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## The Role of PAX5 in ALL

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### 1. Introduction

It is now widely acknowledged that, like other cancers, acute lymphoblastic leukaemia is caused by the acquisition of mutations in somatic cells. The first hit in childhood ALL has been documented to occur in most (if not all) cases before birth (Golub, 2007). The progress in genomics technology holds the promise of making the complete characterization of the 'cancer genome' possible by a systematic search for submicroscopical mutations.

Molecular studies on recurrent chromosomal translocations in ALL have indicated that, beside the constitutive activation of tyrosine kinases, the aberrant expression of transcription factors plays a central role in the pathobiology of lymphoid leukaemia. This aberrant expression leads to abnormal proliferation and differentiation arrest of lymphoid progenitors (O'Neil & Look, 2007). In some cases, recurrent chromosomal translocations generate fusion transcription factors with new functions, enabling them to target genes other than those recognized by the endogenous factors.

In the past few years, the *PAX5* gene has been demonstrated to be a recurrent target of genetic alterations in B-lineage ALL, both in adult and paediatric patients (Mullighan et al., 2007). The role of these aberrancies in human leukemogenesis is still, however, poorly understood (Cobaleda et al., 2007a).

This chapter will review the role of *PAX5* gene involvement in B-lineage ALL to outline the biological and functional effects of different genetic aberrations affecting this new master gene in leukaemia. Moreover, we will address whether *PAX5* alterations are driver or passenger lesions and finally what their potential prognostic impact can be.

### 2. Role of the *PAX5* transcription factor in normal B-cell precursor development

The *PAX5* gene, located on 9p13, belongs to the paired box (*PAX*) gene family of transcription factors, essential for B lymphoid cell commitment (Cobaleda et al., 2007a). Nine mammalian *PAX* transcription factors have been described, but *PAX5* is the only *PAX* protein expressed in the haematopoietic system. In addition, *PAX5* is expressed in the nervous system at the midbrain-hindbrain boundary and in adult testes.

In B-cells, *PAX5* fulfils a unique function by controlling the identity of B lymphocytes throughout B-cell development, from the pro-B to the mature B-cell stage. It functions both

as a transcriptional activator and as a repressor on different target genes, which are involved in lineage development (Busslinger, 2004; Matthias & Rolink, 2005).

## 2.1 B-cell development

Haematopoiesis is an ideal system for investigating the developmental relationships between cells of an organ system. All lineages can be reconstituted from a single bone marrow-derived haematopoietic stem cell (HSC) (Ceredig et al., 2009). According to the classical “tree model” by Weissman, (Akashi et al., 2000; Manz et al., 2002) the haematopoietic system constantly generates a large number of specialized cell types from pluripotent haematopoietic stem cells (PHSCs), which have a self-renewal potential and give rise to different progenitors with a more restricted differentiation capacity (Busslinger, 2004; Matthias & Rolink, 2005).

One of the earliest differentiated precursors is the multipotential progenitor (MPP), which is at the junction between the myeloid and lymphoid lineages. MPPs can differentiate into common myeloid progenitors (CMPs), common lymphoid progenitors (CLPs) or, as recently identified, early lymphoid progenitors (ELPs) (Matthias & Rolink, 2005).

It is generally thought that CLPs have the potential to develop into lymphoid cells (B cells, T cells and NK cells), while the CMPs differentiate either 1) into granulocyte-monocyte progenitors (GMPs), (which subsequently produce mature granulocytes (neutrophils, basophils and eosinophils) and monocyte/macrophages,) or 2) into megakaryocyte-erythrocyte progenitors (MEPs), which give rise to platelets and red erythrocytes (Akashi et al., 2000; Manz et al., 2002).

However, the idea that CLPs have exclusively lymphoid but not myeloid potential has been questioned by several studies (Akashi et al., 2000; Adolfsson et al., 2005; Balciunaite et al., 2005; Benz & Bleul, 2005). It has been suggested that immune-cell progenitors have both lymphoid and myeloid potential and a “circular model” has been proposed accordingly (Ceredig et al., 2009). These cells have been called early progenitors with lymphoid and myeloid potential (EPLMs), and they express B220, cKit (also known as CD117), IL-7 receptor  $\alpha$ -chain (IL-7 $\alpha$ ; also known as CD127), FLT3 (Fms-related tyrosine kinase 3, also known as CD135 and Flk2) and CD93, but do not express CD19 or NK1.1.

Indeed, *CD19* expression is completely controlled by *PAX5*, whose expression starts later in precursor B-cells. Research on the specific role of the *PAX5* gene in haematopoietic development has demonstrated its requirement exclusively in B-cell development. A knock out mouse model has shown a complete block in B-cell differentiation, which is immediately downstream of the block that is seen in the absence of E2A or EBF (Nutt et al., 1999). Remarkably, *PAX5*<sup>-/-</sup> pre-BI cells have extraordinary developmental plasticity showing haematopoietic stem cell features such as multipotency and a self renewing capacity (Rolink et al., 2002a). In fact, it has been reported that EPLMs resemble *PAX5*<sup>-/-</sup> pro-B cells, since they can both differentiate *in vitro* into myeloid cells (macrophages and dendritic cells), NK cells and T cells (Balciunaite et al., 2005).

The plasticity of EPLMs is greater than that of *PAX5*<sup>-/-</sup> pro-B cells because EPLMs can express *PAX5* and thereby generate B-cells. It is possible that EPLMs are a mixture of committed progenitors and their fate is directed by signals and growth factors (Ceredig et al., 2009).

Overall, these features support the key role of *PAX5* in B-cell commitment and differentiation. Further details on its function will be discussed in paragraph 2.2.3.

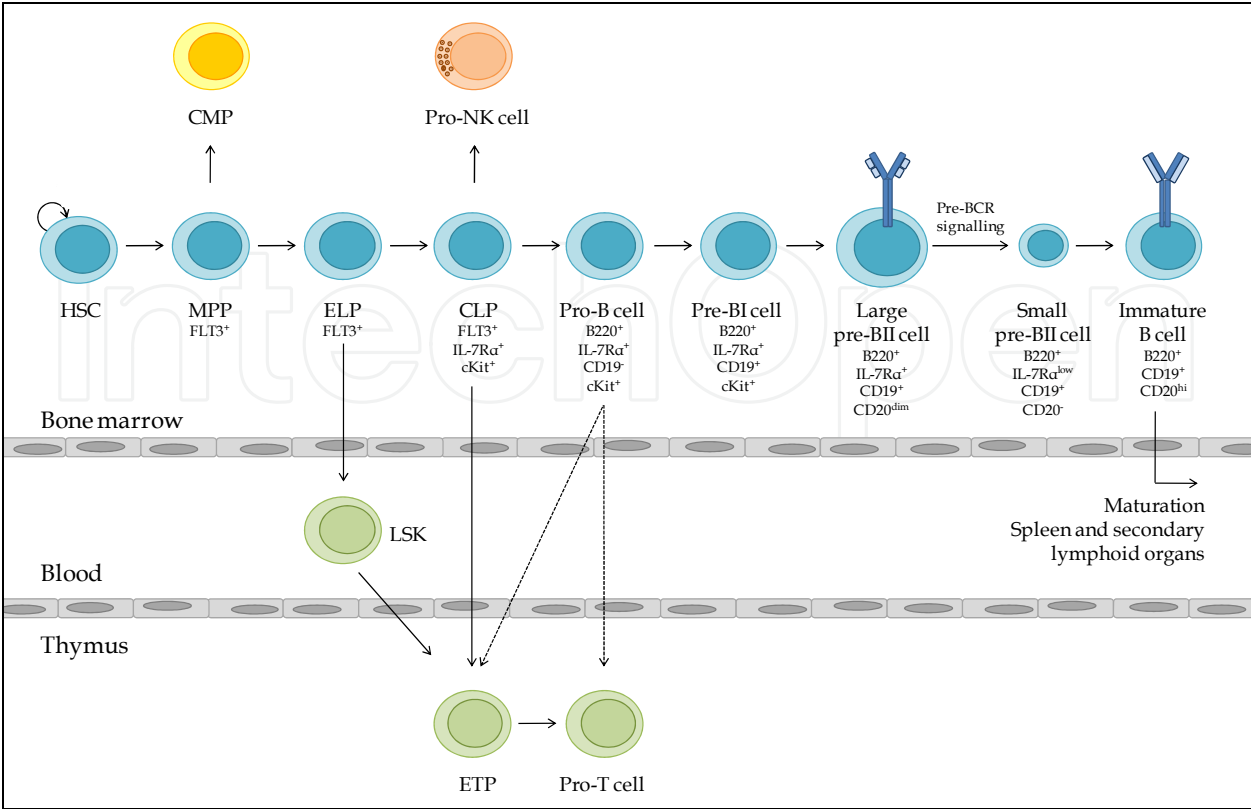


Fig. 1. B-cell development. HSC, haematopoietic stem cell; MPP, multipotential progenitor; ELP, early lymphoid progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; LSK, Lin<sup>-</sup> Sca<sup>hi</sup> cKit<sup>hi</sup>; ETP, early T progenitor.

