Characterization of signaling pathways in normal and malignant hematopoietic cells by microarray technologies

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Contents

Abbreviations

ABC	activated B-cell
ALL	acute lymphoblastic leukemia
APC	antigen presenting cell
BAC	bacterial artificial chromosome
BCR	B-cell receptor
BFU-E	erythroid burst forming unit
BL	Burkitt lymphoma
BM	bone marrow
BMP	bone morphogenetic protein
B-NHL	B-cell Non-Hodgkin's lymphoma
CD	cluster of differentiation (antigens)
CFU-E	erythroid colony forming units
CGH	comparative genomic hybridization
CKI	cyclin dependent kinase inhibitor
CLL	chronic lymphocytic leukemia
CLP	common lymphoid progenitor
DLBCL	diffuse large B-cell lymphoma
Epo	erythropoietin
EpoR	erythropoietin receptor
FACS	fluorescence-activated cell sorter
FCM	flow cytometry
FDC	follicular dendritic cell
FISH	fluorescence in situ hybridization
FL	follicular lymphoma
GC	germinal center
GCB	germinal center B-cell
HC	heavy chain
HD	Hodgkin’s disease
HLH	helix-loop-helix
HPC	hematopoietic progentior cell
HSC	hematopoietic stem cell
ID	inhibitor of DNA binding

IFN	interferon
Ig	immunoglobulin
IGH	immunoglobulin heavy-chain
IKK	IkB kinase
IL	interleukin
KIT	stem cell factor receptor
LC	light chain
MALT	mucosa associated lymphatic tissue
mbp	mega base pairs
MCL	mantle cell lymphoma
MHC	major histocompatibility complex
MM	multiple myeloma
PC	plasma cell
PI3K	phosphoinositide-3-kinase
SCF	stem cell factor
TGF-β	transforming growthfactor β
Th	T-helper

1 List of publications

I. Galteland E, Sivertsen EA, Svendsrud DH, Smedshammer L, Kresse SH, Meza-Zepeda LA, Myklebost O, Suo Z, Mu D, Deangelis PM, Stokke T. Translocation t(14;18) and gain of chromosome 18/BCL2: effects on BCL2 expression and apoptosis in B-cell non-Hodgkin's lymphomas. Leukemia. 2005 Dec;19(12):2313-23.

II. Sivertsen EA, Galteland E, Mu D, Holte H, Meza-Zepeda L, Myklebost O, Patzke S, Smeland EB, Stokke T. Gain of chromosome 6p is an infrequent cause of increased PIM1 expression in B-cell non-Hodgkin's lymphomas. Leukemia. 2006 Mar;20(3):539-42.

III. Sivertsen EA, Hystad ME, Gutzkow KB, Dosen G, Smeland EB, Blomhoff HK, Myklebust JH. PI3K/Akt-dependent Epo-induced signalling and target genes in human early erythroid progenitor cells. Br J Haematol. 2006 Oct;135(1):117-28.

IV. Sivertsen EA, Huse K, Hystad ME, Kersten C, Smeland EB, Myklebust JH. Inhibitory Effects and Target Genes of BMP-6 in human T-cells. *Submitted*

2 Introduction

The development of new techniques in molecular biology during the last decades has opened for new experimental possibilities and, to a large extent, changed the way biological research is performed. The human genome is now fully sequenced (1). Use of high throughput technologies, like the microarray technology, generate enormous amounts of reliable data in a very short time (2). The functional interpretation of such data is still challenging, but use of the new technologies has without doubt greatly improved our biological understanding. For example, it is already proved that genome wide expression profiling is a tool that can be used to define new disease entities in lymphomas (3). Hopefully, the better molecular understanding of diseases will also lead to improved therapy. In this thesis, microarray technology has been used to characterize how selected signaling pathways in hematopoietic cells are affected by physiological and pathological processes.

2.1 The hematopoietic system

The function of the hematopoietic stem cell system is to produce and maintain mature cellular components of blood and lymph (4). The various tasks performed by the fully differentiated cells include transport of oxygen, defense against infection and hemostasis. Mature blood cells have limited life spans and are continuously produced from a pool of multipotent hematopoietic stem cells (HSCs). Homeostasis is achieved through tight regulation of proliferation, differentiation and apoptosis of stem cells, progenitors and mature cells.

2.1.1 Hematopoietic stem cells

Stem cells have three unique properties:

- Stem cells have the ability to self-renew. By cell division stem cells can produce daughter cells that are identical to the parent cells.
- Stem cells are not specialized cells. They do not perform any other tasks than to serve as parent cells for other cell types.
- Stem cells differentiate into multiple specialized cell types.

Different classes of stem cells are responsible for renewal of the various tissues. The concept of plasticity, meaning that one class of adult stem cells can trans-differentiate and generate differentiated cells of another tissue, is debated (5-7). Likewise, while embryonic stem cells are pluripotent and can differentiate into all cell-types of the body, the presence of pluripotent stem cells in the adult bone marrow (BM) has yet to be proven.

Hematopoietic stem cells are responsible for renewal of all types of blood cells (8). These stem cells are multipotent meaning that they can differentiate into mature blood cells of all lineages within this organ. HSCs also have life-long self-renewal potential. Thus, HSCs have the ability to repopulate the bone marrow when transplanted into recipients who have received myoablative chemotherapy or irradiation. Hematopoietic progenitor cells (HPCs) are the immediate progeny of HSCs. In contrast to HSCs, HPCs have lost the ability to self-renew. As these cells divide and move toward terminal differentiation, they also gradually loose their multipotential and proliferative capacity.

2.1.2 Erythropoiesis

Erythropoiesis is the process by which multipotent hematopoietic stem cells differentiate into mature erythrocytes. In adult humans erythropoiesis occurs in the central sinus beds of medullary marrow. The erythroid burst forming units, BFU-E, are the first cells of the erythroid lineage that can be recognized. These cells are progeny of hematopoietic progenitor cells called CFU-GEMM, which can differentiate into granulocytes, monocyte and megakaryocyte as well as erythrocyte lineages *in vitro*. BFU-E express CD34, erythropoietin receptor, EpoR, and start to express transferrin receptor, CD71. BFU-E give rise to erythroid colony forming units, CFU-E. These cells have lost expression of CD34, but not of EpoR and CD71. CFU-E give rise to proerythroblasts, that express glycophorin A, GPA, and CD71 (9). The next steps in the development are erythroblasts, reticulocytes and finally, erythrocytes. The differentiation is characterized by a reduction in cell volume, condensation of chromatin, extrusion of the nucleus and increased synthesis of hemoglobin. A mature erythrocyte functions to transport O_2.

Erythropoietin, Epo, is produced in the kidneys in response to low O_2 tension in the blood and is the key cytokine regulator of erythropoiesis. It exerts its effects on erythroid progenitors upon binding to EpoR. EpoR expression begins at the BFU-E stage. The progression through the CFU-E stage and further developmental stages is completely dependent on Epo signaling (10;11). To obtain efficient erythropoiesis, several other cytokines are necessary of which the most prominent is stem cell factor, SCF, the ligand of the receptor c-kit (12). Efficient signal transduction through both EpoR as well as c-kit is necessary for survival, proliferation and differentiation of erythroid progenitors (13).

2.1.3 B-cell development

B-lymphocytes produce and secrete soluble antibodies that recognize and bind to non-self antigens, typically antigens of an invading micro organism (14). An antibody is the secreted form of the B-cell receptor. B-cells develop in the bone marrow through interaction with stromal cells, but they mature to functional cells in peripheral lymphoid organs like lymph nodes and spleen. A B-cell may develop to a long lived memory cell with a unique receptor that recognizes one specific antigen. The terminally differentiated cell is a plasma cell that secretes antibodies of unique specificity. Different processes in the B-cell development ensure that a vast antibody repertoire is available to the individual.

The B-cell receptor, BCR, consists of two pairs of polypeptide chains, the immunoglobulin heavy and light chains (HC and LC). In the BM, B-cell progenitors rearrange their immunoglobulin genes as a prerequisite for the expression of a functional B-cell receptor. This first phase of B-cell development is antigen independent, but the microenvironment in the BM provides the signals necessary for proper lymphopoiesis. Interestingly, it was recently shown that CD10+ stromal dendritic cells form maturation niches for B-lymphocytes in the BM (15). In the adult BM, B-cells arise from multipotent HSCs that develop through discrete stages that are identified by rearrangement of the immunoglobulin HC (*IGH*) and LC genes, by expression of surface molecules and by expression of enzymes involved in rearrangement of receptor genes (16). The common lymphoid progenitor cells (CLPs) can develop into B-, T- or NK-cells. CLPs express *TDT* and *RAG* genes. Activity of these enzymes is important for the gene rearrangements that are required to generate a functional HC. Rearrangement of Diversity (DH) and Joining (JH) gene segments is followed by rearrangement of the Variable (VH) gene segment at the pro-B cell stage, resulting in a VDJ HC gene (VDJ_H), which marks commitment to the B-lineage. A functional VDJ_H rearrangement is essential for differentiation into the next stage, the pre B-cell stage. Cells that undergo successful rearrangement of the VDJ_H are positively selected, whereas failure to rearrange a functional VDJ_H results in apoptosis. In the pre-B cell, the assembled VDJ heavy chain forms together with a temporarily expressed surrogate light chain and the signal transducing heterodimer IgA/IgB the pre-B cell receptor. Signaling through this receptor promotes light chain (VJ_L) rearrangement and allelic exclusion at the *IGH* locus. Once a successful VJ_L rearrangement has taken place, LCs are expressed and combine with HCs as well as IgA/IgB to form the BCR that is expressed on immature B-cells. Now the cell is tested for self reactivity. Self

reactive immature B-cells that are not rescued by rearrangement of a new LC (receptor editing) are negatively selected and undergo apoptosis or become anergic (17). The immature B-cells that are not eliminated leave the BM and enter the blood stream from which they migrate to the peripheral lymphoid organs.

The peripheral lymphoid organs include the spleen, lymph nodes as well as gut and mucosa associated lymphatic tissue, MALT. These organs function to trap antigens from invading microorganisms and to present these antigens to circulating lymphocytes. The lymph node, LN, is composed of the cortex, where most B-cells are found, paracortex (T-cell area) and the central medulla (dominated by macrophages and plasma cells). Antigen and antigen presenting cells (APCs) from the periphery enter the lymph node via afferent lymphatic vessels.

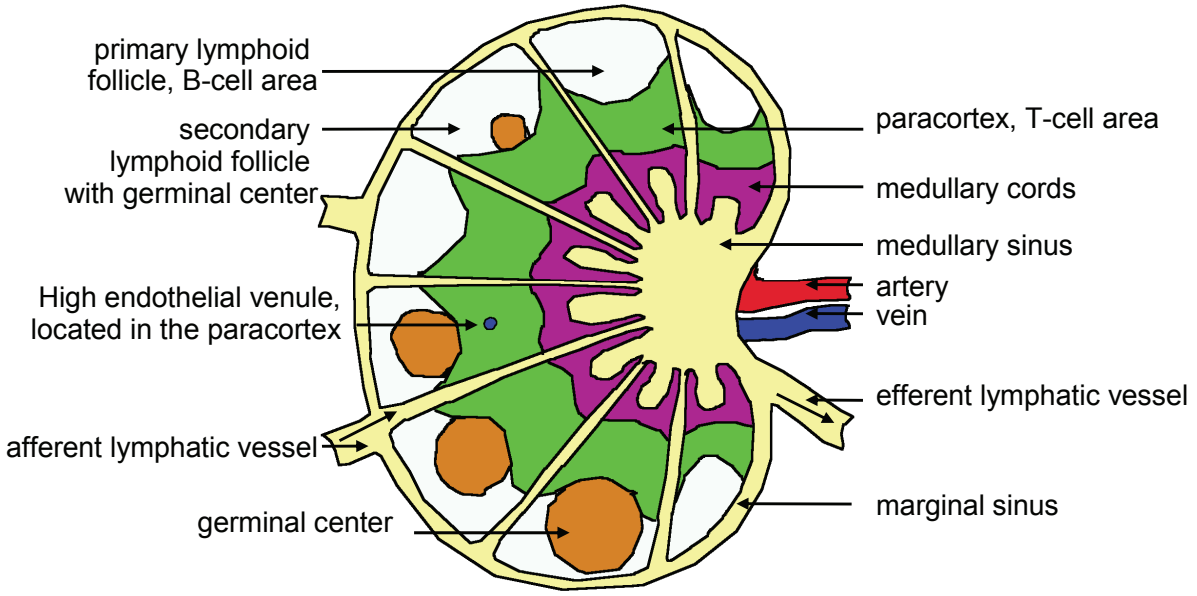


Figure 1 The structure of a lymph node. Antigen and APCs are brought to the LN via afferent lymphatic vessels. Lymphocytes enter through high endothelial venules. The fate of the lymphocyte depends on whether it encounters its antigen. Lymphocytes and plasma cells leave the LN via the efferent lymphatic vessels. Memory B-cells and plasma cells with increased affinity to antigen are generated in the GC. Modified from Janeway et al. (14)

The naïve B-cells circulate from the blood into the peripheral lymphoid organs, which they enter by passing between the specialized endothelial cells of the high endothelial venules. Upon entry to a peripheral lymphoid organ, the fate of a B-cell depends on whether it encounters and is able to bind an antigen to its receptor.

A B-cell that has not bound antigen migrates quickly to the B-cell zone. In the primary follicles of this zone essential survival signals are provided to the naïve B-cell without which

it cannot survive, and thus, if the B-cell is unable to enter a primary follicle it will undergo apoptosis in a few days. Having received survival signals in the primary follicle, the B-cells will enter the blood stream again and circulate between blood and peripheral lymphoid organs until it encounters an antigen and becomes activated, or undergoes apoptosis after a few weeks in circulation (14).

B-cells that have bound antigen become activated and are trapped in the T-cell areas. Here they interact with T-cells with specificity for the same antigen that is presented on the B-cells MHC class II molecules (18;19). These T- and B-cells form a primary focus of proliferation and clonal expansion, and some B-cells differentiate into plasma cells that migrate to the medullary chords (or to the red pulp in the spleen) and secrete IgM antibodies. Alternatively, the antigen-primed B-cells may migrate into primary follicles in the B-cell area which now are referred to as secondary follicles. Seven to ten days after initial antigen priming, a germinal center, GC, is established in the secondary follicle (19). The function of the GC is to generate memory B-cells and plasma cells with increased affinity to antigen. It is mainly a B-cell area, but very important signals are delivered to the B-cells by helper T-cells. Follicular dendritic cells, FDCs, that bind immune complexed antigens at their surface are also found here, providing the B-cells with survival signals.

In the GC intense proliferation takes place displacing the resting B-cells of the primary follicle towards the periphery, the mantle zone. The GC itself is polarized with proliferating B-cells called centroblasts in the dark zone, proximal to the T-cell area, and more resting B-cells, centrocytes, in the light zone. Three crucial activities take place in the GC:

- B-cell receptor diversification in the dark zone. In a process called somatic hypermutation the affinity of the B-cell receptor to its antigen is altered. Normally only the Ig variable region genes undergo this process, but aberrant hypermutation may take place in lymphomas (20). Whether receptor editing also plays a role in the diversification process is still debated, but the cells express low amounts of rearrangement enzymes (21) .

- Selection of high affinity B-cells in the light zone. B-cells with increased affinity to the antigen, present in the light zone as immune complexes on FDCs, are selected for. The selected cells either exit the GC reaction to become memory B-cells or plasma

cells or reenter the GC cycle. Cells with lower affinity undergo apoptosis and are cleared by macrophages.

- Isotype switching. By deletion of the DNA region between the V region and various C regions of the HC locus, the effector function of the antibody is changed whereas the affinity to the antigen is unaltered.

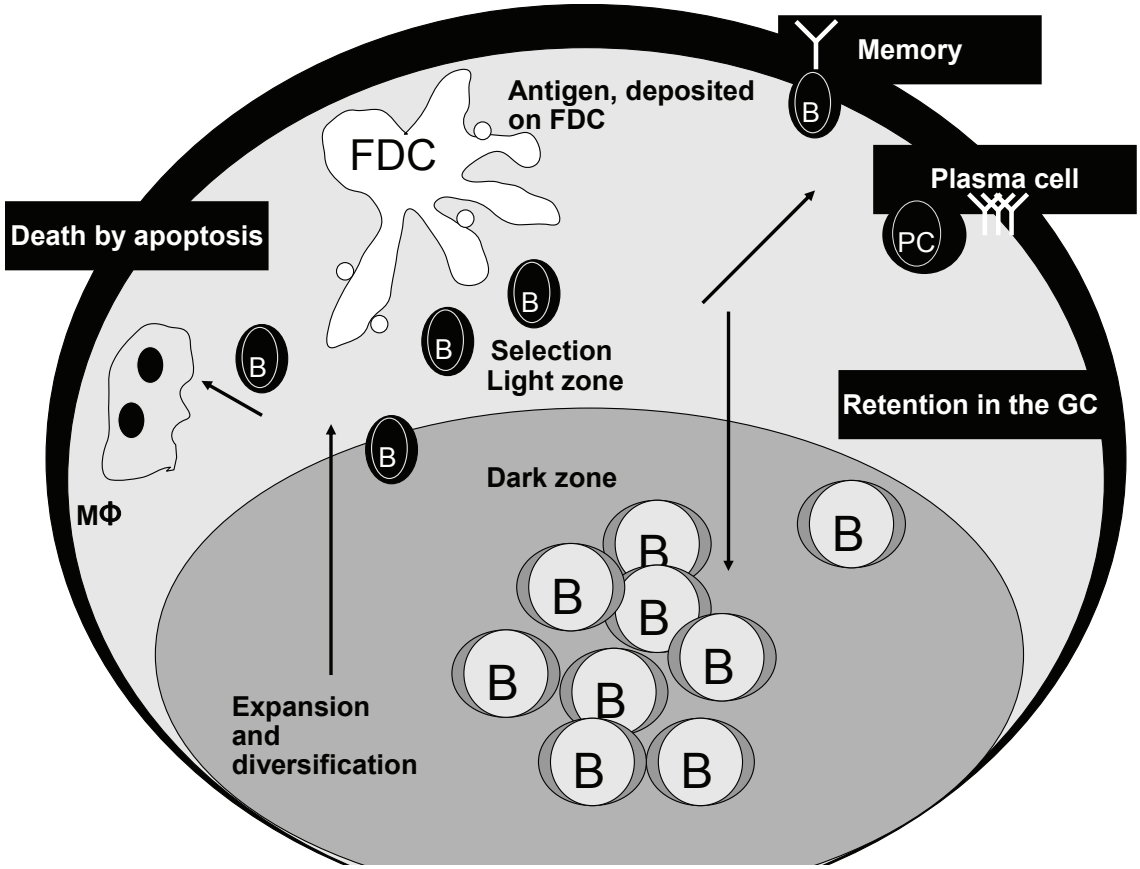


Figure 2 The function of the germinal center is to generate memory B-cells and plasma cells with increased antigen affinity. Antigen specific B-cells are recruited into the follicles of the cortex of the LN. Secondary follicles are formed where rapid clonal expansion takes place. The GC reaction is defined by the polarization of the secondary follicle; rapidly dividing centroblasts localized in the region proximal to the T-cell area and resting centrocytes apical. The cells are cycling between the dark zone and the light zone. Expansion and diversification take place in the dark zone. Selection takes place in the light zone where antigen is present deposited on FDCs. Negatively selected cells undergo apoptosis and are cleared by tingle body macrophages. The positively selected cells may re-enter the dark zone or exit the cycle as memory B-cells or plasma cells. Modified from McHeyzer-Williams et al. (18).

2.1.4 T-cell development

T-cell development has many parallels to that of B-cells. However, T-cells develop in the thymus from progenitor cells that migrate there from the BM. T-cells also differentiate into

distinct populations of cells characterized by expression of different receptors; The α:β and the γ:δ receptors. The majority of T-cells become α:β T-cells and they further differentiate into CD4+ T helper cells and CD8+ cytotoxic cells that play different roles in the immune response (14).

As in B-cells, the developmental stages of α:β T-cell development are marked by changes in receptor genes and in the expression of surface antigens. Initially, cells express neither CD4 nor CD8. Upon successful rearrangement of the beta chain locus, the cells become double positive, i.e. they express both CD4 and CD8. Then the alpha chain locus is rearranged, and the mature T-cell receptor is expressed. Cells that develop receptors compatible with self MHC are positively selected through interaction with the thymic stromal cells, whereas cells that interact too strongly with the MHC complex bound to self antigen, die by apoptosis and are negatively selected (22). Cells that survive the selection process become single positive CD4+ or CD8+ cells. Naïve single positive T-cells leave the thymus and enter the blood stream. They then start recirculation between the blood and the peripheral lymphoid organs.

If the T-cells encounter their specific antigen in a peripheral lymphoid organ, they become activated, undergo clonal expansion and differentiate into effector T-cells. Antigens located in the cytoplasm of all cells in the organism are carried to the surface on MHC I molecules. In this context the antigens can be recognized by CD8+ T-cells which, upon activation, differentiate into effector cytotoxic T-cells. Professional APCs ingest antigens which are carried to the surface on MHC class II molecules. Antigens from pathogens that proliferate in the vesicular compartment of these cells are also presented in this way. These antigens are presented to CD4+ T helper cells. T helper cells may differentiate into either Th1 cells or Th2 cells, depending on various factors, among them the character of the antigen and the external stimuli from the cytokines in the milieu (23). Th1 cells mainly produce IFN-γ, activate macrophages and are important players in cell-mediated immunity. Th2 cells produce various cytokines, including IL-4, and activate B-cells and the humoral immune response.

2.2 B-cell non-Hodgkin's lymphomas

B-cell non-Hodgkin's lymphomas are malignant tumors that arise from mature B-cells. Like other cancers, B-NHL is a genetic disease caused by activation of proto-oncogenes and loss of tumor suppressor genes resulting in a growth advantage of the affected cells. The processes

that generate antibody diversity in B-cells jeopardize the DNA integrity in the BM as well as in the GC (24). At the molecular level VDJ recombination, class switch recombination and somatic hypermutation involve DNA double strand breaks. When such breaks are aberrantly resolved, translocations can occur. Translocations that typically involve the *IGH* locus are a hallmark for lymphomas and are also thought to be the initial step for tumorigenesis. The most common translocation found in malignant lymphomas is the t(14;18)(q32;q21). Here the *BCL2* gene is brought under control of the enhancer region of the *IGH*, resulting in aberrant regulation and overexpression *BCL2* (25).

2.2.1 Classification of B-NHLs

B-NHLs constitute a heterogeneous group of tumors. This heterogeneity is also reflected in clinical outcome and response to treatment. The classification systems aim to create subgroups that are more homogeneous in order to optimize the treatment and improve clinical outcome. Ideally, the different classes of B-NHLs should represent recognizable normal cellular counterparts and be well defined disease entities with distinct clinical presentations. Historically, tumors were first classified on a purely morphological basis and numerous systems were in use. The Kiel classification incorporated immunological techniques in the classification system. In the REAL classification and the current WHO classification, lymphomas are classified according to morphology, immunophenotype, genetic features and clinical features (26;27). By applying this approach, the categories created by the classification systems are more reproducible and hopefully they represent real disease entities. However, there is still substantial variation in survival within different subgroups of B-NHL, indicating that the current subgroups may contain distinct disease entities.

Although morphological and immunophenotypical features of B-NHL suggest the cell of origin of the tumor, further analyses that take into account the genetic changes that take place during the germinal center reaction allow for a more precise categorization of these tumors. Thus, information regarding the mutational status of the Ig variable genes (*IGVH*) can be useful to determine the cellular origin of the tumors. Tumors with hypermutated *IGVH* genes originate from cells that have gone through the GC reaction whereas tumors with ongoing somatic hypermutation are arrested in the GC stage (28). In B-cell chronic lymphocytic leukemia, CLL, the *IGVH* mutation status indicates whether the tumor has arisen pre or post the GC reaction and moreover, is of prognostic significance (29).

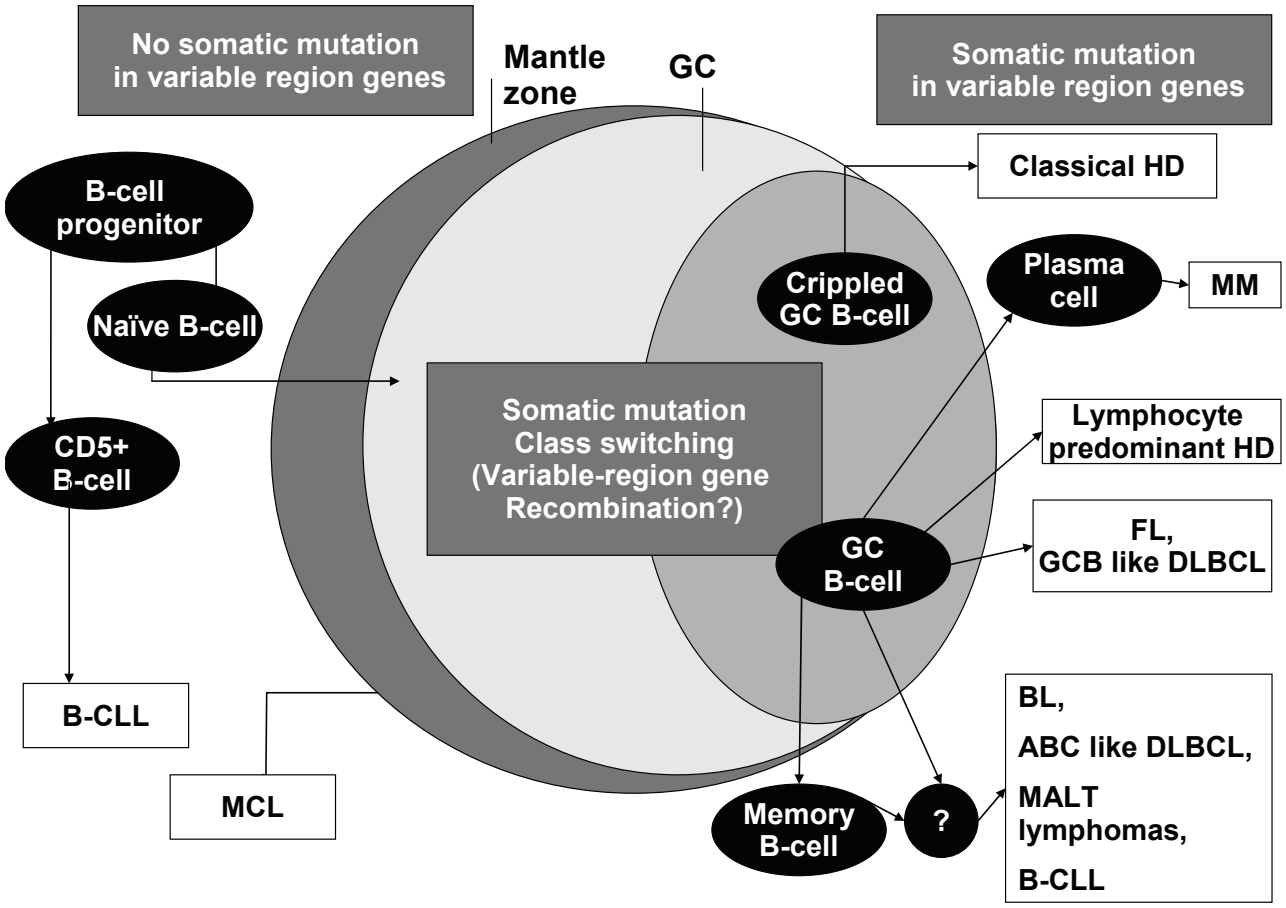


Figure 3 Common malignant lymphomas of B-cell origin (white boxes) and their normal cellular counterparts (black ellipses). Naïve B cells establish germinal centers upon recognition of antigen. The processes that jeopardize the genomic DNA of these cells are indicated in the central grey box. The mutational status of the *IGVH* genes is different according to the cell of origin. Somatic mutations may be absent or present. If they are present, there may be intraclonal *IGVH* gene diversity indicating an ongoing process as in follicular lymphomas. If such diversity is absent the tumor may be derived from post–germinal-center (memory) B cells. Abbreviations: FL follicular lymphoma, CLL chronic lymphocytic leukemia, MM multiple myeloma, HD Hodgkin's disease, MCL mantle cell lymphoma, BL Burkitt lymphoma, DLBCL diffuse large B-cell lymphoma. Modified from Kuppers *et al*. (21).

Although new techniques have made lymphoma diagnosis more accurate, it is conceivable that the currently recognized subgroups contain several entities. Genome wide expression studies of diffuse large B-cell lymphomas, DLBCLs, have shown that this subgroup can be divided into a germinal center B-cell (GCB) like and an activated B-cell (ABC) like subtype based on the expression profiles of the tumors (3;30;31). A third subtype, the primary

mediastinal B-cell lymphoma, has also been identified by genome wide expression profiling (32).

High-resolution techniques, like genome wide expression profiling, are useful to classify current subgroups into new and smaller entities, but it is also of interest to group lymphomas into larger classes that share clinical features in spite of histological differences. Sánches-Beato *et al.* proposed a model where lymphomas are grouped into low-growth fraction

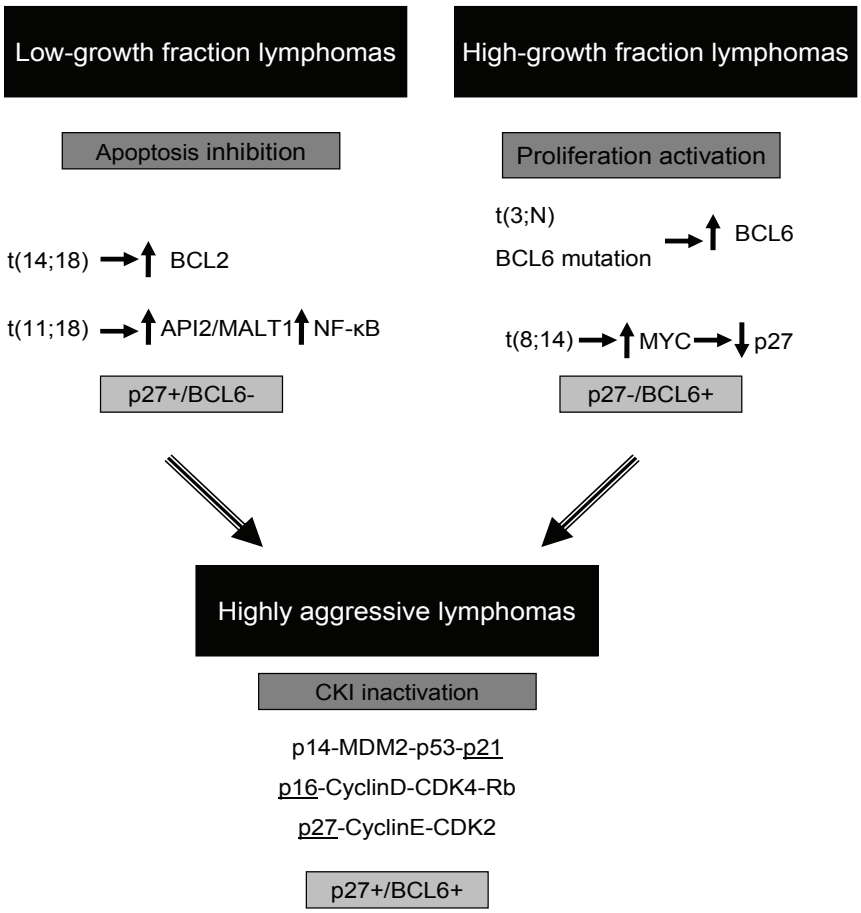


Figure 4 Different primary oncogenic events characterize low-growth fraction and high-growth fraction lymphomas. Examples of primary oncogenic events that lead to apoptosis inhibition in low-growth fraction lymphomas include t(14;18), resulting in *BCL2* overexpression, and t(11;18), resulting in the *API2/MALT1* fusion gene and protein that induce NF-κB activation. These aberrations are common in FL and MALT lymphomas, respectively. Gain of 18q could lead to upregulation of *BCL2* and *MALT1* and lead to apoptosis inhibition. Examples of primary oncogenic events that activate proliferation in high-growth fraction lymphomas include activating translocations of *BCL6*, common in DLBCL, and t(8;14) resulting in c-myc overexpression. t(8;14) is a hallmark of BL and is present in 10% of DLBCL. By acquiring secondary mutations that lead to CKI inactivation both tumor types may evolve to highly aggressive lymphomas. Modified from Sánchez-Beato *et al*. (33).

lymphomas and high-growth fraction lymphomas (33). Low-growth fraction lymphomas typically have mutations in apoptosis-regulating genes, e.g. *BCL2*; whereas high-growth fraction lymphomas have alterations in genes involved in proliferation control e.g. *MYC*.

Both these types of lymphomas may progress to highly aggressive lymphomas by acquiring mutations in genes that affect major tumor suppressor pathways. Most frequently this leads to inactivation of cyclin dependent kinase inhibitors (CKIs), and mutations in *TP53* are typical examples.

The current view on lymphoma diagnosis and treatment regards the tumor bulk as the target for therapy. Reduction in tumor size in response to treatment is thus considered as a criterion for successful treatment. However, it is known that reduction in tumor volume and even complete remission, not always can be translated into a survival advantage (34). This is the case for follicular lymphoma, FL, where a watch and wait approach does not lead to inferior survival compared to start of treatment at the time of diagnosis (35). The concept of tumor stem cells proposes an explanation to this paradox. In this view, the tumor contains a small number of cells with the ability to self-renew that give rise to all the other differentiated, malignant cells of the tumor (36). Unless the tumor treatment is able to eliminate also the cells with ability to self-renew one should not expect cure of the patient. Experimental evidence for this theory exists for several tumor types, including lymphoid malignancies. In multiple myeloma it has been shown that the plasma cells that constitute the bulk of the tumor arise from a small population of less differentiated cells that resemble post-GC B-cells (37)

It is clear that the tumor stem cell hypothesis, if further confirmed, will have significant impact on classification as well as treatment of malignant lymphomas.

2.2.2 Prognostic indicators

The different recognized classes of lymphomas have different clinical outcome. Thus, the classification system in use, which includes morphological as well as immunological criteria, serves as a prognostic indicator. In general, patients with high grade lymphomas, as defined by morphological criteria, have very poor prognosis if left untreated, whereas some patients with low grade lymphomas may survive for years without therapeutic intervention. However, within the recognized classes there is substantial variation with regard to outcome and this probably also warrants different treatment. The use of clinical and molecular markers has been shown to stratify patients within a B-NHL category into different prognostic classes. For

aggressive as well as follicular lymphoma simple prognostic indices have been proposed, i.e. the international prognostic index (IPI) and the follicular lymphoma international prognostic index (FLIPI) (38;39). These take into account several clinical parameters such as age, stage, performance status and standard blood tests.The strength of these indices is the simplicity, but they give no insight into tumor biology.

2.2.2.1 Expression profiles

In the recent years the microarray technology has made genome wide expression profiling feasible. This technology has also been used to obtain gene expression profiles of lymphoma-biopsy specimens in several studies (3;30;31;40;41). Studies of diffuse large B-cell lymphomas, DLBCLs, have shown that clinically significant subtypes can be identified based on variation in gene expression. Interestingly, these subtypes share expression features with different cellular counterparts in the immune system, e.g. the germinal center B-cell and the activated B-cell, suggesting a different cell of origin in the transformation process (42). Moreover, the prediction of survival based on gene expression has been shown to be independent from the clinical parameters in the international prognostic index (IPI) (3;41). In mantle cell lymphomas, MCL, expression profiling of proliferation genes could substitute for measurement of tumor cell proliferation and identify patient subsets with different outcome (41). Furthermore, in FL gene expression of the tumor infiltrating cells at the time of diagnosis was shown to correlate with survival (40). These examples show that characterization of the molecular variation in lymphomas gives useful information regarding prognosis and tumor biology, the latter being very important for development of therapy that may target specific pathogenetic processes.

The microarray technology is costly and it requires relatively large amount of RNA and thus tumor samples. Based on the results of genome wide expression profiling experiments of lymphomas, it has been shown that measurement of mRNA expression of a very limited amount of genes in DLBCL by real time quantitative PCR is able to add prognostic information to the IPI score (43;44). At the protein level it has also been shown that immunostaining of tissue samples with a small panel of antibodies is able to determine the subtypes of DLBCL that have been defined by genome wide expression profiling (45). For diagnostics at the RNA level, it would be interesting to investigate the benefit of smaller cancer type specific microarrays in routine diagnostics of lymphomas.

2.2.2.2 Analyses at the genomic DNA level

Analyses of lymphoma biopsies at the DNA level have also been shown to give useful prognostic information. Cytogenetic analysis requires culturing of tumor cells *in vitro* followed by visualization of metaphase chromosomes. Historically, use of this technique revealed that distinct chromosomal abnormalities correlate with certain histological subtypes, the most well known example being the t(14;18), which is found with a frequency of 80% in FL. Gains or losses of chromosomes or chromosomal arms detected by this method have also been shown to be of prognostic value (46). In the recent years comparative genomic hybridization (CGH) has been used to test tumor DNA from interphase cells for gains and losses of specific genomic regions. Such aberrations have been correlated to clinical outcome in several studies. Stokke *et al.* found that loss of chromosome 11q21–23.1 and 17p and gain of chromosome 6p are independent prognostic indicators in B-NHL (47). Other studies have demonstrated the negative prognostic significance of 18q gains or amplifications and 17p losses in DLBCL (48), whereas loss of material on chromosomal bands 6q25q27 is associated with poor outcome in FL (49). In contrast to cytogenetic studies, CGH does not provide information about the chromosomal organization of the tumor genome. Therefore chromosomal translocations cannot be detected by this method.

Fluorescence in situ hybridization (FISH) with locuc specific probes to interphase cells is a method that allows for the evaluation of numeric aberrations of single genes as well as translocation of chromosomal regions, depending on the probes in use. This method is very sensitive and specific and, in contrast to CGH, it gives information at the single cell level. The method is well suited to detect common translocations in malignant lymphomas, which can aid the diagnosis as well as have prognostic significance. Numeric aberrations like loss of 17p as well as gain of 18q are examples of genomic aberrations associated with poor prognosis that can be detected by FISH (48;50;51).

Point mutations of crucial DNA sequences could also lead to activation of proto-oncogenes and inactivation of tumor suppressor genes. So far, mostly direct sequencing of the genome and different types of techniques that are based on differential electrophoretic migration of wild type and mutant DNA molecules have been used for detection of mutations. Both these methods are labor intensive, and thus, studies to detect the significance of point mutations in lymphomas have been concentrated to a limited amount of genes. The most consistent finding in malignant lymphomas is that mutations in the *TP53* gene are of negative prognostic value

(52-54). As novel techniques that facilitate high throughput mutation detection are developed, one would expect that that the prognostic significance in lymphomas of point mutations of many other genes will be clarified (55;56).

2.3 Signaling pathways and targets for therapy

The way an external stimulus is translated into a biological response in a cell is referred to as signal transduction. Depending on the stimulus, different signaling pathways may be activated or inhibited. The stimuli comprise unspecific changes in the physical environment like changes in temperature or nutrition as well as highly specific binding of ligands to receptors of the target cell. Cytokines are a group of ligands that function as inter-cellular signaling molecules, and many cytokines play critical roles in the development of hematopoietic cells. Cytokine stimulation often leads to activation or inhibition of signaling pathways that are important for proliferation, apoptosis and differentiation. Alterations of such signaling pathways are important features of malignant cells that may lead to uncontrolled growth. Targeted cancer therapy often aims to interfere with these pathways (35;57-59).

2.3.1 PI3K

Phosphoinositide-3 kinases, PI3K, comprise a subfamily of lipid kinases. These enzymes are heterodimers and consist of a catalytic and a regulatory subunit (60). PI3K phosphorylate phophoinositides (PI) at the 3' position of the inositol ring , and this generates PI(3)P, $PI(3,4)P_2$ and $PI(3,4,5)P_3$. These lipid products serve as second messengers and activate a plethora of targets, including the protein kinase B/Akt. The PI3K activity is counteracted by several lipid phosphatases, including PTEN that converts $PI(3,4,5)P_3$ back to $PI(3,4)P_2$. The PI3K/Akt pathway has been shown to play a central role in regulation of apoptosis and proliferation in several systems, including normal erythroid progenitors and B- and T-lymphocytes (61-63).

2.3.1.1 PI3K mediated Epo signaling

Activation of the signaling pathways upon Epo binding to EpoR is dependent on conformational changes of the receptor that bring together the dimerized receptor and JAK2 (64). Autophosphorylation of JAK2 followed by phosphorylation of cytoplasmic tyrosine residues of the EpoR results in an activated ligand-receptor complex that recruits several signaling molecules and adapter proteins which act as substrates for JAK2. Thus, several signaling cascades are involved in mediating the Epo induced changes in gene expression and

subsequent effects on differentiation, proliferation and survival (65). The PI3K-signaling cascade is necessary for maturation of erythroid progenitors (66;67). In these cells PI3K can be activated by direct binding of the p85 regulatory subunit to the activated EpoR, and indirectly through binding to adapter molecules. Akt kinase, which is activated by $PI(3,4,5)P_3$ or $PI(3,4)P_2$, phosphorylates both GATA-1 and Foxo3a. These transcription factors are of crucial importance in erythropoiesis (61;67;68).

2.3.1.2 PI3K in lymphocytes

In lymphoid cells it has been shown that the PI3K signaling cascade is triggered in response to antigen-receptor activation (69-71). In addition, activation of PI3K plays a role upon binding to co-stimulatory receptors (72). A number of cytokines, including IL-3, IL-4 and IL-5, have also been shown to activate PI3K (73). By mediating these physiological stimuli, PI3K controls proliferation and apoptosis. PI3K has also been associated with malignant transformation. Mice with enhanced PI3K activity in lymphocytes show increased incidence of lymphomas (72;74). Analogous to PI3K activation by Epo signaling, activation of receptor complexes in lymphocytes recruits PI3K to the cell surface where the enzyme generates the active lipid products. In lymphocytes this activates several signaling cascades. In B-cells, PI3K signaling facilitates activation of phospholipase C-γ, PLCγ2, by Bruton tyrosine kinase, leading to cleavage of $PI(4,5)P_2$ and generation of inositol triphosphate, IP_3, and diacylglycerole, DAG. This leads to sustained Ca2+ mobilization by IP_3, characteristic of lymphocyte proliferation. Moreover, DAG and Ca2+ activates protein kinase C β, PKCβ which leads to activation of the NF-κB pathway (63). As in erythroid progenitors, activation of Akt is also important for PI3K mediated signaling in lymphocytes (75). Important downstream targets for Akt in B-cells include FOXO and mTOR (63). FOXO is a transcription factor, whose activity is reduced upon phosphorylation by Akt. This leads to reduced expression of FOXO target genes, which include *CDKN1B* ($p27^{KIP1}$) and *TNFSF10* (TRAIL), and subsequently reduced apoptosis and increased proliferation (76). Another downstream target of Akt, the kinase mTOR, is important for growth and proliferation of lymphocytes (63).

2.3.2 The TGF-β superfamily signaling system

The TGF-β superfamily comprises TGF-β, bone morphogenetic proteins (BMP) and activins. Members of this superfamily play important roles in controlling cellular proliferation, differentiation and apoptosis as well as migration. TGF-β has been intensively studied in

normal and malignant hematopoietic cells and is one of the most potent endogenous negative regulators known to date (77). BMP signaling is critical for self-renewal of embryonic stem cells and plays a role in controlling the number of HSC (78;79). In mature B-cells it has been shown that BMP-6 has a negative regulatory role (80).

Members of the TGF-β superfamily signal through ligation and heterodimerization of type I and type II serine/threonine kinase receptors. This leads to phosphorylation of the type I receptor, which is now activated and may phosphorylate receptor Smad proteins (R-Smads). The R- Smads then form complexes with the co-Smad (Smad4) which are translocated into the nucleus to exert gene regulation. The gene regulation and resulting physiological response is context dependent. This is due to the diversity allowed for by the combinatorial interactions of type I and type II receptors and Smads. Smads also cooperate with many different sequence-specific transcription factors (81). The R-Smad activation is regulated by numerous receptor binding proteins as well as the inhibitory Smads, Smad6 and Smad7.
In addition to Smad dependent signaling and transcriptional regulation, the activated receptor complex may activate non-Smad signaling pathways, such as MAPK and TAK1/MEKK (81).

The founder member of the TGF-β superfamily is also the best investigated. Given its critical role as a negative regulator in non-malignant cells, it is logical to predict that disruption of the TGF-β signaling pathway may promote malignant transformation. Indeed, many epithelial neoplasms, including colon, breast, pancreatic, and hepatocellular carcinoma have been characterized by alterations in TGF-β pathway genes, including *SMAD2*, *SMAD4*, and *TGFBR2* (77). Mutational inactivation of this pathway is uncommon in hematological malignancies, but other mechanisms may confer reduced TGF-β responsiveness in these diseases. In Burkitt lymphoma with t(8;14), *MYC* expression is under the control of the *IGH* promoter. In normal cells downregulation of c-myc protein is a critical event for TGF-β induced growth inhibition and the *MYC* promoter contains a Smad-responsive element (82). By the translocation, however, this regulation is disrupted. *CDKN1B*, which encodes $p27^{KIP1}$, is an example of another target of TGF-β that can be altered in lymphomas and thus modulate TGF-β responsiveness (83;84).

Given that TGF-β functions as a brake, opposing many stimulatory factors that promote proliferation and clonal expansion of normal lymphoid cells, modulation of this pathway is an interesting therapeutic strategy in malignancies with loss of TGF-β sensitivity.

2.3.3 NF-κB

The NF-κB proteins are transcription factors that can be activated by several stimuli. Activation of NF-κB is crucial in the inflammatory response and is stimulated by pro-inflammatory cytokines like TNF-α and IL-1. In addition to its role in inflammation, NF-κB proteins are also important in the regulation of apoptosis and proliferation as well as in oncogenesis (85;86).

