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Molecular basis of CLL

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Abstract

B-cell chronic lymphocytic leukemia (CLL), the most common leukemia in the Western world, results from an expansion of a rare population of CD5+ mature B-lymphocytes. CLL occurs in two forms, aggressive and indolent. For the most part indolent CLL is characterized by low ZAP-70 expression and mutated IgH V_H; aggressive CLL shows high ZAP-70 expression and unmutated IgH V_H. Although clinical features and genomic abnormalities in CLL have been studied extensively, molecular mechanisms underlying disease development are still emerging. In the last few years, several important insights were reported in this area. *Mir-15/16* targeting *BCL2* and *MCL1* and *DLEU7* targeting TNF pathway were proposed as tumor suppressors at 13q14, a commonly deleted region in indolent CLL. Molecular details of how activation of *TCL1*, a critical oncogene in aggressive CLL, results in the initiation of this malignancy were clarified. Importance of these pathways was supported by investigations of several mouse models of CLL. Here, we present what has been learned from these new pathways, discuss mouse CLL models and how these mouse models recapitulate the molecular mechanisms of this common leukemia.

Keywords

CLL; *TCL1*; *miR-15/16*; *DLEU7*

Introduction

Chronic lymphocytic leukemia (CLL) is the most common human leukemia, accounting for ~10,000 new cases diagnosed each year in the United States and representing ~30% of all leukemia cases [1]. CLL patients can often survive for a number of years showing relatively mild symptoms [1]. Indeed, a significant proportion of CLL cases are diagnosed incidentally. CLL leukemic cells usually show morphologically mature appearance and usually do not proliferate *in vitro* [1,2]. This disease occurs in two forms, aggressive and indolent, both forms are characterized by the clonal expansion of CD5 positive B-lymphocytes [1,2]. For the most part aggressive CLL is characterized by high ZAP-70 expression and unmutated IgH V_H, while indolent CLL shows low ZAP-70 expression and mutated IgH V_H [1,2]. For a number of years it was generally accepted that CLL is caused by an inherent defect in apoptosis, and that malignant lymphocytes accumulate due to diminished cell death [1,2]. Consistent with this theory, CLL cells are relatively inert, do not proliferate, and accumulate until levels not supportable by a patient. However, this original

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view has been challenged in the last ten years. Several reports showed that high lymphocyte count in CLL patients is caused not only by the prolonged survival, but also by proliferating cells from the bone marrow, spleen or lymph nodes [3–5]. Immune incompetence is another important feature of CLL. Most patients develop severe hypogammaglobulinemia and impaired immune response to antigens [6].

Our understanding of molecular events and prognostic markers in CLL was significantly improved by using molecular genetic approaches such as PCR, fluorescence *in situ* hybridization and microarray analysis. Genomic aberrations were detected in over 80% of CLL cases. These common chromosomal abnormalities include 13q, 11q, 17p and 7q deletions, and trisomy 12 [7]. The 13q14 deletion represents the most common CLL aberration reported in ~50% of all cases (mostly in indolent CLL), on the other hand, ~10–20% of CLL cases do not show chromosomal aberrations [7].