Schematically, B-cell development initiates in progenitors when they start expressing the recombination-activating gene 1 (Rag1) and Rag2 and the process of rearrangement at the immunoglobulin heavy chain (IgH) locus occurs. Indeed, the stages in primary B-cell development are defined by the sequential rearrangement and expression of heavy- and light-chain immunoglobulin genes. Intermediate differentiation stages have been distinguished on the basis of the expression of other cell-surface proteins, together with direct DNA analysis of the state of the immunoglobulin gene loci (Janeway et al., 2001). More specifically, expression of the B-cell marker B220 by a subset of progenitors, known as pro-B cells, coincides with their entry into the B-cell differentiation pathway. Subsequently, CD19 is expressed and the IgH diversity (DH)-to-joining (JH) gene-segment rearrangement is completed, identifying pre-BI cells. The IgH locus then continues to rearrange its variable (V)-region gene segments until productive VH-DJH alleles are generated in large pre-BII cells. These cells cease to express Rag1 and Rag2, and they display the product of the rearranged *IgH* gene at the cell surface. There, they assemble with the surrogate immunoglobulin light chains (IgLs), VpreB and λ5, together with the signalling molecules Igα (which is encoded by the *MB-1* gene) and Igβ (which is encoded by the *B29* gene) to form a pre-B-cell receptor (pre-BCR). The expression of the pre-BCR is a crucial check-point in early B-cell development, and its signalling stimulates a proliferative clonal expansion of large pre-BII cells, which is followed by the re-expression of RAGs and rearrangement at the *IgL* locus in small pre-BII cells.

Once an immature B cell expresses IgM on its surface (sIgM), its fate is guided by the nature of the signals it receives through its antigen receptor. Binding to self molecules in the bone

marrow can lead to the death or inactivation of immature B cells; otherwise they leave the bone marrow, enter the periphery and reach the spleen (Janeway et al., 2001). There they further differentiate through three transitional stages T1, T2 and T3, on the basis of the expression of various cell-surface markers, their short half-life (2–4 days) and their sensitivity to apoptosis induced by antibodies specific for IgM. Indeed transitional B cells can be negatively selected in the periphery. The tumour-necrosis-factor family member B-cell-activating factor (BAFF; also known as BLYS) and its receptor play a crucial role in regulating the transition of immature B cells into mature B cells (Rolink & Melchers, 2002b; Rolink et al., 2002c). Transitional, follicular and even memory B cells can give rise to marginal-zone B cells (responsible for antibody response to blood pathogens) and/or be recruited into this compartment. In mice, most mature B cells are follicular B cells. These cells are mainly responsible for generating humoral immune responses to protein antigens. With the help of T cells, they form germinal centres. Germinal-centre B cells proliferate rapidly, undergo somatic hypermutation of their immunoglobulin variable gene segments and undergo isotype-switch recombination of immunoglobulin genes. Subsequently, germinal centres slowly vanish, and memory B cells and effector plasma cells are generated (Matthias & Rolink, 2005).

### 2.1.1 Comparison between mouse and human B-cell development

Most of our understanding of the initiation and regulation of Ig gene rearrangements in precursor B-cell differentiation comes from studies on mouse models. Genome-wide gene expression profiling has been performed in human precursor B-cells, contributing enormously to the understanding of lymphocyte differentiation. Therefore, Van Dongen and colleagues (van Zelm et al., 2005) purified cells in the five main stages of human precursor B-cell differentiation, pro-B, pre-BI, pre-BII large and small, and immature B-cells. In correspondence with each stage, the authors characterized the IGH and IGK/IGL rearrangements independently from each other and independently from the selection processes. Altogether they have deepened the knowledge about the different stages of human B-cell development, which seems to follow mechanisms similar to that of mice.

This confirms the previous studies reviewed by Ghia (Ghia et al., 1998), in which they concluded that the main mechanisms of selection and the key events during B lymphopoiesis appear to be strikingly similar in murine and human bone marrow.

There are some differences in surface marker expression (e.g. the absence of CD10 and CD34 in mouse pre-BI cells and the absence of CD25 in human pre-BI cells) as well as different growth requirements. Human pre-BI cells are mainly unresponsive to IL-7; they can be grown only 3–4 weeks *in vitro* in presence of IL-7 and do not show a proliferative expansion comparable to mouse cells (Ghia et al., 1998).

### 2.1.2 Transcription factors in B-cell precursor development

The comprehension of the different stages of B-cell development is based on results from multiple levels of analysis including immunophenotype, status of immunoglobulin gene rearrangement, *in vitro* or *in vivo* cell differentiation properties (Matthias & Rolink, 2005) as well as gene-targeting studies of many regulatory molecules in mice. By these studies, B lymphopoiesis has emerged as one of the leading models for research on lineage specification (induction of a lineage-specific gene-expression program) and commitment (repression of alternative gene expression programs).



Transcription factors play a central role in this process. B-cell development, maturation and function are coordinated by a 'battery' of transcription factors and signal-transduction molecules that regulate the sequential execution of the different steps (Matthias & Rolink, 2005; Nutt & Kee, 2007).

The generation of lymphoid progenitors depends on signalling through the c-Kit, FLT3 and IL-7 receptors; in their absence, early B cell progenitors in the bone marrow are severely affected (Nutt & Kee, 2007).

In addition, developmental control of early B lymphopoiesis is exerted by a regulatory network of key transcription factors that include PU.1 (an Ets-family member), Ikaros, Bcl11a (a zinc finger transcription factor), E2A (a helixloop-helix protein), EBF (early B-cell factor) and PAX5 (Fuxa & Skok, 2007).

In particular, three transcription factors have been found to be essential for the differentiation of CLPs into specified pro-B cells: transcription factor E2A, early B-cell factor (EBF; also known as OLF1) and paired box protein 5 (PAX5; also known as BSAP). Absence of any one of these factors leads to an early block in B-cell development at the pro-B-cell or pre-B-cell stage. These three factors seem to work in collaboration, and together, they form a master control switch for engaging B-cell differentiation (Matthias & Rolink, 2005). E2A and EBF are considered primary B-cell fate determinants and co-ordinately activate the expression of B-cell specific genes (e.g. both can initiate the remodelling of MB-1 promoter chromatin) (Fuxa & Skok, 2007). In the absence of E2A or EBF, B-cell development was blocked at early progenitor stages of development, however, EBF can activate the B-cell lineage program in absence of E2A or PU.1. Conversely, in the absence of EBF, B-cell development was not rescued by enforced expression of PAX5 (Hagman & Lukin, 2006).

The ability of EBF to mediate activation of the B-cell program suggests that it has the properties of a 'pioneer' factor (i.e. a protein capable of initiating the activation of transcriptional quiescent genes) (Hagman & Lukin, 2005).

However, the mere activation of the B lymphocyte transcription program is not sufficient to commit B cell progenitors to the B lymphoid lineage in the absence of the paired domain protein PAX5 (Fuxa & Skok, 2007).

## **2.2 PAX5, the sentinel of B cells: identity and function**

The *PAX5* gene is a member of the paired box (PAX) gene family of transcription factors and it is essential for B lymphoid lineage commitment (Morrison et al., 1998; Nutt et al., 1999; Souabni et al., 2002; Cotta et al., 2003) since it controls the identity of B lymphocytes throughout B-cell development from the pro-B to the mature B-cell stage (Busslinger, 2004; Matthias & Rolink, 2005).

This factor has been implicated in the direct transcriptional regulation of several B-cell-specific genes, such as those encoding *CD19*, *Iga* and *BLNK/SLP-65* (Matthias & Rolink, 2005).

