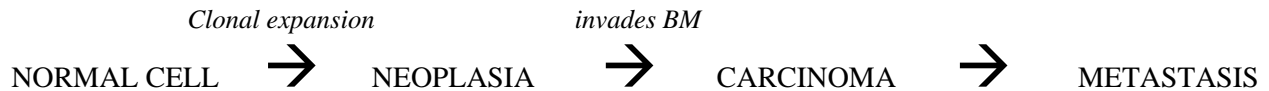


Oncogenes and Neoplasia

This lecture covers the basic biology of taking a normal cell and turning it into a malignancy in the human body. Much of the original work in this area was done in animal models in different species.

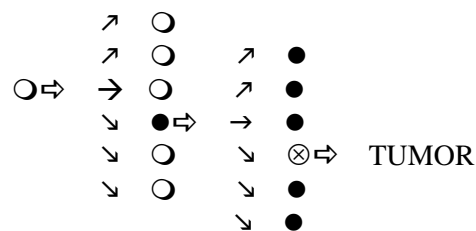
➤ MECHANISM OF TUMOR DEVELOPMENT:



A **normal** cell (stem cell or partially differentiated cell) *clonally expands* to a **neoplasia**, recruiting vasculature and changing its environment to enable growth. The neoplasm then acquires the property to *invade the basement membrane* and leave its local environment, at which point it becomes a **carcinoma** (for epithelial tumors). This invasion then allows **metastasis** to local lymph nodes and local organs, and eventually to the rest of the body.

Clonal Expansion

No one genetic thing is solely responsible for the clonal expansion that leads to malignancy. A combination of oncogenic activation, tumor suppressor inactivation, and other changes in the way the cell is programmed to behave leads to a cell acquiring the properties that will then lead to malignancy.



A cell in a normal cell line may suffer an abnormal genetic or epigenetic event. This leads to an inherited difference in behavior of this cell from its normal sister cells. This difference is then reiterated several times before there are tumor developments. There is always a latency period after the genetic change, because there are other things that must go on inside a cell in combination with the genetic change to allow for tumor growth. This lecture discusses oncogenes as single agents causing cancer, but we should remember that there is always this latency period.

➤ VIRAL ONCOGENES:

- Viral oncogenes can be derived from two kinds of viruses ...
 1. RNA Tumor Virus (retrovirus) – ex. Rous Sarcoma Virus that was discovered in 1911 to cause tumors in chickens.
 2. DNA Tumor Virus (DNA viruses coated with protein) – ex. Rabbit Papilloma Virus that causes warts that move on to become invasive tumors.
- Some basic retro virus biology...

Gene products that are proteins in the lipid bilayer (envelope) of the retrovirus are used to recognize and infect the cell. Inside the nuclear protein core are two copies of single stranded RNA, which make up the retrovirus genome.
- The basic viral life cycle (AVIAN LEUKOSIS VIRUS (ALV) as an example)...

This is the virus in which the **Src** oncogene is inserted. ALV infects most cell types in chickens. The virus binds to the receptors on the host cell membrane and then fuses to enter the cell. The basic life cycle (Dr. Parson's says we don't have to memorize)...

Virus enters cell and capsule breaks down → reverse transcriptase synthesizes a DNA copy of the RNA genome → then a second DNA copy is synthesized → double stranded DNA → part of POL gene has integrase function that integrates the viral genome into the host cell → genome can be transcribed to generate viruses that can produce new virions.

It is important to understand this process, because the biology that enables a virus to integrate into the genome is linked to the way in which the virus becomes oncogenic. LTRs are long terminal repeats that act as enhancers and promoter elements, leading to transcription and translation of GAG, POL and ENV genes. These genes are required for viral production and assembly.

➤ **HOW ARE TRANSDUCED ONCOGENES RENDERED MALIGNANT?**

1. Quantitative effects: Strong promoters (LTRs) can regulate gene transcription to cause inappropriate expression of the transduced gene. (ex. MYC)
2. Qualitative effects: An oncoprotein can be expressed in a truncated or fused form, or the oncoprotein may acquire point mutations during viral replication. (ex. v-Src in RSV has several point mutations and is truncated)