***MiR-15/16* and *DLEU7* at 13q14: a unique collaboration of coding and noncoding genes in indolent CLL**

***MiR-15/16* at 13q14, discovery and function**—Since 13q14 deletions also occur in mantle cell lymphoma, myeloma, and prostate cancer [1,2], it is possible that one or more tumor suppressor genes at 13q14 are important in the pathogenesis of CLL and other malignancies. To identify tumor suppressor gene (or genes) at 13q14 several groups have used positional cloning, and a region of more than 1 Mb has been fully sequenced and characterized [8,9]. However, in spite of extensive research, none of the known genes of this region were found to be inactivated in CLL by deletions, mutations or promoter hypermethylation [8–11]. In order to finally find the elusive tumor suppressor gene at 13q14, in 2001 we generated somatic cell hybrids between mouse and CLLs cells carrying 13q14 deletion and translocation. Using these hybrids we finally identified a 30-kb region of deletion between exons 2 and 5 of *LEU2* gene [12,13]. The translocation breakpoint was mapped in the same region [12]. Since *LEU2* had previously been excluded as a likely candidate tumor suppressor gene in CLL [8,9,11,14] we continued to search for genes within the region and finally discovered that a cluster of two noncoding microRNA genes *miR-15a* and *miR-16-1*, is located precisely within the deleted region ant at the translocation breakpoint [12]. Interestingly, this *miR-15a/miR-16-1* cluster is deleted or down-regulated in ~66% of CLL cases [12]. In contrast, expression of other genes in the region such as *DLEU1*, *DLEU2*, *RFP5* did not seem affected by 13q14 deletions [8,9,13]. To determine possible mechanism of *miR-15/16* tumor suppressor function, subsequent study examined expression levels of *miR-15/16* and *BCL2* (a predicted target of both, *miR-15* and *miR-16*) in CLL [15]. These results showed that *miR-15a* and *miR-16-1* expression is inversely correlated to Bcl2 expression in CLL and that both microRNAs negatively regulate *BCL2* at a posttranscriptional level [15]. Consequently, *BCL2* repression by these microRNAs induces apoptosis in a leukemic cell line [15], and *miR-15/16* showed a tumor suppressor function *in vivo* by inhibiting the growth of tumor engraftments of leukemic cells in nude mice [16]. These findings suggested that deletions of *miR-15/16* result in Bcl2 up-regulation, contributing to the pathogenesis of CLL. The importance of *miR-15/16* was recently confirmed by a report studying CLL development in New Zealand black (NZB) mice [17]. Since these mice are susceptible to CLL, Raveche et al. carried out linkage analysis and discovered that mouse genomic region homologous to 13q14 is one of the *loci* associated with CLL. DNA sequencing identified a point mutation in *miR-15/16* precursor and levels of *miR-16* were decreased in NZB lymphoid tissues [17].

***DLEU7*, a second tumor suppressor at 13q14**—Recently, a high-resolution map of 13q14 deletions using 171 CLL samples was reported [18]. These study used microarray based technology to determine the minimal deleted region at 13q14 in CLL. Interestingly, in

addition to *miR-15/16*, this region also contained *DLEU7* gene located telometic to *miR-15/16* [18], and previously identified as a candidate tumor suppressor gene at 13q14 [19]. *DLEU7* encodes a 221 amino acid protein with no homology to known proteins. Sequencing of *DLEU7* coding exons failed to find mutations in 45 CLL samples, although *DLEU7* promoter was methylated in 61% of CLL [19]. Since *DLEU7* is the only protein coding gene located within the minimal deleted region at 13q14, very recently we investigated whether *DLEU7* can function as a tumor suppressor and cooperate with *miR-15/16* [20]. We first examined expression of *DLEU7* in CLL samples and normal CD19+ B-cells by real time RT-PCR results. All CLL samples examined showed decreased *DLEU7* expression, while over 50% showed decrease of ten fold or more. Expression of both, *miR-15* and *miR-16*, was also decreased in over 90% of CLL samples examined [20]. These results were consistent with previous study showing that *DLEU7* is methylated and in essence is not expressed in CLL [19].

Recent studies of CLL animal models as well as mechanistic studies demonstrated the importance of the NF- κ B pathway in the pathogenesis of CLL (reviewed in [21]). For instance, transgenic expression of a proliferation-inducing TNF ligand (APRIL), a member of the tumor necrosis factor (TNF) superfamily involved in NF- κ B activation resulted in the development of a disease very similar to CLL (see below) [22]. In our recent report we found that Tcl1 activation caused CLL by activation of NF- κ B pathway and inhibition of AP-1 [23]. Since these studies suggested a significant role for the NF- κ B pathway in the pathogenesis of CLL [21], we investigated whether Dleu7 might function as an inhibitor of NF- κ B, in particular in the pathway involving APRIL.

APRIL and its close relative BAFF are recent members of TNF superfamily expressed almost exclusively in lymphoid cells [24]. Interestingly, both BAFF and APRIL show elevated expression levels in various B-cell malignancies including diffuse large cell lymphoma, mantle cell lymphoma and CLL [21,24]. APRIL binds with high affinity to two receptors, BCMA (B-cell maturation antigen) and TACI, both are members of TNF receptor superfamily (Figure 1) [24]. BCMA is mostly expressed in mature B- and T-cells, while TACI is detected in activated T-cells and subpopulations of B-cells [24]. They stimulate NF- κ B pathway by interaction with various TRAFs [24]. Thus, NF- κ B activation through TACI and BCMA could be important in the pathogenesis of CLL [21]. Since *DLEU7* is located within the 13q14 deleted region we investigated whether its expression has an effect on NF- κ B activation by TACI and BCMA. Transfection experiments confirmed our hypothesis: Dleu7 expression inhibited NF- κ B activation by BCMA over 5 fold, similarly, NF- κ B activation by TACI was inhibited over 4 fold [20]. Thus, we demonstrated that Dleu7 functions as NF- κ B inhibitor in a pathway critical for the pathogenesis of CLL (Figure 1).

