The phenomenology of malignant transformation, invasion and metastasis

Review of two papers: Hanahan & Weinberg, (2000), Cell V100: 57-70; ibid. (2011) Cell V144: 646:674

Cancer is a disease which proceeds to malignant transformation and metastasis by inducing dynamic changes in the genome. Since 1970, research has revealed much about which changes in cellular and intra-tissue homeostatic mechanisms result in such a transformation.

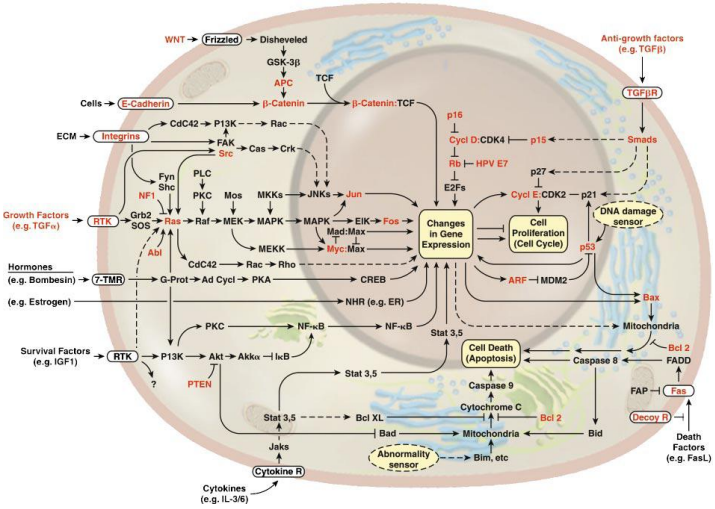
90% of deaths from cancer occur due to metastasis, so whereas it is necessary to understand disease initiation and establishment in-situ, it is also important to understand how metastasis occurs. The reason metastasis kills is that most therapeutic interventions post-metastasis usually fail.

The goal of these two seminal papers was to argue that the complexities of cancer can be understood in terms of a small number of underlying mechanisms.

Why is such a simplification possible at all? Mammalian cells carry a molecular machinery that carefully regulates the proliferation, differentiation and death of cells. There is evidence that this machinery is compromised in cancer and that tumors evolve using a process that resembles Darwinian evolution. Tumorigenesis seems to be a multistep process, the risk of cancer has an age dependence which suggests that 4-6 stochastic events are necessary, specific alterations are seen in many transformed cells and mouse models, pathological evaluation reveals lesions representing intermediate stages in tumorigenesis etc.

Questions:

- How many and which circuits in each cell type need to be disrupted to get cancer?
- Are the same circuits involved in all cancers?
- Are the circuits autonomous to the cell or are they coupled to signals in the microenvironment?
- Do the changes have to be acquired in a specific sequence?



The H&W thesis is that the processes responsible for the malignant phenotype are:

- *Self Sufficiency in growth signals*
- *Insensitivity to growth inhibitory signals*
- *Evasion of apoptosis*
- *Limitless replicative potential*
- *Sustained Angiogenesis*
- *Tissue Invasion and Metastasis*

We will discuss these one at a time

Self Sufficiency in growth signals:

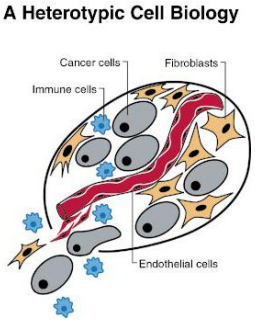
Normal cells die in the absence of growth signals. They also need growth signals to move from quiescence to mitosis. These signals are transduced into cells by trans-membrane receptors with specific functions. The

receptors respond to: diffusible growth factors (GFs), extracellular matrix components, cell-to-cell adhesion molecules.

- Normal cells in culture (eg. Fibroblasts) proliferate only if supplied with mitogenic (growth) factors and a substrate. Normal fibroblasts stop growing in-vitro when they come into contact with the walls of the dish (contact inhibition).
- Tumor cells show a reduced dependence on mitogens. They also lose contact inhibition and keep growing independent of contacts with the edges of the dish, often forming elevated foci.

How do tumor cells do this?

