

Oncogene collaboration revisited from a historical perspective

Master Thesis

Author: Anirudh Cadapa Prahallad,

Guide/Supervisor: Prof. Dr Rene Bernards

*Dept of Molecular Carcinogenesis, The Netherlands Cancer
Institute (NKI-AVL)*

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Chapter 1: Underpinning the origins of human cancer

Introduction

In 1983, a seminal work from the lab of Robert Weinberg entitled “Tumorigenic conversion of embryo fibroblast requires two cooperating oncogenes” opened up a new dimension for cancer research. It discusses the mechanism of how one could convert a primary cell into a malignant one by introducing defined genetic elements (oncogenes *RAS* and *myc*), which cooperate to induce cancer. I will describe in this thesis that this model of oncogene collaboration had its roots in earlier work on how the viral oncogenes of DNA and RNA tumor viruses cooperate to induce cancer. Nevertheless, it was a landmark discovery that cellular oncogenes also act in synergy to cause malignant transformation.

With the aid of recombinant DNA technology in the late 1970s, it was possible to dissect the genome of cancer cells that led to the identification of new cellular oncogenes and tumor suppressor genes. Peeling the onion skin of truth underlying human cancer had begun. Many laboratories across the world are focusing on different aspects of cancer research with the hope that cancer can be cured one day. However, in the 21st century, it would be worthwhile to take one step back and try to get an insight and appreciate the research, which uncovered the myths of the genetic origins of cancer. These include the seminal discovery of oncogenes and tumorsuppressor genes. Exploring this, will make us realize how the field had emerged, and how it is being pursued and tackled in the present day. With this in mind, I will explore here the history of oncogene research starting with the beginning of the 20th century.

There were two schools of thought on the origins of human cancer. One came from a group of scientists who believed that the occurrence of cancer was due to the exposure to harmful chemical carcinogens; this led to the chemical

carcinogenesis theory. The second school of thought involved the research on tumor viruses that gave an insight into cellular transformation, which created a major impact on the field of cancer research. I will discuss both these theories in brief.

The chemical carcinogenesis theory

At the dawn of the 20th century, it had been recognized that certain chemicals cause cancer. A wide variety of cancers were found to be associated directly with specific occupations. There were unusual lung cancers seen in pitchblende miners, and skin and muscle cancers on the abdomens of Kashmiries who warmed themselves through the Himalayan winter by carrying around portable charcoal braziers under their robes. A London surgeon, Percivall Pott, described frequent cancers of the scrotum in men who worked as chimney sweeps. Researchers working on the recently invented X-ray machines began to show skin cancers and leukemias in high numbers. All these stories converged on a single idea: Certain factors or agents seemed to enter the human body from outside and trigger cancer in vulnerable tissues with predictable frequency.

The first proof of principle experiments on the chemical carcinogenesis theory came from Yamagiwa, a Japanese scientist, whose work drew on the Percivall Pott stories about London chimney sweeps. Yamagiwa took extracts of coal tar and applied them directly to the skin of 137 rabbits every two or three days a month. After a year, seven invasive carcinomas appeared at the sites of treatment. As it turned out, the coal tar used by Yamagiwa to create the tumors was a complex mixture of hundreds of uncharacterized chemicals (Loeb and Harris 2008). The particular chemical species responsible for the rabbit tumors could not be pinpointed.

Chemical carcinogenesis theory of Bruce Ames

In 1975, Bruce Ames working at University California Berkeley had tested about 300 carcinogens and non-carcinogens of a wide variety of chemical types

for mutagenicity in the simple Salmonella. This test used bacteria as sensitive indicators of DNA damage. Using mammalian liver cell extracts, the chemical carcinogens were metabolically converted to their active mutagenic forms and tested for mutagenicity in Salmonella. From this experiment, a high correlation was observed between carcinogenicity and mutagenicity where, 90% of carcinogens were mutagenic in the test. Ames had established a rather clear notion that carcinogens create cancer through their ability to induce mutations. This hypothesis had an important message to convey: mutant genes are present within the genomes of cancer cells, and these mutations are critical in driving the cancer process (Ames, McCann et al. 1975).

Theory of Tumor viruses and their role in cancer

Cancer was thought to be primarily an infectious disease. This dates back to the reports in 1908, on a mysterious agent that was able to transmit leukemia from one chicken to another. Later on, Peyton Rous at the Rockefeller institute identified an infectious agent from a ground up muscle tumor of a chicken. This agent when injected into healthy hens was able to induce tumors. It was discovered later to be a RNA tumor virus/Rous sarcoma virus (RSV). Several infectious agents are considered to be causes of cancer in humans. Worldwide, most cases of cervical cancer, hepatocellular cancer, stomach cancer and lymphomas (adult T cell lymphoma, Burkitts lymphoma and Hodgkin lymphomas) are thought to have a viral origin. The estimated total of cancers infection attributable to infection in the year 2002 was 1.9 million cases, or 17.8% of the global cancer burden. The principal agents are the bacterium *Helicobacter pylori* (5.5% of all cancer), the human papilloma viruses (5.2%), the hepatitis B and C viruses (4.9%), Epstein-Barr virus (1%), human immunodeficiency virus (HIV) together with the human herpes virus 8 (0.9%)(Parkin 2006).

Current cancer research has its roots in several distinct lines of work on tumor viruses. The papovaviruses, polyoma and SV40, and the Rous sarcoma retrovirus provided the seminal observations that launched this field by showing that cultured cells infected by these viruses acquire many of the phenotypes of a

cancer cell, including tumorigenicity. Many have discovered different viruses that were able to either induce tumors in susceptible host or transform cells in culture or do both.

The research on the viral origins of cancer has clearly unraveled a wealth of information and established a number of concepts that helps us understand the human origins of cancer today. I shall discuss here the genetic factors (viral oncogenes) that reside in viral genomes, and how they interact with the host proteins to induce cancer. The concept of 'oncogene collaboration' will be the main focus of this thesis.

Chapter 2: Malignant transformation by Tumor viruses

Introduction

Cancer causing viruses can be separated into two classes based upon their genetic material: the DNA tumor viruses and the RNA tumor viruses. This distinction is also relevant because the oncogenes of each virus type have very different origins (Levine 1988). The DNA tumor viruses which include papovavirus, papilloma virus, adenovirus, herpes virus, hepadnavirus and poxvirus have been shown to be associated with tumors in mammals. Among the several RNA viruses, only the retroviruses have cancer causing sequences. These sequences share homology with cellular genes (proto-oncogenes), unlike the DNA tumor viruses that bear little or no homology to cellular genes.

RNA tumor viruses (retroviruses) and their oncogenes

The RNA tumor viruses, now called the retroviruses, were among the first recognized oncogenic agents of any kind. They were identified as filterable leukemogenic and sarcomagenic factors in chickens. An intensive attack on the oncogenic mechanisms employed by these viruses, however, began in parallel with the study of papovaviruses, stimulated by the development of assays for transformation of cultured cells and biochemical methods for analysis of viral genomes and proteins. It was through this area of cancer research that investigators first came in direct contact with cellular genes that could be implicated in cancer.

One of the distinct features of retroviruses is that, they have two identical single strands of RNA which are capable of encoding 1-10 polypeptides. They consist of three coding regions: ***gag*** for the major structural proteins of the viral nucleocapsid, ***pol*** for enzymes (reversetranscriptase, integrase and polymerase) found in virus particles and ***env*** for envelops glycoproteins. The oncogenes of

retroviruses are called *v-oncs*, which are clearly derived from normal cellular genes *c-oncs*. (*C-oncs* are one set of the large class of genes known as proto-oncogenes). Figure 1 describes in chronological order the seminal discoveries made in the field of retroviruses.

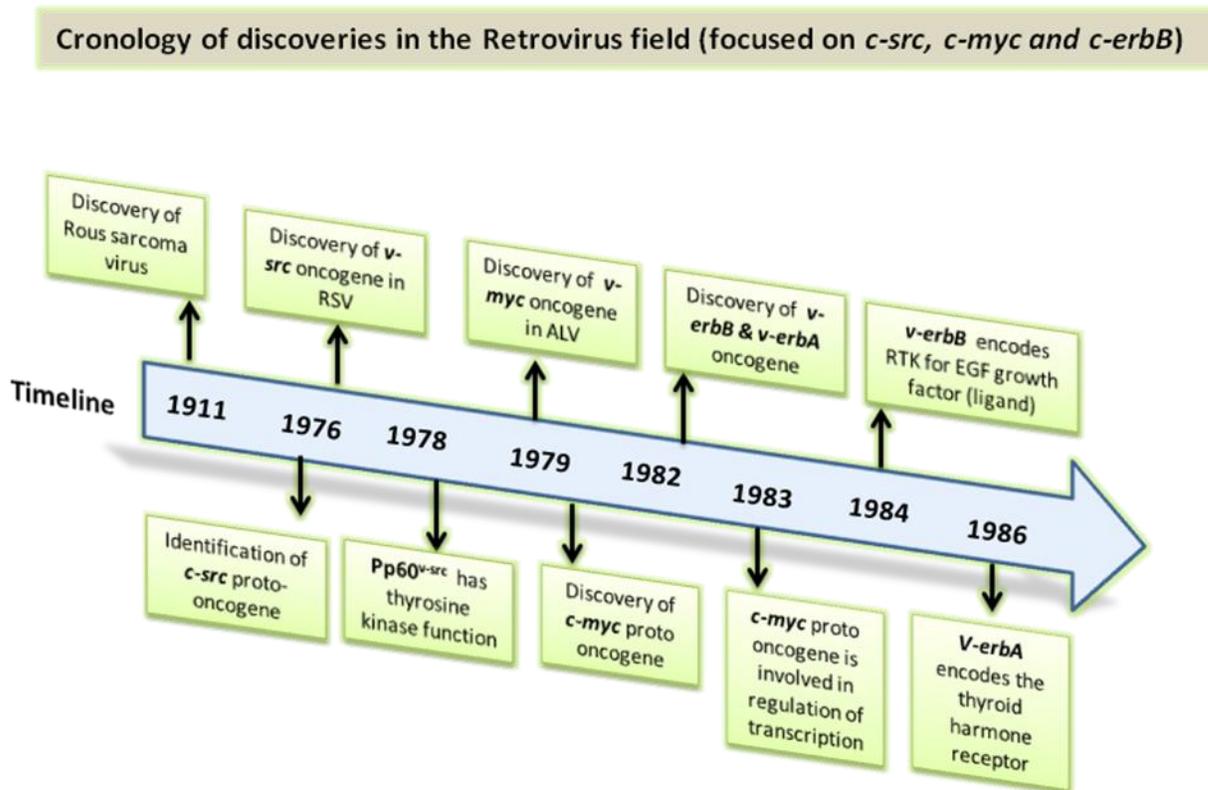


Figure1: Timeline showing the seminal discoveries made in the field of retroviruses (emphasized on *c-src*, *c-myc* and *erbB/erbA* protooncogenes).

