The Fourth Comprehensive Cancer Research Training Program (CCRTP) at Stanford University

presented by
Stanford Cancer Center
Stanford University School of Medicine

Course Directors:

Amato Giaccia, Ph.D.
Ginna Laport, M.D.
Karl Blume, M.D.

Quadrus Conference Center

2400 Sand Hill Road
Menlo Park, California

September 13-17, 2010

STANFORD
CANCER CENTER

4th Annual

Comprehensive Cancer Research Training Program At Stanford University

Monday, September 13 – Friday, September 17, 2010

at Quadrus Conference Center

Menlo Park, CA

Presented by

Stanford University School of Medicine
Philip A. Pizzo, MD, Dean

Stanford Cancer Center
Beverly S. Mitchell, MD, Director

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The 2010 Comprehensive Cancer Research

Course Overview

In September 2007, 2008, and 2009 Stanford Cancer Center hosted the first three annual Comprehensive Cancer Research Training Program (CCRTP) events. This educational initiative was exceedingly well received. Selected responses from trainees who attended the first three courses are included in this brochure.

The Stanford Cancer Center invites graduate students, post-doctoral fellows, residents, clinical fellows, and research fellows from Stanford University, the University of California at San Francisco (UCSF), and San Francisco State University (SFSU) to attend the fourth annual CCRTP. In particular, trainees from the respective Schools of Medicine, Schools of Engineering, and the Schools of Humanities and Sciences from the three universities are encouraged to participate. The Stanford Cancer Center will host this course Monday, September 13 through Friday, September 17, 2010, at the Quadrus Conference Center at 2400 Sand Hill Road in Menlo Park, California.

This dynamic, educational event features a daily plenary session on a topic of general interest followed by nine half-day sessions on two scientific themes, each with three to four presentations. Nationally and internationally renowned faculty from Stanford University and UCSF will present 30 didactic lectures on current topics of basic, translational, and clinical cancer research. Each didactic lecture is followed immediately by a discussion.

Registered trainees are invited to present their own research posters on September 14, 2010. Trainee research poster submissions will be reviewed and selected by the three course directors. A total of 20 posters will be selected. In addition, a poster session featuring the core services that support cancer research at Stanford University will be presented on September 15, 2010.

Three months in advance of the course, registered trainees will receive a printed course syllabus in the mail. The syllabus will provide an illustrated summary and reading list for each lecture. An electronic syllabus featuring color illustrations will be available on the CCRTP web site. Trainees are encouraged to use this syllabus to prepare for active participation during lecture discussions.

During each lecture, the speaker will present two to three multiple-choice questions. Trainees will answer these questions anonymously using an audience-response system. The trainee responses will assist the lecturer to emphasize key issues related to the presentation topic and will guide the post-lecture discussion. This approach is expected to further increase the interaction between trainees and faculty.

Evaluation

After the course, the directors will send a limited number of questions to the participants allowing formative and summative evaluations of the course. These evaluations will be mailed electronically to the course participants at three time points: two weeks, three months, and nine months after completion of the course. Participants are strongly encouraged to provide feedback to the course directors.

Registration

Attendance is limited to 120 participants. Early registration is encouraged. Attendees may register by mail or fax until June 21, 2010. A registration fee of $75.00 is required and may be paid by check or department transfer. Complete registration instructions are located on the registration form in this brochure.

Registered participants will receive a copy of the syllabus three months prior to the course and have access to all lectures, daily breakfast and lunch, and enjoy the reception at the end of the first day of the course. Each trainee in attendance will receive a certificate of participation at the end of the course, Friday, September 17, 2010.

Tuition

No tuition fees will be charged. In order for trainees to fully participate in the course, their Laboratory Directors, Division Chiefs, and Department Chairs are asked to provide the trainees with free educational time without clinical responsibilities for the week of September 13 through September 17, 2010. In turn, the organizers expect that the CCRTP trainees will attend the event in its entirety.

Housing

Trainees traveling a long distance may choose to stay at the housing facility of the Stanford Linear Accelerator that is located across the street from the Quadrus Conference Center. The cost per night is approximately $100.00. This expense must be covered by the trainee or his/her department.

Monday, September 13, 2010

7:45am **Breakfast**
8:15am **Introduction into the Course** *Karl Blume, M.D.*
8:30am **Plenary Lecture 1: Systems Biology of Cancer** *Sylvia Plevritis, Ph.D.*

STEM CELL RESEARCH

9:25am **Control of Proliferation and Differentiation of Stem Cell Lineages** *Margaret Fuller, Ph.D.*
10:15am **Coffee Break**
10:35am **Self-renewal of Normal and Cancer Stem Cells** *Michael F. Clarke, M.D.*
11:25am **Radioresistance of Cancer Stem Cells** *Maximilian Diehn, M.D., Ph.D.*
12:15pm **Lunch**

CELLULAR THERAPY OF CANCER

1:15pm **Biology and Use of Hematopoietic Cell Transplantation** *Robert Lowsky, M.D.*
2:05pm **Cellular Immunotherapy** *Jonathan Benjamin, M.D., Ph.D.*
2:55pm **Dendritic Cell Therapy in Cancer** *Edgar Engleman, M.D.*
3:45pm **Reception**
Adjourn

Training Program at Stanford University

Tuesday, September 14, 2010

7:45am Breakfast
8:30am Plenary Lecture 2: Genomics and Proteomics of Cancer *Frank McCormick, Ph.D.*

PHARMACOGENOMICS/MOLECULAR THERAPEUTICS

9:25am Cancer Pharmacogenomics *Russ Altman, M.D., Ph.D.*
10:15am Coffee Break
10:35am Anti-angiogenic Therapies *Calvin Kuo, M.D.*
11:25am Molecular Therapies Targeting Oncogenes *Dean Felsher, M.D., Ph.D.*
12:15pm Lunch
1:00pm **Poster Session I: Presentation of Research Posters by CCRTP Trainees**

CANCER BIOLOGY

2:15pm Wnt-signaling and Cancer *Roeland Nusse, Ph.D.*
3:05pm Gene Regulation and Cancer *Howard Chang, M.D.*
3:55pm Engineering Protein-based Cancer Diagnostics and Therapeutics *Jennifer Cochran, Ph.D.*
4:45pm Adjourn

Wednesday, September 15, 2010

7:45am Breakfast
8:30am Plenary Lecture 3: How a Cancer Center Can Support Your Research *Beverly Mitchell, M.D.*

CANCER GENES

9:25am Cancer Genomics and Cancer Gene Discoveries *Jonathan Pollack, M.D., Ph.D.*
10:15am Coffee Break
10:35am High-throughput Gene Sequencing and Cancer *Hanlee Ji, M.D.*
11:25am Cancer Genes and Clinical Diseases *James Ford, M.D.*
12:15pm Lunch
1:00pm **Poster Session II: Stanford Cancer Center Cores**

RADIATION THERAPY

2:15pm The Impact of Radiation Biology Research on Cancer Radiotherapy *Amato Giaccia, Ph.D.*
3:05pm Molecular Imaging in Radiation Therapy *Edward Graves, Ph.D.*
3:55pm Radiation Therapy Trials in Pediatric Malignancies *Sarah Donaldson, M.D.*
4:45pm Adjourn

Poster Session Presenters:

