protein is the only member known to be involved in a different class of processes. Its activity consists of binding to m5CpG X TpG mismatches, which most likely helps to minimize mutations at methyl CpG sites [5]. Recently, another methyl CpG-binding protein called Kaiso was proven to be a member of the methyl CpG-binding protein family. Kaiso mediates the movement of the p120ctn/E-cadherin complex from the cytoplasm to the nucleus to regulate the expression of methylated target genes [7]. The MBD proteins play an important role in recruiting histone deacetylases (HDAC), histone methyl transferases (HMT), and chromatin remodelling complexes to the methylated DNA. In conclusion, the activity of MBD proteins is dependent on association with different partners, which they recruit to methylated DNA, acting as co-activators as well as co-repressors.

**Chromatin modifications**

The basic unit of chromatin is the nucleosome. It contains 147bp of DNA twice wrapped around an octamer of the core histones (two of each histone H2A, H2B, H3 and H4) [43]. Each nucleosome is connected to each other by linker DNA with a 10-60bp length.
The chromatin structure regulates gene expression. Putting it simply, gene transcription is inhibited when the chromatin is in a compact, heterochromatic form that results in a poor template for biochemical reactions. In contrast, when the chromatin structure is more open (euchromatin) and therefore accessible for various enzymes, gene transcription can be activated. There are three different ways for the chromatin structure to be altered: nucleosome remodelling, covalent modification of the histones and local deposition of histone variants [44].

**Nucleosome remodelling**

The ability to bind transcription factors is critical for the activation of gene expression. Transcription factors need to find the specific DNA binding sequences and gain access to them. They can get help from chromatin-remodelling complexes which are present at the positions of the nucleosomes. These chromatin-remodelling complexes are able to mobilize the nucleosomes in such way that the histone octamers can move short distances along the DNA [45]. SWI/SNF is a chromatin-remodelling complex with ATPase activity to provide the necessary energy. Mutations in these factors affect both the DNA and the histone methylation. Their role is not understood properly yet, but probably they facilitate the access to the DNA. The SWI2/SNF2 family is divided into at least 8 subfamilies: SNF2, ISWI, CHD1, INO80, ERCC6, RAD54, DDM1, and MOT1 [46, 47]. Only a few of them were shown to be implicated in cancer. For example the mutations in PASG, a member of the DDM1 subfamily, was shown to be identified in 40-60% of the cases of acute myelogenous leukemia and lymphoblastic leuke-
Somatic loss of function mutations in ATRX, which is a PAD45-like protein, was implicated in a very rare form of myelodysplasia; pre-leukemic blood disorder [49]. There are other proteins belonging to this family, like BRG1 showing implications in solid tumors [50, 51]. There is also evidence that one of the core subunits of SNF2 complexes, SNF5, acts as a classical tumor suppressor gene [52].

Histone modifications

Histone tails are subjects to a wide range of post-translational modifications. There are 8 histone modifications known to date, the most known ones are acetylation of lysines, methylation of lysines (K) and arginines (R), ubiquitination lysines and phosphotylation of serines (S) and threonines (T) [43]. Most probably there are other histone modifications that remain undiscovered. The modification of histone tails is very complex. They contain different modifications, like methylation or acetylation, but some of them can also co-exist. To make it even more complicated they can appear in different forms; mono-, di- or even tri- methylated and where the genes will be active or repressed will depend on all the features above taken together. For example, di-methylation of histone 3 (H3) lysine 9 (K9) as well as H3-K27 will lead to heterochromatin formation and gene silencing. However methylation of H3-K4, H3-K36 and H3-K79 is associated with active chromatin [53]. Recent publication also suggested that some histone modifications can cooperate in preparation for gene activation [54].

Histone methyltransferases

A variety of histone modification enzymes have been identified. At least 17 histone methyltransferases (HMT) are known to be involved in methylation of lysine (K) residues on the N-terminal tail of histone 3 (H3) [55]. Several
HMT were shown to be implicated in blood tumors. EZH2 is a H3-K27 histone methyltransferase (HMT) and is a part of the repressive PcG complexes PRC2 and PRC and are highly expressed in lymphomas [56]. SUV39H1 is a HMT for H3-K9 and is also associated with gene silencing. Approximately 30% of SUV39H1 knock-out mice develop B-cell lymphomas. SMYD3 is a HMT for H3-K4 and is associated with gene activation [57].

**Histone demethylases**

For a very long time it was believed that histone methylation was a stable and irreversible post-translational modification. However, with discovery of LSD1[58] this dogma was changed and a new prospect in epigenetics was opened. LSD1 specifically demethylates H3K4me2 and H3K9me2 to H3K4me and H3K9me respectively. This means, that LSD1 can act, depending on accompanying co-factors, as a transcriptional repressor or activator [58, 59]. Since the LSD1 discovery, the whole family of histone demethylases was characterised. The Jumonji (JmjC) domain protein family contains 27 different JmjC domain members, which are divided into 8 clusters, depending on JmjC domain and demethylation specificity [60]. The FBXL10 demethylase was shown to be involved in demethylation of H3K4me3 [61] and also H3K36 which would lead to transcriptional repression. The FBXL11 is a member of the same cluster as FBXL10, but demethylates only of H3K36 [62]. JARID cluster, consisting of JARID1A, JARID1B, JARID1C and JARID1D, are involved in demethylation of H3K4 and transcriptional repression [63-68]. JMJD2 cluster members JMJD2A, JMJD2B, JMJD2C and JMJD2D are demethylases for both H3K9 and H3K36 and can act as transcriptional co-repressor and co-activator [69-72]. However, several studies have shown that not only the JmjC domain can interact with specific areas of chromatin, but also other domains like PHD and Tudor of the Jumanji proteins. JMJD2A is interacting via the Tudor domain by binding to H3K4me3, H3K9me3 and also H4K20me3, acting as transcriptional co-activator or co-repressor depending on modification [73-75]. The JMJD1 cluster contains 3 members, JMJD1A, JMJD1B and JMJD1C, and are demethylases for H3K9 and transcriptional co-repressors [76-78]. JMJD3 and UTX demethylases were identified to be specific for H3K27me3 and me2 [79-82]. The only demethylase identified so far for demethylation of arginines (H3R2me2 and H3R3me2) is JMJD6 [83]. H3R2me2 was shown also to be an antagonist for H3K4me3 leading to transcriptional repression [84, 85]. There are also other demethylases that have been identified; however their biological or functional role was not determined yet. Histone methylation and demethylation is a dynamic process. There is evidence of genes present in a bivalent stage with presence of both active and repressive marks and upon stimulation one of them would be removed followed either by induction or repression of the genes [86, 87].
**Histone acetyltransferases and deacetylases**