Nuclear factor of activated T-cells (NFAT) can also be activated by TACI and BCMA, same molecules responsible for NF- κ B activation (Figure 1) [25]. Previous reports showed that nuclear activated NFAT is a hallmark of unstimulated CLL cells and that NFAT is a critical factor for CD5 expression in B-cells [26,27]. Since Dleu7 inhibits NF- κ B activation by TACI and BCMA, we hypothesized that Dleu7 can also inhibit the ability of TACI and BCMA to induce NFAT mediated transactivation. Indeed, we showed that Dleu7 expression inhibited this activation ~8 fold and concluded that Dleu7 functions as NFAT inhibitor [20]. Further co-immunoprecipitation experiments confirmed that Dleu7 functions as TACI and BCMA inhibitor by direct association with these molecules [20]. Although these data strongly indicate that Dleu7 might function as a tumor suppressor in CLL it is important to show this function in cellular assays. To show that this Dleu7 can indeed function a tumor suppressor we used adenovirus expressing Dleu7 and GFP, as a control. These experiments showed that Dleu7 expression results in >2 fold decrease of cells in the S phase and in >3 fold induction in apoptosis as measured by Annexin V incorporation [20]. All these data

clearly indicate we identified *DLEU7* as a second tumor suppressor in 13q14 in addition to *miR-15/16*.

In summary, recent investigations determined that 13q14 contains two cooperating tumor suppressors, *miR-15/16* and *DLEU7*. *miR-15/16* deletions result a constitutive increase of Bcl2 expression, *DLEU7* inactivation causes induction of TNF signaling through TRAFs. Intriguingly, these two events cause CLL phenotype closely resembling oncogenic events in *TRAF2DN/BCL2* transgenic mouse model (see below) [20].

***TCL1*, a critical oncogene in aggressive CLL**

Activation of the *TCL1* oncogene is arguably one of most studied initiating events in the pathogenesis of aggressive CLL. *TCL1* (T cell leukemia/lymphoma 1) was first identified as a target of translocations and inversions at 14q32.1, common chromosomal aberrations in T-cell prolymphocytic leukemias [28]. Expression analyses of *TCL1* revealed that this gene is mostly expressed in B-cells and to a much lesser extent in T-cells [28]. In B-cells, Tc11 is expressed in all stages of development, except mature B-cells [29,30]. Since our study demonstrating that *TCL1* deregulation in mouse B-cells results in aggressive CLL [31], several additional reports have indicated that Tc11 is critical molecule in the CLL pathogenesis [13,32]. Importantly, high Tc11 expression in human CLL correlates with aggressive CLL phenotype showing unmutated immunoglobulin variable region genes and ZAP70 positivity [32].

***TCL1* function in CLL**—The molecular mechanism of Tc11 mediated oncogenesis was not known until ten years ago when we and others reported that Tc11 functions as an activator PI3K - Akt(PKB) oncogenic pathway [33,34]. Akt kinase encoded by the *AKT* oncogene plays an important role in the regulation of a number of signaling pathways involved in cell proliferation, survival, and death in numerous cell types including B- and T-cells [35]. Our results showed that Tc11 and Akt physically interact and that PH domain of Akt is responsible for this binding [33]. Using *in vitro* and *in vivo* kinase assays we showed that Akt bound to Tc11 exhibits significant increase in the kinase activity [33,34]. We also demonstrated that Tc11 mediates the nuclear translocation of Akt [33].

More recently we investigated whether *TCL1* expression in CLL is regulated by microRNAs [36]. Since Tc11 is up-regulated in aggressive CLL, we were interested in microRNAs down-regulated in aggressive CLL that potentially target *TCL1*. These experiments revealed that expression of *miR-29b* and *miR-181b* significantly inhibits Tc11 expression and that *miR-29b* and *miR-181b* target *TCL1* expression at mRNA and protein levels [36]. Intriguingly, a microRNA signature was previously reported with 13 microRNAs that differentiate aggressive and indolent CLL [37,38]. In this signature only four microRNAs were down-regulated in aggressive CLL *versus* indolent CLL. Remarkably, of the four, three are isoforms of *miR-29* (*miR-29a-2*, *miR-29b-2* and *miR-29c*) [36]. This confirms the importance of the interaction between *miR-29* and *TCL1* in aggressive CLL.

