PAX5 and B-cell neoplasms: transformation through presentation

Andrei Thomas-Tikhonenko[†] *& Diana Cozma*

[†]*Author for correspondence*
Department of Pathobiology and Mari Lowe Center for Comparative Oncology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia PA 19104–6051, USA
Tel.: +1 215 573 5138;
Fax: +1 215 746 0380;
andreit@mail.vet.upenn.edu

'Most diffuse large B-cell lymphomas and other non-Hodgkin lymphomas are derived from mature B cells, which express B-cell receptors and have often undergone maturation in germinal centers.'

What ever Pax5 soweth...

The salient features of B-cell development are immunoglobulin gene rearrangements, class switching recombinations (CSRs) and somatic hypermutations (SHMs). Collectively, these processes account for the diversity of antibodies, as well as for affinity maturation of antibodies in germinal centers. However, the propensity of B cells to undergo genetic alterations has unintended consequences – that is, a high frequency of oncogenic events. These events clearly inform lymphomagenesis: translocated and otherwise altered genes inevitably turn out to be oncogenes playing an important role in tumor initiation. For example, Burkitt's and some diffuse large B-cell lymphomas (DLBCLs) carry the t(8;14) translocation that places the MYC proto-oncogene under control of the Igh enhancer (for a recent review see [1]).

Another transcription factor affected by translocations is Pax5. Pax5 belongs to a family of nine nuclear proteins that are thought to control the tissue-specific transcription necessary for many types of cell differentiation. Pax5 is the only family member found within the hematopoietic system, and its expression is restricted to certain stages during B-cell differentiation [2]. It is largely absent from multipotent progenitors and common lymphoid progenitors [3], but its expression is initiated in pre-pro-B cells and then maintained throughout subsequent stages of B-cell development [4] before it is downregulated in plasma cells [5]. However, Pax5 is unique among other lineage-specific transcription factors in that it is both a driving force behind and one of the primary beneficiaries (or perhaps sufferers) of genomic alterations.

Indeed, one of its important targets is the *Rag-2* gene encoding recombination-activating protein 2 [6,7]. During V_H–DJ_H recombination, the RAG1/RAG2 complex introduces double-stranded DNA breaks that ultimately rejoin to generate functional heavy chain cassettes. The same process is thought to underlie illegitimate recombination, during which Ig heavy chain enhancers will be juxtaposed to one of the B-cell oncogenes such as *Myc*, *Bcl2*, *Bcl1* and so on. In addition to activating the *Rag2* gene, Pax5 occupies V_H genes in early human and mouse B-lineage cells, induces Igh locus contraction [8], and physically interacts with the RAG1/RAG2 complex, thus promoting recombination events [9]. Importantly, the V_H–DJ_H recombination cannot happen in the presence of lysine-9-methylated histone H3, and the removal of this inhibitory signal in B cells is an important function of Pax5 [10]. Consequently, only a minor fraction of lymphocytes in Pax5-null mice have completed V_H–DJ_H rearrangement and most are arrested at the earlier, pro-B stage [11]. Recent data indicate that Pax5 might also play a role in light chain locus contraction [12]. Additionally, Pax5 is known to be required for the expression of Igα [13], a sIgM-associated signaling molecule, which is the key component of the B-cell receptor (BCR; reviewed in [14]).

CSR, which might also contribute to oncogenic translocations, and SHM are less well-understood processes. However, recent work has implicated activation-induced cytidine deaminase (AID) in both phenomena. Interestingly, Pax5 is known to activate AID [15,16] and can therefore contribute to CSR and SHM also. Overall, while Pax5 might be a guardian of B-cell identity [2], it is a curiously permissive one with respect to genome integrity.