Acetylation of histones is balanced by activities between histone acetyltransferases (HAT) with transcriptional co-activator and histone deacetylases (HDAC) associated with transcriptional repression. HATs can be divided into 3 groups: CBP/p300, Gcn5/PCAF and MYST. They form multiprotein complexes and are required to be bound to specific promoters via DNA bound transcriptional factors [88]. HATs act as transcriptional co-activators. It was shown that HATs as CBP, p300, Moz and MORF are involved in fusion proteins that arise from chromosomal translocations and are associated with leukemia [89].

There are 18 HDAC’s encoded in the human genome divided into three different classes: reduced potassium dependency 3 (Rpd3), histone deacetylase 1 (Hda1) and silent information regulator (Sir1). Many of HDAC’s are components of the multiprotein complexes and act as transcriptional repressors [90]. HDAC’s are playing an important role in cancer development and it is especially important when recruitment of HDAC’s occur independently from DNA methylation. In that case HDAC’s are recruited to tumor-suppressor genes via oncogenic DNA binding factors. An example of such mediation is recruitment of HDAC together with co-repressors to AML1-ETO fusion protein which will lead to a block in myeloid development and leukaemic transformation [91]. A similar mechanism is involved in the actions of the PML-RARα fusion protein [92].

**Histone variants**

Not only histone modification but also histone variants can play an important role in chromatin modification. Several variants of histone H3 were identified, such as Cse4/CENP-A, which are essential for centromere function and assembly [93]. Another variant of H3 is H3.3 which replaces the H3 and generates a mark of a transcription event [94]. In case of histone H2A, the macro-H2A functions in X-chromosome inactivation are restricted to metazoans, but another H2ZA (also known as H2A.F/Z or H2AvD) is present in all eukaryotes [95].

**Epigenetic regulation of transcription**

In order for transcription to occur upon the transcription factors (TF), they need to bind to transcription factor responsive elements at/or close to the transcriptional start site (TSS) or on enhancer elements. There are two epigenetic mechanisms which alter transcriptional activation: DNA methylation
and histone modifications. In general, DNA methylation is a mark of the repressive state of the gene transcription. DNA methylation is normally absent in the promoter region and at the enhancer elements and present in the body of the gene, in the repetitive sequences and in the imprinted genes. However, in cancer, aberrant DNA methylation may occur in the promoter regions of the genes. A methyl group at CpG dinucleotides prevents the TFs from binding to the DNA and activating the transcription. Histone modification influences the chromatin state in two ways; directly by attracting corepressor complexes or indirectly by folding chromatin into heterochromatin.

The IRF family

The IFN regulatory factors (IRF) belong to a family of transcriptional factors involved in IFN signalling and in development and differentiation of the immune system [96, 97]. The IRF family consists of 9 members; IRF1, IRF2, IRF3, IRF4, IRF5, IRF6, IRF7, ICSBP/IRF8 and ISGF3γ/IRF9 which share a DNA binding domain (DBD) situated in the N-terminal region. The DBD is characterized by a winged type helix-loop-helix motif with five tryptophan repeats, three of which contact DNA, recognizing the AANGAAA sequence [98]. The IRF association domain (IAD) in the C-terminal region is shared by some members of the IRF family, like IRF3, IRF4, IRF5, ICSBP/IRF8 and IRF9. The members of the IRF family are constantly expressed throughout B cell development and in activated and resting cells. The members of the IRF family regulate the transcription by forming hetero- or homodimers. The IRF activity varies dependent on partners resulting in either activation or repression of the transcription. The IFN-stimulated response elements (ISREs) are DNA sequences which enable IRFs to bind target DNA [99]. The IRF3, IRF5 and IRF7 genes are known to be essential in the inducible expression of type I IFN genes in infected cells. The IRF3 gene is constitutively expressed in all cell types [100]. The IRF5 gene is more restricted to B-cells and DC, but can also be induced by IFN-α [101]. The IRF family proteins are mostly located in the cytoplasm, but cells with high expression of IRFs in the nucleus can also be found. It was also shown that IRF3 and IRF7 protein can interact with histone acetyltransferases. The IRF3 interacts with CBP/p300 and the IRF7 with the p300-CBP-associated factor (PCAF) [102]. The IRF9 protein forms a transcriptional complex with the signal transducer and activator of transcription 1 (Stat1) and Stat 2 in response to IFN-α stimuli. A newly formed complex can also bind and regulate genes containing the ISRE binding site in their promoters [103].
The ICSBP/IRF8 and IRF4 genes