The five mammalian NF-κB family proteins, RelA, RelB, c-Rel, NF-κB1, and NF-κB2, form homo- and heterodimers that activate characteristic sets of genes. When the dimers are bound to inhibitory proteins of IκB type, they are sequestered in the cytoplasm and their activity is prevented. In response to a stimulus, like TNF-α receptor binding, the IκB proteins are phosphorylated, ubiquitinylated and subsequently degraded by the proteasome complex. Not bound to IκB, the active subunit of NF-κB (phospho-p65) may translocate to the nucleus and regulate gene transcription (87). The regulatory step in this signaling pathway is the phosphorylation of IκB performed by a specific serine/threonine kinase IκB kinase (IKK). The mechanism by which external stimuli induce IKK activity is not clear, but it could involve a IκB kinase kinase that is activated by receptor binding and subsequently phosphorylates and activates IKK (87). Although it induces apoptosis in some cases, NF-κB activity in general promotes survival (86). The mechanism for this is not completely established, but the genes regulated by NF-κB include the apoptosis regulating *BCL2* and *BLC2L1* that encodes for BCLXL and BCLXS, several members of the inhibitor of apoptosis (IAP) family as well as genes involved in proliferation like *MYC* and *CCND2* (cyclin D_2) (88). Interestingly, it has been shown that NF-κB is also involved in the regulation of *PIM1* (89). The expression of this gene is highly correlated to the ABC subtype of DLBCL (90;91).

Constitutive activation of NF-κB is a hallmark of many cancer types including malignant lymphomas and leukemias. However, many chemotherapeutic agents, like antracyclins, also trigger the NF-κB pathway, which could decrease their effect (86). The therapeutic potential of modulating the NF-κB pathway is therefore promising. Several agents that interfere with NF-κB activity at different levels have been described. They act by decreasing NF-κB DNA binding, blocking IκB degradation by the proteasome, inhibiting phosphorylation of IκB by IKK or blocking nuclear translocation of NF-κB (88). The selective proteasome inhibitor bortezomib, which prevents release of NF-κB from IκB by blocking degradation of the latter (92;93), is now approved for the treatment of multiple myeloma in Norway.

3 Purpose of the present study

This study was designed to:

1. establish the consensus regions for the common gains of chromosomes 6p and 18q in B-NHL using FISH and array CGH technologies.
2. correlate gains of specific genomic regions to cellular and clinical phenotypes.
3. examine how Epo via activation of PI3K/Akt exerts its role in development of erythroid progenitors from CD34+ cells, and to identify early Epo target genes in CD34+CD71+CD45RA-GPA- erythroid progenitors by microarray technology.
4. identify novel target genes using microarray technology and to characterize functional effects of BMP-6 in human T-cells.

4 Summary of publications

Publication I Translocation t(14;18) and gain of chromosome 18/BCL2: effects on BCL2 expression and apoptosis in B-cell non-Hodgkin's lymphomas.

FISH and array CGH were used to examine numeric alterations of chromosome 18 in B-NHL. Long range PCR and FISH were used to detect t(14;18). Gain of 18q was found in 34% of lymphomas with t(14;18) and in 20% of lymphomas without t(14;18). In lymphomas with t(14;18), the consensus 18q gain was located centromeric of the *BCL2* locus, and thus did not include it. In lymphomas lacking t(14;18), the gain was centered around 18q21, including the *BCL2* locus in all cases. *PMAIP1* and *MALT1* were included in the consensus region of gain in both groups. Gain of *BCL2* was mainly low level. BCL2 protein expression was quantified by immunoblotting in the tumor samples. The cases with both t(14;18) and *BCL2* gain had higher expression of BCL2 than the tumors with t(14;18) only. The latter cases had higher expression than the ones with *BCL2* gain only which again had higher expression levels than the tumors with no *BCL2* alterations. There was a highly significant, but weak, correlation between BCL2 expression and levels of spontaneous apoptosis in the tumor cells. The fraction of apoptotic tumor cells was, however, not correlated to the *BCL2* genotype. In contrast to this, the apoptotic fraction of the normal cells in t(14;18) positive tumors was increased compared to the corresponding tumor cell apoptotic fraction. No difference between tumor and normal cell apoptotic fractions was found for tumors with *BCL2* gain only or no *BCL2* alterations. When comparing the apoptotic fractions of normal cells in tumors with different genotype, t(14;18) positive tumors exhibited increased apoptic fractions compared to tumors with no *BCL2* alterations, whereas the normal cells of tumors with *BCL2* gain only showed intermediate apoptotic fractions. These findings indicate that the tumor genotype may influence on factors in the tumor microenvironment of importance for cell survival.

Publication 2 Gain of chromosome 6p is an infrequent cause of increased PIM1 expression in B-cell non-Hodgkin's lymphomas.

Array CGH and FISH was used to examine an amplicon located at 6p in the B-NHL cell line U698. A 10-fold amplification of a narrow region at 6p21.2 (~1.5mbp) encompassing the *PIM1* gene (~20 copies) was demonstrated. High levels of *PIM1* mRNA and protein in U698 cells was shown by northern- and immunoblotting. Array CGH and FISH was employed to analyze chromosome 6p alterations in a series of B-cell non-Hodgkin's lymphomas. These studies revealed that the consensus region was 6p22.1-pter, not including the *PIM1* locus. In

an independent set of DLBCLs for which genome wide expression data were publicly available, *PIM1* mRNA expression was correlated to poor outcome. There was no significant correlation between *PIM1* copy number, as determined by FISH, and expression level in a panel of 35 of these tumors. Taken together, these data suggest that increased *PIM1* expression, although possibly oncogenic, is only infrequently caused by gene amplification in B-NHL, and that *PIM1* is not the target oncogene for lymphomas with 6p gain.

Publication III PI3K/Akt-dependent Epo-induced signaling and target genes in human early erythroid progenitor cells.

We employed microarray technology and immunoblotting of cell cycle related proteins to examine Epo-induced signaling in CD34+ progenitor cells from human bone marrow. Identification of PI3K dependent regulation was achieved by use of the specific PI3K inhibitor LY294002. Epo alone was able to induce cell cycle progression as demonstrated by upregulation of cyclins D_3, E and A, leading to hyperphosphorylation of RB. These effects were completely counteracted by the PI3K inhibitor LY294002, whereas enforced expression of an activated form of the PI3K target Akt kinase highly augmented Epo induced erythropoiesis. FACS-sorted CD34+CD71+CD45RA-GPA- erythroid progenitors stimulated with Epo in the presence or absence of LY294002 were subjected to gene expression profiling. Several novel target genes of Epo were identified, and the majority was regulated in a PI3K dependent manner, including *KIT* (CD117) and *CDH1* (E-cadherin). FACS analysis of Epo-stimulated erythroid progenitors showed that the increased mRNA expression of *KIT* and *CDH1* was accompanied by an induction of the corresponding proteins CD117 and E-cadherin. Taken together, the data show that ligand binding to the Epo receptor in early CD34+ progenitors initiates a plethora of signaling cascades. The physiological effects of Epo on survival, proliferation and differentiation at this stage are completely dependent on functional PI3K. The observed expression changes of important target genes reflect this dependency.

Publication IV Inhibitory effects and target genes of BMP-6 in human T-cells.

We explored the role of BMP-6 in the pre-T ALL cell line Jurkat TAg and in human CD4+ T-cells. Expression of BMP receptors was determined by FACS analysis, and human T-cells were found to express BMP type I and type II receptors. BMP induced signaling was examined using immunoblotting, luciferase reporter assay, real time RT-PCR and microarray

analysis. BMP-6 rapidly induced phosphorylation of Smad1/5/8. BMP-6 also induced phosphorylation of p38 in peripheral blood CD4+ T-cells and of p38 and ERK1/2 in Jurkat TAg cells. The transcriptional response to BMP-6 stimulation included potent upregulation of *ID1*, *ID2* and *ID3*. Id1 and Id3 were also induced at the protein level in Jurkat TAg cells. Other transcriptional targets that were identified in BMP-6 stimulated Jurkat TAg cells were *NOG* (noggin) and *SMAD6*, in addition to several genes involved in transcriptional regulation including *NFKBIA*, *HEY1*, *DLX2*, *KLF10* and *EGR1*. The functional response to BMP-6 stimulation in Jurkat TAg cells and CD4+ T cells was characterized by various assays. In Jurkat TAg cells, BMP-6 exerted an antiproliferative effect as demonstrated by ^{3}H-Thymidine incorporation and CFSE cell division tracking. This effect was counteracted by *ID1* siRNA. In CD4+ T-cells, BMP-6 significantly inhibited the differentiation into IL-4 producing effector cells from naïve precursors albeit less potently than TGF-β. Taken together, BMP-6 is a novel regulator of human T-cell responses.

5 General discussion

5.1 Methodological considerations

5.1.1 The cell systems

In the present study we have analyzed signaling pathways in cells from lymphoid tumors (paper I and II), in hematopoietic cancer cells (paper II and IV) and in primary hematopoietic cells (paper III and IV). Each of these cell systems have certain advantages and disadvantages with regard to the feasibility of the experimental procedures and the conclusions that can be drawn from the data. The use of these three different systems gave us the opportunity to test our hypotheses more thoroughly than use of only one system would have done.

Two sets of tumor samples were included in this study. In the first set, 93 tumors were randomly selected based on the diagnosis of B-NHL and on the availability of frozen cell suspensions in 10% DMSO. The preparation of single cell suspensions is described by Kvaløy *et al*. (94). In this set the tumor cell fraction had previously been determined by flow cytometry (FCM) as described by Stokke *et al*. (47). Since the patient samples were subjected to batch analyses like array CGH (study I and II) and immunoblotting (paper I), this was very useful when we interpreted the results from those experiments. The ploidy, i.e. DNA index, of each patient sample had also been determined by FCM (47). This was taken into account when we used array CGH to determine absolute gene copy number alterations. Material suitable for mRNA expression analysis was only available for a limited number of the patient samples in the first set. In the second set, 35 DLBCLs were selected based on availability of expression data from a microarray study (30) and of tumor samples for DNA copy number analysis. For this set we had no information regarding ploidy or fraction of normal cells. As stated, tumors of both sets were randomly selected based on availability of material suitable for analysis. This availability was, however, dependent on several factors, including viability of tumor cells and sample size, which could have lead to selection bias.

The use of cancer cell lines in this study had three main advantages. First, the amount of cells and thus protein and RNA, was not a limiting factor. Second, the population of cells from a cell line is relatively homogeneous. Third, the feasibility of genetic manipulation is a prerequisite for loss-of and gain-of function studies. The obvious disadvantage with regard to

use of laboratory cell lines is the questionable physiological relevance of the results of the experiments.

The primary cells used in this study were first positively selected from bone marrow aspirates or blood from healthy donors by use of immunomagnetic beads coated with monoclonal antibodies against a surface antigen (CD34, CD4). This procedure yields high purity of cells with regard to the antigen selected for (>90%) and is not thought to alter the functional capacity or activation status of the cells (95). However, CD34+ cells from bone marrow and CD4+ cells from blood certainly constitute heterogeneous cell populations. In some of the experiments we therefore needed to examine smaller subpopulations. In study III, we stained the positively selected CD34+ cells with anti-CD71, anti-CD45RA and anti-GPA and performed FACS-sorting to obtain CD71+CD45RA-GPA- erythroid progenitors. Furthermore, in study IV we stained CD4+ cells with anti-CD45RA and anti-CD45RO followed by FACS sorting to obtain CD45RA+CD45RO- naïve CD4+ T-cells. This procedure reduced, but did not eliminate, the problem with heterogeneous cell populations. The additional *ex vivo* manipulation of the cells implicated by this procedure could also influence on the results of the experiments, especially the RNA expression studies, which will be discussed in section 5.1.4. In addition to intrasample heterogeneity, donor dependent intersample heterogeneity is an important issue for analyses of primary cells.

With respect to the purification of erythroid progenitors, the amount of mRNA that could be obtained from per individual donor was limited. Thus, amplification of RNA was necessary. The relatively small amount of cells that can be obtained from isolation procedures also imposes difficulties on gain-of and loss-of function studies. Although transfection of primary cells can be feasible, as demonstrated in paper III, it is generally difficult to genetically manipulate mature lymphocytes. To conclude, studies on primary cells from human donors are preferable with regard to the physiological relevance of the conclusions that are drawn. However, the heterogeneity of the cell populations, the limited amount of biological material that can be obtained and difficulties with genetic manipulation are important obstacles.

To summarize, it is necessary to have in mind the pitfalls of each of the different cell systems when evaluating the experimental data. One also needs to be especially careful not to extrapolate conclusions drawn from experiments in one system to another. However, experimental results from cell line studies are very valuable for generation of hypotheses, which subsequently can be tested in primary cells of normal or malignant origin.

5.1.2 Detection of genomic aberrations.

Various techniques are in use to detect genomic aberrations, either at the whole genome level or at specific regions. In traditional karyotyping of tumor cells, metaphase chromosomes of cultured cells are stained and evaluated for numerical and structural changes. Thus, reciprocal translocations as well as genomic gains and losses can be detected in the tumor cells. The main disadvantages of this method are the need for cell culturing and the limited resolution provided by the staining technique, the banding resolution. The development of the comparative genomic hybridization, CGH, technique has improved the analysis of copy number changes in tumors (96). In conventional CGH, tumor DNA and normal DNA are labeled with different fluorescent dyes and then hybridized in situ to normal human metaphase spreads. Detection of gains or losses of chromosomal regions relies on competitive hybridization of the two samples. The sensitivity of the method is determined by the size of the region involved as well as the copy number. In general, in case of single copy changes the size of the affected region must exceed 5-10 mega base pairs, mbp, to be detected. High-level gains may be detected in smaller regions (5-10 fold amplifications of ~1mbp) (97;98). To improve the limited resolution of conventional CGH, the microarray based CGH has been developed (99;100). In this technique, the generation of labeled probes is identical to that of conventional CGH, but the probes are now hybridized to a microarray with spotted DNA sequences. The spotted probes on the array can be PCR products of cDNA clones or BAC clones, or chemically synthesized oligonucleotides. In paper I and II we used BAC arrays with a resolution of about one mbp to profile the tumor samples. This resolution determined how accurate the boundaries of the gains and losses could be defined. We also tried to perform CGH on cDNA arrays with limited success, due to weak signals. However, in paper II, we were able to identify *PIM1* as target of the 6p amplification in U698 using this method.

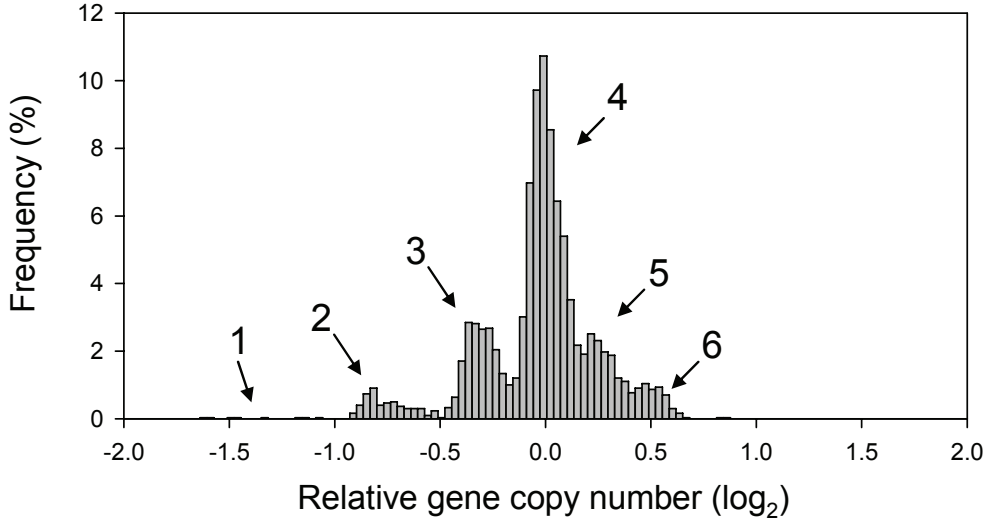
The quantification of numerical changes of genomic regions relies on the size of the fluorescence ratio detected by the scanning equipment. Thus, the log2 transformed fluorescence ratio is referred to as the relative copy number. Limitations of the technique impose difficulties on the interpretation of the data. CGH techniques are batch analyses, i.e. data regarding tumor heterogeneity are missed. The amount of normal cells in the tumor influences on the sensitivity since they bring the detected fluorescence ratio towards unity. Aneuploidy is also a matter of concern. The tumor DNA used in the hybridization is constant, and in the normalization procedure the fluorescence ratio baseline is assumed to correspond to the copy number of most loci of the genome in conventional CGH or to the average of the

spots in array CGH. In diploid and tetraploid tumors this is not an important issue, but in hyperdiploid and hypotriploid tumors knowledge of ploidy is important to determine the threshold for gains or losses (101).

Data regarding tumor cell fraction and ploidy are normally not taken into account when evaluating conventional CGH data, possibly due to lack of knowledge of these parameters. Normally, fixed thresholds for scoring gains, (ratio>1.15), high-level gains (ratio>1.5) and losses (ratio<0.85) are used. In our array CGH experiments we wanted to quantify the gains and losses more precisely. First we performed control experiments with normal male and female DNA to assess the dynamics of the assay. We observed that double amount of DNA (XX vs XY) resulted in a log2 transformed ratio of 0.7-0.8. Furthermore, comparisons of the experimentally obtained ratios of genomic regions with known high-level gains (i.e. *PIM1* in U698) with copy numbers obtained by the independent method FISH, indicated that the assay was linear for practical purposes. By taking into account the linearity and the dynamics of the assay as well as the ploidy and tumor cell fraction of the samples we scored each individual clone, representing different regions of chromosome 18 and 6, as gained or lost for each tumor. Moreover, this method allowed us to suggest an absolute copy number for the region represented by the clone. How this was performed is illustrated in Figure 5.

To assess specific DNA sequences in single tumor cells we applied the FISH method. In this technique gene sequences are labeled with a fluorochrome and hybridized to nuclei in interphase before evaluation on a cell-to-cell basis. FISH can be used to quantify gene copy number changes and was used in paper I and II to verify the array CGH findings. In addition, FISH can be used to assess structural genomic changes. With respect to this, t(14;18) translocation was assessed by FISH in paper I. This was done by scoring co-localized *BCL2* (red) and *IGH* (green) fusion signals as *BCL2* rearrangement. Assessment of t(14;18) was also performed by conventional and long-range PCR in paper I. PCR is extremely sensitive in determining the presence or absence of the genomic sequences tested for, and the data obtained by FISH and PCR in paper I showed concordance with the exception of two cases.

A



B

Gene copy number	1	2	3	4	5	6
Theoretical ratio	-2	-1	-0.42	0	0.32	0.58
Observed ratio	-1.3	-0.8	-0.3	0	0.25	0.5

C

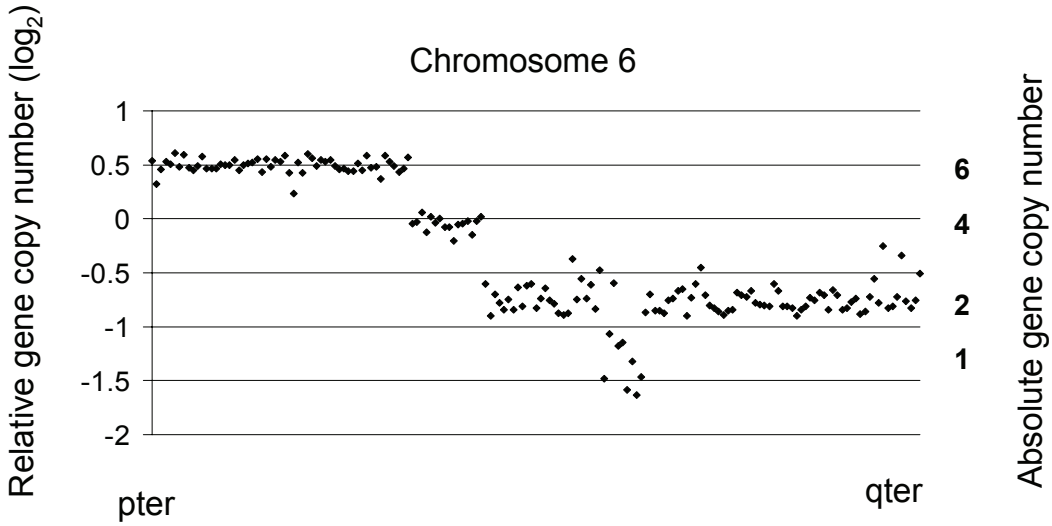


Figure 5 A) Frequency histogram for fluorescence ratios of tumor 358/87. This sample had a DNA index of 2.0 and consisted of 89% tumor cells. Absolute copy numbers for given ratios are indicated. B) Relationship between absolute gene copy number, theoretical ratio and observed ratio. C) BAC array CGH profile of chromosome 6 for tumor 358/87. Gains are scored as 6 copies and losses as 1 or 2 copies.

To conclude, the whole genome techniques used in this study, conventional and array CGH, are sensitive with regard to copy number changes, but give no information with regard to structural changes. Locus specific FISH is practical and sensitive for the analysis of known recurrent aberrations, but is not suitable to screen tumor samples for many different genomic aberrations.

5.1.4 Expression analysis: General aspects

The genome itself determines the phenotype of a cell or an organism in interplay with external stimuli. The differences in morphology and function of various cell types in the human body, which possess identical DNA, depend on changes in gene expression. Thus, the concept of gene regulation is central in understanding biological function. Gene expression is physiologically controlled at several levels. The RNA is subjected to transcriptional control, RNA processing control (splicing), RNA transport control (export to cytoplasm) as well as mRNA degradation control. Proteins can be controlled at the translational level or can be posttranslationally activated or inactivated. Transcriptional control of gene expression seems to be predominant for most genes (87).

To understand the biological role of a gene it is logical to characterize the expression (102). Traditionally, measurement of gene or protein expression has been performed at a single target level, but the development of microarray technology has made measurement of the whole transcriptome in one single experiment feasible. Several important issues regarding measurement of expression levels are common for the different technologies.

- In general, the specificity of the RNA assays relies on complementarity of the sequences of the probe and the target. In northern blotting additional information regarding the size of the mRNA transcript is obtained. The methods used for analysis of protein expression in this study rely on antibody-antigen recognition. The specificity of both the RNA and the protein assays are dependent on several experimental factors that influence on the stringency of the hybridization.
- Microarray, northern blotting, RT-PCR and immunoblotting are batch analyses. Cellular heterogeneity may influence on the experimental obtained data and reduce the sensitivity of the assay.
- To compare the measured gene expression between biologically different samples one needs to normalize for differences in the amount of RNA or protein added to the experiment as well as for other factors that may affect the measurements, for example

degradation of RNA. This normalization can be performed by counting the cells or by measuring the amount of RNA or protein. In single target experiments it is common to normalize the measurements by use of an endogenous control gene. In the phosphorylation experiments, the total level of the same protein represents an ideal control. For the RT-PCR experiments we used *PGK1*. Experiments performed in our lab and published data (103) indicate that the expression of *PGK1* varies less between samples of lymphoid cells than other commonly used endogenous controls like *ACTB* and *GAPDH*.

Knowledge on how the mRNA and protein levels of a given gene are regulated is important when hypothesizing about its function. However, gene silencing followed by examination of the cellular phenotype is a more powerful tool for understanding gene function and should be performed when feasible (104).

5.1.5 Expression analysis: Microarray technology

Genome wide expression profiling at the RNA level is now feasible in one single experiment (2). While measurements of single variables typically are experiments that are hypothesis-driven, the data obtained by the measurement of tens of thousands of variables in one microarray experiment are nonselective. Thus, such experiments have the potential to discover completely new characteristics of the biological system and to generate new hypotheses. Additionally, the parallel expression measurements in microarray experiments allow comparison of patterns of gene expression. By sophisticated statistical approaches, this can be used both to identify subgroups in a heterogeneous population of samples, class discovery, and to identify patterns of gene expression that predict known subgroups, class prediction (105). With regard to malignant lymphomas several new entities have been identified (3;30;31;41;41;91;106;107). In our studies we have used the microarray technology as a hypothesis generating tool.

In paper III and IV we used microarray technology to compare two sample populations. Such analyses consist in principle of three different steps.

- Experimental procedures, data acquisition, filtering and normalization. This step is technology dependent, and was therefore performed differently in paper III and IV. In paper III we used cDNA arrays and in paper IV we used commercially available oligonucleotide arrays (www.affymetrix.com). We chose to use well established experimental procedures and methods for data acquisition, filtering and normalization

for each platform. The output of this step is an array of data that includes expression values of all the genes in all the experiments.

- Identification of differentially expressed genes. The large amount of measurements of gene expression, combined with a limited amount of samples is statistically challenging. The most widely accepted tool for adjustment for multiple comparisons, the Bonferroni correction, implies dividing the required *p*-value with the number of analyses performed (108). This is considered too conservative in microarray experiments and the methods used in paper III and IV are tools that have been evolved to overcome this problem (109;110).
- Functional interpretation of the data. In this step the biological processes affected by the experimental conditions are described. To aid such interpretation automatic procedures have been introduced and in paper III we used the PubGene tool (www.pubgene.com) that has been developed at our institution (111).

The experimental procedures included in the first step are crucial. A microarray experiment is complicated, and numerous technical factors may influence on the inter-assay variability in addition to the biological factors tested for. This includes preparation of the biological sample, RNA extraction, labeling technique and hybridization conditions. A high degree of cautiousness is therefore required to secure reliable data. Unsupervised hierarchical clustering can be used to detect how variations in experimental procedures may influence on expression data. We have previously performed mRNA expression profiling of isolated B-cell precursors. These experiments were performed at two time points, and the differences in the procedures included; pooling vs. individual samples, different fluorescence-activated cell sorters and different batches of arrays. The results of unsupervised hierarchical clustering of the samples are shown in Figure 6.

Firestein *et al*. has suggested four general issues that should be considered when evaluating microarray data (112):

1) Reproducibility has to be demonstrated. This means that measurements of fold change in one experiment are not sufficient.
2) Statistical analysis with correction for multiple testing is required.
3) To reduce the complexity of analysis, homogeneous cell populations should be studied.
4) A reasonable number of genes should be evaluated by an independent method.

All these criteria seem to be fulfilled in paper III and IV.

Finally, although gene expression data obtained by microarray experiments generally are reliable, it is necessary to perform additional experiments to confirm their validity and biological importance.

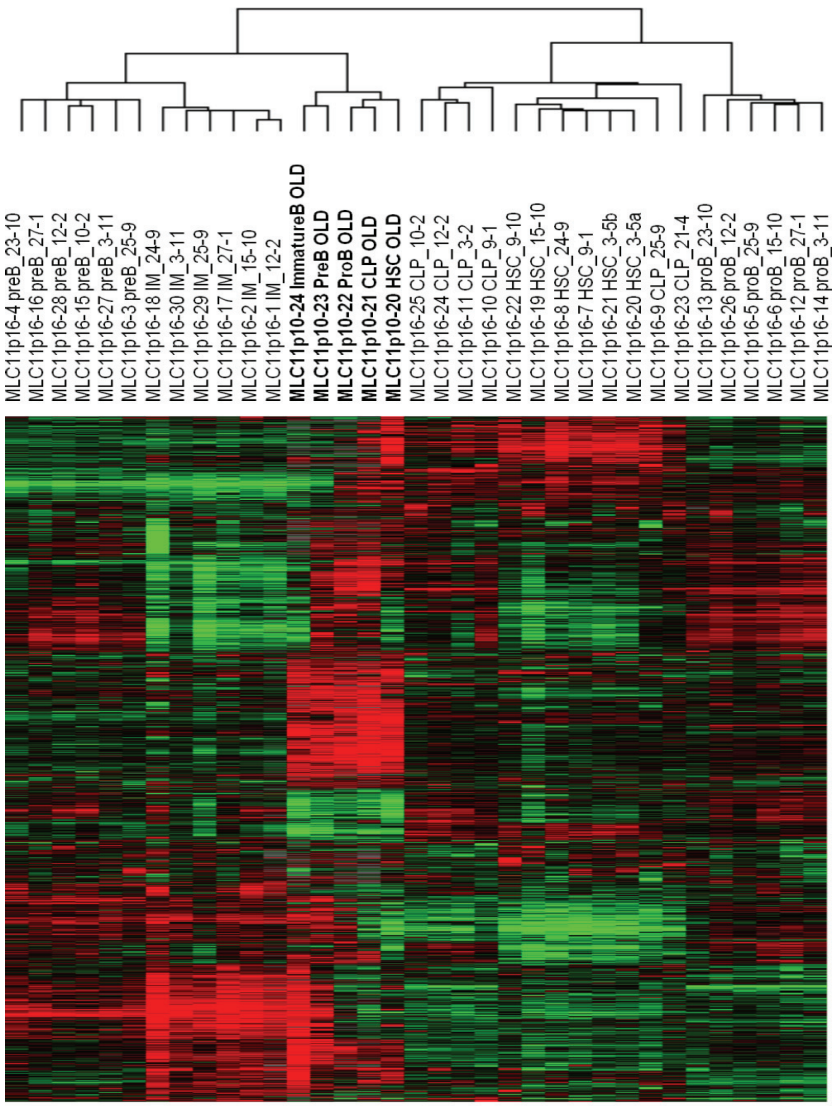


Figure 6 Unsupervised hierarchical clustering of B-cell precursors using the 2943 clones in the experiment set with highest standard variation. Samples from two time points cluster separately due to differences in technical procedures, whereas the other samples from the different developmental stages predominantly cluster together. The samples marked as "OLD" exhibit higher expression levels of NF-κB target genes.

5.2 Genetic aberrations and impact on tumor phenotype

Cancer involves activation of proto-oncogenes and loss of tumor suppressor genes. Proto-oncogenes can be converted to oncogenes by; A) Point mutations in coding sequence leading to a hyperactive protein, B) Gene amplification leading to overproduction of the protein or C) Chromosome rearrangements leading to overproduction or a hyperactive fusion protein (87). Chromosomal translocations are particular common in B-cell lymphoproliferative diseases (113). By use of the CGH technique recurrent numerical alterations have also been identified in malignant lymphomas and, in some cases, found to be significantly correlated to clinical outcome (47;48;114-121). The selection mechanisms, i.e. target genes, for most of these alterations are at present poorly understood.

5.2.1 Gain of chromosome 18 and BCL2

In paper I we examined numerical alterations of regions of chromosome 18 in NHL. We defined the minimal common region gained in the tumors negative or positive for the t(14;18). In lymphomas lacking t(14;18) *BCL2* was included in this region in all cases. In tumors with t(14;18) the 18q gain was more frequent than in t(14;18) negative tumors, but *BCL2* was not included in the minimal common region. Typically the chromosome 18 gain was caused by one extra der18t(14;18) and did thus not include the *BCL2* locus. In some cases an extra copy of the entire chromosome 18 was gained in t(14;18) positive tumors.

The observed gain of 18q in our material was low-level with the highest copy numbers of *BCL2* detected being 6 in a tetraploid tumor and 5 in a diploid tumor. Other studies have found high-level gain of *BCL2* in B-NHL more frequently (48;122). The observed gain was, however, associated with increased BCL2 protein expression. The cases with both t(14;18) and *BCL2* gain had higher expression of BCL2 than the tumors with t(14;18) only. The latter cases had higher BCL2 levels than the ones with *BCL2* gain only, which again had higher BCL2 levels than the tumors with no *BCL2* alterations. Increased BCL2 expression probably confers an oncogenic effect by increasing tumor cell survival. The observed gene dosage effect on BCL2 expression, although less pronounced than in a previous study (123), suggests that *BCL2* is an important target for the 18q gains that include this locus.

There was a weak, but significant, correlation between expression of BCL2 protein and levels of spontaneous apoptosis in the tumor cells, but the latter levels were not different in the cases with both t(14;18) and *BCL2* gain, t(14;18), *BCL2* gain and no *BCL2* alterations. However,

levels of spontaneous apoptosis in the normal cells in the t(14;18) positive tumors were higher than the corresponding tumor cell apoptotic fractions.

In line with a previous study of DLBCLs, gain of *BCL2* only was correlated to poor prognosis (48). However, in our material *BCL2* gain had no independent prognostic value, when assessed together with the prognostic parameters found in an earlier study (47). Our material was heterogeneous with respect to histological subtypes and this may have contributed to the discordance in the results of the two studies.

Although the minimal common region of gain of tumors with t(14;18) did not include the *BCL2* locus, it was found to overlap with the consensus gain of tumors without t(14;18). The overlapping region included *PMAIP* and *MALT1*, proto-oncogens that have been suggested as candidate targets in lymphomas with chromosome 18 gain (124;125).

To conclude, tumors with 18q gain exhibit phenotypes that in part may be caused by gain of the *BCL2* gene. It is likely that additional genes are important for the selection mechanism and resulting phenotype.

5.2.2 Gain of 6p and *PIM1*

In paper II we characterized the gain of genomic material on chromosome 6p in the DLBCL line U698 (126) as well as in primary non-Hodgkin's B-cell lymphomas. In U698 cells we found a narrow (about one mbp), high-level amplification at 6p21.2 that included the oncogene *PIM1* (15-20 copies). The increased DNA content was reflected in high expression levels of the corresponding mRNA and protein, and these observations suggest that *PIM1* is the target for the amplification. Previously there have been sporadic reports on amplica at 6p21 in a lymphoma cell line as well as in primary tumors (115;116;118;127). Gain of 6p is, however, a common finding in B-NHL and has been detected in 10% of FL by karyotyping (128). The incidences found are generally more frequent, up to 25%, when chromosomal or array CGH techniques are applied on materials of DLBCLs or mantle cell lymphomas (MCL) (47;48;119;129). Using array CGH and FISH we found 6p gain in 13% of the tumors in our material and defined the minimal consensus region for gain as 6p22.1-ter. This consensus region did not include *PIM1*, which is in line with other studies of DLBCL and MCL (119;130). By FISH analysis, 10 out of 92 analyzed tumors exhibited increased copy number of *PIM1* relative to the DNA index. The gain was low-level.

We also analyzed *PIM1* copy numbers by FISH in a set of 35 DLBCLs that was independent from the previously analyzed B-NHL. Genome wide expression data for these tumors were publicly available (30). On average, the 7 tumors with increased copy number of *PIM1* exhibited 20% higher expression levels of *PIM1* compared to the ones without gain. This difference was, however, not significant (95% confidence interval 0.74-1.96, p=0.41). The observed increase in mRNA expression levels is in agreement with the findings of Pollack *et al*. (131). In their study of primary human breast tumors they found that a 2-fold change in DNA copy number is associated with a corresponding 1.5-fold change in mRNA levels. Pollack *et al*. employed cDNA microarray technology to determine the mRNA expression levels, as was done in paper II, and it has been suggested that this platform underestimates the expression differences (132).

Increased expression of *PIM1* has previously been associated with development of lymphomas (133-135). The serine/threonine kinase encoded by *PIM1* has been shown to inhibit apoptosis in hematopoietic cells (136-138). The functional role of a moderately increased *PIM1* expression in malignant lymphomas is not clear, but one cannot rule out that it may confer an anti-apoptotic effect which may influence on the tumor phenotype. However, as will be discussed in section 5.3, other mechanisms for *PIM1* upregulation than mere gain of *PIM1* DNA, seem to be more important.

We have previously demonstrated a negative prognostic effect of 6p gain in the material that now was subjected to array CGH analysis (47). There was no difference in survival between the 7 tumors with and the 28 without *PIM1* gain in the analyzed DLBCL material. The prognostic effect of 6p gain has not been confirmed in other recently published lymphoma studies (48;129;139). The prognostic significance of 6p gain in malignant lymphomas is thus not clear.

5.3 Dysregulation of the NF-κB pathway in malignant lymphomas

Constitutive active nuclear NF-κB has been found in many types of cancer (140). NF-κB can suppress apoptosis through induction of numerous anti-apoptotic proteins (141). Through this mechanism, constitutive active NF-κB may affect response to treatment and clinical outcome in cancer patients.

Subcellular localization of the NF-κB component c-Rel can be investigated by immunohistochemistry. Nuclear c-Rel suggests constitutive activation of NF-κB (142).

Several NF-κB gene signatures, that include target genes identified by microarray analysis of manipulated cell lines, have also been defined (88;142-144). Such signatures have been used to test for NF-κB activity in clinical samples (142). Among the genes included in NF-κB signatures are *BCL2* and *PIM1*. With regard to malignant lymphomas, NF-κB dysregulation has been shown in Hodgkin's disease, ABC like DLBCL and primary mediastinal B-cell Lymphoma (88;142;143). Moreover, a pathogenetic role for the NF-κB activation has also been demonstrated.

In paper II we found no significant difference in mRNA expression of *PIM1* between tumors with and without gain of the gene. The expression was, however, highly dependent on the DLBCL subtype and mainly found in ABC like DLBCL, which are characterized by constitutive activation of the NF-κB pathway (30). Recently it was shown that treatment of ABC DLBCL cell lines with inhibitors of IκB kinase (IKK) was toxic for the cells and accompanied by changes in gene expression that included downregulation of *PIM1* (88). This suggests that the high expression levels of *PIM1* seen in ABC like DLBCL, at least partly, are caused by transcriptional activation and dysregulation of the NF-κB pathway.

In paper I we quantified protein expression levels of BCL2, which is a NF-κB target gene, in B-NHL. The expression levels were shown to be dependent on the *BCL2* genotype. Only a proportion of the B-NHLs in this study were DLBCLs. In this subgroup the protein expression showed a similar trend as in the whole material with regard to dependency on *BCL2* genotype. Although we investigated the translocation status, we had no information regarding DLBCL subtypes; neither did we have any direct information regarding NF-κB activity in these tumors. The relatively weak correlation between the expression of BCL2 and levels of spontaneous apoptosis in the tumor cells probably reflects that other mediators contribute to the regulation of apoptosis in transformed B-cells, possibly dependent on activation of NF-κB (140). With regard to DLBCLs, BCL2 expression varies between subgroups as does t(14;18) (145).Interestingly it was recently shown that BCL2 expression had a significant adverse effect on overall survival within the ABC subgroup. No such effect was observed in the GCB subgroup (146). The authors suggested increased copy number of 18q and constitutive activation of NF-κB as alternative mechanisms to t(14;18) for upregulation of BCL2 (146).

Several genetic mechanisms may account for constitutive activation of NF-κB in malignant lymphomas (140;143), but interestingly, gain and high expression of the NF-κB component c-

Rel in malignant lymphomas, does not lead to increased expression of NF-κB target genes (30). In paper I we demonstrated that *MALT1* was included in the consensus region of the frequent gain of 18q in lymphomas. *MALT1* is the target of t(11;18) in MALT lymphomas. Both the fusion protein API2/MALT1 and mere increased expression of MALT1 protein can activate NF-κB through interaction with BCL10 (140;147;148). The increased copy number of *BCL2* itself as well as increased expression of MALT1 protein and subsequent activation of NF-κB could thus mediate the increased expression of BCL2 protein in lymphomas with gain of 18q.

5.4 The PI3K/Akt pathway; role in erythroid progenitors and malignant lymphomas

As introduced in section 2.3.1, various ligand-receptor interactions in non-malignant hematopoietic cells lead to activation of PI3K. Akt is an important downstream target of the lipid products of PI3K, and the activity of Akt has profound impact on cell growth, proliferation and apoptosis in normal hematopoietic cells. Given its physiological role, it is not surprising that the PI3K/Akt pathway also plays an important role in cancer pathogenesis. In line with this, it has been shown that Akt is over-expressed or constitutively active in several cancers (59;149). With regard to malignant lymphomas it was recently shown that such constitutive activation also contributes to the pathogenesis of mantle cell lymphoma (MCL), and is accompanied by phosphorylation of several downstream targets of Akt including BAD and mTOR (150). Furthermore, inhibition of the PI3K/Akt pathway in MCL cell lines led to cell cycle arrest and apoptosis. The authors suggested that loss of the negative regulator *PTEN*, but not activating somatic mutations in the *PI3KCA* gene, is a possible mechanism for the observed activation (150). Activation of the PI3K/Akt pathway has also been shown in other lymphoma types including anaplastic large cell lymphomas and Hodgkin's lymphoma (84;151).

Previously, our group and others have demonstrated the critical role of PI3K in Epo mediated cell cycle progression and differentiation of CD34+ progenitors (66;67;152). In paper III we showed that activation of Akt is essential in this process. We also investigated how the activation of this signaling pathway influenced on the expression of cell cycle proteins, and showed that Epo induced upregulation of cyclins D_3, E and A in a PI3K dependent manner, whereas the protein expression of $p27^{Kip1}$ and $p21^{Cip1}$ largely were unaffected. The impact of PI3K/Akt on expression of cyclins and CKI varies from cell type to cell type. In a study of

erythroid progenitors that represented a later developmental stage than ours, PI3K dependent downregulation of $p27^{Kip1}$, which was mediated by degradation by the E3 ligase SCF^{SKP2}, was observed (67). Recently, a role for Akt mediated downregulation of $p27^{Kip1}$ was also implicated in anaplastic large cell lymphoma and MCL (150;153). However, in another study Akt transformed keratinocytes did not show alterations of $p27^{Kip1}$ expression (154). In conclusion, PI3K/Akt activation invariably promotes cell cycle progression but how this is achieved at the level of cyclin and CKI expression seems to be context dependent.

In paper III we concluded that the cyclins were not regulated at the transcriptional level. None of the cyclins that we tested in real time RT-PCR experiments showed significant changes in response to Epo stimulation. Neither were any cyclins or CKI among the significant changed genes identified by gene expression profiling. Previous studies have shown that Cyclin D expression is controlled post-transcriptionally via a PI3K/Akt dependent pathway (155;156). In ovarian cancer cells upregulation of cyclins at the protein level was mediated by the downstream target of Akt, mTOR (156).

The PI3K/Akt pathway controls transcriptional regulation by phosphorylation of specific transcription factors. In this respect it has been shown that members of the Forkhead transcription factor family are targets of PI3K/Akt in Epo mediated signaling and in lymphocytes as well as in lymphomas (76;150;157). Upon phosphorylation, these transcription factors are released to the cytosol and transcription of growth promoting genes takes place. In paper III we identified novel transcriptional targets of Epo in erythroid progenitors. *KIT* (CD117) mRNA as well as protein was up-regulated in response to Epo treatment. This regulation was dependent on PI3K activity, and interestingly, inhibition of PI3K in untreated cells also led to reduction in *KIT* expression. *KIT* is down-regulated (repressed by GATA-1) in later stages of erythroid differentiation, but the levels in CD34+ progenitors are high. c-kit signaling is crucial for survival and proliferation of hematopoietic progenitors and with respect to this, inactivation of FOXO3 by a PI3K/Akt dependent pathway has been shown to be essential (158). Thus, the increased expression of *KIT* and the corresponding protein in response to PI3K/Akt activity indicates a possible positive feedback mechanism in cells located in a microenvironment where SCF is abundant, as in the BM, and in cells where c-kit signaling is constitutive due to *KIT* mutations, as in gastrointestinal stromal tumors (GISTs) and in acute myelogeneous leukemias, AML (159;160). With regard to this, it was recently shown that treatment of GIST cell lines with the c-kit inhibitor Imatinib

induced apoptosis, and this effect was mediated via PI3K (161). The role of c-kit expression in malignant lymphomas is debated. In DLBCL c-kit expression seems to be infrequent (162). A recent study of MM found c-kit expression in 42% of the patient samples, but treatment with Imatinib had no effect (163).

Most identified Epo responsive genes in paper III were shown to be regulated in a PI3K dependent manner. This included the tissue specific genes *GYPA* (glycophorine A) and *TFRC* (CD71) as well as non-tissue specific genes. However, it is noteworthy that the anti-apoptotic gene *PIM1*, as expected, was up-regulated independently of PI3K (164).

Given the role of the PI3K/Akt pathway in cancer pathogenesis, Akt and other molecules in the regulatory cascade are attractive targets for antitumor therapy. Oral inhibitors of the Akt downstream target mTOR are already in phase II/III clinical evaluation (57;59). Inhibitors of Akt itself are also being investigated, but problems with specificity and bioavailability have so far prevented clinical trials with such components (58).

5.5 BMP-6 signaling in malignant lymphocytes and normal T-cells

It was recently shown that high expression levels of BMP-6 predict poor outcome in DLBCL (30). BMP-6 expression has also been correlated to adverse outcome in other cancer types, including prostate cancer (165). Several studies have pointed towards the role of infiltrating non-tumor cells in malignant lymphomas (166-170). In FL it has been suggested that the prognosis depends on the gene expression profile of the infiltrating non-tumor cells (40;171). These observations have raised several questions with regard to BMP-6 signaling in normal and malignant lymphocytes:

- Which cells produce BMP-6 in malignant lymphomas?
- Is BMP-6 mRNA translated into protein in lymphocytes?
- Which cells are targets for BMP-6 in malignant lymphomas?
- What is the functional role of BMP-6 signaling in malignant lymphomas?
- Which molecular mechanisms mediate the effect of BMP-6 in the target cells?

Several of these questions have been addressed in previous studies of our group (80;172). In this thesis the focus was on BMP-6 signaling in T-lineage cells.

5.5.1 BMP-6 signaling in malignant lymphocytes

In paper IV we showed that BMP-6 exerted an anti-proliferative effect in the pre-T ALL cell line Jurkat TAg. This reduction in DNA synthesis is in accordance with previous findings

concerning BMP effects on malignant lymphoid cells, although others also have demonstrated apoptosis induction (173). We have recently shown such inhibitory effects on immature as well as mature B-cells (80;172). Similar effects were also demonstrated for BMP-2, -4, -6 and -7 in primary isolated human myeloma cells (174;175). In other cellular systems BMPs have been shown to stimulate tumor growth by directly stimulating the malignant cells (176). Thus, the effect of BMPs seems to be context dependent. Preliminary data from our group indicate that the inhibitory effect of BMPs on transformed B-cell lines varies considerably, and seems to be partly dependent on the cell lines' expression levels of BMPs. The majority of investigated lymphoma cell lines showed high expression levels of BMP-7, and they were all resistant to BMP-7 induced growth inhibition. Fewer of the cell lines expressed detectable levels of BMP-6, and the resistance to this cytokine was more unpredictable (June Myklebust, personal communication). On basis of these data one could speculate that high BMP-6 levels are a marker of loss of BMP sensitivity that potentially could mediate adverse outcome in malignant lymphomas.