### **2.2.1 Cloning of PAX5**

PAX5 or the B-cell specific activator protein (BSAP) was independently discovered as a DNA-binding protein with the same DNA sequence specificity as the sea urchin transcription factor TSAP. Biochemical purification and cDNA cloning showed that BSAP is encoded by the *PAX5* gene and is expressed in B lymphocytes, the developing CNS, and adult testes (Cobaleda et al., 2007a).

The human *PAX5* gene is located on chromosome 9p13, is organized in 10 exons and encodes for a transcript of 8536 bp, giving rise to a coding sequence of 1176 bp, which in turn gives rise to a protein of 391 aa, a protein completely homologous to the *Mus musculus* *PAX5* gene. Indeed, *PAX5* retains a high degree of homology between humans and mice (Ghia et al., 1998).

2.2.2 *PAX5* protein structure

*PAX5* is a homeodomain protein, which is a member of a class of transcription factors that contains a DNA-binding domain with homology to *Drosophila melanogaster* homeodomain regulatory proteins. This DNA-binding domain contains a helix–turn–helix motif, which binds to a distinct half-site of the *PAX5* recognition sequence in adjacent major grooves of the DNA helix (Cobaleda et al., 2007a).

The defining feature of the PAX protein family is the conserved ‘paired’ domain, which functions as a bipartite DNA-binding region consisting of an N- and a C-terminal domain. The bipartite nature of the paired domain is responsible for its degenerate consensus recognition sequence, as each half-site independently contributes to the overall affinity of a given binding site (Czerny, 1995).

The transcriptional activity of *PAX5*, which is responsible for regulating its target genes, is determined by the interaction of distinct partner proteins with the central and C-terminal protein interaction motifs of *PAX5*. The partial homeodomain of *PAX5* associates with the TATA-binding protein of the basal transcription machinery, while a C-terminal transactivation domain regulates gene transcription most likely by interacting with histone acetyltransferases (HAT), such as the co-activator CBP or SAGA complex.

In parallel, *PAX5* acts as a repressor, and not as a transcriptional activator, through the binding of its conserved octapeptide motif to co-repressors of the Groucho protein family, which are part of a larger histone deacetylase (HDAC) complex (Cobaleda et al., 2007a).

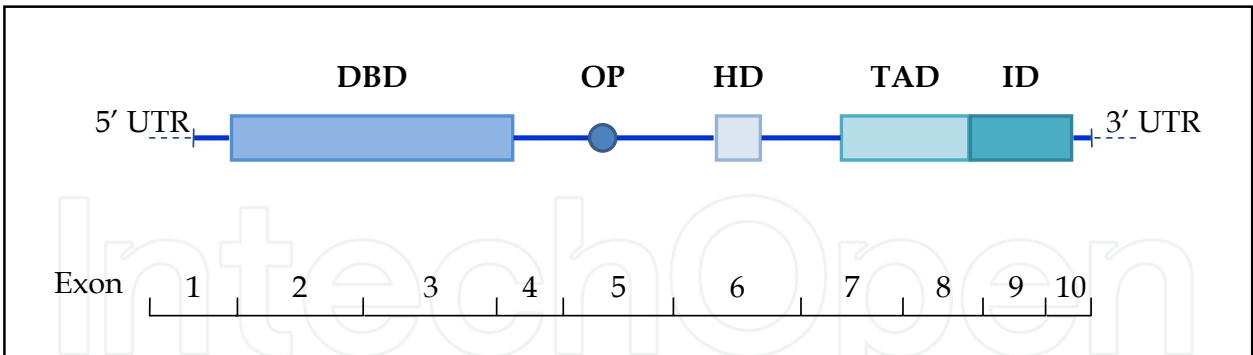


Fig. 2. *PAX5* structure, showing functional domains and corresponding exons. DBD, DNA-binding paired domain; OP, conserved octapeptide; HD, partial homeodomain; TAD, transactivation domain; ID, inhibitory domain.

2.2.3 *PAX5* is essential in B-cell development

*PAX5* is a transcription factor and localizes in the nucleus. In the haematopoietic system it is expressed exclusively in the B-cell compartment, where it is essential for the development of the specific lineage (Matthias & Rolink, 2005; Cobaleda et al., 2007a).

The *PAX5* knock out mouse model leads to a complete block in B-cell differentiation, immediately downstream of the block that is seen in the absence of E2A or EBF (their

expression is normal in PAX5<sup>-/-</sup> B cells) (Nutt et al., 1999). The rearrangement at the IgH locus has already been initiated (Matthias & Rolink, 2005); although DJ gene segments are rearranged normally in these cells, rearrangement of VH gene segments is severely impaired. PAX5 is required not only for rearrangement of VH gene segments and for expression of genes that are required for progression to the pre-B-cell stage but also for commitment to and maintenance of the B-cell differentiation pathway.

Both *in vitro* and *in vivo* studies have demonstrated that, in the absence of PAX5, pre-BI cells show an extraordinary degree of plasticity demonstrating multipotency and a self renewing capacity. After stimulation with the appropriate cytokines, they can in fact differentiate *in vitro* into macrophages, osteoclasts, DCs, granulocytes or NK cells. *In vivo*, however, they develop into all the lineages, even into T cells, and erythrocytes (Rolink et al., 2002a).

From these studies, PAX5 fulfils a dual role in B lineage commitment by activating B cell specific genes while simultaneously repressing lineage-inappropriate genes (Nutt et al., 1998).

More recently, Cobaleda and colleagues (Cobaleda et al., 2007b) showed that conditional PAX5 deletion in mice allows mature B cells from peripheral lymphoid organs to de-differentiate *in vivo* back to early uncommitted progenitors in the bone marrow, which rescues T lymphopoiesis in the thymus of T-cell-deficient mice. These B-cell-derived T lymphocytes carry immunoglobulin heavy- and light-chain gene rearrangements but also participate as functional T cells in immune reactions. The mice lacking PAX5 in mature B cells in the study also developed aggressive lymphomas, which were identified by their gene expression profile as progenitor cell tumours. Hence, they concluded that the loss of PAX5, in the context of strong BCR signalling results in forward differentiation of mature B cells into plasma cells, whereas PAX5 inactivation initiates the reversal of differentiation into uncommitted progenitors, in the absence of BCR signalling.

Thus, in summary, the PAX5 protein is essentially required from pre-B cells to mature B cells, as schematically represented in Fig 1.

#### 2.2.4 PAX5 target genes

The PAX5 transcription factor represses B lineage-inappropriate genes and activates B-cell specific genes in B lymphocytes, functioning both as a transcriptional activator and as a repressor on different target genes (Nutt et al., 1998).

While screening for PAX5-repressed genes, Delogu et al. (Delogu et al., 2006) recently estimated that PAX5 represses 44% and activates 56% of the genes, which are differentially expressed in PAX5<sup>+/+</sup> and PAX5<sup>-/-</sup> pro-B cells.

Examples of PAX5-activated genes are co-receptors CD21 (Horchner et al., 2001) and CD19 (Kozmik et al., 1992), MB-1/CD79a (Maier et al., 2003) and BLNK (Schebesta et al., 2002). PAX5-repressed genes are MCSFR (Morrison et al., 1998), NOTCH1 (Souabni et al., 2002) and FLT3 (Holmes et al., 2006). Among them, one of the most important targets is CD19, which is expressed by B lymphocytes starting from pre-BI cells and lasting to mature B cells (Horchner et al., 2001). Its expression is directly controlled by the PAX5 gene; indeed it has been demonstrated that a sequence consensus for PD of PAX5 is present in the CD19 promoter region (Kozmik et al., 1992).

Delogu and colleagues (Delogu et al., 2006) identified 110 PAX5-repressed genes, demonstrating that PAX5 regulates diverse biological activities including receptor signalling, cell adhesion, migration, transcriptional control, and cellular metabolism during



B-cell commitment. The T lymphoid or myeloid expression of these genes demonstrates that PAX5<sup>-/-</sup> pre-BI cells and common lymphoid progenitors display lymphoid and myeloid promiscuity of gene expression. These lineage-inappropriate genes require continuous PAX5 activity for their repression because they are reactivated in committed pro-B cells and mature B cells following conditional PAX5 deletion (Cobaleda et al., 2007b).