Animal Tumor Models *Yanru Chen-Tsai, Ph.D., Jeewon Kim, Ph.D.*
Bioscience Screening *David Solow-Cordero, Ph.D., James Chen, Ph.D. (Poster presented by Amato Giaccia, Ph.D.)*
Tissue Procurement *Jonathan Pollack, M.D., Ph.D., Reetesh Pai, M.D., Chuong Hoang, M.D.*
Cancer Imaging *Timothy Doyle, Ph.D., Christopher Contag, Ph.D.*
Cell Sciences Imaging *Jon Mulholland, M.S., Steve Smith, Ph.D.*
Flow Cytometry *Marty Bigos, Ph.D. and Garry Nolan, Ph.D.*
Genomics *John Coller, Ph.D., Mike Echart, Ph.D., Catherine Ball, Ph.D.*
Proteomics *Allis Chien, Ph.D., Joshua Elias, Ph.D.*
Human Immune Monitoring *Holden Maecker, Ph.D., Mark Davis, Ph.D.*
Biostatistics *Philip Lavori, Ph.D., Tze Lai, Ph.D.*
Clinical Protocol and Data Management *Miriam Bischoff, M.S., M.B.A., George Fisher, M.D., Ph.D.*

Thursday, September 16, 2010

7:45am Breakfast
8:30am Plenary Lecture 4: Hodgkin's Disease: A Paradigm for Clinical Research *Saul Rosenberg, M.D.*

TRANSLATIONAL/CLINICAL CANCER RESEARCH I

9:25am Therapeutic Targeting of Human Acute Myeloid Leukemia Stem Cells *Ravi Majeti, M.D.*
10:15am Coffee Break
10:35am Mechanisms of Epithelial Tumorigenesis *Paul Khavari, M.D., Ph.D.*
11:25am Head and Neck Cancers *John Sunwoo, M.D.*
12:15pm Lunch

TRANSLATIONAL/CLINICAL CANCER RESEARCH II

1:00pm Prostate Cancer *James Brooks, M.D.*
1:50pm Early Detection of Ovarian Cancer Using Targeted Micro-bubble Enhanced Ultrasound *Amelie Lutz, M.D.*
2:40pm Coffee Break
2:55pm Molecular Targets in Breast Cancer *Mark Moasser, M.D.*
3:45pm The Hedgehog Signaling Pathway for Anti-cancer Therapeutics Including Lung Cancer *Rajat Rohatgi, M.D.*
4:45pm Adjourn

Friday, September 17, 2010

7:45am Breakfast
8:30am Plenary Lecture 5: Cancer Survivorship Studies *David Spiegel, M.D.*

SOCIAL ASPECTS OF CANCER AND CANCER RESEARCH

9:25am Community Cancer Education *Pamela Priest-Naeve, B.A.*
10:15am Coffee Break
10:35am Psychosocial Impact of Cancer in Children *Michael Amylon, M.D.*
11:20am Social Adaptation of Cancer Patients *Ginna Laport, M.D.*
12:05pm Sexuality and Cancer *D. Kathryn Tierney, Ph.D.*
12:50pm Closing Remarks *Karl Blume, M.D.*
Certificates to Trainees
12:55pm Lunch
Adjourn

Cancer Systems Biology

Sylvia Plevritis, PhD
Associate Professor of Radiology
Stanford University

Cancer systems biology is a new research field that applies the principles of systems biology to the study of cancer. Systems biology is itself a growing discipline that views intra- and intercellular processes as a part of an integrated, complex system, rather than a collection of individual genes, proteins or metabolites operating in isolation. To unravel the complex, interconnected molecular mechanisms underlying cellular behavior, systems biology applies both experimental and computational methods toward the dual purposes of hypothesis synthesis and hypothesis testing. The rapid rise of systems biology has been fueled by the rapid accumulation of high throughput –omics data (i.e. genomics, transcriptomics, proteomics, metabolomics), advances in biotechnological methods to probe molecular processes (i.e. small molecular inhibitors, knockdowns by RNAi, overexpression by transfections), new developments in computation and statistics (i.e. artificial intelligence and informatics), and accessibility to massive computer power. To date, research in systems biology has mostly focused on reconstructing regulatory networks in simple organisms such as E. Coli and yeast. However, advances are being made with the application of systems biology to the study of more complex systems, including mammalian cells, and complex diseases such as cancer. Systems biology of cancer has emerged from a view of cancer as the perturbation of interconnected cellular processes, driven by genetic and epigenetic alternations that can serve as key targets of molecular therapies.

This review will focus on a single aspect of systems biology: namely the inference of global transcriptional regulatory networks in cancer. However, systems biology is a broad field that embraces transcriptional networks, protein interaction networks, signaling networks, metabolic networks, and their interactions, as well as the interactions among cells, such as the interaction of cancer cells and the host [1-3].

Inferring Gene Regulatory Networks (GRNs)

Systems biology has made substantial progress in inferring (or "reverse-engineering") global transcriptional regulatory networks from high-throughput data. In these networks, genes (or modules of genes) serve as the nodes; an edge exists between a pair of nodes if a direct or indirect interaction can be inferred from the data. The inferred network is then used to generate hypotheses of molecular interactions that can be experimentally tested. Such networks have been shown to predict cis-regulatory elements in a genome, to identify the target genes of transcription factors, to infer the conditions under which a given transcription factor is activated or repressed, and to analyze regulation by multiple transcription factors[4]. In drug development, these networks are increasingly being used to elucidate the functional properties of candidate genes that are targeted for therapeutic and preventative interventions, as a means to assess potential on-target and off-target effects [5].

A variety of computational approaches can be taken to infer gene regulatory networks, dependent on the questions being asked, and the experimental methods available [6]. This review will only briefly discuss on networks inferred using machine learning approaches that do not necessarily rely on prior knowledge of direct interactions [7].

> ***Relevance Networks:*** Relevance networks (RNs) are the simplest approach to gene network reconstruction. RNs rely on "guilt by association," which suggests a strong association between a pair of genes that are similarly expressed. RNs are typically reconstructed by computing the similarity in terms of the correlation or mutual information between a pair of genes. If the similarity of a gene-pair is above a threshold, then the gene-pair is connected by an edge. While RNs are easy to implement, they have several drawbacks. The most significant drawback is that high similarity may result from an indirect relationship and produce an edge between two genes that tells us little about the underlying biological mechanism. One way to reduce the number of indirect relationships is to evaluate the association between a gene-pair in the presence or one or more other genes. Here the basic idea is that if two genes are regulated through a third

gene, then given the third gene, the first two are not related. This idea of "conditional independence" is captured in many different ways, including a commonly used algorithm called ARACNE [8].

Bayesian Networks: Bayesian networks are a type of conditional independence model, where the relationships between genes are described by a directed acyclic graph (DAG). In a DAG, the state of a gene is jointly determined by its parental nodes in a probabilistic manner. Because Bayesian network can become computationally prohibitive when testing all possible DAGs across a large number genes, heuristic methods are often used. One example for Bayesian networks are Module Networks that encode modules of jointly regulated genes, their common regulators, and the condition under which the regulator occurs [9]. This approach generates testable hypotheses in the form "regulator X regulates module Y under condition W."

The validation of network-derived biological findings is best handled experimentally. However, when the experiment needed is not possible or feasible, the findings may be viewed as robust if they are statistically significant and can be reproduced in additional datasets similar, but distinct, to the one used to derive the result.