...he shall also reap

The cruel irony is that while Pax5 loyally acts on behalf of B cells to ensure their genetic diversity, it is one of the first to suffer from that diversity. Indeed, either an erroneous V_H–DJ_H rearrangement or CSR are likely responsible for the relatively rare [17], but persistent, t(9;14) (p13; q32) translocation [18–20] associated with aggressive

10.2217/14796694.4.1.5 ISSN 1479-6694

B-cell non-Hodgkin lymphomas [21]. This translocation usually brings together the coding sequences of PAX5 and a strong enhancer from the Igh locus, ensuring robust transcription of the translocated *PAX5* allele. In addition to disturbing Pax5-guided gene expression during lymphocyte differentiation, the translocation can potentially lead to failed PAX5 repression at the onset of plasma cell differentiation [2].

'In contrast, most of B-progenitor acute lymphoblastic leukemia (so-called A1 and A2 types) are derived from immature pro- or pre-B-cells lacking B-cell receptors.'

In addition to genomic rearrangements, the *Pax5* gene is also affected by SHM. In the high-profile 2001 study, more than 50% of human germinal center-derived DLBCLs were found to contain mutations in the proto-oncogenes *PIM*, *MYC*, *RhoH*/*TTF* (*ARHH*) – and Pax5 [22]. In the latter, SHM were identified downstream of both transcription initiation sites, predominantly around exon 1B, at the frequency of 0.15 per 100 bp. Although three missense mutations were detected within the short coding portion of exon 1B, most of the changes involved 5′ untranslated and other noncoding sequences [22]. This distribution implied that the main consequence of these gain-of-function SHMs is transcriptional activation of Pax5 and, plausibly, B lymphomagenesis.

Two studies using shRNA-mediated gene knockdown appear to corroborate this notion. The RNAi experiments were performed *in vitro* on two cell lines, SUDHL-4 and -6, belonging to the germinal center B cell-like subclass of DLBCL [23]. In the first study, the authors achieved knockdown of the key downstream effector of Pax5 Igα (also known as CD79a) [24]; in the second study, from our laboratory, the *Pax5* gene itself was targeted [25]. Under both experimental conditions, decreases in cell accumulation were observed, supporting the oncogenic role of *Pax5*. However, other recent data suggest that the picture might be much more complicated.

Acute lymphoblastic leukemia versus non-Hodgkins lymphoma: why does it matter?

The surprising findings came from the analysis of B-progenitor acute lymphoblastic leukemia (B-ALL), a common pediatric malignancy. It turned out that Pax5 is important for B-ALL pathogenesis – but not in the way one would expect. In their December 2006 *Blood* paper, Bousquet *et al*. report a novel t(7;9)(q11;p13) translocation in two B-ALL patients, resulting in the fusion of the *Pax5* and elastin (*ELN*) genes [26]. Fusions between *Pax5* and various translocation partners are not new. For example, the previously described t(9;12)(q11;p13) translocation generates a fusion transcription factor containing DNA-binding domains of both *Pax5* and ets variant gene 6 (*ETV6*)/translocation, ets, leukemia (*TEL*) [27]. What is interesting regarding the *Pax5–ELN* fusion is that not only is it loss-of-function, but it also acts as a dominant-negative isoform in transient expression assays [26].

In parallel, Mullighan *et al*. embarked on the genome-wide analysis of B-ALL using high-resolution SNP arrays and direct genome sequencing. Surprisingly, the two most commonly mutated genes were *Pax5* and its upstream regulator, early B cell factor 1 (*EBF1*) [28]. Again, the mutations identified were, without exception, loss-of-function: monoallelic deletions of *EBF1* and *Pax5*, cryptic transloctions removing the Pax5 activation domain, frameshift mutations in *Pax5*, and so on. Importantly, both transient expression assays and the analysis of *PAX5* target genes confirmed that Pax5 function is consistently reduced (although not completely abolished) in B-ALL cases. The authors conclude that subtle changes in the dosage of *PAX5*, rather than its overexpression, underlie the pathogenesis of ALL [28]. While this might certainly be the case, an important question is whether gain-of-function mutations in the *Pax5* gene play any role in B lymphomagenesis at all.