The ICSBP/IRF8 and IRF4 genes are two members of the IRF family which share similar features: their expression is restricted to immune cells and they are involved in regulation of myeloid, lymphoid and dendritic cells development. The ICSBP/IRF8 gene is expressed in haematopoietic cells, macrophages, myeloid and B-cells, but at a very low level in T-cells and can be induced by IFN-γ [104, 105]. The expression of the IRF4 gene is not stimulated by IFN-γ compared to the ICSBP/IRF8 gene. Both ICSBP/IRF8 and IRF4 genes are sharing the unique interaction with PU.1-Spi1. The PU.1 belongs to the Ets family which is an important regulator in myeloid and lymphoid cell differentiation [106]. PU.1 can form a stable complex with IRF4 and ICSBP/IRF8 and binds to EICE binding site [107]. Several studies revealed that IRF4 and the ICSBP/IRF8 proteins form a complex with PU.1, on the DNA-protein level. This interaction is required to have a stable complex formation, at least in vitro [108]. Depending on interacting partners, the ICSBP/IRF8 gene can be either repressor of transcription via ISRE binding site or an activator via EICE binding site [21, 109]. Loss of the ICSBP/IRF8 gene in human myeloid leukemia and ICSBP/IRF8 knock-out mice are impaired in macrophages development and manifest a chronic myeloid leukemia-like syndrome [110, 111]. Deficiency in ICSBP/IRF8 expression has been associated with increased resistance to apoptosis and defect in myeloid cell differentiation [112]. However, ICSBP/IRF8 double-mutant was able to compete for the interaction of ICSBP/IRF8 with either IRF or non-IRF members, such as PU.1 and led to apoptosis of only haematopoietic cells [113]. Less is known about the ICSBP/IRF8 gene impact for B-cell tumors. It has been reported that the ICSBP/IRF8 gene also play an important role in B-cell differentiation together with the IRF4 gene [114]. In primary effusion lymphoma (PEL) disease with B-cell origin, the ICSBP/IRF8 gene has been lost together with PU.1, Oct-2 and Pax5 genes expression, which can contribute to lymphomagenesis [115]. The IRF4 gene is known in 5% of all multiple myeloma cases to be translocated to the immuno-globulin heavy-chain region and over expressed. Microarray analysis revealed 35 IRF4 target genes [116], some of which are involved in cell cycle regulation. But the most important was strong interaction with MYC gene, which is known to play a key role in cellular mechanisms and proliferation [117]. Knock-out of IRF4 gene using shRNA was shown to be toxic for myeloma cells, opening a new therapeutic target for multiple myeloma (MM) treatment [116].
Hematopoietic tumors

Leukemia

Leukemia is an accumulation of malignant haematopoietic precursors. There are several mechanisms at work in leukemia development. For example, loss of function dominant negative alterations in genes that regulate the normal haematopoietic differentiation. Another mechanism has to do with expression of critical regulators of haematopoietic development occurring in the wrong lineage or at an inappropriate stage of differentiation [118].

Table 1. Genes that are altered in leukemia during haematopoietic development. Adapted with permission from review article: Izraeli S., “Leukemia- a developmental perspective”, Br J of Haematol Volume 126 Page 3 - July 2004

<table>
<thead>
<tr>
<th>Gene(s) names</th>
<th>Normal haematopoietic development</th>
<th>Leukaemic involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCL (TAL1)</td>
<td>Haemangioangioblast specification. Erythro- and megakaryopoiesis</td>
<td>T-ALL</td>
</tr>
<tr>
<td>LMO1/2</td>
<td>Similar to SCL</td>
<td>T-ALL</td>
</tr>
<tr>
<td>NOTCH</td>
<td>T lymphocytes</td>
<td>T-ALL</td>
</tr>
<tr>
<td>HOX11</td>
<td>Spleen</td>
<td>T-ALL</td>
</tr>
<tr>
<td>E2A</td>
<td>T and B lymphocytes</td>
<td>BCP-ALL</td>
</tr>
<tr>
<td>PAX5</td>
<td>B lymphocytes</td>
<td>BCP-ALL, B-NHL</td>
</tr>
<tr>
<td>SLP-65</td>
<td>B lymphocytes</td>
<td>BCP-ALL</td>
</tr>
<tr>
<td>TEL</td>
<td>Bone-marrow haematopoietic stem cells</td>
<td>BCP-ALL, rarely myeloid malignancies</td>
</tr>
<tr>
<td>RUNX1 (AML1, CBFA2)</td>
<td>Definite haematopoiesis. Megakaryopoiesis and T lymphocytes</td>
<td>BCP ALL, AML (M0-M1) Hereditary FPD/AML</td>
</tr>
<tr>
<td>CBFB</td>
<td>Same as RUNX1</td>
<td>AML (M4e)</td>
</tr>
<tr>
<td>C/EBP 1-3</td>
<td>Myeloid cells</td>
<td>AML (M1, M2)</td>
</tr>
</tbody>
</table>
Gene(s) names | Normal haematopoietic development | Leukaemic involvement \\
---|---|---
*PU.1* | Myeloid and lymphoid stem cells | AML \\
*GATA1* | Erythropoiesis, megakaryopoiesis and mast cells | AML (M7) associated with trisomy 21 \\
*FLT3* | Haematopoiesis and lymphopoiesis | AML and ALL \\
*MLL* | Haematopoietic stem cells | AML and ALL \\

ALL, acute lymphoblastic leukemia; BCP, B-cell precursor; AML, acute myeloid leukemia (M0–M7 = FAB classification); FPD/AML, familial platelet disorder with associated myeloid malignancy (OMIM no. 601399).

