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From Hen House to Bedside: Tracing Hanafusa's Legacy from Avian Leukemia Viruses to SRC to ABL and Beyond

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Abstract

The discovery of the *Src* oncogene was the first step on a long journey toward improved cancer chemotherapy. In this review, we explore Src and BCR-ABL, signal transduction, and recent advances in oncogene addiction and celebrate Hidesaboro Hanafusa and the many researchers who ushered in the age of target-directed therapy against tyrosine kinase oncoproteins.

Keywords: Hanafusa, Src, Abl

Introduction

In 1911, Peyton Rous discovered that chickens could transmit sarcomas to one another, thereby implying that tumors could be caused by transmissible agents. Subsequently, he showed that cell-free filtered tumor extracts could transfer sarcomas through the avian sarcoma virus.[1] The significance of this finding was hotly contested because of the lack of obvious infectious tumors in humans. Nevertheless, the mystery over this cause of cancer was lifted after several decades when the nonreceptor tyrosine kinase *src* became the first oncogene identified, thereby ushering in a new paradigm in basic and clinical research.

v-src and *c-src*

The tumor retrovirus genetics field flourished in the mid-20th century when several groups searched for the component of the Rous sarcoma virus (RSV) responsible for tumor propagation (for detailed reviews of Src, see Martin[2,3] and Vogt[4]). In the 1960s, working in Harry Rubin's laboratory, Hanafusa discovered that the RSV preparation contained an additional associated virus.[5] He purified the RSV-associated virus and was able to uncouple the ability of RSV to infect and transform cells.[6] Hanafusa's ability to segregate defective viruses from replication-competent viruses was a technical *tour de force*, testimony to his experimental prowess, and one of his most seminal contributions. In the early 1970s, the Duesberg and Martin laboratories used temperature-sensitive mutants of RSV to map the transforming region of the virus, allowing for the identification of *v-src*.[7-9] In 1976, Varmus and Bishop identified these sequences in cells using a complementary library of deletions of *Src* as probes.[10] Around the same time, Hanafusa's laboratory infected chickens with a transformation-defective virus containing large deletions in the v-src region. The transformation was rescued by a homologous recombination event from within the cell.[11] The discovery and characterization of the recovered viruses suggested appropriation of cellular oncogenes through viral recombination events.[12] Finally, in the 1980s, viral and cellular src was sequenced, proving that the drivers of cancer could be mutated or overexpressed genes.[13,14] Moreover, these findings pointed to the cellular origin for cancer and the coining of the term "proto-oncogene."

More genes containing similar sequences were cloned, including *v-fps* and *v-crk* from Hanafusa's laboratory.[15-17] Tony Pawson and Hanafusa compared the sequences and identified similarities between the oncogenes they cloned that contributed to characterization of the Src homology domains (SH1-SH4).[15,18-20] Src was identified to be a tyrosine kinase (SH1) that also contained a region that enables binding to phosphorylated tyrosines (SH2).[15,21,22] Another domain binds to proline-rich regions located in other proteins (SH3). Subsequently, 8 more Src family members (Fgr, Blk, Fyn, Lck, Hck, Yrk, Yes, and Lyn) were identified as they share the same structural format with Src, besides their unique domain (SH4).[23,24] These and other modular domains became critical in classifying a function to newly identified genes.

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CML and ABL

The groundbreaking work on *Src* and fundamental advances in molecular biology were paralleled by the discovery of another oncogene, *v-abl*, in the Abelson murine leukemia virus. In 1970, *v-abl* was found to cause lymphosarcoma of a nonthymic origin in mice.[25] This virus model ultimately contributed to an understanding of the oncogenic driver of chronic myelogenous leukemia (CML), a clonal disorder marked by an increase in mature myeloid cells in the blood. This myeloproliferative disorder can progress toward an acute or blast phase, which is characterized by a sudden increase in immature cells in the bone marrow and the blood.