One possible answer is that it does, but only at certain stages of B-cell development. Most DLBCL and other non-Hodgkin lymphomas are derived from mature B-cells, which express BCR and have often undergone maturation in germinal centers [29]. In contrast, most of B-ALL (so-called A1 and A2 types) are derived from immature pro- or pre-B-cells lacking BCR [30]. Given the contribution of *Pax5* to BCR signaling (see above), it seems plausible that *Pax5* can only exert its oncogenic effects when this pathway is functional. However, an experimental verification of this simple model has been slow in coming.

A man or a mouse?

Needless to say, dissecting the causative role of putative oncogenes in spontaneous human

tumors can be tricky. However, the knock-out/knock-in mouse technology tends to be helpful in this regard. With this goal in mind, the entire Pax5-coding sequence has recently been knocked into the Igh locus, thus recreating the t(9;14) translocation in the mouse. The animals did develop overt neoplasms, but rather unexpectedly developed T, and not B lymphomas [31]. Apparently, the germline insertion of the *Pax5* gene and its ensuing activation throughout the lymphoid system arrests T-cell development and leads to malignant T lymphomas. This rather artificial system is nevertheless reminiscent of idiopathic activation of Pax5 in forebrain-derived astrocytomas [32] and a subset of highly malignant neuroblastoma cell lines [33].

However, to assess its role in B lymphomagenesis, new approaches were in order. Our laboratory took advantage of a previously described cell line (Myc5) derived from a p53-null, Myc-overexpressing lymphoma [34]. The original Myc5 tumor was Pax5-positive, but upon culturing *in vitro*, Pax5 expression was spontaneously extinguished and B-cell markers were lost. Instead, the cultured cells readily engulfed latex beads and provided T-cell help such as *bona fide* macrophages [35]. Nevertheless, upon re-expression of Pax5, Myc5 neoplastic cells expressed both surface IgM and surface IgD [36], which is characteristic of mature B cells (the so-called fraction F [37]). We reasoned that this system is well-suited to test the role of BCR signaling in Pax5-assisted lymphomagenesis.

Antigen presentation & neoplastic transformation

To analyze the immediate effects of Pax5 on B lymphomagenesis, we generated Myc5 subclones expressing the hydroxytamoxifen (4OHT)-inducible variant of Pax5. Upon re-injection into syngeneic recipients, these clones formed much larger tumors in 4OHT-treated than in control mice [25]. To reveal the mechanism of Pax5-dependent lymphomagenesis, we profiled mRNAs in Pax5-sufficient and -deficient tumors. We discovered that the repertoire of Pax5-activated genes was highly enriched in components of BCR signaling: Igα, CD19 antigen, B-cell linker (BLNK), Bruton agammaglobulinemia tyrosine kinase (Btk), and so on. In addition, two crucial inhibitors of BCR signaling, CD22 and paired-Ig-like receptor B, were repressed. What role do all these genes play in B lymphomagenesis?

To answer this question, we used the fusion protein containing the cytoplasmic regions of Igα and Igβ and constitutively targeted this to the plasma membrane [38]. Transduction of parental Myc5 cells with the Igα/β cassette resulted in strong stimulation of tumor growth. Conversely, forced expression of CD22, which antagonizes BCR activity [39], completely canceled the effects of Pax5 on tumor growth. Similar attenuation of tumor growth could be achieved through the use of pharmacological inhibitors of Syk, a BCR-associated tyrosine kinase [25].

Future directions: Pax5 & BCR as therapeutic targets

It has been proposed that BCR signaling (ligand-dependent or -independent) can promote survival of neoplastic B-lymphoma cells, for instance, via activation of antiapoptotic Mcl1 [40]. Increased BCR signaling was indeed observed in cultured B-lymphoid cells [41], and appeared to contribute to their growth [24]. However, the role of BCR signaling in tumor growth *in vivo* only has been inferred from circumstantial evidence: its persistent expression in most non-Hodgkin lymphomas, the retention of functional Ig alleles in lymphomas with IgH translocations and the discovery of autoreactive BCR in some neoplasms (reviewed in [42]).