ID1-ID3 are central transcriptional targets of BMP-6 in Jurkat TAg cells as demonstrated by the microarray data in paper IV. This was associated with Smad phosphorylation.This finding is in accordance with others that have demonstrated that BMP induced *ID* upregulation is dependent on Smad phosphorylation (177). The functional effect was, at least partly, dependent on induction of the Id1 protein. Id1 also mediates the effect of BMPs in other cellular systems. In endothelial cells BMP-6 stimulated migration and tube formation via Id1 upregulation (177). The functional effects of BMP-2 in a lung cancer cell line were also related to Smad1 dependent upregulation of Id1 (176). Id1 and other Id proteins act by binding to other helixloop-helix, HLH, transcription factors inhibiting their DNA binding activity. Therefore, the effects of Id proteins are dependent on expression of other HLH transcription factors. In lymphoid cells E-proteins are crucial targets for Id proteins, which can modulate these transcription factors in regulating survival, proliferation and differentiation (178;179). In this respect, loss of *E2A* and overexpression of *ID1* and *ID3* have been shown to suppress T-cell development (180-182). Importantly, the effect of Id proteins is different in other cellular systems. Id1 seems to play a role in tumorigenesis and has been found to be over-expressed in several cancer types where it frequently is associated with poor outcome (183-185). The role of Id proteins in B-NHL is largely unexplored, but Id1 has been suggested to play a role in MM (186). Enforced expression of Id1 or Id2 in T-cell progenitors also results in lymphomagenesis (178;187).

In paper IV, *NFKBIA*, the gene encoding IκBα, was identified by the microarray analysis as a transcriptional target for BMP-6 in Jurkat TAg. IκBα is a negative regulator of the critical transcription factor NF-κB and functions by sequestering NF-κB in the cytoplasm (85;86). It has previously been shown that increased transcription of IκBα opposes NF-κB activity in a B-cell line (188). As discussed in section 5.3, dysregulation of NF-κB activity is an important feature of subgroups of B-NHL. Whether BMP-6 also promotes such activity in lymphoma cells is not known, but it is noteworthy that high BMP-6 expression is correlated to the ABC like DLBCL phenotype, in which NF-κB target genes are highly expressed (30).

The physiological effects of BMPs are regulated also by extracellular antagonists that can block BMP-receptor binding. With regard to cancer biology, it was recently shown that one such antagonist, gremlin 1, was expressed by non malignant stromal cells in tumors where the malignant cells had high BMP expression. Moreover, expression of gremlin 1 promoted self-renewal of the tumor cells, inhibiting the differentiation signals from BMP (189). In paper IV we showed that the gene that encodes noggin (*NOG*), another extracellular BMP antagonist, was a transcriptional target for BMP-6 in Jurkat TAg cells. BMP-6 also induced expression of noggin protein in rat osteoblasts (190). Induction of *NOG* and *SMAD6* represents negative feedback mechanisms of BMP-6 signaling. These molecules are thus potential targets for manipulation of BMP-6 signaling *in vivo*. Whether noggin protein is expressed in malignant lymphomas, either by the tumor cells or by the non-malignant infiltrating cells, remains to be tested.

5.5.2 BMP-6 signaling in normal T-cells

Recent studies have reported physiological effects of BMPs in hematopoietic stem cells, normal B-progenitors and mature B cells (80;172;191). Less is known regarding possible effects of BMPs in T-lineage cells, although a role of BMPs have been implicated in thymopoiesis (192-194).

In paper IV we investigated the role of BMP-6 in mature human T-cells. In contrast to the effect on normal B-progenitors and mature B-cells, BMP-6 did not affect DNA synthesis in PHA- or anti-CD3/anti-CD28 activated CD4+ T cells as determined by 3HT incorporating assays. Neither did BMP-6 promote the generation of CD25+CD4+ regulatory T-cells as determined by expression of the transcription factor *FOXP3* in real time RT-PCR assays. However, BMP-6 significantly inhibited the differentiation into IL-4 producing Th2 effector cells from naïve precursors. This inhibitory effect of BMP-6 was somewhat weaker than that

of TGF-β. BMP-6 stimulation led to a rapid phosphorylation of Smad1/5/8 in CD4+ T-cells and *ID1-3* were identified as transcriptional targets. This is in line with findings in B-lineage cells and in Jurkat TAg cells. The role of Id proteins in mediating the inhibitory effect in CD4+ T cells is, however, elusive as no changes in the expression of Id proteins was detected upon BMP-6 stimulation. This could be due to sensitivity problems of the immunoblot assay. With respect to receptor expression, it is noteworthy that CD4+ T cells constitute a heterogeneous population with the expression of the BMP receptors Alk-3 and BMPRII confined to smaller subpopulations.

Other molecular mechanisms than Id protein mediated inhibition of HLH transcription factors could, however, account for the inhibitory effect of BMP-6 on Th2 differentiation. GATA-3 is the crucial transcription factor for Th2 differentiation. It has been shown that TGF-β directly inhibits the expression of *GATA3* in T-cells (195). Further, NF-κB activity is also important for proper induction of *GATA3* expression. It has been shown that inhibition of NF-κB activity could prevent expression of this transcription factor and the resulting Th2 cytokine production in developing Th2 cells (196). In paper IV we did not identify *GATA3* as an immediate transcriptional target of BMP-6 in Jurkat TAg, but interestingly, the NF-κB inhibitor *NFKBIA* (IκBα) was identified as an up-regulated gene in the microarray analysis. One could therefore speculate that the inhibitory effect of BMP-6 in CD4+ T-cells on Th2 differentiation is mediated via an upregulation of *NFKBIA* and subsequent reduction in NF-κB activity and GATA-3 expression.

The inhibition of Th2 differentiation of naïve CD4+ T-cells induced by BMP-6 argues for an immunomodulatory role of this cytokine on T-cells. This could be of importance with regard to anti tumor immune responses. Data on how the Th1/Th2 balance affects lymphoma risk are not consistent. In clinical settings with generally reduced Th1 activity, such as HIV infection, use of TNF-α inhibitors or other immunosuppressive agents, an increase in NHL incidence is observed (197). Epidemiological studies of non-immune deficient people indicate, however, that persistence of a Th2 dominated immune response, as in patients with atopic diseases, is associated with reduced risk of NHL (198). In a recent study an impaired differentiation to effector T-cells was found in patients with B-cell NHL (199). The tumor infiltrating lymphocytes in this study were predominantly found to be of an immature phenotype. A potential immunoregulatory role of BMP-6 in T cells with implications for lymphoid malignancies will be a subject for future investigations.

6 Conclusions

- Array CGH with genomic BAC clones can detect numeric genomic alterations with high sensitivity and resolution, thus it is a powerful tool for establishing consensus regions for common gains and losses in malignant lymphomas.
- Whole or partial gains of chromosome 18 are common in B-NHL. In lymphomas with t(14;18) the consensus 18q gain did not include *BCL2*. In lymphomas lacking t(14;18) the gain was centered around 18q21, including the *BCL2*.
- Low-level gain of *BCL2* led to elevated expression of the corresponding protein. t(14;18) and, to a lesser extent, gain of *BCL2* of the tumor cells was associated with increased normal cell apoptotic fraction, compared to the normal cell apoptotic fractions of tumors with no *BCL2* alterations.
- The consensus region in lymphomas with 6p gain was 6p22.1-pter, not including the *PIM1* locus. *PIM1* expression in DLBCL was correlated to the ABC like subtype, and was not dependent on copy number.
- In CD34+ progenitor cells, Epo alone was able to induce cell cycle progression, and this effect was dependent on PI3K activity. Enforced expression of an activated form of Akt kinase highly augmented Epo induced erythropoiesis.
- The majority of Epo target genes, identified by microarray analysis, were regulated in a PI3K dependent manner, including *KIT* (CD117) and *CDH1* (E-cadherin).
- A functional BMP signaling pathway was present in human T-cells and the transcriptional response to BMP-6 stimulation included potent upregulation of *ID1*, *ID2* and *ID3*. Id1 and Id3 were also induced at the protein level in Jurkat TAg cells.
- Stimulation with BMP-6 exerted an antiproliferative effect in Jurkat TAg that was counteracted by *ID1* siRNA.
- In CD4+ T-cells, BMP-6 significantly inhibited the differentiation into IL-4 producing effector cells from naïve precursors albeit less potently than TGF-β.

7 Perspectives

Genome wide expression profiling has proven valuable for characterization of signaling pathways. Technical development in array based technologies will provide the scientific community with better tools for high-throughput analyses at the DNA, RNA and protein levels. At the RNA level arrays with transcript specific probes will be useful in analysis of mRNA splicing in gene regulation. Small miRNA arrays are already in use, and more comprehensive arrays will be interesting tools for analysis of the role of miRNA in normal hematopoietic cells as well as in lymphomas. Tiling path BAC clone arrays are already available for the analysis of copy number changes at the DNA level as are arrays for analysis of single nucleotide polymorphisms and high-throughput mutation detection. The expanded possibilities to analyze biological samples in a genome wide manner are challenging with regard to planning of experiments and organization of studies. With regard to profiling of lymphomas international cooperation is probably necessary to secure sample sets that are sufficiently large.

The combined use of technologies on the same samples is appealing. A dataset with information on copy number changes and expression profiles for a large set of lymphomas could serve several purposes. First, it could be used to identify novel oncogenes and tumor suppressor genes. Second, with such data it would also be possible to determine to what extent the expression of a given gene is dependent on gene copy number, or is regulated by unrelated mechanisms. Third, one could also identify the copy number alterations associated with constitutive activation of given signaling pathways, defined by gene signatures.

In this study we showed that BMP-6 may have an immunomodulatory role in T-cells. This finding combined with the recognized role of BMP-6 as a negative prognostic factor in DLBCL warrants further exploration of BMP-6 signaling in lymphomas. In characterizing both the tumor cells and the infiltrating non-malignant cells with respect to BMP-6 production and effects, use of microarray based technologies will be valuable.

8 References

(1) Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG et al. The sequence of the human genome. Science 2001; 291(5507):1304-1351.

(2) Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 1995; 270(5235):467-470.

(3) Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling [see comments]. Nature 2000; 403(6769):503-511.

(4) Spangrude GJ, Smith L, Uchida N, Ikuta K, Heimfeld S, Friedman J et al. Mouse hematopoietic stem cells. Blood 1991; 78(6):1395-1402.

(5) Li WC, Yu WY, Quinlan JM, Burke ZD, Tosh D. The molecular basis of transdifferentiation. J Cell Mol Med 2005; 9(3):569-582.

(6) Masson S, Harrison DJ, Plevris JN, Newsome PN. Potential of hematopoietic stem cell therapy in hepatology: a critical review. Stem Cells 2004; 22(6):897-907.

(7) Roybon L, Ma Z, Asztely F, Fosum A, Jacobsen SE, Brundin P et al. Failure of transdifferentiation of adult hematopoietic stem cells into neurons. Stem Cells 2006; 24(6):1594-1604.

(8) Ogawa M. Differentiation and proliferation of hematopoietic stem cells. Blood 1993; 81(11):2844-2853.

(9) Nakahata T, Okumura N. Cell surface antigen expression in human erythroid progenitors: erythroid and megakaryocytic markers. Leuk Lymphoma 1994; 13(5-6):401-409.

(10) Wu H, Liu X, Jaenisch R, Lodish HF. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. Cell 1995; 83(1):59-67.

(11) Krantz SB. Erythropoietin. Blood 1991; 77(3):419-434.

(12) McNiece IK, Langley KE, Zsebo KM. Recombinant human stem cell factor synergises with GM-CSF, G-CSF, IL-3 and epo to stimulate human progenitor cells of the myeloid and erythroid lineages. Exp Hematol 1991; 19(3):226-231.

(13) Munugalavadla V, Kapur R. Role of c-Kit and erythropoietin receptor in erythropoiesis. Crit Rev Oncol Hematol 2005; 54(1):63-75.

(14) Janeway CA, Travers P, Walport M, Schlomchik M. Immunobiology: The Immune System in Health and Disease. 6th ed. New York: Garland Publishing, 2004.

(15) Torlakovic E, Tenstad E, Funderud S, Rian E. CD10(+) stromal cells form B-lymphocyte maturation niches in the human bone marrow. J Pathol 2005; 205(3):311-317.

(16) LeBien TW. Fates of human B-cell precursors. Blood 2000; 96(1):9-23.

(17) Melchers F, Rolink A, Grawunder U, Winkler TH, Karasuyama H, Ghia P et al. Positive and negative selection events during B lymphopoiesis. Curr Opin Immunol 1995; 7(2):214-227.

(18) McHeyzer-Williams LJ, Driver DJ, McHeyzer-Williams MG. Germinal center reaction. Curr Opin Hematol 2001; 8(1):52-59.

(19) McHeyzer-Williams MG, McHeyzer-Williams LJ, Fanelli PJ, Bikah G, Pogue-Caley RR, Driver DJ et al. Antigen-specific immunity. Th cell-dependent B cell responses. Immunol Res 2000; 22(2-3):223-236.

(20) Pasqualucci L, Neumeister P, Goossens T, Nanjangud G, Chaganti RS, Kuppers R et al. Hypermutation of multiple proto-oncogenes in B-cell diffuse large-cell lymphomas. Nature 2001; 412(6844):341-346.

(21) Kuppers R, Klein U, Hansmann ML, Rajewsky K. Cellular origin of human B-cell lymphomas. N Engl J Med 1999; 341(20):1520-1529.

(22) Hogquist KA, Baldwin TA, Jameson SC. Central tolerance: learning self-control in the thymus. Nat Rev Immunol 2005; 5(10):772-782.

(23) Knutson KL, Disis ML. Tumor antigen-specific T helper cells in cancer immunity and immunotherapy. Cancer Immunol Immunother 2005; 54(8):721-728.

(24) Kuppers R. Mechanisms of B-cell lymphoma pathogenesis. Nat Rev Cancer 2005; 5(4):251-262.

(25) Tsujimoto Y, Cossman J, Jaffe E, Croce CM. Involvement of the bcl-2 gene in human follicular lymphoma. Science 1985; 228(4706):1440-1443.

(26) Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J et al. The World Health Organization classification of neoplasms of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting--Airlie House, Virginia, November, 1997. Hematol J 2000; 1(1):53-66.

(27) Harris NL, Jaffe ES, Stein H, Banks PM, Chan JK, Cleary ML et al. A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. Blood 1994; 84(5):1361-1392.

(28) Lossos IS, Alizadeh AA, Eisen MB, Chan WC, Brown PO, Botstein D et al. Ongoing immunoglobulin somatic mutation in germinal center B cell-like but not in activated B cell-like diffuse large cell lymphomas. Proc Natl Acad Sci U S A 2000; 97(18):10209-10213.

(29) Damle RN, Wasil T, Fais F, Ghiotto F, Valetto A, Allen SL et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood 1999; 94(6):1840-1847.

(30) Rosenwald A, Wright G, Chan WC, Connors JM, Campo E, Fisher RI et al. The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. N Engl J Med 2002; 346(25):1937-1947.

(31) Shipp MA, Ross KN, Tamayo P, Weng AP, Kutok JL, Aguiar RC et al. Diffuse large B-cell lymphoma outcome prediction by gene-expression profiling and supervised machine learning. Nat Med 2002; 8(1):68-74.

(32) Rosenwald A, Wright G, Leroy K, Yu X, Gaulard P, Gascoyne RD et al. Molecular diagnosis of primary mediastinal B cell lymphoma identifies a clinically favorable subgroup of diffuse large B cell lymphoma related to Hodgkin lymphoma. J Exp Med 2003; 198(6):851-862.

(33) Sanchez-Beato M, Sanchez-Aguilera A, Piris MA. Cell cycle deregulation in B-cell lymphomas. Blood 2003; 101(4):1220-1235.

(34) Huff CA, Matsui W, Smith BD, Jones RJ. The paradox of response and survival in cancer therapeutics. Blood 2006; 107(2):431-434.

(35) Peterson CG, Kahl BS. Follicular lymphoma: management options in the era of targeted therapy. Curr Treat Options Oncol 2005; 6(4):297-308.

(36) Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature 2001; 414(6859):105-111.

(37) Matsui W, Huff CA, Wang Q, Malehorn MT, Barber J, Tanhehco Y et al. Characterization of clonogenic multiple myeloma cells. Blood 2004; 103(6):2332-2336.

(38) The International Non-Hodgkin's Lymphoma Prognostic Factors Project. A predictive model for aggressive non-Hodgkin's lymphoma. N Engl J Med 1993; 329(14):987-994.

(39) Solal-Celigny P, Roy P, Colombat P, White J, Armitage JO, Arranz-Saez R et al. Follicular lymphoma international prognostic index. Blood 2004; 104(5):1258-1265.

(40) Dave SS, Wright G, Tan B, Rosenwald A, Gascoyne RD, Chan WC et al. Prediction of Survival in Follicular Lymphoma Based on Molecular Features of Tumor-Infiltrating Immune Cells. N Engl J Med 2004; 351(21):2159-2169.

(41) Rosenwald A, Wright G, Wiestner A, Chan WC, Connors JM, Campo E et al. The proliferation gene expression signature is a quantitative integrator of oncogenic events that predicts survival in mantle cell lymphoma. Cancer Cell 2003; 3(2):185-197.

(42) Ma C, Staudt LM. Molecular definition of the germinal centre stage of B-cell differentiation. Philos Trans R Soc Lond B Biol Sci 2001; 356(1405):83-89.

(43) Lossos IS, Jones CD, Warnke R, Natkunam Y, Kaizer H, Zehnder JL et al. Expression of a single gene, BCL-6, strongly predicts survival in patients with diffuse large B-cell lymphoma. Blood 2001; 98(4):945-951.

(44) Lossos IS, Czerwinski DK, Alizadeh AA, Wechser MA, Tibshirani R, Botstein D et al. Prediction of survival in diffuse large-B-cell lymphoma based on the expression of six genes. N Engl J Med 2004; 350(18):1828-1837.

(45) Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Delabie J, Ott G et al. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood 2004; 103(1):275-282.

(46) Yunis JJ, Mayer MG, Arnesen MA, Aeppli DP, Oken MM, Frizzera G. bcl-2 and other genomic alterations in the prognosis of large-cell lymphoma. N Engl J Med 1989; 320(16):1047-1054.

(47) Stokke T, DeAngelis P, Smedshammer L, Galteland E, Steen HB, Smeland EB et al. Loss of chromosome 11q21-23.1 and 17p and gain of chromosome 6p are independent prognostic indicators in B-cell non-Hodgkin's lymphoma. Br J Cancer 2001; 85(12):1900-1913.

(48) Bea S, Colomo L, Lopez-Guillermo A, Salaverria I, Puig X, Pinyol M et al. Clinicopathologic Significance and Prognostic Value of Chromosomal Imbalances in Diffuse Large B-Cell Lymphomas. J Clin Oncol 2004; 22(17):3498-3506.

(49) Viardot A, Moller P, Hogel J, Werner K, Mechtersheimer G, Ho AD et al. Clinicopathologic Correlations of Genomic Gains and Losses in Follicular Lymphoma. J Clin Oncol 2002; 20(23):4523-4530.

(50) Galteland E, Holte H, Stokke T. c-MYC, RB-1, TP53, and centromere 8 and 17 copy number in B-cell non-Hodgkin's lymphomas assessed by dual-color fluorescence in situ hybridization. Cytometry 1999; 38(2):53-60.

(51) Iqbal J, Neppalli VT, Wright G, Dave LJ, Horsman DE, Rosenwald A et al. BCL2 expression is a prognostic marker for the activated B-cell-like type of diffuse large B-cell lymphoma. J Clin Oncol 2006; 24(6):961-968.

(52) Stokke T, Galteland E, Holte H, Smedshammer L, Suo Z, Smeland EB et al. Oncogenic aberrations in the p53 pathway are associated with a high S phase fraction and poor patient survival in B-cell Non-Hodgkin's lymphoma. Int J Cancer 2000; 89(4):313-324.

(53) Chen PM, Chiou TJ, Hsieh RK, Fan FS, Chu CJ, Lin CZ et al. p53 gene mutations and rearrangements in non-Hodgkin's lymphoma. Cancer 1999; 85(3):718-724.

(54) Ichikawa A, Kinoshita T, Watanabe T, Kato H, Nagai H, Tsushita K et al. Mutations of the p53 gene as a prognostic factor in aggressive B-cell lymphoma. N Engl J Med 1997; 337(8):529-534.

(55) Shendure J, Mitra RD, Varma C, Church GM. Advanced sequencing technologies: methods and goals. Nat Rev Genet 2004; 5(5):335-344.

(56) Ragoussis J, Elvidge G. Affymetrix GeneChip system: moving from research to the clinic. Expert Rev Mol Diagn 2006; 6(2):145-152.

(57) Houghton PJ, Huang S. mTOR as a target for cancer therapy. Curr Top Microbiol Immunol 2004; 279:339-359.

(58) Barnett SF, Bilodeau MT, Lindsley CW. The Akt/PKB family of protein kinases: a review of small molecule inhibitors and progress towards target validation. Curr Top Med Chem 2005; 5(2):109-125.

(59) Bianco R, Damiano V, Tortora G. Combination of biological therapies in non-small cell lung cancer. Ann Oncol 2006; 17 Suppl 2:ii52-ii54.

(60) Vanhaesebroeck B, Waterfield MD. Signaling by distinct classes of phosphoinositide 3-kinases. Exp Cell Res 1999; 253(1):239-254.

(61) Uddin S, Kottegoda S, Stigger D, Platanias LC, Wickrema A. Activation of the Akt/FKHRL1 Pathway Mediates the Antiapoptotic Effects of Erythropoietin in Primary Human Erythroid Progenitors. Biochem Biophys Res Commun 2000; 275(1):16-19.

(62) Haseyama Y, Sawada Ki, Oda A, Koizumi K, Takano H, Tarumi T et al. Phosphatidylinositol 3-Kinase Is Involved in the Protection of Primary Cultured Human Erythroid Precursor Cells From Apoptosis. Blood 1999; 94(5):1568-1577.

(63) Fruman DA. Phosphoinositide 3-kinase and its targets in B-cell and T-cell signaling. Curr Opin Immunol 2004; 16(3):314-320.

(64) Remy I, Wilson IA, Michnick SW. Erythropoietin Receptor Activation by a Ligand-Induced Conformation Change. Science 1999; 283(5404):990-993.

(65) Richmond TD, Chohan M, Barber DL. Turning cells red: signal transduction mediated by erythropoietin. Trends Cell Biol 2005; 15(3):146-155.

(66) Myklebust JH, Blomhoff HK, Rusten LS, Stokke T, Smeland EB. Activation of phosphatidylinositol 3-kinase is important for erythropoietin-induced erythropoiesis from CD34+ hematopoietic progenitor cells. Exp Hematol 2002; 30(9):990-1000.

(67) Bouscary D, Pene F, Claessens YE, Muller O, Chretien S, Fontenay-Roupie M et al. Critical role for PI 3-kinase in the control of erythropoietin-induced erythroid progenitor proliferation. Blood 2003; 101(9):3436-3443.

(68) Kadri Z, Maouche-Chretien L, Rooke HM, Orkin SH, Romeo PH, Mayeux P et al. Phosphatidylinositol 3-Kinase/Akt Induced by Erythropoietin Renders the Erythroid Differentiation Factor GATA-1 Competent for TIMP-1 Gene Transactivation. Mol Cell Biol 2005; 25(17):7412-7422.

(69) Marshall AJ, Niiro H, Yun TJ, Clark EA. Regulation of B-cell activation and differentiation by the phosphatidylinositol 3-kinase and phospholipase Cgamma pathway. Immunol Rev 2000; 176:30-46.

(70) Genot EM, Arrieumerlou C, Ku G, Burgering BM, Weiss A, Kramer IM. The T-cell receptor regulates Akt (protein kinase B) via a pathway involving Rac1 and phosphatidylinositide 3-kinase. Mol Cell Biol 2000; 20(15):5469-5478.

(71) Koyasu S. The role of PI3K in immune cells. Nat Immunol 2003; 4(4):313-319.

(72) Fruman DA, Cantley LC. Phosphoinositide 3-kinase in immunological systems. Semin Immunol 2002; 14(1):7-18.

(73) Gold MR, Duronio V, Saxena SP, Schrader JW, Aebersold R. Multiple cytokines activate phosphatidylinositol 3-kinase in hemopoietic cells. Association of the enzyme with various tyrosine-phosphorylated proteins. J Biol Chem 1994; 269(7):5403-5412.

(74) Krasilnikov MA. Phosphatidylinositol-3 kinase dependent pathways: the role in control of cell growth, survival, and malignant transformation. Biochemistry (Mosc) 2000; 65(1):59-67.

(75) Otero DC, Omori SA, Rickert RC. Cd19-dependent activation of Akt kinase in B-lymphocytes. J Biol Chem 2001; 276(2):1474-1478.

(76) Yusuf I, Zhu X, Kharas MG, Chen J, Fruman DA. Optimal B-cell proliferation requires phosphoinositide 3-kinase-dependent inactivation of FOXO transcription factors. Blood 2004; 104(3):784-787.

(77) Kim SJ, Letterio J. Transforming growth factor-beta signaling in normal and malignant hematopoiesis. Leukemia 2003; 17(9):1731-1737.

(78) Ying QL, Nichols J, Chambers I, Smith A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell 2003; 115(3):281-292.

(79) Zhang J, Niu C, Ye L, Huang H, He X, Tong WG et al. Identification of the haematopoietic stem cell niche and control of the niche size. Nature 2003; 425(6960):836-841.

(80) Kersten C, Sivertsen EA, Hystad ME, Forfang L, Smeland EB, Myklebust JH. BMP-6 inhibits growth of mature human B cells; induction of Smad phosphorylation and upregulation of Id1. BMC Immunol 2005; 6(1):9.

(81) Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 2003; 425(6958):577-584.

(82) Yagi K, Furuhashi M, Aoki H, Goto D, Kuwano H, Sugamura K et al. c-myc is a downstream target of the Smad pathway. J Biol Chem 2002; 277(1):854-861.

(83) Lim MS, Adamson A, Lin Z, Perez-Ordonez B, Jordan RC, Tripp S et al. Expression of Skp2, a p27(Kip1) ubiquitin ligase, in malignant lymphoma: correlation with p27(Kip1) and proliferation index. Blood 2002; 100(8):2950-2956.

(84) Rassidakis GZ, Feretzaki M, Atwell C, Grammatikakis I, Lin Q, Lai R et al. Inhibition of Akt increases p27Kip1 levels and induces cell cycle arrest in anaplastic large cell lymphoma. Blood 2005; 105(2):827-829.

(85) Baldwin AS. Control of oncogenesis and cancer therapy resistance by the transcription factor NF-kappaB. J Clin Invest 2001; 107(3):241-246.

(86) Kucharczak J, Simmons MJ, Fan Y, Gelinas C. To be, or not to be: NF-kappaB is the answer--role of Rel/NF-kappaB in the regulation of apoptosis. Oncogene 2003; 22(56):8961-8982.

(87) Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Molecular Biology of the Cell. 4 ed. New York: Garland, 2002.

(88) Lam LT, Davis RE, Pierce J, Hepperle M, Xu Y, Hottelet M et al. Small Molecule Inhibitors of I{kappa}B Kinase Are Selectively Toxic for Subgroups of Diffuse Large B-Cell Lymphoma Defined by Gene Expression Profiling. Clin Cancer Res 2005; 11(1):28-40.

(89) Zhu N, Ramirez LM, Lee RL, Magnuson NS, Bishop GA, Gold MR. CD40 signaling in B cells regulates the expression of the Pim-1 kinase via the NF-kappa B pathway. J Immunol 2002; 168(2):744-754.

(90) Sivertsen EA, Galteland E, Mu D, Holte H, Meza-Zepeda L, Myklebost O et al. Gain of chromosome 6p is an infrequent cause of increased PIM1 expression in B-cell non-Hodgkin's lymphomas. Leukemia 2006; 20(3):539-542.

(91) Wright G, Tan B, Rosenwald A, Hurt EH, Wiestner A, Staudt LM. A gene expression-based method to diagnose clinically distinct subgroups of diffuse large B cell lymphoma. Proc Natl Acad Sci U S A 2003; 100(17):9991-9996.

(92) Montagut C, Rovira A, Albanell J. The proteasome: a novel target for anticancer therapy. Clin Transl Oncol 2006; 8(5):313-317.

(93) Olivier S, Robe P, Bours V. Can NF-kappaB be a target for novel and efficient anti-cancer agents? Biochem Pharmacol 2006; 72(9):1054-1068.

(94) Kvaloy S, Godal T, Marton PF, Steen H, Brenhovd IO, Abrahamsen AF. Spontaneous (H)-thymidine uptake in histological subgroups of human B-cell lymphomas. Scand J Haematol 1981; 26(3):221-234.

(95) Smeland EB, Funderud S, Kvalheim G, Gaudernack G, Rasmussen AM, Rusten L et al. Isolation and characterization of human hematopoietic progenitor cells: an effective method for positive selection of CD34+ cells. Leukemia 1992; 6(8):845-852.

(96) Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F et al. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science 1992; 258(5083):818-821.

(97) Bentz M, Plesch A, Stilgenbauer S, Dohner H, Lichter P. Minimal sizes of deletions detected by comparative genomic hybridization. Genes Chromosomes Cancer 1998; 21(2):172-175.

(98) Forozan F, Karhu R, Kononen J, Kallioniemi A, Kallioniemi OP. Genome screening by comparative genomic hybridization. Trends Genet 1997; 13(10):405-409.

(99) Pinkel D, Segraves R, Sudar D, Clark S, Poole I, Kowbel D et al. High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. Nat Genet 1998; 20(2):207-211.

(100) Pollack JR, Perou CM, Alizadeh AA, Eisen MB, Pergamenschikov A, Williams CF et al. Genome-wide analysis of DNA copy-number changes using cDNA microarrays. Nat Genet 1999; 23(1):41-46.

(101) Rosenberg C, Schut TB, Mostert MC, Tanke HJ, Raap AK, Oosterhuis JW et al. Comparative genomic hybridization in hypotriploid/hyperdiploid tumors. Cytometry 1997; 29(2):113-121.

(102) Noordewier MO, Warren PV. Gene expression microarrays and the integration of biological knowledge. Trends Biotechnol 2001; 19(10):412-415.

(103) Lossos IS, Czerwinski DK, Wechser MA, Levy R. Optimization of quantitative real-time RT-PCR parameters for the study of lymphoid malignancies. Leukemia 2003; 17(4):789-795.

(104) Dykxhoorn DM, Novina CD, Sharp PA. Killing the messenger: short RNAs that silence gene expression. Nat Rev Mol Cell Biol 2003; 4(6):457-467.

(105) Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek M, Mesirov JP et al. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 1999; 286(5439):531-537.

(106) Ek S, Hogerkorp CM, Dictor M, Ehinger M, Borrebaeck CA. Mantle cell lymphomas express a distinct genetic signature affecting lymphocyte trafficking and growth regulation as compared with subpopulations of normal human B cells. Cancer Res 2002; 62(15):4398-4405.

(107) Staudt LM. Gene expression profiling of lymphoid malignancies. Annu Rev Med 2002; 53:303-318.

(108) Altman DG. Practical Statistics for Medical Research. London: Chapman Hall, 1991.

(109) Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A 2001; 98(9):5116-5121.

(110) Breitling R, Armengaud P, Amtmann A, Herzyk P. Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. FEBS Lett 2004; 573(1-3):83-92.

(111) Jenssen TK, Laegreid A, Komorowski J, Hovig E. A literature network of human genes for high-throughput analysis of gene expression. Nat Genet 2001; 28(1):21-28.

(112) Firestein GS, Pisetsky DS. DNA microarrays: boundless technology or bound by technology? Guidelines for studies using microarray technology. Arthritis Rheum 2002; 46(4):859-861.

(113) Harris NL, Stein H, Coupland SE, Hummel M, Favera RD, Pasqualucci L et al. New approaches to lymphoma diagnosis. Hematology Am Soc Hematol Educ Program 2001;194-220.

(114) Bea S, Zettl A, Wright G, Salaverria I, Jehn P, Moreno V et al. Diffuse Large B-Cell Lymphoma Subgroups Have Distinct Genetic Profiles that Influence Tumor Biology and Improve Gene Expression-Based Survival Prediction. Blood 2005;106(9):3183-90.

(115) Bentz M, Werner CA, Dohner H, Joos S, Barth TF, Siebert R et al. High incidence of chromosomal imbalances and gene amplifications in the classical follicular variant of follicle center lymphoma. Blood 1996; 88(4):1437-1444.

(116) Dierlamm J, Rosenberg C, Stul M, Pittaluga S, Wlodarska I, Michaux L et al. Characteristic pattern of chromosomal gains and losses in marginal zone B cell lymphoma detected by comparative genomic hybridization. Leukemia 1997; 11(5):747-758.

(117) Galteland E, Smedshammer L, Suo Z, DeAngelis P, Stokke T. Proliferation-dependent expression and phosphorylation of pRB in B cell non-Hodgkin's lymphomas: dependence on RB1 copy number. Leukemia 2002; 16(8):1549-1555.

(118) Knuutila S, Bjorkqvist AM, Autio K, Tarkkanen M, Wolf M, Monni O et al. DNA copy number amplifications in human neoplasms: review of comparative genomic hybridization studies. Am J Pathol 1998; 152(5):1107-1123.

(119) Monni O, Joensuu H, Franssila K, Knuutila S. DNA copy number changes in diffuse large B-cell lymphoma--comparative genomic hybridization study. Blood 1996; 87(12):5269-5278.

(120) Monni O, Oinonen R, Elonen E, Franssila K, Teerenhovi L, Joensuu H et al. Gain of 3q and deletion of 11q22 are frequent aberrations in mantle cell lymphoma. Genes Chromosomes Cancer 1998; 21(4):298-307.

(121) Viardot A, Moller P, Hogel J, Werner K, Mechtersheimer G, Ho AD et al. Clinicopathologic correlations of genomic gains and losses in follicular lymphoma. J Clin Oncol 2002; 20(23):4523-4530.

(122) Rao PH, Houldsworth J, Dyomina K, Parsa NZ, Cigudosa JC, Louie DC et al. Chromosomal and Gene Amplification in Diffuse Large B-Cell Lymphoma. Blood 1998; 92(1):234-240.

(123) Monni O, Joensuu H, Franssila K, Klefstrom J, Alitalo K, Knuutila S. BCL2 overexpression associated with chromosomal amplification in diffuse large B-cell lymphoma. Blood 1997; 90(3):1168-1174.

(124) Martinez-Climent JA, Alizadeh AA, Segraves R, Blesa D, Rubio-Moscardo F, Albertson DG et al. Transformation of follicular lymphoma to diffuse large cell lymphoma is associated with a heterogeneous set of DNA copy number and gene expression alterations. Blood 2003; 101(8):3109-3117.

(125) Sanchez-Izquierdo D, Buchonnet G, Siebert R, Gascoyne RD, Climent J, Karran L et al. MALT1 is deregulated by both chromosomal translocation and amplification in B-cell non-Hodgkin lymphoma. Blood 2003; 101(11):4539-4546.

(126) Nilsson K, Sundstrom C. Establishment and characteristics of two unique cell lines from patients with lymphosarcoma. Int J Cancer 1974; 13(6):808-823.

(127) Matteucci C, La Starza R, Crescenzi B, Falzetti D, Romoli S, Emiliani C et al. Interpretation of the complex karyotype and identification of a new 6p amplicon by integrated comparative genomic hybridization and fluorescence in situ hybridization on the U937-I cell line. Cancer Genet Cytogenet 2002; 135(1):28-34.

(128) Hoglund M, Sehn L, Connors JM, Gascoyne RD, Siebert R, Sall T et al. Identification of cytogenetic subgroups and karyotypic pathways of clonal evolution in follicular lymphomas. Genes Chromosomes Cancer 2004; 39(3):195-204.

(129) Allen JE, Hough RE, Goepel JR, Bottomley S, Wilson GA, Alcock HE et al. Identification of novel regions of amplification and deletion within mantle cell lymphoma DNA by comparative genomic hybridization. Br J Haematol 2002; 116(2):291-298.

(130) Tagawa H, Karnan S, Suzuki R, Matsuo K, Zhang X, Ota A et al. Genome-wide array-based CGH for mantle cell lymphoma: identification of homozygous deletions of the proapoptotic gene BIM. 2004; 24(8):1348-1358.

(131) Pollack JR, Sorlie T, Perou CM, Rees CA, Jeffrey SS, Lonning PE et al. Microarray analysis reveals a major direct role of DNA copy number alteration in the transcriptional program of human breast tumors. Proc Natl Acad Sci U S A 2002; 99(20):12963-12968.

(132) Yuen T, Wurmbach E, Pfeffer RL, Ebersole BJ, Sealfon SC. Accuracy and calibration of commercial oligonucleotide and custom cDNA microarrays. Nucleic Acids Res 2002; 30(10):e48.

(133) Moroy T, Verbeek S, Ma A, Achacoso P, Berns A, Alt F. E mu N- and E mu L-myc cooperate with E mu pim-1 to generate lymphoid tumors at high frequency in double-transgenic mice. Oncogene 1991; 6(11):1941-1948.

(134) Moroy T, Grzeschiczek A, Petzold S, Hartmann KU. Expression of a Pim-1 transgene accelerates lymphoproliferation and inhibits apoptosis in lpr/lpr mice. Proc Natl Acad Sci U S A 1993; 90(22):10734-10738.

(135) Selten G, Cuypers HT, Berns A. Proviral activation of the putative oncogene Pim-1 in MuLV induced T-cell lymphomas. EMBO J 1985; 4(7):1793-1798.

(136) Lilly M, Kraft A. Enforced expression of the Mr 33,000 Pim-1 kinase enhances factor-independent survival and inhibits apoptosis in murine myeloid cells. Cancer Res 1997; 57(23):5348-5355.

(137) Lilly M, Sandholm J, Cooper JJ, Koskinen PJ, Kraft A. The PIM-1 serine kinase prolongs survival and inhibits apoptosis-related mitochondrial dysfunction in part through a bcl-2-dependent pathway. Oncogene 1999; 18(27):4022-4031.

(138) Pircher TJ, Zhao S, Geiger JN, Joneja B, Wojchowski DM. Pim-1 kinase protects hematopoietic FDC cells from genotoxin-induced death. Oncogene 2000; 19(32):3684-3692.

(139) Hough RE, Goepel JR, Alcock HE, Hancock BW, Lorigan PC, Hammond DW. Copy number gain at 12q12-14 may be important in the transformation from follicular lymphoma to diffuse large B cell lymphoma. Br J Cancer 2001; 84(4):499-503.

(140) Karin M, Cao Y, Greten FR, Li ZW. NF-kappaB in cancer: from innocent bystander to major culprit. Nat Rev Cancer 2002; 2(4):301-310.

(141) Karin M, Lin A. NF-kappaB at the crossroads of life and death. Nat Immunol 2002; 3(3):221-227.

(142) Feuerhake F, Kutok JL, Monti S, Chen W, LaCasce AS, Cattoretti G et al. NFkappaB activity, function, and target-gene signatures in primary mediastinal large B-cell lymphoma and diffuse large B-cell lymphoma subtypes. Blood 2005; 106(4):1392-1399.

(143) Davis RE, Brown KD, Siebenlist U, Staudt LM. Constitutive nuclear factor kappaB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells. J Exp Med 2001; 194(12):1861-1874.

(144) Shaffer AL, Rosenwald A, Hurt EM, Giltnane JM, Lam LT, Pickeral OK et al. Signatures of the immune response. Immunity 2001; 15(3):375-385.

(145) Huang JZ, Sanger WG, Greiner TC, Staudt LM, Weisenburger DD, Pickering DL et al. The t(14;18) defines a unique subset of diffuse large B-cell lymphoma with a germinal center B-cell gene expression profile. Blood 2002; 99(7):2285-2290.

(146) Iqbal J, Neppalli VT, Wright G, Dave BJ, Horsman DE, Rosenwald A et al. BCL2 expression is a prognostic marker for the activated B-cell-like type of diffuse large B-cell lymphoma. J Clin Oncol 2006; 24(6):961-968.

(147) Akagi T, Motegi M, Tamura A, Suzuki R, Hosokawa Y, Suzuki H et al. A novel gene, MALT1 at 18q21, is involved in t(11;18) (q21;q21) found in low-grade B-cell lymphoma of mucosa-associated lymphoid tissue. Oncogene 1999; 18(42):5785-5794.

(148) Lucas PC, Yonezumi M, Inohara N, McAllister-Lucas LM, Abazeed ME, Chen FF et al. Bcl10 and MALT1, independent targets of chromosomal translocation in malt lymphoma, cooperate in a novel NF-kappa B signaling pathway. J Biol Chem 2001; 276(22):19012-19019.

(149) Nicholson KM, Anderson NG. The protein kinase B/Akt signalling pathway in human malignancy. Cell Signal 2002; 14(5):381-395.

(150) Rudelius M, Pittaluga S, Nishizuka S, Pham TH, Fend F, Jaffe ES et al. Constitutive activation of Akt contributes to the pathogenesis and survival of mantle cell lymphoma. Blood 2006; 108(5):1668-1676.

(151) Dutton A, Reynolds GM, Dawson CW, Young LS, Murray PG. Constitutive activation of phosphatidyl-inositide 3 kinase contributes to the survival of Hodgkin's lymphoma

cells through a mechanism involving Akt kinase and mTOR. J Pathol 2005; 205(4):498-506.

(152) Schmidt E, Fichelson S, Feller S. PI3 kinase is important for Ras, MEK and Erk activation of Epo-stimulated human erythroid progenitors. BMC Biology 2004; 2(1):7.

(153) Rassidakis GZ, Feretzaki M, Atwell C, Grammatikakis I, Lin Q, Lai R et al. Inhibition of Akt increases p27Kip1 levels and induces cell cycle arrest in anaplastic large cell lymphoma. Blood 2005; 105(2):827-829.

(154) Segrelles C, Moral M, Lara MF, Ruiz S, Santos M, Leis H et al. Molecular determinants of Akt-induced keratinocyte transformation. Oncogene 2006; 25(8):1174-1185.

(155) Muise-Helmericks RC, Grimes HL, Bellacosa A, Malstrom SE, Tsichlis PN, Rosen N. Cyclin D expression is controlled post-transcriptionally via a phosphatidylinositol 3-kinase/Akt-dependent pathway. J Biol Chem 1998; 273(45):29864-29872.

(156) Gao N, Flynn DC, Zhang Z, Zhong XS, Walker V, Liu KJ et al. G1 cell cycle progression and the expression of G1 cyclins are regulated by PI3K/AKT/mTOR/p70S6K1 signaling in human ovarian cancer cells. Am J Physiol Cell Physiol 2004; 287(2):C281-C291.

(157) Kashii Y, Uchida M, Kirito K, Tanaka M, Nishijima K, Toshima M et al. A member of Forkhead family transcription factor, FKHRL1, is one of the downstream molecules of phosphatidylinositol 3-kinase-Akt activation pathway in erythropoietin signal transduction. Blood 2000; 96(3):941-949.

(158) Engstrom M, Karlsson R, Jonsson JI. Inactivation of the forkhead transcription factor FoxO3 is essential for PKB-mediated survival of hematopoietic progenitor cells by kit ligand. Exp Hematol 2003; 31(4):316-323.

(159) Martelli AM, Nyakern M, Tabellini G, Bortul R, Tazzari PL, Evangelisti C et al. Phosphoinositide 3-kinase/Akt signaling pathway and its therapeutical implications for human acute myeloid leukemia. Leukemia 2006; 20(6):911-928.

(160) Joensuu H. Gastrointestinal stromal tumor (GIST). Ann Oncol 2006; 17 Suppl 10:x280-x286.

(161) Tarn C, Skorobogatko YV, Taguchi T, Eisenberg B, von Mehren M, Godwin AK. Therapeutic effect of imatinib in gastrointestinal stromal tumors: AKT signaling dependent and independent mechanisms. Cancer Res 2006; 66(10):5477-5486.

(162) Vakiani E, Cattoretti G, Colovai AI, Murty VV, Alobeid B, Bhagat G. CD117 expression in diffuse large B-cell lymphomas: fact or fiction? Pathol Int 2005; 55(11):716-723.

(163) Dispenzieri A, Gertz MA, Lacy MQ, Geyer SM, Greipp PR, Rajkumar SV et al. A phase II trial of imatinib in patients with refractory/relapsed myeloma. Leuk Lymphoma 2006; 47(1):39-42.

(164) Shirogane T, Fukada T, Muller JM, Shima DT, Hibi M, Hirano T. Synergistic roles for Pim-1 and c-Myc in STAT3-mediated cell cycle progression and antiapoptosis. Immunity 1999; 11(6):709-719.

(165) Autzen P, Robson CN, Bjartell A, Malcolm AJ, Johnson MI, Neal DE et al. Bone morphogenetic protein 6 in skeletal metastases from prostate cancer and other common human malignancies. Br J Cancer 1998; 78(9):1219-1223.

(166) Yang ZZ, Novak AJ, Ziesmer SC, Witzig TE, Ansell SM. Attenuation of CD8(+) T-cell function by CD4(+)CD25(+) regulatory T cells in B-cell non-Hodgkin's lymphoma. Cancer Res 2006; 66(20):10145-10152.

(167) Ansell SM, Stenson M, Habermann TM, Jelinek DF, Witzig TE. Cd4+ T-cell immune response to large B-cell non-Hodgkin's lymphoma predicts patient outcome. J Clin Oncol 2001; 19(3):720-726.

(168) Lee AM, Clear AJ, Calaminici M, Davies AJ, Jordan S, Macdougall F et al. Number of CD4+ Cells and Location of Forkhead Box Protein P3-Positive Cells in Diagnostic Follicular Lymphoma Tissue Microarrays Correlates With Outcome. J Clin Oncol 2006; 24(31):5052-5059.

(169) Carreras J, Lopez-Guillermo A, Fox BC, Colomo L, Martinez A, Roncador G et al. High numbers of tumor-infiltrating FOXP3-positive regulatory T cells are associated with improved overall survival in follicular lymphoma. Blood 2006; 108(9):2957-2964.

(170) Farinha P, Masoudi H, Skinnider BF, Shumansky K, Spinelli JJ, Gill K et al. Analysis of multiple biomarkers shows that lymphoma-associated macrophage (LAM) content is an independent predictor of survival in follicular lymphoma (FL). Blood 2005; 106(6):2169-2174.

(171) Harjunpaa A, Taskinen M, Nykter M, Karjalainen-Lindsberg ML, Nyman H, Monni O et al. Differential gene expression in non-malignant tumour microenvironment is associated with outcome in follicular lymphoma patients treated with rituximab and CHOP. Br J Haematol 2006; 135(1):33-42.