Is involvement of Tc11 in Akt activation important in the pathogenesis of CLL? The fact that transgenic mice overexpressing activated Akt in T-cells develop T-cell leukemias [39] provides an important evidence that Akt contributes to lymphomagenesis in T-cells initiated by *TCL1* activation (Figure 2). On the other hand, transgenic mice expressing activated Akt in B-cells do not develop lymphoid malignancies (YP and CMC, unpublished results), and conditional Pten knockout in mouse B-cells expressing constitutively active Akt did not cause B-cell transformation [40]. Thus, it is likely that Tc11 mediated CLL pathogenesis is caused by mechanism(s) other than Akt activation.

In 2008 we were able to uncover such mechanism [23]. We hypothesized that some CLL samples might have gain of function mutations in *TCL1*, and that functional analysis of these mutations could point to molecular mechanisms of Tc11 mediated B-cell transformation. Thus, we sequenced the *TCL1* gene in 600 CLL samples and identified two heterozygous mutations resulting in amino acid substitutions, T38I and R52H. Interestingly, RT-PCR results showed that the T38I mutant *TCL1* mRNA was the major expressed allele in the CLL of origin, accounting for ~80% of the *TCL1* mRNA, and the R52H allele was the only allele expressed [23]. Since NF- κ B activation is critical in CLL development, we first examined if *TCL1* mutants show gain of function in NF- κ B activation. Using reported system we showed that Tc11 activated NF- κ B activity 2–4 fold by a mechanism independent of Akt. However, in these experiments the Tc11 mutants did not exhibit gain of function in the activation of NF- κ B [23].

After examining several other pathways we focused on activator protein 1 (AP-1) dependent transcription [41]. The classical AP-1 complex contains c-Jun and c-Fos, although other Jun proteins (JunB and JunD) and Fos proteins (Fra-1, Fra-2 and FosB) have also been reported [42]. Several studies reported that AP-1 induces apoptosis by activating pro-apoptotic genes [43–45]. For example, activation of AP-1 was reported in lymphoid cells after serum deprivation and this induction was associated with apoptosis [46]. Also, mouse knockout studies showed that JunB deficiency caused induction of B-cell leukemias [47]. Thus, we investigated whether Tc11 can inhibit AP-1 dependent transcription. Using reported system we showed that Tc11 expression inhibited AP-1 dependent transactivation ~2.5-fold while Tc11 T38I caused a dramatic ~100-fold inhibition, and the R52H mutant showed ~4 fold inhibition [23]. Similar results were obtained using individual components of the AP-1 complex, such as c-Jun, c-Fos and JunB. In all these cases Tc11 T38I mutant inhibited more potently than WT Tc11 strongly indicating that Tc11 mutants show gain of function effect in AP-1 inhibition [23]. To elucidate the mechanism of this inhibition we carried out a series of co-immunoprecipitation experiments. These results revealed that Tc11 specifically interacts with c-Jun, c-Fos and JunB. Thus, we concluded that Tc11 physically interacts with AP-1 components and functions as an AP-1 inhibitor [23]. The fact that both Tc11 mutants identified in CLL patients show gain of function properties in this pathway suggests the ability of Tc11 to inhibit AP-1 dependent transcription is critical in the pathogenesis of CLL (Figure 2).

In summary, in the last ten years we discovered two critical pathways involving Tc11 (Figure 2). In T-cells, Tc11 activates Akt, this leads to increased proliferation, inhibition of apoptosis and T-cell transformation [33]. In B-cells, Tc11 functions as transcriptional regulator, it activates NF- κ B and inhibits AP-1 [23]. This leads to defects in apoptosis, increase in cell survival, and CLL pathogenesis.

Mouse models of CLL

Although dysregulation of a specific pathway (or gene) in CLL suggests possible involvement in this disease, the final proof of the critical role of this pathway (or gene) in the pathogenesis of CLL requires generation of animal models showing CLL phenotype. In this chapter we discuss animal models of CLL which confirmed the importance of molecular mechanisms we have described above.

***TCL1* driven CLL mouse model**—Several years ago, we produced transgenic mice in which the expression of *TCL1* was under the control of a V_H promoter-IgH-E μ enhancer that targets expression of the transgene to immature and mature B-cells [31]. The immunophenotypic profile of peripheral blood lymphocytes (PBLs) from these mice between one and nine months of age detected an expansion of the B220/IgM⁺ population, in particular