According to the results from the Cancer Genome Project (CGP) (www.sanger.ac.uk/CGP), there are at least 367 cancer genes identified so far, which represent more than 1% of all the genes in the human genome. 70% of all known cancer genes are associated with leukemia and lymphoma. The majorities of the 367 cancer genes are protein kinase genes and have previously been shown to be implicated in tumorigenesis. However, the proteins implicated in leukemogenesis are often proteins involved in transcriptional regulation and encoded by cancer genes which are activated by chromosomal translocations. Two major types of translocations have been found; translocations involving the immunoglobulin heavy-chain locus, and translocations generating fusion genes resulting in proteins with new functions. The CGP list does not contain genes with changes of the methylation pattern in the promoter region. However, there are several reports revealing the implication of methylation changes in tumorigenesis, like p73 in ALL or p16 in CLL [119, 120]. An early role for epigenetic changes, affecting progenitor cells, later developing into malignant tumors, has been suggested [121]. However, a causative role for epigenetic aberrations of specific genes in the development of a tumor has been more difficult to prove.

Multiple Myeloma
Multiple myeloma (MM) is a malignant haematological disease that arises from malignant B-cell (monotypic plasmablasts-plasma cells) in the bone
Patients with MM suffer from bone destruction, renal failure, anaemia and pain. MM cells are very dependent on the BM microenvironment for survival, growth and differentiation [122]. In approximately 60% of all MM cases, translocations to a heavy-chain gene locus were found. There have been at least 5 genes identified as primary, non-random translocation partners which include Bcl-1/PRAD-1/cyclins D1 (11q13) [123], cyclin D3 (6p21) [124], FGFR3-MMSET (4p16.3) [125], c-Maf (16q23) [126] and mafB (20q11) [127]. Deletions of chromosome 13 is also common and appear in early stage of multiple myeloma development [128]. During progression of the disease several additional instabilities, mutations and dysregulations in expressions of the genes like c-myc, N-ras, K-ras, FGFR3 and p53 occur [129]. The genetic changes in remaining the 40% are not known, but may also include epigenetic changes. A few genes have been shown to be silenced by methylation in MM, e.g. SOCS-1, p16, p53, DAP-kinase [130-132].

Acute Promyelocytic Leukemia (APL)

Acute promyelocytic leukemia (APL) is one of the well studied forms of acute myelogenous leukemia (AML). APL is developed by chromosomal translocation of retinoic acid receptor-α (RARα) gene on chromosome 17 and promyelocytic leukemia (PML) gene on chromosome 15, which results in formation of a PML-RARα fusion protein. The formation of this novel...
protein will lead to blockage at the promyelocytic stage of myeloid differentiation [133]. 95% of the APL patients had PML-RARα translocation. However, other types of fusion protein formations were detected where the PML gene was substituted by the promyelocytic leukemia zinc finger (PLZF) or nucleophosmin (NPM) or nuclear mitotic apparatus (NUMA) or the signal transducer and activator of transcription 5b gene (Stat5b) [134].

Due to formation of the PML-RARα fusion protein, the normal functions of both PML and RARα are disrupted. Retinoid acid receptors consist of two groups, one is retinoid receptor (RAR) with 3 isotypes α, β and γ; and the second group is rexinoid receptors (RXR) [135]. All-trans retinoic acid (ATRA) is a natural derivative of vitamin A. Upon ATRA treatment receptors from either homo- (RXR/RXR) or heterodimes (RAR/RXR), bound to genes containing RARE binding sites, activate transcription and cellular responses [135-137]. In absence of a ligand, the ATRA responsive genes are repressed via recruitment of HDAC-containing complexes, which include NCoR and SMRT [138]. Binding of these co-repressor complexes leads to deacetylation of histones, chromatin condensation and gene silencing. However, with RA binding, HDAC-containing complexes disintegrate and HAT-containing complexes are recruited by co-activators of the p160 [139-141] family and histone acetyltransferases CBP/p300 [142, 143], which lead to acetylation of histones, chromatin de-condensation and expression of the ATRA responsive genes. Demethylation of H3K27me3 and removal of Polycomb-group proteins was shown to be essential upon ATRA stimulation. JMJD3 demethylase activation is connected to dissociation of SMRT co-repressor, which will lead to demethylation of H3K27me3 and removal of the Polycomb-group proteins [144]. Another demethylase, the UTX while activated by ATRA stimulation, replaced histone methylase EZH2 from ATRA-responsive genes and H3K27me3 demethylation [79, 145].

The formation of PML-RARα fusion protein also disrupts normal localization of co-called PML oncogenic domains (PODs) [135, 146]. The PML gene is believed to be a tumor suppressor gene and mediates inhibition of tumor growth and pro-apoptotic effect [147, 148]. Upon ATRA stimulation, the PML-RARα fusion protein is degraded and PODs are reassembled [149]. The PML pro-apoptotic effect is regulated via Fas- and caspase-dependent pathway and induced in response to TNFα induction [147].