More recently, Schebesta and colleagues (Schebesta et al., 2007) have identified 170 PAX5-activated genes, of which 66 (some, already known and some, newly identified) were further confirmed to be expressed under PAX5 control. The PAX5-activated genes code for key regulatory and structural proteins involved in B cell signalling, adhesion, migration, antigen presentation, and germinal-centre B cell formation. Thus they reveal a complex regulatory network that is activated by PAX5 to control B-cell development and function. Six PAX5-activated genes (*CD157*, *CD44*, *CD55*, *CD97*, *Sdc4*, and *Tnfrsf19*) code for cell-surface molecules, and nine genes (*Bcar3*, *Capn2*, *Eps8*, *Fhod3*, *Gsn*, *Myh10*, *Mylip*, *Nedd9*, and *Pard3*) code for intracellular proteins, which have been implicated in cell migration and adhesion. PAX5 has been implicated in the control of pre-BCR signalling by the previously characterized PAX5 target genes (*CD19*, *CD79a/Iga*, *BLNK*, etc...) already mentioned above. The identification of the PAX5-activated genes *Vpreb3*, *Igk*, *Slamf6*, *Siglecg/CD22*, *Lcp2*, *Plekha2*, *Prkd2*, *Ikzf2*, and *Spib2* has now extended the notion that PAX5 controls signalling from the pre-BCR on the cell surface to transcription in the nucleus at multiple levels. Comparing T lymphoid and myeloid genes repressed by PAX5 in a specular manner, the PAX5-activated genes require continuous PAX5 activity for normal expression in pro-B and mature B cells (conditional mutagenesis experiments). Expression of the PAX5-activated genes is either absent or significantly reduced upon PAX5 loss in plasma cells.

In conclusion, target genes that are activated by PAX5 code for essential components of pre-B- and B-cell receptor (pre-BCR and BCR, respectively) signalling pathways; and when PAX5 acts as a transcriptional repressor, its function is to limit lineage choices that differ from B cells (Fuxa & Skok, 2007). However, conditional deletion of *PAX5* in pro-B and mature B cells causes the aberrant reactivation of these repressed targets, thus creating the need for continuous repression by PAX5. Surprisingly, even at the transition to the plasma cell stage, when PAX5 is physiologically lost during terminal differentiation, repressed genes are reactivated and contribute to the plasma cell transcriptional program (Fuxa & Skok, 2007).

### 3. The *PAX5* gene in haematological tumours

In haematological malignancies, *PAX5* was initially described as involved in lymphomas, as a target either of mutation in diffuse large B cell lymphomas (DLBCL) (Busslinger et al., 1996) or of translocation t(9;14)(p13;q32) in non-Hodgkin's lymphoma (Lida et al., 1996).

As recently reviewed (Cobaleda et al., 2007a), in DLBCL, which are germinal-centre B-cell-derived tumours, exon 1B of *PAX5* is the target of misdirected class-switch recombination and somatic hypermutations (SHM). SHMs are essential for the affinity maturation of immunoglobulins in germinal-centre B cells, but can be potentially misdirected to generate oncogenic mutations or chromosomal translocations involved in lymphomagenesis. However, the alternatively transcribed exon 1A, as well as the second *PAX5* allele, are normally expressed; indeed they escape SHMs. The generation of the t(9;14)(p13;q32) translocation is due to a misguided class-switch recombination, which is mainly associated with aggressive B cell non-Hodgkin's lymphoma (Souabni et al., 2007). This translocation

brings one allele of the *PAX5* gene under the control of strong enhancers from the IGH locus, leading to its increased expression. The consequence is tumour formation when the *PAX5*-dependent gene expression program alters, due to increased *PAX5* transcription in B cells or failed *PAX5* repression at the onset of plasma cell differentiation. The human t(9;14) translocation was recently reconstructed in a knock-in mouse by inserting a *PAX5* minigene into the mouse's IgH locus. IgH-*PAX5* knock-in mice develop aggressive T-lymphoblastic lymphomas, demonstrating that even the T cell lineage is particularly sensitive to the oncogenic action of *PAX5* (Souabni et al., 2007).

### 3.1 PAX5 role in leukaemia

Abnormalities of the short arm of chromosome 9 (9p) have been described in approximately 10% of childhood ALL, with a higher incidence in T-ALL (Harrison, 2001). The majority of 9p abnormalities results in wide-spread (frequently complete) deletion of the chromosome's short arm, which usually includes the cell cycle regulatory genes *p14*, *p15* and *p16*. More specifically, deletions of *p16*, also known as *CDKN2A*, have been detected by molecular analysis and FISH in approximately 80% of childhood T-ALL and 20% of common-pre-B ALL.

More recently, thanks to technological improvements, a new genetic lesion has been identified on 9p, affecting the *PAX5* gene in about 30% of BCP-ALL cases (Mullighan et al., 2007), which can be considered one of the most common alterations. In addition to deletion, the *PAX5* gene has been reported as a recurrent target of mutation (about 7%) and translocation (2-3%), both in adult and in childhood B-Cell Precursor-ALL (BCP-ALL) cases with similar incidence (Familiades et al., 2009).

Both in adult and childhood cases, *PAX5* deletions seem to be secondary events, since they are frequently associated with other lesions, such as ETV6/AML1 (Mullighan et al., 2007), BCR/ABL1 or TCF3/PBX1 (Paulsson et al., 2008; Den Boer et al., 2009; Familiades et al., 2009; Iacobucci et al., 2010).

To further confirm the similar *PAX5* genetic profile of adult and paediatric BCP-ALL, translocations were found in both cohorts, even with the same fusion gene, such as *PAX5/ELN*, *PAX5/FOXP1* and *PAX5/ETV6*, the last, being the most frequent translocation compared to the others. Details about the different cohorts and the frequency of partner genes are reported in table 1.

Although the role of these aberrancies is still poorly understood, a different biological consequence for mutations/deletions and translocations can be hypothesized.

#### 3.1.1 Deletions, point mutations and amplifications

Deletion is the most frequent *PAX5* aberrancy, occurring in about 25% of patients. Three types of deletions have been found: a) wide-range deletions, involving the full length *PAX5* gene and flanking genes, or even the whole short arm of chromosome 9, extended over *CDKN2A-2B*; b) focal deletions involving a subset of *PAX5* exons extending to its 3' region, thus leading to a prematurely truncated gene/protein; c) focal deletions involving only a subset of internal *PAX5* exons, determining different *PAX5* isoforms lacking functional domains, such as the DNA binding domain, the octamer or the transcriptional regulatory domain. In the larger study in childhood ALL, deletions were monoallelic in 53/192 of the cases (27.6%), and among them 25 were focal (13%), while the complete *PAX5* was lost in 28 cases (14.6%); only 3/192 (1.6%) were biallelic (Mullighan et al., 2007). In addition, in the

study of adult BCP-ALL, *PAX5* is target of exclusively monoallelic deletions in 27/117 cases (23.1%) (Familiades et al., 2009).

Similarly, aberrant splicing variants have been described as alternative mechanisms of deletion, both in adults and in children (Santoro et al., 2009). Different isoforms have been described, the most frequent lacking either only exon 2, causing frame shift and premature stop; or exon 5, corresponding to the octamer domain; or both exons 8 and 9 which encode the transactivation domain. In leukemic blast cells, these variants are more abundant compared to full length wild type *PAX5*, and are predicted to code for less functional or even completely non-functional *PAX5* proteins.

Point mutations have been found in about 7% of both adult and paediatric cases. In childhood leukaemia cases, point mutations were hemizygous in 14/192 of the cases (7.3%) while only 1 case was homozygous (1/192, 0.5%) (Mullighan et al., 2007). A recent study reported a higher rate of *PAX5* point mutations compared to the previous investigations. Point mutations were found in 9/50 (18%) adults and in 14/50 (28%) children, showing novel sites of mutations scattered along all the exons (Santoro et al., 2009). Modelling studies using the *PAX5* crystal structure suggested that point mutation should either impair DNA binding, alter transcriptional regulation, or cause frame shift, splice site, or missense mutations. A single case with an exon 1B frame shift mutation resulted in a prematurely truncated ten-residue peptide.

Altogether, point mutations are hemizygous, somatically acquired; they result in reduced expression of *PAX5* mRNA, and lost or altered DNA-binding or transcriptional regulatory function (Mullighan et al., 2007; An et al., 2008). Therefore they generate hypomorphic *PAX5* alleles with reduced gene function that could lead to haploinsufficiency.

An overview of point mutations is represented in Fig. 3.

