Oncogenes and the Revolution in Cancer Research: Homage to Hidesaburo Hanafusa (1929-2009)

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La mémoire ne nous servirait à rien, si elle fut rigoureusement fidèle.

—Paul Valéry

I would like to express my gratitude for the invitation to open this celebration of Saburo's life. It is a great honor, and I am deeply touched by this event.

Hidesaburo Hanafusa was a man of towering achievements, of deep originality and imagination, a master of rigorous analysis. He was a man of great dignity, openness, generosity, and humility. To his postdocs and students, he was an inspiring teacher and model; to his colleagues, he was a warm and supportive friend. He earned a degree of affection and loyalty that is as rare as it is precious.

But no scientist works in a vacuum; each is a link in a chain of explorations and discoveries, of lives lived and of developments started and ended. Saburo was an integral part of the great revolution in cancer research that unfolded in the past century. That revolution had its origin in the study of the smallest and simplest organisms—in the study of viruses. I will pay homage to Saburo by reflecting on his work in this larger historical context.

The dawn of virology dates to the beginning of the past century. In 1911, Peyton Rous, working at the Rockefeller University, discovered the first virus that could cause solid tumors—the virus now known as the Rous sarcoma virus.¹ That was the spark that started tumor virology. In short order, researchers in Europe identified several other avian tumor viruses. Initially, however, these discoveries appeared to represent primarily confirmations of Rous's work that were more lateral arabesque than progress. Unfortunately, few of these viruses have survived. Those that were preserved ultimately turned out to be far more than simply insignificant duplications of archival interest. Here, I will only mention the Mill Hill 2 virus, which was found to contain the novel oncogenes *myc* and *raf*, and of course there was the CT10 virus, the source of the unique *crk* oncogene, to which I will return later. (In the 1970s, I asked all of my friends at the National Institute for Medical Research Mill

Hill, London and the Houghton Poultry Research Station to check their freezers for old tumor samples, and besides the Mill Hill 2 virus, I got the PRCII and PRCIV sarcoma viruses, which also yielded new oncogenes.)

Tumor virology had a slow start. In the early 1900s, there was a lack of appropriate techniques for studying the fundamental aspects of viral oncogenesis, and the scientific attitudes prevalent during this time were not conducive for the kind of analysis that was needed. The question was also raised whether virus-induced tumors in chickens would be relevant to cancer in mammals. But then mammalian tumor viruses appeared on the scene. In 1933, Richard Shope, also at Rockefeller, discovered papilloma viruses in rabbits.² Viral papillomas, with their benign to malignant transition, presented a far more varied and seemingly more interesting neoplasm than the simple and straightforward Rous sarcoma. Papillomas are a biologically more tractable disease than are avian sarcomas and could be much more easily analyzed with the available tools. Peyton Rous was attracted by the pathology of papillomas and devoted a great deal of his efforts to studying these mammalian neoplasms.

Peyton Rous was a great scientist who has my deep admiration and respect. I met him when he was in his late 70s. He was chairing a tumor virus symposium at the National Academy of Sciences. I gave a talk at this symposium, and I fondly remember his kindness and support. But Peyton Rous, like many great men, could be quite opinionated, and he was certainly not always right. His most stunning error came in 1959, when he published a combative review in *Nature* under the title "Surmise and Fact on the Nature of Cancer."³ In this he declared his strident opposition to the idea that

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somatic mutations could cause cancer. He was mainly concerned with cancer as an infectious process. Little did he suspect that the Rous sarcoma virus and its relatives are among the most potent somatic mutagens known and that this same Rous sarcoma virus induces cancer by picking up a cellular gene, mutating it and introducing it into the next host cell.

The first half of the past century was a stagnant period for animal virology, characteristic of eras that often precede and precipitate a scientific revolution. That revolution was already brewing in a related but distinct field, that of phage research. Modern virology owes its intellectual origins to the phage school. Phage research in the 1940s was taken over by a very tightly knit, almost clannish, group of scientists that was led by Max Delbrück and Salvador Luria in the United States and by Andre Lwoff in Europe. These researchers were giants, the pioneers who gave the phage school its focus, direction, and discipline.