**NFκB signalling**

Tumor necrosis factor α (TNFα) has been shown to play an important role in inflammatory and immunological responses, as well as in cell proliferation and apoptosis [150]. TNFα has also been proposed to function as an efficient anti tumor agent [151]. Upon TNFα induction nuclear factor-κB (NFκB)
signalling is activated. The NFκB signalling pathway consists of several components; the NFκB/Rel family, which include p65, RelB, c-Rel, NFκB1 and NFκB2, all sharing Rel homology domain (RHD) responsible for DNA binding and homo- and heterodimers formation. The second component is the IκB family, which includes IκBα, IκBβ, IκBε, IκBγ, Bcl-3 and IκBζ, shares the ankyrin repeat domain (ANK). The third component is IKK complex, which includes 3 members, IKKα, IKKβ and IKKγ which are responsible for degradation of IκB upon activation [152]. Without stimuli, NFκB transcriptional factors are bound to IκB proteins and localised in the cytoplasm [153]. Upon stimulation the IκB proteins are phosphorylated and ubiquitinated, which would lead to release of TFs dimers and transport them to the nucleus. In the nucleus the TFs dimers would bind to the promoter or enhancer region of the NFκB pathway genes and initiate the transcription. The IKKα and IKKβ are the mediators of IκB proteins and have 70% protein similarity. However, they have a different function. There are two distinct NFκB signalling pathways: one is called canonical pathway, which is activated by IKKβ, and leads through TNFR-associated factor (TRAF)/RIP complexes and is responsible for the innate immunity. The noncanonical pathway is activated by IKKα, through TRAFs and NFκB inducing kinase (NIK) and is responsible for adaptive immunity and lymphoid organogenesis [154]. In the canonical pathway IκBα, IκBβ and IκBε are phosphorylated and ubiquitinated. The IKKγ is required for IKK complex formation and essential for activation of the classical pathway [155]. The alternative pathway proceeds via proteasomal processing of p100 and activation of p52:RelB dimers [156]. The transcription of the genes is further regulated by post-translational modifications. In addition to the phosphorylation of IκBα, the activity of the NFκB signalling pathway is controlled via IKKα-induced phosphorylation of RelA/p65 leading to the removal of HDAC1 and SMRT-HDAC repression complexes, followed by recruitment of co-activator complexes, such as p160s [157], and the CBP/p300 complex [158-161]. NFκB also activates the expression of JMJD3 demethylase and also activates the BCL2 gene which is silenced by the Polycomb-protein group [80]. The TNFα stimuli activate the canonical NFκB signalling pathway via the TNF receptor. There are other nuclear receptors known to activate the canonical NFκB signalling pathway: Toll/IL-1 receptor [162, 163], T-cell receptor (TCR) and B-cell receptor (BCR) [164, 165]; and DNA damage [166, 167].
Present investigation

Aims

The aims of this thesis were: 1) to analyse epigenetic changes and their influence on gene transcription in the ICSBP/IRF8 gene in multiple myeloma and 2) to analyse the influence of TNFα on histone modifications in the human monoblastic U-937 cell line.

Specific aims:

- To determine the relationship between DNA methylation and histone modification in regulation of ICSBP/IRF8 gene expression. (Paper I)

- To analyse the epigenetic state and possible repression by DNA methylation of the ICSBP/IRF8 gene in a panel of MM cell lines and primary tumors. (Paper II)

- To determine the role of the NFκB signalling pathway in the control of differentiation and cell cycle in ATRA induced U-937 cell line. (Paper III)

- To determine the mechanism behind combined TNFα and ATRA stimulation and its association with histone modification in RA-responsive genes. (Paper IV)
Paper I: “Positive histone marks are associated with active transcription from a methylated ICSBP/IRF8 gene”

Epigenetics has been suggested to play a key role in tumorigenesis [121, 168-170]. There are two epigenetic mechanisms which influence gene expression: DNA methylation and chromatin remodelling. Methylation of DNA takes place at CpG dinucleotides at the C5 position of the pyrimidine group, and is mediated by a family of enzymes called DNA methyl transferases (DNMTs) [171]. There is another class of proteins, methyl CpG-binding proteins (MBD proteins) [39], which bind to methylated DNA and repress or activate the transcription depending on the activity of the associating partners. As a general rule, methylation of DNA is associated with gene silencing. A few exceptions to this paradigm have been reported, in most cases involving the methylation of regions distal to the promoter [13, 14, 16, 172]. However, direct evidence demonstrating methylation of a transcriptionally active promoter is very rare, and has been suggested to be a consequence of an activator with the capacity to bind methylated DNA, RFX [21] or affecting repression by MBD displacement by an activator [18], or the conversion of MBD2 to an activator by associated proteins [6].

Aim: The aim of this study was to analyse the relation between gene expression and methylation status of the ICSBP/IRF8 gene in the human monoblastic U-937 cell line.

Results: We found that the ICSBP/IRF8 gene is expressed in the U-937 cell line. Paradoxically, methylation analysis of the CpG island in the ICSBP/IRF8 gene promoter revealed complete methylation of all 39 CpG positions. However, chromatin immunoprecipitation (ChIP) analysis demonstrated acetylation of histone 3-lysine 9 and trimethylation of histone 3-lysine 4 in the ICSBP/IRF8 promoter.

The finding that the ICSBP/IRF8 promoter was completely methylated in U-937 cells was unexpected. To establish that transcription was initiated at the previously identified transcriptional start site (P1) we analysed the transcripts by 5’-RACE. A second promoter region (P2) was identified 18kbp upstream from P1. The P2 promoter does not have a CpG island. Transcription from P2 produces a RNA containing a perfect match for a splice site that adds a small exon to the previously identified exon 1, constituting a part of the 5’-UTR. However, transcription initiated at P2 only constituted a minor fraction of the transcripts, making it an unlikely explanation for the
ICSBP/IRF8 expression in U-937 cells. Presence of RNA-Polymerase II at a major transcriptional start site, analysed by ChIP assay, suggests active gene transcription.

