Retroviral insertional mutagenesis and characterization of the frequently activated PIM kinases
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Chapter VI

Summarizing discussion
The origin of cancer can be brought back to the sequential alteration of genes, either oncogenes or tumor suppressor genes. This means that tumorigenesis is a complex process of accumulating genomic mutations and each alteration yields a selective advantage that is additive to the already existing mutations. In mice, tumorigenesis can be induced by infection with slow transforming retroviruses, such as the Molony Murine Leukemia Virus (M-MuLV) (reviewed in Chapter 1). These types of RNA viruses give rise to tumors by insertion of a DNA copy of their RNA genome into the host genome (here: mouse). This is an obligatory step in the life cycle of retroviruses. Insertion of the viral genome is relatively random and, therefore, viruses can insert in the proximity of proto-oncogenes and/or tumor suppressor genes. If viruses insert adjacent to proto-oncogenes, the upregulation of these genes via either the enhancer and/or promoter sequences of the virus or stabilization using a poly-adenylation signal of the virus provides a selective advantage to the cell carrying this insertion. A selective growth advantage might also be conferred by virus insertion within a tumor suppressor gene generating an inactivated tumor suppressor allele via disruption of the gene. Since the viruses can repeatedly insert their DNA into the genome of a single cell and its descendants, multiple distinct virus-mediated alterations will be found in the resulting clonal tumor. The insertions likely will mark multiple genes that collaborate in the tumorigenic process. In view of the requirement to select for collaborating mutations, the initiating mutation will have a determining role in the mutations that are subsequently selected in the process.

This is illustrated by the identification of E2a as a provirus target in T cell lymphomas derived from mice that express a c-Myc transgene (Chapter IV). The provirus insertions in the E2a promoter yield enhanced E2A protein levels. Prior to this finding, it was generally accepted that E2a acts as a tumor suppressor in T cells. Mice deficient for E2A develop T cell lymphomas (Bain et al., 1997), and upon re-introduction of E2A in the E2a-deficient T cell lines these tumor cells undergo apoptosis (Engel and Murre, 1999; Yan et al., 1997). High MYC levels seem to be a prerequisite in lymphomagenesis. The levels of c-Myc expressed from the EμMyc transgene in the T cell compartment are too low for tumor development. In order to overcome this, MYC expression has to be increased. One of the ways to boost expression of the Myc transgene is E2A overexpression. E2A is known to bind to the transgene's immunoglobulin enhancers and transactivates expression of the downstream gene. Since overexpression of MYC but also E2A induces apoptosis, these MYC and E2A overexpressing tumors should have acquired an anti-apoptotic alteration. The identification of the gene(s) that can inhibit MYC as well as E2A-induced apoptosis would be highly relevant. Unfortunately, the nature of these genes has remained elusive despite extensive analysis of the tumors carrying E2a insertions.

One of the first proto-oncogenes identified as a retroviral target in retrovirus-induced hematopoietic tumors was the provirus insertion site of M-
MuLV (Pim) (Cuypers et al., 1984). The PIM proteins belong to a distinct family of serine/threonine kinases, consisting of the members PIM1, PIM2 and PIM3 (reviewed in Chapter II). At the start of this thesis-research, downstream targets for PIM were not known, but during this thesis-work a number of in vitro substrates have been published. However, the relevance for these proteins in the context of lymphomagenesis is still unknown. To identify components downstream of PIM signaling that are relevant to the oncogenic function of PIM, we set up a genetic screen that is analogous to the enhancer screens utilized in the fruit fly to dissect the RAS pathway (Chapter III). This screen takes advantage of two features of the Pim proto-oncogenes: 1) The Pim genes are a frequent provirus target in M-MuLV-induced lymphomas. 2) The Pim genes collaborate very efficiently with the Myc genes in lymphomagenesis as indicated by the observation that tumors carrying an activation of a Myc gene, either c-Myc or N-Myc, frequently contain Pim activations (Selten et al., 1984), and as shown by the observation that Myc;Pim1 or Pim2 double transgenic mice succumb to pre-B cell leukemias very early in life (Allen et al., 1997; Verbeeck et al., 1991). Since MYC synergizes efficiently with PIM, the introduction of a lymphoid-specific c-Myc transgene should select for co-operating oncogenic events, such as activation of Pim, in M-MuLV-induced lymphomas. Indeed, the tumors induced in an EμMyc background carry very frequently insertions near either Pim1 (~40%) or Pim2 (~10%) (van der Lught et al., 1995, Chapter III). Previously, it was also shown that the frequency of Pim2 activations increased up to 90% of the tumors if Pim1 was knocked out and c-MYC overexpressed through the c-Myc transgene (van der Lught, et al., 1995). This study underscored the requirement for activation of the PIM pathway in the presence of high c-MYC levels for lymphomagenesis. Therefore, M-MuLV-induced lymphomagenesis in EμMyc;Pim1−/−:Pim2−/− mice was expected to still require activation of another Pim gene, a downstream effector of PIM or a PIM-like pathway.