- *Tumor cells often synthesize GFs to which they themselves are responsive*. For example, Glioblastomas and Sarcomas produce PDGF (platelet derived growth factor) and TGFα (Tumor growth factor alpha).
- *Tumors alter cell surface receptors that transduce proliferation signals.*
    - Overexpress such receptors to become hypersensitive to GFs (EGF-R/erBb is overexpressed in stomach and brain cancers)
    - Induce structural changes in receptors to induce intra-cellular proliferative signals: EGF receptor truncates its cytoplasmic domain to fire its growth signals constitutively, independent of GFs.
    - Switch the type of extracellular matrix receptor (integrin) to favor those that transmit growth signals. This often involves activation of the SOS-RAS-Raf-Map kinase pathway. 25% tumors have a RAS mutation – ½ of colon cancers have it and the rest may have other mutations that induce a phenocopy of the RAS mutation.
- *Recruit ancillary cells to provide growth signals*. In normal tissue, cells are instructed to grow by their neighbors (paracrine signals) or via systemic (endocrine) signals (eg. From blood vessels). These signals are compromised in tumors. Many tumors seem to grow and invade by inducing growth signals by co-opting stromal cells or by recruiting inflammatory and immune cells (which induce angiogenesis).



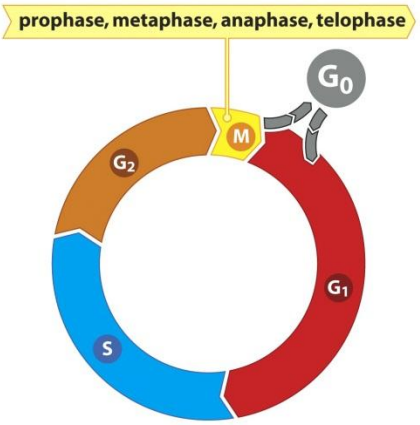
NEW (2011):

- *Somatic mutations activate additional pathways:* For example 40% of melanomas contain activating mutations affecting the B-Raf protein, causing constitutive proliferating signaling to mitogen activated protein (MAP) Kinase pathway.
- *Disruptions of Negative Feedback that inhibit proliferation:* PTEN is a tumor suppressor which regulates PI3K signaling by degrading its product PIP_3. Loss of function in PTEN often occurs via promoter methylation.
- *Excessive Proliferative Signaling can trigger senescence:* Excessive signaling by onco-proteins like RAS, MYC and RAF can induce cell senescence or trigger apoptosis. The intensity of oncogene activity in cells may be a compromise between stimulation and anti-proliferative defenses.

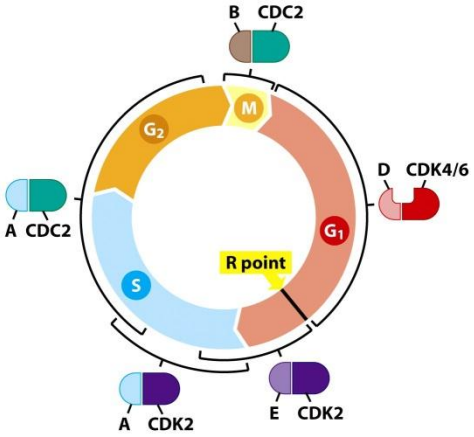
<u>Insensitivity to Anti-growth signals:</u>

Anti-growth signals maintain quiescence and homeostasis. The signals involve both soluble growth inhibitors and immobilized inhibitors in the extra-cellular matrix (ECM). The effort to understand them is difficult and nascent because it is like inferring the presence of the Cheshire Cat in “Alice In Wonderland” by looking for its smile, but without the smile.

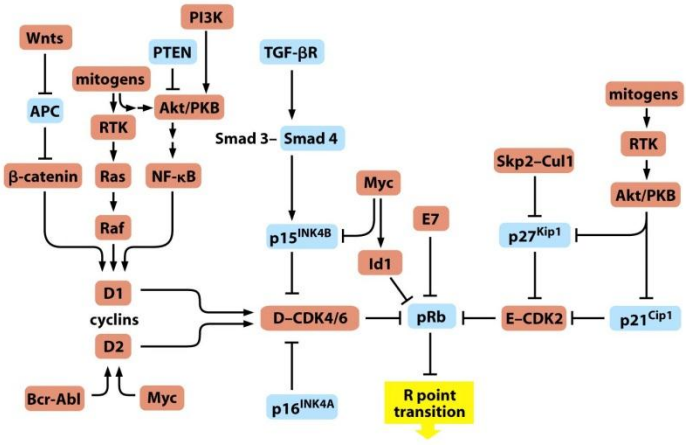
Anti-growth signals can block proliferation in two ways. They can transition the cell to G_0 phase (reversible) or cause the cell to enter a post-mitotic state (irreversible) such as in hair, rods/cones, heart muscle, neurons.