To investigate the chromatin structure at the ICSBP/IRF8 promoter we used a ChIP assay, analysing the H3K9-ac, H3K4me3 and H3K9me3. We found that the ICSBP/IRF8 promoter was associated with H3-K9-ac and H3K4me3 in U-937 cells, consistent with an actively transcribed gene. In contrast, as would be expected, the methylated ICSBP/IRF8 promoter in a non-expressing cell (the U-266-1984 myeloma cell line) was associated with trimethylation of H3-K9, suggesting a transcriptionally silent chromatin. This raises the question by which mechanism the methylated ICSBP/IRF8 promoter is activated in U-937 cells? It was recently shown that RFX, a transcription factor involved in the regulation of MHC class II expression, is capable of sequence-specifically binding to methylated DNA and can mediate transcriptional activation from a methylated HLA-DR promoter [21]. The fact that the ICSBP/IRF8 promoter contains a consensus RFX binding site 1500bp upstream from TSS (http://www.genomatix.de/), and that U-937 cells express MHC class II and can express RFX [173] makes this an attractive explanation. Another possibility is suggested by the recent observations that MBD2 can interact with MBD2in [6] or TACC3 [20], in both cases relieving transcriptional repression and activating a methylated promoter. However, the expression of MBDin and TACC3 in U-937 cells is not known. The effect of reducing promoter methylation on ICSBP/IRF8 expression is interesting. With 1μM DAC treatment we could decrease the methylation by 25 % and with 10μM DAC treatment by 75%, but at the same time this resulted in a decrease in the expression level of the ICSBP/IRF8 gene (Tshuikina, unpublished observation). This could either mean that treatment with a demethylation drug, such as DAC which will rather unspecifically affect the methylation of the whole genome, has secondary effects leading to decreased expression, or that transcriptional activation of the ICSBP/IRF8 gene requires methylation of the promoter to function. U-266-1984 treated with 1μM and 10μM DAC showed a decrease of the DNA methylation level, but also an increase in the ICSBP/IRF8 gene expression [24], suggesting that differences in activation of ICSBP/IRF8 could also be cell specific.

In conclusion, our results suggest that the U-937 cell line contains an unidentified activity that mediates histone modifications (H3-K9-ac and H3K4me3) which recruit the RNA-Polymerase II at the ICSBP/IRF8 promoter, resulting in a chromatin structure permissive for transcription, which over-rides the silencing effect of DNA-methylation.
Paper II: "Epigenetic silencing of the interferon regulatory factor ICSBP/IRF8 in human multiple myeloma."

Multiple myeloma (MM) is presently an incurable and very heterogeneous malignant haematological disease. The clonal expansion of malignant plasma cells/plasmablasts takes place in bone marrow (BM) and MM cells and is very dependent on the BM microenvironment for survival, growth and differentiation. Several non-random translocations were shown to be implicated in development of MM. Several genes, such as p15INK4B, p16INK4A, DAP-kinase, SOCS-1 and genes involved in the Wnt pathway, show hypermethylation of their promoter regions. This was shown to occur in 20-40% of MM patients. However, to what extent epigenetic changes occur and contribute to pathogenesis of MM is still widely unknown. The IRF proteins are a family of transcriptional regulators involved in haematopoietic cell development as well as the early immune response to pathogens. Apart from the high expression of IRF4, which in approximately 5% of MM cases is the result of t(16;14), expression of other IRFs have not been systematically investigated.

Aim: The aim of this study was to analyse the expression of the IRF gene family members in a panel of human MM cell lines and a series of other B-cell leukemias, and investigate if a potential loss of expression was the result of silencing by DNA-hypermethylation.

Results: We analysed the IRF family expression by western blot and realtime qRT-PCR in a panel of 13 human MM cell lines. The expression of IRF3, IRF4, IRF5 and IRF9 was found to be uniformly high in all lines, whereas IRF1, IRF2 and IRF7 displayed a heterogeneous pattern of expression. Strikingly, expression of the ICSBP/IRF8 mRNA and protein was absent or low in MM cells as compared to normal PBMC. A CpG island was identified in the ICSBP/IRF8 gene and analysis of DNA-methylation showed methylation of a 308bp region, containing 39 CpG positions, at the transcriptional start site. There was a strong correlation between gene expression and methylation pattern among the cell lines examined. The ICSBP/IRF8 and IRF4 gene’s expression and methylation status were analysed in nine CD138+ purified primary tumors from MM patients. One out of 9 patients showed partial methylation in the promoter region. In vitro methylation of the ICSBP/IRF8 promoter repressed transcriptional activity, and demethylation by treatment of MM cell lines with 5-deoxy-2’-azacytidine (DAC) induced endogenous ICSBP/IRF8 expression.

The finding that ICSBP/IRF8 gene expression was undetectable in a majority of the MM cell lines and very low in U-1958, EJM, Karpas 707 and
RPMI 8226 cell lines indicated that absence of ICSBP/IRF8 could be part of the MM phenotype. This was supported by the panel of B cell leukemia cell lines, arranged in order of increasing differentiation according to antigen expression, which displayed increasing expression of ICSBP/IRF8, suggesting that mature B cells do express the ICSBP/IRF8. In contrast, expression of ICSBP/IRF8 has been reported to be higher in CD19⁺ B cells than in plasma cells; supporting the interpretation that down-regulation occurs at later stages of normal B cell differentiation [174].

So what could be the consequences of a loss of the ICSBP/IRF8 expression? Absence of ICSBP/IRF8 would lead to a shift in the IRF transcription factor network affecting the expression of a large number of genes, which ultimately may contribute to malignant transformation. Knock-out of ICSBP/IRF8 in mice results in a chronic myelogenous leukemia-like syndrome [110], and a lack of ICSBP/IRF8 expression has also been associated with human leukemia [111]. Moreover, deficiency in the ICSBP/IRF8 expression has been associated with increased resistance to apoptosis and defects in myeloid cell differentiation [112].