(172) Kersten C, Dosen G, Myklebust JH, Sivertsen EA, Hystad ME, Smeland EB et al. BMP-6 inhibits human bone marrow B lymphopoiesis--upregulation of Id1 and Id3. Exp Hematol 2006; 34(1):72-81.

(173) Ishisaki A, Yamato K, Hashimoto S, Nakao A, Tamaki K, Nonaka K et al. Differential inhibition of Smad6 and Smad7 on bone morphogenetic protein- and activin-mediated growth arrest and apoptosis in B cells. J Biol Chem 1999; 274(19):13637-13642.

(174) Baade RT, Utne HR, Brenne AT, Hjorth-Hansen H, Waage A, Hjertner O et al. Bone morphogenetic protein-5, -6 and -7 inhibit growth and induce apoptosis in human myeloma cells. Oncogene 2004; 23(17):3024-3032.

(175) Hjertner O, Hjorth-Hansen H, Borset M, Seidel C, Waage A, Sundan A. Bone morphogenetic protein-4 inhibits proliferation and induces apoptosis of multiple myeloma cells. Blood 2001; 97(2):516-522.

(176) Langenfeld EM, Kong Y, Langenfeld J. Bone morphogenetic protein 2 stimulation of tumor growth involves the activation of Smad-1/5. Oncogene 2006; 25(5):685-692.

(177) Valdimarsdottir G, Goumans MJ, Rosendahl A, Brugman M, Itoh S, Lebrin F et al. Stimulation of Id1 expression by bone morphogenetic protein is sufficient and necessary for bone morphogenetic protein-induced activation of endothelial cells. Circulation 2002; 106(17):2263-2270.

(178) Murre C. Helix-loop-helix proteins and lymphocyte development. Nat Immunol 2005; 6(11):1079-1086.

(179) Rivera R, Murre C. The regulation and function of the Id proteins in lymphocyte development. Oncogene 2001; 20(58):8308-8316.

(180) Qi Z, Sun XH. Hyperresponse to T-cell receptor signaling and apoptosis of Id1 transgenic thymocytes. Mol Cell Biol 2004; 24(17):7313-7323.

(181) Bain G, Engel I, Robanus Maandag EC, te Riele HP, Voland JR, Sharp LL et al. E2A deficiency leads to abnormalities in alphabeta T-cell development and to rapid development of T-cell lymphomas. Mol Cell Biol 1997; 17(8):4782-4791.

(182) Heemskerk MH, Blom B, Nolan G, Stegmann AP, Bakker AQ, Weijer K et al. Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix loop helix factor Id3. J Exp Med 1997; 186(9):1597-1602.

(183) Schoppmann SF, Schindl M, Bayer G, Aumayr K, Dienes J, Horvat R et al. Overexpression of Id-1 is associated with poor clinical outcome in node negative breast cancer. Int J Cancer 2003; 104(6):677-682.

(184) Schindl M, Oberhuber G, Obermair A, Schoppmann SF, Karner B, Birner P. Overexpression of Id-1 protein is a marker for unfavorable prognosis in early-stage cervical cancer. Cancer Res 2001; 61(15):5703-5706.

(185) Ouyang XS, Wang X, Lee DT, Tsao SW, Wong YC. Over expression of ID-1 in prostate cancer. J Urol 2002; 167(6):2598-2602.

(186) Hudlebusch HR, Theilgaard-Monch K, Lodahl M, Johnsen HE, Rasmussen T. Identification of ID-1 as a potential target gene of MMSET in multiple myeloma. Br J Haematol 2005; 130(5):700-708.

(187) Kim D, Peng XC, Sun XH. Massive apoptosis of thymocytes in T-cell-deficient Id1 transgenic mice. Mol Cell Biol 1999; 19(12):8240-8253.

(188) Arsura M, Wu M, Sonenshein GE. TGF beta 1 inhibits NF-kappa B/Rel activity inducing apoptosis of B cells: transcriptional activation of I kappa B alpha. Immunity 1996; 5(1):31-40.

(189) Sneddon JB, Zhen HH, Montgomery K, van de RM, Tward AD, West R et al. Bone morphogenetic protein antagonist gremlin 1 is widely expressed by cancer-associated stromal cells and can promote tumor cell proliferation. Proc Natl Acad Sci U S A 2006; 103(40):14842-14847.

(190) Gazzerro E, Gangji V, Canalis E. Bone morphogenetic proteins induce the expression of noggin, which limits their activity in cultured rat osteoblasts. J Clin Invest 1998; 102(12):2106-2114.

(191) Bhatia M, Bonnet D, Wu D, Murdoch B, Wrana J, Gallacher L et al. Bone morphogenetic proteins regulate the developmental program of human hematopoietic stem cells. J Exp Med 1999; 189(7):1139-1148.

(192) Hager-Theodorides AL, Outram SV, Shah DK, Sacedon R, Shrimpton RE, Vicente A et al. Bone morphogenetic protein 2/4 signaling regulates early thymocyte differentiation. J Immunol 2002; 169(10):5496-5504.

(193) Bleul CC, Boehm T. BMP signaling is required for normal thymus development. J Immunol 2005; 175(8):5213-5221.

(194) Graf D, Nethisinghe S, Palmer DB, Fisher AG, Merkenschlager M. The developmentally regulated expression of Twisted gastrulation reveals a role for bone morphogenetic proteins in the control of T cell development. J Exp Med 2002; 196(2):163-171.

(195) Gorelik L, Fields PE, Flavell RA. Cutting edge: TGF-beta inhibits Th type 2 development through inhibition of GATA-3 expression. J Immunol 2000; 165(9):4773-4777.

(196) Das J, Chen CH, Yang L, Cohn L, Ray P, Ray A. A critical role for NF-kappa B in GATA3 expression and TH2 differentiation in allergic airway inflammation. Nat Immunol 2001; 2(1):45-50.

(197) Yun AJ, Lee PY. The link between T helper balance and lymphoproliferative disease. Med Hypotheses 2005; 65(3):587-590.

(198) Grulich AE, Vajdic CM, Kaldor JM, Hughes AM, Kricker A, Fritschi L et al. Birth order, atopy, and risk of non-Hodgkin lymphoma. J Natl Cancer Inst 2005; 97(8):587-594.

(199) Anichini A, Mortarini R, Romagnoli L, Baldassari P, Cabras A, Carlo-Stella C et al. Skewed T-cell differentiation in patients with indolent non-Hodgkin lymphoma reversed by ex vivo T-cell culture with gammac cytokines. Blood 2006; 107(2):602-609.

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Translocation t(14;18) and gain of chromosome 18/*BCL2*: effects on BCL2 expression and apoptosis in B-cell non-Hodgkin's lymphomas

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Gain of chromosome 18q and translocation t(14;18) are] frequently found in B-cell non-Hodgkin's lymphomas (B-NHL). Increased *BCL2* transcription and BCL2 protein expression have been suggested to be the result of the gain. We utilized FISH, PCR and array CGH to study *BCL2* and chromosome 18 copy number changes and rearrangements in 93 cases of B-NHL. BCL2 protein was expressed in >75% of the tumor cells in 92% of the cases by immunohistochemistry. Gain of *BCL2* was associated with a 25% increase in BCL2 expression levels (immunoblotting), whereas t(14;18) resulted in a 55% increase in BCL2 levels compared to cases without *BCL2* alterations. The tumor cell (spontaneous) apoptotic fractions were similar for the cases with different *BCL2* genotypes. However, the normal cell apoptotic fractions were *higher* for the tumors with t(14;18) compared to the tumors without *BCL2* alterations, while the tumors with gain of *BCL2* only showed intermediate levels. Low-level gains of parts of chromosome 18 were found in 14 of the 38 B-NHL cases with t(14;18), with a consensus region 18pter-q21.33 that did not include the *BCL2* gene. The 11 cases with 18q gain only showed a consensus region encompassing 18q21.2–18q21.32 and 18q21.33, which contain *PMAIP1*/*MALT1* and *BCL2*, respectively.
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Introduction

Translocation of the *BCL2* gene (18q21.3) to the *IGH* transcriptional enhancer, as a result of the t(14;18)(q32;q21), causes constitutive overexpression of the antiapoptotic BCL2 protein.[1] Translocation t(14;18) is the primary tumorigenic event in most follicular lymphomas (FL) and some diffuse large B-cell lymphomas (DLBCL).[2–4] Increased BCL2 levels confer extended survival to B-cells, and may thus cause accumulation of cells likely to be targets for additional oncogenic events and transformation.[5,6]

Gain of chromosome 18 material has been detected frequently in non-Hodgkin's lymphomas (NHL), lymphomas with t(14;18) included.[7–18] It has been suggested that *BCL2* could be the target gene in the DLBCL cases with gain of 18q.[19,20] BCL2 protein expression was enhanced in the tumors with either t(14;18) or *BCL2* amplification; the latter two aberrations appeared to be exclusive.[19] This is in agreement with the study of Horsman *et al*,[17] showing that der(18)t(14;18), not containing the *BCL2* coding sequences, was frequently gained in FL cases with *BCL2* rearrangement. In contrast, the *BCL2* gene was found to be included in the 18q amplica in lymphomas with t(14;18).[21] The proto-oncogene *MALT1*, also involved in the t(11;18)(q21;q21) translocation,[22] is another possible target in some B-cell non-Hodgkin's lymphomas (B-NHL) with 18q21 gain.[20] The expression of *PMAIP1* at locus 18q21.3 was upregulated in transformed FL with 18q21 gain,[21] as well as in some cases of DLBCL.[23]

We have studied the impact of *BCL2* genotypes on phenotypes such as BCL2 expression, apoptosis, proliferation and survival in 93 cases of B-NHL. Copy number alterations on chromosome 18 were also assessed by array CGH and FISH.

Patients and methods

Tumor samples

Cell suspensions in DMSO prepared from 93 individual lymph node biopsies, histologically and immunologically typed as B-NHL, were included in this study. Preparation of the cell-suspensions, as well as the clinical characteristics and treatment of the patients, has been described in previous studies.[15,24,25] Table 1 gives the classification of lymphomas according to WHO, as well as previously published data for gains and losses of chromosome 18 by conventional CGH, DNA index and malignant/normal cell apoptotic fractions.[15,24]

Detection of t(14;18) by conventional and long-range PCR

The oligonucleotide primer for the 150-bp MBR was 5′-AAC TCT GTG GCA TTA TTG-3′ and for the mcr 5′-GAC TCC TTT ACG TGC TGG TAC-3′. The primers were combined with a consensus *IGH* joining region primer: 5′-ACC TGA GGA GAC GGT GAC C-3′ in the conventional PCR experiments. Long-range PCR, as described by Akasaka *et al*,[26] was applied to detect translocations outside the MBR and mcr as well. A primer hybridizing to a sequence 5′ of the MBR (5′-CAC AAG TGA AGT CAA CAT GCC TGC CCC AAA CAA AT-3′) was used together with a primer specific for the Eμ region in the *IGH* gene (5′-CTA GGC CAG TCC TGC TGA CGC CGC ATC GGT GAT TC-3′) for the detection of translocations with breakpoints in the MBR and 3′-MBR. 5′-mcr and mcr translocations were detected with a primer recognizing a sequence approximately 9.5 kbp 5′ of the mcr (5′-GGT AGA GGT GAA TAC CCC AGG GCT GAG CAG GAA GG-3′) and the Eμ-primer as above. Cases with MBR or mcr translocations only detectable by long-range PCR,

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Table 1 *BCL2* genotypes and phenotypes in 93 cases of B-NHL

Case	*Lymphoma subtype (WHO)*	*DNA index*[a]	*CGH gain Chr. 18*[a]	*PCR t(14;18)*	*FISH*				*IHC*[b] *BCL2*	*IB*[b] *BCL2*	*Apoptosis*[c]	
					# red signals	*# fusion red/green signals*	*# blue signals (cen 18 copy #)*	*BCL2 copy # (derived)*[d]			*Tumor cells*	*Normal cells*
t(14;18) positive												
377/83	FL I/II	1.00	No	3′-MBR	3	2	2	2	>75	1.69	0.2	0.6
364/86	FL I/II+SLL	1.02	18pter–q21	MBR	4	3	3	2	>75	1.88	1.8	2.7
369/86	FL I	1.02	No	mcr	3	2	2	2		1.15	1.4	2.1
021/87	FL I/II	1.00	18pter–q21.1	MBR	4	3	3	2	>75	1.66	0.6	2.0
241/87	FL I/II	1.02	No	mcr	3	2	2	2	>75	1.00	1.3	4.0
345/87	FL II	1.00	18q	MBR	4	2	3	3	>75	1.38	0.4	0.4
018/88	FL II	1.00	No	mcr	3	2			>75	1.10	2.2	2.0
041/88	FL II	1.03	18q12.3–qter	MBR	4	2	3	3	>75	1.42	0.9	1.9
046/88	FL II	0.97	No	MBR	3	2	2	2	>75	1.37	0.3	
047/88	FL I/II	1.00	No	5′-mcr	3	2	2	2		1.29	0.7	1.6
176/88	FL II	1.04	18q12–q22	MBR	4	3	3	2	>75	1.66	0.3	1.6
233/88	FL II	1.00	No	MBR	3	2	2	2		1.42	0.6	18.8
275/88	FL I/II	1.00	No	MBR	3	2	2	2	>75	1.32	3.4	1.5
284/88	FL II	1.02	No	MBR	3	2	2	2	>75	1.29	2.2	2.5
381/88	FL II	1.03	18	mcr	4	2	3	3	>75		3.4	4.5
399/88	FL III	1.00	No	MBR	3	2	2	2	>75	0.91	2.5	2.0
468/88	FL II	1.00	No	MBR	3	2	2	2	0	0.59	2.2	2.1
064/89	FL II	1.26	No	MBR	5	4	3	3	>75	1.45	0.9	1.1
287/89	FL I	1.03	No	MBR	3	2	2	2	>75	0.84	0.5	20.0
311/89	FL II	1.05	No	MBR	3	2	2	2	>75	1.36	0.8	
521/89	FL II	1.00	No	5′-mcr	3	2	2	2	>75	1.07	0.3	0.7
140/90	FL II	1.03	18	MBR					>75	1.19	0.8	4.0
372/90	FL II	1.07	No	3′-MBR	3	2	2	2	>75	0.68	3.3	3.2
377/90	FL II	1.06	No	5′-mcr	3	2	2	2	>75	0.74	3.5	1.2
416/90	FL II	1.00	No	3′-MBR	3	2	2	2	>75	0.98	2.7	5.4
581/90	FL II	1.22	18q12.3–q21	5′-mcr	4	3	3	2	>75	1.19	1.8	1.2
635/90	FL I/II	1.00	No	3′-MBR	3	2	2	2		1.18	0.8	6.4
382/91	FL II	1.02	No	MBR	3	2	2	2	0	0.38	0.6	15.4
436/91	FL II	1.04	18,**18pter–q21**	MBR	5	4	4	2	>75	1.54	1.3	12.4
489/91	FL I/II	1.03	No	0	3	2	2	2	>75	1.30	1.6	8.0
021/92	FL II	1.02	No	MBR	3	2	2	2	>75	0.87	2.8	1.2
103/92	FL II	1.00	No	MBR	3	2	2	2	>75	1.20	0.5	4.1
130/92	FL I/II	1.03	18pter–q21	MBR	5	3	4	3	>75	2.14	3.3	5.7
287/88	DLBCL	1.00	18cen–q22	mcr	4	3	3	2	>75	1.46		
454/88	DLBCL	1.02	No	mcr	3	2	2	2	>75	1.12	0.9	0.5
34/90	DLBCL+SLL	1.16	No	3′-MBR	3	0	3	3	>75	2.22	1.2	2.3
472/90	DLBCL	1.16	18pter–q21	MBR	4	3	3	2	>75	1.33	0.2	2.3
340/91	DLBCL	1.03	No	MBR	3	2	2	2	>75	1.30	0.0	1.8
BCL2 gain only												
051/90	SLL	1.05	18q12.3–qter	0	4	0	2	4	>75	1.09	0.8	0.6
154/88	MCL	0.95	No	0	3	0	2	3	>75		0.1	1.0
265/88	MCL	1.00	18q12–qter	0	3	0	3	3	>75	1.14	0.4	4.9
309/89	MCL	1.83	No	0	4	0	4	4	>75	0.89	0.5	0.4
383/91	MCL	0.97	No	0	3	0	2	3	>75	0.75	8.0	1.8
122/84	DLBCL	1.23	No	0	3	0	3	3	>75	1.00	2.5	1.9
255/85	DLBCL	1.04	18q21–qter	0	3	0	3	3	>75	1.09	0.6	0.2
131/89	DLBCL	2.23	18	0	6	0	5	6	>75	1.02	0.9	0.8
525/89	DLBCL	1.07	18q21–qter	0	3	0	2	3	>75	0.49	3.0	3.7
050/93	DLBCL	1.00	No	0	5	0	2	5		1.25	1.4	3.7
448/91	MZL	1.05	18	0	3	0	3	3	>75	1.02	0.2	0.5
8/92	Unclass.	1.00	18	0	3	0	3	3		0.72	3.1	2.8
No BCL2 alterations												
577/90	FL II	1.97	No	0	4	0	4	4	5_15	0.24	7.6	7.6
533/91	FL II	1.00	No	0	2	0		2	>75	0.64	1.8	1.3
300/92	FL II	1.00	No	0	2	0	2	2	>75	0.74	8.8	0.9
140/83	SLL	1.00	No	0	2	0		2	>75	0.97	2.1	0.5
086/85	SLL	1.00	No	0	2	0		2	>75	1.07	1.5	0.6
320/88	SLL	1.00	No	0	2	0		2	>75	0.81	0.7	0.7
325/88	SLL	1.00	No	0	2	0		2	>75	1.31	0.8	0.4
416/88	SLL	1.03	No	0	2	0		2	>75	0.74	0.2	0.1
470/88	SLL	1.02	No	0					>75	0.88	3.4	1.6

Table 1 *Continued*

Case	Lymphoma subtype (WHO)	DNA index[a]	CGH gain Chr. 18[a]	PCR t(14;18)	FISH				IHC[b] BCL2	IB[b] BCL2	Apoptosis[c]	
					# red signals	# fusion red/green signals	# blue signals (cen 18 copy #)	BCL2 copy # (derived)[d]			Tumor cells	Normal cells
191/89	SLL	1.02	No	0	2	0		2	>75	0.79	0.2	
339/89	SLL	1.00	No	0	2	0		2	>75	0.77	1.1	2.1
445/89	SLL	1.02	No	0	2	0		2	>75	0.64	1.3	2.9
452/89	SLL	1.00	No	0	2	0	2	2	>75	1.08	0.4	0.3
159/90	SLL	1.04	No	0	2	0		2		0.71	1.1	1.9
571/90	SLL	1.00	No	0	2	0		2	>75	0.50	0.7	0.3
156/91	SLL	1.00	No	0	2	0		2	>75	0.70	0.4	2.1
244/91	SLL	1.00	No	0	2	0		2		1.00	1.2	0.3
462/91	SLL	1.00	No	0	2	0		2	>75	0.80	0.5	0.9
225/92	SLL	1.03	No	0	2	0		2	>75	0.63	0.0	0.0
315/92	SLL	1.00	No	0	2	0	2	2	>75	1.16	0.3	
538/92	SLL	1.00	No	0	2	0	2	2	>75	0.75	6.5	1.9
123/84	MCL	1.04	No	0	2	0	2	2	>75	1.00	0.7	8.7
358/87	MCL	1.94	No	0	4	0	4	4	>75	0.90	1.2	2.8
010/89	MCL	1.00	No	0	2	0		2	>75	0.69	2.7	14.4
129/89	MCL	1.02	No	0	2	0		2	>75	0.82	1.5	1.2
428/91	MCL	2.00	No	0	4	0	4	4	>75	0.98	0.4	0.3
037/92	MCL	1.00	No	0	2	0		2	>75	0.96	7.2	8.7
067/92	MCL	0.98	No	0	2	0		2	>75	0.95	8.4	6.0
245/92	MCL	1.03	No	0	2	0	2	2	>75	1.05	0.3	0.0
377/87	DLBCL	1.03	No	0	2	0		2	>75	0.58	0.9	3.5
399/87	DLBCL	1.06	No	0	2	0		2	>75	0.46	3.8	1.9
258/88	DLBCL	1.07	No	0	2	0		2	>75	0.52	2.6	1.3
438/89	DLBCL	1.00	No	0	2	0		2	>75	0.45	3.2	2.2
612/90	DLBCL	1.04	No	0	2	0		2	15_75	0.39	0.7	2.0
070/92	DLBCL	1.01	No	0	2	0		2	15_75	0.61	1.5	0.5
117/92	DLBCL	1.00	No	0	2	0	2	2	>75	0.89	0.8	
277/89	BL	1.04	No	0	2	0	2	2	5–15	0.14	6.7	15.3
458/88	BL-like	1.03	No	0	2	0	2	2	>75	0.98	1.6	9.1
42/92	BL-like	1.03	No	0	2	0	2	2	>75	0.25	21.5	4.9
315/88	MZL	1.00	No	0	2	0		2	>75	0.95	0.5	0.3
237/91	MZL	1.00	No	0	2	0		2	>75	0.87	0.5	0.0
214/88	Unclass	1.00	No	0	2	0		2	>75	0.63	2.9	2.4
Loss of BCL2:												
390/87	DLBCL	1.00	Loss 18q	0	1	0	2	1	0	0.31	7.7	2.2

Bold indicates high-level gain.
[a]The DNA indices (by flow cytometry) and conventional CGH results for these cases have been published.[15]
[b]IHC = immunohistochemistry; IB = immunoblotting.
[c]The data for apoptosis were published previously.[24]
[d]The *BCL2* copy number was derived as described in 'Patients and methods'.

and not by conventional PCR, were assumed to have breakpoints in the 3′-MBR or 5′-mcr, respectively. As a control for DNA integrity, a 10 560 bp germline sequence 5′ of the mcr was amplified employing the primers 5′-GCG GCT ATT GAG CAC TGA AAT GTG ACT GAA ATG AC-3′ and 5′-CAT CCA ACT GGC TTA GGA GGC AAG ACA GAA ACC TT-3′.

Translocation t(14;18) and BCL2/MALT1/PMAIP1/ centromere 18 copy number analysis by FISH

A dual-color translocation probe (LSI *IGH* Spectrum Green/LSI *BCL2* Spectrum Orange) from Vysis (Downers Grove, IL, USA) was hybridized as previously described[27,28] to detect t(14;18) and *BCL2* copy number. This probe set yields red (*BCL2* locus) and green (*IGH* locus) signals. The red and green signals are split and the two colors are colocalized if a translocation event has occurred. The *BCL2* probe covers the *BCL2* gene and extends approximately 250 kbp 5′ as well as 3′ of the gene (http://www.vysis.com/ProbeMap_5235.asp). A blue fluorescent centromere 18 probe (CEP 18 Spectrum Aqua, VYSIS) was cohybridized with the t(14;18) probe set. The numbers of total red, cohybridized red/green and blue signals were assessed in a correlated manner.[27] der(14)t(14;18), and not the der(18)t(14;18), contains the *BCL2* coding sequences in the cases with breakpoints 3′ of the *BCL2* coding sequences (assuming no other complicated rearrangements). The number of fusion signals was assumed to represent the total number of derivative chromosomes, while the number of blue centromere 18 signals was used to assess the total number of chromosome 18 and der(18)t(14;18). If there are no local amplica including the *BCL2* gene, an assumption that will be discussed in 'Results and discussion', the number of *BCL2* coding sequence copies in the cases with t(14;18) can be calculated as follows: #*BCL2*

coding sequences = 2 × #red signals −#red/green fusion signals −#cen 18 signals.

FISH was also applied to examine the copy numbers of sequences corresponding to the RP11-108P20 (MALT1), RP11-103A19 (PMAIP1), RP11-46D1, RP11-563B11 and RP11-154H12 clones on chromosome 18, as well as the RP11-12F16 clone covering PAC820M16 at the telomere of chromosome 14 (BACPAC Resources, Oakland, CA, USA). The DNA was isolated, nick translated and labelled with Cy3-dUTP (Amersham Biosciences, Buckinghamshire, UK).[29] Hybridization was performed with 40 ng of labelled probe and 1 μg Cot-1 DNA (Invitrogen, Carlsbad, CA, USA) in 10 μl 50% formamide, 2 × SSC, 10% dextran sulfate.

Chromosome 18 copy number alterations examined by array CGH

The genomic microarrays printed covered the human genome at ~1 mbp resolution, and contained 77 BAC (RPCI-11 library[30]) and one PAC clone for chromosome 18 (Wellcome Trust Sanger Institute, Cambridge, UK). The localization of each clone was obtained from Ensembl. DNA isolation, amplification by DOP-PCR and preparation of microarrays were carried out as described previously.[31] The PCR products were arrayed in quadruplicate onto amine-binding slides (CodeLink, Amersham Biosciences). Two sets of duplicate spots were printed in separate areas of the array.

Sequence-verified human cDNA clones (approximately 40 000 clones) were purchased from Invitrogen. The cDNAs were amplified by PCR, purified, resuspended in 3 × SSC and printed onto two Corning Gamma Amino Propyl Silane (GAPS) slides (Corning, NY, NY, USA) with each of the two slides consisting of 20 000 cDNA clones. The chromosomal position of the printed cDNAs was determined by BLAT analysis of the UCSC human genome browser (http://genome.ucsc.edu/).

DNA (1.5 μg) was digested, purified and labelled with Cy3-dCTP or Cy5-dCTP (Perkin Elmer, Boston, MA, USA) as described previously.[31] Labelled tumor and sex-matched reference DNA were combined and ethanol precipitated together with 135 μg human Cot-1 DNA. The DNA and 400 μg yeast tRNA (Invitrogen) was dissolved in 108 μl 50% formamide, 10% dextran sulfate, 4% SDS, 2 × SSC and denatured for 10 min at 70°C. Hybridization (48 h at 37°C) and washing was performed in an automated hybridization station, GeneTAC (Genomic Solutions/Perkin-Elmer).

Microarrays were scanned using an Agilent G2565BA scanner (Agilent Technologies, Palo Alto, CA, USA). The spots were segmented, local background was subtracted, and the fluorescence intensities and the intensity ratio of the two dyes were calculated for each spot. Further data processing, including filtering and normalization, was carried out using M-CGH, a MATLAB toolbox specifically designed for this purpose.[32]

BCL2 protein analysis by immunohistochemistry (IHC) and immunoblotting (IB)

The protocols for IHC and IB, and the interpretation and evaluation of the data, have been described in detail.[25,28,33,34] The anti-BCL2 antiserum C-15 (Santa Cruz Biotech, Santa Cruz, CA, USA) and a monoclonal anti-BCL2 antibody kindly provided by DY Mason (Oxford, UK) were used for IHC and IB, respectively.

Results and discussion

BCL2 genotype

PCR and long-range PCR were used to detect and characterize the translocation t(14;18) involving the *BCL2* and *IGH* loci (Figure 1a, Table 1). t(14;18) was found by PCR in 32 of 36 FL and in five of 18 DLBCL (Table 1). The *BCL2* breakpoints were within the MBR in 22 cases (59%) and within the mcr in six (16%) cases, that is, t(14;18) was also detected by standard PCR in these cases (standard PCR data not shown). The breakpoints in the remaining nine cases were distributed between these regions (3′-MBR, 5′-mcr) (Table 1). The t(14;18) translocations were also assessed by FISH (Figure 1b, Table 1). *BCL2* rearrangement results by PCR/long-range PCR and FISH showed concordance with the exception of two cases (Table 1; cases 489/91 and 34/90). Case 489/91 may have a t(14;18) involving the 5′ region of *BCL2*, which will not be found by PCR with the primers employed in this work. Case 34/90 may have a more complex rearrangement, with, for example, insertion of a short fragment of the *IGH* gene containing the Eμ enhancer between the MBR and the mcr.

The *BCL2* coding sequence copy number was derived from the FISH counts as described in 'Patients and methods'. Eight of the lymphomas with a translocation t(14;18) involving the *BCL2* locus had increased numbers of red signals (>3) and red/green signals (>2) compared to the majority of the cases with t(14;18) (Table 1; cases: 364/86, 21/87, 176/88, 287/88, 472/90, 581/90, 436/91, 130/92). However, in seven of these eight cases (excluding case 130/92), the number of centromere 18 signals was increased correspondingly (Table 1), indicating that der(18)t(14;18), and not der(14)t(14;18) containing the *BCL2* coding sequence, was gained (Figure 1b, case 21/87). Case 130/92 may additionally have gained an extra-chromosome 18. Three cases with t(14;18) (345/87, 41/88, 381/88) had four red and three blue signals, but only two red/green fusion signals, indicating that an extra copy of chromosome 18 was gained. Case 64/89 had a DNA index of 1.26, and by FISH this case had 5 red signals, four fusion signals and 3 centromere 18 signals. This result can be due to gain of both the translocation-derived chromosomes. Case 489/91 was suggested to have a t(14;18) 5′ of the *BCL2* gene from the PCR and FISH results, and the *BCL2* coding sequences are found on der(18)t(14;18). However, this case showed the expected pattern for a balanced translocation (three red, two red/green and two blue), and the *BCL2* copy number was therefore 2.

In the cases without t(14;18), the number of red spots in the FISH experiments gives the *BCL2* copy number directly. Of 55 lymphoma cases, 12 without t(14;18) had gain of *BCL2* and/or chromosome 18 (five DLBCL, four MCL, one SLL, one MZL, and 1 unclassified B-NHL; Table 1, Figure 1b: case 265/88). Hence, *BCL2* was gained in the seven tumors without *BCL2* translocation that displayed 18q gain by conventional CGH, as well as in five others (122/84, 154/88, 309/89, 383/91, 50/93). The near-tetraploid cases 577/90, 358/87 and 428/91, which had no gain of chromosome 18 by conventional CGH, had four *BCL2*/centromere 18 copies (Table 1). The latter cases with no relative gain of *BCL2* compared to the DNA index were not included in the group with gain of *BCL2*. The suggested composition and number of chromosome 18 derived chromosomes will be discussed in more detail after presentation of the array CGH results (Figure 4, Table 2).

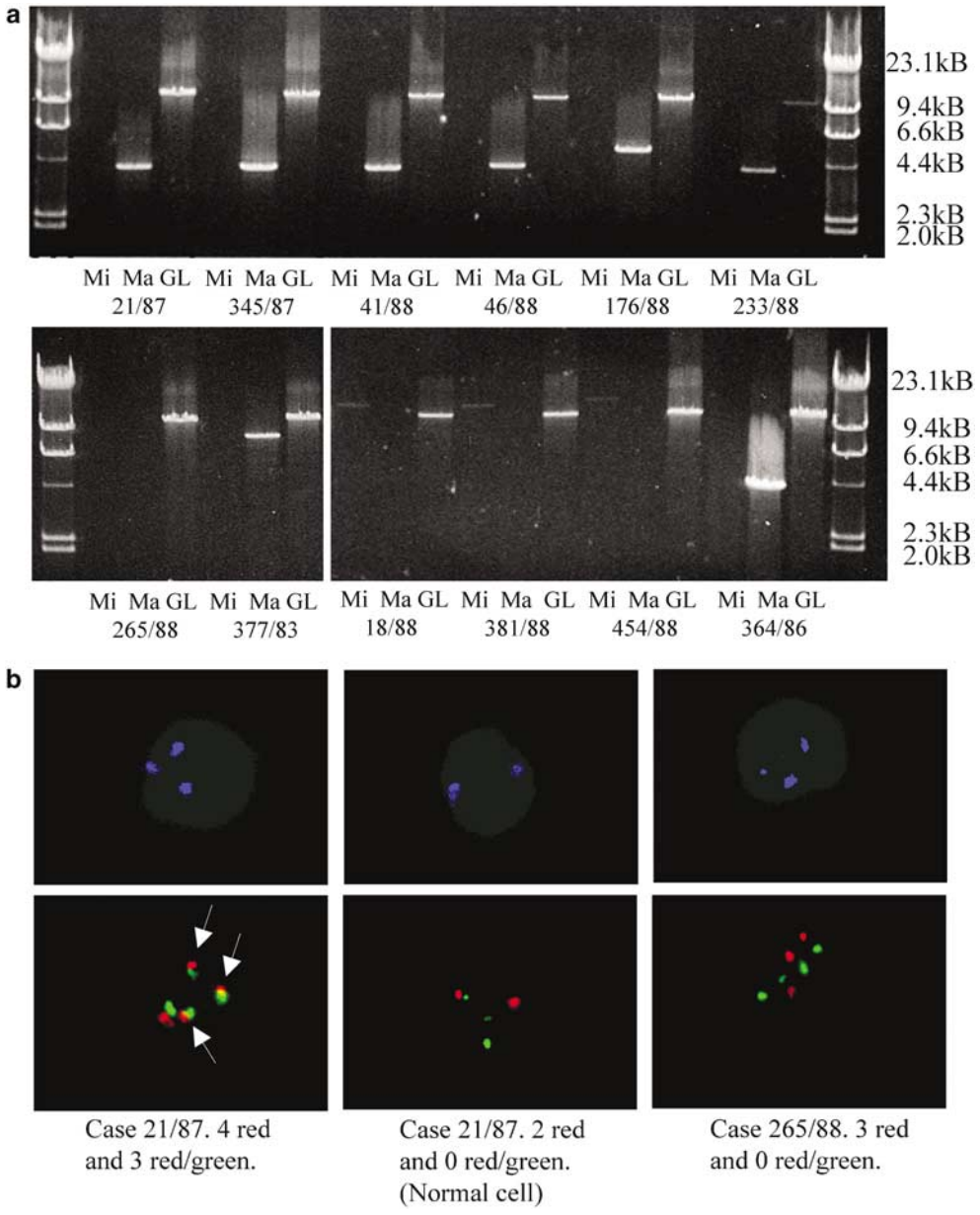

Figure 1 *BCL2* genotypes in B-cell NHL. (a) Long-range PCR was applied for the detection of t(14;18) translocations. PCR was performed with primers for t(14;18) with breakpoints on chromosome 18 within and 3′ of the major breakpoint cluster region (Ma), or within and 5′ of the minor breakpoint cluster region (Mi). The reverse primer was in the Eμ region of the *IGH* gene. The germline product (GL) of length 10 560 bp was amplified with primers for a sequence located in the 3′-MBR region. (b) Detection of t(14,18) and *BCL2*/centromere 18 copy number by FISH. The *BCL2* probe was labelled in red, the *IGH* probe in green and the centromere 18 probe in blue. The upper panels show centromere 18 (blue) and background nuclear fluorescence (green), while the lower panels show *BCL2* (red) and *IGH* (green) for the same fields as shown in the upper panels. Arrows indicate colocalization (translocation).

BCL2 protein levels and dependence on BCL2 genotype

IHC showed that more than 75% of the tumor cells expressed BCL2 in 79 of 86 tumors examined (Table 1). Hence, BCL2 expression is a property of the tumor cells in almost all B-NHL. IB could be used to assess the expression levels of BCL2 (Figure 2a, Table 1), because of the high fraction of tumor cells in the samples (mean: 75%).[15] The cases with both t(14;18) and *BCL2* gain had higher expression of BCL2 (mean: 1.63 relative to p83) than the tumors with t(14;18) only (mean: 1.19; $P=0.008$). The latter cases had higher BCL2 levels than the ones with *BCL2* gain only (mean: 0.96; $P=0.05$), which again had higher BCL2 levels than the tumors with no *BCL2* alterations (mean: 0.77; $P=0.03$; Figure 2b). Case 390/87 with loss of one *BCL2* allele was excluded from this analysis. Hence, gain of *BCL2*, in most cases one extra copy, was associated with a 25% increase in BCL2 expression compared to the cases without *BCL2* alterations, whereas t(14;18) caused a 55% increase in BCL2 expression. There was no difference in the expression of BCL2 for the different translocation regions ($P=0.70$). The expression levels of BCL2 were also compared in 17 cases of DLBCL. Case 34/90 had both t(14;18) and gain of *BCL2*, four cases showed t(14;18) only, five cases carried *BCL2* gain and seven cases had no *BCL2* alteration. The mean BCL2 protein levels in these

Table 2 BAC array and FISH results for the 27 NHL cases with gains on chromosome 18

Case	Lymphoma subtype (WHO)	DNA index	BAC array CGH		FISH				Suggested number of chromosome 18 and derivatives
			Chromosome 18 gain (loss)	Chr14 qter gain (pac820m16)	cen 18 copy #	MALT1 copy #	PMAIP1 copy #	BCL2 copy #	
t(14;18) positive									
364/86	FL I/II+SLL	1.02	pter–ba215a20	+	3	3	3	2	1 × 18+1 × der(14)t(14;18)+2 × der(18)t(14;18)
021/87	FL I/II	1.00	pter–ba215a20	–	3	3	3	2	1 × 18+1 × der(14)t(14;18)+2 × der(18)t(14;18)
345/87	FL II	1.00	pter–qter	–	3	3	3	3	2 × 18+1 × der(14)t(14;18)+1 × der(18)t(14;18)
041/88	FL II	1.03	pter–qter	+	3	3	3	3	2 × 18+1 × der(14)t(14;18)+1 × der(18)t(14;18)
176/88	FL II	1.04	pter–ba215a20	+	3	3	3	2	1 × 18+1 × der(14)t(14;18)+2 × der(18)t(14;18)
381/88	FL II	1.03	pter–qter	–	3	3	3	3	2 × 18+1 × der(14)t(14;18)+1 × der(18)t(14;18)
064/89	FL II	1.26	pter–qter	–	3	3	3	3	1 × 18+2 × der(14)t(14;18)+2 × der(18)t(14;18)
140/90	FL II	1.03	pter–qter	–					2 × 18+1 × der(14)t(14;18)+1 × der(18)t(14;18)
581/90	FL II	1.22	pter–ba215a20	+	3	3	3	2	1 × 18+1 × der(14)t(14;18)+2 × der(18)t(14;18)
436/91	FL II	1.04	pter–ba215a20	+	4	4	4	2	1 × 18+1 × der(14)t(14;18)+3 × der(18)t(14;18)
130/92	FL I/II	1.03	pter–ba215a20	+	4	4	4	3	2 × 18+1 × der(14)t(14;18)+2 × der(18)t(14;18)
287/88	DLBCL	1.00	cen-ba215a20 (pter–cen)	+	3	3	3	2	1 × 18+1 × der(14)t(14;18)+2 × der(18)t(14;18) (loss 18p)
34/90	DLBCL+SLL	1.16	pter–qter	–	3	3	3	3	3 × 18 (complex rearrangement)
472/90	DLBCL	1.16	pter–ba215a20 (ba45a1-qter)	+	3		3	2	1 × 18+1 × der(14)t(14;18)+2 × der(18)t(14;18) (loss 18q23)
Gain only									
051/90	SLL	1.05	ba25c13-qter	–	2	4	4	4	2 × 18 (gain 18q12.3-qter)
154/88	MCL	0.95	ba397a16-ba45a1 (ba169f17-qter)	–	2	3	3	3	2 × 18 (gain 18q21.2–q22.3(?), loss 18q22.3-qter)
265/88	MCL	1.00	cen–qter (pter–cen)	–	3	3	3	3	3 × 18 (loss 18p)
309/89	MCL	1.83	pter–qter	–	4			4	4 × 18
383/91	MCL	0.97	ba116k4-qter	–	2	3	3	3	2 × 18 (gain 18q21.2-qter(?))
122/84	DLBCL	1.23	pter–qter	–	3	3	3	3	3 × 18
255/85	DLBCL	1.04	pter–qter	–	3	3	3	3	3 × 18
390/87	DLBCL	1.00	ba296e23-ba178f10 (rest of 18)	–	2			1	1x18 (gain 18cen-q11.2)
131/89	DLBCL	2.23	pter–qter	–	5	6	6	6	5 × 18 (gain 18q(?))
525/89	DLBCL	1.07	ba116k4-qter	–	2	3	3	3	2 × 18 (gain 18q21.2-qter (?))
050/93	DLBCL	1.00	ba116k4-ba28f1	–	2	3	3	5	2 × 18 (gain 18q21.2–21.33)
448/91	MZL	1.05	pter–qter	–	3	3	3	3	3 × 18
8/92	Unclass.	1.00	pter–qter	–	3	3	3	3	3 × 18

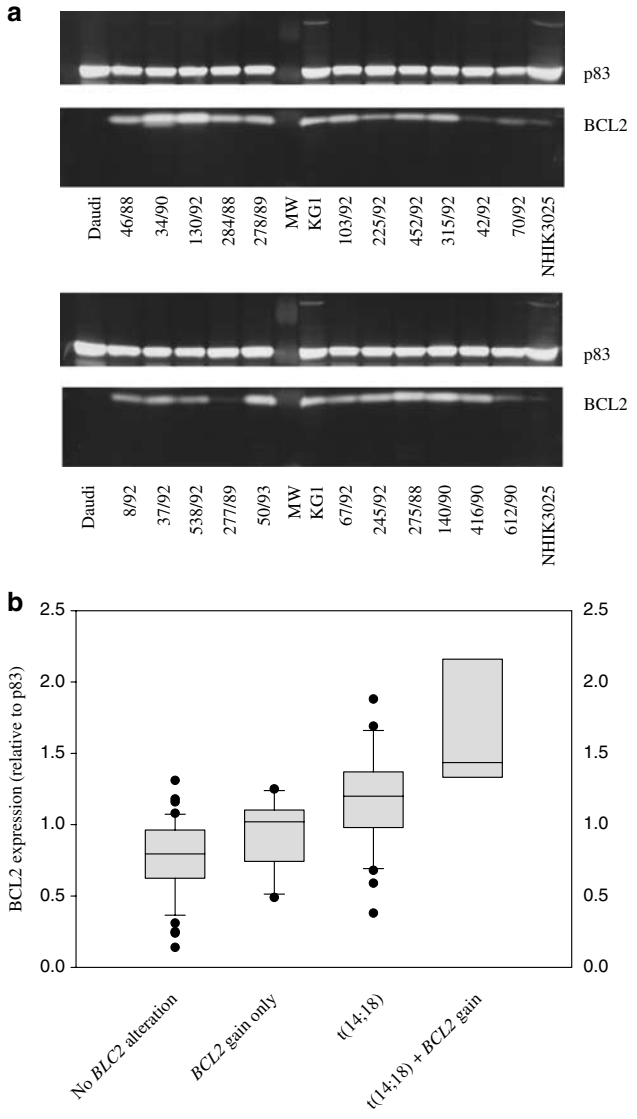

Figure 2 BCL2 protein analysis and dependence on *BCL2* genotype. (a) BCL2 and p83 protein levels were assessed by IB. p83 is an abundantly expressed nuclear protein used as a control for loading. The cell lines KG1 and Daudi served as positive and negative controls, respectively. (b) BCL2 expression according to *BCL2* genotype.

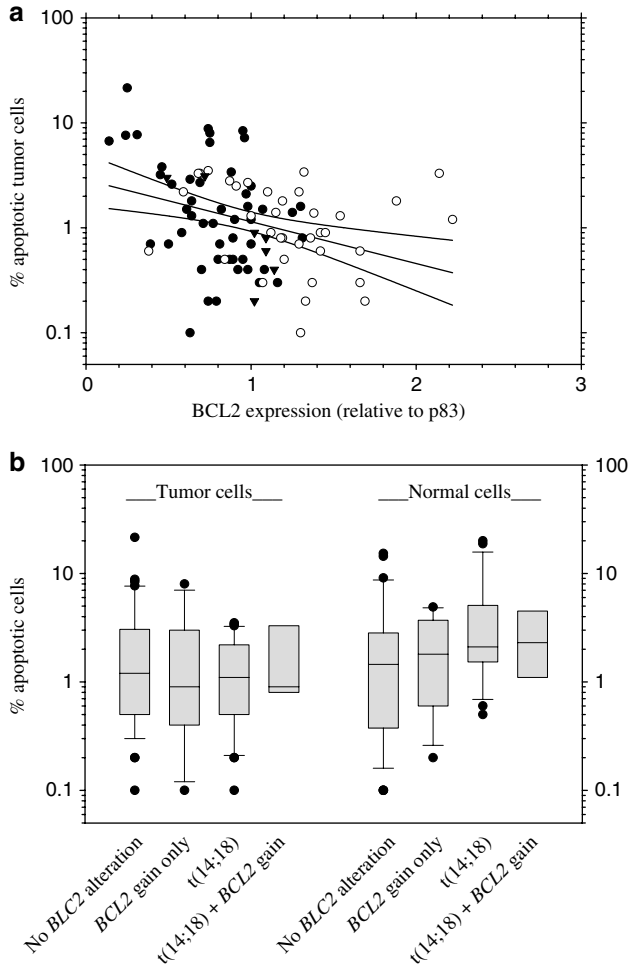

Figure 3 Apoptotic fractions correlated with BCL2 expression and dependence on *BCL2* genotype. (a) There was a negative correlation between apoptosis levels[24] and BCL2 protein levels (Figure 2a) ($P<0.001$, $r^2=0.11$). ● = cases with no *BCL2* alteration; ○ = cases with t(14;18); and ▼ = cases with *BCL2* gain only. The regression line and the 95% confidence interval are also shown. (b) The apoptotic fractions of tumor cells and normal cells according to *BCL2* genotype.

groups of DLBCL cases were 2.22, 1.30, 0.97 and 0.56, respectively, which was similar to the trend observed for the whole material. Hence, the gene dosage effect on BCL2 expression was less pronounced compared to the effect of t(14;18) in this study than in a previous one,[19] where similar BCL2 protein levels were measured in DLBCL with *BCL2* amplification and t(14,18), respectively.