• **ROUS SARCOMA VIRUS:**

Peyton Rous observed spontaneous carcinomas in chickens that he got from the market. We now know that this was an ALV derivative. Rous propagated the sarcomas by passaging them from chicken to chicken within certain breeds. The tumor became more and more invasive as he went on with his experiment, and he collected the most invasive and aggressive forms. In 1911 he prepared a cell-free filtrate from one of his sarcoma explants, using a variety of filters. Rous then inoculated chickens with the filtrate, and the chickens developed sarcomas. Thus beginning the study of this process as a virus causing tumors. Because RSV can cause a rapid sarcoma in infected animals that are fully immunocompetent, it is known as "acutely transforming". RSV causes focus formation in cultured chick fibroblasts. These can be polyclonal in the sense that the virus can hit many targets to yield multiple tumors arising at the same time. This was the beginning of the study of these viruses outside of body, because one could take a culture of embryonic or baby chick fibroblasts and infect them with the virus in the petri dish. Growths different from the normal fibroblast cells are observed to result from the virus.

Why is RSV so tumor-genic in comparison to its parental strain???

RSV is very similar to ALV. Studies were conducted in which random mutations were generated in RSV and then analyzed for their ability to replicate and lose their oncogenic potential. Those mutants that lost the ability to cause tumors had lost their v-Src sequence. Therefore, this sequence, which was not found in other retroviruses (ALV), was associated with tumorigenicity. If a virus is missing v-Src, it won't cause tumors.

In 1977 it was discovered that genomic DNA from normal uninfected chickens harbored a close homolog of v-Src, known as c-Src. This host Src gene (not from any virus) was conserved phylogenetically (could be found in other birds). We now know that c-Src functions normally in animal development. RSV appeared in a spontaneous sarcoma in an ALV infected chicken. RSV, therefore, arose from incorporation of the host's c-Src sequence into the ALV genome. Acquisition of c-Src converted a slowly transforming retrovirus (ALV) into an acutely transforming retrovirus (RSV). Because the virus has the ability to mutate, the v-Src is a mutated version of c-Src (c-Src on its own cannot cause tumors), in which its kinase activity is turned on constitutively. This idea of capturing genes and then altering them so that they are no longer normally regulated but are turned on constitutively is

very important. All of the different signaling machinery (ligands, receptors, adaptor proteins etc.) can be hijacked and manipulated in the virus to then stimulate tumor growth.

- **MYC GENE:**

LTRs act as a transcription unit and have an enhancer sequence in them. The viral genome can randomly integrate into the host cell genome. For example, if it integrates next to a gene important in growth regulation, it can turn that gene on so that it is expressed at higher levels than normal, causing a tumor. This process is associated with the slower acting, non-acute oncogenic factors, because they have to randomly insert through out the genome and hit a site that is important for growth control to cause the growth of a tumor. Therefore, only a rare infection is tumorigenic.

An example of this process is a virus inserting next to the MYC gene. This gene is an important gene that is turned on early in response to growth signals. MYC coordinates the committed entry of the cell into the cell cycle. Thus, the enhancer LTR can act at a distance from the MYC gene and turn on transcription of a new protein that is usually not present, causing the cell to inappropriately enter the cell cycle and cause tumor growth.

- **IN VITRO “Transformed” PROPERTIES OF MALIGNANT CELLS:**

- **Immortality:** If you culture a normal cell, it will grow for about 30-40 generations. The cell will then reach a limit after which it can no longer divide. Tumor cells, however, will continue to divide after many doublings.
- **Decreased Growth Factor requirements**
- **Altered Morphology**
- **Loss of Contact Inhibition:** Normally, when cells grow in culture they will stop dividing when they touch each other and will not grow on top of each other. Tumor cell will grow on top of each other.
- **Loss of Dependence on Anchorage of Cell Growth:** Cells can grow in agar suspension, which normal cells will not do.

These properties correlate with those found in a human tumor and were useful in discovering some very important human oncogenes. **Focus Forming Assays** are ideal for quantification, since you can measure the number of transformed foci. Each original transformation event can yield a single focus, in which the cells behave according to the properties listed above. Then, each focus can be individually picked and recultured, allowing the isolation of a cell clone containing the progeny of the original transformed cell. One can then examine this cloned colony to determine what specifically caused the change in the cell's behavior. An example shown was of chick embryo fibroblasts that when transformed with RSV look rounded instead of spindle-like, do not stick to the substrate below, and grow on top of each other.