Application of Gene Regulatory Networks to Cancer

The value of inferring gene regulatory networks in cancer is becoming evident by an increasing number of related publications. In the study of the transformation of follicular lymphoma (FL) to diffuse large B-cell lymphoma (DLBCL), regulatory module networks revealed a key role for pluripotency-related genes in the transformation process that was also predictive of survival outcomes[10]. Studies in other hematologic malignancies have uncovered key genetic regulatory effects, such as the existence of a NOTCH1-cMYC feed-forward loop that drives proliferation in T-cell acute lymphoblastic leukemia; and validated prediction of specific oncogenic influences, including BCL6, have been made in diverse B-cell malignancies [11] [12, 13]. More recently, network-based approaches have been extended to solid tumors, identifying CEBPb and STAT3 as drivers of the mesenchymal transition in glioblastoma and predictors of poor clinical outcome [14]; the influence of T-cell homing factors on colorectal cancer survival [15]; and rare mutations driving Wnt/TBF-beta cross-talk, Wnt/VEGF signaling and MAPK/focal adhesion kinase pathways in breast, colorectal and brain cancers [16].

Future Directions

As the application of systems biology contributes new insights into cancer, these insights will lead to new questions that will undoubtedly propel methodological advances in the field of systems biology itself. Two such areas of new methodological development will be highlighted here. The first relates to dynamic modeling of regulatory networks. Once the direct gene or gene-product interactions are established, interest shifts to elucidating the network dynamics by monitoring temporal responses in perturbation experiments. The machine learning approaches summarized above can be used to analyze such time course data, but at a loss because they do not utilize the time dependency in the data. Instead dynamic models are being developed to analyze these more complex datasets; these methods are offer greater promise to restore feedback loops and the cross-talk between pathways [17].

Finally, a more comprehensive view of the molecular properties of cells will require an understanding of the orchestration of transcriptional, proteomic and metabolic networks [18]. This will necessitate the development of computational and bioinformatics methods to integrate observations on different temporal and spatial scales. Network-based representations of high-throughput data may provide the most natural means to integrate diverse biological datasets.

Recommended Reading

1. Ivakhno, S., From functional genomics to systems biology. FEBS J, 2007. 274(10): p. 2439-48.
2. Kreeger, P.K. and D.A. Lauffenburger, Cancer systems biology: a network modeling perspective. Carcinogenesis, 2010. 31(1): p. 2-8.
3. Laubenbacher, R., et al., A systems biology view of cancer. Biochim Biophys Acta, 2009. 1796(2): p. 129-39.
4. Li, H. and W. Wang, Dissecting the transcription networks of a cell using computational genomics. Curr Opin Genet Dev, 2003. 13(6): p. 611-6.
5. Lum, P.Y., J.M. Derry, and E.E. Schadt, Integrative genomics and drug development. Pharmacogenomics, 2009. 10(2): p. 203-12.
6. Sun, N. and H. Zhao, Reconstructing transcriptional regulatory networks through genomics data. Stat Methods Med Res, 2009. 18(6): p. 595-617.
7. Markowetz, F. and R. Spang, Inferring cellular networks--a review. BMC Bioinformatics, 2007. 8 Suppl 6: p. S5.
8. Margolin, A.A., et al., ARACNE: an algorithm for the reconstruction of gene regulatory networks in a mammalian cellular context. BMC Bioinformatics, 2006. 7 Suppl 1: p. S7.
9. Segal, E., et al., Module networks: identifying regulatory modules and their condition-specific regulators from gene expression data. Nat Genet, 2003. 34(2): p. 166-76.
10. Gentles, A.J., et al., A pluripotency signature predicts histologic transformation and influences survival in follicular lymphoma patients. Blood, 2009. 114(15): p. 3158-66.
11. Basso, K., et al., Integrated biochemical and computational approach identifies BCL6 direct target genes controlling multiple pathways in normal germinal center B cells. Blood, 2010. 115(5): p. 975-84.
12. Palomero, T., et al., NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. Proc Natl Acad Sci U S A, 2006. 103(48): p. 18261-6.
13. Mani, K.M., et al., A systems biology approach to prediction of oncogenes and molecular perturbation targets in B-cell lymphomas. Mol Syst Biol, 2008. 4: p. 169.
14. Carro, M.S., et al., The transcriptional network for mesenchymal transformation of brain tumours. Nature, 2010. 463(7279): p. 318-25.
15. Mlecnik, B., et al., Biomolecular network reconstruction identifies T-cell homing factors associated with survival in colorectal cancer. Gastroenterology, 2010. 138(4): p. 1429-40.
16. Torkamani, A. and N.J. Schork, Identification of rare cancer driver mutations by network reconstruction. Genome Res, 2009. 19(9): p. 1570-8.
17. Hecker, M., et al., Gene regulatory network inference: data integration in dynamic models-a review. Biosystems, 2009. 96(1): p. 86-103.
18. Huang, S.S. and E. Fraenkel, Integrating proteomic, transcriptional, and interactome data reveals hidden components of signaling and regulatory networks. Sci Signal, 2009. 2(81): p. ra40.

Questions to Consider

Which study (or studies) are most amenable to a systems biology approach?

- **A.** identifying the target genes of a particular transcription factor
- **B.** identifying a gene signature that is predictive of survival outcomes
- **C.** identifying markers upstream and downstream from a gene-product of interest
- **D.** identifying conditions for gene activation or inhibition

How are computationally-derived biological findings best validated?

- **A.** only through experimental methods
- **B.** only if their p-value is less than 0.05
- **C.** only if the findings have been previously-reported in a curated database
- **D.** only if the finding can be reproduced in public-domain datasets not used in the initial study

Which method (or methods) are well developed in the field of systems biology?

- **A.** methods that capture intercellular and intracellular interactions
- **B.** methods that integrate genomic, proteomic and metabolic data
- **C.** methods that capture feedback-back loops in transcriptional and signaling processes
- **D.** methods that quantify the degree of association (or similarity) between genes or modules of co-expressed genes

Control of Proliferation and Differentiation in Adult Stem Cell Lineages

Margaret T. Fuller, PhD
Professor of Developmental Biology and Genetics
Stanford University

Adult stem cell lineages are responsible for long-term maintenance and repair of tissues containing highly specialized, short-lived cell types, including blood, sperm, skin, and intestinal epithelium. Differentiated cells in many other tissues, including breast, lung, skeletal muscle, bladder and prostate, are also produced from adult stem cells in response to physiological changes or damage. Hallmarks of adult stem cells are a committed but relatively undifferentiated state, long-term ability to proliferate, and ability to produce both new stem cells (self-renewal) and differentiating progeny (Figure 1). Many common cancers arise in adult stem cell lineages, and there is increasing evidence that defects in the mechanisms that regulate self-renewal, proliferation and differentiation in adult stem cell lineages can contribute to oncogenesis. The mechanisms that regulate adult stem cell self-renewal and the proliferation and differentiation of stem cell progeny are key for harnessing the potential of adult stem cells for regenerative medicine and for understanding the possible developmental origins of cancer.