We found no genetic aberrations that could explain the lack of ICSBP/IRF8 expression. In contrast, methylation analysis revealed a 308bp CpG island in the promoter region densely methylated in non-expressing MM cell lines. A methylated CpG island in the promoter would be predicted to result in gene silencing by the binding of methyl CpG binding proteins (MBD) and recruitment of histone deacetylases and chromatin remodelling enzymes [39]. The fact that we observed a strong correlation between gene expression and methylation patterns among the analysed MM cell lines is suggestive of a causative relationship. However, two additional findings support that demethylation of the ICSBP/IRF8 promoter is needed for gene expression in MM cells. Firstly, by performing in vitro methylation of a cloned ICSBP/IRF8 promoter and analysing the activity of a luciferase reporter we observe almost complete repression of the transcriptional activity in U-266-84 cells. Secondly, it was possible to restore the endogenous ICSBP/IRF8 gene expression by using the demethylation drug 5-Aza-2’-deoxycytidine (DAC). Combinations of DAC and the deacetylase inhibitor Trichostatin A (TSA) induced expression even further, supporting the notion that the ICSBP/IRF8 gene is actively repressed in MM cell lines. The ICSBP/IRF8 gene could not be induced by IFN-γ in MM cells, despite that the cells are responsive to this cytokine, as judged by the induction of IRF1. A tentative explanation for this observation is that the main mediator of the IFN-γ signal, the transcription factor Stat1, binds to a sequence motif in the promoter in which CpG’s are methylated, possibly interfering with DNA binding.
It should be noted that the question whether silencing of ICSBP/IRF8 by methylation is attributable to the malignant phenotype or associated with the terminal stages of normal B cell differentiation needs to be addressed. The ways by which tumorigenic changes in DNA methylation could be acquired during the process of myeloma development are completely unknown. The DNA methyl transferase DNMT1 has been reported to be over-expressed in myeloma compared to normal bone marrow plasma cells [175]. Moreover, interleukin 6 (IL-6), an important survival factor for myeloma cells, has been shown to induce expression of DNMT1 in a MM cell line KAS 6/1 [176]. However, a higher frequency of DNA-methylation is often found in cultured cell lines than in primary cells [177]. That suggest that there are other epigenetic silencing mechanisms that could be involved in the ICSBP/IRF8 gene inactivation. This was supported by recently described involvement of polycomb group proteins in prostate cancer [178]. Therefore, this issue can only be resolved by analysing purified CD138+ primary myeloma cells and comparing the methylation status of ICSBP/IRF8 to purified normal bone marrow plasma cells.

In conclusion, the expression of ICSBP/IRF8 gene is frequently lost in MM cell lines and our findings suggest that this is a direct consequence of promoter DNA-methylation. The function of the ICSBP/IRF8 gene as a tumor suppressor gene and our finding of DNA methylation in one of the primary tumor samples, suggests that the DNA methylation of the ICSBP/IRF8 may influence the malignant phenotype of MM. In view of the previously reported function of ICSBP/IRF8 in regulating differentiation and apoptosis, as well as its role in myeloid leukemia, it is tempting to speculate that silencing of ICSBP/IRF8 contributes to the biology of myeloma.

Paper III: "Activation of NFκB in all-trans retinoic acid induced differentiation, cell cycle arrest and p21 (CDKN1A/Waf1) expression in U-937 cells."

The NFκB signaling pathway was shown to be induced upon ATRA induction during differentiation of monocytes to macrophages and during differentiation of NB4 cells [179-181]. The NFκB pathway is important for survival of differentiating cells [182, 183] and activated upon TNFα stimuli and all-trans retinoic acid ATRA, where the combination of these drugs showed synergistic effect on myeloid differentiation [184, 185]. The NFκB family consists of homo- and heterodimers, which in inactive form are located in the cytoplasm bound to IκB inhibitory proteins [186]. Upon stimulation the IKK complex phosphorylates IκBα and IκBβ proteins, triggers the IκB pro-
teins ubiquitination and proteosomal degradation. The NFκB dimmers would be released and translocated into the nucleus activating the transcription [186]. There are two distinct NFκB pathways, classical and alternative. The IKKβ is responsible for the activation of the classical pathway [186], and the IKKα is activator of the alternative pathway [153, 187].

Arrest in Go/G1 phase is tightly linked to terminal differentiation. ATRA induce Go/G1 arrest in myeloid differentiation by down-regulating cyclines E, D3 and B and up-regulating CDK inhibitors (CKIs) p21\textsuperscript{WAF1/CIP1} and p27\textsuperscript{KIP1} [188-191]. Inducible expression of p21\textsuperscript{WAF1/CIP1} also regulates monocytic differentiation [189, 192] and also functions as anti differentiation-induced apoptosis [192-195].

Aim: The aim of this study was to investigate the role of NFκB in ATRA induced differentiation.

Results: U-937 sublines containing constitutively expressing the NFκB repressor IkB (S32A/S36A) and control vector were generated. Analysis of induced differentiation in generated sublines in response to ATRA and 1α,25-dihydroxy-vitamin D3 treatment was analyzed by FACS. The IkB (S32A/S36A) sublines showed reduced up-regulation of CD11c marker and also delayed G1 arrest after ATRA treatment. However, expression of IkB (S32A/S36A) by 1α,25-dihydroxy-vitamin D3 treatment did not affect the differentiation and G1 arrest. It was previously shown that there is a link between NFκB activation and p21\textsuperscript{WAF1/CIP1} [183, 193, 196, 197]. The mRNA expression of p21\textsuperscript{WAF1/CIP1} was analyzed by qRT-PCR in sublines IkB:3 and IkB:6 and control luc:1 clones. There was differentiation-associated increase in p21\textsuperscript{WAF1/CIP1} mRNA expression in generated sublines and corresponding lack of expression in the control clone. The activation of NFκB signaling pathway with subsequent activation of p21\textsuperscript{WAF1/CIP1} was shown to be essential in PMA-stimulated U-937 cell lines and functions as protector for anti differentiation-induced apoptosis [183]. However, FACS analysis did not reveal any differences in amount of apoptotic cells between IkB (S32A/S36A) and control clones. The analysis of RARE-induced reporter in sublines showed reduced ectopic expression of IkB (S32A/S36A), showing that activation of NFκB is important for RARE-driven target genes. Inhibition of NFκB would indicate the impairment of p21\textsuperscript{WAF1/CIP1} induction in U-937 cell line. In case of NB4 cell line this impairment does not occur due to PML-RARα fusion protein formation and maturation-associated apoptosis is induced via induction of TRAIL and caspase-8 [181]. U-937 and NB4 cell lines also acquire phenotypical difference after ATRA treatment. U-937 cell line will differentiate into monocytes with a long life span, which will integrate into tissues and survive as macrophages. The NB4 cell line will differen-
entiate into granulocytes with short life span (2-3 days) in circulation system.