Presaging the discovery of the altered ABL oncogene, in 1960, Nowell and Hungerford identified a recurring chromosomal aberration in patients with CML.[26] Over a decade later, Janet Rowley identified this reciprocal chromosomal translocation as t(9;22)(q34.1;q11.23).[27] The *c-ABL1* gene, the normal cellular counterpart to *v-abl*, was cloned and identified fused to a gene in a region in which chromosomal break points were tightly clustered, hence thereafter called the break-point cluster region gene, or *BCR*.[28-30] The *BCR-ABL* fusion was found in 95% of patients with CML and in 10% to 30% of patients with acute lymphoblastic leukemia (ALL), a disorder more similar to the leukemia that develops from the Abelson murine leukemia virus[26,31] than to CML. Moreover, the *BCR-ABL* fusion was shown in a mouse model that it could solely drive CML[32] or ALL.[33] The discovery of the BCR-ABL translocation became the first of many mutations or chromosomal aberrations found in leukemia.[34,35]

The Structure and Activation of Src and Abl

Besides their parallel history, Src and Abl share a similar structure and function. Src and Abl both have conserved SH3, SH2, and tyrosine kinase domains. Both proteins maintain their tightly regulated kinase activity through similar principles of autoinhibition and autoregulation[36] but with slightly differing features. For example, the phosphorylated C-terminal domain of Src self-interacts with its SH2 domain as a "latch" mechanism, constraining an inactive conformation, which is further stabilized by a "clamp" that is mediated by a tight interaction between the SH2 and SH3 domains.[37,38] In comparison, ABL can be inactivated by its N-terminal myristoylation cap locking to the C-lobe of the kinase domain.[39,40] In contrast, when disrupted by chromosomal translocation, the c-ABL N-terminal region is replaced by BCR in the BCR-ABL fusion, and a coiled-coil domain of BCR mediates oligomerization, thus pushing the kinase toward an activated state.[37,41] Besides structural characteristics that modify kinase activity, the C-terminal region of ABL mediates signaling interactions with the cytoskeleton and other signaling adaptors.[42-45]

Src and BCR-ABL Signaling

Signaling via the Src family kinases and BCR-ABL are closely related; a more detailed description can be found within these reviews.[46,47] BCR-ABL and Src family members Hck and Lyn physically interact with each other and play a key role in leukemogenesis.[48-50] The SH2, SH3, and C-terminal domain of BCR-ABL can interact with Hck,[51] which can in return phosphorylate the SH2, SH3, and Grb2 binding site.[49] BCR-ABL can activate Hck, leading to phosphorylation of STAT5.[52] When infected with a BCR-ABL retrovirus, mice lacking Lyn, Hck, and Fgr develop a CML-like disease but not a lymphoid disease.[48] These and other data implicate the Src family of kinases in progression to blast crisis,[53-55] and increased src activity has been observed in a subset of patients resistant to inhibitors.[54] A mutation in *BCR-ABL* within a target residue of the src family kinases can lead to resistance to therapy.[46,56]

Signaling Complexes

Historically, many groups have focused on the signaling cascades downstream of BCR-ABL and receptor tyrosine kinases to elucidate mechanisms for oncogenic transformation. Adaptor molecules such as Grb2/Gab2/Shc, Dok1/Dok2, and CrkI complexes orchestrate the activation of key signaling nodes.[57,58] Hanafusa's laboratory contributed many critical observations to this enormous compendium of data, such as elucidating the role of v-crk in activating the PI3K/Akt signaling pathway.[59,60] CrkI was initially considered dispensable for lymphoid ABL transformation but proved in recent studies to be required for myeloid leukemia.[61,62] A complex interplay of adaptors with kinases contributes to BCR-ABL activation of a variety of signaling nodes, including the c-MYC, PI3K/AKT, RAS, and JAK/STAT pathways.[63-67] These pathways together drive proliferation and maintain survival.

Molecular Targeted Therapy

The discovery of Src and a host of related tyrosine kinase oncoproteins not only enhanced our understanding of cancer's origins but also triggered a revolution in cancer therapy. Prior to the 1990s, CML had a poor prognosis, with allogenic stem transplantation as the only available curative therapy.[68] Cytotoxic drugs such as hydroxyurea were administrated to control symptoms and leukocytosis but did nothing to change the inexorable progression of leukemia to a fatal blast crisis.[69] Although interferon therapy could alter the course of disease and prolong survival for a subset of responders, the majority of patients ultimately succumbed to CML.[70]

Because of the highly conserved nature of the ATP-binding catalytic pocket of tyrosine kinases, conventional wisdom considered it impossible to develop a specific inhibitor against a given kinase without inducing intolerable toxicity. In the late 1980s, scientists at Ciba-Geigy (now Novartis), in collaboration with Dana Farber, performed a screen for inhibitors against a panel of kinases, developing selective inhibitors of PDGFR that later proved to also inhibit BCR-ABL.[71] Being the first molecular lesion consistently associated with and also causative of human