Key Regulatory Points in Adult Stem Cell Lineages

Tissue homeostasis in adult stem cell lineages requires the proper execution and coordination of several key switches in cell state (Figure 1). When adult stem cells divide, the daughter cells must choose between maintenance of stem cell identity or initiation of differentiation. A specialized microenvironments, the stem cell niche, regulates the outcome of this critical cell fate decision in vivo, ensuring that populations of stem cells and differentiating progeny are properly maintained. In several well-studied adult stem cell model systems, stem cell self-renewal relies upon on close range signals from a support cell niche; failure to maintain an intimate association with the niche causes cells to adopt differentiated fates. Discrete anatomical niches provide a strategy for regulating stem cell number, and may defend against cancer by preventing undifferentiated precursors from self-renewing outside of the niche.

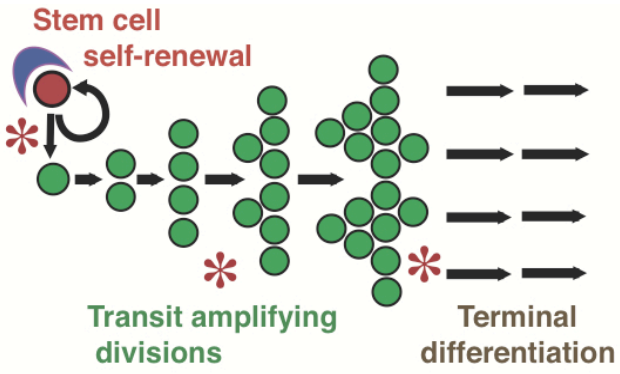

Figure 1. Key decisions in adult stem cell lineages. A stem cell (red) in the niche (blue crescent) divides to both self-renew and produce a daughter that initiates differentiation (green). After a limited number of transit amplifying mitotic divisions (green) the cells cease proliferation and turn on the terminal differentiation program (bank of arrows).

In many adult stem cell lineages, daughter cells that initiate differentiation first execute a series of transit amplifying divisions prior to terminal differentiation, allowing production of many differentiated cells from a single stem cell division (Figure 1). Mechanisms that limit the number of transit amplifying cell divisions comprise another key regulatory point, ensuring that mutations arising in precursor cells are not maintained in the pool of long term proliferating cells. Third, the switch from proliferation to terminal differentiation entails dramatic changes in the cell cycle and transcriptional programs, culminating in expression of terminal differentiation genes. Additionally, many adult stem cell lineages are multipotent, and precursor cells must choose to differentiate along a single, context-appropriate developmental pathway.

Drosophila Spermatogenesis as a Model Adult Stem Cell Lineage

Investigation of germ line stem cell lineages in Drosophila has provided insight into the types of mechanisms that regulate these critical cell fate switches in vivo and paradigms for researchers working in mammalian stem cell systems. In Drosophila, germ line stem cell (GSC) self-renewal depends upon short-range signals from a support cell niche, physical attachment to the niche, and orientation of stem cell divisions with respect to the niche. In addition, transit amplifying cells, although clearly differentiating, retain ability to reoccupy the niche and revert to stem cell function.

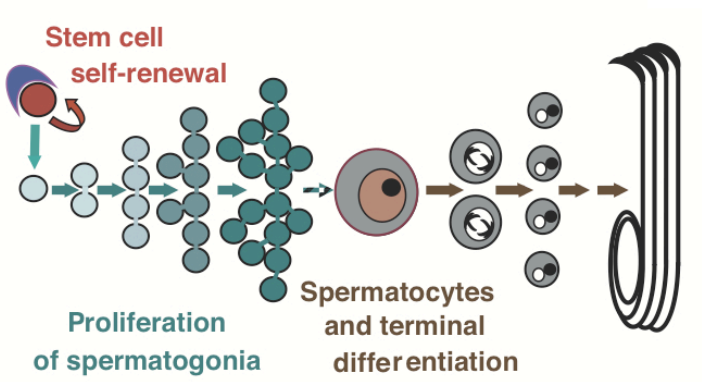

Figure 2. Male germ line stem cell lineage in Drosophila. A stem cell (red) at the tip of the testis divides asymmetrically, producing a new stem cell and a gonialblast, which initiates four rounds of synchronous spermatogonial mitotic divisions with incomplete cytokinesis. The resulting 16 interconnected germ cells undergo premeiotic DNA replication in synchrony and switch to the spermatocyte program of cell growth, meiotic prophase, and transcription of terminal differentiation genes. (all 16 cells become spermatocytes but only one is shown for simplicity).

Male gametes are produced in a unipotent adult stem cell lineage. Spermatogenesis in Drosophila normally initiates with the asymmetric division of a male germ line stem cell (GSC). One daughter maintains stem cell identity while the other initiates differentiation as a gonialblast (Gb), the founder of a clone of transit amplifying spermatogonia (Figure 2). Transit amplifying cells can be clearly distinguished from GSCs on the basis of cell behavior, structure, and gene expression. The transit amplifying spermatogonia descended from a Gb divide synchronously with incomplete cytokinesis producing a cyst of interconnected germ cells joined by cytoplasmic bridges. After a genetically predetermined number of transit amplifying divisions (4 in D. melanogaster), the resulting 16 germ cells together exit the mitotic program, synchronously execute premeiotic DNA synthesis, and switch to the the specialized cell cycle of meiosis and the primary spermatocyte program of cell growth and expression of terminal differentiation genes. After the two meiotic divisions, the resulting 64 haploid spermatids together execute the dramatic cell morphogenesis program that generates mature sperm.

Oriented Spindles and a Local Niche Can Program the Asymmetric Outcome of Stem Cell Divisions

Approximately [8-10] Drosophila male germ line stem cells (GSCs) lie in a rosette around a cluster of somatic support cells called the hub at the apical tip of each testis. Drosophila male germ line stem cells normally divide asymmetrically: the mitotic spindle is set up perpendicular to the hub-GSC interface so that, upon cytokinesis, one daughter remains next to the hub and maintains stem cell identity, while the other daughter is displaced away and initiates differentiation.

GSCs physically attach to the hub by localized adherens junctions. The resulting concentration of E-Cadherin, β-Catenin and APC2, a homolog of the Adenomatous Polyposis Coli tumor suppressor, concentrated at the GSC cortex adjacent to the hub that provide a polarity cue toward which GSCs orient throughout the cell cycle [1]. In G1, the single centrosome in each GSC localizes near the cell cortex where the germ cell attaches to the hub. When the duplicated centrosomes separate in G2, one stays next to the hub, while the other migrates to the opposite side of the cell. The stereotyped position of the centrosomes in turn orients the mitotic spindle perpendicular to the GSC-hub cell interface, ensuring that the outcome of GSC divisions is invariantly asymmetric: one daughter inherits the attachment to the hub, remains subject to local niche signals and renews stem cell identity, while the other is displaced away from the niche and initiates differentiation[1,2]. A checkpoint mechanism blocks progression into mitosis unless a centrosome is properly situated next to the attachment to the hub3,

ensuring a reliably asymmetric outcome of each stem cell division. The two centrosomes in male GSCs have different characters and fates. Differential labeling of mother versus daughter centrosomes by transient expression of a GFP-tagged centriolar protein fragment during centrosome duplication revealed that the mother centrosome normally remains adjacent to the hub and is inherited by the GSC, while the daughter centrosome migrates to the opposite side of the cell and is inherited by the Gb. As a result, male GSCs maintain a centriolar Eve, a centriole that was assembled many cell generations earlier [4].