Cellular and clinical phenotypes and dependence on BCL2 genotype and phenotype

There was a highly significant negative correlation between the expression of BCL2 and levels of spontaneous apoptosis in the tumor cells ($P<0.001$) (Figure 3a). The correlation was not very strong ($r^2=0.11$), probably reflecting the fact that mediators other than BCL2 can regulate apoptosis in transformed B cells.[35–37] However, the median tumor apoptotic fractions in the cases with both t(14;18) and *BCL2* gain (0.9%), t(14;18) (1.1%), *BCL2* gain (0.9%) and no *BCL2* alterations (1.2%) were not different ($P=0.86$; Figure 3b). Surprisingly, levels of spontaneous apoptosis in the normal cells in the t(14;18)-positive tumors without (2.1%) and with (2.3%) *BCL2* gain were higher than the corresponding tumor cell apoptotic fractions ($P=0.002$ for the cases with t(14;18); Figure 3b). No difference in tumor and normal cell apoptotic fractions was found for tumors with *BCL2* gain only and no *BCL2* alterations ($P=0.86$ and $=0.96$, respectively). Furthermore, the normal cells of t(14;18)-positive tumors showed increased apoptotic fractions compared to the normal cells in tumors with no *BCL2* alterations (median: 2.1 and 1.45%, respectively; $P=0.02$). The normal cells in the tumors with *BCL2* gain only showed intermediate apoptotic fractions (median: 1.8%). A mean of 23% ($\pm$s.d. = 11%)/1.2% ($\pm$1.2%), 17% ($\pm$11%)/2.2% ($\pm$1.8), and 23% ($\pm$14%)/1.5% ($\pm$2.4%) of the cells in the suspensions were T lymphocytes/monocytes in the cases with t(14;18)

($N=36$), with *BCL2* gain only ($N=11$) and without *BCL2* alterations ($N=43$), respectively, as assessed by immunofluorescence. Since the average tumor cell fraction was 75%,[15] this means that the large majority of normal cells in the samples were T-lymphocytes. It may be speculated that disruption of an apoptotic pathway (increased BCL2 levels) in the tumor cells may cause the release of proapoptotic mediators causing cell death in normal T-lymphocytes in the affected lymph node.

The median tumor cell S-phase fraction was higher in the B-NHL with *BCL2* gain only (6.2%), compared to the cases with t(14;18) (1.1%; $P<0.001$), and without *BCL2* alterations (1.3%; $P=0.02$). The normal cell S-phase fractions were not different in the lymphomas with various *BCL2* genotypes (0.9–1.5%; $P>0.17$).

Patients with lymphomas with gain of *BCL2* only had a poor prognosis (relative risk: 2.9, $P=0.002$), in agreement with the results of Bea *et al*.[38] Cox multivariate survival analysis was employed to assess the prognostic value of *BCL2* gain when entered together with the prognostic parameters found in an earlier study (TP53 aberrations, 11q21–23.1 deletions, 6p gain, IPI).[15] The results showed that *BCL2* gain only had no independent prognostic value ($P=0.09$), probably due to covariation with the (stronger) prognostic parameters 6p gain ($P=0.007$), and TP53 aberrations ($P=0.05$). The six patients with lymphomas with concomitant *BCL2* rearrangement and amplification, as well as the others with t(14;18) only, survived slightly longer than the remaining patients, but the differences were not significant ($P>0.29$).

Our previous study showed that spontaneous apoptosis in the tumor cells has no prognostic value in this tumor material.[39] The apoptotic fraction of the normal cells did not have any prognostic value for the whole group of patients ($P=0.91$, cutoff at median = 1.9%), or for the patients with FL ($P=0.62$). The gene expression patterns in the normal cells in FL have prognostic value,[40] however, the majority of the genes with prognostic value in this study were not apoptosis associated.

Assessment of DNA copy number changes on chromosome 18 by array CGH and FISH

Array CGH was applied to define amplified regions on chromosome 18 in more detail (Figure 4, Table 2, web Table: radium.no/stokke/FTP/Stokke/Chromosome18arrayCGH.xls). The observed ratios in microarray experiments will generally depend on the linearity and dynamic range of the assay, as well as on the fraction of normal cells in the sample, tumor heterogeneity and the DNA index of the tumor. We used the near-diploid DLBCL cell line U698 to test the dynamics of our array CGH platforms. U698 has a narrow amplicon (~1 mbp) at chromosome 6p, which was observed by both BAC and cDNA array CGH. The expected ratio of approximately 10 was observed with one probe on the BAC array (20 copies of the 6p locus by FISH), whereas the ratio at this locus was only two when U698 cells were examined by cDNA array CGH with several more densely spaced probes. Thus, there was not a one-to-one relationship between copy number and ratio in the cDNA array experiments. There was also noise in the data, and the sensitivity for detection of copy number aberrations was much lower for cDNA arrays compared to BAC arrays. The primary tumors also contained an average of 25% normal cells,[15] which tended to bring the ratios towards unity. Hence, the ratios in the BAC array CGH experiments were expected to be somewhat lower than predicted from the copy numbers in the case of gains. No sharp high-level amplifications were detected by BAC array CGH in any of the 93 tumors, and the $\log_2$ ratios varied between −1.05 and +0.92 (web Table: radium.no/stokke/FTP/Stokke/Chromosome18arrayCGH.xls). The spacing between the probes on the BAC array was approximately 1 mbp. We therefore employed cDNA arrays with a much closer spacing of the probes (~100 kbp) to reveal any high-level amplification of small regions that could have been missed with the BAC arrays. No gains were detected on chromosome 18 by cDNA array CGH in any of the tumors listed in Table 2, indicating that there were no high-level amplification of regions of size larger than ~100 kbp. FISH showed that one to two extra copies of *MALT1* and *PMAIP1* were gained compared to the average copy number of the tumors in Table 2, in agreement with the low-level gains observed by BAC array CGH.

Seven of the tumors with t(14;18) had gains that extended from 18pter to clone ba215a20 at 18q21.33, thus defining the consensus region of gain (defined by at least three tumors). Case 287/88 had gain from the centromere to clone ba215a20. Clone ba215a20 is the closest clone (in this study) centromeric of *BCL2*, and since the segment recognized by the 14qter probe pac820m16 was gained in seven of these eight tumors (Figure 4, Table 2; none of these tumors had any other gains on chromosome 14), it is strongly suggested that one extra copy of der(18)t(14;18) was gained in cases 364/86, 21/87, 176/88, 472/90 and 581/90. Case 472/90 showed loss from ba45a1 to 18qter, indicating that either the der(14)t(14;18) or the untranslocated chromosome 18 had lost this region. Case 287/88 may have gained an extra copy of a der(18)t(14;18) lacking the p-arm, or the p-arm may have been lost from the untranslocated chromosome 18 and 1 of the der(18)t(14;18). Case 436/91 had probably gained two extra copies of der(18)t(14;18), while case 130/92 may have gained one copy of der(18)t(14;18) and one of chromosome 18. These results are in agreement with the study of Horsman *et al*,[17] which showed that der(18)t(14;18) was frequently gained in FL cases with *BCL2* rearrangement. The six other cases with chromosome 18 gain and *BCL2* rearrangement showed gain of the entire chromosome 18. There were no indications of local amplica in the tumors with t(14;18), also justifying the calculation of *BCL2* copy numbers performed in Table 1.

Six of the 12 tumors with *BCL2* gain, but without translocation had gains extending from 18pter to 18qter, suggesting that an extra copy of the whole chromosome 18 was gained in these cases. Case 390/87, with loss of *BCL2*, had a narrow amplicon extending from the centromere to ba17i14. The other lymphomas with 18q gain only defined the consensus region to be 18q21.2 (ba116k4)-18qter (Figure 4). In case 50/93, the ratio was increased for clone ba28f1, which was in agreement with the five copies detected by FISH for this tumor. However, there was a clear decrease in ratio telomeric of ba28f1 also in cases 154/88, 383/91 and 525/89 (Figure 4). Also, the ratios appeared to be lower between ba350k6 and ba28f1 in cases 154/88, 383/91, 525/89 and 50/93. If these fine structures in the amplica were taken into consideration, the consensus regions of gain were 18q21.2 (ba116k4)–18q21.32 (ba350k6) and 18q21.33 (ba28f1).

Although the level of amplification was lower in the present study employing primary tumor samples, we found the same regions of gain as Sanchez-Izquierdo *et al*,[20] centered on the *BCL2* and *MALT1*/*PMAIP1* loci. However, while the cell lines of MCL and DLBCL origin showed preferential amplification of the region containing the *BCL2* locus,[20] we found coamplification of these regions in the primary MCL cases 154/88 and 383/91, and the DLBCL cases 525/89 and 50/93. The high-level gains

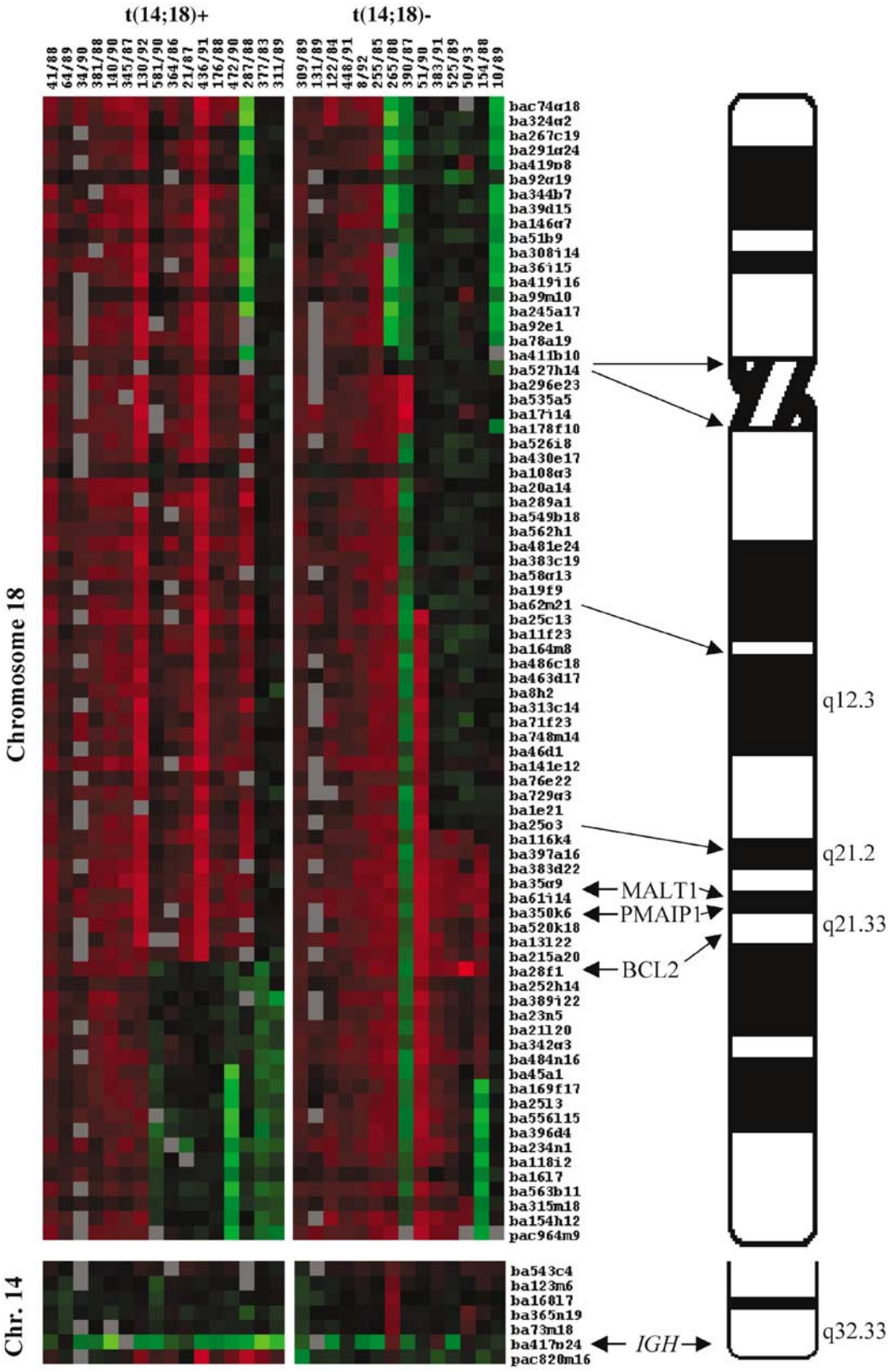

Figure 4 Chromosome 18 copy number changes in B-NHL. BAC arrays covering chromosome 18 with a resolution of approximately 1 mbp (78 clones) were used to assess DNA copy number changes in 93 cases of B-NHL. The 30 cases with alterations on chromosome 18 are shown. The copy number alterations of the seven clones most telomeric on chromosome 14q are also shown. DNA gains and losses are colored in red and green, respectively. The most intense red and green colors represent $\log_2$ ratios of >0.72 and <-0.72, respectively. The ratios varied between -1.05 and $+0.92$.

may be more frequent in B-cell lymphoma lines than in primary tumors,[20] in agreement with the results presented here and in another study.[19]

Interestingly, the majority of tumors with *BCL2* alterations (Figure 4), as well as the ones without (radium.no/stokke/FTP/Stokke/Chromosome18arrayCGH.xls), showed selective loss of the *IGH* probe ba417p24, covering the region between the D-segments and Cα3. This loss was probably the result of V(D)J, and, in some cases, class switch rearrangements.

Conclusions

In this study, we have shown that whole or partial gains of chromosome 18 are common in B-NHL, both with and without t(14;18). There appears to be a *BCL2*-collaborating gene in the region 18pter-q21.33 (excluding *BCL2*), as this was the consensus region of gain for the cases with t(14;18). The low level gain of *BCL2* in primary tumors led to elevated BCL2 levels in the t(14;18)-negative cases. Increased BCL2 expression was associated with increased apoptosis in the normal cells in the tumors. The region 18q21.2–18q21.32 was amplified together with the region containing *BCL2* in the t(14;18)-negative tumors, indicating that the increased expression of another gene in this region, together with the increased BCL2 expression, is of importance in lymphoma development.

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References

1 Tsujimoto Y, Finger LR, Yunis J, Nowell PC, Croce CM. Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science* 1984; **226**: 1097–1099.
2 Yunis JJ, Frizzera G, Oken MM, McKenna J, Theologides A, Arnesen M. Multiple recurrent genomic defects in follicular lymphoma. A possible model for cancer. *N Engl J Med* 1987; **316**: 79–84.
3 Weiss LM, Warnke RA, Sklar J, Cleary ML. Molecular analysis of the t(14;18) chromosomal translocation in malignant lymphomas. *N Engl J Med* 1987; **317**: 1185–1189.
4 Jacobson JO, Wilkes BM, Kwaiatkowski DJ, Medeiros LJ, Aisenberg AC, Harris NL. BCL-2 rearrangements in *de novo* diffuse large cell lymphoma. Association with distinctive clinical features. *Cancer* 2003; **72**: 231–236.
5 Vaux DL, Cory S, Adams JM. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* 1988; **335**: 440–442.
6 McDonnell TJ, Deane N, Platt FM, Nunez G, Jaeger U, McKearn JP *et al*. BCL-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell* 1989; **57**: 79–88.
7 Monni O, Joensuu H, Franssila K, Knuutila S. DNA copy number changes in diffuse large B-cell lymphoma – comparative genomic hybridization study. *Blood* 1996; **87**: 5269–5278.
8 Rao PH, Houldsworth J, Dyomina K, Parsa NZ, Cigudosa JC, Louie DC *et al*. Chromosomal and gene amplification in diffuse large B-cell lymphoma. *Blood* 1998; **92**: 234–240.
9 Berglund M, Enblad G, Flordal E, Lui WO, Backlin C, Thunberg U *et al*. Chromosomal imbalances in diffuse large B-cell lymphoma detected by comparative genomic hybridization. *Mod Pathol* 2002; **15**: 807–816.
10 Bentz M, Werner CA, Döhner H, Joos S, Barth TFE, Siebert R *et al*. High incidence of chromosomal imbalances and gene amplifications in the classical follicular variant of follicle center lymphoma. *Blood* 1996; **88**: 1437–1444.
11 Avet-Loiseau H, Vigier M, Moreau A, Mellerin MP, Gaillard F, Harousseau JL *et al*. Comparative genomic hybridization detects genomic abnormalities in 80% of follicular lymphomas. *Br J Haematol* 1997; **97**: 119–122.
12 Allen JE, Hough RE, Goepel JR, Bottomley S, Wilson GA, Alcock HE *et al*. Identification of novel regions of amplification and deletion within mantle cell lymphoma DNA by comparative genomic hybridization. *Br J Haematol* 2002; **116**: 291–298.
13 Dierlamm J, Rosenberg C, Stul M, Pittaluga S, Wlodarska I, Michaux L *et al*. Characteristic pattern of chromosomal gains and losses in marginal zone B cell lymphoma detected by comparative genomic hybridization. *Leukemia* 1997; **11**: 747–758.
14 Bentz M, Huck K, du Manoir S, Joos S, Werner CA, Fischer K *et al*. Comparative genomic hybridization in chronic B-cell leukemias shows a high incidence of chromosomal gains and losses. *Blood* 1995; **85**: 3610–3618.
15 Stokke T, DeAngelis P, Smedshammer L, Galteland E, Steen HB, Smeland EB *et al*. Loss of chromosome 11q21–23.1 and 17p and gain of chromosome 6p are independent prognostic indicators in B-cell non-Hodgkin's Lymphoma. *Br J Cancer* 2001; **85**: 1900–1913.
16 Viardot A, Möller P, Högel J, Werner K, Mechtersheimer G, Ho AD *et al*. Clinicopathologic correlations of genomic gains and losses in follicular lymphoma. *J Clin Oncol* 2002; **20**: 4523–4530.
17 Horsman DE, Connors JM, Pantzar T, Gascoyne RD. Analysis of secondary chromosomal alterations in 165 cases of follicular lymphoma with t(14;18). *Genes Chromosomes Cancer* 2001; **30**: 375–382.
18 Armitage JO, Sanger WG, Weisenburger DD, Harrington DS, Linder J, Bierman PJ *et al*. Correlation of secondary cytogenetic abnormalities with histologic appearance in Non-Hodgkin's lymphomas bearing t(14;18)(q32;q21). *J Natl Cancer Institute* 1988; **80**: 576–580.
19 Monni O, Joensuu H, Franssila K, Klefstrom J, Alitalo K, Knuutila S. BCL2 overexpression associated with chromosomal amplification in diffuse large B-cell lymphoma. *Blood* 1997; **90**: 1168–1174.
20 Sanchez-Izquierdo D, Buchonnet G, Siebert R, Gascoyne RD, Climent J, Karran L *et al*. MALT1 is deregulated by both chromosomal translocation and amplification in B-cell non-Hodgkin lymphoma. *Blood* 2003; **101**: 4539–4546.
21 Martinez-Climent JA, Alizadeh AA, Segraves R, Blesa D, Rubio-Moscardo F, Albertson DG *et al*. Transformation of follicular lymphoma to diffuse large cell lymphoma is associated with a heterogeneous set of DNA copy number and gene expression alterations. *Blood* 2003; **101**: 3109–3117.
22 Ott G, Katzenberger T, Greiner A, Kalla J, Rosenwald A, Heinrich U *et al*. The t(11;18)(q21;q21) chromosome translocation is a frequent and specific aberration in low-grade but not high-grade malignant non-Hodgkin's lymphomas of the mucosa-associated lymphoid tissue (MALT−) type. *Cancer Res* 1997; **57**: 3944–3948.
23 Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A *et al*. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 2000; **403**: 503–511.
24 Stokke T, Holte H, Smedshammer L, Smeland EB, Kaalhus O, Steen HB. Proliferation and apoptosis in malignant and normal cells in B-cell non-Hodgkin's lymphomas. *Br J Cancer* 1998; **7**: 1832–1838.
25 Stokke T, Galteland E, Holte H, Smedshammer L, Suo Z, Smeland EB *et al*. Oncogenic aberrations in the p53 pathway are associated with a high S phase fraction and poor patient survival in B-cell Non-Hodgkin's lymphoma. *Int J Cancer* 2000; **89**: 313–324.
26 Akasaka T, Akasaka H, Yonetani N, Ohno H, Yamabe H, Fukuhara S *et al*. Refinement of the BCL2/immunoglobulin heavy chain fusion gene in t(14;18)(q32;q21) by polymerase chain reaction amplification for long targets. *Genes Chromosomes Cancer* 1998; **21**: 17–29.
27 Galteland E, Holte H, Stokke T. c-MYC, RB-1, TP53, and centromere 8 and 17 copy number in B-cell non-Hodgkin's lymphomas assessed by dual-color fluorescence *in situ* hybridisation. *Cytometry* 1999; **38**: 53–60.
28 Galteland E, Smedshammer L, Suo Z, DeAngelis P, Stokke T. Proliferation-dependent expression and phosphorylation of pRB in B cell non-Hodgkin's lymphomas: dependence on RB1 copy number. *Leukemia* 2002; **16**: 1549–1555.
29 De Angelis PM, Clausen OP, Schjolberg A, Stokke T. Chromosomal gains and losses in primary colorectal carcinomas detected by

CGH and their associations with tumour DNA ploidy, genotypes and phenotypes. *Br J Cancer* 1999; **80**: 526–535.
30 Osoegawa K, Mammoser AG, Wu C, Frengen E, Zeng C, Catanese JJ *et al.* A bacterial artificial chromosome library for sequencing the complete human genome. *Genome Res* 2001; **11**: 483–496.
31 Fiegler H, Carr P, Douglas EJ, Burford DC, Hunt S, Smith J *et al.* DNA microarrays for comparative genomic hybridization based on DOP-PCR amplification of BAC and PAC clones. *Genes Chromosomes Cancer* 2003; **36**: 361–374.
32 Wang J, Meza-Zepeda LA, Kresse SH, Myklebost O. M-CGH: Analysing microarray-based CGH experiments. *BMC Bioinformatics* 2004; **5**: 74.
33 Holte H, Suo Z, Smeland EB, Kvaløy S, Langholm R, Stokke T. The prognostic value of lymphoma-specific S-phase compared with other cell proliferation markers. *Acta Oncol* 1999; **38**: 495–503.
34 Lømo J, Smeland EB, Krajewski S, Reed JC, Blomhoff HK. Expression of the Bcl-2 homologue Mcl-1 correlates with survival of peripheral blood B lymphocytes. *Cancer Res* 1996; **56**: 40–43.
35 Igney FH, Krammer PH. Death and anti-death: tumor resistance to apoptosis. *Nat Rev Cancer* 2002; **2**: 277–288.
36 Ghia P, Boussiotis VA, Schultze JL, Cardoso AA, Dorfman DM, Gribben JG *et al.* Unbalanced expression of bcl-2 family proteins in follicular lymphoma: contribution of CD40 signaling in promoting survival. *Blood* 1998; **91**: 244–251.
37 Karin M, Lin A. NF-kappa B at the crossroads of life and death. *Nat Immunol* 2002; **3**: 221–227.
38 Bea S, Colomo L, Lopez-Guillermo A, Salaverria I, Puig X, Pinyol M *et al.* Clinicopathologic significance and prognostic value of chromosomal imbalances in diffuse large B-cell lymphomas. *J Clinical Oncol* 2004; **22**: 3498–3506.
39 Stokke T, Smeland EB, Kvaløy S, Holte H. Tumor cell proliferation, but not apoptosis, predicts survival in B-cell non-Hodgkins lymphomas. *Br J Cancer* 1998; **77**: 1839–1841.
40 Dave SS, Wright G, Tan B, Rosenwald A, Gascoyne RD, Chan WC *et al.* Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells. *New Engl J Med* 2004; **351**: 2159–2169.

Gain of chromosome 6p is an infrequent cause of increased PIM1 expression in B-cell non-Hodgkin's lymphomas

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Gains and losses of genomic material are changes that play central roles in tumor development and progression. Comparative genomic hybridization (CGH) allows for the detection of over- and underrepresented sequences in tumor DNA as compared to normal DNA, and such genomic aberrations have been shown to have prognostic value in several studies of primary lymphomas. The serine/threonine kinase *PIM1* is a known oncogene that has previously been shown to be

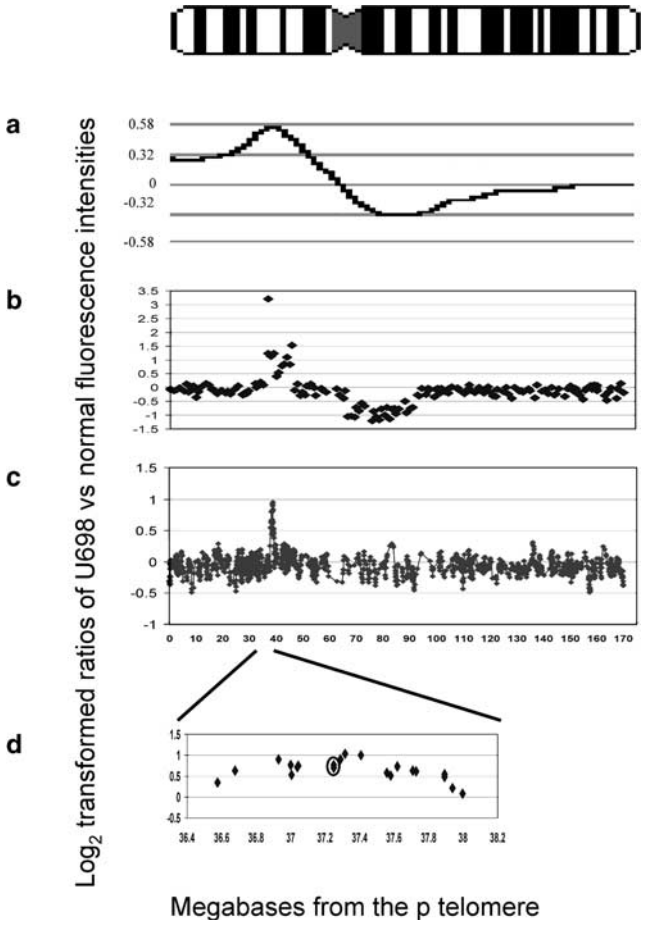

Figure 1 Gain of 6p21 in the lymphoma cell line U698. (**a**) CGH profile of chromosome 6 in U698. (**b**) BAC array CGH profile. (**c**) cDNA array CGH profile. (**d**) Detailed view of the cDNA array CGH profile of the 6p21 amplicon in U698. The two clones representing *PIM1* are encircled.

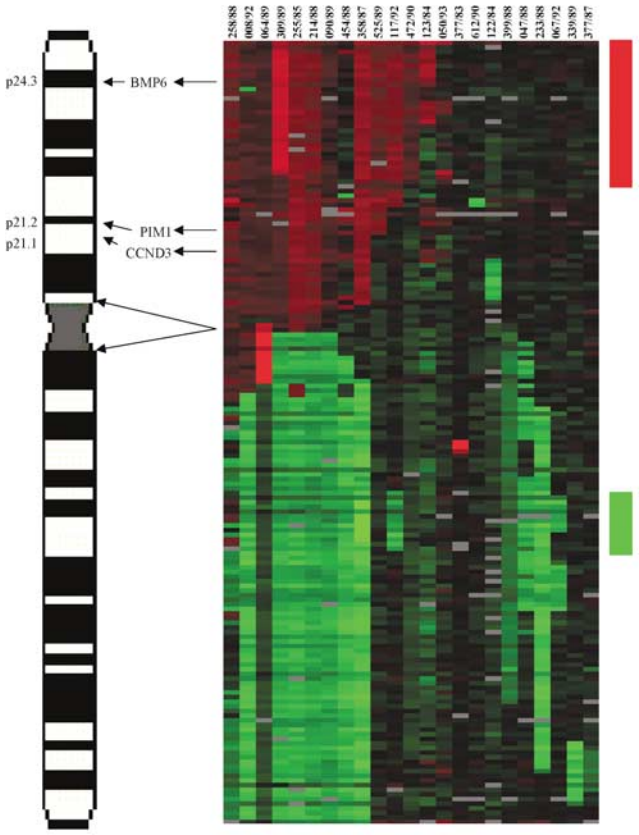

Figure 2 BAC array CGH profile of B-NHLs with chromosome 6 alterations. Genomic gains are shown in red whereas genomic losses are shown in green. The color intensities reflect the experimentally obtained $\log_2$ transformed ratios. The most intense red and green colors reflect values above 1 and below −1, respectively. Regions with normal DNA copy number are shown in black.

upregulated in prostate cancer. Overexpression of *PIM1* in cell lines protects against apoptosis induced by genotoxic agents. The *PIM1* gene is localized to chromosome 6p21.2. Parts of chromosome 6 or the whole 6p arm are gained in a subset of B-cell non-Hodgkin's lymphomas, and these gains are associated with a poor prognosis.[1]

In order to identify genomic aberrations important for tumorigenesis of non-Hodgkin's lymphomas, we characterized in detail the gains and losses of genomic material in the mature B-cell lymphoma cell line U698, originally classified as a lymphoblastic lymphosarcoma.[2] The general procedures of DNA isolation, CGH hybridizations and gene copy number analysis by FISH has been described earlier.[3] A local DNA gain mapping to region 6p21 was identified by conventional CGH analysis (Figure 1a). To identify genes in the amplified region, we performed array CGH analysis using a BAC-array with an average spacing of 1 Mbp between the probes (Figure 1b). This analysis narrowed the region of gain down to nine megabases. The most telomeric clone (RP3-431A14) was located on 6p21.31, whereas the most centromeric clone (RP11-546O15) was located on 6p21.1. A 10-fold gain was observed with the probe RP1-90K10. To further resolve any fine structure in the amplicon, DNA from U698 cells was hybridized to cDNA arrays with an average spacing of 150 kbp (Figure 1c). Although the dynamics and sensitivity of the array CGH analysis was much lower with cDNA arrays than with BAC arrays, the former analysis indicated that the amplicon was approximately 1.5 Mbp. *PIM1* was approximately in the center of this amplicon (Figure 1d).

Dual color interphase FISH studies showed that there were two copies of centromere 6 in both Reh and U698 cells. In contrast, U698 exhibited one normal signal and in addition a cluster of 15–20 signals from the *PIM1* probe (RP3-355M6), probably representing intrachromosomal amplification of the *PIM1* gene (Supplementary Figure 1). Translocations in lymphomas targeting *CCND3* has been described, and high *BMP6* expression has been shown to be of negative prognostic value in DLBCL.[4] Considering these genes as possible targets for chromosomal alterations, we performed FISH with a probe for *CCND3* (RP5-973N23), located at 6p21 and *BMP6* (RP1-119C5) located at 6p24. Whereas there were only two signals from the *BMP6* probe, the *CCND3* probe generated four signals, indicating that this gene is also represented in a proportion of the amplica in U698 cells.

Having established that *PIM1* is included in the high-level amplified region of 6p observed in U698, we investigated whether the increase in DNA content was reflected in high expression levels of the corresponding mRNA and protein. Northern blot analysis revealed that the basal mRNA levels of *PIM1* in U698 are higher than in other cell lines (Supplementary Figure 2). U698 cells also express high amounts of PIM1 protein,

Table 1 B-NHL with chromosome 6 alterations: summary of chromosomal CGH findings, copy number data of *CCND3, PIM1* and *BMP6* obtained by FISH and the copy numbers of the same genes suggested by array CGH. The order of the tumors is the same as in Figure 2.

Case no.	Histology	Tumor fraction %B	Ploidy	CGH		Copy number determined by FISH				Copy number suggested by array CGH		
				Gain	Deletion	Centromere 6	CCND3	PIM1	BMP6	CCND3	PIM1	BMP6
258/88	dlbcl	44	1.07	6p		3	3	3	3	3	3	3
008/92	unclass	81	1	6p	6q	2	3	3	3	3	3	3
064/89	fcl II	69	1.26	6pter-q15		3	3	3	3	3	3	3
309/89	mcl	79	1.83	6p	6q	4	6	6	8	6	6	8
255/85	dlbcl	71	1.04	6p	6q	3	3	3	3	3	3	3
214/88	unclass	84	1.00	6p	6q	2	3	3	3	3	3	3
090/89	fcl III	52	1.21	6p	6q	2	3	3	3	3	3	3
454/88	dlbcl	92	1.02		6q	2	Inconcl.	Inconcl.	2	2	2	2
358/87	mcl	89	1.94	6p	6q	3	6	6	6	6	6	6
525/89	dlbcl	84	1.07	6p		2	2	3	3	2	3	3
117/92	dlbcl	98	1	6p21.3-ter		2	2	2	3	2	2	3
472/90	dlbcl	85	1.16	6p22-ter		2	2	2	3	2	2	3
123/84	mcl	94	1.04			ND	2	2	3	2	2	3
050/93	dlbcl	85	1.00			2	2	2	2	2	2	2
377/83	fcl I/II	78	1			2	ND	2	2	2	2	2
612/90	dlbcl	82	1.04			ND	ND	2	ND	2	2	2
122/84	dlbcl	86	1.23		6q	2	ND	2+5	2+5	2	2	2
399/88	fcl III	54	1		6cen-q24	ND	ND	2	ND	2	2	2
047/88	fcl I/II	72	1.00		6cen-q22	ND	ND	2	ND	2	2	2
233/88	fcl II	90	1.00		6q14-ter	ND	ND	2	ND	2	2	2
067/92	mcl	90	0.98			ND	ND	2	ND	2	2	2
339/89	sll	90	1.00			2	2	2	2	2	2	2
377/87	dlbcl	48	1.03			ND	ND	2	ND	2	2	2

dlbcl = diffuse large B-cell lymphoma; mcl = mantle cell lymphoma; fcl = follicle center lymphoma; ND = not determined; Inconcl. = inconclusive.

whereas the levels are undetectable in Reh cells (immunoblotting data not shown).

We now proceeded to define the core chromosome 6p amplicon in B-NHL to clarify whether *PIM1* could be the target of these gains. 93 B-cell lymphomas were subjected to analysis with array CGH as well as FISH with probes corresponding to *PIM1*, *CCND3*, *BMP6* and centromere 6. These tumors have previously been analyzed by chromosomal CGH, and in this study gain of chromosome 6p was found to have independent negative prognostic value.[1] Compared to the data obtained by chromosomal CGH, the BAC array CGH experiments revealed more copy number changes and could better define the boundaries of the gains and losses. The data of the array CGH experiments are available in Supplementary Table 1. The minimal consensus region for the gain on chromosome 6 was 6p22.1-ter (Figure 2). Loss of the whole arm of 6q was the most common change on 6q, and this was previously also detected by chromosomal CGH.[1] The minimal consensus region for the deletions at 6q was 6q16.2–q21 by BAC array CGH. In addition, we observed high-level gains at 6cen-q13 in tumor 64/89 (ratio = 2.9), 6q15 in 377/83 (ratio = 6.6) and 6p22.2-ter in 309/89 (ratio = 1.7) (Figure 2). The cDNA array CGH experiments did not reveal any high-level gains on chromosome 6 in this material. By FISH analysis, we found that 10 out of 92 successfully analyzed tumors exhibited increased copy number of *PIM1* relative to the DNA index. Nine of these tumors had also gain of 6p detected by chromosomal CGH analysis, whereas one tumor contained a clone of tumor cells with five copies of both *PIM1* and *BMP6* (122/84). Table 1 summarizes the chromosomal CGH data for chromosome 6, the copy number data of *CCND3*, *PIM1* and *BMP6* obtained by FISH, and the copy numbers of the same genes suggested by array CGH.

Given that gain of *PIM1* is a feature of a significant proportion of B-NHL, we wanted to clarify whether this gain is reflected in increased expression levels that might influence on the tumor response to treatment. We therefore proceeded to analyze a set of diffuse large B-cell lymphomas,[4] that was independent from the previously analyzed B-NHL.[1] By assessing the copy number of *PIM1* in 35 of the tumor samples from this study of which we had material available,[4] we first examined whether increased copy number of *PIM1* in the tumor samples could account for the variation in the expression levels of the gene. Seven of the tumor samples had increased copy number of *PIM1*; three tumors had four copies while four had three. On average, these tumors exhibited 20% higher expression levels of *PIM1* compared to the ones without gain. This difference was, however, not significant (95% confidence interval 0.74–1.96, $P = 0.41$).

We then proceeded to investigate the role of *PIM1* expression with respect to DLBCL subtype and prognosis. Analysis of the data revealed that expression higher than the median of *PIM1* is associated with the activated B-cell phenotype ($P < 0.001$). Moreover, patients with high expression levels had significantly poorer outcome ($P = 0.0054$) (Supplementary Figure 3).

Our data indicate that other genes than *PIM1* are the targets for the 6p gain frequently observed in malignant lymphomas, since the gene is not included in the core chromosome 6p amplicon. Moreover, the variation in *PIM1* expression seen in lymphomas cannot merely be explained by an increase of the copy number of the gene. Changes in transcriptional regulation could be a mechanism for *PIM1* upregulation. It has been shown that the NF-kappa B pathway is involved in PIM1 regulation.[5] This would fit well with the fact that high *PIM1* expression mainly is found in activated B-cell like DLBCL (ABC), which are characterized by constitutive activation of the NF-kappa B pathway.[4] Recently, it was shown that that inhibitors of IkappaB kinase (IKK) are toxic for ABC DLBCL cell lines, but not for cell lines derived from the germinal center like DLBCLs. Interest-

ingly, treatment of the cells with such inhibitors was accompanied by changes in gene expression that included down-regulation of *PIM1*.[6] This suggests that the high expression levels of *PIM1* seen in this tumor type, at least partly, are caused by transcriptional activation. However, other mutations of the genome than a mere increase in copy number could also lead to activation of the proto-oncogene, and *PIM1* has been shown to be involved in translocations as well as to be a subject for hypermutations in lymphomas. The highest number of *PIM1* copies detected by FISH in our material of 125 primary lymphomas was five, indicating that high-level gain of this locus must be a rare event in this tumor type. Previously, there have been sporadic reports on amplica at 6p21 in primary lymphomas.[7,8] Our findings in U698 where the high-level gain at this locus was accompanied by high expression levels of *PIM1*, suggest that *PIM1* can be the target of these rare amplifications, and thus in some cases play a role in the tumorigenesis of lymphomas.

The *PIM1* locus was not included in the consensus region of the 6p gain in our study, which is in line with the finding of others. Other candidate target genes for the gain are *BMP6* at 6p24 or *IRF4* at 6p25. High expression of both these genes has been associated to poor prognosis in DLBCL,[4] and they are also included in the core 6p amplicon in our study.

It is now well established that certain gene expression features of the tumor in part cause the clinical heterogeneity of the lymphomas in terms of outcome, and the different tumor phenotypes have also been correlated to certain genotypes. The great variability of lymphomas regarding tumor genotype and phenotype as well as clinical behavior, is a therapeutic challenge. *PIM1* is promising as a therapeutic target, since it is overexpressed in tumors with poor response to therapy, and since silencing of this gene potentially could lead to higher sensitivity of the tumor cells to apoptosis inducing agents. However, since our data indicate that the transcriptional regulation to some extent overrides the gene dosage effect of this gene, the ideal targeting of *PIM1* probably also involves factors upstream in the regulation cascade. It will therefore be interesting to examine the effects of selective *PIM1* knock down combined with inhibitors of IkappaB kinase in B-NHL.

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References

1 Stokke T, DeAngelis P, Smedshammer L, Galteland E, Steen HB, Smeland EB *et al.* Loss of chromosome 11q21-23.1 and 17p and gain of chromosome 6p are independent prognostic indicators in B-cell non-Hodgkin's lymphoma. *Br J Cancer* 2001; **85**: 1900–1913.
2 Nilsson K, Sundstrom C. Establishment and characteristics of two unique cell lines from patients with lymphosarcoma. *Int J Cancer* 1974; **13**: 808–823.
3 Galteland E, Sivertsen EA, Svendsrud DH, Smedshammer L, Kresse SH, Meza-Zepeda LA *et al.* Translocation t(14;18) and gain of chromosome 18//BCL2: effects on BCL2 expression and apoptosis in B-cell non-Hodgkin's lymphomas. *Leukemia* 2005, 10.1038/sj.leu.2403954.
4 Rosenwald A, Wright G, Chan WC, Connors JM, Campo E, Fisher RI *et al.* The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. *N Engl J Med* 2002; **346**: 1937–1947.
5 Zhu N, Ramirez LM, Lee RL, Magnuson NS, Bishop GA, Gold MR. CD40 signaling in B cells regulates the expression of the Pim-1 kinase via the NF-kappa B pathway. *J Immunol* 2002; **168**: 744–754.
6 Lam LT, Davis RE, Pierce J, Hepperle M, Xu Y, Hottelet M *et al.* Small molecule inhibitors of I{kappa}B kinase are selectively toxic for subgroups of diffuse large B-cell lymphoma defined by gene expression profiling. *Clin Cancer Res* 2005; **11**: 28–40.
7 Bentz M, Werner CA, Dohner H, Joos S, Barth TF, Siebert R *et al.* High incidence of chromosomal imbalances and gene amplifications in the classical follicular variant of follicle center lymphoma. *Blood* 1996; **88**: 1437–1444.
8 Dierlamm J, Rosenberg C, Stul M, Pittaluga S, Wlodarska I, Michaux L *et al.* Characteristic pattern of chromosomal gains and losses in marginal zone B cell lymphoma detected by comparative genomic hybridization. *Leukemia* 1997; **11**: 747–758.

bjh research paper

PI3K/Akt-dependent Epo-induced signalling and target genes in human early erythroid progenitor cells

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Summary

Erythropoietin (Epo) is the major regulator of differentiation, proliferation and survival of erythroid progenitors, but the Epo-induced changes in gene expression that lead to these effects are not fully understood. The aim of this study was to examine how Epo, via activation of phosphatidylinositol 3-kinase (PI3K)/Akt, exerts its role in the development of erythroid progenitors from $CD34^+$ cells, and to identify early Epo target genes in human erythroid progenitors. In $CD34^+$ progenitor cells, Epo alone was able to induce cell cycle progression as demonstrated by upregulation of cyclin D_3, E and A leading to hyperphosphorylation of the retinoblastoma protein (RB). These effects were completely counteracted by the PI3K inhibitor LY294002. Furthermore, enforced expression of an activated form of Akt kinase highly augmented Epo-induced erythropoiesis. Fluorescent-activated cell sorting (FACS)-sorted $CD34^+CD71^+CD45RA^-GPA^-$ erythroid progenitors stimulated with Epo in the presence or absence of LY294002 were subjected to gene expression profiling. Several novel target genes of Epo were identified, and the majority were regulated in a PI3K-dependent manner, including *KIT* (CD117) and *CDH1* (E-cadherin). FACS analysis of Epo-stimulated erythroid progenitors showed that the increased mRNA expression of *KIT* and *CDH1* was accompanied by an induction of the corresponding proteins CD117 and E-cadherin.

Keywords: Erythropoietin, phosphatidylinositol 3-kinase, KIT, erythropoiesis, microarray.

Human erythropoiesis takes place in the bone marrow, where multipotent stem cells differentiate into mature erythrocytes. The earliest committed cells of the erythroid lineage, the erythroid burst-forming unit (BFU-E), express the erythropoietin receptor (EpoR), and progression through further developmental stages are completely dependent on signalling through this receptor (Krantz, 1991). Epo alone is able to induce erythroid development, but this process is enhanced by other cytokines, of which the most prominent is stem cell factor (Scf) (McNiece *et al*, 1991).

Activation of the signalling pathways upon ligand binding to EpoR is dependent on conformational changes of the receptor that bring together the dimerised receptor and Janus kinase 2 (JAK2) (Remy *et al*, 1999). Autophosphorylation of JAK2, followed by phosphorylation of cytoplasmic tyrosine residues of the EpoR results in an activated ligand-receptor complex that recruits several signalling molecules and adapter proteins that act as substrates for JAK2. Thus, several signalling cascades are involved in mediating the Epo-induced changes in gene expression and subsequent effects on differentiation, proliferation and survival (Richmond *et al*, 2005). Signal transducer and activator of transcription (STAT)5a/b, but also STAT1 and STAT3, have been shown to play a role in signal transduction, and JAK2 phosphorylation leads to dimerisation of these transcription factors and subsequent gene activation (Wojchowski *et al*, 1999; Richmond *et al*, 2005). The Ras/Raf-1/mitogen-activated protein kinase (MAPK) pathway is activated upon recruitment of the Grb2-Sos adapter molecules to the EpoR and this pathway also participates in mitogenesis (Jacobs-Helber & Sawyer, 2004; Arcasoy & Jiang, 2005).

The phosphatidylinositol 3-kinase (PI3K)-signalling cascade is crucial in mediating signals for survival and proliferation and is necessary for maturation of erythroid progenitors (Myklebust *et al*, 2002; Bouscary *et al*, 2003). PI3K can be activated by direct binding of the p85 regulatory subunit to the activated EpoR, and indirectly through binding to adapter

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molecules. The lipid products of PI3K activate a plethora of targets, including the protein kinase B/Akt. The PI3K-Akt pathway has been shown to play a central role in regulation of apoptosis and proliferation in several systems, including normal erythroid progenitors (Haseyama *et al*, 1999; Uddin *et al*, 2000). In the context of Epo signalling it has been shown that Akt kinase, which is activated by PI(3,4,5)P3 or PI(3,4)P2, phosphorylates both GATA-1 and Foxo3a, transcription factors of crucial importance in erythropoiesis (Uddin *et al*, 2000; Bouscary *et al*, 2003; Kadri *et al*, 2005).

In the present study we have investigated how Epo, by activating PI3K, exerts its effects on cell cycle progression and differentiation in human $CD34^+$ progenitor cells. By use of genome wide expression profiling of Epo-stimulated $CD34^+CD71^+CD45RA^-GPA^-$ erythroid progenitors, we identified novel Epo target genes and confirmed their differential expression by fluorescent-activated cell sorting (FACS) analysis, as well as hypothesise novel roles for signalling pathways that have no previously established function in erythropoiesis.

Materials and methods

DNA expression vectors

Expression vectors for HA-tagged wild type (WT-Akt) or myristylated Akt1 (Myr-Akt) created in pCMV6, were provided as a gift (Ahmed *et al*, 1997). pcDNA3 without insert was used as control vector (Control).