Phage research used a scientific approach that was entirely novel, revolutionary, and—despite ties to bacterial genetics—distinct from that of any scientific enterprise then active. The idea of viruses as model biological organisms, the challenge to understand viral replication, to define the role of the host cell, the strictly reductionist strategy that concentrated on the single cell and the single viral particle—all of these marked a new way of thinking about biological problems. This approach defined an attitude that was compelling for biologists and that attracted distinguished physical scientists as well. It was the major force in the movement that we now recognize as shaping quantitative biology. The Cold Spring Harbor lab, site of the famous annual phage course, was the primary hub for this movement. Caltech—with Max Delbrück—was the focus on the West Coast. By the mid-1950s, Delbrück's disciples had fanned out throughout the country, but their ties to Pasadena remained strong, and regular meetings were held at the parent lab. The spirit of the phage school at Caltech was contagious. Eventually it had to conquer animal virology.

That critical turning point came with the introduction of the plaque assay for cytocidal animal viruses by Renato Dulbecco and Marguerite Vogt.^{4,5} The inspiration for this assay derived from the spectacular success of phage workers in analyzing and understanding virus infections. The plaque assay for animal viruses was an almost direct transfer of phage technology to animal cells and animal viruses. But it also relied on the tremendous advances in cell culture that were pioneered at the National Institutes of Health (NIH) by Harry Eagle. The plaque assay for cytocidal viruses transformed the field of animal virology. Its introduction in 1953 marks the origin of experimental cellular virology and, ultimately, of today's molecular virology.

The next big advance in virology came with the introduction of the focus assay for Rous sarcoma virus, which was created by Howard Temin and Harry Rubin. Today it

seems only a small step from the plaque assay for cytocidal viruses to the focus assay for virus-induced oncogenic transformation. Some of the characteristics and techniques of the two assays are indeed the same, but to move from assaying cell killing to assaying oncogenic transformation was a paradigm shift. Howard Temin was Renato Dulbecco's graduate student at Caltech, but he collaborated mostly with Harry Rubin, a thoughtful, highly analytical animal virologist who had just finished an adventurous stint as a Department of Agriculture veterinarian, working on the foot-and-mouth disease eradication program in Mexico.

Until the late 1950s, Rous sarcoma virus had been studied only in the chicken embryo and in chickens. There was no quantitation. Typical test results showed incredible variation, making conclusions tentative at best, and there was no cell culture system for any tumor virus. The situation changed marginally with a short note by Manaker and Groupe in *Virology*, then the preeminent journal of the field. This brief note suggested that a cell culture-based assay for virus-induced oncogenic transformation might be possible. Temin and Rubin took up the challenge and designed the focus assay in 1958.⁶ This assay, which enabled quantitation of oncogenic activity, was the birth of modern tumor virology.

At this point, I would like to insert a short digression into my personal history. In my last year as a graduate student at the Max Planck Institute of Virology in Tübingen, Germany, I applied to Salvador Luria, then in Urbana, Illinois, for a position as a postdoc. Luria replied immediately with a short handwritten note (I wish I had kept it), saying that he had just decided to move to MIT. He expected to lose at least a year of lab time because he had to equip his new facilities and rebuild his group. He advised me to look elsewhere.

At almost the same time, Harry Rubin came to Tübingen to give a seminar on Rous sarcoma virus and the new focus assay. I remember that all of those present in the tiny seminar room adjacent to the institute library had the feeling that Harry Rubin's talk heralded a new era in tumor virology. The distinguished plant virologist Wendel Stanley had just recruited Rubin from Caltech to the Virus Lab at UC Berkeley. Rubin, who was less concerned than Luria about a possible slowdown caused by the move, accepted me as a postdoc, and I joined his lab in the fall of 1959, almost 50 years ago.

When I came to Berkeley, the lab contained another postdoc, a graduate student and two fabulously efficient, ambidextrous, and highly skilled technicians. There was an atmosphere of high energy, curiosity, and discovery—almost of adventure. The only problem was the focus assay. Quite often it did not work. One week, all experiments would be fine, producing valid data. The next week, we would run reams of experiments only to find no foci: all of our work had

been in vain. Success and failure were not predictable but eventually were linked to certain batches of chicken embryo fibroblasts. Harry Rubin then discovered the explanation and the solution for the perplexing lack of predictability. Some embryos were infected by a virus that caused resistance to Rous sarcoma virus, and this virus was the source of our failures. This Rous-interfering virus was called RIF, for resistance-inducing factor. Testing individual embryos for infection by RIF beforehand eliminated the problem.