'Given the contribution of Pax5 to BCR signaling, it seems plausible that Pax5 can only exert its oncogenic effects when this pathway is functional.'

To determine whether our experimental findings might be applicable to spontaneous human lymphomas, Dr Teresa Marafioti's group stained tissue arrays of human DLCBLs for both PAX5 and phosphorylated BLNK, the key adaptor protein of activated BCR. Her group observed that all DCBCL tumors stained positively for PAX5 and approximately half were also positive for phosphorylated BLNK. Thus, BCR is likely to be constitutively activated in at least some human B-cell tumors, attesting to the important causative role of PAX5 in B lymphomagenesis.

Acknowledgements

The authors thank Dr Martin Carroll (University of Pennsylvania) for critical comments on this manuscript.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. O'Neil J, Look AT: Mechanisms of transcription factor deregulation in lymphoid cell transformation. *Oncogene* 26(47), 6838–6849 (2007).
2. Cobaleda C, Schebesta A, Delogu A, Busslinger M: Pax5: the guardian of B cell identity and function. *Nat. Immunol.* 8(5), 463–470 (2007).

• Most recent and comprehensive review of the roles that Pax5 plays in normal development and neoplastic disease.

3. Fuxa M, Busslinger M: Reporter gene insertions reveal a strictly B lymphoid-specific expression pattern of Pax5 in support of its B cell identity function. *J. Immunol.* 178(12), 8222–8228 (2007).
4. Nutt SL, Eberhard D, Horcher M, Rolink AG, Busslinger M: Pax5 determines the identity of B cells from the beginning to the end of B-lymphopoiesis. *Int. Rev. Immunol.* 20(1), 65–82 (2001).
5. Kallies A, Hasbold J, Fairfax K *et al.*: Initiation of plasma-cell differentiation is independent of the transcription factor Blimp-1. *Immunity* 26(5), 555–566 (2007).
6. Lauring J, Schlissel MS: Distinct factors regulate the murine RAG-2 promoter in B- and T-cell lines. *Mol. Cell Biol.* 19(4), 2601–2612 (1999).
7. Kishi H, Jin ZX, Wei XC *et al.*: Cooperative binding of c-Myb and Pax-5 activates the RAG-2 promoter in immature B cells. *Blood* 99(2), 576–583 (2002).
8. Fuxa M, Skok J, Souabni A, Salvagiotto G, Roldan E, Busslinger M: Pax5 induces V-to-DJ rearrangements and locus contraction of the immunoglobulin heavy-chain gene. *Genes Dev.* 18(4), 411–422 (2004).
9. Zhang Z, Espinoza CR, Yu Z *et al.*: Transcription factor Pax5 (BSAP) transactivates the RAG-mediated V(H)-to-DJ(H) rearrangement of immunoglobulin genes. *Nat. Immunol.* 7(6), 616–624 (2006).
10. Johnson K, Pflugh DL, Yu D *et al.*: B cell-specific loss of histone 3 lysine 9 methylation in the V(H) locus depends on Pax5. *Nat. Immunol.* 5(8), 853–861 (2004).
11. Urbanek P, Wang ZQ, Fetka I, Wagner EF, Busslinger M: Complete block of early B-cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. *Cell* 79(5), 901–912 (1994).

• Classical paper establishing the role of Pax5 in maintaining B-cell identity.