Reagents and antibodies

Recombinant human (rh) Epo was obtained from Boehringer Mannheim (GmbH, Germany) and rh Scf was from R&D Systems (Abingdon, UK). Cytokines were used at predetermined optimal concentrations: 5 U/ml for Epo and 50 ng/ml for Scf or as specified. LY294002 was from Calbiochem (Darmstadt, Germany). The following antibodies (mAbs, anti-human) were used in flow-cytometry analysis: anti-CD34 phycoerythrin (PE), anti-CD117 PE-cyanin 5 (CY5) and anti-CD45RA PE were from Becton Dickinson (San Jose, CA, USA), anti-glycophorin A-PE (GPA PE), and anti-CD71 fluorescein isothiocyanate (FITC) were from DakoCytomation AS (Glostrup, Denmark). Anti E-cadherin was from Calbiochem (San Diego, CA, USA; mouse IgG1, #205601) and anti mouse IgG1 PE (Southern Biotech, Birmingham, AL, USA) was used as secondary layer. The following antibodies were used in immunoblot analysis: anti-cyclin D_3, anti-cyclin A (sc-596), anti-cyclin E (sc-247), anti p21 (sc-397), anti-p27 (sc-528), anti-pRB (Ser780) and from Santa Cruz Biotechnologies (Santa Cruz, CA, USA), anti-Akt (#9272) and anti phospho Akt1 (Ser473, #9271) were from Cell Signaling (Beverly, MA, USA) anti-RB (G3–245) was from Becton Dickinson. Goat anti rabbit IgG-horseradish peroxidase (HRP) or rabbit anti mouse IgG-HRP were used as secondary layer (DakoCytomation).

Cell separation

Bone marrow cells were aspirated from the iliac crest of healthy adult donors with their informed consent and the approval of the Ethics Committee of the Norwegian Radium Hospital. Mononuclear cells were separated by Lymphoprep gradient centrifugation (Axis-Shield PoC AS, Oslo, Norway). Subsequently, $CD34^+$ cells were isolated by the use of immunomagnetic beads coated with CD34 mAbs (Dynal, Oslo, Norway) as previously described (Smeland *et al*, 1992; Myklebust *et al*, 2000). Ninety to 95% of the cells were $CD34^+$.

Cell culture and transient transfection

$CD34^+$ cells, $CD34^+CD71^+GPA^-CD45RA^-$ cells or cytokine-expanded $CD34^+$ cells (day 6 erythroid precursors) were plated in 1 ml X-VIVO 15 containing 1% bovine serum albumin (Stem Cell Inc., Vancouver, BC, Canada), 300 mg/l L-glutamine, 50 μmol/l β-mercaptoethanol (GibcoBRL), 66 mg/l penicillin and 100 mg/ml streptomycin (referred to as X-VIVO 15 complete medium) in 24-well plates in medium alone or cultured in the presence of Epo (5 U/ml or as indicated) with or without LY294002 (25 μmol/l) as specified. Cytokine-expanded $CD34^+$ cells, referred to as day 6 erythroid precursors, were obtained by culture in Epo (5 U/ml), Scf (25 ng/ml) and interleukin 3 (IL-3; 20 ng/ml) for 6 d, with addition of cytokines in new medium also at day 3. Quantification of total number of cultured cells was performed by counting viable cells in a hemocytometer. $CD34^+$ cells were transiently transfected by electroporation using the sNucleofector Device from Amaxa (http://www.amaxa.com/) and the human CD34 transfection kit.

Flow cytometry and cell sorting

$CD34^+$ cells or cultured cells were incubated with the specified mAbs for 30 min at 4°C and then washed with phosphate-buffered saline. The stained cells were analysed on a FACS Calibur flow cytometer with an argon-ion laser tuned at 488 nm [Becton Dickinson (BD), San Jose, CA, USA]. Data acquisition and analysis were performed using CELLQuest (BD) or FlowJo (http://www.flowjo.com). Highly purified $CD34^+CD71^+CD45RA^-GPA^-$ cells were obtained by staining $CD34^+$ cells with anti-CD71-FITC, anti-CD45RA-PE and anti-GPA-PE followed by sorting on a FACS DIVA cell sorter (BD).

Real time reverse transcription polymerase chain reaction (RT-PCR) and gene expression profiling

Total RNA was extracted from $CD34^+CD71^+CD45RA^-GPA^-$ cells (2×10^4–4×10^4) or day 6 erythroid precursors using Absolutely RNA® Microprep or Miniprep Kit (Stratagene) respectively.

cDNA was synthesised using random hexamers and TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster

City, CA, USA). mRNA expression of *KIT*, *CCNA1*, *CCNA2*, *CCNE1*, *CCNE2* and *CCND3* was analysed by real-time quantitative RT-PCR using Taqman technology according to the manufacturers procedure (Applied Biosystems). Pre-developed assay reagents, including primers and probes for the target genes, were supplied by Applied Biosystems and the PCR reactions were performed according to the manufacturer's instructions using Taqman Universal PCR Master Mix. Each measurement was performed in duplicate and the threshold cycle (C_t) was determined. Relative expression levels were calculated following the formula $\Delta C_T = C_T(\text{sample}) - C_T(\text{calibrator})$, where the expression of the target gene in T0 controls was used as a calibrator, and the values used to plot relative expression were calculated using the equation $2^{-\Delta CT}$.

In microarray experiments, total RNA was subjected to two rounds of linear amplification as described by Baugh *et al* (2001) with minor modifications (Nygaard *et al*, 2003).

A total of 42 000 spot cDNA microarrays from the Stanford Functional Genomics Facility were used to measure the relative mRNA expression levels in the samples (http://www.microarray.org/sfgf/jsp/home.jsp). Three micrograms of experimental sample antisense RNA (aRNA) and universal reference RNA (Stratagene, La Jolla, CA, USA) was labelled with Cy5-dUTP and Cy3-dUTP respectively (Amersham Pharmacia Biotech, Piscataway, NJ, USA). aRNA was reverse transcribed with Superscript II (GIBCO BRL) using random hexamer primers (Roche). The labelled probes were hybridised to microarrays in a GeneTAC automatic hybridisation station (http://www.genomicsolutions.com) and scanned with the Agilent DNA microarray scanner (http://www.agilent.com). Data were acquired using the GenePix Pro software (Axon Instruments Inc., Union City, CA, USA). The raw data files are available at Stanford Microarray Database (http://smd.stanford.edu/), and the filtered data used for the manuscript are available through our website: http://radium.no/ebsmeland. Only cDNA spots with a ratio of signal over background of at least 1·5 in both the Cy3 and the Cy5 channel were included. Clones differentially expressed between two cellular populations were identified as previously described (Shen *et al*, 2004). Briefly, clones with a *P*-value in a two tailed *t*-test of less than 0·05 were further analysed using Significance Analysis of Miroarrays (SAM) (Tusher *et al*, 2001).

PubGene analysis

The PubGene Gene Expression Analysis tool (http://www.pubgene.org/) was used to search for literature gene-networks dominated by Epo regulated genes (Jenssen *et al*, 2001). We analysed the clones identified through the SAM analysis and used the difference between the mean $\log_2$-based expression ratios of the Epo and medium only treated cells as input for the PubGene tool. The average of the difference was used when several clones represented the same gene. A tab-delimited text-file with two columns, one with official gene symbols and one with the $\log_2$-transformed ratio of the gene expression values was prepared and submitted to the online analysis. To score gene neighbourhoods, we used the parameter settings 'Score-depth' = 3, 'Neighbourhood size' = 25 and 'Upregulation' as score criteria. In this case, 'Upregulation' corresponded to upregulation in the Epo-treated cells. For 'Calculation scheme', we used 'By gene associations'.

Immunoblot analysis

Cell lysates were prepared by lysing the cells in sodium dodecyl sulphate (SDS) lysis buffer and 10–30 μg total protein loaded on 12% SDS-polyacrylamide mini gels (Bio-Rad, Hercules, CA, USA) as previously described (Myklebust *et al*, 2000).

Results

Erythropoietin induces hyperphosphorylation of RB as well as upregulation of cyclin D_3, E and A by activation of PI3K

Recent studies have recognised the importance of PI3K in Epo-induced proliferation of erythroid progenitors (Myklebust *et al*, 2002; Bouscary *et al*, 2003). Here, we explored the role of PI3K on Epo-induced proliferation in the earliest detectable erythroid progenitors in human bone marrow in more detail. $CD34^+$ haematopoietic progenitor cells were cultured in medium alone or stimulated with Epo in the presence or absence of the PI3K inhibitor LY294002 for various times, and then subjected to Western blot analysis to evaluate the phosphorylation status of RB and the protein expression of cyclins and cyclin-dependent kinase inhibitors (CKIs). Stimulation of $CD34^+$ cells with Epo promoted a prominent hyperphosphorylation of RB (Fig 1A). Similar results were observed using a phospho-specific anti-RB antibody. The hyperphosphorylation of RB was completely blocked by LY294002 (Fig 1A). Furthermore, Epo induced a significant upregulation of cyclin D_3, E and A by 3·5-, 3·9- and 4·9-fold respectively, after 48 h of stimulation. The protein expression of $p21^{Cip1}$ and $p27^{Kip1}$ were not significantly affected (Fig 1B and C). In accordance with the complete inhibitory role of LY294002 on cell cycle progression and on hyperphosphorylation of RB, LY294002 also completely blocked the Epo-induced upregulation of cyclins. Taken together, these data suggest that Epo induces progression of the cell cycle through upregulation of cyclin D_3, E and A, and that the regulation of cyclin expression is dependent on activation of PI3K.

Activation of Akt kinase is essential in Epo-induced erythropoiesis

$CD34^+$ cells could be efficiently transfected by electroporation, as 50–75% of $CD34^+$ cells were GFP^+ 1 d after transfection with pGFP plasmid (Fig 2A). Akt is activated by PI3K, and to explore the role of this kinase in Epo-induced early erythropoiesis, expression vectors containing wild type Akt1 (WT-Akt) or Myristylated-Akt1 (Myr-Akt) were transiently trans-

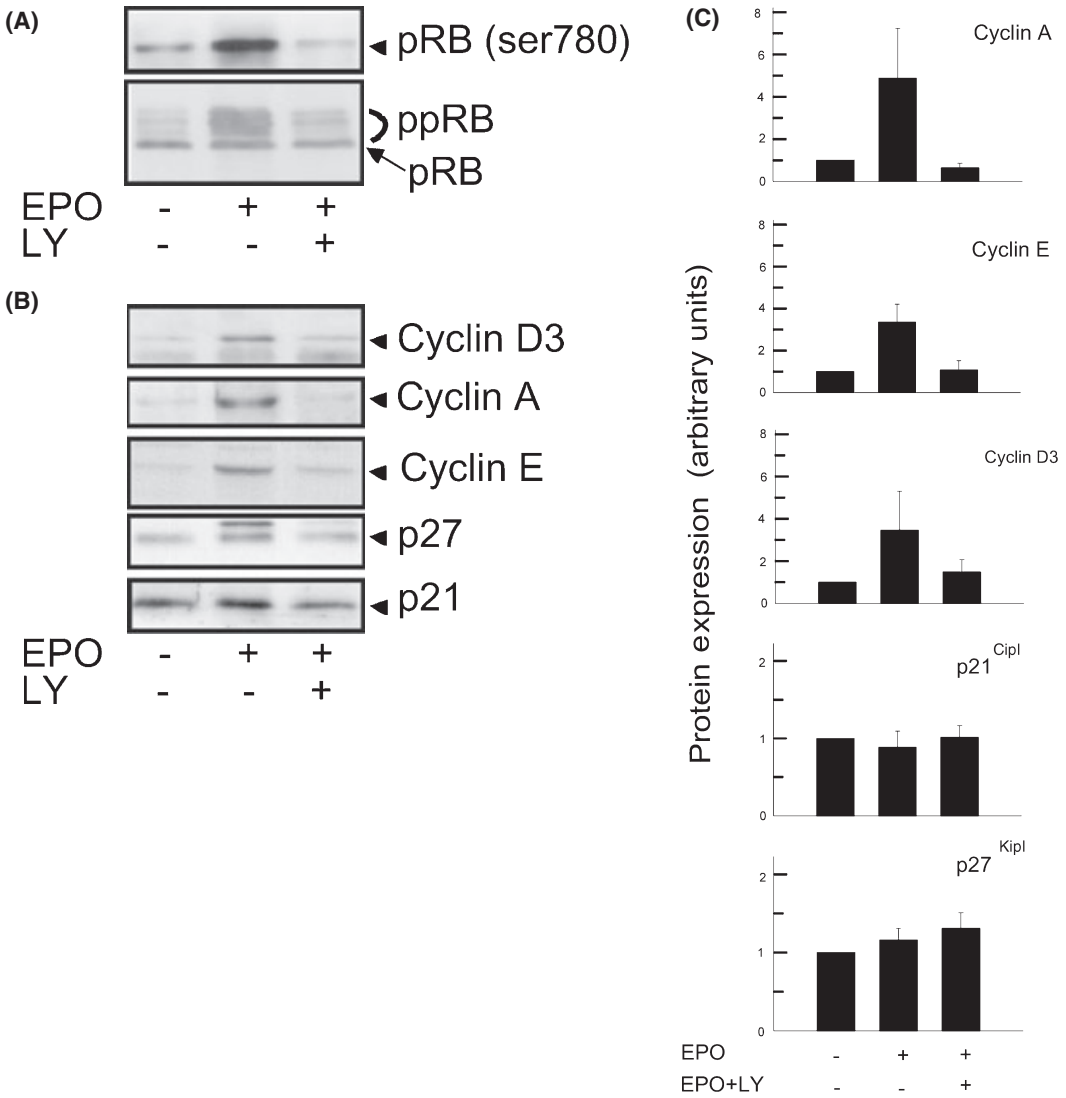

Fig 1. LY294002 inhibits Epo-induced hyperphosphorylation of retinoblastoma protein (RB) and upregulation of cyclin D_3, E and A. $CD34^+$ cells were cultured in medium alone or stimulated with Epo (5 U/ml) in the presence or absence of LY294002 (25 μmol/l) for 2 d before the preparation of total cell lysates, and the expression of phospho-RB, cyclin D_3, E and A, $p21^{Cip1}$ and $p27^{Kip1}$ was determined by Western blot analysis. (A) Phospho-RB (ser 780) or hyperphosphorylated forms of RB, one representative experiment of three separate is shown. (B) One representative experiment, showing that Epo induced upregulation of cyclin D_3, E and A, whereas the levels of $p21^{Cip1}$ and $p27^{Kip1}$ were unchanged. The presence of LY294002 completely blocked the Epo-induced upregulation of the cyclins. (C) Relative protein expression of cyclins and cyclin-dependent kinase inhibitors shown as mean of relative protein expression ± SEM of three or four experiments (cyclin E and cyclin A).

fected into $CD34^+$ haematopoietic progenitor cells. Myr-Akt induced detectable levels of phospho-Akt whereas WT-Akt did not, indicating higher Akt kinase activity in Myr-Akt transfected cells, as expected (Fig 2B). Interestingly, erythropoiesis was more efficiently induced in Myr-Akt transfected cells compared with mock transfected cells in the presence of Epo, as a 4·7-fold increase in the number of GPA^+ cells was detected after 6 d stimulation with Epo (Fig 2C and D; $n = 9$ and $P \leq 0{\cdot}008$). In these experiments, Epo was added 7–20 h after transfection. To investigate whether Myr-Akt could substitute for Epo to induce erythropoiesis, $CD34^+$ cells were transfected with Myr-Akt, control vector or mock and cultured in medium alone or in the presence of various concentrations of Epo. When no Epo was added, Myr-Akt was not able to induce detectable levels of GPA^+ erythroid cells (Fig 2E). However, Myr-Akt markedly reduced the concentration of Epo necessary to induce GPA^+ erythroid cells, compared to cells transfected with control vector or mock (Fig 2E). Taken together, these results suggest that activation of Akt is necessary for effective Epo-induced erythropoiesis.

Identification of Epo responsive genes in erythroid progenitors by cDNA microarray analysis

Erythropoietin promotes differentiation and proliferation of early progenitors, and we wished to characterise the early

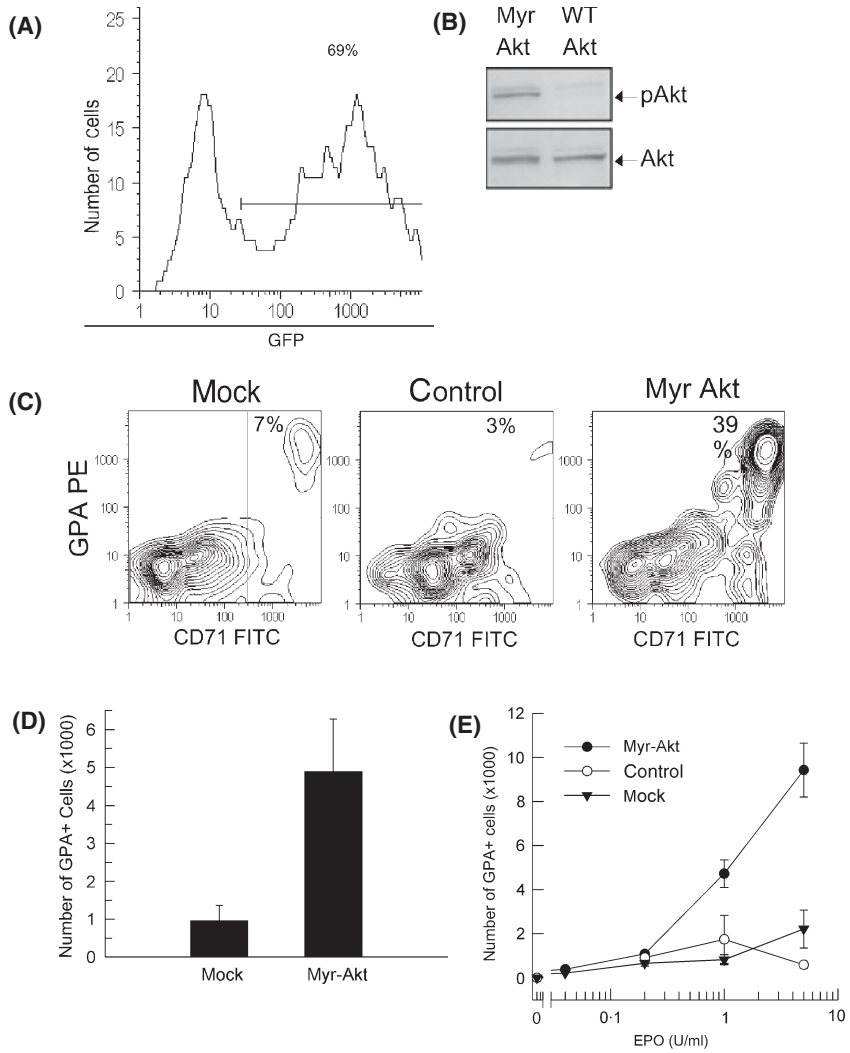

Fig 2. Activation of Akt highly improves Epo-induced erythropoiesis. (A) $CD34^+$ cells can be efficiently transfected by using the nucleofector device from Amaxa as evaluated by the transfection of $CD34^+$ cells with GFP expression plasmid. From 50–75% of the $CD34^+$ cells expressed GFP. (B) $CD34^+$ cells were transfected with pCMV Myr-Akt or pCMV WT-Akt expression plasmid, cultured for 20 h in medium and then stimulated with Epo for 2 d before preparation of total cell lysates. Phospho-Akt1 (Ser 437) and Akt were then detected by Western blot analysis. One experiment of three is shown. (C) $CD34^+$ cells were mock transfected (without vector) or transfected with pCMV Myr-Akt or control vector, cultured for 6–20 h and then stimulated with Epo (5 U/ml) for 6 d before the total number of cells were determined and the expression of glycophorin A (GPA) detected by fluorescent-activated cell sorting (FACS) analysis. Shown is per cent or total number of GPA^+ cells at day 7 after transfection pr 100 000 $CD34^+GPA^-$ cells seeded at day 0; one representative experiment (C) and mean number of GPA^+ cells ± SEM, $n = 9$, $P<0{\cdot}008$ in (D). (E) $CD34^+$ cells were mock transfected or transfected with pCMV Myr-Akt or control vector, cultured for 20 h and then stimulated with various concentrations of Epo (0·04 U/ml −5 U/ml) for 6 d before total number of cells were determined, the expression of GPA detected by FACS analysis and the total number of GPA^+ cells calculated. Shown is the mean number of cells ± SEM of three experiments.

transcriptional changes that precede and lead to these events. $CD34^+CD71^+CD45RA^-GPA^-$ cells, which are highly enriched for early erythroid progenitors (Mayani *et al*, 1993), were separated from three different bone marrow donors. The cells were cultured with or without Epo in combination with LY294002, resulting in a total number of 12 samples that were harvested for mRNA analysis after 4 h. Only one time point was selected due to very limited cell numbers. The RNA samples were subjected to genome wide expression profiling. We first compared the expression profiles of the populations that were cultured in presence of Epo or with medium only. A total of 2253 clones were differentially expressed ($P < 0{\cdot}05$). By applying SAM on this gene list, we identified 394 positively- and 351 negatively-regulated clones, representing 584 unique known genes that exhibited a fold change of more than 1·5. Table I shows the 20 genes with the highest and lowest d score (the most significant genes) from the SAM. Fig 3A shows examples of different expression patterns of some of the differentially expressed genes. The list of all significant genes is available as Supplementary material. Many of the clones represented poorly characterised sequences, but we also found several well-known genes that appeared to be regulated by Epo

Table I. Genes regulated by erythropoietin in $CD34^+$ erythroid progenitors. The genes with the 20 highest and lowest d values (most significant) from the significance analysis of miroarrays are shown.

Symbol	Accession no.	Fold change	Score (d)	Gene description
GNG2	T80856	3·49	2·13	Heterotrimeric G proteins play vital roles in cellular responses to external signals.
PTPRC	AA280209	2·49	2·05	This PTP suppresses JAK kinases, and thus functions as a regulator of cytokine receptor signalling.
SEMA3F	AI970855	4·96	1·98	SEMA3F is a secreted member of the semaphorin III family.The semaphorins are a family of proteins that are involved in signalling.
PIM1	N80481	3·48	1·86	The proto-oncogene PIM1 encodes a protein kinase upregulated in prostate cancer.
CPSF5	AA705275	2·78	1·86	The protein encoded by this gene is one subunit of a cleavage factor required for 3′ RNA cleavage and polyadenylation processing
CD1C	AA002086	2·53	1·74	Expressed on cortical thymocytes, on certain t-cell leukemias, and in various other tissues.
	AI083542	2·87	1·69	
RGS20	AI264190	2·69	1·61	Regulator of G protein signalling (RGS) proteins are regulatory and structural components of G protein-coupled receptor complexes.
ANAPC4	AA436173	2·18	1·53	A large protein complex, termed the anaphase-promoting complex (APC), or the cyclosome, promotes metaphase-anaphase transition.
	AI022958	1·88	1·5	
DHX36	AA430052	2·49	1·49	DEAD box proteins are putative RNA helicases.
DDX5	H27564	2·21	1·47	DEAD box proteins are putative RNA helicases.
FLJ10038	AA935693	2·24	1·46	
CDX2	AA524749	2·51	1·45	This gene is involved in the transcriptional regulation of multiple genes expressed in the intestinal epithelium.
FLJ37034	AA421018	2·9	1·44	
CRSP2	AA282594	2·6	1·43	The protein encoded by this gene is a subunit of the CRSP complex, which is required for efficient activation by SP1
LOC90355	H69538	2·23	1·42	
KIAA0746	AA456569	2·15	1·4	This gene may play a role in notch signalling.
LBR	N54333	1·99	1·36	This gene anchors the lamina and the heterochromatin to the inner nuclear membrane. Can interact with chromodomain proteins.
CTHRC1	AA406425	2·23	1·35	
PRKAG2	AA226716	0·54	−1·31	
TREX1	AI352447	0·48	−1·31	This gene could be involved in DNA replication, repair, and recombination.
PABPN1	N76654	0·39	−1·33	The protein is required for progressive and efficient polymerisation of poly(A) tails on the 3′ ends of eukaryotic genes.
	AA252394	0·48	−1·34	
THG-1	AI049714	0·5	−1·34	Transcriptional repressor (by similarity).
GPR35	AA515711	0·56	−1·38	Orphan receptor
	AA679865	0·47	−1·38	
RAD23A	AA433808	0·48	−1·39	
AAK1	AI688320	0·51	−1·39	The protein encoded by this gene belongs to the Ser/Thr family of protein kinases SNF1 subfamily.
FCGRT	AA642155	0·49	−1·39	This protein binds to the fc region of monomeric immunoglobulins gamma
	AA707488	0·52	−1·4	
KLF8	AI820817	0·45	−1·41	Transcriptional repressor. Binds to caccc-boxes promoter elements.
	AA973568	0·47	−1·42	
MGC13114	AA398922	0·47	−1·42	
	AI081539	0·46	−1·43	
CNOT3	AA449841	0·46	−1·45	Negative regulator of transcription
	R61230	0·44	−1·48	
GCS1	AA291490	0·47	−1·49	Glucosidase I is the first enzyme in the N-linked oligosaccharide processing pathway
	*mitoch. cont.	0·45	−1·51	Mitochondrial contained uncharacterised protein
ITGB5	AA434397	0·42	−1·54	Integrin beta-5 subunit. Integrin alpha-v/beta-5 is a receptor for fibronectin

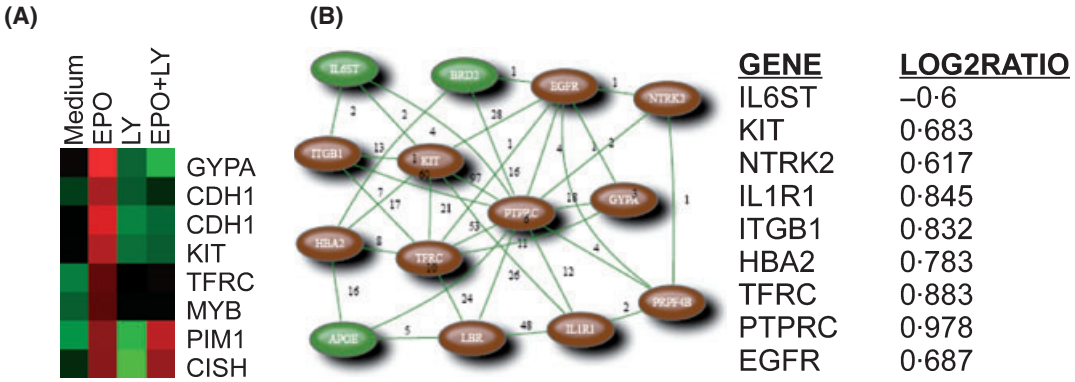

Fig 3. Epo-induced genes in early erythroid progenitors and gene networks. $CD34^+CD71^+CD45RA^-GPA^-$ cells were fluorescent-activated cell sorting from three different donors and cultured in separate experiments in medium alone or with Epo in the presence or absence of LY294002 for 4 h (i.e. four different treatments for each donor, giving 12 samples altogether) before preparation of total RNA. The RNA was subjected to two rounds of linear amplification before hybridisation to Stanford microarray chips. (A) The mRNA expression of *GYPA*, *KIT* and *CDH1* (two independent clones with Accession No W86859 and H97778), *TFRC*, *MYB*, *PIM1* and *CISH* are visualised using TreeView (http://rana.lbl.gov/EisenSoftware.htm). The figure illustrates that *PIM1* and *CISH* expression are induced independently of PI3K. (B) Literature networks were ranked by scores, based on average upregulation of the 745 significantly changed clones induced by Epo compared with medium. Shown is the top-scoring literature neighbourhood of *IL6ST*. Genes in *red* are upregulated by Epo, genes in *green* boxes are downregulated. *Numbers* indicate co-occurrence counts from the literature. Log_2 transformed ratios of Epo *versus* medium treated samples accompany the gene symbols included in the network.

in these cells. Interestingly, several genes associated with transcriptional repression were represented among the down-regulated genes.

To identify the genes that were not regulated in a PI3K-dependent manner, we first performed SAM with the 2253 clones that were changed by Epo (identified by the *t*-test) on the six LY294002 treated samples (three with Epo and three without). This resulted in only three upregulated clones, and suggests that a majority of the Epo responsive genes are regulated in a PI3K-dependent fashion. Indeed, the two genes that were represented by these three clones were *CISH* (cytokine inducible SH2-containing protein) and the serine/threonine kinase *PIM1*, both known to be cytokine inducible in a STAT5-dependent, PI3K-independent manner (Matsumoto *et al*, 1997; Peltola *et al*, 2004).

To examine the biological relationship between genes regulated by Epo, we used the literature-mining tool PubGene, which identifies neighbourhoods that are defined by co-occurrence of the genes in MEDLINE records. It was previously shown that such co-occurrence often reflects biological relationships (Jenssen *et al*, 2001). We evaluated the 745 clones identified through the previously described SAM, and by applying upregulation as the score criterion, genes with neighbours whose expression was induced by erythropoietin obtained the highest score. The literature neighbourhood of interleukin-6 receptor beta chain precursor (*IL6ST*) ranked highest in this analysis (Fig 3B). *IL6ST*, *BRD2* (bromodomain containing 2) and *APOE* (apolipoprotein E) were downregulated genes in this cluster, but the score was dominated by the upregulated genes *GYPA* (glycophorin A), *HBA2* (Haemoglobin alpha 2), *LBR* (laminreseptor B), *EGFR* (epidermal growth factor receptor), *NTRK2* (Neurotrophic tyrosine kinase 2) *ITGB1* (Integrin, beta 1/CD29), *TFRC* (transferrin receptor/CD71, *PTPRC* (Protein tyrosine phosphatase

Table II. Expression changes in $CD34^+$ erythroid progenitors induced by erythropoietin. The cells were cultured in the presence or absence of LY294002 for two days. Shown are the mRNA expression changes determined by microarray analysis for *GYPA*, *CDH1* (two clones) and *KIT* (left panel), and the protein expression changes determined by fluorescent-activated cell sorting analysis of their corresponding gene products Glycophorin A, e-cadherin and CD117 (right panel).

						EPO vs. *Med*		*EPO* + *LY* vs. *LY*	
Symbol	Name	Accession no.	*EPO* vs. *Med* (Fold change)	*EPO* + *LY* vs. *LY* (Fold change)	Protein	Fold change	STDV	Fold change	STDV
GYPA	Glycophorin A (includes MN blood group)	N70285	1·99	0·57	Glycophorin A	3·88	2·89	0·89	0·69
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	W86859	1·77	0·75	E-cadherin	20	3·82	0·71	0·09
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	H97778	1·87	1·06					
KIT	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	N24824	1·61	0·76	CD117	1·33	0·22	0·61	0·21

receptor type C/CD45), *PRPF4B* (pre-mRNA processing factor 4 homolog B) and *IL1R1* (Interleukin-1 receptor type I). Interestingly, *KIT* was also included in this network as a gene upregulated by Epo, and was increased 1·6-fold (Fig 3B and Table II). The results indicate a functional relationship between known and novel target genes regulated by Epo.

KIT is a PI3K dependent, Epo-induced target gene in erythroid progenitors

The mRNA expression of *KIT* was studied in sorted $CD34^+CD71^+CD45RA^-GPA^-$ early erythroid progenitors by real time RT-PCR. Again, in agreement with the microarray experiments, *KIT* was found to be upregulated 1·6-fold by Epo after 4 h, compared with the T0 control, whereas LY294002 decreased *KIT* expression 0·67-fold (Fig 4A). As sorted early erythroid progenitors are limited in numbers, we also expanded $CD34^+$ cells in the presence of Epo, Scf and IL-3 for 6 d (day 6 erythroid precursors). The day 6 erythroid precursors were cytokine-starved for 4 h and then restimulated with Epo for various times before preparation of total RNA, and real time RT-PCR analysis. None of the cyclins tested (*CCND3*, *CCNA1*, *CCNA2*, *CCNE2*, *CCNE2*) showed significant changes at any of the time points tested (0, 1 or 2 h post Epo addition, Fig 4B and data not shown), and were not included in the list of genes that were significantly changed >1·5-fold by Epo from the microarray analysis. Thus, the increased protein expression of cyclin A, E and D3, shown in Fig 1, were most probably not due to transcriptional regulation. In contrast, the expression of *KIT* was increased 1·5-fold after 2 h in Epo-treated cells when compared with T0 control cells. Taken together, these findings further support a role for *KIT* as an Epo-induced target gene in primary erythroid progenitors.

Confirmation of differential expression of selected Epo target genes

The expression of some of the Epo-regulated genes identified by gene expression profiling, were examined at the protein level in our experimental system, including Kit (CD117). We selected genes that were feasible to analyse at the protein level by FACS analysis. The results from the microarray and FACS experiments are summarised in Table II and Fig 5. In these experiments, glycophorin A, served as a positive control. At the RNA level *GYPA* was upregulated 2·0-fold by Epo stimulation, and FACS analysis showed that the upregulation of glycophorin A was restricted to the $CD71^{high}$ cells. LY294002 completely suppressed the Epo-induced upregulation of glycophorin A.

By microarray and real-time RT-PCR analysis, *KIT* expression was upregulated 1·6-fold, (Table II and Fig 4A). We assessed the corresponding gene product CD117 by FACS analysis. Most of the $CD34^+$ cells expressed CD117, but it was evident that, in the Epo-treated populations, the $CD71^{high}$ expressing cells exhibited a higher expression of the protein

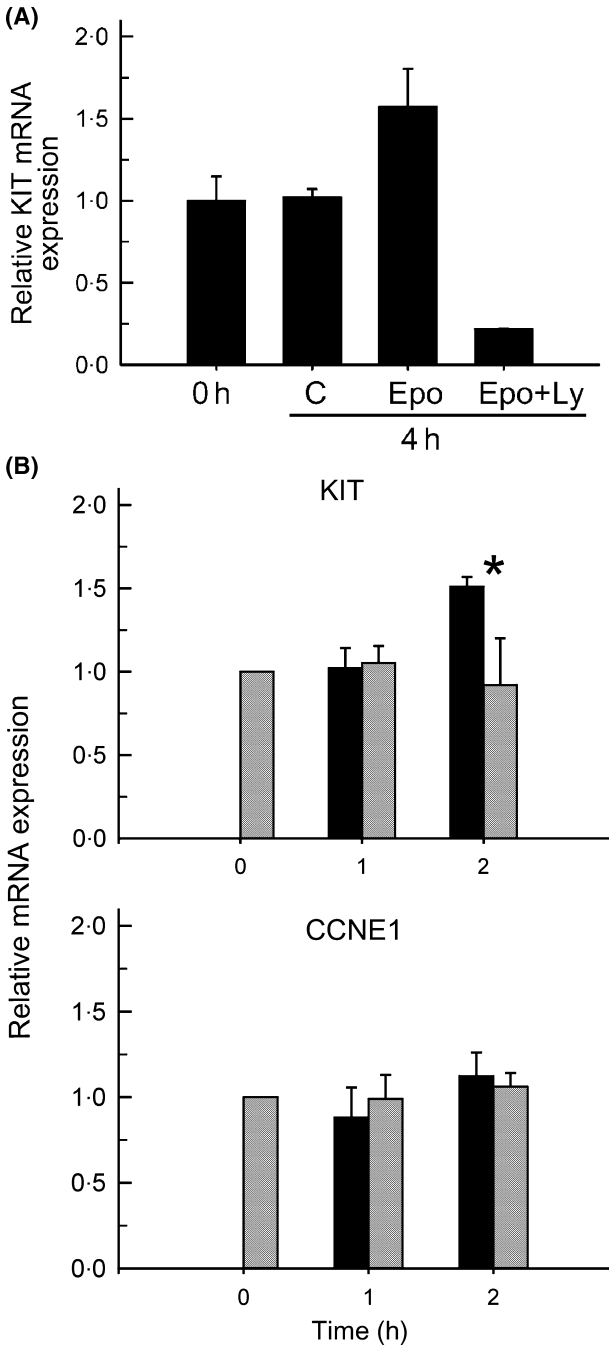

Fig 4. Epo induced upregulation of *KIT* in early erythroid prognitors and in day 6 cytokine expanded erythroid precursors. The mRNA expression in cells stimulated in the presence or absence of Epo with or without LY294002 were analysed by real time RT-PCR. (A) *KIT* mRNA relative expression in $CD34^+CD71^+CD45RA^-GPA^-$ early erythroid progenitors. $\Delta C_T \pm$ SD of duplicates of one representative experiment is shown. (B) Relative mRNA expression of *KIT* and *CCNE1* in *in vitro* expanded d6 erythroid precursors restimulated with Epo for 0, 1 or 2 h. Shown is mean $\Delta C_T \pm$ SEM, $n = 3$, Black bars: Epo stimulated cells, grey bars: cells cultured in medium only. $^*P = 0{\cdot}012$ for Epo vs. T0.

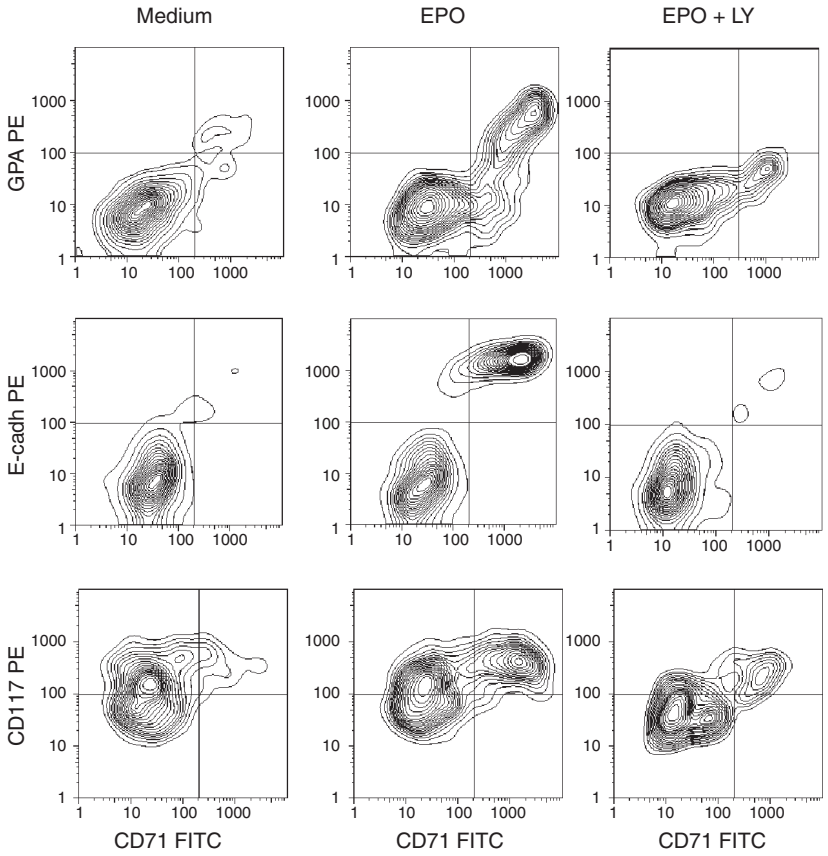

Fig 5. Epo increased the protein expression of CD117 (KIT) and E-Cadherin (CDH1) in early erythroid progenitors. $CD34^+CD71^+CD45RA^-GPA^-$ fluorescent-activated cell sorting (FACS) cells were cultured in medium alone or with Epo in the presence or absence of LY294002 for 2 d before the protein expression of CD71, glycophorin A (GPA), CD117 (KIT) and E-cadherin (CDH1) were analysed by FACS analysis. One representative experiment of three separate ones is shown.

than did the $CD71^{low}$ expressing cells, indicating that Epo induced the expression of this protein as well. LY294002, however, inhibited the expression of CD117 in both the $CD71^{low}$ and $CD71^{high}$ expressing cells.

Two clones represented the gene e-cadherin, *CDH1*. The average fold-change at the RNA level followed by Epo-mediated stimulation was 1·8. At the protein level, the expression of this gene was strongly induced by Epo, and only the $CD71^{high}$ cells were e-$cadherin^{high}$. As for glycophorin A, e-cadherin induction was also almost completely suppressed by LY294002.

Discussion

Erythropoietin provides the essential signals for differentiation and survival to erythroid progenitors. We investigated the role of PI3K-dependent Epo signalling in cell cycle progression of $CD34^+$ progenitors, and showed that Epo alone was able to induce hyperphosphorylation of RB and upregulate cyclin D_3, E and A. Genome wide profiling of $CD34^+CD71^+CD45RA^-GPA^-$ erythroid progenitors showed that Epo initiated a transcriptional programme that led to significant change of expression levels in 584 genes after 4 h. The majority of these genes were apparently regulated in a PI3K-dependent manner, including glycophorin A, e-cadherin and Scf receptor/CD117 (*KIT*).

We have previously shown that Epo-induced differentiation of erythroid cells is dependent on PI3K/Akt signalling pathway (Myklebust *et al*, 2002). Here we showed that enforced expression of Myr-Akt, a constitutively active form of Akt, highly augmented the Epo-induced differentiation. Furthermore, Myr-Akt transfected cells were hypersensitive to Epo. Interestingly, Ghaffari and co-workers recently demonstrated that enforced expression of activated Akt in murine fetal liver progenitor cells overrode the need for Epo to induce erythroid differentiation (Ghaffari *et al*, 2006). They concluded that the enhanced erythroid maturation of activated-Akt-transduced cells was not limited to its anti-apoptotic or proliferative effect as no increase in total cell number was observed. Increased activation of the PI3K/Akt signalling pathway may be particularly relevant to patients with polycythemia vera (PV). PV is a clonal haematopoietic progenitor cell disease characterised by enhanced erythropoiesis and increased number of erythrocytes. PV erythroid progenitors are hypersensitive to several cytokines, including Epo (Casadevall *et al*, 1982). Upon stimulation with Epo or Scf, PV erythroid progenitors showed a marked

increase in phosphorylation of Akt and its downstream targets GSK3α/β, compared with normal erythroid progenitors (Dai *et al*, 2005). Furthermore, the cytokine hypersensitivity and increased phosphorylation of Akt is probably caused by an activating mutation in *JAK2* found in the majority of PV patients (Campbell *et al*, 2005; James *et al*, 2005; Zhao *et al*, 2005).

The molecular events that initiate cell cycle progression and differentiation upon Epo signalling in $CD34^+$ progenitors are not fully understood. Although, several different pathways are activated, the critical role of PI3K has been shown (Myklebust *et al*, 2002; Bouscary *et al*, 2003; Schmidt *et al*, 2004). Here we show that Epo induced upregulation of cyclin D_3, E and A in a PI3K-dependent manner, whereas the protein expression of $p27^{Kip1}$ and $p21^{Cip1}$ largely was unaffected. Bouscary *et al* (2003) also found a PI3K-dependent upregulation of D cyclins upon Epo stimulation and they also observed downregulation of $p27^{Kip1}$, which was mediated by degradation by the E3 ligase SCF^{SKP2} (Bouscary *et al*, 2003). However, in our study, the erythroid progenitors represent an earlier developmental stage (BFU-E). It is possible that Epo-induced effects on cell cycle regulators differ in early *versus* later stages of erythroid development, as late erythroid precursors in response to Epo exit cell cycle during differentiation. Moreover, the regulation of cyclins is most likely not regulated at the transcription level, as none of the cyclins or CKIs were among the significantly changed genes by gene expression profiling. Furthermore, none of the cyclins that we tested in real time RT-PCR experiments showed significant changes in response to Epo stimulation.

Genome wide profiling has been used in several studies of erythroid differentiation (Kolbus *et al*, 2003; Edvardsson *et al*, 2004; Fujishima *et al*, 2004; Welch *et al*, 2004). The findings of these studies are heterogeneous, reflecting the variation in the experimental systems used. By choosing freshly isolated early erythroid progenitors ($CD34^+CD71^+CD45RA^-GPA^-$), and by stimulating them for 4 h with Epo with or without LY294002, our study design aimed to discover early targets of Epo signalling in the human system and to clarify whether their regulation was PI3K-dependent. By choosing a threshold of 1·5-fold regulation and applying a practical two-step filtering procedure, we defined a list of 584 unique known genes that exhibited regulation upon Epo stimulation. This approach has been shown to be practical in defining a list of genes that contains physiologically relevant targets (Shen *et al*, 2004). A number of known Epo target genes were included in our list of upregulated genes. *GYPA* and *TFRC* are associated with erythroid differentiation and were upregulated in a PI3K-dependent fashion. This was shown in the microarray experiments and verified at the protein level by FACS assays. One of the most interesting findings in our study was the upregulation of *KIT* (CD117) expression in response to Epo treatment. Scf acts synergistically with Epo to enhance the generation of glycophorin A positive cells from $CD34^+$ progenitors (Wu *et al*, 1997; Myklebust *et al*, 2002; Munugalavadla *et al*, 2005). Several mechanisms of cooperative action between Epo and Scf have been suggested, and it is well established that Scf can promote an increase in EpoR expression (Lodish *et al*, 1995; Kapur & Zhang, 2001). Upregulation of *KIT* (CD117) in response to Epo is a novel mechanism for such cooperative action. Our data also clearly indicated that the Epo-induced *KIT* upregulation is dependent on activation of PI3K. The PI3K inhibitor LY294002 abrogated the increased *KIT* expression, and in the cultures that were treated with LY294002 only, the levels of *KIT* mRNA and protein were even lower compared with the medium only treated cultures. This suggests that, for $CD34^+$ progenitors, a certain basal level of PI3K activity is required to maintain expression of *KIT*. *KIT* is downregulated (repressed by GATA-1) in the later stages of erythroid differentiation, but the levels in $CD34^+$ progenitors are high (Welch *et al*, 2004; Munugalavadla *et al*, 2005). In our experimental system, Scf could not bypass the requirement for Epo and alone promote erythroid differentiation of $CD34^+$ progenitors (data not shown). Previous data on c-kit dependency of early erythroid progenitors are controversial. Human BFU-Es have been shown to be Scf-dependent when cultured in serum free media (Dai *et al*, 1991), but it has also been shown that c-$Kit^{w/w}$ mice, that are functionally c-kit deficient, can be rescued by transgenic, enforced expression of Epo (Waskow *et al*, 2004).

CDH1 was, in addition to *KIT*, among the intriguing candidate genes from the microarray screening that we decided to analyse at the protein level. This gene encodes for a cell-cell adhesion glycoprotein that is expressed at the erythroblast and the normoblast stages, and it has previously been shown that e-cadherin has a functional role in erythropoiesis (Armeanu *et al*, 2000). We have now shown that e-cadherin is rapidly induced by Epo in early $CD34^+$ progenitors. The protein expression was restricted to the $CD71^{high}$ expressing cells. By use of the PubGene tool we identified literature neighbourhoods that consisted of Epo-regulated genes defined by our SAM. The neighbourhood of *IL6ST*, which obtained the highest score, included both known Epo targets, such as transferrin receptor, haemoglobin alpha 2 and glycophorin A, and also *KIT* that was validated in our study. Upregulation of *IL1R1* and *EGFR*, could provide mechanisms through which survival and proliferation are promoted, in response to interleukin 1 or epidermal growth factor stimulation respectively. Interestingly, *PTPRC* (CD45), was also among the upregulated genes in this cluster. Several studies have shown that CD45 plays an important role in the negative regulation of erythroid differentiation (Harashima *et al*, 2002). Thus, the PubGene tool can be helpful in organising the differentially expressed genes into functionally related gene clusters, and aids the generation of novel hypotheses.