RIF is an avian leukosis virus that is vertically transmitted from the hen through the egg yolk to the chick. It was the first example of a relatively benign retrovirus, and its discovery presaged Saburo's work on helper factors, endogenous retroviral genetic elements that can interact with exogenous virus. I am not going to describe this part of Saburo's research except to say that it elicited awe for its ingenious experimental design and the precision with which it was carried out. A commentary in the News and Views section of *Nature* around 1970 already referred to Saburo as "legendary." The story goes that Saburo's papers on helper factors were understood by only 3 other people: his wife and scientific collaborator Teruko, and the 2 avian tumor virologists Steve Martin and Robin Weiss. I think there is a grain of truth in this.

There was one other noteworthy aspect of the Rubin lab, the chronic conflict between Harry Rubin and Howard Temin. It was the collision of rigorous, disciplined, restrained analysis with intuition, imagination, and vision. The dispute had started over a paper that Temin and Rubin authored and that showed an unexpected and unexplained radio-resistance of Rous sarcoma virus. Temin saw in it some resemblance to lysogenic phage and wanted to apply this model to Rous sarcoma virus, postulating the existence of a provirus. Rubin would have none of this. He strictly confined his interpretations to what could safely be concluded from observed facts. But Temin did not want to settle for a partial understanding; he was determined to commit himself to what appeared, at least initially, a very risky hypothesis. Although the discussions proceeded mostly in good spirit, the fundamental difference between Rubin and Temin was never completely resolved, even after it had become clear that Howard Temin's views had prevailed.

It is easy to fault Harry Rubin today for being wrong and closed-minded, but he had actually produced data that were very difficult to reconcile with the provirus hypothesis. He had found that vertical transmission of avian leukosis virus from one generation to the next is strictly maternal; there is no paternal transmission.⁷ Even today, there are only ad hoc explanations for this puzzling finding. In the Rubin lab, the conflict with Temin was always palpable; it was part of the intellectual atmosphere. This polarity probably touched Saburo and Teruko, but during their tenure in the lab, there was no compelling reason to take sides.

In 1961, when Saburo and Teruko arrived in Berkeley, I had just finished an immunofluorescence study of Rous sarcoma virus infection and, together with Rubin, had isolated a second, nontransforming virus, Rous-associated virus (or RAV), from our virus stocks. I had accepted a job at the Pathology Department of the University of Colorado School of Medicine and left for Denver early 1962. Thus, my time in the Rubin lab overlapped with Saburo and Teruko's stay there for just a few months. For both the Hanafusas and me, Harry Rubin's lab provided the opening of a new world and a lasting formative experience. We always felt deeply grateful to Harry Rubin for his friendship and for the opportunities and the guidance he gave us.

The next time I saw Saburo was in 1964 at the Avian Tumor Virus Meeting at Duke University in Durham, North Carolina. Saburo and Teruko, with their baby daughter Kei, were on their way to France to spend a 2-year exile imposed by the conditions of the J-1 visa. It was the meeting at which Howard Temin first publicly announced the provirus hypothesis, facing almost universal disbelief.⁸ It was also the meeting at which it became clear that Saburo would become a major force in tumor virology. Following up on an initial observation by Howard Temin, Saburo had just demonstrated the defectiveness of the Bryan strain of the Rous sarcoma virus.⁹ Cells could be transformed in the absence of virus production.

This separation of virus maturation from oncogenic transformation was of fundamental importance. It told us that transformation did not depend on the complete set of viral genes and functions and could be induced in the absence of infectious virus production. The example of RAV had taught us that the reverse was also true: you could have vigorous virus replication without oncogenic transformation. These were the first hints of a specific cancer-inducing gene in the virus, a function that was distinct from replicative activities.

Today the defectiveness of Rous sarcoma virus serves as a universal model for the genome structure of highly oncogenic retroviruses. In these viral genomes, the oncogene displaces essential viral information, and as a result, these viruses require a helper virus that provides the missing functions in trans. It is ironic that this replication-defective strain of Rous sarcoma virus studied in the Rubin lab and so illuminatingly analyzed by Saburo is not representative of the majority of Rous sarcoma viruses. Most strains of Rous sarcoma virus are not replication-defective; they are exceptional in being the only rapidly oncogenic retroviruses that are completely replication-competent. This dual competence for replication and transformation, combining all functions in a single viral genome, was of critical importance for the identification of the first oncogene, the *src* gene of Rous sarcoma virus.