A Common Niche Can Maintain Two Adult Stem Cell Populations

The apical hub supports two adult stem cell populations: male GSCs and somatic cyst stem cells (CySCs) (Figure 3), allowing spatially coordinated production of progeny cells that interact and co-differentiate. The cell bodies of CySCs sit distal to the GSCs, and they maintain direct contact with the hub via narrow cytoplasmic processes that intercalate between the GSCs. CySCs self-renew and give rise to daughters that differentiate into somatic cyst cells. Two cyst cells associate with and envelop each gonialblast, forming a discrete packet, or cyst, which remains intact throughout the rest of spermatogenesis. The cyst cells co-differentiate with the germ cells they enclose and continued association and communication between the germ line and the enveloping somatic cyst cells is essential for proper differentiation of both cell types.

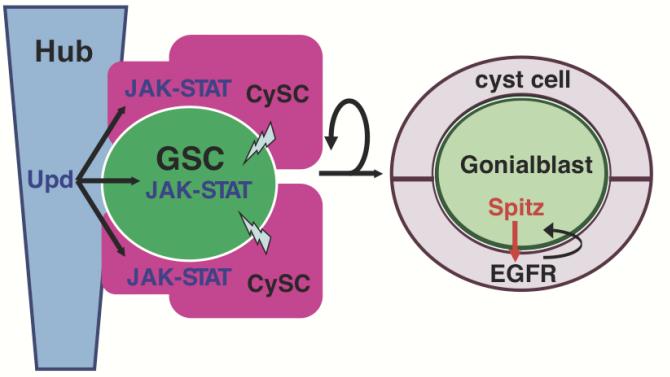

Figure 3. GSCs and CySCs require the *Upd* signal from the hub and the hub and CySCs both contribute to the GSC niche. Left) *Upd* secreted by hub cells (blue) activates signal the JAK-STAT pathway, which is required cell autonomously to maintain attachment of germ line stem cells (GSCs) to the hub and for self-renewal of cyst stem cells (CySC). GSC self-renewal also requires as yet unknown signal(s) (lightning bolt) from the CySCs. Right) Interactions between the progeny of somatic and germ line stem cells set up the functional unit of differentiation, the cyst. A gonialblast (light green), produced by asymmetric division of a GSC, signals via the EGFR ligand *Spitz*, to cysts cells (light pink) produced by asymmetric division of CySCs. Activation of EGFR triggers the somatic cyst cells to envelop the gonialblast and may also induce changes in gene expression in the cyst cells required for the germ cells to execute the TA division program.

The hub supports maintenance of germ line and somatic stem cells by secreting the signaling ligand Unpaired (Upd) (Figure 3). In a cytokine-like signaling pathway, Upd produced by hub cells locally activates the Janus Kinase - Signal Transducer and Activator of Transcription (JAK- STAT) pathway in neighboring GSCs and CySCs. Action of STAT is required cell autonomously in male GSCs for maintenance of attachment to the hub. Male GSCs made homozygous mutant for a null allele of stat fail to maintain stem cell identity and instead differentiate [5,6].

The same cytokine-like signal from the hub that maintains GSCs also maintains the somatic CySCs (Figure 3). Like GSCs, the CySCs over-proliferate when Upd is ectopically expressed in early germ cells (paracrine stimulation) or in early cyst cells (autocrine stimulation) [5-7]. Upd activates the JAK-STAT cascade in CySCs to specify their self-renewal. Action of stat is required cell autonomously for CySC maintenance: somatic CySCs made homogygous mutant for stat fail to maintain stem cell identity and instead commit to differentiation [7]. The transcriptional repressors zfh-1 and chinmo are likely critical downstream targets of STAT in CySCs required for stem cell maintenance [7-8].

Multiple Cell Types Can Contribute to Niche Function

The niche that maintains male GSCs is complex, consisting of both signals from hub cells and signals from the CySCs. Complex niches may be a feature of systems where multiple stem cell types must be maintained in close proximity and coordinated to produce progeny that must differentiate together. Although activation of STAT in GSCs by Upd secreted from the hub is required to maintain attachment of GSCs, it is not sufficient for stem cell self-renewal. However, forced activation of STAT in the cyst

cell lineage resulted in overproliferation of germ line stem cells or their immediate progeny and inhibited germ cell differentiation [7]. Taken together, these data suggest that the cytokine-like signal from the hub instructs GSCs to attach to and orient toward the hub, and that these in turn maintain GSCs within range of a self-renewal signal from the CySCs and ensure an asymmetric outcome of germ line stem cell divisions. (Figure 3).

Transit Amplifying Cells Can Revert to Stem Cell Identitiy

Remarkably, male germ cells undergoing transit amplifying divisions can and indeed often do reoccupy the niche and revert back to stem cell identity [3,9]. Reversion of TA cells to stem cell state to replace stem cells lost by normal turnover or damage may be especially important in cases where stereotyped spindle orientation normally prevents a symmetric outcome of stem cell divisions in the niche. Although transit amplifying cells are clearly morphologically and behaviorally different from GSCs, they are not yet irreversibly committed to differentiation. Dedifferentiation of spermatogonia requires STAT activity. Adult males conditionally mutant for stat maintained stem cells at permissive temperature, but rapidly lost both GSCs and CySCs to differentiation when shifted to non-permissive temperature. However, if the flies were returned to permissive conditions within a few days, transit amplifying spermatogonial cysts adjacent to the hub broke apart and provided germ cells that regained stem cell behavior, reconstituting the vacated niche [9].

Genetic marking experiments demonstrated that transit amplifying cells that had progressed to the 4-cell cyst stage and turned on the TA cell differentiation factor bam can repopulate the niche and revert to stem cell behavior, even without drastic clearing of the niche and manipulations of niche signaling pathways [3]. The frequency of stem cell replacement by de-differentiation of transit amplifying cells increased when flies were irradiated, suggesting that this may be an important mechanism to restore stem cells to the niche after damage. Replacement of stem cells by dedifferentiation of transit amplifying cells also appears to occur during mammalian spermatogenesis [10]. This ability means that transplantation assays may detect not only the most fundamental stem cells, but also possibly early transit amplifying cells that have initiated but are not yet committed to differentiation. It will be interesting to find out if this underlies the recent discovery of two interconverting populations of hematopoietic stem cells, one deeply quiescent and one more rapidly proliferating [11].

The picture emerging from work on germ line stem cell niches in Drosophila points toward a self-sustaining and mutually reinforcing community of several cell types engaged in close range signaling conversations that influence the fate choices of neighbors within the niche. The ability to identify stem cells and trace their descendents in vivo has yielded insights into how self-renewal, proliferation and differentiation are regulated in adult stem cell lineages. Analysis of male germ line stem cells in Drosophila has revealed the importance of local signals from the microenvironment, the stem cell niche, in controlling stem cell behavior. Strikingly, much as LIF and TGFb signaling maintain mouse ES cells, maintenance of stem cell state in the Drosophila male germ line is regulated by cytokine-like signals from hub cells that activate the transcription factor STAT, and TGFb class signals from surrounding support cells, which repress expression of a key differentiation factor. Shared requirements for sustaining signals from the local microenvironment may underscore the relationship between stem cell lineages and cancer. Recent work on basal cell carcinoma indicates that tumor cells are maintained by close range signals from accompanying stromal cells, which provide an ersatz niche [12].

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Questions to Consider

Why might adult stem cell lineages commonly include transit-amplifying divisions?

- **A.** Transit amplifying cells are irreversibly committed to differentiate.
- **B.** To dilute out self-renewal factors.
- **C.** To increase the number of differentiated cells produced by one round of stem cell division.
- **D.** Because adult stem cells always produce more than one type of differentiated progeny cells.