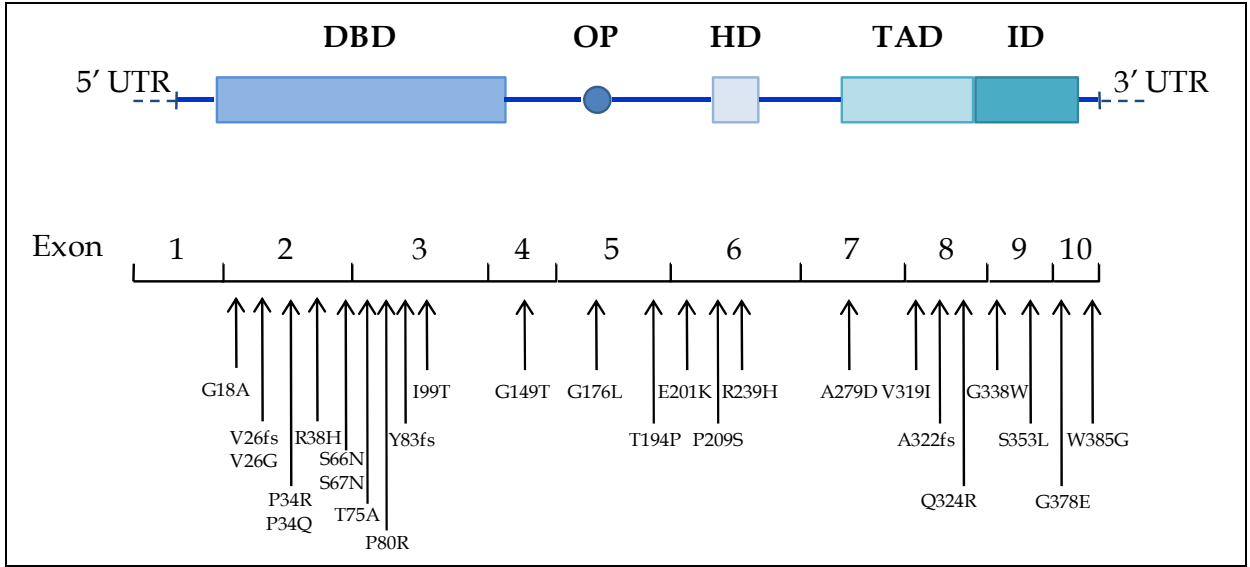


Fig. 3. *PAX5* point mutations. DBD, DNA-binding paired domain; OP, conserved octapeptide; HD, partial homeodomain; TAD, transactivation domain; ID, inhibitory domain.

Amplifications of the *PAX5* gene are less common and include: a) partial amplification, which covers a subset of internal exons (in particular from exon 2 to 5/6); b) complete amplification, which targets the full sequence of the gene (Mullighan et al., 2007; Familiades

et al., 2009). It has been proposed that focal amplification of *PAX5* exons 2–5 is predicted to abolish expression of normal *PAX5* from the amplified allele, although further research is required to clarify the importance of this rare genetic lesion.

Epigenetic mechanisms have been investigated to explain lower *PAX5* gene expression, in absence of classical rearrangements (e.g. point mutations and deletions) (Mullighan et al., 2007). However, although high-levels of the *PAX5* promoter methylation were detected in T-ALL, only a minimal methylation level was observed in BCP-ALL, independently by *PAX5* mutational status.

### 3.1.2 *PAX5* translocations, a new promiscuous gene with several partners

While the t(9;14) translocation in lymphoma gives rise to *PAX5* de-regulated expression, in ALL, *PAX5* is involved in a growing number of chromosomal translocations with several partner genes, resulting in *in frame* fusion genes, with an estimated frequency of 2-3% in paediatric BCP-ALL (Mullighan et al., 2007; Nebral et al., 2009).

A revision of the literature is reported in Table 1.

Since the first case encoding for *PAX5/ETV6* was identified in 2001 (Cazzaniga et al., 2001), as many as 21 known partner genes have been identified in several chromosomes, although chromosomes 7, 12 and 20 are the most frequently involved, with different genes on each chromosome. Interestingly, *PAX5* genetic lesions are typically associated to dicentric chromosomes, although the breakpoint on the short arm of chromosome 9 is very heterogeneous (An et al., 2008). *PAX5* was rearranged in 54 out of 110 paediatric patients carrying a dicentric chromosome: 26/38 of the cases carrying the dic(9;12)(p11~13;p13), 24/59 positive for the dic(9;20)(p11~13;q11) and in 4/13 of the cases harbouring the dic(7;9)(p11;p11~13) (An et al., 2008). Dicentric chromosomes can coexist with established chromosomal changes, for example, dic(7;9) is found in association with t(9;22)(q34;q11) (*BCR/ABL1* fusion), and dic(9;12) occurs with t(12;21)(p13;q22) (*ETV6/RUNX1* fusion), suggesting that these events may cooperate.

A more recent study confirmed the involvement of the *PAX5* gene in dicentric chromosome events in adult and childhood BCP-ALL (Coyaud et al., 2010a). In this study, 40 patients (14 adult and 26 paediatric) harboured a dicentric chromosome involving chromosome 9 with various different partners (such as, 7, 8, 12, 15, 16, 17, 20) and as much as 90% of the cases involved *PAX5* alterations.

The various fusion genes can be classified in different sub-groups according to the molecular function of the *PAX5* partner gene, which can encode either for a transcription factor (e.g. *ETV6/TEL*, *PML*, *FOXP1*, *ZNF521/EVI3*, *BRD1*, *DACH1*), for proteins related to transcription regulation (*HIPK1*, *NCOR1*), structural proteins (*ELN*, *POM121*, *LOC39027*, *KIF3B*), kinases (*JAK2*, *TAOK1*), carriers of molecules (*SLCO1B3*) or co-activator proteins (*ASXL1*). The implicated genes encode less frequently for molecules of unknown function (e.g. *C20orf112*, *AUTS2*, *GOLGA6*). Among this plethora of fusion genes, *PAX5/ETV6* originating from the translocation t(9;12) or dic(9;12) is the most common.

A common feature of *PAX5* translocations is that they result in the fusion of the 5' N-terminal DNA-binding domain of *PAX5* (*PAX5-DBD*) with the 3' C-terminal sequences of the partner gene, whose domains therefore substitute *PAX5* regulatory domains. The breakpoints are mostly located after exon 5 of *PAX5*, or after exon 4, at the end of the *DBD* region. Cases with a more distal *PAX5* breakpoint are less common and retain additional domains, including the highly conserved Octamer domain.