- In G_1 phase, cells monitor their environment to determine whether to proliferate, go quiescent or become post-mitotic. The key gene involved in this go-nogo decision is pRb (and to a lesser extent its homologues p107 and p130). The pRb gene regulates the so called R-checkpoint, which is the point in G_1 phase beyond which entry into S phase cannot be inhibited.
- The cell cycle is regulated by a "clock" (which keeps time). This clock is created by a dynamic regulatory system involving pocket proteins, the pRb gene, Cyclins and Cyclin-dependent kinases. These determine determines entry into and progress through cell cycle.
    - At the beginning of G1 phase the pRb gene is hypo-phosphorylated. In this state it is bound to E2F and recruits histone deacytylases, which inhibit transcription.
    - *Cyclin D's levels are regulated by mitotic signals:* In response to mitotic signals, Cyclin D–CDK4/6 complexes are produced, which sequester pocket proteins p27, p21 to liberate Cyclin E-CDK2 complexes, which then continue phosphorylation of pRb.
    - Other Cyclins and Cyclin dependent kinases complete the hyper-phosphorylation of pRb.
    - Hyper-phosphorylated pRb unbinds from E2F, which then recruits histone acytylases, which in turn recruit the transcription machinery from genes which cause the cell to irreversibly enter cell cycle.



    - At the end of mitosis (beginning of next cell-cycle), pRb is stripped of its phosphate groups (again hypo-phosphorelated).
- Other players:
    - TGF-β (tumor suppressor) elevates p15 which blocks formation of D-CDK4/6 complexes
    - The viral onco-protein E7 (from HPV) can directly binds to pRb to inactivate it.
    - In Retinoblastomas and Sarcomas, pRb is lost through mutation and LOH.
    - In breast cancer, pRb function is lost through overexpression of Cyclin D1.
    - In Melanoma, methylation of p16 turns off this gene, which is a regulator of CDK4/6 levels.

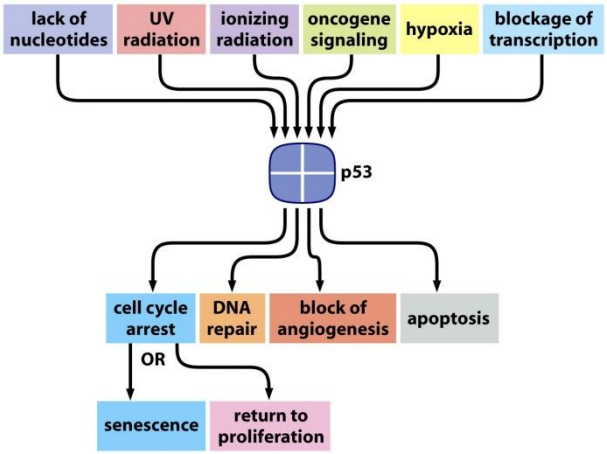


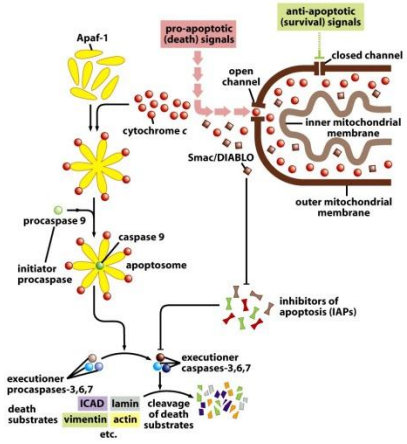
NEW (2011):

- *pRb knockout mice:* Chimeric mice whose cells lack the pRb gene are free of proliferative abnormalities, normal tissue morphogenesis and only develop pituitary tumors late in life.
- *P53 knockout mice:* Develop normally, show proper tissue homeostasis and only develop lymphomas and sarcomas late in life.
- *What does this mean?***:** It must mean that the genome contains redundantly acting mechanisms which constrain inappropriate replication and regulate cells even in the absence of these proliferation suppressors.

- *Mechanisms of contact inhibition:* The gene NF2 is a tumor suppressor whose loss triggers neurofibromatosis. Its product (called Merlin) mediates signaling between E-Cadherin and EGF to ensure that cells maintain contact inhibition.
- *Corruption of the TGF-β pathway promotes malignancy via an EMT transition:* In late stage tumors, TGF-β signaling is redirected to activate a cellular program that causes an Epithelial-Mesenchymal transition.