***v-src* oncogene**

One of the classical examples of RNA bearing oncogenic retroviruses is the Rous sarcoma virus. RSV was one of the first animal viruses to be isolated (Rous 1911). Many of the seminal experiments that established the principle facts about retroviral oncogenes were performed with Rous sarcoma virus. The RSV is unique among retroviruses because it carries all the genetic information required for both replication and efficient transformation. Two kinds of RSV mutants, isolated around 1970. Provided powerful tools for genetic and biochemical analysis of transformation by this virus: (1) temperature sensitive (*ts*) mutants, that grow

normally at 35° and 41°C, but neither initiate nor maintain the transformed phenotype at the higher (nonpermissive) temperature; and (2) nonconditional transformation defective (*td*) mutants that replicate to wild type levels but cannot transform cells as a result of an extensive deletion of genetic information (Martin 1970; Martin and Duesberg 1972). The behavior of these mutants clearly dissociated the replication and transformation specific functions of RSV. Chemical and genetic analysis of wild-type and mutant RNA genomes enabled the identification of the viral oncogene *v-src* that mediated the neoplastic activity in the host. Complementary DNA prepared from the RSV genome that represented the *v-src* region, was able to hybridize to the DNA of all vertebrate species. However, the (*td*) mutants that lacked this region failed to hybridize. This experiment led to the discovery of *c-src* proto-oncogene. The *c-src* proto-oncogene is widely expressed in normal host tissues, often at rather high levels, to produce a protein that differs in small but functionally important ways from that of viral *src* (*v-src*). This cellular *src* gene can be converted to an oncogene through capture (transduction) by a retrovirus (Spector, Varmus et al. 1978; Varmus 1982). Transduction is a process that introduces mutations within the gene and promotes efficient expression under the control of viral regulatory sequences. This process involves the capturing of the upstream regulatory sequences and the normal mRNA initiation sites of a particular cellular proto-oncogene by the retroviruses. As a result, high level transcription of the *c-oncs* is imposed by the retroviral promoter which is likely to result in loss of normal transcriptional regulation of the *c-oncs*. (Stehelin, Varmus et al. 1976). Varmus and Bishop won the 'Nobel Prize' for this seminal work.

v-myc oncogene

The viral *myc* oncogene was first isolated from the avian myelocytomatosis retrovirus MC29. Retrovirus MC29 induces a wide spectrum of neoplastic diseases in chickens, which includes renal and hepatic carcinomas, sarcomas and leukemias. In vitro the virus transforms macrophages, fibroblasts and epithelial cells. The oncogenic potential of this virus is attributed solely to the *myc*

oncogene. The retrovirus MC29 has no other functional genes apart from the *gag* and *env*. The *pol* gene is entirely deleted which makes this virus replication incompetent. As a result a helper virus is needed for propagation. The *v-myc* is homologous to the cellular gene *c-myc* that is present in several avian and mammalian species and has apparently been conserved during the course of vertebrate evolution. Generally, avian leukosis viruses do not possess oncogenes. Almost all of the lymphomas induced by ALVs contain viral DNA integrated in a common domain of the host genome. Within this domain lies a cellular gene whose activity is apparently augmented as a consequence of the insertion of viral DNA. Chickens infected with avian leukosis virus develop brusal tumors after long latency periods. This is due to the integration of the viral genome adjacent to the *myc* oncogene locus, which leads to the enhanced expression of this oncogene. It appears likely that the *myc* gene can exert an oncogenic effect as either a cellular or a viral oncogene. The discovery of the cellular *myc* oncogene was one of the major discoveries in the field. I will describe in later chapters that *c-myc* oncogene collaborates with many other oncogenes to induce cancer.

v-erb-B and v-erb-A oncogenes

v-erb-B was identified in a retrovirus causing avian erythroblastosis (AEV) and erythroleukemia (Vennstrom and Bishop 1982). This avian retrovirus contained two cell derived genes: *v-erbA* and *v-erbB*. Genetic analysis indicated that transforming activity reside in *erbB*. The *v-erb-B* transforming protein is homologous to the transmembrane and cytoplasmic domains of the EGF receptor. It arose by insertion of viral DNA into the EGF receptor gene; this resulted in expression of a protein that lacked the amino terminal ligand binding domain. Other strains of erythroblastosis virus contain progressive C terminal truncations in *erbB*, a change that extends a host range and enhances their transforming activity. The finding that *erbB* retains the central protein tyrosine kinase domain of the EGF receptor implied that constitutive expression of this was the basis for its activity as

a transforming oncoprotein, and its activity in transformed cells is clearly shown to be increased relative to untransformed cells.

Genetic deletion of the ligand binding domain from the EGF receptor is sufficient to create a transforming oncogene. Moreover, mutations within the protein tyrosine kinase domain of *erbB* abolish its transforming activity, whereas mutations in the C terminus do not. Therefore, *erbB* is transforming due to constitutively active protein tyrosine kinase resulting from deletion of the regulatory ligand-binding domain.

DNA tumor viruses and their oncogenes

Before getting into the details of DNA tumor viruses, I would like to introduce the concept of permissive and nonpermissive behavior of cells that is essential for viral replication and transformation. Figure 2 describes in chronological order the seminal discoveries made in the field of DNA tumor viruses.

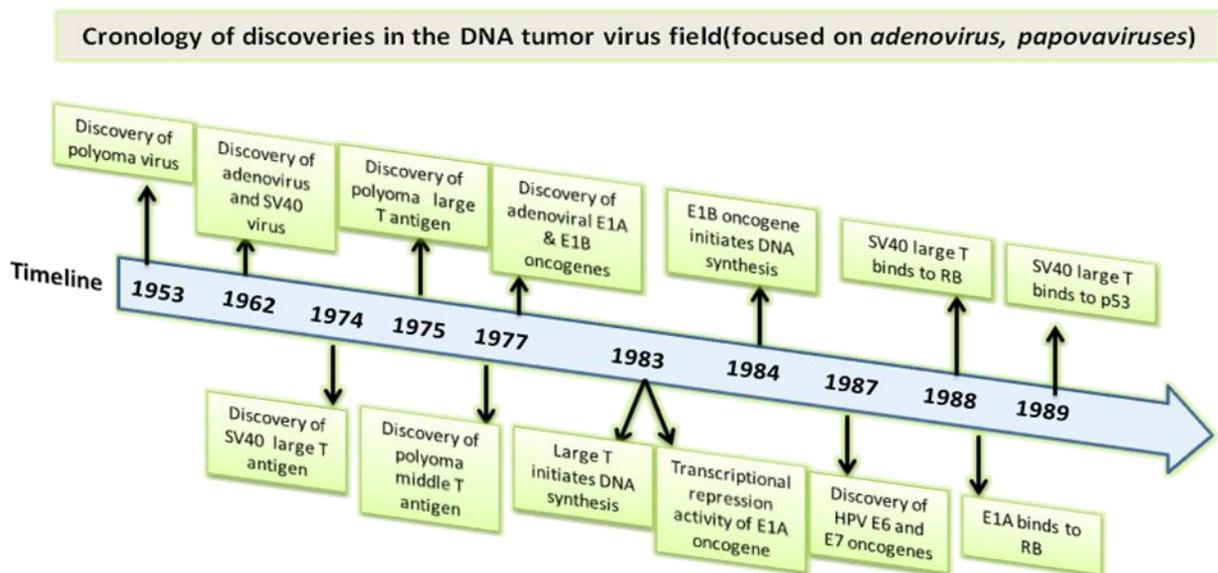


Figure2: Timeline showing the seminal discoveries made in the field of DNA tumor viruses.

Permissive versus Nonpermissive Cells

Cells that support the complete replication of polyoma virus or SV40, and adenovirus, and as a result suffer lysis, are called permissive cells, whereas cells that do not support the replication of these viruses but may be stably transformed by them are called nonpermissive cells. Some cells (e.g., mouse cells, including those of stable lines such as 3T3) are nonpermissive for SV40 but permissive for polyoma virus; other cells (e.g., hamster cells) do not allow either virus to replicate well.

Polyoma virus

Until the beginning of the 1980s, much of the work on these viruses was focused on understanding their transforming potential. The first DNA tumor virus that was isolated and studied for transforming abilities was the polyoma virus by Ludwik Gross in 1953 (Gross 1953). The virus caused adenocarcinomas in the salivary glands (parotid). The polyoma virus got its name from the fact that it was able to induce a variety of other tumors in different cell types (Stewart et al 1958). Polyoma virus can be found in a large fraction of both wild and laboratory bred mice. However, polyoma virus apparently does not produce any detectable disease when it multiplies in adult mice. On the contrary, the virus produces tumors only when it is inoculated into newborn laboratory mice.

Polyoma virus was easily isolated from extracts of whole mice, whereas, extracts of the parotid tumors induced by the virus had virtually no viral particles. These findings led to the speculation that polyoma virus is present in tumor cells as a provirus. The first cultures of highly permissive embryonic mouse cells, that were able to produce polyoma virus which replicated as a lytic virus, created a major breakthrough in the field. This made it possible to accumulate and culture more viruses sufficient for chemical and molecular analysis (Stewart, Eddy et al. 1957; Stewart, Eddy et al. 1958). Some were able to perform this in hamster cells, which after infection gave rise to stable transformed variants that resembled the tumors caused by polyoma virus (Sachs and Winocour 1959) (Dulbecco and Vogt 1960). Compared to the mouse cultures, the hamster cultures gained more

attention because they are semipermissive and do not allow polyoma virus to replicate well; nevertheless, they are transformed by the virus at a higher frequency than are mouse cells, most of which allow the virus to replicate freely in a lytic cycle. This enabled experimentalists to study the primary events of transformation without having to deal with the complications posed by lytic events. 10-20% of early viral RNA molecules transcribed inside the host encode viral tumor antigens. These findings are also found to be similar in the case of SV-40 viruses. The genome of the polyoma virus specifies three early proteins large T, middle T and small T antigens. In the 1970s it was possible to extract these three protein antigens from cellular extracts infected by polyoma virus by treating with sera from animals bearing tumors induced by polyoma virus. This procedure was termed Immunoprecipitation. The three isolated antigens were 21, 000, (small T), 66,000(middle T) and 96,000(Large T) Kda respectively (Benjamin 1970; Feunteun, Sompayrac et al. 1976; Ito, Spurr et al. 1977; Hutchinson, Hunter et al. 1978; Schaffhausen, Silver et al. 1978). These T antigens were responsible for cell transformation. The Middle T antigen of polyoma virus associates with the cell membrane of its host and this association is very much essential for carrying out the transformation functions of middle T antigen(Carmichael, Schaffhausen et al. 1982). The large T antigen of both polyoma virus and SV40 virus localizes in the nucleus of its host. The combined effect of both the T antigens is required to mediate complete transformation.

Simian virus 40 (SV40)

The discovery of the simian virus 40 was essentially a by-product of work with poliomyelitis virus. At that time, vaccines against poliovirus were made using monkey kidney cells, because use of human cells was prohibited as they were thought to harbor undetected human cancer viruses. Systematic studies were therefore initiated to ensure that viruses that belong to monkey cells were not present in any vaccine used to protect human population.

Normally the SV40 virus does not produce any detectable cytopathic effects when it multiplies in the cells of the rhesus monkey, the source of most

cells used to produce vaccines. However, when African green monkeys of the genus *Cercopithecus* were used as a source of test cells, it was here that the existence of SV40 came to light. It was realized later that the batch of polio vaccine was contaminated with the SV40 virus in considerable number. Soon after its discovery (Eddy et al 1962) showed that SV40 induced tumors when injected into newborn hamsters. The SV40 and polyoma virus share similarities in terms of molecular structure, chemical composition and molecular biology of their interaction with permissive and nonpermissive host cells. The organization of the SV40 genome is similar to that of polyoma, with one notable exception: Only two T antigens, small T and large T, are encoded in the early region of SV40 viral genome; there is no middle T.

Human papilloma virus

Human papilloma viruses are small DNA viruses that usually infect the anogenital area in humans. There are over 60 HPV s associate with various clinical lesions (de Villiers, Hirsch-Behnam et al. 1989). Based on the lethality of HPV infection they are classified into two groups. HPV-6 and HPV-11 that are associated with anogenital warts that can very rarely progress towards cancer. Viruses from this class are referred to as low risk viruses. On the other hand, the second class that includes the HPV-16 and HPV-18 is associated with genetic lesions that are high risk for malignant progression. This class of viruses is found to be associated with most of the anogenital carcinomas. The transcriptionally active DNA of these high risk viruses are found in cervical cancer cell lines and also biopsy tissues. The cloned genome of the high risk HPVs are able to transform cells in vitro which suggests that they play an important role in the etiology of human cancer(Schwarz, Freese et al. 1985).