Fusion gene	Chromosomal translocation	PAX5 ex-Partner ex	n° pt	Function of partner gene	Literature Reference
PAX5/HIPK1	t(1;9)(p13;p13)	ex5-ex9	1°	Transcriptional Regulator	(Nebral et al., 2009)
PAX5/FOXP1	t(3;9)(p14;p13)	ex6-ex7	1°	Transcription factor	(Mullighan et al., 2007)
		ex5-ex12	1°		(Kawamata et al., 2008)
		ex6-ex7	2*		(Coyaud et al., 2010a)
PAX5/ELN	t(7;9)(q11;p13)	ex7-ex2	2°*	Structural protein	(Bousquet et al., 2007)
			1#		(Coyaud et al., 2010a)
PAX5/AUTS2	t(7;9)(q11.2;p13.2)	ex6-ex4	1°	Unknown	(Kawamata et al., 2008)
		ex6-ex6	1°		(Coyaud et al., 2010b)
PAX5/LOC392027	dic(7;9)(p12;p13)	ex4-ex2	1°	Structural protein	(An et al., 2008)
PAX5/POM121	t(7;9)(q11;p13)	ex5-ex5	1°	Structural protein	(Nebral et al., 2009)
		ex5-ex4	1°		(Coyaud et al., 2010a)
PAX5/JAK2	t(9;9)(p13;p24)	ex5-ex19	2°	Kinase	(Nebral et al., 2009)
		ex5-ex19	1°		(Coyaud et al., 2010a)
JAK2/PAX5	t(9;9)(p13;p24)	ex18-ex6	2°	Kinase	(Nebral et al., 2009)
PAX5/???	t(9;11)(p13;p?)	?	1°		(Nebral et al., 2009)
PAX5/ETV6	t(9;12)(q11;p13)	ex4-ex3	1*	Transcription factor	(Cazzaniga et al., 2001)
	dic(9;12)(p13;p13)	ex4-ex3	2°		(Strehl et al., 2003)
		ex4-ex3	2°		(Mullighan et al., 2007)
		ex4-ex3	2°		(Kawamata et al., 2008)
		ex4-ex3	7°		(An et al., 2008)
		ex4-ex2	1°		(An et al., 2008)
		ex4-ex3	3°*		(Coyaud et al., 2010a)
PAX5/SLCO1B3	dic(9;12)(p13;p12)	ex4-ex2	1°	Solute carrier transporter	(An et al., 2008)
PAX5/DACH1	t(9;13)(p13;q24)	ex5-ex5	1°	Transcription factor	(Nebral et al., 2009)
PAX5/???	t(9;14)(p13;q32)	?	1°		(Nebral et al., 2009)
PAX5/PML	t(9;15)(p13;q24)	ex6-ex2	2°	Transcription factor	(Nebral et al., 2007)
PAX5/GOLGA6	t(9;15)(p13;q24)	ex6-ex3	1*	Unknown	(Coyaud et al., 2010a)
PAX5-truncated	dic(9;16)(p13;q11)	intr5/6-?	3°**	Unknown partner gene	(Coyaud et al., 2010a)
PAX5/NCoR1	t(9;17)(p13;p11)	ex5-ex43	1°	Component of HDAC	(Coyaud et al., 2010a)
PAX5/TAOK1	t(9;17)(p13;q11)	ex5-intr19	1°	Kinase	(Coyaud et al., 2010a)
PAX5-ZNF521	t(9;18)(p13;q11)	ex7-ex4	1°	Transcription factor	(Mullighan et al., 2007)
PAX5/C20orf112	dic(9;20)(p13;q11)	ex5-ex8	1°	Unknown	(Kawamata et al., 2008)
		ex8-ex3	2°		(Kawamata et al., 2008)
		ex7-ex6	1°		(An et al., 2008)
		ex8-ex8	1°		(Nebral et al., 2009)
PAX5/ASXL1	dic(9;20)(p11;q11)	ex4-ex4	1°	Co-activator for RA receptor	(An et al., 2008)
		3' reg-ex1/4	1°		(An et al., 2009)
PAX5/KIF3B	dic(9;20)(p13;q11)	ex7-ex6	1°	Structural protein	(An et al., 2008)
PAX5-trunc/PLAGL2	dic(9;20)(p13;q11)	intr6/7-ex3	1°	Zinc-finger protein	(Coyaud et al., 2010a)
PAX5/BRD1	t(9;22)(p13;q13)	ex5-ex1	1°	Transcription factor	(Nebral et al., 2009)
PAX5/DACH2	t(X;9)(q21;p13)	ex5-ex3	1°	Transcription factor	(Coyaud et al., 2010a)

°=paediatric patient (<16 years old) ; #=adolescent patient (16-18 years old); \*=adult patient (>18 years old)

Table 1. Reported PAX5 translocations and corresponding fusion genes.



As a further feature, *PAX5* translocations are unbalanced (An et al., 2008), and therefore the reciprocal fusion gene (5' partner gene to 3' *PAX5*) is not preserved, with the sole known exception of the translocation, t(9;9)(p13;p24), encoding for *PAX5/JAK2* (Nebral et al., 2009). *ETV6/TEL*, *ELN* and *FOXP1* are recurrent partner genes, independent of patient age, as well as translocation events giving rise to *PAX5*-truncated isoforms.

Although the biological significance of *PAX5* translocations has not been elucidated yet, it has been demonstrated that patients with dicentric chromosomes carrying either a deletion or a fusion significantly under-expressed the *PAX5* gene, thus indicating that both genetic alterations can result in a similar reduced expression of wild-type *PAX5* (An et al., 2008). These data would suggest that *PAX5* haploinsufficiency has a major consequence in patients. However, this is not supported by the *in vivo* studies, B-cell development being normal in heterozygous *PAX5*<sup>+/-</sup> mice (Cobaleda et al., 2007b). Moreover, inactivation of one *PAX5* allele in the absence of other oncogenic lesions is not sufficient to induce tumour development in heterozygous *Cd19-cre Pax5*<sup>fl/-</sup> mice. Instead, the complete loss of *PAX5* in B cells leads to an aggressive progenitor cell lymphoma, thus identifying *PAX5* as a tumour suppressor gene of the different B-lymphoid lineage malignancies (Cobaleda et al., 2007b). Furthermore, even though in some cases the translocation events lead only to a truncated form of *PAX5*, it can be speculated that the role of *PAX5* in this setting can be different from deletions or point mutations. The fusion protein could retain the functional activities of the partner genes and therefore it may alter the molecular function of the normal counterparts, affecting both the partner gene and wild type *PAX5* functions. Indeed, many of the *PAX5* fusion genes have a dominant negative role on normal *PAX5*; in fact, in leukemic blasts, expression analysis revealed that its transcriptional targets (e.g. *EBF1*, *FLT3*, *ATP9A*, *ALDH1A1*) are repressed in dicentric cases, indicating a reduced activity of wild type *PAX5* (An et al., 2008). Despite these data, *CD19* and *CD79a* expression (direct *PAX5* targets) did not correlate with *PAX5* altered status (Mullighan et al., 2007).

Comprehensively, several fundamental questions on *PAX5* involvement in leukemogenesis are still unresolved. Two points remain to be understood: 1) whether these genetic alterations are responsible for the disruption of *PAX5* functions, generating a haploinsufficiency setting; 2) whether these lesions are more generally responsible for the B-cell development block, through deregulation of *PAX5* control, which is crucial for normal B lymphopoiesis. A more important role for fusion genes cannot be excluded, in which a gain of function could lead to cell transformation. *In vitro* and *in vivo* studies will be helpful to better elucidate the role of fusion genes.

The molecular and functional role of most relevant fusion genes will be addressed in paragraph 4.

#### 4. Molecular and functional analysis of *PAX5* fusion genes

Although a huge amount of work has to be done to characterize the functional role of the *PAX5* fusion proteins, we have revised the functional studies reported in literature to discuss the most corroborated hypothesis, that these fusion proteins could act as aberrant transcription factors, deregulating the physiological pathway of wild type *PAX5*, and potentially the normal function of the partner gene as well.

Since the availability of patient material is always a limiting factor, human and mouse biological *in vitro* models have been developed to make the functional investigations of *PAX5* fusion proteins possible. Among the different models, a common strategy has been

employed to transiently or stably transfect cells by a plasmid that contains the sequence of a variously tagged fusion gene cloned from a patient.

Here, we focus on studies regarding *PAX5/ETV6*, *PAX5/FOXP1*, *PAX5/PML*, *PAX5/ELN* and *PAX5/C20orf112* fusion genes, all of which are in frame genes and are predicted to be translated into protein. These partner genes are representative of different functional classes, being transcription factors (*ETV6*, *FOXP1*, *PML*), structural proteins (*ELN*) or unknowns (*C20orf112*).

The *ETV6* (or *TEL*) gene encodes for a transcription factor with suppressor function, belonging to the highly conserved ETS family, which is fundamental for the haematopoietic system. It is widely acknowledged that it is involved in several translocations giving rise to different fusion genes in various haematological malignancies. Among them, *ETV6/AML1* is the most recurrent in childhood BCP-ALL (Bohlander, 2005).

*Forkhead Box P1* (*FOXP1*) is a member of the FOX family of evolutionarily conserved transcriptional regulators, which have a broad range of functions. *FOXP1* is widely expressed and has been shown to have a role in cardiac, lung and lymphocyte development. In mice, *FOXP1* deficiency is associated with a block in transition from pro-B cells to mature B cells. *FOXP1* is targeted by recurrent chromosome translocations and its over-expression confers a poor prognosis in a number of types of lymphomas, suggesting that it may function as an oncogene (Koon et al., 2007; Myatt & Lam, 2007).

The *Promyelocytic leukaemia* gene (*PML*) encodes for a protein which is a member of the tripartite motif (TRIM) family. The TRIM motif includes three zinc-binding domains, a RING, a B-box type 1 and a B-box type 2, and a coiled-coil region. This phosphoprotein functions as a transcription factor and tumour suppressor. The gene is often involved in translocation with the *Retinoic Acid Receptor alpha* (*RARα*) gene associated with acute promyelocytic leukaemia (APL), a relatively rare form of acute myelogenous leukaemia (AML) (Gregory & Feusner, 2009).