Evasion of Apoptosis (= programmed cell death):





The apoptotic program is present in latent form in every cell of multi-cell Eukaryotes (in fact, the default state of the cell is dead). ***One of the regulators of the apoptotic pathway*** is the gene p53, which integrates a variety of stress signals and decides on a number of possible outcomes (see the picture on left). One of these choices is programmed cell death or apoptosis, which is initiated by the release of cytochrome-c from the mitochondrial membrane, leading to a caspase cascade which in 30-120 minutes leads to disruption of the cellular membrane, breakdown of cytoplasmic and nuclear skeletons, extrusion of the cytosol, degradation of chromosomes and nuclear fragmentation. The immune system is not involved (and not alarmed) and the debris is cleaned up in ~ 24 hours.

The Apoptosis machinery consists of two distinct components:

- The Sensors: These sense the extracellular environment to decide whether the cell should live or die. Survival signals are transduced by receptors such as IGF-1, IGF-2, IL-3R etc. Death signals are transduced by receptors such as FAS, TNF-α, TNF-R1. Intracellular p53 signals to the Bcl-2 family of genes: tumor suppressors Bax, Bak, Bid, Bim which are pro-apoptotic and oncogenes Bcl-2, Bcl-XL, Bcl-W etc which are anti-apoptotic.
- The Effectors such as Apaf1, Cytochrome-c, Caspases, which initiate and complete the suicide program.

How do tumors evade this?

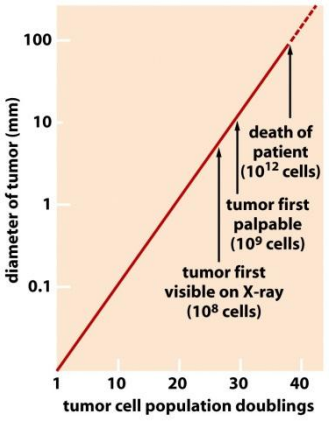
- Disable p53. The TP53 protein functions primarily as a transcription factor and is biologically active as a homotetramer, comprising 4 × 393 amino acid residues. As a result, one mutation reduces its efficacy to 1/16. Many tumors do not like even this reduced efficiency of TP53– preferring to abrogate its effect completely by LOH of the functional allele.
- Overexpress the Bcl-2 family of oncogenes
- Overexpress Myc (but not too much – since too much Myc overexpression seems to trigger apoptosis)
- Activate PI3K→AKT/PKB survival pathway by inducing extracellular factors IGF-1/2, IL-3 or extracellular signaling from RAS or by loss of PTEN

Hopeful direction for the future: Redundant apoptotic pathways seem to remain even after p53 is knocked out. If one can identify what their biology is, they might suggest novel interventions.

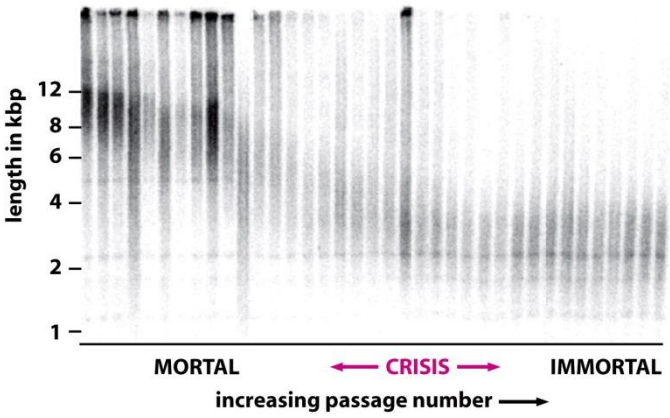
New (2011):

- *Autophagy mediates both tumor survival and death:* Autophagy is a cellular program operating at low levels in normal cells which recycles catabolites for biosynthesis and metabolism in times of nutrition deficiency. It is anti-apoptotic. Survival signals from mTOR, PI3K and AKT that block apoptosis also block autophagy. Loss of survival signaling (eg. low levels of PI3K signaling) can induce autophagy. The key player in autophagy seems to be Beclin-1, which seems to be necessary to induce autophagy. Nutrition starvation, radiotherapy and some cytotoxic drugs seem to increase autophagy and are cytoprotective for cancer cells, impairing the killing action of these stress inducing situations and sometimes sending these cells into dormancy. Thus autophagy seems to have conflicting effects on tumor cells and tumor growth.
- *Necrosis has pro-inflammatory and tumor-promoting potential:* Necrosis or cell death causes the cells to become bloated and explode. It seems to be under some level of genetic control, rather than a random event. Necrosis seems to trigger an immune response (in contrast to autophagy and apoptosis which do not). The normal function of this response seems to be to clean up the debris. However, in tumor cells the immune response elicits angiogenesis, proliferation and invasiveness. Hence tumor cells may deliberately permit some level of necrosis in order to recruit GF signaling into its vicinity.