those that were CD5⁺/B1, whereas such cells are generally infrequent [48]. This expansion was revealed in 100% of the transgenic mice by six months of age, without sign of clinical disease [31]. Fluorescence-activated cell sorting (FACS) analysis detected a phenotypically homogeneous CD5⁺IgM⁺ population significantly expanded in the peritoneal cavity of the transgenic mice starting at two months of age (44%) that became then evident in spleen (9%) by four months and bone marrow by eight months (43%) [21,31]. At this age, Eμ-*TCL1* mice presented a slightly enlarged spleen and a much higher cellularity in the peritoneal cavity, ranging between 50- to 100-fold increase. The cell cycle distribution and the rate of cell proliferation in spleen and peritoneal cavity of these transgenic mice were also investigated and we found that IgM⁺CD5⁺ sorted populations were arrested in the G₀/G₁ phase of the cell cycle and did not actively divide. The onset of a frank leukemia in the elderly mice provided the final evidence of the establishment of a murine model for human CLL [21,31]. All mice between 10 and 20 months of age became visibly ill and presented with spleno- and hepatomegaly associated with high counts of white blood cells (WBCs). Moreover, some mice also developed advanced lympho-adenopathy, another hallmark of human CLL [21,31]. Simultaneously, another Tc11 transgenic mouse model was created to express Tc11 in both, B- and T-cells [49]. The phenotype of CLL like disease in this second mouse was similar to that described for our Tc11 transgenics.

Later on, we studied the spontaneous tumor phenotype of Eμ-*TCL1* transgenic mice more in detail [50]. An extensive pathologic analysis of these transgenic animals revealed that, in addition to CLL leukemias, more than 30% of mice showed the onset of solid malignant tumors, even though the tumors themselves lacked Tc11 expression. Secondary malignancies are also frequent complications in patients with CLL [51] and they are the most common cause of death in these patients [52]. Higher risk of secondary non-hematological neoplasms in patients with CLL has several explanations including genetic predisposition, immune deficiency, carcinogen exposure, and the side-effects of therapeutic treatments [53]. In our CLL model, 25% of secondary cancers were malignant pilomatrixoma, a type of skin cancer extremely rare in mice. Thus far, the Eμ-*TCL1* model seems to be the only one reported that displays the onset of secondary malignancies in CLL-prone mice [21,50].

The homogeneity of the genetic background of a mouse model is an important factor to evaluate the effects of novel therapies. For this reason, we established CLL transplants in syngeneic mice that allowed us at first to propagate, and later to therapeutically treat, the same leukemia in different mice but with the same genetic background. Such a system allows for the study of each leukemia and is a necessary step on the road to find drugs that specifically interfere with the pathways involved in the disease [21,50]. To determine whether the development of mouse CLL is dependent on the Akt pathway, we investigated whether the disruption of Akt signaling through mTOR, by the mTOR inhibitor rapamycin, prevented or cured mouse CLL. The treatment prolonged the life of all treated animals in either the prevention or the therapeutic group. Mammalian TOR normally regulates translation by regulation key components of the protein synthesis machinery, including ribosomal protein S6 kinase. We found that lymph nodes of untreated mice expressed phosphorylated S6, whereas lymphoid tissues from rapamycin-treated mice did not show any phosphorylation. In conclusion, rapamycin inhibited mTOR activity in the Tc11-dependent CLL mouse model [50].

As we reported in the previous chapter, levels of *TCL1* expression in CLL are regulated by *miR-29* and *mir-181* that target 3' UTR of *TCL1*. To determine whether treatment with microRNAs targeting *TCL1* can inhibit CLL in mice, we designed TCL1 transgenic mice using a construct containing the 3' and 5' UTRs of *TCL1* under B cell – specific Eμ promoter (Eμ-*TCL1* Full Length). At the age of 16–20 months, these mice showed a CLL-like disease [54]. Immunophenotyping revealed accumulation of CD5⁺ CD23⁺ B220⁺ population in

spleens and lymph nodes. Our findings showed that CD5⁺ CD23⁺ B cell populations from E μ -*TCL1*FL mice actively proliferated and showed significantly increased levels of phospho-Akt. E μ -*TCL1*FL mice showed immunological abnormalities similar to those present in human CLL, including hypogammaglobulinemia, abnormal levels of cytokines and impaired immune response. Overall, these results revealed biochemical and immunological similarities to Tcl1 driven CLL mouse models and human CLL [54].

In summary, a vast corpus of information has been collected in the last eight years about leukemia developed by the E μ -*TCL1* transgenic mice, demonstrating its clear similarity to human CLL.