The *ELN* gene encodes for the protein elastin, an extracellular matrix macromolecule that imparts arterial elasticity and plays a critical role in maintaining vessel integrity and elastic properties under pulsatile flow. Elastin plays an important role in signalling and regulating luminal endothelial cells and smooth muscle cells in the arterial wall (Waterhouse et al., 2011).

The function of *C20orf112* is totally unknown; it is described as involved in translocation events, with the *RUNX1* gene in AML (Guastadisegni et al., 2010) and with the *PAX5* gene in ALL. Kawamata et al. identified two different rearrangements of *PAX5/C20orf112*, which were defined as long or short respectively, since in one case with dic(9;20), exon 5 of *PAX5* was fused to exon 8 of *C20orf112* (*PAX5/C20L*), and in the other case, with dic(9;20), exon 8 of *PAX5* was fused to exon 3 of *C20orf112* (*PAX5/C20S*) (Kawamata et al., 2008).

#### 4.1 Sub-cellular localization, DNA binding ability and transcription activity of *PAX5* fusion genes

*PAX5* encodes for a transcription factor, which by definition localizes in the nucleus and regulates several target genes through the binding of a specific consensus sequence on DNA (Delogu et al., 2006; Schebesta et al., 2007). Therefore, the first issues to be addressed are where *PAX5* fusion proteins localize in the cell and whether they conserve the ability to bind DNA, with an effect on the transcription process.

By confocal analysis in transfected NIH3T3, PAX5/ELN has been demonstrated to localize exclusively in the nucleus (Bousquet et al., 2007), as we showed for PAX5/ETV6 (Fazio et al., 2008). While PAX5/ELN retains the nuclear localization signal (NLS) of wt-PAX5, this domain is not retained in the PAX/ETV6 fusion protein, but its activity could be exerted by the corresponding ETV6 NLS. By sub-cellular fractionation and western blot analysis in transfected 293T cells, it has been shown that PAX5/ETV6, PAX5/FOXP1 and PAX5/C20S were present mainly in the nucleus and less abundant in the cytoplasm, while PAX5/C20L was exclusively nuclear (Kawamata et al., 2008). In agreement with these data, fluorescent microscopic examination of transiently expressed proteins in HeLa and 293T cells demonstrated that PAX5/PML exhibited a diffuse granular pattern within the nucleus, similar to PAX5 but not to PML (Qiu et al., 2011).

To support the aberrant transcription factor activity, the DNA binding activity of PAX5 fusion genes has been assessed by a direct (through electrophoretic mobility shift assay, EMSA) or indirect (such as the luciferase reporter assay) binding measure. Kawamata demonstrated the specific binding for PAX5/ETV6, PAX5/FOXP1 and PAX5/C20orf112 in parallel to wild type PAX5 (Kawamata et al., 2008). In an analogous setting, it has been demonstrated that PAX5/PML is able to bind to PAX5-responsive elements (Qiu et al., 2011). A formal demonstration of DNA binding by PAX5/ELN is missing in literature, although the transcriptional activity of PAX5 fusion genes, including the PAX5/ELN, has been proven by reporter gene assays (Bousquet et al., 2007; Mullighan et al., 2007; Kawamata et al., 2008; Kurahashi et al., 2011; Qiu et al., 2011).

#### 4.2 Fusion genes have a dominant effect on wild type PAX5

The PAX5 fusion proteins herewith described retained the DNA-binding domain of PAX5 but substituted its regulatory domains with those of the partner gene, which are mostly responsible for transcription regulation.

PAX5/ETV6 fusion merits a special focus for the central role of the ETV6/TEL partner gene in onco-haematology, and because PAX5/ETV6 is the most common PAX5 translocation.

We previously showed that PAX5/ETV6 maintains the transcriptional activity common to both partner genes and it is responsible for the down-regulation of key PAX5-targets, such as CD19, BLNK/SLP-65 and MB-1/CD79a through recruitment of the mSIN3a co-repressor (Fazio et al., 2008). We investigated PAX5/ETV6 function through gene expression profile (GEP) analysis by stable transduction of the PAX5/ETV6 construct in wild type (wt) pre-BI cells, a primary culture of immature B-cells (cKit+/B220+/CD19+) from mouse fetal liver, which express the endogenous wild type PAX5 protein (Fazio et al., 2008). GEP analysis defined a PAX5/ETV6 specific signature, indicating that PAX5/ETV6 acts mainly as a repressor of transcription but, surprisingly, the fusion protein was also able to activate transcription. Among DEGs we identified a reliable number of genes (7%) known to be direct transcriptional targets of PAX5 (Delogu et al., 2006; Schebesta et al., 2007). These results strongly suggest that PAX5/ETV6 primarily affects the PAX5 transcriptional pathway by repressing PAX5-target genes normally activated by wt PAX5 and by up-regulating genes known to be PAX5 wt-repressed. This supports a dual function for PAX5/ETV6 on transcription, which is mainly a repressor but also to a lesser extent, an activator. Therefore, PAX5/ETV6 does not exert a canonical “dominant negative” effect on the endogenous protein whilst it does have an “opposite dominant” function on wt PAX5 (Fazio et al., 2010).

A canonical *dominant negative* effect of fusion over wt PAX5 was demonstrated for PAX5/ELN, PAX5/FOXP1, PAX5/PML and PAX5/C20orf112. As in the context of PAX5/ETV6, various PAX5 transcriptional targets are repressed by PAX5 fusion proteins, such as *CD19*, *MB-1/CD79a*, *BLNK*, *NEDD9*, *NEDD5*, *ATP1B1*, *TCF7L2*, resulting in reduced PAX5 wild type activity.

Certainly, the molecular mechanism through which repression is achieved includes the competition for binding the consensus sequence on DNA between wt PAX5 and its fusion protein. A novel insight has been provided by the study on PAX5/PML (Qiu et al., 2011), in which they showed that PAX5/PML forms homodimer to bind the PAX5 consensus sequence through the Coiled-Coiled (CC) domain of PML. Furthermore, PAX5/PML can form complexes with PAX5/PML as well as wild-type PML. The intranuclear mobility of PAX5/PML is decreased compared with wild-type PAX5, in analogy to other fusion proteins in leukaemia, such as PML/RAR $\alpha$  and AML1/ETO (Dong & Blobe, 2006; Qiu et al., 2011).

Since other PAX5 fusion proteins from ALL patients such as PAX5/FOXP1 and PAX5/EVT6 maintain the dimerization motif of the PAX5 partner, the aberrant molecular mechanism proposed for PAX5/PML can be valid for other PAX5 fusion genes as well.

#### 4.3 Functional consequences of PAX5 fusion genes on cellular processes in precursor B cells

Preliminary results indicate that the wt PAX5 protein is present at lower levels in cells positive for translocation (An et al., 2008). In patients with PAX5 translocation, we see that one PAX5 allele is involved in the translocation event, while the second allele could be partially repressed and also hindered by the fusion protein in its function, by competition and by dynamic deregulation of target genes. Moreover, PAX5 mutations result in reduced levels of PAX5 protein and corresponding activity (Mullighan et al., 2007).

In the B-cell context, the biological consequences of decreased normal PAX5 function plus the opposite dominance of the fusion protein could be various.

A pathway analysis of GEP results revealed the involvement of several cellular processes, specifically: B-Cell Receptor assembly and B-cell maturation, cell adhesion and migration (our unpublished observations). PAX5/ETV6 cells are impaired to complete the IgM rearrangement, and consequently the precursor B cells are immature and unable to complete the differentiation process (Mullighan et al., 2007; Fazio et al., 2008). This could be due to the direct down-regulation of pre-BCR components, such as the  $\mu$  chain itself, Ig $\alpha$ /MB-1, and BLNK/SLP-65. This incapacity to express a pre-BCR on the cell surface has already been demonstrated in primary leukemic ALL blast cells, in the presence of the BCR/ABL1 fusion gene (Trageser et al., 2009). It is possible to speculate that the survival of precursor B cells carrying a PAX5 fusion gene could be guaranteed by alternative signalling pathways.

Overall, these data suggest that PAX5 behaves like a tumour suppressor in early B cells, and that impairment of its function can be associated with the development of ALL (Kawamata et al., 2008).

#### 4.4 Future perspective of functional studies

Preliminary results indicate that PAX5/ETV6 cells have a significant advantage over *in vitro* migration to CXCL12 (Fazio et al., 2008). In humans, cells with aberrant PAX5 could therefore have a greater ability to aggressively infiltrate CXCL12-secreting tissues and organs, and thus proliferate and survive better, as described in several tumours (Burger & Burkle, 2007).