Limitless replicative potential:

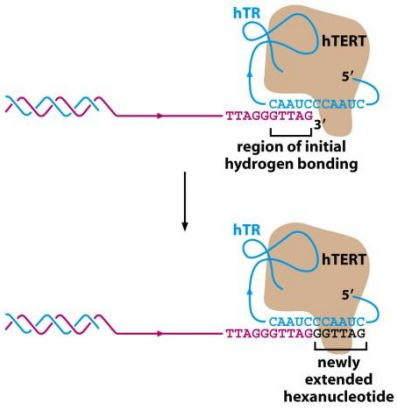


- The work of Hayflick showed that cells have finite replicative potential. There seem to be two phases. After ~ 50-60 doublings, cells seem to stop cycling but are otherwise normal (have normal karyotype). If induced to keep dividing beyond this stage (by circumventing TGFs, p53, pRb etc), they continue to divide for another 20-30 cycles until they reach a state called "crisis", characterized by chromosomal chaos and massive cell death (even without p53). Some cells may emerge from crisis to become immortal, and are then capable of limitless mitotic cycles. All cells in culture are immortal.
- 40-50 doublings would result in $2^{50} – 2^{60}$ cells ~ 10^{15} - 10^{18} cells. The human body contains ~ 10^{13}-10^{14} cells so this number of doublings is sufficient to create a very large tumor. However, tumors are much smaller than this. This suggests that the size of a tumor is NOT necessarily an indication of its age – because a tumor goes through massive attrition/death before becoming established. Tumors seem to stabilize the state of "crisis" that causes chaos and death of normal cells induced to proliferate beyond 50-60 doublings.



- Crisis seems to be due to telomere shortening. Telomeres are repeat sequences at the ends of chromosomes which keep the ends from fraying, much like an end-knot in a rope. In vertebrates the telomere sequence is a 6-bp TTAGGG sequence (in insects it is TTAGG and in higher plants such as Arabidopsis Thaliana it is TTTAGGG). Human telomere lengths vary by chromosome (the bigger the chromosome the greater the number of repeats) but generally range from 7000-10,000 TTAGGG repeats. 50-100 repeats are lost in each cell division. Crisis seems to ensue when the number of repeats falls below ~ 3000-4000.

How do tumors evade crisis? The cells which evade crisis turn on Telomerase. Telomerase is generally not-expressed in somatic cells. However, it is present in embryogenesis and expressed in stem cells, whose telomeres shorten less than 50-100 repeat lengths/division. Telomerase is an enzyme (a ribonucleoprotein) which can add telomere repeats at the ends of chromosomes. It consists of a protein hTERT (human telomerase reverse transcriptase) and an RNA unit call hTR (or hTERC) which carries the reverse template (3'-CAAUCCCAAUC-5') used by hTERT to insert the TTAGGG repeats.



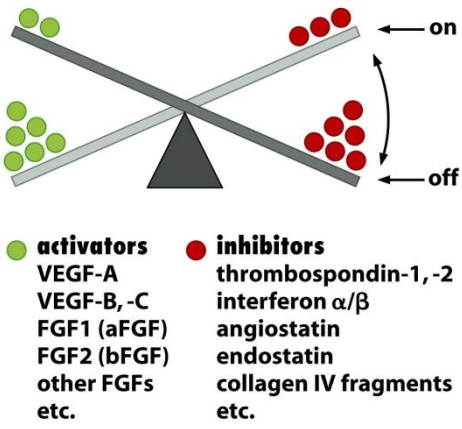
Can the senescence phenotype be induced in tumor cells? Maybe. The problem is that it is unclear whether senescence is a real state of cells in-vivo or whether it is an artificial state of cells in-vitro.