Diverse sets of data contributed to the recognition of *src*. First, there was the distinction between replication and

transformation that was discovered by Saburo. At about the same time, Temin and the geneticist Boris Ephrussi isolated a mutant of Rous sarcoma virus that induced a morphologically distinct cellular transformation, indicating that at least some aspects of oncogenesis are under the genetic control of the virus.¹⁰

Then came the isolation of temperature-sensitive mutants of Rous sarcoma virus. The first report of such mutants came from Saburo's compatriot Kumao Toyoshima and myself, followed shortly by publications from Steven Martin's lab in Berkeley and Saburo's group in New York.¹¹⁻¹³ These viral mutants could not induce transformation at elevated temperatures, but they were still able to reproduce. The inescapable conclusion from this work was that a particular viral protein controlled the oncogenic transformation process.

The temperature-sensitive mutants had been obtained with replication-competent strains of Rous sarcoma virus, and they made these strains the universal standards. Helper viruses were no longer needed to sustain viral replication and presumably could be eliminated. But no matter how hard one tried to clone the virus biologically, the progeny virus always contained, in addition to the transforming virus, a virus that was replication-competent but nontransforming. This latter agent represented transformation-defective genetic variants that spontaneously arose from the transforming virus. A comparison of the RNA genomes of transforming and transformation-defective viruses carried out by Peter Duesberg and myself revealed an interesting size difference: the genome of the virus that could transform and replicate was about 20% larger than the genome of the transformation-defective virus that could only replicate. It was tempting to speculate that this difference represented the physical entity of the viral transforming gene.¹⁴

At this time, RNA fingerprinting with RNase T1 came into use, and Peter Duesberg and his lab, collaborating with both Saburo and with me, determined the gene order of Rous sarcoma virus, provided an estimate of the size of the transforming sequences, and positioned this *src* gene toward the 3' of the genetic map.^{15,16} The transformation-defective mutants were merely terminal truncations of this gene sequence.

The discovery of reverse transcriptase by Howard Temin and David Baltimore in 1970 turned the central dogma of molecular biology on its head, proved the provirus hypothesis, and generated the essential tools that were needed for developing the molecular genetics of retroviruses.^{17,18}

David Baltimore had been fascinated by the unexplained sensitivity of RNA tumor viruses (as these retroviruses were then called) to inhibitors of DNA synthesis. In 1969, Peter Duesberg and I found that the DNA in question was viral, not cellular, and John Kates and Brian McAuslan had discovered that polymerases could be essential components of

viral particles. So David Baltimore requested a sample of Rous sarcoma virus from me, and the rest is history. In his characteristically incisive style, Baltimore became a pioneer of retrovirology and of oncogenes. He greatly admired Saburo; they shared an interest in oncogene-mediated cellular signaling and collaborated in groundbreaking work on modular protein interaction domains.¹⁹ For both, the Rockefeller University was an academic home.

Reverse transcriptase rapidly turned into a formidable force for discovery. Michael Bishop and Harold Varmus used this new tool to generate a DNA probe specific for viral *src*. They applied subtractive hybridization to DNA transcripts from transforming and transformation-defective viruses, obtaining sequences specific for the transforming virus and hence *src* specific. With this probe, they established the cellular origin of *src*, a fundamental observation that was extended to all retroviral oncogenes.²⁰ Indeed, being of cellular origin has become one of the defining criteria for retroviral oncogenes. There are exceptions to this rule, but they are very few. Today, cellular oncogenes are recognized as the major driving forces of tumor formation.

The realization that oncogenes are derived from the cell raised an important question: how are these genes acquired by the viral genome? As yet, we have no definitive answer. We have plausible models with some experimentally verified steps but no reproducible and efficient cell-based or organismal system for the acquisition of cellular genes by purely retroviral genomes. Saburo was strongly attracted to this problem. It was obvious from the onset that it would not be an easy task. But Saburo, ingenious and resourceful, decided to divide the problem into its components and then solve these separately. One of these components was to prove that the virus, by integrating into the cell genome, could pick up cellular sequences, provided there was some homology between virus and cell. He induced partial deletions in viral *src*, generating a transformation-defective virus. Upon passage in chickens and in cell culture, this virus acquired the missing *src* sequences and regained the ability to transform cells. These recovered sarcoma viruses provided convincing demonstrations of the ultimate origin of retroviral oncogenes.²¹