In the near future, the *in vivo* mouse models will be crucial for studying the role of translocation on leukemogenesis. Currently, the xenotransplantation of human primary patient cells into immune-deficient mice is the 'gold standard' assay to identify human leukemic stem cells and functionally assess their tumorigenicity *in vivo*. The availability of primary samples from patients carrying a *PAX5* fusion gene will shed light on the human setting.

Among the main questions in leukaemia research is how and in what temporal sequence the genetic lesions lead to abnormal proliferation and differentiation arrest of lymphoid progenitors.

Beside a dissection of the role of *PAX5* translocations in leukaemia, several additional questions on *PAX5* aberrancies in ALL are still unresolved, namely:

- Are *PAX5* translocations responsible for blocking B-cell development?
- Are *PAX5* translocations per se sufficient in leukemogenesis?

## 5. Are *PAX5* alterations driver or passenger lesions?

Studies on ALL patients and/or *in vitro* models concentrated the efforts to understand whether *PAX5* alterations are part of a complex scenario of cooperating genetic lesions or whether they are a unique genetic aberration event that drives leukemogenesis.

Patients carrying *PAX5* deletion have a more complex karyotype than patients with translocation or dicentric chromosomes. Frequently, classical deletion events or dicentric chromosomes giving rise to prematurely truncated *PAX5* transcripts have been described as co-existing in leukemic blast cells together with other *PAX5*-unrelated genetic lesions, such as *ETV6/AML1* (Mullighan et al., 2007), *BCR/ABL1* and *TCF3/PBX1* fusion genes (Paulsson et al., 2008; Den Boer et al., 2009; Familiades et al., 2009; Iacobucci et al., 2010). This observation led us to hypothesize that there is a cooperative role for *PAX5* deletions over the main genetic lesion. Similarly to micro-deletions on fundamental genes in haematopoiesis (such as *IKZF1*, *EBF1*, *TCF3*, *LEF1*) (Mullighan et al., 2007; Paulsson et al., 2008), *PAX5* aberrancies can contribute to blocking cell development in B cell precursors, as reflected in the mouse model of homozygous *PAX5* deletion, which is characterized by complete blockage at an early stage of differentiation (Nutt et al., 1999).

By contrast, patients with *PAX5* fusion genes (as a consequence of translocations) were mainly reported as negative for the most common genetic aberrations found in childhood (Nebral et al., 2009; Coynaud et al., 2010a).

A high resolution Copy Number Abnormality (CNA) analysis on diagnostic samples with *PAX5* fusion genes revealed that in both childhood and adult ALL, *PAX5*-translocated cases have a simple karyotype (Coynaud et al., 2010a), with a mean of only three lesions in addition to the translocation event itself (our unpublished observation).

In conclusion, it becomes evident that *PAX5* translocations giving rise to fusion genes have a completely different impact on the biology and development of leukaemia compared to *PAX5* deletions. It can be argued that gene fusions occur early, whereas deletions can be regarded as a late/secondary event (Coynaud et al., 2010a). We can therefore presume that *PAX5* fusion genes are driver genetic lesions, whereas *PAX5* deletions can be a cooperative aberrancy, which require further alteration to determine the disease.

The final word on the real impact of *PAX5* alterations will come in the near future from robust functional studies, including the development of an appropriate *in vivo* leukemogenesis model.



## 6. Are PAX5 alterations associated to prognosis and/or outcome? Is PAX5 a new target for leukaemia treatment?

Although the frequency and the molecular nature of PAX5 aberrancies are analogous, the complexity and biological differences between adult and childhood B-ALL may reflect another example of the importance of PAX5 alterations on the disease course.

Gene expression profiling (GEP) of childhood ALL has identified a genetic subtype of childhood ALL with a poor outcome, that clusters close to the subgroup characterized by the *BCR/ABL* fusion gene (thus called '*BCR/ABL* like'). Patients in this group carry mutations in genes that are fundamental in B-cell development, such as *IKZF1*, *PAX5*, *VPREB1*, *TCF3*, *EBF1* and pre-*BCR* (Den Boer et al., 2009; Mullighan et al., 2009).

A study by Mullighan et al. showed that *PAX5* abnormalities were not associated with an unfavourable prognosis, in contrast to the *BCR/ABL*-like subtype (Mullighan et al., 2007). Deletions in the *BCR/ABL*-like group not only affect the *PAX5* gene locus but often include larger regions on chromosome 9p and other genes involved in B-cell development. This suggests that the *BCR/ABL*-like group does not exactly overlap with the subgroup of BCP-ALL patients carrying exclusively *PAX5* abnormalities.

A genome-wide DNA copy number analysis on matched diagnosis and relapse samples from paediatric patients with ALL detected an increasing number of additional regions of deletion at relapse, including the *PAX5* and *IKZF1* genes. This suggests that genomic abnormalities contributing to ALL relapse, including *PAX5* alterations, are selected during treatment, paving the way for designing a new therapeutic intervention to target these abnormalities (Mullighan et al., 2008).

Even in adult ALL, *PAX5* alterations are not associated to outcome (Familiades et al., 2009), except for a reported trend toward a higher incidence of relapse in the structural mutant *PAX5* subgroup.

Indeed, deletions of *PAX5* were not significantly correlated with overall survival, disease-free survival or cumulative incidence of relapse in *BCR/ABL1*-positive ALL adult cases treated with conventional or investigational therapy including TKI (imatinib or dasatinib) (Iacobucci et al., 2010).

In conclusion, the state of the art on *PAX5* alterations and prognosis gives evidence that no significant correlation exists in paediatric or in adult BCP-ALL. Nevertheless, considering the biology of the different subgroups of genetic lesions (point mutations vs. deletions vs. translocations), we cannot completely exclude the possibility that the sole translocation event may have a different significance and influence on the clinical course of the disease, although the limited number of cases in the reported studies hamper a statistically significant analysis.

We need further clinical data to show whether *PAX5* could be a new target for leukaemia treatment, and to inspire the design of a strategy to target the molecule itself or to interfere with its aberrant activity.

## 7. Conclusion

The *PAX5* gene, encoding for a transcription factor fundamental for B-cell development, is the most recurrent target of genetic lesions in BCP-ALL disease, both in the adult and paediatric population. Its alterations are a hallmark which defines a new molecular subgroup of patients.

Although in general, PAX5 translocations do not influence the prognosis or outcome by themselves but in association to other molecular aberrancies, we can hypothesize that they could play a different role from deletions and point mutations.

Further clinical investigations are needed to assess the real impact of PAX5 alterations on disease progress. In parallel, we believe that *in vitro* and *in vivo* models will help to clarify the biological basis of leukemogenesis driven by PAX5, especially in presence of its fusion genes.

If we are able to fill these biological and clinical gaps, we could recognize the altered PAX5 molecule as a novel target to hit, with the final purpose of eradicating leukaemia.

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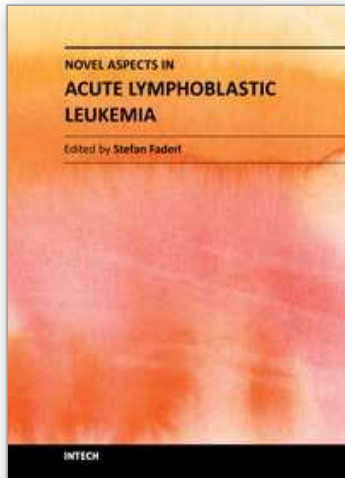
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Acute lymphoblastic leukemia (ALL) has turned from a universally fatal to a highly curable disease in little more than four decades. Even though differences in outcome continue to exist between children and adults, intense efforts are under way to overcome this discrepancy and improve the prognosis of adult patients as well. This exemplary progress in ALL therapy has been possible by the combination of an increasingly better understanding of the biology of the disease, availability of a range of effective drugs, and astute designs and relentless executions of many clinical trials. ALL is a complex disease requiring complex therapy. Whereas this book cannot provide a comprehensive review of every one of its many facets, the chapters from many investigators from around the world nevertheless cover a number of relevant topics: aspects of the epidemiology of ALL in Hispanics, ophthalmologic manifestations of ALL, overviews of current therapy and drug-resistance mechanisms, novel biological pathways and targets, new drugs in development, and long-term consequences of CNS prophylaxis and therapy. The publishers and editor therefore hope that the prospective readers will find enough insight and information for their own endeavors.

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