Most papilloma viruses are able to replicate only in epithelial cells. However, some are able to induce fibropapillomas. Fibroblasts can be transformed by these viruses invitro. Infection by papillomaviruses probably begins in the cells of the basal layer, which can be transformed but are nonpermissive for virus replication. As the cells differentiate during their transit to the surface, they

become able to support virus growth. Genetic dissection of the HPV genome has revealed 2 essential early genes whose open reading frames are found to be highly intact. These genes code for the 15.5-kD E6 protein that is found in both nuclear and membrane fractions of transformed cells and E7 protein that has a transcriptional trans- activation function. Both the proteins have implications in cancer. The E6 and E7 oncoproteins of the high risk HPVs is responsible for its primary transforming activity. The E6 and E7 proteins act cooperatively in the development of HPV- induced cancers, with the action of one factor complementing that of the other. The expression of E7 protein of the high risk HPV can immortalize human keratinocytes whereas E6 has no such activity. However, the combination of E6 and E7 proteins can efficiently immortalize most types of primary cells.

Human Adenoviruses

In 1962, (Yabe, Trentin et al. 1962) discovered that adenovirus 12 induces tumors following injection into newborn hamsters. This paved way for further investigations into the adenovirus field. There are many serotypes of adenovirus each classified based upon the potential to transform their host. In 1963, (Huebner 1963) discovered that, twelve of thirty one adenoviruses transform cells in culture or induce tumors. Serotypes 12, 18 and 31 are highly oncogenic and induce tumors rapidly in the majority of hamsters into which they are inoculated. On the other hand, serotypes 3, 7, 14 and 16 are weakly oncogenic; they induce tumors in a smaller number of animals after a longer latent period. Still other serotypes 1, 2, 5 and 6 are not oncogenic.

All adenoviruses, including those that do not cause tumors, can transform rodent cells in vitro. Unlike cells transformed by papovaviruses, cells transformed by adenovirus are free of viral particles. Adenoviral infection in non-permissive cells, leads to the production of a viral specific T antigen. The T antigen is also made during early stages of the lytic infection cycle. This provided the first clue that the viral DNA persists in the genome and is essential to induce tumors. Using

DNA –RNA hybridization procedure, it was able to prove that the adenoviral DNA took residence in the host genome.

Adenovirus serotypes 2 and 5 can replicate quite well in hamster cells(Williams 1973). Dissecting the genomes of rat or hamster cells transformed by adenovirus-2 revealed that, only partial sequences of adenovirus could be retrieved. This may be due to the fact that not all virus sequences are required to transform the cells, and/or that some sequences may prevent transformation. Either possibility may explain why only partial sequences were retrieved from the transformed cells. To achieve transformation in hamster cells which are semipermissive by Ad 2 serotype, it is necessary to use viruses disabled either by UV irradiation or the presence of a temperature sensitive mutation(Lewis, Rabson et al. 1974). Experimental evidence suggested that, in highly oncogenic adenoviruses, the continuous expression of 11% of the left terminal region of the genome is required and this region consisted of two early genes E1A and E1B. This partial stretch of the adenoviral genome comprises of left hand 14% of the viral genome, encoding some of the early expressed genes.

There are four early regions expressed in Adenovirus infections: E1-E4, where, only E1 has oncogenes E1A and E1B. This was found true in another type of adenovirus -5 transformed cells that harbored the left hand 10% of the genome. In 1975, Alex van der Eb and Graham (Graham, van der Eb et al. 1974) showed that the left hand 10% of the Ad5 viral genome was able to transform rat embryo cells in vitro. (Graham, Harrison et al. 1978) showed that mutations in the left hand 1-11% of early region blocked transformation. This early region 1 consists of two transcriptional units: E1A and E1B (Chow, Lewis et al. 1980; Ross, Flint et al. 1980). E1A encodes for proteins having molecular weights of 44,000 and 54,000 kDa and E1B encodes for two proteins of molecular weights 15,000 and 55,000 kDa (Lewis, Atkins et al. 1976; Halbert, Spector et al. 1979).

An observation was made which showed that both E1A and E1B were required for cellular transformation. Among adenoviruses, Ad12 strain readily transformed cells and has the ability to form tumors when compared to the Ad5 counterpart that can only transform cells. Genetic dissections of their genomes

shed light on the mechanism of their action during transformation. The E1A region of the Ad12 adenovirus encoded proteins that suppress the expression of Class 1 transplantation antigens evading the elimination by host cytotoxic T cells. The E1A region of Ad5 adenovirus did not execute the same function. The E1B regions of both the strains were also distinguishable in the sense that it determined the oncogenicity of the transformed cells. Experiments performed using recombinant Ad12/Ad5 adenovirus in which all E1 genes were derived from Ad12 and the remainder of the viral genome from Ad5 showed that this virus did not cause tumors in hamsters, demonstrating that tumor induction in vivo is dependent on sequences outside the transforming E1 (Bernards, de Leeuw et al. 1984).

Virus and host cell interactions during transformation

In the previous sections, I described the properties and characteristics of different kinds of tumor viruses. With the aid of recombinant DNA technology, the important oncogenes that reside in the genomes of tumor causing viruses were discovered. However, understanding the biochemical function of the proteins encoded by these oncogenes will enable us to understand the detailed principles of transformation. I will review in brief the cellular protein interactions of the RNA and DNA tumor viruses.

The biochemical properties of oncogenes and protooncogenes were elucidated using three experimental approaches: nucleotide sequencing of cloned genes to deduce the primary sequence of oncoproteins; the use of antibodies in combination with protein biochemistry, and cell fractionation procedures to determine the size, cellular location and chemical modifications of encoded proteins; and tests for proteins (e.g., for enzymatic or binding activities) to seek biochemical properties that might explain the biological consequences of oncogene expression.

There are three major biochemical strategies by which the products of viral oncogenes might act:

Protein modification

Both proteins and phospholipids are potential substrates for phosphorylation. The transforming protein may be a factor that elicits phosphorylation or it may be a catalytic kinase itself. In each of these instances the primary action occurs at the plasma membrane where, the responsible protein would strike at the cell surface, spans the plasma membrane, or reside at the inner surface of the membrane (Hunter 1985). The 60 kD product of *v-src* (pp60^{v-src}) of the Rous sarcoma virus was the first transforming protein to have a biochemical activity attributed to it. pp60^{v-src}, catalyzes the phosphorylation of proteins on the tyrosine residues and resides in the cytoplasm by tethering to juxtannuclear membrane of the golgi apparatus (Brugge and Erikson 1977). The members of this enzymatic family have so far taken two forms: transmembrane receptors for growth factors, or cytoplasmic proteins affiliated with membranes.

The product of *v-erb-B* is a truncated version of the receptor for epidermal growth factor (EGF). Protein tyrosine kinase activity has been attributed to the product of *v-erb-B* (Downward, Yarden et al. 1984). The protein encoded by *v-erb-B* represents the EGF receptor short of both its extracellular domain (which binds the ligand) and either 32 or 71 amino acids from its carboxy terminus. The principle remnants include a sequence of 71 amino acids that might produce from the cell surface, a hydrophobic domain that presumably spans the plasma membrane; and a shortened cytoplasmic domain that must carry the protein-tyrosine kinase activity. The truncations suffered by this protein have several consequences any of which might contribute to neoplastic transformation (Hayman and Beug 1984). After ligand binding, the EGF receptor is returned to the interior of the cell. A regulatory device is seemingly designed to protect the cell from a surfeit of stimulus. By contrast, the product of *v-erb-B* cannot bind ligand and may therefore be exiled permanently to the surface of the cell. The third consequence is that the EGF receptor displays the full force of its protein kinase active only after binding ligand. The *v-erb-B* product is unleashed from this dependence and is constitutively active. This constitutively active nature of this receptor makes it potently oncogenic.

During the examination of middle T antigen of polyoma virus, it was shown that middle T antigen exhibited protein kinase activity by phosphorylating tyrosine residue. Like the protein tyrosine kinases encoded by the retroviruses, the middle T antigen was found to be localized at the plasma membrane. However, it was shown later that the phosphorylation of middle T antigen is carried out by pp60^{c-src} (the cellular analog of pp60^{v-src}), which is tightly bound to a portion of middle T antigen in cells transformed or lytically infected by polyoma virus. The binding of middle T to pp60^{c-src} appears to augment the specific enzymatic activity of pp60^{c-src} by manyfold and is necessary but not sufficient for transformation by polyoma virus (Ito, Brocklehurst et al. 1977). Polyoma middle T also binds to other cellular proteins that may affect properties of transformed cells. In middle T immunoprecipitates there is association of phosphatidylinositol (PtdIns) kinase activity. The presence of this activity, like that of pp60^{c-src}, is closely correlated with transforming activity of middle T. since PtdIns kinase activity is involved in transduction of signals across cell membranes, it is possible that middle T affect cellular signaling through this mechanism.

SV40 LT is mainly responsible for cell transformation by SV40 virus. Under some conditions, small T is also required, but large T alone is sufficient in most cases. The large T antigen plays a role in the nucleus as well as in the plasma membrane during transformation. Mutations in SV40 large T antigen that affect its nuclear localization, can elicit a full transformation in established cell cultures indicating that, the membranous form of large T antigen might have biological potency of its own (Fischer-Fantuzzi and Vesco 1985). By contrast, transformation of primary cultures requires that large T antigen be present in the nucleus as well as on the plasma membrane. It was discovered that the membrane bound form of SV40 large T antigen acts through phosphorylation. The kinase affiliated with SV40 large T antigen phosphorylates serine and threonine residues.

Initiation of DNA synthesis

Unrestrained synthesis of DNA is an inevitable component of the neoplastic phenotype. Some transforming proteins allegedly elicit this property by

acting directly to initiate DNA synthesis. The large T antigens of papovaviruses initiate the synthesis of viral DNA. They might also serve as a lash for the synthesis of cellular DNA and thus propel neoplastic growth (Rigby and Lane, 1983). It has been shown that microinjection of the large T antigen of SV40 into the nucleus of quiescent cells stimulates these cells to synthesize DNA. The mutant versions of SV40 large T antigen that has lost its ability to establish residence in the nucleus cannot initiate viral or cellular DNA replication or transform primary cultures of embryonic cells. However, the same mutants readily transform established lines of cells, which in contrast to primary cultures apparently have no need for the sustained mitogenic stimulus offered by the wild-type version of large T antigen (Lanford, Hyland et al. 1985). The 21 kD product of E1B of adenovirus stabilizes cellular DNA during the course of productive infection by adenoviruses. It permits viral DNA to replicate in cells that are not dividing, and accounts in large part for the mitogenic effect of infection by adenoviruses (White, Blose et al. 1984).