MiR-29 driven mouse model of CLL—We recently found that *miR-29* is over-expressed in indolent human CLL, compared to aggressive CLL and normal CD19⁺ B-cells. To elucidate the role of *miR-29* in B-cell leukemias we created transgenic mice over-expressing *miR-29* in B-cells and recently we reported the phenotype of this new mouse model [55]. Immunophenotypic profile of spleen lymphocytes from *miR-29* transgenics showed increased populations of CD5⁺CD19⁺IgM⁺ B-cells, a typical feature of CLL. Between the age of 12 and 24 months a markedly expanded CD5⁺ B-cell population was evident in spleens of 34 of 40 (85%) *miR-29* transgenic mice; ~50% of B-cells in these transgenics were CD5 positive [55]. Interestingly, of 20 *miR-29* transgenic mice followed to 24–26 months of age, only 4 (20%) developed frank leukemia and died of the disease. Since almost all *miR-29* transgenics showed expanded CD5⁺CD19⁺IgM⁺ B-cell populations, but only 20% developed a frank leukemia, we concluded that *miR-29* transgenics developed a disease similar to indolent CLL [55]. Moreover, *miR-29* mice showed significant increases in % of leukemic cells with age. In mice younger than 15 months, CD5⁺ leukemic cells were only ~20% of total B-cells; on the other hand, at the age of 20–26 months, >65% of all B-cells were CD5⁺. These results showed a gradual progression of indolent CLL in *miR-29* transgenics [55]. To determine whether leukemic cells from *miR-29* mice divide, we did measure the proliferative capacity of CD5⁺ leukemic B-cells compared to WT CD19⁺ spleen lymphocytes. BrdU incorporation experiments showed significant proliferation in *miR-29* transgenic B-cells, while no proliferation was found in CD19⁺ WT lymphocytes [55]. These results suggest that *miR-29* over-expression plays an important role in promoting B-cell proliferation. Because progressive hypogammaglobulinemia and immune incompetence are important features of human CLL [6], we analyzed serum levels of immunoglobulins and the immune response to SRBC antigen in the serum of *miR-29* mice and wild type littermates. We showed that in *miR-29* transgenics immune response to SRBC antigen and serum levels of immunoglobulins were significantly decreased [55]. Thus, these findings confirmed our initial observations that *miR-29* transgenics develop a disease mimicking the indolent human counterpart.

MiR-15a/16-1 cluster knockout mouse model of CLL—As recently reviewed [56], the literature on the importance of miRNAs deregulation in creation of mouse models of lymphocytic leukemias is becoming increasingly richer. Early this year, Dalla-Favera and colleagues published the genetic validation that deletion of *miR-15a/16-1* leads to CLL in mice [57]. In order to identify the genetic elements targeted by the putative 13q14 tumor suppressor and to determine their contribution to the pathogenesis of CLL, they generated knockout mice that carried conditional alleles that either mimicked the deletion of the minimal deleted region (MDR), previously described in human CLL [9,58] and comprising the entire *Dleu2* gene, or that specifically deleted the *miR-15a/16-1* cluster without affecting the expression of *Dleu2*. At the age of 2–4 months, homozygous *Mdr*^{-/-} and *miR-15a/16-1*^{-/-} mice showed normal percentages of B and T cell subpopulations and normal development of lymphoid organs. However, one year old *Mdr*^{-/-} mice showed higher percentages of CD5⁺B220 B cells among mononuclear cells in the peritoneal cavity (50%

vs. 15% in controls). Similar observations were made in *miR-15a/16-1*^{-/-} rodents. Thus, deletions of the *Mdr* and *miR-15a/16-1* are associated with expansion of CD5⁺B220 cells in the peritoneal cavity. In a fraction of the animals, clonal CD5⁺ B cell populations were accompanied by significant infiltration of lymphoid organs with histopathologic features resembling human CLL [56,57]. These mice displayed an enlargement of the spleen white pulp due to the expansion or accumulation of small lymphocytes with a pattern similar to CLL. Discrete aggregates of small lymphocytes were also observed in the bone marrow. Overall, 27% of *Mdr*^{-/-} and 21% of *miR-15a/16-1*^{-/-} mice developed CLL. Altogether 42% of *Mdr*^{-/-} and 26% of *miR-15a/16-1*^{-/-} mice, 15–18 months of age, developed some type of clonal B cell lymphoproliferation. *Mdr*^{-/-} mice also showed a trend toward developing B cell tumors (24%), suggesting that monoallelic deletion of the *Mdr* can cause disease. This trend is not evident in *miR-15a/16-1*-deleted mice, probably due to the lower penetrance of the phenotype. *Mdr*^{-/-} mice died earlier than their wild type littermates, suggesting that the homozygous mice eventually succumb to their tumors at an advanced age [56,57]. On the contrary, the survival of *miR-15a/16-1*^{-/-} mice was not significantly different from wild type littermates, suggesting that these mice show a milder disease course than their *Mdr* counterparts. In conclusion, both *Mdr*^{-/-} and *miR-15a/16-1*^{-/-} mice developed lymphoproliferations with an indolent disease course reminiscent of human CLL, with *Mdr*^{-/-} mice displaying a more aggressive phenotype. The significantly more aggressive phenotype displayed by *Mdr*^{-/-} mice suggests that additional genetic elements within the MDR locus may contribute to the tumor suppressive function. One candidate for such a role is the *DLEU2* gene itself. Besides providing the primary transcript for the production of *miR-15a/16-1*, it produces a spliced cytoplasmic RNA that may have important regulatory functions not yet detected [56,57].