It soon became clear that *src* was not the only oncogene. Avian leukemia viruses of various sorts yielded *myc*, *myb*, *erbA*, and *erbB*. *Ras* was discovered in rodent sarcoma viruses. The interest in finding new oncogenes became intense and was directed toward viruses that had been overlooked or were entirely new. Saburo investigated the Fujinami sarcoma virus and found its oncogene. I went into chicken slaughterhouses, collected tumors, isolated viruses, and found *jun*, a component of the AP1 transcription factor complex. In the meantime, Saburo had gotten hold of the historical virus CT10 and identified *crk*.²²

Crk was an entirely new sort of oncogene. It encodes a protein that has no enzymatic activity but contains several modular protein-protein interaction domains, thereby functioning as a linker, as an adaptor protein. These modular domains had been discovered by Tony Pawson when he compared the sequence of several sarcoma viral oncogenes.²³ The modular domains are now part of the canonical cellular signaling code in which Saburo's work on *crk* not only reinforced Tony Pawson's findings but also made important contributions to our understanding of modular protein interaction domains, leading to functional assignments. To understand the significance of *crk*, we have to return to *src*.

The Src protein had been identified by Joan Brugge and Ray Erikson,²⁴ and Tony Hunter and Bartholomew Sefton²⁵ had shown that it functions as a protein tyrosine kinase. The Crk protein and its modular interaction domains turned out to be a kind of Rosetta stone that ties protein kinase activity to signaling, facilitating the transmission of information to a specific cellular target.

Saburo's investigations of modular domains in *src* illuminated yet another aspect of that remarkable protein, its autoregulation and the ingenious way in which viral evolution was able to eliminate this obstacle to constitutive activity.

Saburo's work forms the basis of much of current cancer research. It also has provided the starting point for a development that is beginning to transform cancer treatment: targeted therapy. Development of Gleevec, Iressa, and Tarceva—kinase inhibitors now used in cancer therapy—depended on the insights that came from the study of oncogenic viral kinases. The remaining challenges are daunting, but the rewards could be immense. We live in a time when therapeutically effective kinase inhibitors are still the exception, when lipid kinases are considered the most drugable targets of the day, when the control of transcription factors seems remote. But it is a time of great opportunities and of new departures made possible by Saburo's achievements.

Before closing, I would like to clarify one point. I have mentioned Teruko a few times during this talk but not as consistently as I should have. So let me say here that Teruko and Saburo were a team; they were complementary in the most ideal way. Teruko was a highly distinguished virologist, and it does not detract from Saburo's accomplishments to say that Teruko had an essential part in all of them and made critical contributions to Saburo's discoveries.

After Teruko passed away, Saburo was fortunate to find a new companion, Emiko. During this difficult period, when increased administrative duties and an active research program had to be balanced against failing health, Emiko stood at Saburo's side and was his constant and devoted support.

Saburo and I were very good friends. It was one of those friendships that grew out of the type of intense competition that generates deep respect and then turns into genuine, mutual affection.

The last time I saw Saburo in Osaka, about 4 years ago, his health was poor; at times he had to use a wheelchair. As a man accustomed to being intensely active, he was not reconciled to the state of his body.

However, I prefer to remember Saburo by another encounter years earlier at a meeting in Japan when he was still at the Rockefeller University and in good health. We were sitting in a Japanese teahouse, and in one corner of that tiny place there was a table for playing Go. The rules of Go are simple, but the strategies are frighteningly complex; they require much thought and planning—far more than chess. We started a game of Go, and for quite a while I fooled myself into thinking that I was doing pretty well. Little did I realize that Saburo was teasing and provoking me. All of a sudden, the tide turned, and I was quickly and soundly defeated. It is clear to me now that Saburo knew all along that he could beat me in a few minutes but that that would not have been fun. I think he must be smiling now.

Acknowledgments

The descriptions of scientific developments in this address reflect personal recollections and interpretations; they should not be considered critically established history. Literature references are intentionally selective. I am greatly indebted to Elizabeth Adler for editorial assistance. She offered numerous insightful suggestions that greatly improved the manuscript and put it into publishable form.

Note

Text of an address in memory of Hidesaburo Hanafusa. Presented at the Scientific Symposium, June 26, 2009, The Rockefeller University, New York, NY.

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