Regulation of transcription

Several of the transforming proteins may influence transcription from cellular genes, either by stimulation or by inhibition. They could do so by interacting with other proteins, with promoters for transcription, or with enhancers. Illegitimate stimulation of transcription from cellular promoters could give rise to unwanted gene expression and thus produce the neoplastic phenotype. The nucleus houses a substantial variety of transforming proteins. These include the products of the retroviral oncogenes *v-fos*, *v-myb* and *v-myc*; the large T antigens of papovaviruses; and the products of E1A. All of the nuclear transforming proteins are found in the nuclear matrix and at least several (E1A, *myb* and *myc*) have short half lives (30-120 min) as befits proteins thought to serve regulatory functions (Klempnauer, Ramsay et al. 1983). For example E1A can both positively and negatively regulate transcription. E1A can repress the action of selected transcriptional promoters and enhancers. The inhibition of transcription from MHC-1 genes by the E1A region of the tumorigenic adenoviruses is an example of transcriptional repression by adenoviral E1A (Schrier, Bernards et al. 1983). E1A

binds to certain transcriptional co-activators, co-repressors and cell-cycle-regulatory proteins. E1A interacts with components of the general and specific transcriptional machinery, including the TATA-binding protein (TBP), several TBP-associated factors (TAFs), and numerous transcription factors (such as ATF-2, c-Jun).

Interaction with CBP/p300

P300 and CBP (CREB-binding protein) are highly related coactivators of transcription that are recruited to many promoters through their association with DNA-binding transcription factors. Using their histone acetyltransferase activity (HAT), CBP/p300 activates transcription by acetylating the histone tails. The amino terminus of adenoviral E1A binds p300/CBP directly at the TRAM (transcriptional adaptor motif) domain. This interaction inhibits the HAT activities of p300/CBP and prevents certain interactions with transcription factors.

Interactions with Retinoblastoma tumor suppressor protein (RB)

The *RB* tumor suppressor gene is the founder of the *RB* family, which comprises two additional structurally and functionally related genes, namely *p107* and *p130*. All three *RB* family gene products act in the control of cell cycle progression between the G1 and S phases through the interaction with E2F family of transcription factors. Adenoviral E1A, human papilloma virus E7, polyomavirus and SV40 large T oncoproteins share the LXCXE motif, through which they physically interact with all of the members of the *RB* family proteins. Binding of the E1A, E7 or large T oncoproteins to the *RB* protein, results in physical displacement of physiologically interacting partners. In this way, the viral oncoproteins strongly compromise the regulatory properties of the *RB* proteins, thereby allowing the cells to converge to an unscheduled progression to the S phase of the cell cycle.

Interaction with P53 tumor suppressor protein

TP53 is a key tumor suppressor gene. It was identified as a T antigen binding protein, and physically interacts with SV40 large T, adenoviral E1B and HPV E6 oncoproteins. The E6/P53 interaction promotes P53 degradation via ubiquitin-dependent pathway, leading to the abrogation of P53 dependent control of growth arrest and apoptosis. E6 induced P53 degradation is regarded as the functional equivalent of an inactivating mutation of *P53* gene.

Chapter 3: Collaborating oncogenes.

Introduction

The discovery and characterization of different kinds of tumor viruses has uncovered the means by which they can induce tumors in mammalian cells. However it should be noted that cancer is a multistep process and it requires a collaborative effort of different oncogenes in order to achieve the malignant phenotype. With the discovery of gene transfer techniques, it was possible to introduce individual or combinations of desired oncogenes into cells and study their functions during cellular transformation. In this chapter, I will go through a series of experiments, which were performed in vitro using oncogenes of RNA and DNA tumor viruses, which uncovered their potential to transform cells in culture. I will review the collaborating viral oncogenes as this led to a number of concepts that set a platform for understanding oncogene collaboration in context with cellular oncogenes (figure3 describes in chronology the discoveries that established the concept of oncogene collaboration). However, it is important to first understand the differences between a primary cell and a cell line since most of the experiments that I will describe in the following sections have been performed in either of these cell cultures.

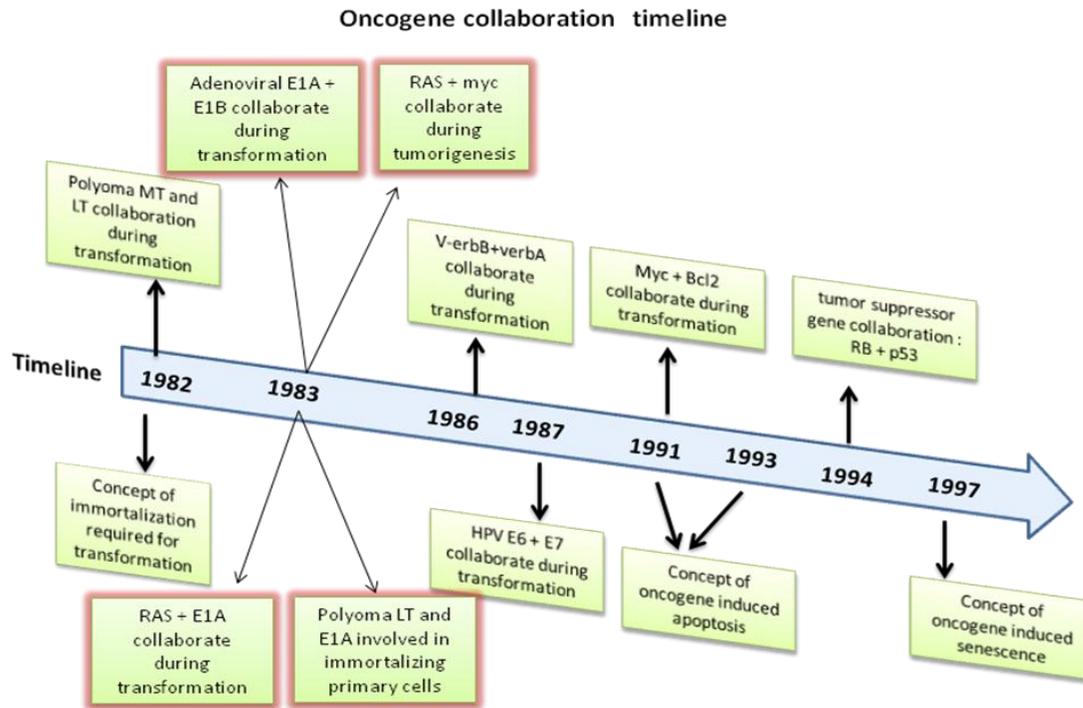


Figure 3: Timeline showing the seminal observations that led to the development of the concept of oncogene collaboration. The boxes highlighted in red are landmark discoveries.

Primary cells versus cell lines

Rodent fibroblasts have been utilized for a number of experiments to study the functions of oncogenes. Two types of these cells were used: these are cells which were explanted or derived freshly from embryos. These cells are called primary cells. When rodent embryo cells (primary cells) are placed in culture, they grow for 30-50 doublings before they enter a phase, termed senescence or crisis. The genetic basis of senescence will be discussed in later sections. However, emerging from the large population of cells in crisis may be a small number of cell clones that once again show strong and steady replicative potential. These clones can be expanded and will have an unlimited number of doublings in culture. Such cell clones have undergone a process variously termed establishment or immortalization, and their descendants now constitute a cell line. The frequency of these usually rare events can be increased by treatment of rodent cells with mutagenic carcinogens, suggesting that immortalization in culture is a result of a genetic change.

Collaborating oncogenes in retroviruses

Retroviruses harbor multiple oncogenes, which collaborate with each other during host cell transformation. One of the most interesting examples can be reviewed from the avian erythroblastosis virus (AEV). AEV carries two distinct oncogenes, *erbA* and *erbB*, which collaborate to create erythroblastosis. *erbB*, which happens to be an altered form of the normal chicken gene encoding the epidermal growth factor receptor, is able to induce proliferation of erythroid stem cells, but these tend to differentiate spontaneously into red cells. *erbA*, derived from the gene specifying the normal triiodothyronine receptor, does little on its own; however, in the presence of *erbB*, it is able to block differentiation, trapping the *erbB* transformants in a stem cell state having unlimited proliferating potential (Frykberg, Palmieri et al. 1983). Interestingly, other oncogenes encoding cytoplasmic proteins, like *v-src* and *v-Ha-RAS* are able to replace *erbB* by cooperating with *erbA* in erythroid transformation (Kahn, Frykberg et al. 1986). Other models like the MH2 retrovirus, carries both the *RAF* and *myc* oncogenes that collaborate to confer potent and rapid tumorigenicity.

Collaborating oncogenes of the DNA tumor viruses

Transformation in vivo and in vitro requires at least two distinctive genetic changes. Because the precise targets of these genetic changes are usually obscure, it would be valuable to reconstruct the stages of tumor progression using precisely defined genetic elements. A number of researchers have attempted to mimic one or both steps of tumor formation by introducing well studied oncogenes into cultured cells. These experiments have provided insights into the distinctive workings of different oncogenes and the abilities of these genes to collaborate in tumorigenesis.

The identification of the oncogenes from the polyoma virus and the adenovirus showed their potential to transform cells upon viral infection. However, there was still a lack of understanding of why two oncogenes for example (middle T, small T and large T in case of polyoma virus, E6 and E7 in human

papillomavirus and E1A and E1B in case of adenoviruses) were required in order to get the fully transformed state, and why not achieve it by having only one? This question paved way for determining the functions of each of these oncogenes and what role they play in cellular transformation.

Recombinant DNA technology and efficient gene transfer techniques, which includes the calcium phosphate precipitation mediated transfection, helped solve this major question. In 1981, (Treisman, Novak et al. 1981) constructed a recombinant polyoma virus vector that only encoded the middle T antigen. Transfection of the modified viral DNA into cultured rat cells efficiently induced the formation of transformed cell, which could give rise to tumors upon injection into Fischer rat. This showed that the function of one oncogene, which is in this case the middle T, could satisfactorily induce tumors. However, it can be argued that these kind of cell lines constitutively expressed establishment functions that can substitute for those of the virus. The same experiment when performed using dense monolayer cultures of primary cells had little if any effect on the behavior of the cell population. The use of immortal cell lines in performing such experiments has set researchers on a wrong foot by suggesting that a single oncogene could transform cells. The apparent incomplete transformation of embryo cells by middle T antigens provoked a search for other agents or oncogenes that could push this process to completion. In the case of polyoma virus it was previously known that the whole genome of polyoma virus induced full malignant transformation of rodent embryo fibroblasts, indicating that one of the other viral genes (large T antigen) expressed in transformants must collaborate with the middle T gene in this process.

Immortalization of primary cells by adenoviral E1A, polyoma large T and myc oncogenes

Transformation of primary cells requires at least two separate functions. The first, an establishment function, which is concerned with immortalization of cells, while the second, the transformation function, which is required for the full expression of the oncogenic phenotype. Establishing a primary cell in culture is

possible by the expression of the adenoviral E1A oncogene or largeT antigen of polyoma virus. (Houweling, van den Elsen et al. 1980) Expression of these oncogenes immortalizes the cells by allowing them to grow indefinitely in culture, providing the first step towards oncogenic transformation (fig 4). Several oncogenes have been isolated from established human tumors, using the NIH3T3 transformation assay, but in most cases these oncogenes failed to transform primary cell cultures. Given the requirement for two viral functions for transformation of primary cells by polyoma and adenoviruses, it can be considered that cellular oncogenes also require establishment functions before they can execute their oncogenic potency. Therefore, the oncogenes that scored in the NIH3T3 assay carry transformation function but may lack establishment functions. This is because NIH3T3 cells have already been established. The use of primary cells was beneficial for categorizing and testing certain viral and cellular oncogenes for their ability to transform or establish primary cells.

The same holds true for the cellular homolog of the *v-myc* oncogene. Introduction of *c-myc* into rat embryo fibroblasts established them in culture. The E7 oncoprotein of the Human papilloma virus also has the establishment function associated with it. These experiments precisely showed the genetic factors that were responsible for immortalizing primary cells, which were just one step towards achieving complete transformation. Nevertheless, the expression of oncogene E1B of adenovirus together with E1A, middle T antigen of polyoma virus together with large T antigen and the E6 protein of HPV together with the E7 protein, achieves complete transformation.