This type of assay was used to take rodent fibroblasts and transfect them with human DNA. The donor DNA from a human tumor (or control DNA from normal human cells) can be introduced into a host. The host that was used most for identifying oncogenes was the NIH3T3 cell, which itself is not purely normal. These cells are not yet tumors, but they have become immortal. These cells can, however, be pushed to become tumorigenic by transfection with an oncogene. When human tumor genomic DNA is transfected into these cells, a very low rate of transformed foci results. Occasionally though, a tumor will arise with two orders of magnitude increase in the number of transformed cells (20% of tumors, such as sarcomas, carcinomas and leukemia). The genomic DNA of transformed cells is active in subsequent in vitro transformation assays. DNA from a transformed cell can be used to transform other NIH3T3 cells. Therefore, these transformed cells have somehow inherited the transformation property.

This type of experiment was used to identify a really important class of human oncogenes. Using a human bladder carcinoma cell line, DNA was transformed into the NIH3T3 cells. The human DNA is then substantially diluted out, because the mouse genome is what is dividing. The experiment is then repeated several times by taking the transformant and transforming it again. The human DNA is being diluted out, but each time the oncogene that is causing the NIH3T3 cell to become a tumor is being

selected for. The tertiary transformant should contain the transforming sequence but little other human DNA.

➤ **WHAT IS THE TRANSFORMING AGENT IN THE HUMAN TUMOR DNA?**

Human DNA fragments can be isolated, cloned, and tested for their ability to transform NIH3T3 in vitro. The transforming activity from the cell line could be ascribed to a single fragment of DNA. Sequencing of that DNA then revealed that the transforming agent is the cellular version of the h-Ras gene. This was a retroviral oncogene that had already been identified in animal studies and was found to be in human tumors. A variety of Ras genes were found to be oncogenic in these types of experiments (c-H-Ras, N-Ras, c-K-Ras).

Ras Genes from normal DNA do not have the ability to transform NIH3T3. Somehow, the Ras gene of these cells is malignantly activated. The sequence of the transforming Ras genes from the tumor can be compared to non-transforming Ras genes from normal human cells. Each transforming Ras gene harbors a point mutation that yields an amino acid substitution at residue 12, 13 or 61. The mutation of the same residues is also observed in retrovirally transduced Ras oncogenes.

• **RAS SIGNALING PATHWAY:**

EGFR → SOS → RAS → RAF → ERK → JUN → MYC → CYCLIN D

Ras is part of the mitogenic signaling pathway. A lot of oncogenes discovered using retrovirus analysis fit on this pathway. These include the oncogene of the EGF receptor (ERB-b), mutated forms of RAS and RAF, and retroviruses that activate the JUN gene. Normal activation of a dormant cell with a ligand can activate the EGF receptor and stimulate signaling in the cell to up-regulate MYC levels, which up-regulate Cyclin D and begin the cascade of gene expression that allows S phase entry of the cell. Functionally, a lot of oncogenes are activating the mitogenic pathway in a way that causes cells to no longer need to respond to an extra cellular cue. K-Ras mutations are the most common in human tumors (50% of sporadic colorectal cancers and sporadic adenomas). These mutations are not inherited but are acquired during the cell's life. These mutations affect only one allele (they are dominant) and inactivate the GTPase activity of Ras.

(see figure on page 181 in text) Inactive Ras binds GDP. When a growth factor binds the receptor, Ras is induced to expunge the GDP and bind GTP. RAS then becomes active and catalyzes the removal of the terminal phosphate of GTP → GDP. When Ras is mutated, the GTP can be loaded onto the protein, but Ras is no longer catalytic. Therefore, Ras is stuck in its active form in a complex with GTP and is continually signaling the cell to stimulate cell cycle progression.

• **TRANSLOCATIONS:**

Translocations are another important oncogenic event. Translocations can occur in a variety of sites and can activate chromosomes. These translocations are typically reciprocal between two chromosomes. An example is the (9:22)(q34;q11) translocation, resulting in two non-germ-line chromosomes, of which one is the "Philadelphia chromosome" that is associated with chronic myelogenous leukemia. When the break point of this new chromosome was cloned and isolated, the BCR-ABL gene was identified. This new fusion gene had the transcriptional promoter of the BCR gene. The ABL gene is a non-receptor protein tyrosine kinase. Phosphorylation of ABL substrates promotes cell growth. The enzymatic activity of normal ABL is tightly controlled. The fusion retains the tyrosine kinase activity of ABL, but the enzyme activity is deregulated such that the enzyme is always active and constitutively promotes cell growth. BCR-ABL is a malignantly activated form of the ABL proto-oncogene. ABL had also been isolated as a retroviral oncogene in animals. This fusion protein can infect animals to cause a CML like disease.