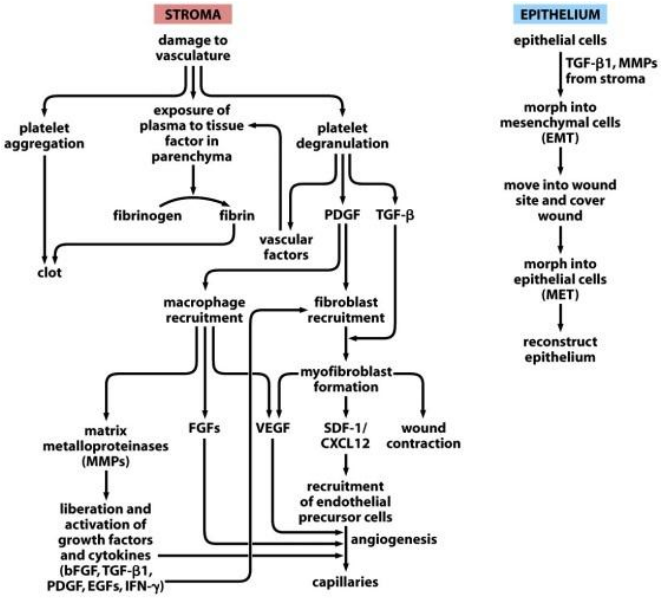
New (2011):

- *Reassessing replicative senescence:* Induction of senescence in cultured cells can be delayed and probably eliminated by the use of improved cell cultures, suggesting that induced senescence in-vitro may be an artifact. However, mice lacking telomerase have shortened telomeres and can shunt premalignant cells into a senescent state with reduced oncogenic potential (of course they have other problems – such as multi-organ failure, early onset of global senescence etc).
- *Delayed activation of telomerase may both limit and foster neoplastic progression:* The development of human neoplasms may be aborted by telomere induced crisis long before they become macroscopic lesions. However, in the absence of TP53, shortened telomeres often lead to karyotype chaos via breakage-fusion-breakage (BFB) cycles which can lead to a malignant phenotype. However, tumors often seem to turn on telomerase later in the development of a tumor, presumably to stabilize a karyotype that is more likely to progress to malignancy (much like selection for a beneficial trait).
- *New functions for telomerase:* Telomerase seems to have functions unrelated to telomere maintainence. TERT seems to amplify signaling by the Wnt pathway by serving as a cofactor in the β-catenin pathway, increse cell proliferation, induce resistance to apoptosis, is involved in DNA damage repair and in RNA-dependent polymerase function.

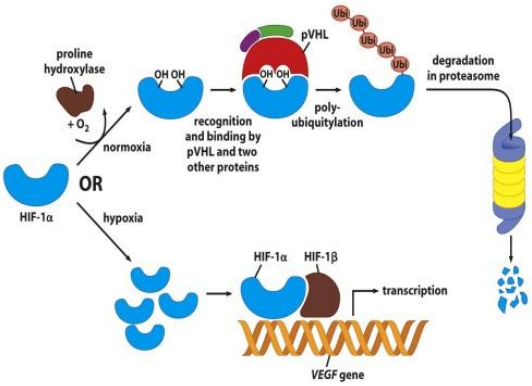
<u>Sustained Angiogenesis (=blood vessel growth):</u>

All cells must live within 100 µm of a cappillary. Cells within a growing tumor initially lack angiogenic capability. They must turn it on to grow beyond a certain size. Two key pathway available to them for this purpose are the VEGF (Vascular Endothelial Growth Factor) and the FGF1/FGF2 (Fibroblast Growth Factor) pathways. These growth factors bind to specific tyrosene-kinase receptors on endothelial cells to promote their proliferation. The shift to Angiogenesis seems to be subtle,





involving regulatory changes in gene levels rather than an on/off switch (see diagram). In most cases, the angiogenic pathway is regulated by shifts in the expression of several genes. However, in some cases, it is an on/off switch. For example, in kidney cancer, the VHL gene is mutated. In concert with HIF1- α, VHL regulates the angiogenic switch induced when conditions change from normoxia to hypoxia. Loss of VHL frees the tumor from this regulation, allowing angiogenesis free reign in the absence of hypoxia. Several VEGF pathway inhibitors are in late stage clinical trials (in 2000).