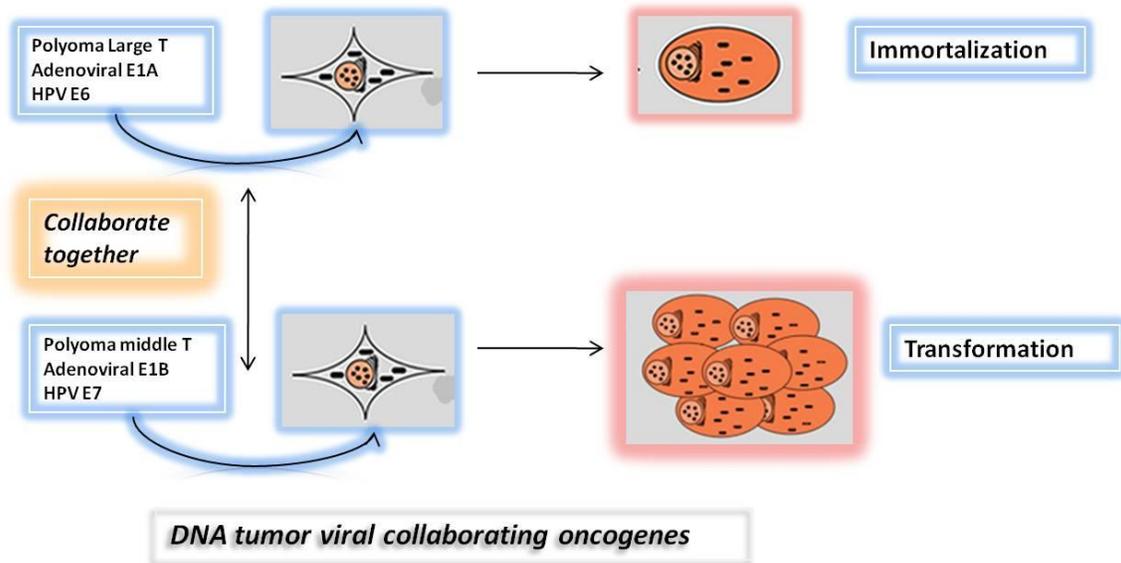


Figure 4: genetic factors that can immortalize primary cells (LT, E1A and E6). Genetic factors (MT, E1B and E7) that can achieve complete transformation in collaboration with (LT, E1A and E6).

Isolation of RAS cellular oncogene

In 1982, the group of Robert Weinberg (Parada, Tabin et al. 1982) isolated the first cellular oncogene from the human EJ bladder carcinoma cell line. This oncogene represents a variant of the human c-Ha-RAS proto-oncogene and encodes a 21,000-molecular weight protein which was termed EJ-RAS oncogene. This EJ-RAS oncogene stands as a model for other human oncogenes as it is a member of the RAS gene family and is thus closely related to the Ki-RAS and N-RAS oncogenes found to be activated in several different tumor types. It was later shown that the EJ-RAS oncogene was actually a mutated version of the cellular Ha-RAS gene, and for this reason these cellular counterparts of oncogenes were named proto-oncogenes. They demonstrated that certain point mutations in the DNA that encodes for a critical domain of the protein would make the proto-oncogene execute the functions of an oncogene.

Identification of tumor suppressor gene p53 and its interactions with viral oncoproteins

Earlier work on the SV40 virus and adenoviruses have revealed that the large T antigen of SV40 and E1B-55kd protein of adenovirus was found to form stable a stable complex with a cellular protein of approximately 53kd which became known as the p53 protein. The exact function of this protein was not clearly understood. Earlier work on p53 suggested that it may be implicated in the promotion of cell proliferation. The growth arrested mouse 3T3 cells, as a result of serum deprivation exhibited very low levels of p53 mRNA and protein. When the cell was induced to grow by serum stimulation, the level of p53 mRNA and the rate of p53 protein synthesis increased markedly, reaching a peak near the G1/S boundary just prior to the initiation of DNA replication. Similar experiments performed with normal resting T lymphocytes (Milner and McCornick 1980) and normal diploid fibroblasts (Mercer, Avignolo et al. 1984) showed that p53 expression is always concomitant with induction of cell growth. This observation, added to other characteristics of the p53 protein (short half life, nuclear localization), led to the notion that wild type p53 could play a positive role in cell proliferation.

It was observed that a large variety of transformed cells have elevated levels of p53 which includes the cells transformed by SV40, adenovirus and some of the chemically transformed cells. This strong correlation between tumorigenicity and high levels of p53 predicts an important role of p53 in tumorigenesis. It has been shown that only expressing p53 does not transform the rat embryo fibroblast. However two groups showed that co transfection of p53 with EJ-RAS oncogene into REFs readily transforms them leading to a cooperative effect of both p53 and EJ-RAS (Eliyahu, Raz et al. 1984; Parada, Land et al. 1984) A third group demonstrated that murine p53 could immortalize normal rat chondrocytes leading to cells sensitive to RAS transformation (Jenkins, Rudge et al. 1984; Jenkins, Rudge et al. 1985). It was later discovered that the p53 cDNA used in these experiments had a mutation that inactivated the tumor suppressive function of the wild type p53. We now know that the expression of a cloned wild type p53 gene

does not promote cellular transformation. Therefore, co-transfection of wild-type p53 along with oncogenic EJ-RAS failed to transform primary rat embryo fibroblasts. P53 protein executes its function through the formation of a multimeric complex. Therefore, when a mutant form of p53 is overexpressed in a cell line, it can act in a trans-dominant fashion to inactivate the endogenous wild-type p53 function.

The p53 protein has a lifetime of 20-30 min. it's interesting to speculate how the oncogenic tumor viruses cope with a protein having such a short half-life? The viral oncoproteins like SV40 large T and adenovirus E1B sequester p53 in the form of inactive complexes that increase its steady state but block its entry into the nucleus where it executes its function. On the other hand, the human papilloma virus deals with it in a slightly different manner. The viral E6 protein sequesters the p53 molecules and tags it for proteosomal degradation, which is ubiquitin dependent (Levine 1990; Scheffner, Werness et al. 1990; Werness, Levine et al. 1990)

The retinoblastoma tumor suppressor gene (Rb) and its interactions with viral oncoproteins

The retinoblastoma gene is located on the q14 band of chromosome 13 and is deleted frequently in retinoblastomas. Using a series of chromosome-13-specific DNA probes it was possible to isolate the Rb-1 locus as a molecular clone, which comprises more than 190 kilobases of DNA and at least 27 exons. The use of DNA probes derived from various parts of this gene has revealed a number of distinct types of mutations that serve to inactivate the RB-1 locus. The use of DNA probes to analyze cellular DNA and RNAs, as well as antisera reactive with 105 kilodalton RB protein, has shown that this gene and its encoded products are altered in many more tumors than retinoblastomas. The classification of the retinoblastoma gene as a tumor suppressor was made based on the observation that mutations in the wild type alleles were recessive in tumor predisposition. A number of sarcomas, notably osteosarcomas, have been found to carry inactive

Rb-1 alleles. Defective Rb-1 alleles have been found in several other tumor types; including small cell lung carcinomas (SCLC), bladder carcinomas and mammary carcinomas (Hansen and Cavenee 1988). This led to the conclusion that retinoblastoma gene was an antioncogene since tumor formation ensues when these antioncogenes are inactivated

The Rb-1 protein may play a role in mechanisms involving tumorigenesis. This was made clear in 1988 that the transforming mechanisms of several DNA tumor viruses directly involve the Rb-1 encoded protein, p105. The oncoproteins of the adenovirus E1A, SV40 large T (LT) and the human papilloma virus E7 genes were each found complexed with the RB-1 protein in virus transformed cells (Whyte, Buchkovich et al. 1988).

The association of adenoviral E1A, SV40 LT and HPV E7 with the 105kd retinoblastoma protein has important implications. In normal circumstances, Rb executes its function as a growth repressor and prevents the entry of cells into the S phase. The advantages that adenoviral E1A or SV40 LT or HPV E7 gain when they bind to the RB protein is that it antagonizes the functions of this protein, thereby stimulating DNA synthesis by pushing the cells to S phase. This shows that, these two classes of genes, one acting positively (E1A) and the other acting negatively (RB) on cell growth, directly cooperate with each other by the binding activity of their encoded proteins during cellular transformation. Therefore, it can be suggested that oncogenes and anti-oncogenes are the same constituents of a common regulatory pathway.

This is very striking since adenovirus E1A, SV40 and HPV having no structural similarity yet have acquired a common tropism for p105-Rb (Weinberg 1991). However they share a common LXCXE structural motif that enables their interaction with the RB protein. These observations have highlighted the importance of RB showing that the gene and its encoded protein sits at a central and critical point in the cells growth regulatory pathway, and involved in several distinct mechanisms leading to cancer.

Oncogene collaboration 1: it takes two to tango.

After the identification and isolation of the EJ-RAS oncogene, it was soon being tested for its transforming abilities in culture. Transfection of EJ-RAS oncogene into NIH3T3 cells readily transformed them in a single step. This was because the NIH3T3 cells were partially transformed and it just required another driving step to undergo malignant transformation. However, transfection of primary diploid rat fibroblast with EJ-RAS did not transform these cells. On the contrary, carcinogen induced immortal hamster fibroblast variants were successfully transformed with EJ-RAS, which in conclusion shows that immortalization of primary cells, is a prerequisite for malignant transformation (Newbold and Overall 1983). Although primary cell cultures can apparently become tumorigenic in a single step after infection in vitro by tumor viruses such as polyoma or adenovirus, this does not qualify the norms of multistep carcinogenesis as, genetic dissection of the genomes of these viruses have revealed that viruses carry at least two genes harboring distinct functions both of which must be expressed in order to obtain a malignant transformed phenotype. The multistep model had an explanation at the genetic level where in, each step required the activation of a distinct gene and the final outcome required the concomitant expression of the previously activated genes.

Another line of evidence came from the experiments performed by Earl Ruley at the Cold Spring Harbor laboratory. They performed a series of experiments with cloned viral and cellular oncogenes and tested them individually and in combinations in primary rat kidney cells to determine whether viral establishment functions were required by cellular oncogenes to transform primary cells in culture. Transfection of vectors containing the polyoma middle T, adenovirus E1B and the EJ-RAS individually did not transform the primary cells. However, when adenoviral E1B and E1A, polyoma middle T and large T and adenoviral E1A and EJ-RAS vectors were co-transfected, they readily transformed the primary cells. This confirmed the requirement of E1A and large T for establishing cells in culture prior to full transformation induced by middle T, E1B,

and EJ-RAS (Ruley 1983). This was further proved by the fact that polyoma middle T, adenoviral E1B and EJ-RAS could transform a variety of established cell lines. An interesting point to be highlighted here is that activated EJ-RAS being a cellular oncogene was unable to transform primary baby rat kidney cells, but was only able to transform by collaborating with the function of adenoviral E1A oncogene.

Collaborating MYC and RAS cellular oncogenes

The multistep process by which a tumor virus induces transformation in primary cells has been elucidated in previous sections. I have also described how certain DNA tumor viral oncogenes like adenoviral E1A and polyoma large T, can collaborate with the EJ-RAS oncogene in order to push the cells towards malignant transformation. These experiments convey the same message: malignant transformation cannot be achieved by a single oncogene. However, it was important to understand what genetic alterations existed in human tumor samples that collaborated with EJ-RAS to induce transformation.

Work on promyelocytic leukemia and American Burkitt's lymphoma had shown the co-existence of an altered version of the *myc* gene as well as activated versions of a second cellular oncogene, termed N-RAS (Murray, M.J et al. 1983). This line of evidence probed to examine whether the *myc* oncogene, could collaborate together with EJ-RAS oncogene to create a tumor cell.