To understand the mechanism by which the deletions lead to clonal B lymphoproliferations, Klein et al. [57] investigated whether *miR-15a/16-1* deletions could affect proliferation of mouse B cells. BrdU incorporation assays, by measuring active DNA-synthesis, demonstrated that *miR-15a/16-1*^{-/-} B cells begin to synthesize DNA earlier than wild type B cells. Mitogen-stimulated B cells purified from *miR-15a/16-1*^{-/-} or *Mdr*^{-/-} and wild type mice were analyzed for levels of phosphorylated retinoblastoma (p-Rb) protein, an indicator of entry into the cell cycle. P-Rb could be revealed at earlier time points in both *miR-15a/16-1*^{-/-} and *Mdr*^{-/-} compared with wild type B cells. Consistently, the expression of the cell cycle inhibitory protein cyclin-dependent kinase inhibitor p27-Kip1 appeared to follow similar kinetics. To unravel the individual contributions of *DLEU2* versus the *miR-15a/16-1* cluster to the lymphoproliferative phenotype, the authors created an inducible *in vitro* system in which the *DLEU2* transcript and the *miR-15a/16-1* cluster were separately re-expressed in the human I83E95 cell line that was derived from a 13q14^{-/-} CLL. The results showed that *miR-15a/16-1* expressing cells, but not those expressing *DLEU2*, were impaired in proliferation. In accord with this observation, *miR-15a/16-1* expressing cells had a higher fraction of cells in the G0/G1 phase compared with both *DLEU2* and the control, as documented by BrdU incorporation assays. Thus, expression of the *miR-15a/16-1* cluster seems to control cellular proliferation, possibly by inhibiting the G0/G1 phase transition. Altogether, the results of the knockout and re-expression experiments suggest that the *miR-15a/16-1* cluster exhibits negative control of proliferation both in human and mouse B cells. Conversely, the *DLEU2* transcript *per se* did not affect proliferation of 13q14 homozygous deleted cells [56,57]. Because their findings suggested that *miR-15a/16-1* may affect the G0/G1-S phase transition in B cells, the authors sought to identify candidate G0/G1-S phase-related genes among inferred *miR-15a/16-1* targets predicted by different computational target prediction algorithms. Seven proteins (cyclins Ccnd2, Ccnd3, and Ccne1, and cyclin-dependent kinases Cdk4, Cdk6, Chk1, and Mcm5) known to be involved in the regulation of the G0/G1-S phase transition, were downregulated in I83E95 13q14^{-/-} cells upon *miR-15a/16-1* expression. Similarly, the same gene products

showed higher expression levels in anti-IgM-stimulated *miR-15a/16-1*-deficient mouse B cells. Though it remains to be determined whether the proliferation-associated genes identified represent direct or indirect *miR-15a/16-1* targets, the concurrent results obtained in two independent genetically defined *miR-15a/16-1* knockout systems strongly indicate that these miRNAs regulate the expression of multiple genes involved in the G0/G1-S phase transition in both human and mouse B cells [56,57].