How does spindle orientation specify the asymmetric outcome of a stem cell division?

- **A.** The stem cell inherits β-catenin.
- **B.** Centrosome orientation is random during interphase.
- **C.** STAT is activated in the stem cell.
- **D.** One daughter cell remains in the niche while the other daughter is displaced away.

The complex niche for germ line stem cells in the *Drosophila* testis consists of?

- **A.** Self-stimulating (autocrine) signals from the germ line stem cells.
- **B.** Signals from the hub specify attachment while signals from the somatic stem cells specify self-renewal.
- **C.** Attachment to basal lamina by integrins.
- **D.** Circulating reproductive hormones.

Cancer Stem Cells

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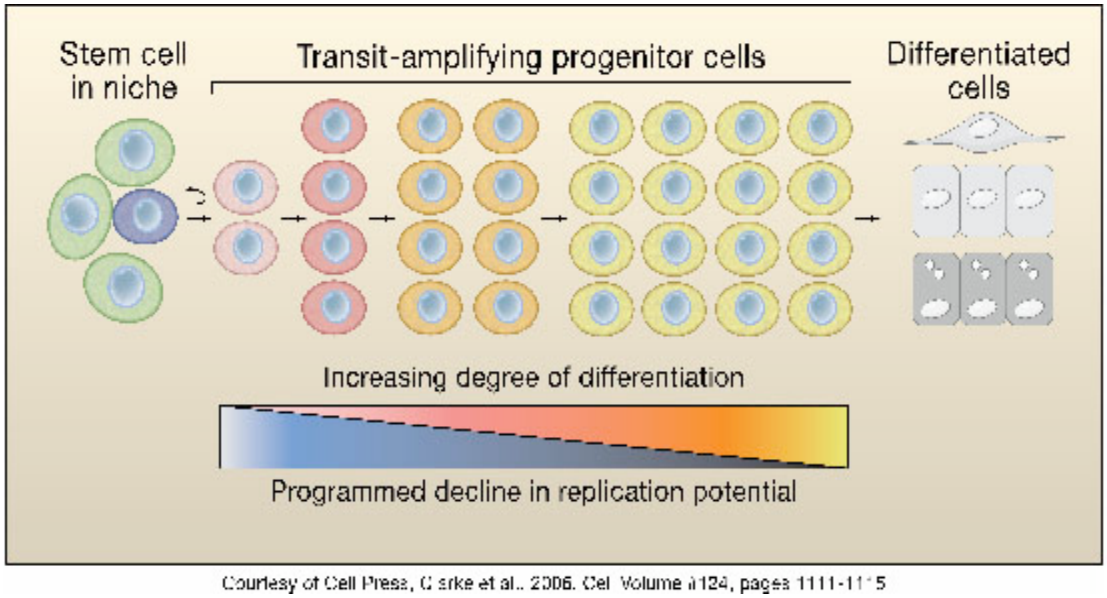
Several aspects of stem cell biology are relevant to cancer. Common Cancers arise in tissues that contain a large sub-population of proliferating cells that are responsible for replenishing the short-lived mature cells. In such organs, cell maturation is arranged in a hierarchy in which a rare population of stem cells gives rise to the mature cells (Figure 1). To do this, stem cells have 2 functions. First, they need to self-renew in order to maintain the stem cell pool whose size is under strict genetic regulation. Second, they must undergo differentiation to maintain a constant pool of mature cells in normal conditions and to produce increased numbers of a particular lineage in response to stresses (Reya et al., 2001).



Courtesy of Cell Press, Clarke et al., 2006. Cell Volume 124, pages 1111-1115

Figure 1. Interactions between normal stem cells and the tissue stroma (niche). In normal tissues, the niche cells (pale green) provide signals enabling normal stem cells (blue) to self-renew (curved arrow). Transit-amplifying progenitor cells (pink, yellow, orange) do not receive this signal and their proliferation is constrained by cellular mechanisms that count the number of mitotic divisions. With each cell division, the proliferation capacity of these daughter cells declines (programmed decline in replication potential), and their degree of differentiation increases.

By contrast, cancer cells have escaped this homeostatic regulation and the number of cells within a tumor that have the ability to self renew is constantly expanding, resulting in the inevitable growth of the tumor. Differences in self-renewal pathways between cancer stem cells and normal stem cells can be exploited to more effectively treat cancer. Self-renewal is a cell division in which one or both of the daughter cells remain undifferentiated and retain the ability to give rise to another stem cell that has the same capacity to proliferate as the parental cell. Proliferation does not require either daughter cell to be a stem cell nor retain the ability to give rise to a differentiated progeny. A single HSC can restore the blood system for life, but a progenitor cell is destined to eventually stop proliferating. Thus, normal stem cells share with at least some of the cancer cells within a tumor the ability to replicate without losing the capacity to proliferate.

Not surprisingly, some oncogenes function to promote stem cell self-renewal. Adult stem cells of the hematopoietic and neuronal tissues express the proto-oncogene Bmi-1, a member of the polycomb family involved in the epigenetic repression of target genes. Although the number of HSCs in the fetal liver of Bmi-1-/- mice was normal, the number of HSCs was markedly reduced in postnatal Bmi-1-/- mice(Park et al., 2003). Transplanted fetal liver and bone marrow cells obtained from Bmi-1-/- mice contibuted only transiently to hematopoiesis. There was no detectable self-renewal of adult hematopoietic stem cells, indicating a cell autonomous defect in Bmi-1-/- mice. A gene expression analysis revealed that the expression of stem cell associated genes4, cell survival genes, transcription factors, and genes modulating proliferation including p16Ink4a and p19Arf was altered in bone marrow cells of the Bmi-1-/- mice. Expression of p16Ink4a and p19Arf in normal HSCs resulted in proliferative arrest and p53-dependent cell death, respectively. Similarly, Bmi-1 regulates self-renewal of adult neuronal stem cells partly via p16Ind4a. Thus, expression of Bmi-1 is essential for the generation of self-renewing adult hematopoietic and neuronal stem cells(Molofsky et al., 2003).

HSCs maintain themselves for the lifetime of the organism because of their ability to self-renew. However, MPPs lack the ability to self-renew, therefore their mitotic capacity and expansion potential are limited and they are destined to eventually stop proliferating after a finite number of cell divisions1, 2. The molecular mechanisms that limit the proliferation capacity of MPPs and other more mature progenitors are not fully understood 2, 3. Bone marrow cells from mice deficient in three genes genetically downstream of Bmi1, p16Ink4a, p19Arf, and Tp53 (triple mutant mice) have an approximately ten-fold increase in cells able to reconstitute the blood long-term. This increase is associated with the acquisition of long-term reconstitution capacity by cells whose phenotype is c-kit+Sca-1+Flt3+CD150-CD48-Lineage-, which defines MPPs in wild type mice4-6. The pattern of triple mutant MPP response to growth factors resembles that of wild type MPPs but not wild type HSCs. These results demonstrate that Ink4a/Arf and Tp53 play a central role in limiting the expansion potential of MPPs. These pathways are commonly repressed in cancer, suggesting a mechanism by which early progenitor cells could gain the ability to self-renew and become malignant with further oncogenic mutations(Akala et al., 2008).