Taken together, our data show that ligand binding to the Epo receptor in early $CD34^+$ progenitors initiates a plethora of signalling cascades. The physiological effects of Epo on survival, proliferation and differentiation at this stage are completely

dependent on functional PI3K, and the observed expression changes of important target genes reflect this dependency.

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References

Ahmed, N.N., Grimes, H.L., Bellacosa, A., Chan, T.O. & Tsichlis, P.N. (1997) Transduction of interleukin-2 antiapoptotic and proliferative signals via Akt protein kinase. *Proceedings of the National Academy of Sciences of the United States of America*, **94,** 3627–3632.

Arcasoy, M.O. & Jiang, X. (2005) Co-operative signalling mechanisms required for erythroid precursor expansion in response to erythropoietin and stem cell factor. *British Journal of Haematology*, **130,** 121–129.

Armeanu, S., MUller, C.A. & Klein, G. (2000) Involvement of e-cadherin in the development of erythroid cells; subject heading. *Hematology*, **5,** 307–316.

Baugh, L.R., Hill, A.A., Brown, E.L. & Hunter, C.P. (2001) Quantitative analysis of mRNA amplification by in vitro transcription. *Nucleic Acids Research*, **29,** E29.

Bouscary, D., Pene, F., Claessens, Y.E., Muller, O., Chretien, S., Fontenay-Roupie, M., Gisselbrecht, S., Mayeux, P. & Lacombe, C. (2003) Critical role for PI3-kinase in the control of erythropoietin-induced erythroid progenitor proliferation. *Blood*, **101,** 3436–3443.

Campbell, P.J., Scott, L.M., Buck, G., Wheatley, K., East, C.L., Marsden, J.T., Duffy, A., Boyd, E.M., Bench, A.J., Scott, M.A., Vassiliou, G.S., Milligan, D.W., Smith, S.R., Erber, W.N., Bareford, D., Wilkins, B.S., Reilly, J.T., Harrison, C.N. & Green, A.R. (2005) Definition of subtypes of essential thrombocythaemia and relation to polycythaemia vera based on JAK2 V617F mutation status: a prospective study. *Lancet*, **366,** 1945–1953.

Casadevall, N., Vainchenker, W., Lacombe, C., Vinci, G., Chapman, J., Breton-Gorius, J. & Varet, B. (1982) Erythroid progenitors in polycythemia vera: demonstration of their hypersensitivity to erythropoietin using serum free cultures. *Blood*, **59,** 447–451.

Dai, C.H., Krantz, S.B. & Zsebo, K.M. (1991) Human burst-forming units-erythroid need direct interaction with stem cell factor for further development. *Blood*, **78,** 2493–2497.

Dai, C., Chung, I.J. & Krantz, S.B. (2005) Increased erythropoiesis in polycythemia vera is associated with increased erythroid progenitor proliferation and increased phosphorylation of Akt/PKB. *Experimental Hematology*, **33,** 152–158.

Edvardsson, L., Dykes, J., Olsson, M.L. & Olofsson, T. (2004) Clonogenicity, gene expression and phenotype during neutrophil versus erythroid differentiation of cytokine-stimulated CD34+ human marrow cells in vitro. *British Journal of Haematology*, **127,** 451–463.

Fujishima, N., Hirokawa, M., Aiba, N., Ichikawa, Y., Fujishima, M., Komatsuda, A., Suzuki, Y., Kawabata, Y., Miura, I. & Sawada, K. (2004) Gene expression profiling of human erythroid progenitors by micro-serial analysis of gene expression. *International Journal of Hematology*, **80,** 239–245.

Ghaffari, S., Kitidis, C., Zhao, W., Marinkovic, D., Fleming, M.D., Luo, B., Marszalek, J. & Lodish, H.F. (2006) AKT induces erythroid-cell maturation of JAK2-deficient fetal liver progenitor cells and is required for Epo regulation of erythroid-cell differentiation. *Blood*, **107,** 1888–1891.

Harashima, A., Suzuki, M., Okochi, A., Yamamoto, M., Matsuo, Y., Motoda, R., Yoshioka, T. & Orita, K. (2002) CD45 tyrosine phosphatase inhibits erythroid differentiation of umbilical cord blood CD34+ cells associated with selective inactivation of Lyn. *Blood*, **100,** 4440–4445.

Haseyama, Y., Sawada, K.i., Oda, A., Koizumi, K., Takano, H., Tarumi, T., Nishio, M., Handa, M., Ikeda, Y. & Koike, T. (1999) Phosphatidylinositol 3-kinase is involved in the protection of primary cultured human erythroid precursor cells from apoptosis. *Blood*, **94,** 1568–1577.

Jacobs-Helber, S.M. & Sawyer, S.T. (2004) Jun N-terminal kinase promotes proliferation of immature erythroid cells and erythropoietin-dependent cell lines. *Blood*, **104,** 696–703.

James, C., Ugo, V., Le Couedic, J.P., Staerk, J., Delhommeau, F., Lacout, C., Garcon, L., Raslova, H., Berger, R., Bennaceur-Griscelli, A., Villeval, J.L., Constantinescu, S.N., Casadevall, N. & Vainchenker, W. (2005) A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature*, **434,** 1144–1148.

Jenssen, T.K., Laegreid, A., Komorowski, J. & Hovig, E. (2001) A literature network of human genes for high-throughput analysis of gene expression. *Nature Genetics*, **28,** 21–28.

Kadri, Z., Maouche-Chretien, L., Rooke, H.M., Orkin, S.H., Romeo, P.H., Mayeux, P., Leboulch, P. & Chretien, S. (2005) Phosphatidylinositol 3-kinase/Akt induced by erythropoietin renders the erythroid differentiation factor GATA-1 competent for TIMP-1 gene transactivation. *Molecular and Cellular Biology*, **25,** 7412–7422.

Kapur, R. & Zhang, L. (2001) A novel mechanism of cooperation between c-Kit and erythropoietin receptor. Stem cell factor induces the expression of Stat5 and erythropoietin receptor, resulting in efficient proliferation and survival by erythropoietin. *Journal of Biological Chemistry*, **276,** 1099–1106.

Kolbus, A., Blazquez-Domingo, M., Carotta, S., Bakker, W., Luedemann, S., von Lindern, M., Steinlein, P. & Beug, H. (2003) Cooperative signaling between cytokine receptors and the glucocorticoid receptor in the expansion of erythroid progenitors: molecular analysis by expression profiling. *Blood*, **102,** 3136–3146.

Krantz, S.B. (1991) Erythropoietin. *Blood*, **77,** 419–434.

Lodish, H.F., Hilton, D.J., Klingmuller, U., Watowich, S.S. & Wu, H. (1995) The erythropoietin receptor: biogenesis, dimerization, and intracellular signal transduction. *Cold Spring Harbor Symposia on Quantitative Biology*, **60,** 93–104.

Matsumoto, A., Masuhara, M., Mitsui, K., Yokouchi, M., Ohtsubo, M., Misawa, H., Miyajima, A. & Yoshimura, A. (1997) CIS, a cytokine inducible SH2 protein, is a target of the JAK-STAT5 pathway and modulates STAT5 activation. *Blood*, **89,** 3148–3154.

Mayani, H., Dragowska, W. & Lansdorp, P.M. (1993) Cytokine-induced selective expansion and maturation of erythroid versus myeloid progenitors from purified cord blood precursor cells. *Blood*, **81,** 3252–3258.

McNiece, I.K., Langley, K.E. & Zsebo, K.M. (1991) Recombinant human stem cell factor synergises with GM-CSF, G-CSF, IL-3 and epo to stimulate human progenitor cells of the myeloid and erythroid lineages. *Experimental Hematology*, **19,** 226–231.

Munugalavadla, V., Dore, L.C., Tan, B.L., Hong, L., Vishnu, M., Weiss, M.J. & Kapur, R. (2005) Repression of c-Kit and its downstream substrates by GATA-1 inhibits cell proliferation during erythroid maturation. *Molecular and Cellular Biology*, **25,** 6747–6759.

Myklebust, J.H., Smeland, E.B., Josefsen, D. & Sioud, M. (2000) Protein kinase C-alpha isoform is involved in erythropoietin-induced erythroid differentiation of CD34+ progenitor cells from human bone marrow. *Blood*, **95,** 510–518.

Myklebust, J.H., Blomhoff, H.K., Rusten, L.S., Stokke, T. & Smeland, E.B. (2002) Activation of phosphatidylinositol 3-kinase is important for erythropoietin-induced erythropoiesis from CD34+ hematopoietic progenitor cells. *Experimental Hematology*, **30,** 990–1000.

Nygaard, V., Loland, A., Holden, M., Langaas, M., Rue, H., Liu, F., Myklebost, O., Fodstad, O., Hovig, E. & Smith-Sorensen, B. (2003) Effects of mRNA amplification on gene expression ratios in cDNA experiments estimated by analysis of variance. *BMC Genomics*, **4,** 11.

Peltola, K.J., Paukku, K., Aho, T.L.T., Ruuska, M., Silvennoinen, O. & Koskinen, P.J. (2004) Pim-1 kinase inhibits STAT5-dependent transcription via its interactions with SOCS1 and SOCS3. *Blood*, **103,** 3744–3750.

Remy, I., Wilson, I.A. & Michnick, S.W. (1999) Erythropoietin receptor activation by a ligand-induced conformation change. *Science*, **283,** 990–993.

Richmond, T.D., Chohan, M. & Barber, D.L. (2005) Turning cells red: signal transduction mediated by erythropoietin. *Trends in Cell Biology*, **15,** 146–155.

Schmidt, E., Fichelson, S. & Feller, S. (2004) PI3 kinase is important for Ras, MEK and Erk activation of Epo-stimulated human erythroid progenitors. *BMC Biology*, **2,** 7.

Shen, Y., Iqbal, J., Xiao, L., Lynch, R., Rosenwald, A., Staudt, L., Sherman, S., Dybkaer, K., Zhou, G., Eudy, J., Delabie, J., McKeithan, T. & Chan, W. (2004) Distinct gene expression profiles in different B-cell compartments in human peripheral lymphoid organs. *BMC Immunology*, **5,** 20.

Smeland, E.B., Funderud, S., Kvalheim, G., Gaudernack, G., Rasmussen, A.M., Rusten, L., Wang, M.Y., Tindle, R.W., Blomhoff, H.K. & Egeland, T. (1992) Isolation and characterization of human hematopoietic progenitor cells: an effective method for positive selection of CD34+ cells. *Leukemia*, **6,** 845–852.

Tusher, V.G., Tibshirani, R. & Chu, G. (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proceedings of the National Academy of Sciences of the United States of America*, **98,** 5116–5121.

Uddin, S., Kottegoda, S., Stigger, D., Platanias, L.C. & Wickrema, A. (2000) Activation of the Akt/FKHRL1 pathway mediates the antiapoptotic effects of erythropoietin in primary human erythroid progenitors. *Biochemical and Biophysical Research Communications*, **275,** 16–19.

Waskow, C., Terszowski, G., Costa, C., Gassmann, M. & Rodewald, H.R. (2004) Rescue of lethal c-KitW/W mice by erythropoietin. *Blood*, **104,** 1688–1695.

Welch, J.J., Watts, J.A., Vakoc, C.R., Yao, Y., Wang, H., Hardison, R.C., Blobel, G.A., Chodosh, L.A. & Weiss, M.J. (2004) Global regulation of erythroid gene expression by transcription factor GATA-1. *Blood*, **104,** 3136–3147.

Wojchowski, D.M., Gregory, R.C., Miller, C.P., Pandit, A.K. & Pircher, T.J. (1999) Signal transduction in the erythropoietin receptor system. *Experimental Cell Research*, **253,** 143–156.

Wu, H., Klingmuller, U., Acurio, A., Hsiao, J.G. & Lodish, H.F. (1997) Functional interaction of erythropoietin and stem cell factor receptors is essential for erythroid colony formation. *Proceedings of the National Academy of Sciences of the United States of America*, **94,** 1806–1810.

Zhao, R., Xing, S., Li, Z., Fu, X., Li, Q., Krantz, S.B. & Zhao, Z.J. (2005) Identification of an acquired JAK2 mutation in polycythemia vera. *Journal of Biological Chemistry*, **280,** 22788–22792.

Supplementary material

The following supplementary material is available for this article online:

Table SI. Filtered expression values from 12 microarray experiments. The filtered expression values of the microarray experiments in this study are listed. Column 1 contains the clone identifier, which is a unique number. Column 2 contains the official gene symbol. Column 3 contains the gene name. Column 4 contains the Unigene cluster ID. Column 5 contains the GenBank accession number. Columns 6 to 17 contain expression values of the 12 experiments.

Table SII. EPO regulated genes. This table contains the expression data of the clones identified by the t-test statistics.

Table SIII. Data from the SAM analysis This table contains the data from the SAM analysis.

Table SIV. Pubgene input. This table contains the input data in the pubgene analysis. Column 1 contains the gene symbol whereas the Column 2 contains the average difference of the log2 transformed expression values between the EPO-stimulated *versus* the medium-treated cells.

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Inhibitory Effects and Target Genes of BMP-6 in human T-cells

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Abbreviations:

BMP bone morphogenetic protein

Id Inhibitor of DNA binding

HLH helix-loop-helix

Abstract

Bone morphogenetic proteins (BMPs) are multifunctional cytokines that belong to the TGF-β superfamily. BMPs have been shown to regulate hematopoietic stem cells, B-lymphopoiesis and early thymocyte differentiation. In the present study we explored the role of BMP-6 in human T-cells. Human T-cells were found to express BMP type I and type II receptors, and BMP-6 rapidly induced phosphorylation of Smad1/5/8. BMP-6 also induced phosphorylation of p38 in peripheral blood CD4+ T-cells and of p38 and ERK1/2 in Jurkat TAg cells. The transcriptional response to BMP-6 stimulation in both cell systems included potent upregulation of ID1, ID2 and ID3 but Id1 and Id3 were only induced at the protein level in Jurkat TAg cells. Genome wide expression profiling of BMP-6 stimulated Jurkat TAg cells identified other transcriptional targets known to be involved in BMP-6 signalling, including Noggin (NOG) and Smad6. Several genes involved in transcriptional regulation were also identified, including NFKBIA, HEY1, DLX2, KLF10 and EGR1. Stimulation with BMP-6 exerted an antiproliferative effect in Jurkat TAg that was counteracted by Id1 siRNA. In CD4+ T-cells, BMP-6 significantly inhibited the differentiation into IL-4 producing effector cells from naive precursors albeit less potently than TGF-β. Taken together, BMP-6 is a novel regulator of human T-cell responses.

Introduction

Bone morphogenetic proteins (BMPs) are cytokines that belong to the transforming growth factor β (TGF-β) superfamily. BMPs were originally identified by their ability to induce ectopic cartilage and bone formation, but it has been shown that these cytokines also control cellular proliferation, migration and apoptosis in various cellular systems [1;2]. Members of the TGF-β superfamily signal through ligation and heterodimerization of type I and type II serine-threonine kinase receptors [3]. This leads to phosphorylation and activation of the type I receptor which subsequently phosphorylates receptor Smad proteins (R-Smads). The R-Smads then form complexes with the co-Smad (Smad4), which translocate into the nucleus where they exert gene regulation. The gene regulation and resulting physiological response are context dependent. This is in part due to the diversity allowed for by the combinatorial interactions of type I and type II receptors and Smads. Smads also cooperate with many different sequence- and tissue specific transcription factors [3]. The R-Smad activation is regulated by numerous receptor binding proteins as well as the inhibitory Smads, Smad6 and Smad7. Smad1/5/8 are mainly involved in BMP signalling and Smad2/3 are restricted to TGF-β/Activin [4]. In addition to Smad dependent signalling and transcriptional regulation, the activated receptor complex may activate non-Smad signalling pathways, such as MAPK and TAK1/MEKK1 which leads to phosphorylation of p38 and ERK [3]. Several studies have pointed toward members of the Id protein family, a class of helix-loop-helix (HLH) proteins, as important targets for BMP signalling [5-8]. HLH proteins act as transcriptional regulators and are essential factors for lymphoid development and function [9-11].

TGF-β is the member of the superfamily that has been most thoroughly studied in hematopoietic cells of normal and malignant origin, and it is now recognized as a major endogenous negative regulator [4;12]. TGF-β has an inhibitory effect on B- and T-cells through several mechanisms [12;13]. In T-cells, TGF-β exerts its inhibitory effects partly by reducing the ability of CD4+ T-cells to differentiate into Th1 or Th2 effector cells [12]. Furthermore, upregulation of ID2 and downregulation of ID3 at the mRNA level have been observed during Th2 differentiation, and interestingly, this was counteracted by TGF-β stimulation [14;15]. Mature Th1 cells produce IFN-γ and are crucial for cellular immune responses, whereas Th2 cells produce other cytokines, including IL-4, that mobilize humoral immunity. In contrast to the recognized role of TGF-β, the role of BMPs in regulating cells of the immune system is not

thoroughly investigated. However, we recently showed that BMP-6 inhibits growth of human B progenitors and mature B-cells [16;17]. In multiple myeloma cells BMP-2, -4, -6 and -7 have been shown to have antiproliferative and proapoptotic effects [18;19]. Interestingly, BMP-2 and BMP-4 were shown to inhibit early thymocyte differentiation [20], whereas transgenic expression of the BMP antagonist Noggin implicated that BMP signalling was required for normal thymus development [21].

Given the negative effects of BMPs in B lineage cells and early T-cell development, we wanted to further explore the role of BMP-6 in human T-cell biology. We have characterized BMP receptor expression and examined the BMP-6 signalling transduction pathway in the Jurkat TAg cell line and in mature CD4+ T-cells. Furthermore, downstream transcriptional targets of BMP-6 were identified in both cell types, and functional effects were characterized.

Materials and Methods

Reagents and Antibodies

Carrier free recombinant human (rhu) BMP-6 (500 ng/ml, if not specified otherwise), rhu BMPR-II/Fc Chimera (5 μg/ml), rhu Act-RIA/Fc Chimera (5 μg/ml), recombinant mouse (rm) Noggin (5 μg/ml), anti-Act-RIA/Alk2 (BAF637), anti-BMP-RIA/Alk3 (BAF829), anti-BMP-RIB/Alk-6 (BAF505), anti-BMPR-II (BAF811), anti-Act-RIIA (BAF340) and anti-Act-RIIb (BAF339) ab were purchased from R & D Systems (Abingdon, UK). Goat serum (negative control) was from Sigma and Streptavidin PE was from Dakocytomation (Glostrup, Denmark). Anti-CD45RA PE, anti-CD45RO FITC, anti-CD4 FITC, and isotype matched irrelevant ab (FITC and PE) that served as negative controls were from Dakocytomation, and anti-IL-4 PE (554516) and IFN-γ FITC (340449) were from Becton Dickinson (www.bdeurope.com).

Anti-pSmad1/5/8 (9511), anti-phospho-p38 and anti-p38 were from Cell Signalling, whereas anti-Smad1 (06-653), anti-phospho-p42/p44 and anti-p42/p44 were from Upstate, and anti-Id1 (sc-488), anti-Id2 (sc-489) and anti-Id3 (sc-490) were from SantaCruz Biotechnology (Heidelberg, Germany). As secondary antibodies for immunoblot analysis served anti-mouse, anti-goat or anti-rabbit IgG-horseradish peroxidase (HRP) from Dakocytomation AS.

Cell isolation, culture and transfections

Buffy coats from peripheral blood were provided by the Blood Bank at Ullevål Hospital with informed consent from the blood donors and formal approval by the regional ethics committee. Highly purified resting human CD4+ T-cells were isolated from peripheral blood by the use of CD4 Dynabeads (Invitrogen, Oslo, Norway). The pre-T ALL cell line Jurkat TAg was maintained in RPMI (supplemented with 10% FCS, 100 units/ml penicillin G, and 100 units/ml streptomycin sulphate). Prior to experiments, Jurkat TAg cells were cultured over night in X-VIVO 15™ . In all experiments, CD4+ T-cells, CD4+CD45RA+CD45RO- naive T-cells and Jurkat-TAg cells were cultured in X-VIVO 15™ serum-free medium (BioWhittaker, Verviers, Belgium) at 37 °C and 5% CO_2 in air.

In the transfection experiments Jurkat TAg cells were electroporated at 200 V for 70 ms using an ECM 830 Electro Square Porator (BTX Inc., San Diego, CA, USA). The final concentrations of the nucleic acids: 500 nM of ID1 siRNA (5'-CUCGGAAUCCGAAGUUGGAtt-3') or scrambled siRNA (Ambion Inc, Austin, TX, USA), 5 μg/ml of the BRE-Luc vector (a gift from Dr. Peter ten Dijke) and 0.25 μg/ml of pRL-CMV

(renilla luciferase) (Promega, Madison, WI, USA). The transfected cells were transferred to prewarmed X-VIVO 15™ medium and incubated at 37 °C for 2-4 hours before BMP-6 was added in various concentrations.

Cell proliferation and CFSE cell division tracking

For estimation of DNA synthesis, JurkatTAg cells were cultured in triplicates in 96 well round-bottom plates, 20 000 cells/well in 200 µl, and incubated for 3 days. ^{3}H-Thymidine (3.7×10^{4} Bq) were added the 4 last hours of incubation and the ^{3}H-Thymidine incorporation determined as previously described [16]. Cell division tracking was performed by labelling Jurkat TAg cells with 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA) as described earlier [22].

Determination of cell death

Cell death was measured by vital dye exclusion test as described [16].

Induction of Th1 and Th2 differentiation in vitro

CD4+CD45RA+CD45RO- naive T-cells were cultured at 140 000/200 µl in X-VIVO 15™ serum-free medium and preincubated in medium alone or with BMP-6 (500 ng/ml) or TGF-β (10 ng/ml) for 1-20 hours prior to the primary activation using CD3CD28 T-cells expansion Beads (Invitrogen) at the bead:cell ratio of 0.3. To direct Th1 differentiation, rhu IL-12 (20 ng/ml) and anti-IL-4 ab (10 µl/ml) were added, and to direct Th2 differentiation, rhu IL-4 (40 ng/ml) and anti IFN-γ (2.5 µg/ml) were added. After 3 days, the cells were resuspended well and moved to 24 well plates containing 1.8 ml medium with IL-2 (10 ng/ml) and the cells were expanded for another 5 days.

FACS sorting and analysis

All incubations with antibodies were performed in the dark at 4 °C for 30 minutes. To perform intracellular cytokine staining, the cultured cells were activated with PHA (1 µg/ml) and ionomycine (0.5 µg/ml; Calbiochem, Darmstadt, Germany) for 5 hours. Brefeldin A (BD) was added for the last 3 hours of activation. The cells were then permeabilized by 1% paraformaldehyde in PBS for 10 min at RT, followed by permeabilization in 100% methanol for at least 20 min at –20° C. After washing twice with PBS, the cells were stained with anti-IL-4 PE and anti-IFN-γ FITC or corresponding isotype matched ab. The stained cells were analysed in a FACSCalibur flowcytometer (BD). CD4+CD45RA+CD45RO- naive T-cells were obtained by

staining of CD4+ cells with anti-CD45RA PE and anti-CD45RO FITC for 30 min and the CD45RA+CD45RO- cells were sorted on a FACSDiva Cell sorter (BD).

Western-blot analysis

Jurkat TAg or CD4+ T-cells were lysed in SDS-lysis buffer and run on 10-12 % SDS/PAGE gels as previously described [16].

Luciferase reporter assay

Jurkat TAg cells were transfected with the BRE-Luc plasmid (specific BMP/Smad transcriptional reporter [23]), in combination with pRL-CMV (renilla luciferase) that was used as an internal transfection control. After transfection, the cells were treated with various concentrations of BMP-6 over night. The respective activities of firefly and renilla luciferase were determined sequentially using the Firelite dual luminescence reporter gene assay (Perkin-Elmer, Rodgau-Jügesheim, Germany) and a Top Count liquid-scintillation counter (Packard, Meriden, CT, USA).

RNA isolation

For the RNA expression analyses, total RNA was isolated using Absolutely RNA™ RT-PCR Miniprep Kit (Stratagene Europe, Amsterdam, Netherlands) according to the manufacturer's instructions. Quantification of RNA was achieved by using spectrophotometric OD_{260} measurements (Nanodrop, Wilmington, DE, USA).

Real-time RT-PCR

ID1, ID2 and ID3 mRNA expression was analyzed by real-time quantitative RT-PCR using Taqman technology according to the manufacturer's procedure (Applied Biosystems, Foster City, CA, USA) and as previously described [16]. The expression was normalized to the expression of PGK1, and calibrated to the expression levels in Ramos cells.

MicroArray experiments

Jurkat TAg cells were cultured with or without 500 ng/ml BMP-6 for 2 or 16 hours. The experiments were performed in triplicates resulting in 15 samples, including the T_0 samples, which were subjected to genome wide profiling. Three µg of total RNA was used to generate cRNA, subsequently hybridized to HG-U133A Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA) according to the Affymetrix GeneChip Expression Analysis Technical Manual (http://www.affymetrix.com), and scanned using the Affymetrix GeneChip Scanner 3000. Data were acquired using the Affymetrix software, GeneChip Operating Software1.3. The data were RMA normalized by the use of the free Software R version 2.1.1 (http://www.r-project.org/) and

BioConductor 1.7 Packages, Affy version 1.8.1.(http://www.bioconductor.org/download/bioc1.7/). The filtered data are available as supplementary material.

Statistical analysis

Differentially expressed genes were identified by the rank products (RP) test [24]. Briefly rank products are calculated for all probe sets. By a permutation-based estimation procedure it is determined how likely it is to observe a given RP value or better in a random experiment. The algorithm also provides an estimate of the percentage of false positive genes at a given cut off level, denoted false discovery rate (FDR). In our experiments FDR less than 0.1 were considered significant. Elsewhere, the statistical significance of differences between groups was determined using the paired two-tailed Wilcoxon nonparametric test, by applying SPSS12.0.1 software (SPSS Inc., Chicago, IL, USA). *p* values less than 0.05 were considered significant.

Results

The pre-T ALL cell line Jurkat TAg and human CD4+ T-cells express receptors for BMP-6

We used FACS analysis to determine the expression of the BMP type I receptors Alk-2, Alk-3 and Alk-6 and the type II receptors ActRIIa, ActRIIb and BMPRII on the Jurkat TAg cell line as well as on normal CD4+ T-cells from peripheral blood. As shown in Figure 1, Jurkat TAg cells expressed Alk-2, Alk-3 and BMPRII. CD4+ T-cells exhibited a more heterogeneous pattern as shown in Table 1. However, 58% of T-cells expressed Alk-2, and 11% expressed BMPRII. Since BMP-6 mainly utilizes the Alk-2 and BMPRII receptors, these data show that BMP-6 signalling is feasible in T-cells [18].

BMP-6 signalling in JurkatTAg and human CD4+ cells

Upon BMP-6 binding and subsequent activation of the receptor complex, several downstream signalling pathways can be triggered. First, we examined the effect of BMP-6 on Smad phosphorylation by western blotting. Smad1/5/8 phosphorylation was induced after 5 minutes, and was sustained for 24 hours in Jurkat TAg cells as well as in CD4+ cells (Figure 2A and 2B), but the level of pSmad was lower in CD4+ cells than in Jurkat TAg cells. BMPs have been shown to induce activation of p38 and ERK in other systems [25]. In Jurkat TAg we observed an increase in the levels of phospho p38 and phospho ERK1/2 after one hour of stimulation, and this increase was sustained for at least 24 hours (Figure 2A). In CD4+ cells the levels of phospho p38 were increased upon BMP-6 stimulation (Figure 2B), whereas the levels of phospho ERK1/2 remained undetectable (not shown).

The BRE-Luc reporter contains binding sites for phosphorylated Smad proteins and is selectively activated by BMPs. To examine whether BMP-6-induced Smad phosphorylation can induce transcriptional responses, this reporter was transfected into Jurkat TAg cells followed by stimulation with BMP-6 in various concentrations. The BRE-Luc reporter was induced in Jurkat TAg cells in a dose dependent manner (Figure 3). Taken together, our data show that T-cells contain the components necessary to transduce the BMP-6 signal from the cell membrane to the nucleus.

Identification of BMP-6 target genes

To characterize the transcriptional changes that precede and lead to the functional responses of BMP-6 in cells of T-cell origin, we performed genome wide expression profiling of the Jurkat TAg cell line. Cells were stimulated for 2 or 16 hours with BMP-6 or cultured in medium alone,

followed by isolation of RNA and microarray experiments. The 2 hours time point was chosen to detect predominantly early transcriptional effects of BMP-6. A false discovery rate of 0.1 or lower was chosen as threshold for significant change. 45 probesets fulfilled this criterion on at least one time point (Figure 4A). Interestingly, the highest ranked probesets represented genes encoding, ID1, ID2 and ID3. The transcription of these genes was induced 18, 10 and 4.6 fold, respectively, after two hours of stimulation. Other identified genes known to be involved in BMP signalling included the BMP antagonist Noggin (NOG) and the inhibitory Smad6. Notably, also several genes involved in transcriptional regulation were changed including NFKBIA, HEY1, DLX2, KLF10 and EGR1.

We next determined the levels of the Id proteins by western blot analysis in Jurkat TAg cells. Interestingly, increased Id1 and Id3 protein expression correlated well to the mRNA data, and was detectable one hour after BMP-6 addition (Figure 4B). In contrast to the mRNA data, we did not observe any significant change in the level of Id2 protein upon BMP-6 stimulation. Taken together these data indicate that both Id1 and Id3 could be target genes, mediating the effects of BMP-6 in Jurkat TAg cells.

We next tested for the upregulation of ID1-ID3 in human CD4+ cells by real-time RT-PCR. The cells were stimulated for two hours with BMP-6, TGF-β or cultured in medium alone followed by isolation of RNA. TGF-β was chosen as a positive control since it was previously shown that this cytokine induces increased expression of ID3 in lymphocytes [26]. Interestingly, we observed a 2.8, 3.4 and 3.4 fold upregulation of ID1, ID2 and ID3 respectively (n=2) (Figure 5). However, Id1 protein was not detectable, and no change in Id2 and Id3 protein levels was observed upon BMP-6 stimulation in CD4+ T –cells (data not shown).

BMP-6 inhibits proliferation of Jurkat TAg cells

To examine the effect of BMP-6 on the DNA synthesis of Jurkat TAg, we treated the cells for 3 days with various concentrations of BMP-6 and measured ^{3}H-thymidine incorporation. BMP-6 reduced the spontaneous proliferation of the cells in a dose-dependent manner (Figure 6A). The BMP-6 effect was reversed by addition of the extracellular inhibitor Noggin or by addition of a combination of soluble receptors for BMP-6 (Figure 6B). Reduced ^{3}H-thymidine incorporation could result from reduced viability in the cell cultures or increased cell cycle length. BMP-6 stimulation for 48 or 72 hours did not significantly affect the viability of Jurkat TAg cells, as determined by propidium iodide (PI) staining (data not shown). In contrast, high-resolution cell

division tracking using the CFSE method showed that T-cells treated with BMP-6 exhibited increased cell cycle length (Figure 6C).

Id1 mediates the inhibitory effect of BMP-6 in Jurkat TAg cells

Id proteins may affect proliferation and differentiation of lymphocytes through binding of E-proteins, thus inhibiting these proteins' ability to bind DNA. We tested whether the endogenous Id proteins, induced by BMP-6, mediated the inhibitory effect of BMP-6 in Jurkat TAg cells. Following transfection with siRNA targeting ID1 or scrambled siRNA, we stimulated the cells with BMP-6. Notably, transfection with siRNA targeting ID1 blocked the BMP-6 induced upregulation of the corresponding proteins as detected by western blot analysis (Figure 7A). We then measured ^{3}H-thymidine incorporation to assess the DNA synthesis of the cells upon BMP-6 stimulation. Whereas cells transfected with scrambled siRNA responded to BMP-6 stimulation in a similar manner as untransfected cells and reduced the spontaneous proliferation by 60%, ID1 siRNA partly counteracted the inhibitory effect of BMP-6 (Figure 7B). In these cells ^{3}H-thymidine incorporation was reduced by only 35%.

Taken together, our data suggest that Id1 is an important downstream target of BMP-6 in mediating the inhibitory effect of this cytokine on Jurkat TAg cells.

BMP-6 inhibits the generation of IL-4 producing Th2 cells from naive CD4+ T-cells

The striking effects of BMP-6 on the pre-T ALL cell line Jurkat TAg and the findings that BMP-6 also activate Smad1/5/8 and p38 in normal CD4+ T-cells, prompted us to investigate the role of this cytokine on normal CD4+ T-cells. BMP-6 stimulation did not significantly affect the proliferative capacity of CD4+ T-cells activated with PHA or anti-CD3/anti-CD28 (data not shown). Furthermore, the viability of CD4+ T-cells was also unaffected by BMP-6 stimulation (data not shown). However, as it previously was shown that TGF-β inhibited the generation of IL-4 producing Th2 as well as IFN-γ producing Th1 cells from naive CD4+ T-cells [12], we wanted to explore the effects of BMP-6 on in vitro induced differentiation of naive CD4+ T-cells. Interestingly, BMP-6 had a significant inhibitory effect on the generation of Th2 cytokine producing effector cells as the percentage of IL-4 positive cells was reduced by 36% compared to control cells (SEM=8.6, n=5, $p<0.05$, Figure 8A). In contrast, BMP-6 had no significant effect on the generation of IFN-γ producing cells. For comparison, TGF-β inhibited both Th2 and Th1 differentiation to a similar extent, with a 51% (SEM=13.1, n=5) and 57% (SEM=12.7, n=3)

reduction in the percentage of IL-4 and IFN-γ positive cells, respectively. Taken together, this suggests that BMP-6 signalling may exert immunomodulatory actions in normal CD4+ T-cells.

Discussion

Members of the BMP superfamily play important roles in the regulation of growth and differentiation of different hematopoietic cells. Recent studies have reported effects of BMPs in hematopoietic stem cells, B-cell malignancies, and in normal B-progenitors and mature B-cells [16-18;27;28]. Less is known regarding possible effects of BMPs in T-lineage cells, although a role of BMPs has been implicated in thymopoiesis [20;21;29]. In this study we have demonstrated the presence of a functional BMP-6 signalling pathway in mature CD4+ T-cells as well as in the pre-T ALL cell line Jurkat TAg. The transcriptional response to BMP-6 stimulation included potent upregulation of ID1, ID2 and ID3, both in Jurkat TAg cells and in CD4+ T-cells. Additionally, the corresponding Id1 and Id3 proteins were induced by BMP-6 in Jurkat TAg cells. In these cells, stimulation with BMP-6 exerted an antiproliferative effect that was suppressed by silencing of ID1. Interestingly, in CD4+ T-cells stimulation with BMP-6 did not affect proliferation, but significantly inhibited the differentiation into IL-4 producing effector cells, suggesting immunomodulatory effects of BMP-6 in mature T-cells.

Activation of the R-Smads is one of the major signalling pathways triggered by BMP stimulation [3;30]. BMP induced phosphorylation of R-Smad has been associated with various functional effects depending on the cellular systems. In normal and malignant B-lineage cells BMPs have previously been shown to inhibit proliferation via R-Smad phosphorylation [16-18;31]. In our study we detected a rapid phosphorylation of Smad1/5/8 in both peripheral CD4+ T-cells as well as in Jurkat TAg cells in response to BMP-6, although the level of pSmad1/5/8 was higher in the latter. Phosphorylation of p38 and ERK1/2 are events representing activation of other signalling pathways triggered by BMP-6 stimulation. Whereas p38 phosphorylation was observed both in CD4+ and Jurkat TAg cells, ERK1/2 phosphorylation was only detected in the Jurkat TAg cells and only at later time points. This indicates that activation of different signalling pathways could, at least in part, account for the different functional effects detected in the two cellular systems. However, it should be noted that in contrast to Jurkat TAg cells, peripheral CD4+ cells constitute a heterogeneous population with the expression of Alk-3 and BMPRII confined to smaller subpopulations. Furthermore, the expression pattern of BMP receptors on CD4+ T-cells was different from that on mature B cells as the latter cell type expressed Alk-6, but not Alk-3 [16]. Thus, the regulation of receptor expression could be important for controlling BMP effects in different subpopulations or at different stages of differentiation.

The transcriptional response to stimulation with BMPs has been analyzed in different cellular systems [5;32-34] , but not previously in cells of lymphoid origin. Upregulation of ID genes is an important event in this response, and has been demonstrated to be dependent on Smad phosphorylation [8]. We used a reporter assay to demonstrate this also in Jurkat TAg cells. The BRE-Luc reporter contains elements from the ID1 promoter and is selectively activated by BMP specific R-Smads [23]. This reporter was induced in Jurkat TAg cells by BMP-6 concentrations lower than normally used in functional assays, indicating that phosphorylation of R-Smads is an efficient signalling pathway in this cell line. The microarray and real time PCR data in our study confirm the central role of the ID genes as targets for BMP signalling, and indicate that ID1, ID2 as well as ID3 are targets of BMP-6 in cells of the T-cell lineage.

In addition to IDs, several other genes that were identified by the microarray experiments are known to be involved in transcriptional regulation, including NFKBIA, HEY1, KLF10 and EGR1. NFKBIA codes for the protein IκBα which is a negative regulator of the critical transcription factor NF-κB and functions by sequestering NF-κB in the cytoplasm, thus preventing transcriptional activation of NF-κB target genes [35;36]. Although the levels of the IκB inhibitors mainly are regulated by proteasomal degradation [37], increased transcription of this gene could lead to higher levels of the protein, and thus oppose NF-κB activity as shown in a B-cell line [38]. Early growth response-1 (EGR1) was also represented by two probesets, and the expression levels were significantly reduced after 16 hours. This gene codes for a zinc-finger transcription factor. EGR proteins play central roles in controlling the differentiation program initiated by pre-TCR signalling [39;40] and in mature CD4+ T-cells it was recently shown that EGR1 is required for CD154 (CD40 ligand) transcription [41]. The transcriptional regulators HEY1 and KLF10 are involved in regulation of cell fate decisions and proliferation, respectively, but have yet not been investigated in T-lineage cells. CD24 was represented by two probesets on our list of significant genes and was upregulated after 16 hours. CD24 is normally expressed on the surface of developing T-cells. Later on it is rapidly induced by engagement of the TCR-CD3 complex. CD24 expression on T-cells has been shown to be required for optimal homeostatic proliferation [42]. The function of this gene in relation to BMP signalling is unknown. Furthermore, the known negative regulators of BMP signalling, Noggin and Smad6, were also among the upregulated genes in Jurkat TAg identified in this study. This indicates that BMP-6 stimulation activates several negative feedback mechanisms. Interestingly, both Noggin and

Smad6 were previously shown to be targets for BMP-6 in a prostate cancer cell line [32]. In addition, increased expression of Noggin induced by BMP-6 has also been demonstrated in rat osteoblasts [43].

The reduction in proliferative capacity observed in Jurkat TAg cells in this study is in accordance with previous findings concerning effects of BMPs on lymphoid cells. We have recently demonstrated the inhibitory effect of BMP-6 on immature as well as mature cells of B-cell origin [16;17]. Similar effects were demonstrated for BMP-2, -4, -6 and -7 in human myeloma cells [18;19]. Several studies have shown that BMP signalling may play a role in thymocyte differentiation. Studies of fetal thymic organ cultures indicate that BMP-2 and -4 may act directly on the developing thymocytes. However, other studies suggest that it is the thymic stroma that depends on BMP signalling [20;21;44]. In Jurkat TAg cells the functional effect of BMP-6 was, at least partly, dependent on induction of the Id1 protein. The dependency on Id1 upregulation is in accordance with studies of BMP effects in other cellular systems. In endothelial cells it has been shown that BMP-6 stimulated migration and tube formation via Id1 upregulation [8]. The functional effects of BMP-2 in a lung cancer cell line were also related to Smad1 dependent upregulation of Id1 [6]. Id proteins are known to negatively regulate another class of HLH proteins, the E-proteins, by inhibiting these proteins ability to bind DNA. The E-proteins play crucial roles in survival, proliferation and differentiation of lymphocytes, and interestingly, they have been shown to both promote and inhibit growth at different stages of lymphocyte development [9;10]. It has been shown that T-cell development is partially blocked at the CD4-CD8- double negative 1 stage in E2A deficient mice [45]. Furthermore, Id1 transgenic mice are T-cell deficient and their thymocytes undergo massive apoptosis. Their thymus is characterized by an accumulation of CD4-CD8-CD44+CD25- thymocytes [46]. Enforced expression of Id3 in human T lineage precursor cells also suppresses thymocyte development [47]. Thus, induction of Id1 expression leading to inhibition of E protein activity is a likely mechanism for the growth inhibitory effect of BMP-6 on Jurkat TAg. In addition to the effect of BMP-6 on upregulation of Id1, the Smad-independent signalling through the ERK1/2 and p38 MAPK pathways that was demonstrated in this study could also contribute to the antiproliferative effect. Activation of the p38 MAPK is thought to mediate BMP induced apoptosis [30] whereas activation of both these pathways was shown to play a role in selection T-lymphocytes in the thymus [48].

As we did not detect Id1 protein expression in CD4+ cells and Id2 and Id3 protein levels were unchanged after BMP-6 stimulation, the role of Id proteins in mediating the effect of BMP-6 on Th2 differentiation of naive CD4+ T-cells remains elusive. However, it has been shown that TGF-β directly inhibits expression of the transcription factor GATA-3, which is crucial for Th2 differentiation [49]. Additionally, several signalling pathways need to be activated for GATA-3 induction and subsequent Th2 differentiation, and it has been shown that inhibition of NF-κB activity could prevent GATA-3 expression and Th2 cytokine production in developing Th2 cells [50]. GATA-3 was not an immediate target of BMP-6 in our microarray experiments of Jurkat TAg cells. Whether it is a target in CD4+ T-cells remains to be tested. However, as we showed that the NF-κB inhibitor NFKBIA (IκBα) is a target gene for BMP-6 in Jurkat TAg cells, it will be of interest to investigate whether the effect of BMP-6 in CD4+ T-cells is mediated via an upregulation of NFKBIA and subsequent reduction in NF-κB activity and GATA-3 expression.

The inhibition of Th2 differentiation of naive CD4+ T-cells induced by BMP-6 argues for an immunomodulatory role of this cytokine on T-cells. This could be of importance with regard to anti tumour immune responses. Previously, BMPs have been shown to stimulate tumour growth by directly stimulating the malignant cells [6]. Inhibition of tumour specific T-cells could be another mechanism for BMP effects on tumour growth. In follicular lymphomas, gene expression profiling identified that the signature of non-malignant immune cells had implications for the prognosis, and in particular, the immune-response 1 signature that included several T-cell–restricted genes correlated with good outcome [51]. In addition, high expression of BMP-6 has been correlated to poor outcome in diffuse large B cell lymphomas [28]. Thus, a potential immunoregulatory role of BMP-6 in T cells with implications for lymphoid malignancies will be a subject for future investigations.

Acknowledgements

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Supplemental material

Supplemental material is available through the following website:

http://radium.no/ebsmeland/?k=review

Password=oslo123

References

1. **Wozney,J.M., Rosen,V., Celeste,A.J., Mitsock,L.M., Whitters,M.J., Kriz,R.W., Hewick,R.M., and Wang,E.A.,** Novel regulators of bone formation: molecular clones and activities. *Science* 1988. **242**: 1528-1534.

2. **Chen,D., Zhao,M., Harris,S.E., and Mi,Z.,** Signal transduction and biological functions of bone morphogenetic proteins. *Front Biosci.* 2004. **9**: 349-358.

3. **Derynck,R. and Zhang,Y.E.,** Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 2003. **425**: 577-584.

4. **Dennler,S., Goumans,M.J., and ten Dijke,P.,** Transforming growth factor beta signal transduction. *J.Leukoc.Biol.* 2002. **71**: 731-740.

5. **Kowanetz,M., Valcourt,U., Bergstrom,R., Heldin,C.H., and Moustakas,A.,** Id2 and Id3 define the potency of cell proliferation and differentiation responses to transforming growth factor beta and bone morphogenetic protein. *Mol.Cell Biol.* 2004. **24**: 4241-4254.

6. **Langenfeld,E.M., Kong,Y., and Langenfeld,J.,** Bone morphogenetic protein 2 stimulation of tumor growth involves the activation of Smad-1/5. *Oncogene* 2006. **25**: 685-692.

7. **Ying,Q.L., Nichols,J., Chambers,I., and Smith,A.,** BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* 2003. **115**: 281-292.

8. **Valdimarsdottir,G., Goumans,M.J., Rosendahl,A., Brugman,M., Itoh,S., Lebrin,F., Sideras,P., and ten Dijke,P.,** Stimulation of Id1 expression by bone morphogenetic protein

is sufficient and necessary for bone morphogenetic protein-induced activation of endothelial cells. *Circulation* 2002. **106**: 2263-2270.

9. **Engel,I. and Murre,C.,** The function of E- and Id proteins in lymphocyte development. *Nat.Rev.Immunol.* 2001. **1**: 193-199.

10. **Murre,C.,** Helix-loop-helix proteins and lymphocyte development. *Nat.Immunol.* 2005. **6**: 1079-1086.

11. **Rivera,R. and Murre,C.,** The regulation and function of the Id proteins in lymphocyte development. *Oncogene* 2001. **20**: 8308-8316.

12. **Gorelik,L. and Flavell,R.A.,** Transforming growth factor-beta in T-cell biology. *Nat.Rev.Immunol.* 2002. **2**: 46-53.

13. **Lomo,J., Blomhoff,H.K., Beiske,K., Stokke,T., and Smeland,E.B.,** TGF-beta 1 and cyclic AMP promote apoptosis in resting human B lymphocytes. *J.Immunol.* 1995. **154**: 1634-1643.

14. **Lund,R., Ahlfors,H., Kainonen,E., Lahesmaa,A.M., Dixon,C., and Lahesmaa,R.,** Identification of genes involved in the initiation of human Th1 or Th2 cell commitment. *Eur.J.Immunol.* 2005. **35**: 3307-3319.