***BCL2* and *TRAF2* transgenic mouse model of CLL**—As mentioned previously in this review, a link between genomic abnormalities and high expression levels of Bcl2 in the majority of CLL patients was established by the discovery of the role of *miR-15a/16-1* in the disease. Thus, deregulation of *BCL2* is most likely a major contributor to the pathogenesis of CLL, specifically the indolent form of CLL that is often accompanied by 13q14 deletions. A study by Dr. Reed's laboratory provided an important evidence in favor of this hypothesis [59]. The study was based on two previous models, a mouse over-expressing Bcl2 in the lymphoid system [60] and a transgenic line over-expressing an isoform of TRAF2 in murine B- and T-cells [61]. TNF receptor associated factor 2 (TRAF2) belongs to a family of adaptor proteins that bind to TNF receptor family members and mediate the activation of NF- κ B and JNK by the tumor necrosis factor proteins (Fig. 2) [62]. Increase in lymphocyte proliferation and survival is led by TNF-mediated signaling [24]. *TRAF2* transgenic mice over-expressed *TRAF2* mutant, lacking the N-terminal RING and zinc finger domains located at the N- terminal of the molecule (*TRAF2DN*), which mimics *TRAF1* [21,61]. The mice developed splenomegaly, lympho-adenopathy and a higher number of B-cells but failed to develop a clear blood neoplasia. *BCL2* transgenics were generated by using a construct that mimics t(14;18) translocation juxtaposing *BCL2* oncogene with the immunoglobulin heavy-chain locus at 14q32 observed in human follicular lymphomas. These mice did not develop any malignant phenotype, but displayed polyclonal expansions of B-cells and a more pronounced *in vitro* B-cell survival [21,60]. Contrary to the single transgenic animals, *TRAF2DN/BCL2* double transgenics developed severe splenomegaly, and most rodents developed a B-cell leukemia similar to CLL with B-cell blood count as high as 40 times normal [59]. The mice died prematurely at 6–14 months of age, though Bcl2 or *TRAF2DN* single transgenics had a regular lifespan. As in the case of the *TCL1* transgenic mice, malignant cells were B220⁺CD5⁺, with a small number of animals having B-cell expansions of a different subtype. In conclusion, it is still uncertain if the *TRAF2DN/BCL2*-driven leukemia resembles an indolent or an aggressive form of CLL [21].

***APRIL* driven mouse model of CLL**—NF- κ B is a downstream target of TRAFs (Figure 1). Hence, it is possible that activation of NF- κ B plays a role in the pathogenesis of CLL. Thus, a study described another mouse model for CLL suggesting the involvement of NF- κ B. The study reported a transgenic line expressing APRIL (a proliferation-inducing ligand) under control of the T-cell specific lck promoter [22]. APRIL is a secreted protein, thus its over-expression in T-cells caused elevated APRIL levels in serum. In fact, APRIL works systemically in these transgenic mice affecting proliferation and survival of B-cells [22]. These expanded mature B-cells revealed significant increase in survival *in vitro* in comparison to normal B-lymphocytes [21,22]. The expansion of B220⁺CD5⁺ cells was observed in ~40% of transgenic animals in contrast with other described CLL mouse models where the penetrance was approximately 100%. Generally, APRIL transgenics showed much milder phenotype than *TCL1* or *TRAF2DN/BCL2* transgenic animals. *APRIL* mice showed only mild expansion of splenic B-cells, they did not develop high blood white cells count and did not die prematurely from leukemia or lymphoma. Hence, it is possible that APRIL predisposes CD5⁺ B-cells to malignancy, but this occurrence would require also a second hit, such as Bcl2 or Tcl1 up-regulation.

Conclusions

In the last few years, several important studies uncovering molecular mechanisms of CLL pathogenesis were reported. In addition to *miR-15/16* targeting *BCL2*, *DLEU7* was identified as second tumor suppressor at 13q14 [20]. Also, we demonstrated that *Tcl1* functions as a transcriptional regulator in the pathogenesis of aggressive CLL [23]. Importance of *miR-15/16* and *TCL1* in CLL was supported by generating *TCL1* transgenic and *miR-15/16* knockout mouse models, both showing CLL phenotype. Activation of NF- κ B pathway has evolved as another critical event in CLL pathogenesis since NF- κ B is activated in three CLL mouse models, *APRIL* driven, *BCL2* and *TRAF2* driven, and *TCL1* driven (*Tcl1* functions as NF- κ B activator) (Figure 1).

BCL2/TRAF2DN transgenic mouse model represents a very interesting parallel and confirmation for the idea of two cooperating tumor suppressors at 13q14. Although *BCL2* or *TRAF2DN* single transgenics do not develop CLL, *TRAF2DN/BCL2* double transgenic mice over time developed B-cell leukemias of B220+CD5+ phenotype similar to CLL.[59] Since human 13q14 region contains two cooperating tumor suppressors, *miR-15/16* and *DLEU7*, 13q14 deletions cause inactivation of both genes. *MiR-15/16* inactivation causes a constitutive increase of Bcl2 expression, *DLEU7* inactivation causes induction of TNF signaling through TRAFs. These two events cause the CLL phenotype closely resembling the oncogenic events in *TRAF2DN/BCL2* transgenic mouse model (Figure 1) [20].

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Figure 1.
Schematic representation of molecular pathways in CLL and animal models of CLL.

Figure 2.
Schematic representation of Tcl1 oncogenic function in T-cells and in B-cells.