Recently, tumorigenic and non-tumorigenic subsets of cancer cells have been isolated from human breast cancer tumors, providing the first direct evidence for cancer stem cells in solid tumors(Al-Hajj et al., 2003). Remarkably, a gene signature derived from breast cancer stem cells has prognostic power in patients with early stage breast cancer(Liu et al., 2007). These observations suggest that in order to eradicate a tumor, the cancer stem cells must be eliminated by a particular therapy. Indeed, recent evidence shows that the cancer stem cells in solid tumors are resistant to common therapeutic agents. It has recently been shown that central nervous system stem cells and haematopoietic stem cells and early progenitors contain lower levels of ROS than their more mature progeny, and that these differences are critical for maintaining stem cell function. We proposed that epithelial tissue stem cells and their cancer stem cell (CSC) counterparts may also share this property. We found that normal mammary epithelial stem cells contain lower concentrations of ROS than their more mature progeny cells. Notably, subsets of CSCs in some human and murine breast tumours contain lower ROS levels than corresponding non-tumorigenic cells (NTCs). Consistent with ROS being critical mediators of ionizingradiation- induced cell killing, CSCs in these tumours develop less DNA damage and are preferentially spared after irradiation compared to NTCs. Lower ROS levels in CSCs are associated with increased expression of free radical scavenging systems. Pharmacological depletion of ROS scavengers in CSCs markedly decreases their clonogenicity and results in radiosensitization. These results indicate that, similar to normal tissue stem cells, subsets of CSCs in some tumours contain lower ROS levels and enhanced ROS defences compared to their non-tumorigenic progeny, which may contribute to tumour radioresistance (Diehn et al., 2009).

The mechansisms regulating stem cell self-renewal and for cancer stem cell drug resistance will be discussed.

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Questions to Consider

Which of the following statements has been shown about self-renewal in normal tissues?

- **A.** All cells in a tissue have unlimited replication potential
- **B.** Mature blood cells can dedifferentiate and become stem cells with stress
- **C.** Stem cells in the gastrointestinal tract are always quiescent
- **D.** Polycomb plays a role in self renewal of blood stem cells

Which is true about self-renewing cancer cells?

- **A.** They are derived from normal stem cells
- **B.** They share the use of some self renewal pathways used by normal stem cells
- **C.** Genes that limit self renewal of normal cells are commonly mutated in cancer
- **D.** Both b & c are true

Which statement is true about the role of microRNAs in the regulation of stem cells?

- **A.** microRNAs play no role in self renewal
- **B.** microRNAs regulate self renewal pathways
- **C.** microRNAs do not play a role in cancer

Therapeutic Resistance of Cancer Stem Cells

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It has been recognized for decades that only a small proportion of malignant cells within most tumors can form colonies in clonogenic assays and that cancer cells within the same tumor can display strikingly distinct histologic and molecular properties. Two predominant models have been developed to explain tumor heterogeneity (Figure 1). The first, known as the stochastic model, is the classical view and argues that all malignant cells within a tumor have the potential to give rise to new tumors, but that this can only occur under ideal conditions and therefore is a relatively rare event. In this model, histologic and molecular heterogeneity are attributed to microenvironmental differences or to genomic instability of cancer cells. The second model, known as the cancer stem cell (CSC) model, argues that like the normal tissues from which they are derived, tumors contain a cellular hierarchy in which only a subpopulation of malignant stem cells can proliferate indefinitely and thus give rise to new tumors. While the remaining cancer cells, often called non-tumorigenic cancer cells, can proliferate, the number of divisions they can undergo is limited and they cannot form new tumors. In the cancer stem cell model, the histologic and molecular heterogeneity seen in most tumors is largely explained by the partially intact differentiation programs that exist within tumors and which attempt to recapitulate the normal development of the tissue of origin.

As discussed below, a growing body of evidence indicates CSCs play critical roles in formation and maintenance of human malignancies and that these cells have important implications for cancer therapy.

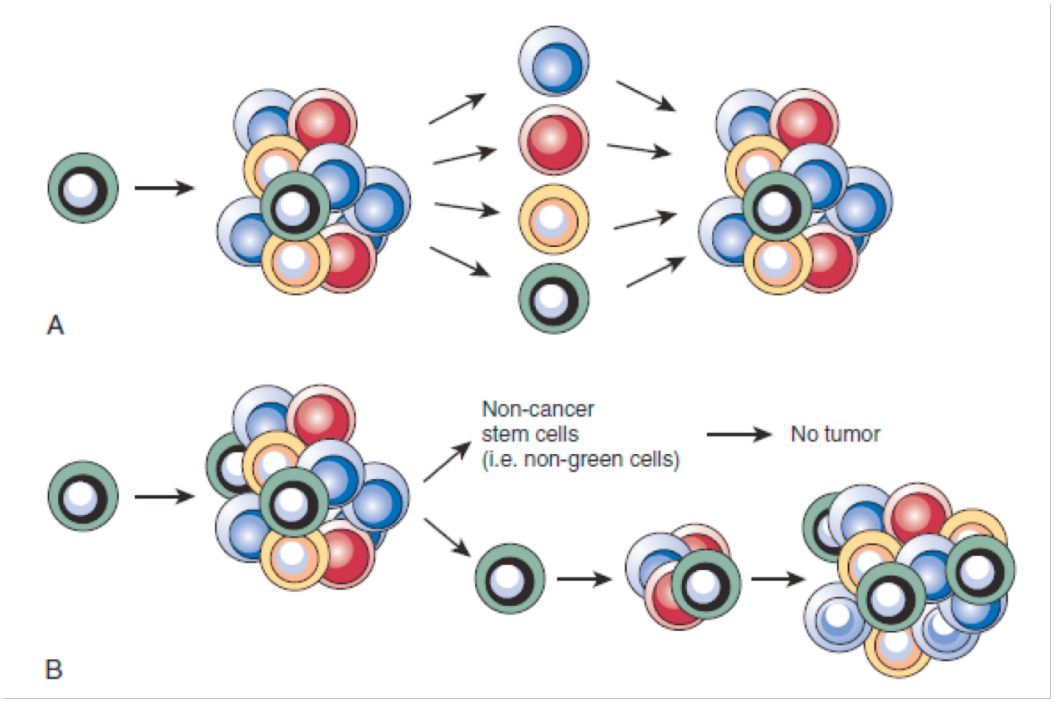

Figure 1. Models of tumor formation and maintenance. (A) The classical stochastic model. In this model all of the phenotypically diverse cells of the tumor have similar tumor-forming abilities. B, The cancer stem cell model. In this model, only the cancer stem cells (green cells) can form new tumors. Other cancer cells, called non-tumorigenic cancer cells, are unable to give rise to a tumor.

Identification of Cancer Stem Cells in Solid Tumors

The identification of CSCs was accomplished by applying principles and tools of normal stem cell biology to cancer research. In order for a cell to be considered a CSC it must be able to self renew and give rise to differentiating progeny with limited proliferative potential. Self renewal describes a type of mitotic division in which daughter cells retain the stem cell phenotype. Alternatively, progeny resulting from stem cell divisions can differentiate, thus losing the ability to replicate indefinitely. The balance between self renewal and differentiation must be strictly regulated in normal tissues and becomes dysregulated during tumorigenesis. The gold standard assay for proving the existence of CSCs within a subpopulation of tumor cells is purification of the putative stem cells using cell sorting followed by transplantation into mice. By definition, CSCs are contained in subpopulations that can form tumors while non-tumorigenic cells are present within subpopulations that cannot. Importantly, the cancer stem cell hypothesis does not require CSCs to make up only a small subpopulation of tumor cells, although in many human tumors this does appear to be the case.