15. **Lund,R., Aittokallio,T., Nevalainen,O., and Lahesmaa,R.,** Identification of novel genes regulated by IL-12, IL-4, or TGF-beta during the early polarization of CD4+ lymphocytes. *J.Immunol.* 2003. **171**: 5328-5336.

16. **Kersten,C., Sivertsen,E.A., Hystad,M.E., Forfang,L., Smeland,E.B., and Myklebust,J.H.,** BMP-6 inhibits growth of mature human B cells; induction of Smad phosphorylation and upregulation of Id1. *BMC.Immunol.* 2005. **6**: 9.

17. **Kersten,C., Dosen,G., Myklebust,J.H., Sivertsen,E.A., Hystad,M.E., Smeland,E.B., and Rian,E.,** BMP-6 inhibits human bone marrow B lymphopoiesis--upregulation of Id1 and Id3. *Exp.Hematol.* 2006. **34**: 72-81.

18. **Baade,R.T., Utne,H.R., Brenne,A.T., Hjorth-Hansen,H., Waage,A., Hjertner,O., Sundan,A., and Borset,M.,** Bone morphogenetic protein-5, -6 and -7 inhibit growth and induce apoptosis in human myeloma cells. *Oncogene* 2004. **23**: 3024-3032.

19. **Hjertner,O., Hjorth-Hansen,H., Borset,M., Seidel,C., Waage,A., and Sundan,A.,** Bone morphogenetic protein-4 inhibits proliferation and induces apoptosis of multiple myeloma cells. *Blood* 2001. **97**: 516-522.

20. **Hager-Theodorides,A.L., Outram,S.V., Shah,D.K., Sacedon,R., Shrimpton,R.E., Vicente,A., Varas,A., and Crompton,T.,** Bone morphogenetic protein 2/4 signaling regulates early thymocyte differentiation. *J.Immunol.* 2002. **169**: 5496-5504.

21. **Bleul,C.C. and Boehm,T.,** BMP signaling is required for normal thymus development. *J.Immunol.* 2005. **175**: 5213-5221.

22. **Bryder,D. and Jacobsen,S.E.,** Interleukin-3 supports expansion of long-term multilineage repopulating activity after multiple stem cell divisions in vitro. *Blood* 2000. **96**: 1748-1755.

23. **Korchynskyi,O. and ten Dijke,P.,** Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. *J.Biol.Chem.* 2002. **277**: 4883-4891.

24. **Breitling,R., Armengaud,P., Amtmann,A., and Herzyk,P.,** Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. *FEBS Lett.* 2004. **573**: 83-92.

25. **Gallea,S., Lallemand,F., Atfi,A., Rawadi,G., Ramez,V., Spinella-Jaegle,S., Kawai,S., Faucheu,C., Huet,L., Baron,R., and Roman-Roman,S.,** Activation of mitogen-activated protein kinase cascades is involved in regulation of bone morphogenetic protein-2-induced osteoblast differentiation in pluripotent C2C12 cells. *Bone* 2001. **28**: 491-498.

26. **Kee,B.L., Rivera,R.R., and Murre,C.,** Id3 inhibits B lymphocyte progenitor growth and survival in response to TGF-beta. *Nat.Immunol.* 2001. **2**: 242-247.

27. **Bhatia,M., Bonnet,D., Wu,D., Murdoch,B., Wrana,J., Gallacher,L., and Dick,J.E.,** Bone morphogenetic proteins regulate the developmental program of human hematopoietic stem cells. *J.Exp.Med.* 1999. **189**: 1139-1148.

28. **Rosenwald,A., Wright,G., Chan,W.C., Connors,J.M., Campo,E., Fisher,R.I., Gascoyne,R.D., Muller-Hermelink,H.K., Smeland,E.B., Giltnane,J.M., Hurt,E.M., Zhao,H., Averett,L., Yang,L., Wilson,W.H., Jaffe,E.S., Simon,R., Klausner,R.D., Powell,J., Duffey,P.L., Longo,D.L., Greiner,T.C., Weisenburger,D.D., Sanger,W.G., Dave,B.J., Lynch,J.C., Vose,J., Armitage,J.O., Montserrat,E., Lopez-Guillermo,A., Grogan,T.M., Miller,T.P., LeBlanc,M., Ott,G., Kvaloy,S., Delabie,J., Holte,H., Krajci,P., Stokke,T., and Staudt,L.M.,** The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. *N.Engl.J.Med.* 2002. **346**: 1937-1947.

29. **Graf,D., Nethisinghe,S., Palmer,D.B., Fisher,A.G., and Merkenschlager,M.,** The developmentally regulated expression of Twisted gastrulation reveals a role for bone morphogenetic proteins in the control of T cell development. *J.Exp.Med.* 2002. **196**: 163-171.

30. **Nohe,A., Keating,E., Knaus,P., and Petersen,N.O.,** Signal transduction of bone morphogenetic protein receptors. *Cell Signal.* 2004. **16**: 291-299.

31. **Nakahara,T., Tominaga,K., Koseki,T., Yamamoto,M., Yamato,K., Fukuda,J., and Nishihara,T.,** Growth/differentiation factor-5 induces growth arrest and apoptosis in mouse B lineage cells with modulation by Smad. *Cell Signal.* 2003. **15**: 181-187.

32. **Haudenschild,D.R., Palmer,S.M., Moseley,T.A., You,Z., and Reddi,A.H.,** Bone morphogenetic protein (BMP)-6 signaling and BMP antagonist noggin in prostate cancer. *Cancer Res.* 2004. **64**: 8276-8284.

33. **Peng,Y., Kang,Q., Cheng,H., Li,X., Sun,M.H., Jiang,W., Luu,H.H., Park,J.Y., Haydon,R.C., and He,T.C.,** Transcriptional characterization of bone morphogenetic proteins (BMPs)-mediated osteogenic signaling. *J.Cell Biochem.* 2003. **90**: 1149-1165.

34. **Sekiya,I., Vuoristo,J.T., Larson,B.L., and Prockop,D.J.,** In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. *Proc.Natl.Acad.Sci.U.S.A* 2002. **99**: 4397-4402.

35. **Baldwin,A.S.,** Control of oncogenesis and cancer therapy resistance by the transcription factor NF-kappaB. *J.Clin.Invest* 2001. **107**: 241-246.

36. **Kucharczak,J., Simmons,M.J., Fan,Y., and Gelinas,C.,** To be, or not to be: NF-kappaB is the answer--role of Rel/NF-kappaB in the regulation of apoptosis. *Oncogene* 2003. **22**: 8961-8982.

37. **Alberts,B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P.,** *Molecular Biology of the Cell*. Garland, New York 2002.

38. **Arsura,M., Wu,M., and Sonenshein,G.E.,** TGF beta 1 inhibits NF-kappa B/Rel activity inducing apoptosis of B cells: transcriptional activation of I kappa B alpha. *Immunity.* 1996. **5**: 31-40.

39. **Carleton,M., Haks,M.C., Smeele,S.A., Jones,A., Belkowski,S.M., Berger,M.A., Linsley,P., Kruisbeek,A.M., and Wiest,D.L.,** Early growth response transcription factors are required for development of CD4(-)CD8(-) thymocytes to the CD4(+)CD8(+) stage. *J.Immunol.* 2002. **168**: 1649-1658.

40. **Bettini,M., Xi,H., Milbrandt,J., and Kersh,G.J.,** Thymocyte development in early growth response gene 1-deficient mice. *J.Immunol.* 2002. **169**: 1713-1720.

41. **Cron,R.Q., Bandyopadhyay,R., Genin,A., Brunner,M., Kersh,G.J., Yin,J., Finkel,T.H., and Crow,M.K.,** Early growth response-1 is required for CD154 transcription. *J.Immunol.* 2006. **176**: 811-818.

42. **Li,O., Zheng,P., and Liu,Y.,** CD24 expression on T cells is required for optimal T cell proliferation in lymphopenic host. *J.Exp.Med.* 2004. **200**: 1083-1089.

43. **Gazzerro,E., Gangji,V., and Canalis,E.,** Bone morphogenetic proteins induce the expression of noggin, which limits their activity in cultured rat osteoblasts. *J.Clin.Invest* 1998. **102**: 2106-2114.

44. **Tsai,P.T., Lee,R.A., and Wu,H.,** BMP4 acts upstream of FGF in modulating thymic stroma and regulating thymopoiesis. *Blood* 2003. **102**: 3947-3953.

45. **Bain,G., Engel,I., Robanus Maandag,E.C., te Riele,H.P., Voland,J.R., Sharp,L.L., Chun,J., Huey,B., Pinkel,D., and Murre,C.,** E2A deficiency leads to abnormalities in alphabeta T-cell development and to rapid development of T-cell lymphomas. *Mol.Cell Biol.* 1997. **17**: 4782-4791.

46. **Qi,Z. and Sun,X.H.,** Hyperresponse to T-cell receptor signaling and apoptosis of Id1 transgenic thymocytes. *Mol.Cell Biol.* 2004. **24**: 7313-7323.

47. **Heemskerk,M.H., Blom,B., Nolan,G., Stegmann,A.P., Bakker,A.Q., Weijer,K., Res,P.C., and Spits,H.,** Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix loop helix factor Id3. *J.Exp.Med.* 1997. **186**: 1597-1602.

48. **Sugawara,T., Moriguchi,T., Nishida,E., and Takahama,Y.,** Differential roles of ERK and p38 MAP kinase pathways in positive and negative selection of T lymphocytes. *Immunity.* 1998. **9**: 565-574.

49. **Gorelik,L., Fields,P.E., and Flavell,R.A.,** Cutting edge: TGF-beta inhibits Th type 2 development through inhibition of GATA-3 expression. *J.Immunol.* 2000. **165**: 4773-4777.

50. **Das,J., Chen,C.H., Yang,L., Cohn,L., Ray,P., and Ray,A.,** A critical role for NF-kappa B in GATA3 expression and TH2 differentiation in allergic airway inflammation. *Nat.Immunol.* 2001. **2**: 45-50.

51. **Dave,S.S., Wright,G., Tan,B., Rosenwald,A., Gascoyne,R.D., Chan,W.C., Fisher,R.I., Braziel,R.M., Rimsza,L.M., Grogan,T.M., Miller,T.P., LeBlanc,M., Greiner,T.C., Weisenburger,D.D., Lynch,J.C., Vose,J., Armitage,J.O., Smeland,E.B., Kvaloy,S., Holte,H., Delabie,J., Connors,J.M., Lansdorp,P.M., Ouyang,Q., Lister,T.A., Davies,A.J., Norton,A.J., Muller-Hermelink,H.K., Ott,G., Campo,E., Montserrat,E., Wilson,W.H., Jaffe,E.S., Simon,R., Yang,L., Powell,J., Zhao,H., Goldschmidt,N., Chiorazzi,M., and Staudt,L.M.,** Prediction of Survival in Follicular Lymphoma Based on Molecular Features of Tumor-Infiltrating Immune Cells. *N Engl J Med* 2004. **351**: 2159-2169.

Figure legends

Figure 1 Jurkat TAg cells and CD4+ T-cells express BMP type I and type II receptors. Jurkat TAg cells and CD4+ T-cells were stained with various biotin-labelled anti-BMP-receptor antibodies or isotype control antibodies, using streptavidin-PE as a secondary layer. Shown are histogram plots of one representative experiment of at least four. Anti-BMP-receptor antibody is shown as black line. Control is shown as grey line.

Figure 2 Effect of BMP-6 on phosphorylation of Smad1/5/8, p38 and ERK1/2. Jurkat TAg (A) or CD4+ T-cells (B) were treated with BMP-6 for various times before preparation of total protein lysates that were subjected to western blot analysis. One representative experiment of three is shown.

Figure 3 BMP-6 signalling activates the BMP/Smad specific reporter BRE-Luc in a dose dependent manner. Jurkat TAg cells were transfected with the BRE-Luc reporter in combination with the pRL-CMV (renilla luciferase) plasmid used as an internal transfection control. Shown is relative luciferase activity ± SD of duplicates from one experiment.

Figure 4 BMP-6 target genes in Jurkat TAg. A) Cells stimulated with BMP-6 were compared to cells cultured with medium alone. The heatmap shows 45 probesets with a false discovery rate of 0.1 or lower from at least one time point. Detailed information about the probesets is found in supplementary table 1. B) Id1 and Id3 are induced by BMP-6 at the protein level. Western blot analysis of one representative experiment of three is shown.

Figure 5 BMP-6 induced upregulation of ID1, ID2 and ID3 in CD4+ T-cells. Cells were cultured in X-VIVO 15 over night before treatment with BMP-6 or TGF-β for 2 hours. ID1, ID2 and ID3-expression was analysed by real-time RT-PCR; values are normalised to the expression level of PGK1 mRNA and calibrated to expression levels of the ID genes in the cell-line Ramos. Data are shown as mean relative mRNA expression ± SEM of two experiments

Figure 6 BMP-6 inhibits proliferation of Jurkat TAg cells. A) Dose dependent inhibition of BMP-6, one representative experiment is shown (mean cpm ± SD of triplicates). B) Preincubation of Noggin or soluble Alk-2 and BMPRII receptors with BMP-6 (100 ng/ml) counteracts the effects of BMP-6. Shown is relative proliferation obtained by normalizing the mean cpm for each stimulation to the mean cpm obtained for Jurkat TAg cultured in medium alone, one representative experiment (relative expression ± SD of triplicates). C) The number of cell divisions was obtained by culturing $CFSE^{mean}$ FACS-sorted cells in X-VIVO 15 medium for

3 days in the presence or absence of BMP-6 (100ng/ml) followed by FACS analysis. Shown is one representative experiment of two.

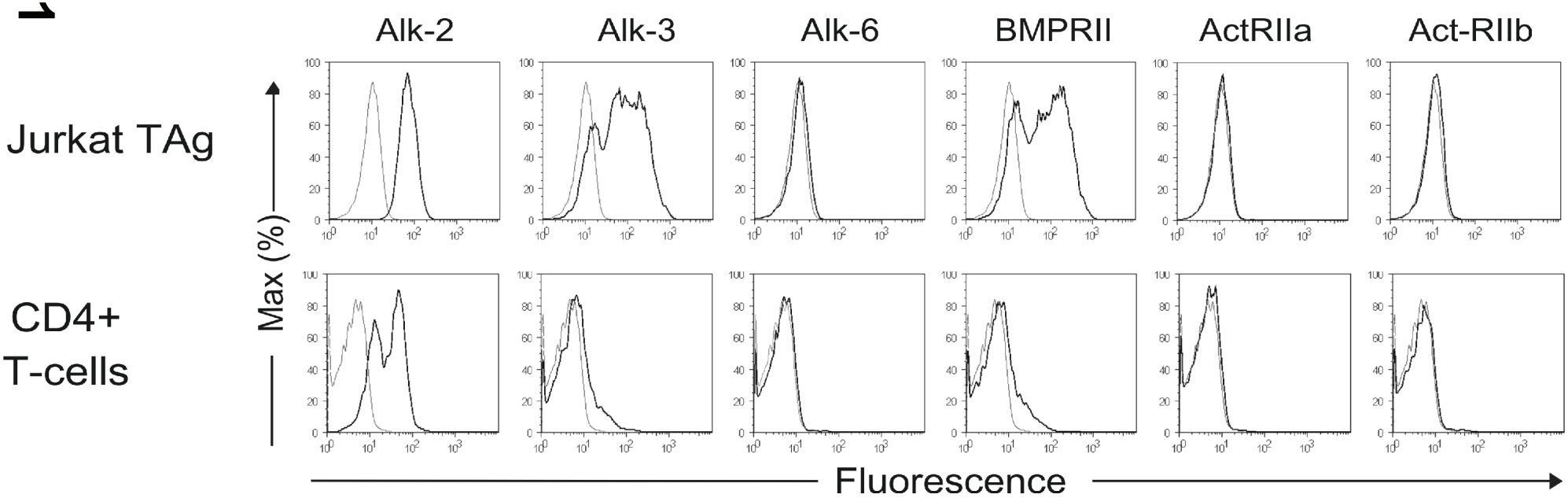
Figure 7 Id1 mediates the antiproliferative effect of BMP-6 in Jurkat TAg. Jurkat TAg were transiently transfected with siRNA targeting ID1 or scrambled siRNA, followed by stimulation with BMP-6. A) Western blot of one representative experiment. B) ^{3}H thymidine incorporation of transfected Jurkat TAg. Data are given as relative proliferation obtained by normalizing the cpm for each transfected sample stimulated with BMP-6 to the mean cpm obtained for the transfected sample cultured in medium alone. Shown is mean relative proliferation (%) ± SEM of six experiments ($p<0.05$).

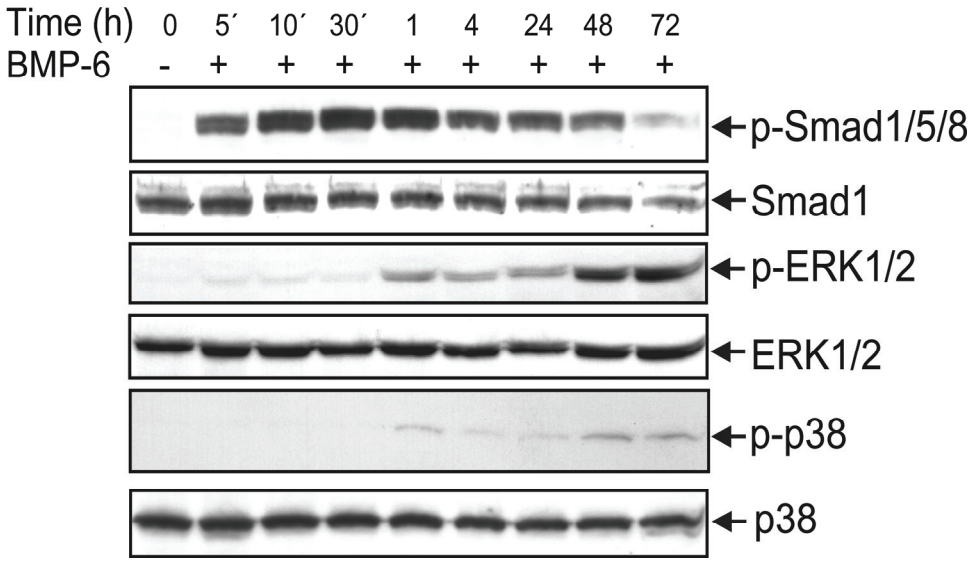
Figure 8 BMP-6 inhibits the generation of IL-4 producing Th2 cells from naive CD4+ cells. CD4+CD45RA+CD45RO- naive T-cells were isolated, preincubated alone or with BMP-6 (500 ng/ml) or TGF-β (10 ng/ml) before activation and culturing in conditions directing Th1 or Th2 differentiation. A) Mean percentage of IL-4 positive cells developed under Th2 conditions (± SEM, $n = 5$, $p<0.05$ for both stimulations). B) Mean percentage of IFN-γ positive cells developed under Th1 conditions (± SEM, $n = 5$ for BMP-6, $n=3$ for TGF-β).

Table 1 Expression of BMP receptors on CD4+ T-cells from peripheral blood (n=4-6).

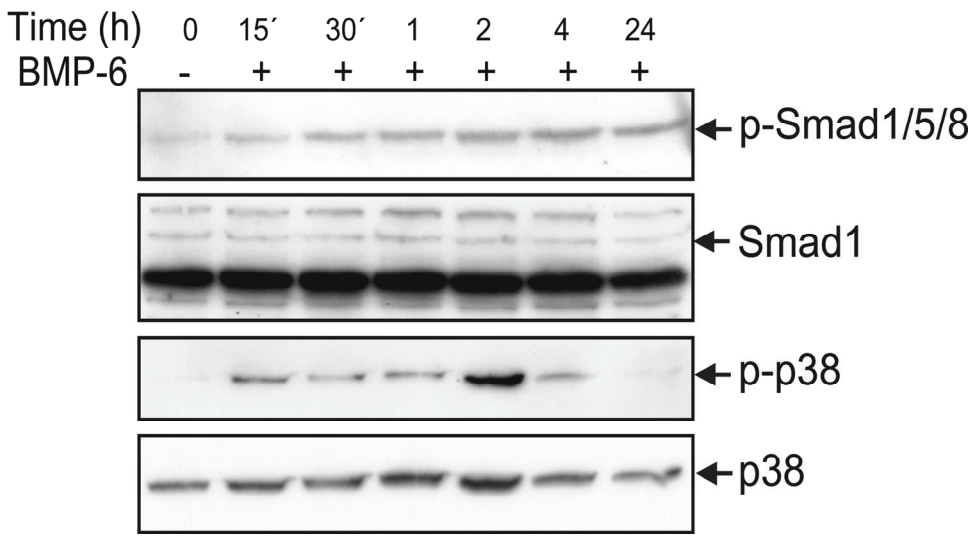
	Positive Cells (%)					
	Alk-2	Alk-3	Alk-6	BMPRII	ActRIIA	ActRIIB
mean (SEM)	58 (5)	12 (2)	1 (0)	11 (3)	1 (1)	4 (3)

Figure 1


Alk-2
Alk-3
Alk-6
BMPRII
ActRIIa
Act-RIIb
Jurkat TAg
CD4+
T-cells
Max (%)
Fluorescence


Time (h) 0 5´ 10´ 30´ 1 4 24 48 72
BMP-6 - + + + + + + + +
p-Smad1/5/8
Smad1
p-ERK1/2
ERK1/2
p-p38
p38

A


Time (h) 0 15´ 30´ 1 2 4 24
BMP-6 - + + + + + +
p-Smad1/5/8
Smad1
p-p38
p38

B

Figure 2

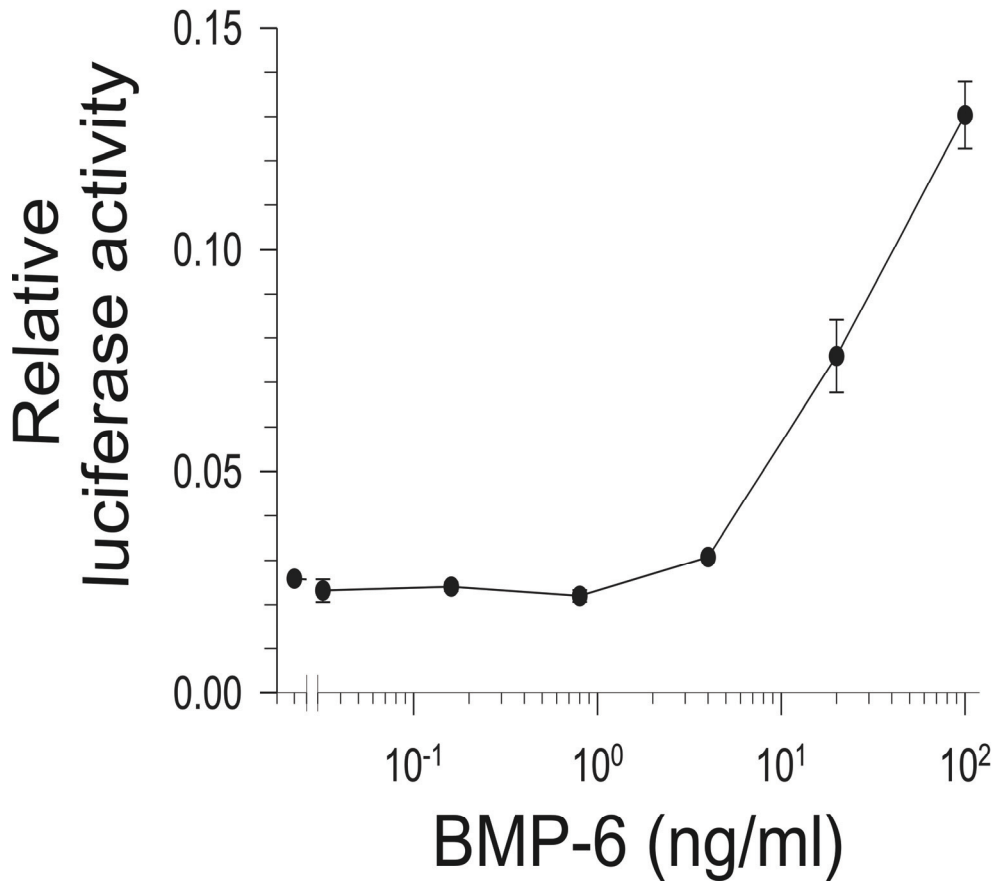

Relative
luciferase activity
0.15
0.10
0.05
0.00
10^{-1}
10^{0}
10^{1}
10^{2}
BMP-6 (ng/ml)

Figure 3


2 Hours
16 Hours
Gene symbol
ID1
ID2
ID2 /// ID2B
ID3
HEY1

FLJ25076
NOG
RGS3
NFKBIA
DLX2

RIPK4
SMAD6
RIPK4
TIPARP
KLF10
HEY1
LOC129607
RGS3
HRH1
CPEB2
ID2 /// ID2B
CD24

CD24
GPR18
LGMN
EGR1
EGR1
CRIP2
ALDH1A2
PLCL3
HKDC1
FUS
CHRNA3
PDLIM7
LOC143381
C9
ALDH1A2
SLC39A8
NUPL1
NEBL
RAG2
ITGAL

Figure 4A

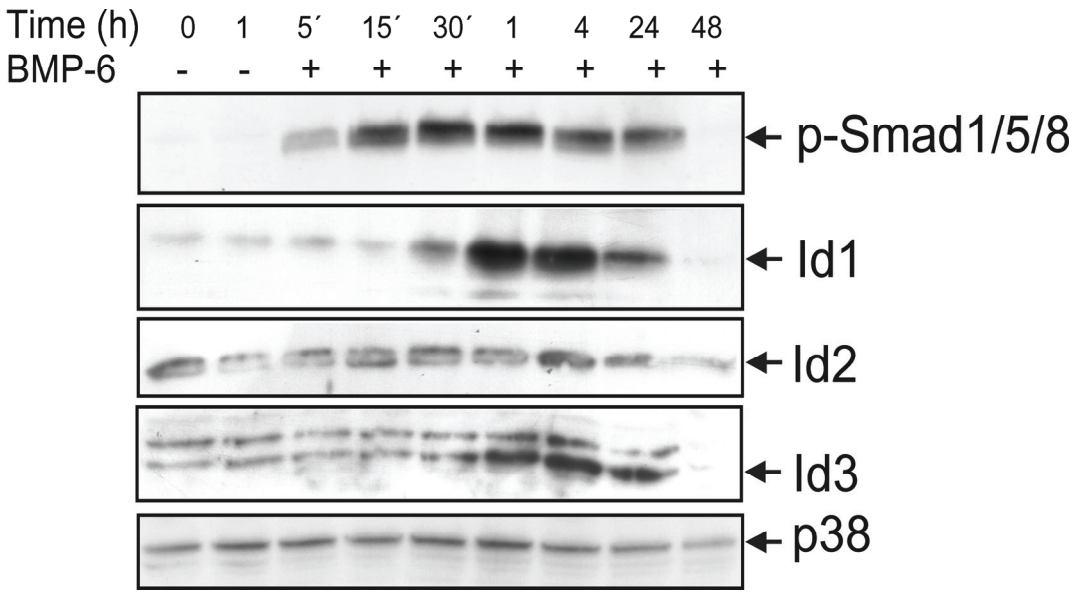


Figure 4B

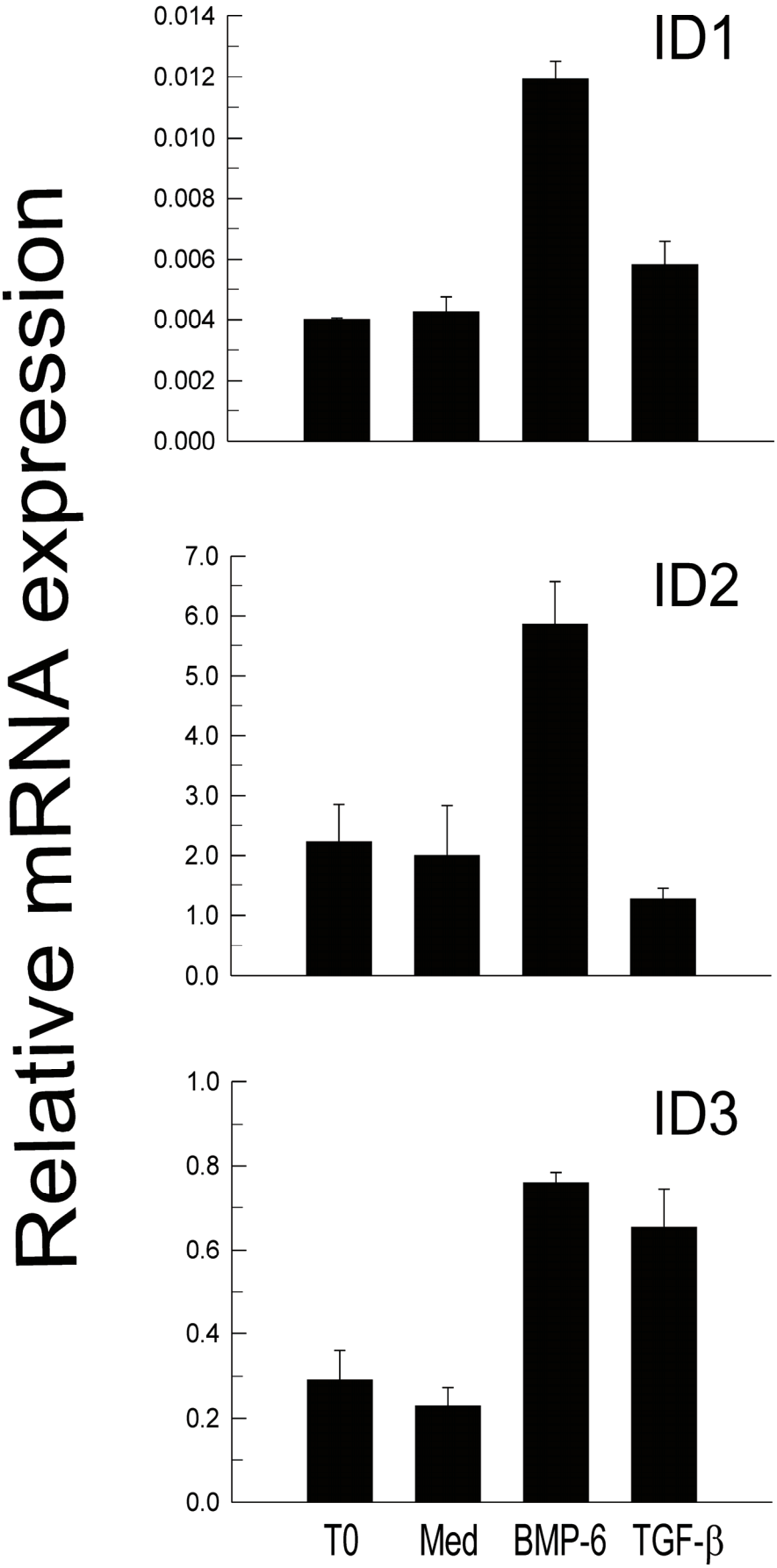
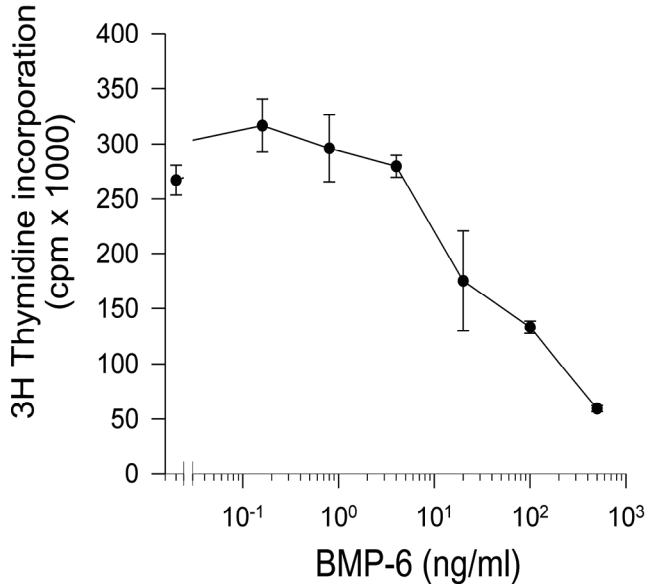
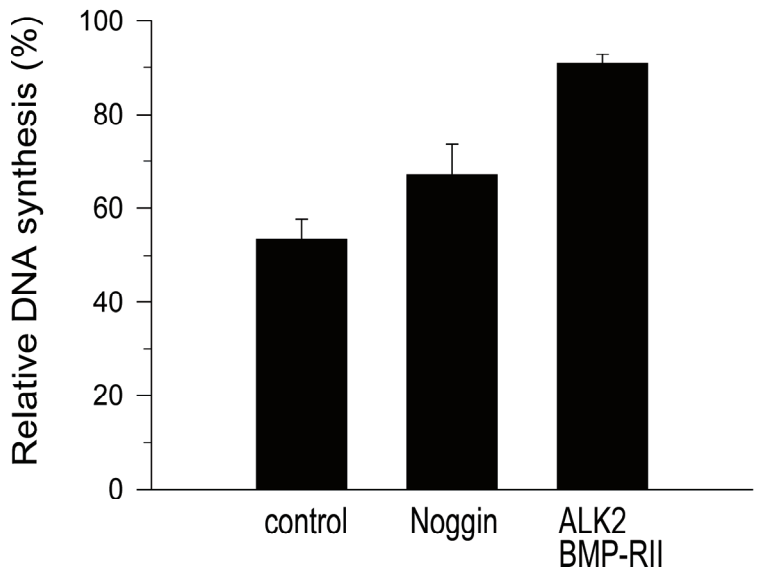

Relative mRNA expression
ID1
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
ID2
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
ID3
1.0
0.8
0.6
0.4
0.2
0.0
T0
Med
BMP-6
TGF-β

Figure 5

A


3H Thymidine incorporation
(cpm x 1000)
400
350
300
250
200
150
100
50
0
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
BMP-6 (ng/ml)

B


Relative DNA synthesis (%)
100
80
60
40
20
0
control
Noggin
ALK2
BMP-RII

C

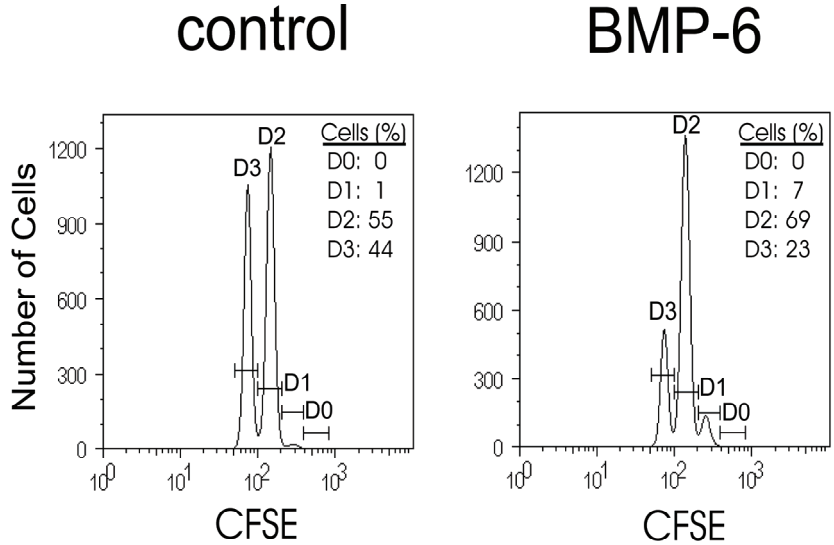

control
BMP-6
Number of Cells
1200
900
600
300
0
D2
D3
D1
D0
Cells (%)
D0: 0
D1: 1
D2: 55
D3: 44
Cells (%)
D0: 0
D1: 7
D2: 69
D3: 23
10^{0}
10^{1}
10^{2}
10^{3}
CFSE
CFSE

Figure 6

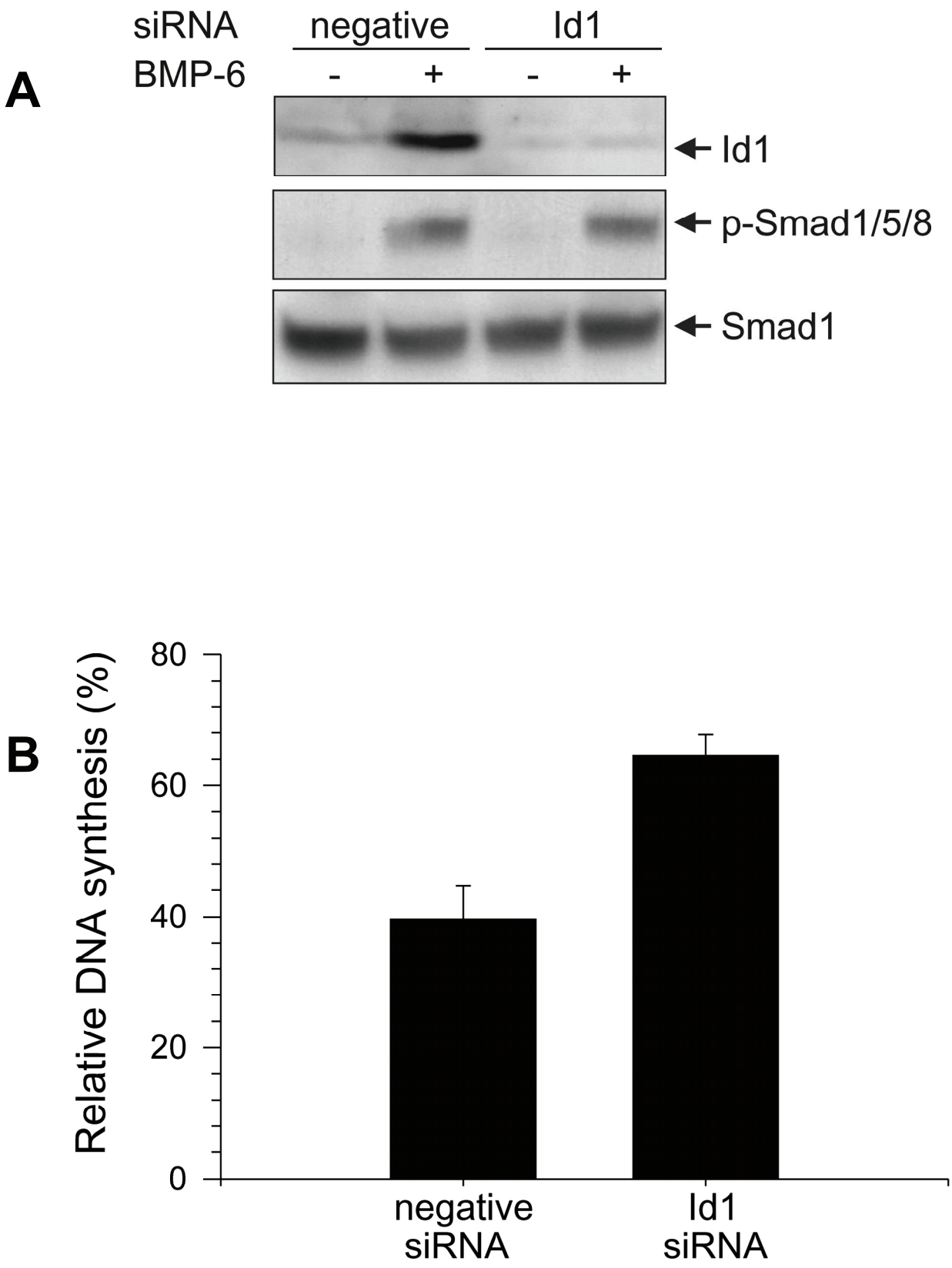
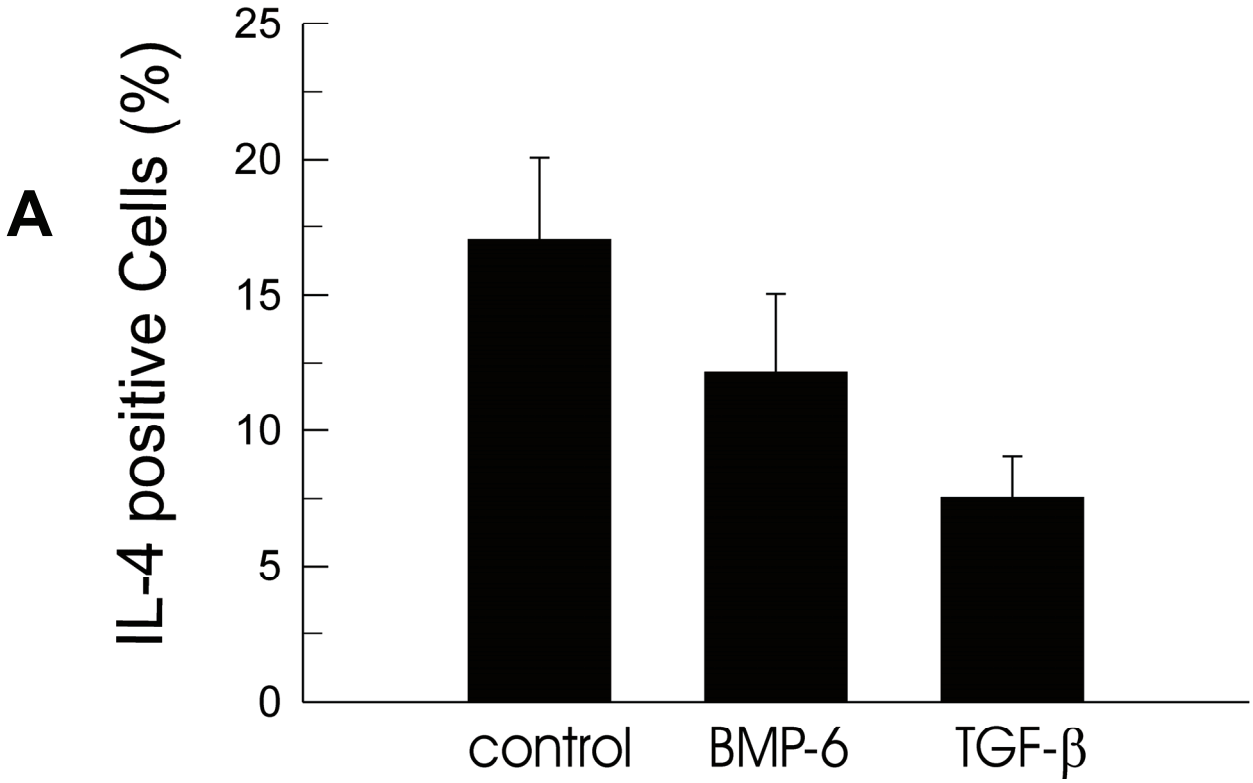

A
siRNA
negative
Id1
BMP-6
-
+
-
+
Id1
p-Smad1/5/8
Smad1
B
Relative DNA synthesis (%)
80
60
40
20
0
negative siRNA
Id1 siRNA

Figure 7


A
IL-4 positive Cells (%)
25
20
15
10
5
0
control
BMP-6
TGF-β

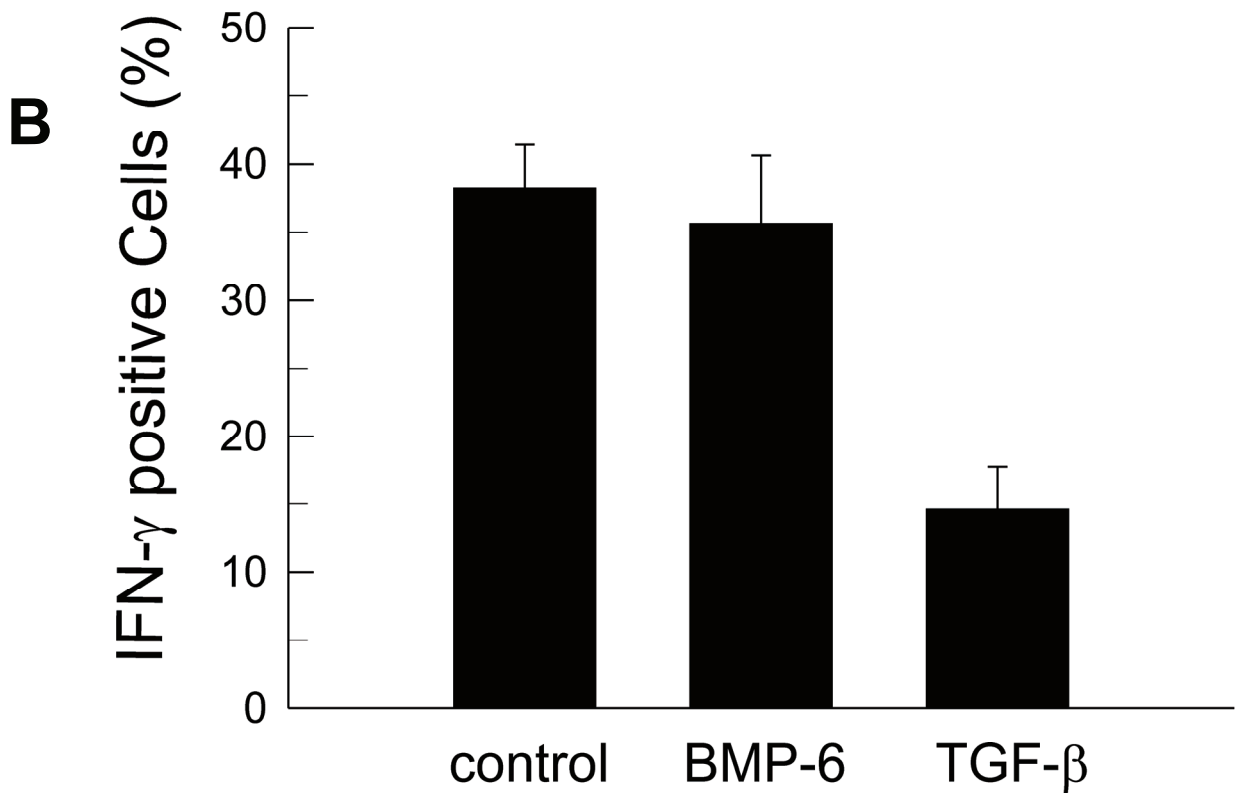

B
IFN-γ positive Cells (%)
50
40
30
20
10
0
control
BMP-6
TGF-β

Figure 8