CML,[32] the BCR-ABL kinase was considered an attractive drug target. The potent ABL inhibitor known as STI-571 was identified and tested in human cell lines and patient cells in Brian Druker's laboratory[72] and shown to induce death in human cancer cell lines.[72-74] Serendipitously, this inhibitor also exhibited favorable pharmacokinetic properties and was readily translated to the clinic. Despite the structural similarities between ABL and Src, STI-571 had no effect on tumors driven by v-src.[75] The drug, later dubbed imatinib, was subsequently shown to bind tightly within the ATP-binding cleft of ABL, trapping the kinase in an inactive conformation.[76] Structural differences between ABL and Src determined that imatinib was specific to the former but not the latter.[76] Druker, Charles Sawyers, Moshe Talpaz, and Novartis, which now owned the compound, conducted a phase I study to test its efficacy. The drug showed impressively low toxicity and a remarkable hematological response rate of >90%.[77] Over the past decade, imatinib (Gleevec, Novartis, Basel, Switzerland) has evolved into the standard of care, transforming CML from an inevitably fatal malignancy to a manageable chronic condition for most patients.[78] Moreover, it has been shown to be effective in treating other malignancies such as gastrointestinal stromal tumors driven by mutated c-kit and other translocations containing PDGF fusions.[79,80]

Resistance to Targeted Therapy

Even though imatinib has yielded dramatic results, some patients nonetheless develop resistance or convert to blast crisis.[78] Investigation of the mechanisms of resistance has revealed mutations within BCR-ABL that reactivate the kinase and abrogate drug binding.[81] The reactivation of kinase activity, as opposed to activation of alternative pathways, highlights the essential function of ABL kinase activity as the main driver of disease. Despite the success of imatinib, the many mutations identified in patients compelled the development of next-generation inhibitors that are more potent and active against imatinib-resistant mutations. Nilotinib (Novartis) and Dasatinib (Bristol-Myers Squibb, New York, NY), a dual SRC/ABL inhibitor, have proven their efficacy as second-line therapies in CML.[82-84] Of note, dual SRC/ABL inhibitors have also been considered in solid malignancies.[85] But one challenge remains—the T315I mutation,[86] which places a bulky isoleucine residue in the kinase domain, blocking the access of inhibitors to the active site while also creating conformational changes that activate the kinase itself.[86] Inhibitors like ponatinib (AP24534), currently in phase II clinical trials, and HG-7-85-01, currently preclinical, are aimed at targeting the T315I mutation.[83,87,88] Moreover, ABL inhibitors that act allosterically, like GNF-2, are being combined with Nilotinib (Novartis) and show activity against T315I.[89] In addition to the search for more potent inhibitors that mitigate resistance, alternative strategies are being developed to target the quiescent BCR-ABL stem cell population that appears insensitive to kinase inhibition and thus acts as a reservoir from which relapse can occur.[90,91]

Oncogene Addiction

Early work on viral oncogenes suggested that a single gene could hijack multiple pathways, leaving the cell dependent upon the oncogene for growth and survival signals. Indeed, another aspect of the legacy of Src biology is the concept of oncogene addiction: cancer cells become "addicted" to signals emanating from a particular oncoprotein, such that abrogation of that pathway via therapeutic targeting could potentially affect only cancer cells while sparing normal cells.[92,93]

A particularly good validation of the concept of oncogene addiction stems from the remarkable activity of imatinib in the context of CML.[93-96] The exquisite sensitivity of leukemia cells to imatinib, and their death in response to drug treatment, suggested that the cells had indeed become entirely dependent on oncogenic signaling and had somehow lost the natural controls on cell proliferation and survival. Given that the BCR-ABL oncoprotein represents a "gain of function," one might have supposed that drug inhibition would merely revert cells to their normal routine. Instead, in many experimental models, cell death is a consequence of drug treatment. Nevertheless, whereas oncogene addiction might describe the effectiveness of imatinib against rapidly amplifying progenitor pools in CML patients, the more quiescent hematopoietic stem cell pool harboring the BCR-ABL translocation is not eliminated by imatinib, arguing that the stem cell compartment is not suffering addiction, which helps explain the challenge in eradicating leukemic clones.[97] In addition to BCR-ABL, oncogene addiction has also been reported in other cancer signaling pathways such as Myc or Ras.[98,99] Alternatively, "oncogenic shock" has been invoked to explain tumor cells' sensitivity upon the loss of oncogenic signals,[93] whereby acute removal of the oncogene can lead to rapid apoptosis and cell cycle arrest.[93,96] Whether addiction or shock, the effectiveness of target-directed chemotherapy owes to epigenetic changes in the cellular landscape that renders cells dependent upon a given oncogenic signal, thereby allowing for therapeutic targeting and the sparing of normal tissues.