12. Hodawadekar S, Wei F, Yu D, Thomas-Tikhonenko A, Atchison M: Epigenetic histone modifications do not control Igk locus contraction and intranuclear localization in cells with dual B cell-macrophage potential. *J. Immunol.* 177(9), 6165–6171 (2006).
13. Fitzsimmons D, Hodsdon W, Wheat W, Maira SM, Wasylyk B, Hagman J: Pax-5 (BSAP) recruits Ets proto-oncogene family proteins to form functional ternary complexes on a B-cell-specific promoter. *Genes Dev.* 10(17), 2198–2211 (1996).
14. Monroe JG: ITAM-mediated tonic signalling through pre-BCR and BCR complexes. *Nat. Rev. Immunol.* 6(4), 283–294 (2006).
15. Gonda H, Sugai M, Nambu Y *et al.*: The balance between Pax5 and Id2 activities is the key to AID gene expression. *J. Exp. Med.* 198(9), 1427–1437 (2003).
16. Oppezzo P, Dumas G, Lalanne AI *et al.*: Different isoforms of BSAP regulate expression of AID in normal and chronic lymphocytic leukemia B cells. *Blood* 105(6), 2495–2503 (2005).
17. Cook JR, Aguilera NI, Reshmi-Skarja S *et al.*: Lack of PAX5 rearrangements in lymphoplasmacytic lymphomas: reassessing the reported association with t(9;14). *Hum. Pathol.* 35(4), 447–454 (2004).
18. Busslinger M, Klix N, Pfeffer P, Graninger PG, Kozmik Z: Deregulation of PAX-5 by translocation of the Eμ enhancer of the IgH locus adjacent to two alternative PAX-5 promoters in a diffuse large-cell lymphoma. *Proc. Natl Acad. Sci. USA* 93(12), 6129–6134 (1996).
19. Iida S, Rao PH, Nallasivam P *et al.*: The t(9;14)(p13;q32) chromosomal translocation associated with lymphoplasmacytoid lymphoma involves the PAX-5 gene. *Blood* 88(11), 4110–4117 (1996).

•• Ref. 18 and 19 are first independent reports of translocations involving Pax5.

20. Morrison AM, Jager U, Chott A, Schebesta M, Haas OA, Busslinger M: Deregulated PAX-5 transcription from a translocated IgH promoter in marginal zone lymphoma. *Blood* 92(10), 3865–3878 (1998).
21. Poppe B, De PP, Michaux L *et al.*: PAX5/IGH rearrangement is a recurrent finding in a subset of aggressive B-NHL with complex chromosomal rearrangements. *Genes Chromosomes Cancer* 44(2), 218–223 (2005).
22. Pasqualucci L, Neumeister P, Goossens T *et al.*: Hypermutation of multiple proto-oncogenes in B-cell diffuse large-cell lymphomas. *Nature* 412(6844), 341–346 (2001).

•• First demonstration that the *Pax5* gene is affected by somatic hypermutations in diffuse large B-cell lymphoma.

23. Alizadeh AA, Eisen MB, Davis RE *et al.*: Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 403(6769), 503–511 (2000).
24. Gururajan M, Jennings CD, Bondada S: Cutting edge: constitutive B cell receptor signaling is critical for basal growth of B lymphoma. *J. Immunol.* 176(10), 5715–5719 (2006).
25. Cozma D, Yu D, Hodawadekar S *et al.*: PAX5 promotes lymphomagenesis through the stimulation of B-cell receptor signaling. *J. Clin. Invest.* 117(9), 2602–2610 (2007).

•• Recent reports from the authors' laboratory demonstrating that B cell receptor signaling is crucial for Pax5-dependent B lymphomagenesis.

26. Bousquet M, Broccardo C, Quelen C *et al.*: A novel PAX5–ELN fusion protein identified in B-cell acute lymphoblastic leukemia acts as a dominant negative on wild-type *PAX5*. *Blood* 109(8), 3417–3423 (2007).

• Demonstrates that some Pax5 fusion proteins act as dominant-negative mutants to inhibit function of endogenous Pax5 in acute lymphoblastic leukemia.

27. Cazzaniga G, Daniotti M, Tosi S *et al.*: The paired box domain gene *PAX5* is fused to *ETV6/TEL* in an acute lymphoblastic leukemia case. *Cancer Res.* 61(12), 4666–4670 (2001).