Taking together the results, the NFκB signaling pathway is essential for ATRA-induced terminal differentiation in the U-937 cell line.

Paper IV: “TNFα induces a reduction of histone 3 lysine 9 trimethylation and dimethylation (H3K9me3 and H3K9me2) at all-trans retinoic acid target genes.”

TNFα plays an important role in many regulatory mechanisms, cell proliferation and apoptosis and also in inflammatory and immunological responses, functioning as anti tumor agent [150, 151]. The NFκB signalling pathway is activated upon TNFα stimulation via TNFR [198, 199]. The combination of TNFα and all-trans (ATRA) retinoic acid synergistically increase the activation of haematopoietic cell differentiation and TNFα-induced apoptosis [184, 200, 201].

There are several histone modifications that were shown to be triggered by both TNFα and ATRA: phosphorylation and ubiquitination of IkB upon stimulation, leads to phosphorylation of RelA/p65, which would lead to removal of HDAC1 and SMRT-HDAC co-repressor complexes and recruitment p160s and CBP/p300 of co-activator complexes [157-161]. With ATRA treatment HAT-containing complexes are recruited by co-activators of p160, which will also lead to acetylation of histones [139]. Demethylation of H3K27me3 by JMJD3 and UTX and removal of Polycomb-protein group by ATRA, was shown to play an important role in activation of ATRA-responsive genes [79, 144, 145]. However, the histone modification which would influence TNFα induced synergy in combination with ATRA is still not known.

**Aim:** The aim of this study was to investigate the mechanism and associated histone modification in response to TNFα treatment of RA-responsive genes.

**Results:** mRNA kinetic expression was analysed in five, CD38, C/EBPα, RARβ and CDKN1A, RA-induced genes in response to ATRA, TNFα and a combination of both drugs in U-937 and NB4 cell lines. In U-937 cell line the expression with ATRA increased already after 6 hours of treatment. TNFα alone did not increase gene expression except for CDNK1A. However, the combined treatment of both drugs affected the dynamic expression in a complex way. There was an enhanced increase in expression already after 6 hours in C/EBPα, CDNK1A and RARβ, followed by decrease at 24h,
and increase again, resembling biphasic pattern. The expression pattern in
the NB4 cell line was different from that in U-937, but rapid increase after
6h was common for both of them.

ATRA induced G1 arrest in U-937 cell line 72 h after treatment. In combin-
ing both drugs, it was clearly shown that ATRA rescues U-937 cells from
TNFα induced apoptosis. Cell cycle analysis of the NB4 cell line did not
reveal any changes with or without treatment. Different responses to stimuli
could be due to PML-RARα fusion protein in the NB4 cell line. It was pre-
viously shown that higher doses of ATRA in combination with HDAC in-
hibitors are needed [202]. Also due to PML differences in oligomerization
several co-repressor complexes are attracted [203].

We analysed the histone modifications in CD38 and C/EBPε genes in re-
ponse to ATRA, TNFα and combination of both drugs. There was increase
in H3K9 acetylation after ATRA and ATRA/TNFα stimulation. Our results
are supporting previously shown data. It was shown that in absence of a RA-
ligand the ATRA responsive genes are repressed by HDAC co-repressor
complexes [138]. Upon ATRA treatment HAT-containing complexes are
recruited, which would lead to histone acetylation [139]. H3K4me3 and
H3K9me3 were decreased by ATRA in the combination of both drugs. That
could be explained by the recent publications showing ATRA induced re-
cruitment of demethylases JMJD3 and UTX, removing H3K27me3 and
Polycomb-group proteins [79] or also recruitment of LSD1 demethylase in
combination with HMT/HDM [204].

TNFα alone did not increase H3K9-ac. However, it showed a decrease in
H3K4me3 and H3K9me3 and even more pronounced decrease of H3K4me2
and H3K9me2 in both CD38 and C/EBPε genes in the U-937 cell line. Simi-
lar decrease in H3K9me2 could be seen in the NB4 cell line as well. Our
results suggest that TNFα alone can trigger demethylation in ATRA respon-
sive genes. The LSD1 demethylase was shown to be associated with nuclear
receptors, but it is specific for demethylation of H3K9me2/H3K9me1 and
H3K4me2/H3K4me1 [205]. Since we saw the demethylation of H3K9me3
and H3K4me3, another demethylase should be tested.
Figure 4. Suggested mechanism for TNFα function in activation of ATRA responsive genes. In the absence of a ligand the RA-responsive genes are silenced by HDAC and co-repressor complexes. The chromatin is also in heterochromatic stage. LSD1 is present. Upon TNFα treatment, no gene activation occurs, however, the chromatin structure changes from heterochromatin to euchromatin, H3K9me3 to H3K9me2 and H3K4me3 to H3K4me2, but the lysine demethylase (KDM-X) still remains unknown. Upon RA ligand binding, HAT and co-activator complexes are recruited. Demethylation of H3K9me2, H3K4me2 and H3K9-ac has occurred. RA-responsive genes are expressed.

Taking all results together, we can conclude that combination of ATRA and TNFα enhance RAR-responsive genes expression at early time points, but not TNFα alone. However, TNFα alone can trigger demethylation of H3K4me3/H3K4me2 and H3K9me3/H3K9me2 and that may pave the way for subsequent ATRA induced transcription.
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References


113. Hashmueli, S., et al., A truncated IFN-regulatory factor-8/IFN consensus sequence-binding protein acts as dominant-negative, inter-


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