The paradigm of inhibiting kinases remains a dominant focus of today's therapeutic cancer treatments. To expand the current collection of targets, several groups have surveyed the entire kinome in cancer cell lines.[100-103] Interestingly, addiction does not necessarily depend on overexpression or mutation of one particular gene.[104] For example, when STK33 is knocked down in K-RAS–dependent tumors, rapid apoptosis occurs specifically in cells addicted to K-RAS but not N-RAS.[105] These findings suggest that a specific kinase or a particular checkpoint within a pathway could be required uniquely within the cellular context for survival.

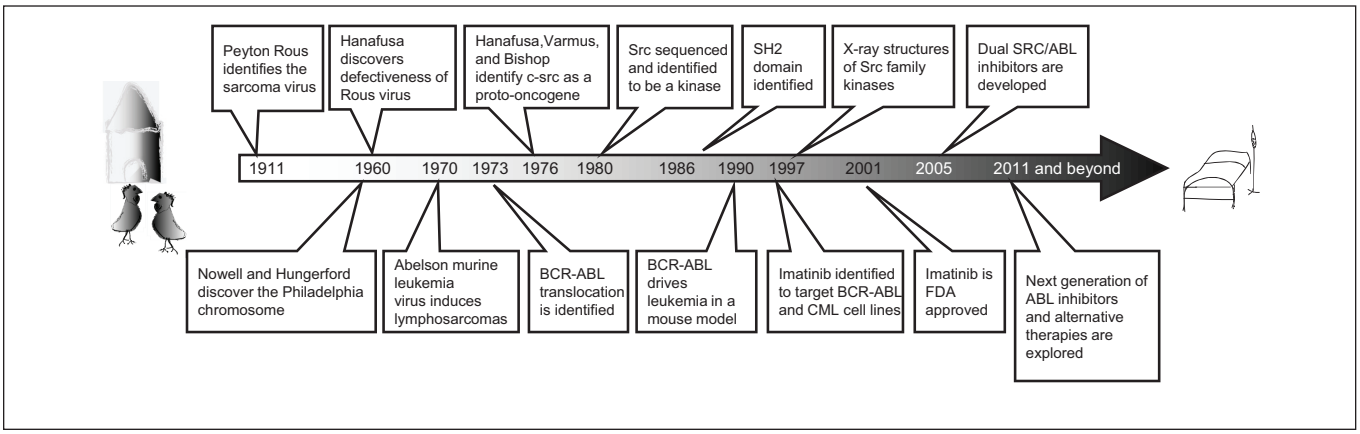

Figure 1. Timeline: the legacy of Hanafusa.

Novel Targets

Besides the activation of kinases, hematopoietic transformation results in massive perturbations of the transcriptional and translational networks. Modifications of myeloid-specific transcription factors such as CCAAT/enhancer binding protein (CEBP) and PU1 are known contributors to leukemia.[106,107] Besides these transcription factors, RNA binding proteins can also regulate leukemia progression as shown in a recent study that identified the RNA binding protein MUSASHI-2 (MSI2) to regulate translation in myeloid leukemias. Moreover, *MSI2* can be utilized as a diagnostic marker for aggressive AML and CML-BC.[108,109] MSI2 blocks the translation of Numb and regulates myeloid differentiation and proliferation. Human myeloid leukemic cells are addicted to high MSI2 levels, based on the results of differentiation and apoptosis in cell lines that have been depleted for MSI2. Admittedly, while transcription factors and RNA binding proteins provide a novel class of therapeutic targets, it is still currently difficult to develop inhibitors against them. Potential strategies include therapeutic inhibitory RNAs and/or small molecules targeting interactions between DNA or RNA and proteins.

Conclusion

It has been a century since avian viruses were first identified in the sarcomas of chickens. Hanafusa was one of the titans of cancer research in that last century, during which he and many others through the study of Src pioneered the field of oncogenic signaling, which ultimately resulted in molecularly targeted cancer therapy (Fig. 1). One of us (G.Q.D.) was privileged to have worked for a brief period as a young medical student in the Hanafusa laboratory and to have witnessed both this great scientist's command of experimental detail and deep appreciation for the complexities of biology. His legacy will long be respected.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the authorship and/or publication of this article.

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