28. Mullighan CG, Goorha S, Radtke I *et al.*: Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature* 446(7137), 758–764 (2007).
•• Establishes that *EBF1* and *Pax5* are the two most frequently mutated genes in acute lymphoblastic leukemia and that most of these mutations are loss-of-function.
29. Shaffer AL, Rosenwald A, Staudt LM: Lymphoid malignancies: the dark side of B-cell differentiation. *Nat. Rev. Immunol.* 2(12), 920–932 (2002).
30. Staudt LM: It's ALL in the diagnosis. *Cancer Cell* 1(2), 109–110 (2002).
31. Souabni A, Jochum W, Busslinger M: Oncogenic role of *Pax5* in the T-lymphoid lineage upon ectopic expression from the immunoglobulin heavy-chain locus. *Blood* 109(1), 281–289 (2006).
• Demonstrates the propensity of Pax5 to transform T cells when expressed in the germline.
32. Stuart ET, Kioussi C, Aguzzi A, Gruss P: PAX5 expression correlates with increasing malignancy in human astrocytomas. *Clin. Cancer Res.* 1(2), 207–214 (1995).
33. Baumann Kubetzko FB, Di PC, Maag C *et al.*: The *PAX5* oncogene is expressed in N-type neuroblastoma cells and increases tumorigenicity of a *S*-type cell line. *Carcinogenesis* 25(10), 1839–1846 (2004).
34. Yu D, Thomas-Tikhonenko A: A non-transgenic mouse model for B-cell lymphoma: *in vivo* infection of p53-null bone marrow progenitors by a Myc retrovirus is sufficient for tumorigenesis. *Oncogene* 21(12), 1922–1927 (2002).
35. Hodawadekar S, Yu D, Freedman B, Sunyer JO, Atchison M, Thomas-Tikhonenko A: B-lymphoma cells with epigenetic silencing of Pax5 trans-differentiate into macrophages, but not other hematopoietic lineages. *Exp. Cell Res.* 313(2), 331–340 (2007).
36. Yu D, Allman D, Goldschmidt M, Atchison M, Monroe JG, Thomas-Tikhonenko A: Oscillation between B-lymphoid and myeloid lineages in Myc-induced hematopoietic tumors following spontaneous silencing/reactivation of the EBF/Pax5 pathway. *Blood* 101(5), 1950–1955 (2003).
37. Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K: Resolution and characterization of pro-B and pre-pro-B-cell stages in normal mouse bone marrow. *J. Exp. Med.* 173(5), 1213–1225 (1991).
38. Bannish G, Fuentes-Panana EM, Cambier JC, Pear WS, Monroe JG: Ligand-independent signaling functions for the B-lymphocyte antigen receptor and their role in positive selection during B lymphopoiesis. *J. Exp. Med.* 194(11), 1583–1596 (2001).
39. Nitschke L: The role of CD22 and other inhibitory co-receptors in B-cell activation. *Curr. Opin. Immunol.* 17(3), 290–297 (2005).
40. Petlickovski A, Laurenti L, Li X *et al.*: Sustained signaling through the B-cell receptor induces Mcl-1 and promotes survival of chronic lymphocytic leukemia B cells. *Blood* 105(12), 4820–4827 (2005).
41. Irish JM, Czerwinski DK, Nolan GP, Levy R: Altered B-cell receptor signaling kinetics distinguish human follicular lymphoma B cells from tumor infiltrating non-malignant B cells. *Blood* 108(9), 3135–3142 (2006).
42. Kuppers R: Mechanisms of B-cell lymphoma pathogenesis. *Nat. Rev. Cancer* 5(4), 251–262 (2005).

Affiliations

- *Andrei Thomas-Tikhonenko*
  Department of Pathobiology and Mari Lowe Center for Comparative Oncology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia PA 19104–6051, USA
  Tel.: +1 215 573 5138
  Fax: +1 215 746 0380
  andreit@mail.vet.upenn.edu
- *Diana Cozma*
  Envision Pharma, Inc., 123 South Broad Street, Suite 1670, Philadelphia PA 19109, USA
  Tel.: +1 215 239 6605
  Fax: +1 215 545 0240
  Diana.Cozma@envisionpharma.com