To isolate large numbers of provirus insertion sites in a high throughput fashion, we first adapted a splinkerette-based PCR strategy to amplify virus flanking host genomic sequences followed by comparing these sequences with the annotated mouse genome sequences at Celera and Ensembl (Chapter III). This approach yielded 477 informative flanks derived from 27 EμMyc and 38 EμMyc;Pim1−/−:Pim2−/− tumors. In order the estimate the probability that loci that were occupied more than once in independent tumors marked genes involved in the tumorigenic process we performed a statistical analysis assuming random insertions of retroviruses. Previously, a tumor locus (common insertion site (CIS)) was defined by two or more independent provirus insertions within a genomic region that can be analyzed by Southern blotting. Since the number of insertions screened was relatively small, finding two or more insertions in a relatively small DNA fragment was highly unlikely unless a selective advantage (tumor growth) was associated with this insertion. However, with the current approach, the chance that two viruses land into the same genomic region will increase with the number of retrovirus insertion sites. For our panel of ~500 insertion, a CIS was defined by the occurrence of two provirus insertions within 26 Kb or 3 or more within 250 Kb. If retroviruses insert the genome randomly, which is very unlikely as has been demonstrated for transposons (for review see Craig, 1997), these criteria would yield 0.5% and 0.1% falsely labeled CISs carrying 2 or 3 independent
insertions, respectively. The total number of CISs as identified by the 477 insertions was 52. Out of these 52 tumor loci, 39 had never been identified. Comparison of the RISs isolated from this tumor panel with the RISs isolated from two different tumor panels (total number of RISs ~ 2000) revealed another 49 CISs (Lund et al., 2002; Suzuki et al., 2002). Thus, 230 RISs represented 92 CISs, indicating that approximately half of the insertion sites isolated from the tumors have contributed to the oncogenic process. These results demonstrate the accumulative power of retroviral insertional mutagenesis now the mouse genome has been fully sequenced and annotated. But how long will it take before such a RIS database becomes saturated? The distinct retroviral insertional mutagenesis strategies applied so far have shown that there is only a partial overlap of RISs between different tumor panels. The overlap seems therefore to be influenced by tumor type and the predisposing oncogenic mutation in the germline of the host. Since insertional mutagenesis is not only limited to hematopoietic tumors (Chapter I) and only a very few predisposed oncogenic settings have been subjected to retroviral insertional mutagenesis, saturation is not expected shortly.

Although 477 RISs were identified and the identification of 92 tumor loci was quite appealing, the screen was set up to identify genes that can substitute for \textit{Pim} in lymphomagenesis. To qualify for the \textit{Pim}-complementation group, the \textit{Pim}-substituting genes should not be found in PIM-proficient tumors. This means that the activation of these genes should be absent in a total of 89 \textit{E\textsubscript{mu}Myc}, \textit{E\textsubscript{mu}Myc;Pim1\textsuperscript{"\textminus"}} and \textit{E\textsubscript{mu}Myc;Pim2\textsuperscript{"\textminus"}} tumors. These criteria were met by 10 of the 52 CISs (0.0001 \(\leq P \leq 0.088\)). The most frequently found CIS, activated \textit{Pim3}. The identification of \textit{Pim3} as a gene that is preferentially activated in tumors deficient for its family members \textit{Pim1} and \textit{Pim2} provided evidence for the pathway-specificity of this screen. Three other genes corresponding to the loci replacing \textit{Pim} in lymphomagenesis were the serine/threonine kinase \textit{Tpl2} (~18\%), the tyrosine kinase receptor \textit{Kit} (~8\%) and the cell-cycle regulator \textit{Ccn2} (~13\%). Although the other genes substituting for \textit{Pim} have not been identified, the diversity of the genes identified in this screen points towards a central role for PIM in a complex network of oncogenic signals. Since the identified targets act either downstream of PIM or in a parallel pathway, it seems that PIM is required for growth factor signaling. KIT signaling can activate TPL2 and induce \textit{CYCLIND2} expression. Furthermore, \textit{Pim} expression is induced by a number cytokines that synergize with KIT signaling. A model fitting this would be that PIM acts as a modulator for the cross-talk between synergizing growth factor pathways. Experiments in which a KIT-mutant mast cell proliferation defect can be rescued by the introduction of a \textit{Pim} transgene have indeed suggested a role for PIM either by acting upstream or downstream of KIT signaling (H.M. unpublished results). However, PIM might be involved in other signaling pathways as well. In order to show that the genes near the \textit{Pim}-specific insertions indeed can substitute for PIM deficiency, overexpression of these genes should be able to substitute for \textit{Pim} in lymphomagenesis.