In 1983, seminal work by Robert Weinberg and coworkers showed that the *myc* oncogene derived from the avian MC29 virus, which is usually associated with cancers of the hematopoietic lineage, was able to induce transformation when cotransfected along with the EJ-RAS oncogene in Rat embryo fibroblasts. The combinatorial effects of *myc* and EJ-RAS indeed fully transformed the REFs in culture. This was the first evidence that showed two cooperating oncogenes both from the cellular origin that induced malignant transformation (Land, Parada et al. 1983) (fig 5). This cooperative effect with c-*myc* oncogene was indeed found true with both NRAS and HRAS oncogenes.

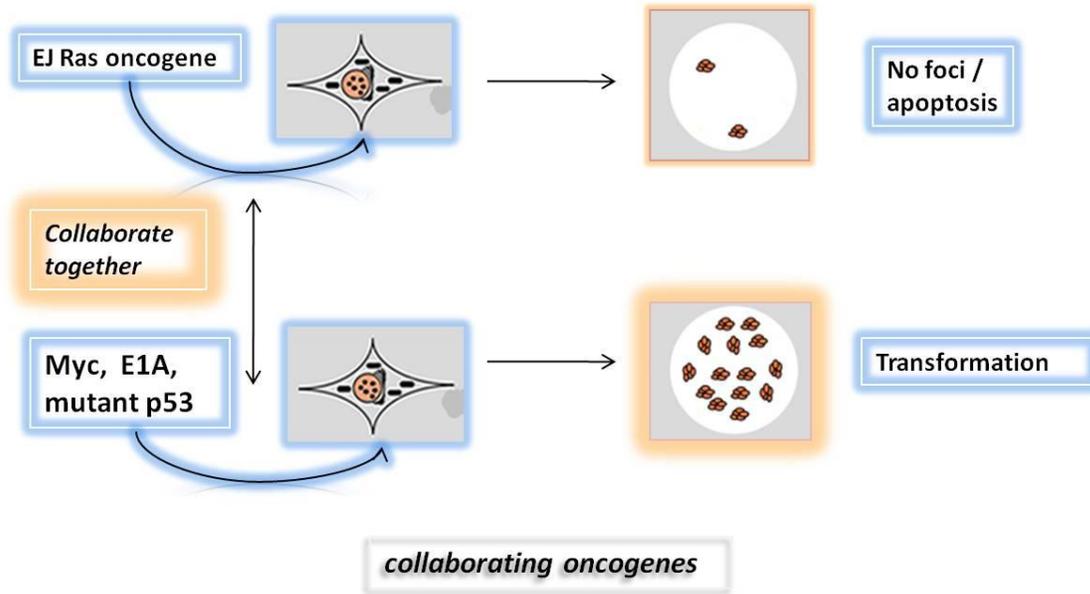


Figure 5: RAS alone cannot transform primary cells. RAS in collaboration with either E1A or myc can induce complete transformation.

A repeating theme in many of these examples is that the collaborating oncogenes act at different cellular sites. Oncogenes like *myc*, polyoma middle T, p53, *erbA* and E7 have gene products in the nucleus and hence are called nuclear oncogenes. The proteins specified by *RAS*, polyoma middle T, *src* and *erbB* are called cytoplasmic oncogenes.

From the above discussion it is apparent that two oncogenes are required to completely transform primary cells instead of one. There are two reasons that can explain this mechanism of oncogene collaboration; 1. 'Escape from apoptosis' and 2. 'Escape from senescence'. These are discussed below

Oncogene collaboration 2: escape form apoptosis.

An observation was made by Earl Ruley and colleagues showing that over expression of EJ *RAS* oncogene in primary rat embryo fibroblasts resulted in growth arrest of the cells. This growth arrest can be rescued by the expression of another oncogene that effectively collaborates with oncogenic *RAS* and proceeds with cell proliferation. The growth arrest is mediated since the expression of the

oncogene exceeded the threshold levels in the cell. These high intensity oncogenic signals are sensed by the p53 tumor suppressor gene which mediates the growth arrest. This can be confirmed by the Introduction of a mutant p53 cDNA that can rescue the growth arrest (Hirakawa and Ruley 1988; Friedman, Kern et al. 1990).

It is now clear that the p53 is a tumor suppressor gene, as a number of alterations were detected in p53 in several tumor samples (Hollstein, Sidransky et al. 1991). Mutations in the p53 gene occur quite frequently in colorectal cancer. In fact elegant work by the group of Bert Vogelstein and co-workers showed that introduction of a wild type p53 gene into the colorectal cancer cell lines suppressed their tumorigenic phenotype which stopped their aggressive growth. This again support the notion that wild type p53 severely opposes proliferation in cells (Baker, Markowitz et al. 1990).

Oncogenes induce apoptosis

Expression of adenoviral E1A alone can stabilize p53 in untransformed cells. P53 stabilization is usually observed when it complexes with adenoviral E1B or SV40 large T antigen. Studies by Scott Lowe and co-workers have shown that p53 levels increased within 40 hours after adenoviral E1A expression. As a consequence, there was a dramatic reduction in cell viability which resembled apoptosis. The apoptosis induction was dependent on p53, because p53 levels reverted back to normal in surviving cells that had lost the E1A expression. This was also found true with the expression of oncogenic *RAS* in rat embryo fibroblasts which induced apoptosis in a p53 dependent fashion. The binding of adenoviral E1B to p53 protects the cells from the toxic effects of expressing E1A alone (Lowe and Ruley 1993).

E2F

The cell cycle (supplementary info fig 8) is heavily secure and a highly regulated process. Each phase of the cell cycle is regulated by different kinds of

checkpoint proteins. One of them is the RB protein binding partner: E2F transcription factor. E2F acts as a positive regulator and signals the S phase entry of cells. On the other hand p53 monitors the G1 phase of the cell cycle and responds to DNA damage. It has been shown that expression of recombinant E2F together with a temperature sensitive allele of p53 (expresses WT at 32°C and mutant at 39°C) cooperates to induce apoptosis at 32°C but fails to do so when shifted to 39°C at which p53 has lost its wild type function. This led to the conclusion that the cell cycle utilizes an interactive and communicative pathway between E2F-RB-p53 (Wu and Levine 1994). Further investigation into this intercommunication between the RB and p53 pathways has strengthened its existence.

Over expression of E2F in quiescent fibroblasts, passed the cells into the S phase by inducing DNA synthesis but did not favor the complete replication of cellular DNA. On the contrary, E2F overexpression drives the cells towards p53 mediated apoptosis (Kowalik, DeGregori et al. 1995). E2F induced apoptosis was suppressed when co expressed with wild type RB or mutant p53 but continued exhibiting the apoptotic phenotype when expressed with mutant RB or wild type p53(Phillips, Bates et al. 1997). Therefore the p53 and the RB proteins are very essential or critical regulators of the cell cycle.

c-myc

In follicular B-cell lymphoma, there is a chromosomal translocation, which juxtaposes the Bcl-2 gene with the immunoglobulin heavy gene locus. It was seen that this translocation gave rise to elevated levels of *Bcl-2* gene product that inhibited the cells programmed cell death i.e. apoptosis. The 25000kd inner mitochondrial membrane *Bcl-2* was classified as a proto-oncogene (Nunez, Hockenbery et al. 1991). Gerard Evan and co-workers working on *c-myc* oncogene, showed that in serum starved rat-1 fibroblasts that expressed *c-myc* constitutively, were unable to undergo growth arrest in spite of serum starvation and instead showed signs of apoptotic cell death because of the substantially decreasing cell number. This indeed showed that constitutive over expression of

an oncogene in this case either *c-myc* or E1A alone triggered programmed cell death. Therefore, the *c-myc* proto-oncogene implicated in cell transformation, differentiation and cell cycle progression also has a central role in mediating apoptosis. These opposing roles of *c-myc* in cell growth and apoptosis could be that different genes dictate the outcome of *c-myc* expression in a cell (Evan, Wyllie et al. 1992). One such cooperative interaction is elucidated here.

The *Bcl-2* translocation, which commonly occurs in lymphoid tumors, is usually expressed in higher levels. It was seen that *c-myc* induced apoptosis is opposed by *bcl2* overexpression. This is an elegant example which describes the consequences of *myc* induced apoptosis that is inhibited by collaborating with *Bcl-2* oncogene in order to drive cellular transformation. (Bissonnette, Echeverri et al. 1992; Fanidi, Harrington et al. 1992)

Oncogene collaboration 3: escape from senescence

Oncogenes induce senescence

Normally we think of cellular senescence in the context of telomere shortening, which leads to the growth arrest due to telomere erosion. This is called replicative senescence. However, in 1997 the group of Serrano showed that senescence could be provoked prematurely by an oncogene. This concept was termed oncogene induced senescence. I discussed previously that oncogenic *RAS* can transform established or immortal cell lines; however, most of the primary cells require a cooperating oncogene like *myc* or E1A or an inactivation of a tumor suppressor gene to attain a transformed phenotype. They showed that expression of an oncogenic *RAS* invoked cellular senescence prematurely. The term premature senescence is used here as senescence occurs without the occurrence of telomere shortening. This unscheduled signaling within the cell by the protein products of oncogenes activates a tumor suppressor network, which is accompanied by higher levels of p53 and p16 (fig 6 B). However, when either p53 or p16 are inactivated, oncogenic *RAS* does not induce senescence.

Cellular senescence can be triggered by a combination of at least three mechanisms (fig 6A): 1. Telomere shortening, 2. Up regulation of the *INK4A* (see supplementary information fig 9) gene locus which encodes p16^{INK4A} and p19^{ARF} and 3. Accumulation of DNA damage. I have discussed the seminal finding from Serrano, which showed for the first time senescence induced by oncogenic *RAS* in primary cells. This concept emerged to be a putative tumor suppressive mechanism that is similar to the oncogene-induced apoptosis. These in vitro findings were corroborated by in vivo studies using inducible mouse models. In these models, a knock in of an oncogenic version of *RAS* can be expressed endogenously by the induction of an external stimulus. The induction of the oncogenic *RAS* did not show signs of tumor formation in mice. This proved to be true in various lung, colon and pancreatic mouse models suggesting that endogenously expressed oncogenic *RAS* induces senescence. The senescent cells share the key features and cellular markers with cells undergoing replicative senescence: total loss of proliferation activity, a flat and enlarged cellular morphology and increased SA-β- GAL activity. Moreover, oncogene induced senescence is accompanied by the activation or induction of p16^{INK4A} and ARF both encoded by the *CDKN2A* locus. (Hara, Smith et al. 1996; Kamijo, Zindy et al. 1997; Serrano, Lin et al. 1997; Collado and Serrano 2010)

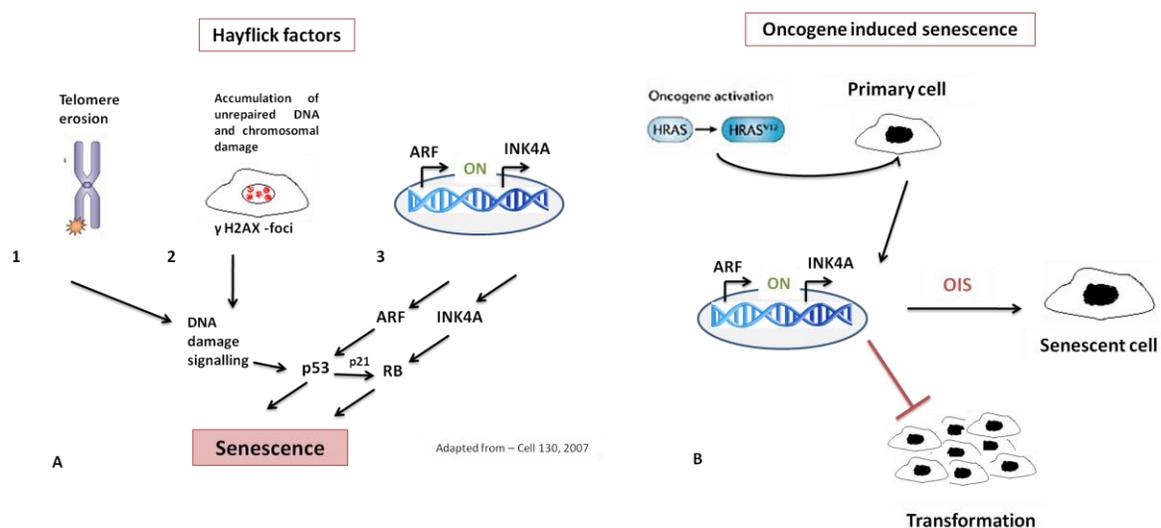


Figure 6: A. hayflick factors that induce senescence. Telomere erosion repeated DNA damage and expression of ARF and INK4A can induce senescence in cells. B. in primary cells expression

of an activated version of *RAS* oncogene can prematurely induce senescence through the up regulation of the *INK4A* locus.