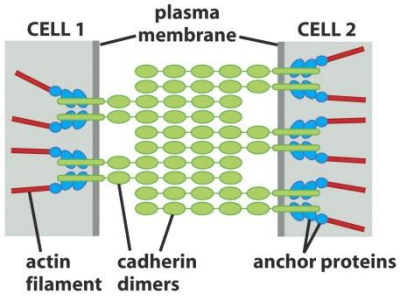
New (2011):

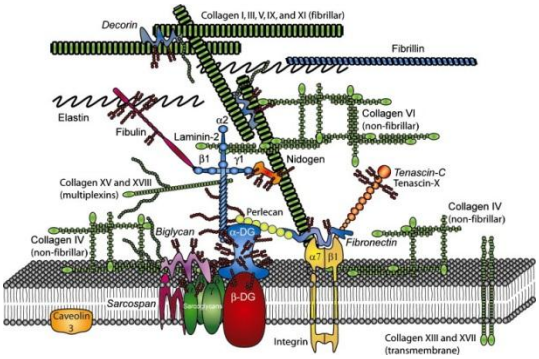
- *Blood Vessels in tumors are aberrant:* Tumor microinvasion blood vessels are typically leaky, have precocious cappilliary sprouting, convoluted and excessive branching, distorted vessels, erratic blood flow, abnormal levels of endothelial cells and apoptosis.
- *Angiogenesis is induced early in tumors:* Histological analysis of premalignant tumors suggests early tripping of the angiogenic switch.
- *Heterogenetity of angiogenesis across tumor types:* Some tumors (eg pancreatic adenocarcinomas) are hypovascularized and have large stromal deserts. Other tumors (eg renal, pancreatic neuroendocrinal) are densely vascularized.
- *Pericytes are important components of tumor vasculature:* Pericytes are cells apposed to the outer surface of endothelial tubes in normal tissue. Tumor vessels lack appreciable coverage of these cells, making them leakier. Leaking vessels may help metastatis. However, modeling has shown that pericytes are important to maintain tumor neovasculature.
- *Bone marrow cells contribute to tumor angiogenesis:* Macrophages, neutrophils, mast cells and myeloid progenitor cells infiltrating tumors can trip the angiogenic switch. They also protect the vasculature from the effects of drugs targeting endothelial signaling.

Tumor Invasion and Metastasis:

Key molecules whose functions must be compromised in tumor invasion are the ECM molecules such as E-Cadherin, Laminin, Collagen etc. The normal signaling function of these cells is to transmit non-proliferative signals via cytoplasmic contacts with genes such as β-catenin to intra-cellular circuits.

E-Cadherin dimers creat cell to cell anchors between cells in epithelial surfaces and with the basement membrane. In the majority of epithelial tumors, E-Cadherin junctions are lost either by mutation, inactivation of β-catenin, transcriptional repression, or the proteolysis of the external E-Cadherin domain. Mouse models show that induced expression of E-Cadherin impairs invasion and the metastatic phenotype.





Integrins are receptors that mediate the attachments between a cell and the tissue that surrounds it. They pass messages to the cell about the ECM matrix surrounding it. They are hetero-dimers made from two distinct chains (α and β). In humans, there are 18 α type and 8 β type integrins (ITGA1, ITGA2,…, ITGA18 and ITGB1, ITGB2…, ITGB8). These also have other confusing names such as LFA-1, MAC-1, MFI7 for ITGB2 and

VNRA, MSK8, ITGAV for ITGA16. *Metastatic tumor cells alter the integrins they use to those which are adapted to the microenvironment at the metastatic site. The large number of integrins confounds the efforts of the modeling community to explain the cell biological effects of integrins in terms of a small number of mechanistic rules.*

Extracellular Proteases. These are a complex and heterogeneous superfamily of enzymes which play key role in many pathophysiologic processes including cancer, inflammatory diseases, and cardiovascular conditions such as atherosclerosis and restenosis. Whereas matrix metalloproteinases are their best known members, many others are becoming better known. They include metalloproteinases (matrix metalloproteinases, adamalysins, or pappalysins), serine proteases (elastase, coagulation factors, plasmin, tissue plasminogen activator, urokinase plasminogen activator), and cysteine proteases (such cathepsins). In addition to their matrix degradation capabilities, they have other, less well known biologic functions that include angiogenesis, growth factor bioavailability, cytokine modulation, receptor shedding, enhancing cell migration, proliferation, invasion, and apoptosis. In normal tissue they perform a variety of functions, such as breaking down food to allow digestion, create blood clotting cascases etc. They are turned on/off by tumors to degrade the ECM to facilitate invasion or to create barriers against normal cells. Sometimes, the proteases used by tumors are conscripted from surrounding stromal and inflammatory cells.