- **OTHER CLASSES OF ONCOGENES IMPORTANT IN CANCER:**

- **Epidermal Growth Factor Receptor:**

Normally this receptor is silent. The EGF ligand induces dimerization and signaling into the cell. Erb-B is the retroviral oncogene of this receptor and has been found to be amplified up to 50 fold in human cancers. (Amplification is the process during tumor growth in which multiple copies of the genome are repeated with in a single locus or multiple little mini-chromosomes accumulate.) This process results in fifty or more copies of the gene in the tumor, resulting in the production of a lot of EGF receptor that is sufficient to stimulate cell growth. This phenomenon often occurs in combination with the mutation of the receptor into an active form that no longer requires the binding of ligand to stimulate the signaling cascade. This process is common in glioblastoma.

- **MYC**

The MYC oncogene was originally isolated as part of an avian retrovirus. MYC can also be activated, however, by pro-viral integration of a retrovirus that activates MYC transcription. When the immunoglobulin enhancer is placed near the MYC gene via chromosomal translocation, MYC transcription is turned on and over-expressed and Burkitt's lymphoma results.

- **AKT**

AKT was identified from a mouse retrovirus that causes T-Cell lymphoma. AKT is a serine-threonine kinase that regulates apoptotic proliferation, cell size, and angiogenic pathways. Amplification of this gene occurs in human ovarian and pancreatic cancer. This kinase is normally silent. When it is a retrovirus, the kinase is turned on and tethered to the membrane constitutively.

- **ONCOGENES WITH OUT RETROVIRAL COUNTERPARTS:**

- **Cyclin D-1:** Cyclin D-1 is critical in normal cells for cell cycle regulation (associates with CDK4 and CDK6 to regulate entry into S phase). Cyclin D-1 is activated by amplification in breast cancer and is over expressed by translocation in parathyroid cancer.

- **MDM 2:** MDM 2 is amplified in sarcoma and inactivates the P53 tumor suppressor.

- **BCL 2:** BCL 2 regulates apoptosis in the mitochondria. BCL 2 is activated by immunoglobulin enhancer due to translocation and accounts for the majority of follicular lymphomas.

- **MECHANISMS OF ONCOGENE ACTIVATION**

- **POINT MUTATIONS:** can affect the regulation of an oncoprotein ex. Ras

- **TRANSLOCATION:** can affect the amount of an oncoprotein (over-expression) ex. MYC or BCL 2 or can generate a novel fusion protein ex. BCL-ABL

- **AMPLIFICATION:** multiple copies of a normal sequence ex. Cyclin D-1 or EGF Receptor (mutation in combination with amplification)

- **DNA TUMOR VIRUSES:**

DNA tumor viruses are a diverse class of viruses, including Human Papilloma Viruses, Adenoviruses, Polyoma Viruses (rodent), and SV40 (monkey). These viruses are typically lytic viruses that stimulate the cell into S phase and suppress apoptosis in order to replicate. The virus needs all the equipment and machinery of a dividing cell to replicate itself and generate many copies of its genome. Therefore, if these viruses encounter a host in which they are unable to replicate, the virus has simply caused a host cell to enter S phase and become resistant to apoptosis. In general, DNA tumor virus oncogenes inactivate tumor suppressors. The SV 40 large T antigen inactivates Rb and P53 by binding them and sequestering them from their normal function. HPV E6 inactivates a P53, and HPV E7 inactivates Rb. Adenovirus E1A inactivates Rb, and Adenovirus E1B inactivates P53. These different viruses have evolved the same mechanism to stimulate the cell to become a tumor. One of the few examples in which the tumor virus proteins actually stimulate a tumor pathway directly, rather than inactivating a suppressor, is Polyoma Middle T Antigen. This tumor virus acts as an oncogene in its own right by activating PI3 Kinase and Src.

➤ **THE BIG PICTURE:**

This is the pathway that is affected by DNA/RNA retroviruses and their oncogenes to cause cancer...

MITOGENS (EGF receptor and signaling molecules)



CYCLIN-D induction



CYCLIN-D + CDK 4/CDK 6 (kinase complex)



Rb tumor suppressor (phosphorylated)

(When not phosphorylated Rb sequesters the E2F transcription factor and keeps the cell from entering the cell cycle.)



E2F transcription factor (released)



turns on genes like **CYCLIN-E**



cell enters **S PHASE**