BRAF^{V600E} induced senescence

The BRAF protein is a downstream effector of the RAS signaling pathway. Mutation in the V600 amino acid can convert the protein to a constitutively active form BRAF^{V600E}. BRAF^{E600} induces premature senescence in human diploid fibroblasts and primary human melanocytes. Primary human melanocytes that overexpress BRAF^{E600} show a transient and moderate increase in proliferative activity before entering senescence which is in contrast to the more rapid cell cycle arrest mediated by HRAS^{V12}. Although both BRAF^{E600} and RAS^{V12} induce p16^{INK4A}, the subsequent premature senescence cannot be prevented by p16^{INK4A} knockdown, at least in cultured human cells. In contrast, human fibroblasts harboring a small homozygous germline deletion in p16^{INK4A} are resistant to RAS^{V12}-induced senescence, but still enter senescence induced by BRAF^{E600} (Brookes, Rowe et al. 2002). Neither RAS^{V12} nor BRAF^{E600} induces ARF expression in human cells (Wei, Hemmer et al. 2001; Michaloglou, Vredeveld et al. 2005). p21^{CIP1}, a key transcriptional target of p53, is not induced by BRAF^{E600} (Gray-Schopfer, Cheong et al. 2006), and consistently, p53 knockdown is not sufficient to prevent HRAS^{V12} or BRAF^{E600}-induced senescence. Furthermore, in mouse models, melanocyte-specific overexpression of HRAS^{V12} (Chin, Pomerantz et al. 1997) or NRAS^{K61} (Ackermann, Fruttschi et al. 2005) cooperates with loss of either p16^{INK4A} or ARF in the induction of melanoma.

PTEN induced senescence

Senescence induction in majority of the cases is caused by oncogene activation. However, recent reports suggest that loss of tumor suppressor genes can activate a senescence program in vivo. This can be exemplified by the loss of tumor suppressor gene PTEN which is among the most commonly mutated tumor suppressors in human cancers. PTEN encodes a phosphatase catalyzing the conversion of the membrane lipid PIP3 to the PI3K substrate PIP2, fueling

downstream signaling cascades, including the AKT pathway. Pandolfi and co-workers have demonstrated that a loss of single PTEN allele acts mitogenically whereas loss of both alleles instead sets in motion a senescence program which is p53 dependent (Alimonti, Carracedo et al. 2010; Alimonti, Nardella et al. 2010).

Tumor suppressor gene collaboration

The p53 and Retinoblastoma (RB) are two of the best-studied tumor suppressor genes. It has been suggested that the coupled inactivation of p53 and Rb may be important for transformation in some instances. As I have described earlier, several DNA tumor viruses, including SV40, adenovirus and human papilloma virus encode proteins that bind and presumably inactivate both Rb and p53, and both of these functions are required for efficient transformation by these viral proteins *in vitro* and *in vivo*. High frequency of mutation is observed in both p53 and Rb in several human tumor cell types, including certain sarcomas (Friend, Bernards et al. 1986) and carcinomas of the lung (Rygaard, Sorenson et al. 1990), breast (Prosser, Thompson et al. 1990), cervix and pancreas. This suggested that a loss of function of both these tumor suppressors collaborate during tumorigenesis.

It is possible to study the cooperative effects of *p53* and *Rb* loss during tumorigenesis using mouse models. Mice heterozygous for *Rb*^{+/-} developed intermediate lobe pituitary tumors and medullary carcinoma of the thyroid. *Rb*^{+/-} mice has a life expectancy of 9-15 months. However, *Rb*^{-/-} mice die prematurely between 13.5 and 15.5 days with defects in fetal haematopoiesis and widespread neuronal cell death. Mice heterozygous for *p53*^{+/-} have a tumor incidence of 25% within 16 months of age and develop sarcomas and lymphomas. Homozygous *p53*^{-/-} mutation causes even more pronounced cancer susceptibility where 90% of mice develop one or more tumors by three or six months of age. In the case of both *Rb*^{+/-} heterozygous and *p53*^{+/-} heterozygous mice, the tumor spectrum was comparable to that of the *Rb*^{+/-} heterozygotes. These mice were associated with several tumor types: leiomyosarcoma, anaplastic sarcomas, islet cell sarcomas and pinealoblastoma. Another strain of mice that was both heterozygous for *Rb*^{+/-}

and homozygous for *p53*^{-/-} displayed 15 separate lesions affecting eight different tissues which included pinealoblastoma and islet cell tumors. The DNA analyzed in these tumors indicated that these mice were found to have lost the remaining wild type allele of *Rb*. These data imply that certain tissues in the mouse are susceptible to transformation upon inactivation of both the *p53* and *Rb* tumor suppressor genes. This provided the first example of collaboration between two tumor suppressors in murine tumorigenesis (Bart O. Williams et al. 1994).

These experiments suggests that *Rb* and *p53* are critical tumor suppressors and both act as negative regulators of growth in a redundant fashion and thus, the loss of both *p53* and *Rb* is necessary for tumorigenesis in susceptible tissues. *p53* plays a major role in maintaining genomic stability. Thus, the loss of *p53* would increase the likelihood of *Rb* mutations in the relevant cell types. Another major role attributed to *p53* is oncogene induced apoptosis. Absence of the *Rb* function in context to a developing embryo, results in wide spread cell death in the presence of wild-type *p53*. Therefore, elimination of *p53* function would allow cells to bypass the apoptotic program.

Absence of p53 function increases Rb mutation rate



Absence of p53 function allows survival of Rb-/- cells

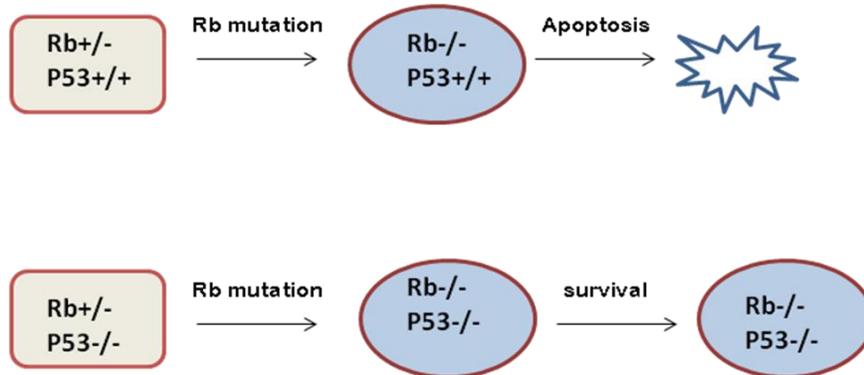


Figure 7: absence of p53 function increases Rb mutation rate. Mutant Rb and WT p53 cells die due to apoptosis, where as mutation in p53 in cells which have a mutated Rb continue to proliferate

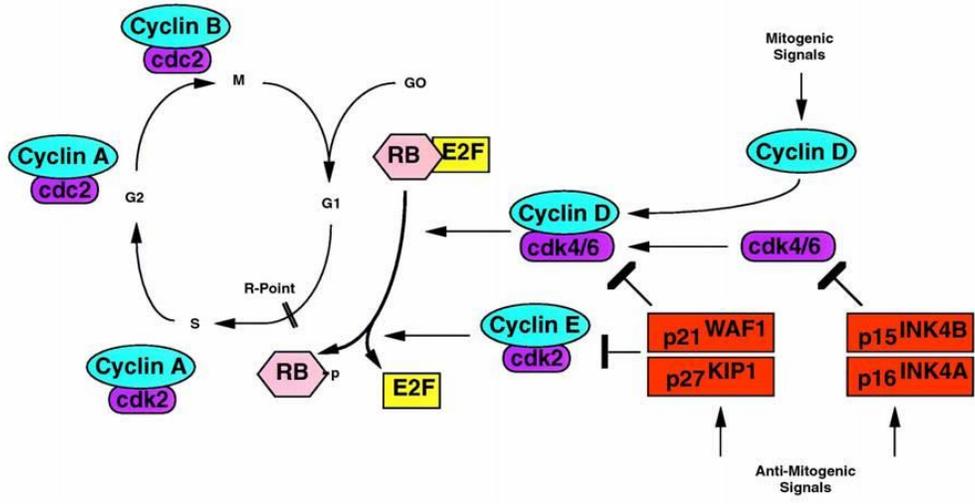
Supplementary information

Cell cycle

The main core components of the cell cycle machinery are the cyclin dependent kinases (CDKs) and the cyclins. The cyclins and the CDKs interact together to form functional complexes, which phosphorylate specific cellular substrates. During the progression of the cell cycle a variety of cyclin-CDK complexes are made in order to drive specific parts of the cell cycle. The critical phases of the cell cycle are G₁, S, G₂ and M. Cells that are in G₀ i.e. quiescence, need mitogenic stimuli to progress through the cell cycle. The G₁ phase is critically regulated by RB. RB, when hypophosphorylated remains highly active and suppresses cell proliferation. Upon mitogen stimulation in quiescent cells, the synthesis of D type cyclin begins. The D type cyclin associates with CDK4 and/or CDK6. CyclinD together with CDKs modulate the function of RB by phosphorylation. Phosphorylation inactivates RB function, but cyclinD/CDK4/6 complexes alone cannot accomplish this phosphorylation. In late G₁, cyclin E is synthesized, which forms a complex with CDK2 and this complex can add additional phosphorylations to RB, leading to the release of E2F from RB. This moment is thought to coincide with the restriction point, the point at which the cells become autonomous for progression through the rest of the cell cycle. (Lundberg and Weinberg 1998).

. Another level of regulation of CDKs is exerted by the CDK inhibitors (CKIs). One class belongs to the INK4A family that binds to CDK4 and 6 and prevents their association with their cyclin regulatory proteins. P16^{INK4A}, p15^{INK4B}, p18^{INK4C} and p19^{INK4D} belong to this class.

The second class of CKIs is the CIP/KIP family. p21^{CIP1}, p27^{KIP1} and p57 belong to this class. p21^{CIP1} was the first to be discovered. p21^{CIP1} and p27^{KIP1} inhibit cyclinE/CDK2 and cyclinA/CDK2, but are required for assembly of cyclinD/CDK4/6 complexes, and are thus an activator of that complex. (Hunter 1993).