New (2011):

- *An EMT program broadly regulates invasion and metastasis:* This seems to happen through a developmental program which is referred to as the "epithelial-mesenchymal transition" whereby transformed epithelial cells acquire the ability to invade, resist apoptosis and disseminate broadly. This program is activated by co-opting steps in embryonic morphogenesis and wond healing and can be activated transiently or stably. A number of transcription factors seem to be involved in the EMT program, such as Snail, Slug, Twist and Zeb1/2. They are associated with supression/loss of adhesion junctions, conversion from a polygonal epithelial shape to a spindly fibroblastic shape, increased motility and resistance to apoptosis, expression of matrix degrading enzymes. Often cells at the periphery of primary tumors seem to transform, suggesting that the EMT phenotype involves signals from the tumor microenvironment. It seems that EMT can induce all changes necessary for metastasis *EXCEPT* colonization.
- *Stromal cells are involved in invasion and metastasis:* Mesenchymal stem cells (MSCs) in stroma secrete CCL5/RANTES in response to signalling from cancer cells, which then acts reciprocally on the tumor cells to stimulate invasion. Macrophages in the tumor periphery supply metalloproteins and cystein-cathepsin proteases to further stimulate invasion. Studies suggest that high grade malignancy does not arise in a strictly cell-autonomous manner.
- *Distinct forms of invasion may underlie different cancer types:* Invasion seems to be of at least two types: Collective invasion (rately metastatic), where nodules of cancer advance en masse into adjacent tissue (squamous cell carcinomas) or "Amoeboid" invasion, where the tumor cells slither through existing interstices in the ECM. It is not clear what the difference in biological programs of these two invasive forms is.
- *Metastatic colonization is a complex process:* Tumors must invade and adapt to successfully metastatize. Adaptation of tumor cells in foreign tissue microenvironments is necessary for successful colonization. Many patients have micrometastases which never progress to macrometastic tumors. Some tumors release systemic suppressors that render them dormant, as evidenced by their explosive growth after resection of the primary. Others (breast cancer and melanoma) may persistently attempt to colonize and succeed many years after micrometastasis is first established.
- *Nutrient starvation and anti-growth signals can induce dormancy:* An intense autophagic response to nutrition starvation can make tumors go dormant. Such cells can resume proliferation when the tissue

microenvironment allows access to nutrients. ECM induced anti-growth signals can also induce a dormant tumor state.

2000: Emerging Hallmark:

- *Genomic instability:* Editing and repair mechanisms ensure that mutations are rare events. Regulatory processes such as cell cycle arrest, DNA repair and apoptosis ensure that even these rare events do not upset tissue homeostasis. Loeb has argued that these mechanisms are so good that tumor cells must acquire increased mutability to create a tumor in 5-6 decades. Genomic instability is one general process by which such an increased mutation rate is achievable. This is achieved by alterations in the DNA repair programs – mutations in genes such as BRCA1, BRCA2, 53BP1 etc as well as by loss of p53 function. Other mechanisms also exist – such as telomere shortening and synthesis of abberant DNA in cells which phagocytose the residue of apoptotic and necrotic cells.

2011: Emerging Hallmarks:

- *Genomic Instability and mutation*
- *Tumor promoting inflammation*
- *Reprogramming energy metabolism*
- *Evading immune destruction*
- *Cancer Stem Cells (CSC):* These are defined operationally through their ability to seed new tumors upon inoculation into host mice and expression of stem cell specific markers found on normal stem cells.

Closing Comments from H&W (2000)

Two decades from now (i.e. in 2020), *"it will be possible to lay out the complete "integrated circuit of the cell" upon its current outline. We will then be able to apply the tools of mathematical modeling to explain how specific genetic lesions serve to reprogram the integrated circuit in each of the constituent cell types so as to manifest cancer. With holistic clarity of mechanism, cancer prognosis will become a rational science, unrecognizable by current practitioners. It will be possible to understand with precision how and why treatment regimes and specific antitumor drugs succeed or fail."*

"One day, we imagine that cancer biology and treatment – at present a patchwork quilt of cell biology, genetics, histopathology, biochemistry, immunology and pharmacology – will become a science with a conceptual structure and logical coherence that rivals that of chemistry or physics."

Closing Comments from H&W(2011)

The following are likely to play an important role in our understanding of cancer going forward:

- *Aerobic glycolysis*
- *Chromatin in control of transcription*
- *Epigenetic regulation*
- *MicroRNA*
- *Circuit diagram of heterotic cell-cell interaction*
-