\textit{Pim} is likely involved in the cross-talk between different growth factor signaling pathways. Since growth factor signals are crucial for the development of any organism, this would suggest that animals that lack all PIM family members should exhibit a defect in development. The generation of a mouse that is deficient for \textit{Pim1}, \textit{Pim2} and \textit{Pim3} is described in Chapter
V of this thesis. Although the Pim-mutant mice are viable and fertile, they display a reduced body size, which is primarily a result of Pim1 and Pim3 loss illustrating the redundancy in function of these two Pim members, at least for this specific feature. Further analysis showed that the reduction in body mass was caused by a reduction in the number of cells indicating a deficiency in either proliferation or anti-apoptosis. A decrease in size from birth on normally represents a defect in growth hormone (GH) signaling. Pim-mutant mice are already smaller at birth, however, indicating rather a defect in IGF/insulin signal transduction pathways (for review see Accili et al., 1999). Since the Pim genes are regulated by a range of distinct hematopoietic growth factors, we also studied the response of PIM-mutant cells to these growth factors. Bone marrow colony assays demonstrated an impaired ability of Pim-mutant cells to form colonies in response to IL-3, IL-5, SCF and TPO as the number as well as size of the cells was reduced. Remarkably, the response to GM-CSF of which the downstream signaling is similar to that of IL-3 was unaltered. This observation indicates that although both cytokines are potent inducers of Pim transcription, PIM is only important for IL-3 mediated signaling. Whether PIM affects proliferation, differentiation or apoptosis in this response remains, however, to be investigated. The early transcription of Pim in response to GM-CSF, IL-5, TPO and IL-3 is likely regulated by JAK2/STAT5 signaling (Lilly et al., 1992; Temple et al., 2001; Nagata and Todokoro, 1995; Domen et al., 1993). The partial overlap in phenotype between Pim-mutant and Stat5a;5b-mutant bone marrow cells with respect to colony formation suggests that some of the phenotypes ascribed to STAT5 signaling is mediated through transactivation of the Pim genes. In addition to these cytokine-dependent aberrations, we observed a proliferation defect of pre-B cells in response to IL-7 in vivo as well as in vitro. These defects were apparent in Pim1 and Pim1;Pim2;Pim3-mutant animals and to a lesser extent in Pim3-deficient mice. This indicates that Pim1 and Pim3 can also act redundantly in this setting, but that Pim1 is the most crucial Pim member here. Since the proliferating fraction of B cell precursors is higher in Pim-mutant animals in comparison to wild-type animals, this would suggest that rather the proliferation than apoptosis is affected in Pim-deficient B cells. We also checked whether Pim-mutant T cells show an impaired response to TCR activation and IL-2. Pim1;Pim2;Pim3-mutant peripheral T lymphocytes do not exhibit a significant altered proliferation in response to TCR activation. However, the synergism between TCR and IL-2 seems to be absent in Pim1;Pim2;Pim3-mutant T cells, and to a lesser extent in Pim1- or Pim2-mutant T cells. This finding is consistent with the model for PIM functioning as emerging from the genetic screen discussed above. Culturing T cells in the presence of TCR/IL-2 will provide an in vitro system that might be instrumental in addressing PIM signal transduction. Future biochemical experiments might teach us about the signaling pathways downstream of the PIM proteins important for the synergism of TCR and IL-2 signaling.

In summary, two distinct genetic approaches described in this thesis demonstrate, independently, that PIM proteins likely play a role in the cross-talk between growth factor signaling pathways. This thesis further illustrates the power of retroviral insertional mutagenesis to identify new genes involved in tumorigenesis and to dissect pathways collaborating in this process.
Discussion

References