Cell cycle and its regulation

Adapted –Weinberg et al

Figure 8: Schematic representation of cell cycle

INK4A tumor suppressor locus

The INK4A locus encodes two different proteins from overlapping reading frames, which are critical tumor suppressors. The alpha transcript encodes P16^{INK4A}; it inhibits the cyclin D/CDK complexes that act on RB-E2F complexes (Kamb, Gruis et al. 1994). The beta transcript bears no homology to the alpha transcript and encodes a completely different protein p19^{ARF} (in mouse) or p14^{ARF} (in humans) that activates p53 by binding to Mdm2 (Stone, Jiang et al. 1995). Expression of this locus regulates the activities of two tumor suppressor pathways i.e. the RB and the p53, which is disrupted in most cancers.

The INK4A locus is most commonly mutated in human cancer. In 1994 the group of David Beach showed that cell lines acquired from tumors ranging from lung, esophagus, liver colon and pancreas all harbored deletions in the INK4A genetic locus. Moreover, introduction of a cDNA encoding p16^{INK4A} into these cell lines inhibits their colony forming efficiency. Ectopic overexpression of any of the INK4A proteins in proliferating cells can inhibit the activity of the cyclin dependent kinase and induces G1 arrest (Serrano, Hannon et al. 1993).

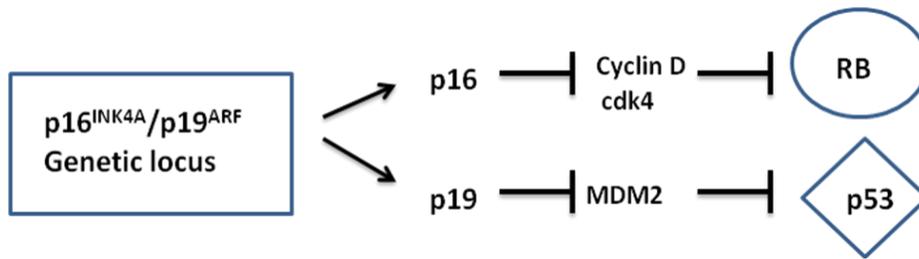


Figure 9: the INK4A genetic locus encodes p16 and p19 that regulates p53 and RB two critical tumor suppressors.

Cells that are transformed by the cooperation of *RAS* and *myc* can be reverted by the over expression of INK4A proteins. However, transformation induced by *RAS* and E1A cannot be reverted. This is because the ultimate goal of p16 is to inhibit the phosphorylation of pRB, which is mediated by CDKs. Therefore, it is logical to assume that cells transformed by E1A and RAS cannot be arrested by expression of p16^{INK4A} as they are already immortalized through binding of E1A to RB. We also see that inactivation of p16 and pRB are mutually exclusive in human tumors since both are a part of the same regulatory pathway (Weinberg et al 2000).

Chapter 5: Multistep carcinogenesis, the Genomic era and its benefits in cancer treatment

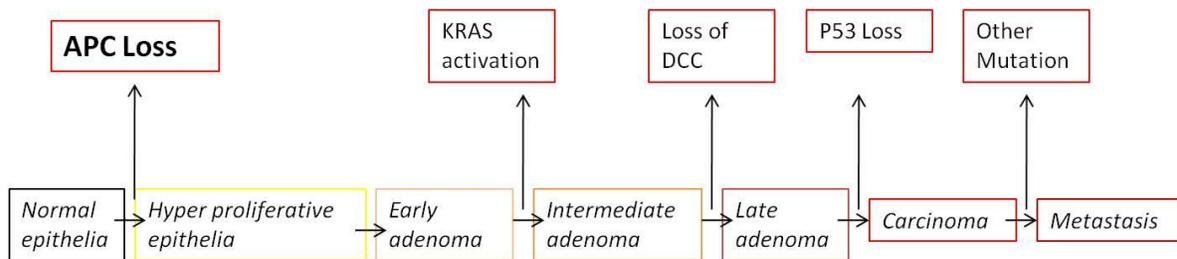
Introduction

Research on the DNA tumor viruses has shown the precise mechanisms of tumorigenesis. I also described the layout of the cells security grid that prevents tumorigenesis, and also discussed about the critical tumor suppressors that are often mutated in cancer. This brings us to the final part of this topic related to oncogene collaboration where, firstly I would like to discuss briefly how cancer begins by accumulating several series of mutation till it becomes malignant in one's lifetime i.e. the theory of multistep carcinogenesis and secondly, discuss about oncogene addiction and synthetic lethality as a therapeutic option.

Multistep carcinogenesis model

In humans, the development of cancer involves a complex succession of events that usually occur over many decades, as each step in this process is a distinct physiological barrier that needs to be breached in order to proceed further. During this process, several mutations are acquired over time in proto-oncogene and tumor suppressor genes, which is the reason why the genomes of cancer cells become rather disorganized during oncogenesis. Different combinations of mutations of these mutant alleles are found in the genomes of many distinct types of human cancer. Cancer cells are often genetically unstable, as they have lost critical DNA repair and maintenance proteins. A classical study on colorectal cancer progression by the group of Bert Vogelstein has shown how colon cancer progresses during time by accumulating genetic alteration during each distinct stage.

Colorectal cancer begins with the appearance of small polyps in the villus of the large intestine. These are benign tumors called adenomatous polyps. Histopathological and genetic studies of these polyps revealed a great majority of them acquiring an inactivating mutation in the APC tumoursuppressor gene that makes it inactive. The next stage is the transition form adenomatous polyps to form intermediate sized adenomas. About half of the tissue biopsies harbored an activated *RAS* oncogene. The third and the final step in colorectal cancer progression lead to the formation of adenocarcinoma, which is highly malignant. These samples frequently had a p53 tumor suppressor mutation along with the previously described APC and *RAS* mutations. This step-by-step inactivation of genes and the gradual transition from one form to another determines the characteristics of multistep carcinogenesis. One critical observation that has to be made here is that the cancer cells try to acquire mutations in those genes whose collaboration in the end shows the characteristics of a fully transformed state, which is illustrated in this classical example where mutations in *RAS* or APC alone cannot be fully transformed. Whereas, a combination of *RAS*, APC and p53 mutations easily collaborate to fully transform the cells.



Multistep Carcinogenesis in Colorectal cancer

Adapted –Vogelstein et al

Figure 10: model of multistep carcinogenesis in colorectal cancer

Oncogene addiction

Oncogene addiction describes the phenomenon by which some cancers that contain multiple genetic and epigenetic abnormalities remain dependent on (addicted to) one or a few genes for both maintenance of the malignant phenotype

and cell survival(Weinstein and Joe 2008). Evidence form genetically engineered mouse models of human cancer, mechanistic studies in human cancer cell lines and clinical trials involving specific molecular targeted agents support this concept. The best demonstration comes from a transgenic mouse model, where switching on the *c-myc* oncogene in the hematopoietic cell led to the development of T-cell and myeloid leukemias. However, switching off this gene dramatically ceased the proliferation of the leukemia cells(Felsher and Bishop 1999). Another interesting line of evidence comes from the increasing number of examples of the therapeutic efficacy of anticancer drugs or antibodies that target specific oncogenes in human cancer. Trastuzumab, an antibody that targets the receptor tyrosine kinase HER-2/NEU in patients is one of the earliest examples. Other recent examples include imatinib which targets *bcr-abl* oncogene in chronic myeloid leukemia (Gorre, Mohammed et al. 2001), gefitinib and erlotinib which target the epidermal growth factor receptor (EGFR) in non small cell lung cancer, pancreatic cancer and glioblastoma (Pao, Miller et al. 2005).

Cancer cells depend on the continuous expression of certain oncogenes in order to maintain their oncogenic phenotype (Weinstein 2002). For example, pancreatic cancer carries a mutant *KRAS* oncogene, several other cancers overexpress the cyclin D1 gene (Weinstein 2000) etc. When *KRAS* or cyclinD1 is inhibited by antisense oligonucleotides, it preventes the proliferation of the cancer cells and reverted them back to normalcy. However, these reverted cancer cells still continue to express high levels of the relevant oncogenes, indicating that the parental cancer cells were addicted to the particular oncogene. The continuous oncogene expression is required to maintain the malignant phenotype. Moreover, if an activated or overexpressed oncogene exerts its effects by causing genomic instability (which leads to other critical mutations), then blocking its expression may not reverse the cancer phenotype (Jain, Arvanitis et al. 2002).

Likewise, many tumors have lost their tumor suppressor genes (E.g. p53, Rb or APC). Reintroduction of the wild-type tumor suppressor gene into the respective cancer cells promotes marked inhibition of growth. However, if these cancer cells have evolved through a multistep process, then correcting one

mutation might just give a modest inhibitory effect. Thus some cancers are hypersensitive to the growth inhibitory effects of specific tumor suppressor genes. The concept of oncogene addiction can be exemplified by the fact that the subset of patients with chronic myeloid leukemia treated with imatinib later suffered relapse. This was because the leukemia cells had developed de novo mutation in the kinase domain of the BCR/ABL protein which blocked the inhibitory activity of imatinib. This de novo mutation in BCR/ABL protein that drives leukemia helps to acquire resistance to its inhibitor. Likewise, In NSCLC, secondary mutations have been observed in the EGFR (T70M) that acquired resistance to gefitinib or erlotinib therapy (Pao, Miller et al. 2005). This shows that there is a strong selective pressure for the emergence of cells that carry de novo mutations in the respective oncogenes indicates the remarkable dependence of these neoplastic cells on specific oncogenes.

From this we can understand that the phenomenon of oncogene addiction and tumor suppressor gene hypersensitivity is not simply a summation of the individual effects of oncogene activation and tumor suppressor gene inactivation. This is consistent with the fact the proteins encoded by these genes often play multiple roles in complex and interacting networks and exhibit both positive and negative feedback control. Furthermore, throughout the multistage process the cancer cells has to maintain homeostasis between the positive signals and negative signals in order to maintain structural integrity, viability and normal replication(Weinstein 2002).

Synthetic lethality as a therapeutic option

Two genes are said to be synthetic lethal if mutation in one gene alone selects for cell viability whereas simultaneous mutation of both genes causes cell death(Kaelin 2005). Mutations acquired by cancer cells affect certain regulatory pathways. As a consequence, the ability of the cancer cells to respond to perturbations may be affected. Loss of function genetic screens is a powerful tool that helps in the identification of synthetic lethal interactions(Bernards, Brummelkamp et al. 2006). Therefore, knowledge of synthetic lethal interactions

can provide insights into the 'Achilles heel' of cancer cells. Knowing this, it is easy to design drugs that targets the weak spot in cancer cells and kills it. Although synthetic lethal interactions are used to uncover new drug targets, it can also be used to identify potential drug response biomarkers. These are called chemical synthetic lethality screens where one can search for genes whose suppression renders the cancer cells more sensitive to the given drug (Bernards 2010).

One of the examples that illustrate the synthetic lethal interaction can be learnt in tumors having an inactivation in the Rb gene. RB1 inactivation leads to increased E2F activity, which can stimulate S-phase entry but also can promote p53 dependent apoptosis. Therefore, a tumor which previously had a mutation in TP53 will derive additional benefits by mutating RB1 but at the cost of becoming addicted to the p53 mutation. This can be exploited therapeutically by administering the cancer drugs with certain drugs that can reactivate p53 function and lead the RB1 inactivated cancer cells to p53 dependent apoptosis(Adams and Kaelin 1996; Sherr 2000). Several drugs like RITA (reactivation of tumor suppressor activity) and NUTLIN that can restore the function of p53 are already being used in clinical trials.

Using RNAi as a tool many researchers have performed many screens that have uncovered synthetic lethal interactions in cancer cells. These kinds of screens help us to identify biomarkers which improve the diagnosis of cancer.

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