The existence of CSCs was first documented in acute myelogenous leukemia (AML). In the majority of patients with AML, leukemic stem cells reside in the $CD34^{+}CD38^{-}$ subpopulation, which are the only these cells that can lead to engraftment of human leukemia in NOD/SCID mice. The more numerous leukemic blasts, generally displaying the CD34-CD38+ immunophenotype, are unable to transplant the disease. Soon after these discoveries, other investigators showed that CSCs can be prospectively isolated from solid tumors. This was first accomplished for human breast cancers where tumorigenic activity was found to be highly enriched in cancer cells with the $CD44^{+}CD24^{-/low}Lineage^{-}$ immunophenotype. Transplantation of as few as 100 of these cells led to tumor formation in mice while tens of thousands of the remaining cancer cells did not [1]. Importantly, tumors arising from $CD44^{+}CD24^{-/low}Lineage^{-}$ cells contained the entire diversity of cancer cells found in the primary tumor, including cells with the CSC phenotype and a large population of cells with the non-tumorigenic cell phenotype. Re-transplantation of cells from first generation xenografts similarly led to tumors displaying the phenotypic diversity of the primary tumor. These data indicate that human breast cancers contain a hierarchy of cancer cells that originates from $CD44^{+}CD24^{-/low}Lineage^{-}$ CSCs, which have the capacity to self-renew.

More recently, CSC-enriched populations have been isolated from many other human malignancies including those arising in the brain, prostate, colon, head and neck, pancreas, bladder, and liver. Thus, many human tumors appear to be maintained by subpopulations of CSC that are able to self-renew and to give rise to non-tumorigenic cells with limited proliferative potential. Additionally, CSCs have been found in mouse models of leukemia and breast cancer.

Cancer Stem Cells and Resistance to Chemotherapy

Many of the most frequently used cancer therapies induce cell death by producing DNA damage. These agents are also toxic to normal stem cells and their progeny. Fortunately, normal stem cells are protected from genotoxic stress through a variety of mechanisms and this makes them relatively resistant to cell killing with cytotoxic agents. The recognition of apparent similarities between normal stem cells and CSCs led to the hypothesis that CSCs may be relatively resistant to chemotherapeutic agents compared to their more differentiated non-tumorigenic counterparts [2]. Mounting evidence suggests that this is the case.

Multiple studies have shown that "CSC-like cells" present in some established cell lines and in long-term xenografts appear to be relatively resistant to cytotoxic chemotherapeutics. Other investigators have studied chemotherapy resistance of CSCs in early passage xenograft models that may more closely recapitulate the biology of CSCs in human patients. For example, on study analyzed human $CD44^{+}ESA^{+}$ colon CSCs in early passage xenografts and found that these were enriched in xenografts that had been treated with cyclophosphamide compared to untreated control tumors [3]. Enrichment was documented both as an increased percentage of CSCs as measured by flow cytometry and as higher tumor forming capacity in limiting dilution assays.

Observations of CSC resistance to chemotherapy have also been extended to the clinical setting. In one study, the frequency of CSCs was analyzed in patients before and after they received neoadjuvant

chemotherapy for locally advanced breast cancer. It was found that CSCs accumulated after treatment with cytotoxic chemotherapy but not after exposure to certain biologically-targeted agents [4].

Cancer Stem Cells and Resistance to Radiotherapy

Radiobiologists were among the earliest investigators studying CSCs through the use of local control assays, which test the ability of tumors to regrow after treatment. By definition, CSCs are the only cells that can lead to significant regrowth of tumors and thus local control assays test the ability of CSCs to survive a given therapy. A significant body of data in the radiobiologic literature suggests that CSC content (as assessed by *in vivo* and *in vitro* clonogenic assays) and their intrinsic radiosensitivity correlate with radioresistance.

More recently, investigators have begun to apply the tools of stem cell biology to the question of CSC radiosensitivity. Attention has particularly focused on models of breast and brain tumors, which were the first solid tumors for which CSC markers became available and for both of which clinical treatment plans often include radiotherapy. Most of these studies focused on cancer-stem-like cells within established cell lines that had previously been selected for *in vitro* growth for many generations, thus raising concerns that results may not necessarily reflect the behavior of CSCs in primary tumors. However, taken in aggregate, these studies indicate that in many tumors, irradiation *in vivo* or *in vitro* leads to a relative enrichment of CSCs as measured by flow cytometry. For example, CSCs from the most common adult brain tumor, glioblastoma multiforme (GBM) have been recently been found to be radioresistant [5]. The CSCs in at least some of these tumors can be identified by expression of CD133, and these cells accumulated by a factor of ~2-3 fold after irradiation *in vitro* and *in vivo* (i.e. xenografts). Molecular surrogates of radiation-induced cell killing such as apoptosis and DNA damage, were also lower in the CSCs.

We have documented similar radiation resistance in CSCs from breast cancers [6]. Specifically, we found that CSCs from murine breast tumors accumulated after *in vivo* irradiation and were relatively radioresistant in clonogenic assays *in vitro* (Figure 2). DNA damage assays confirmed this sensitivity difference.

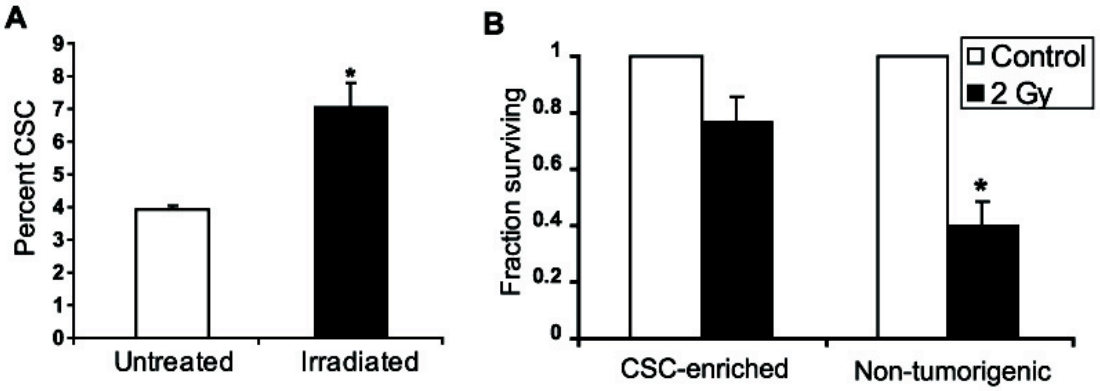

Figure 2. Enrichment of breast CSCs after irradiation. (A) Mice bearing murine breast tumors were irradiated or left untreated and the percentage of CSCs was quantified by flow cytometry 72 h after the last fraction was delivered. (n=6, *p=0.008). (B) Clonogenic survival of CSC-enriched cells and non-tumorigenic cells before (open bars) and after treatment with 2 Gy of ionizing radiation (filled bars) in vitro (n=3, *p=0.001).

Mechanisms of Cancer Stem Cell Treatment Resistance

What are the mechanisms by which CSCs resist chemotherapy- or radiotherapy-induced cell killing? One possible explanation involves cell cycle kinetics of CSCs. Rapidly dividing cells have long been known to be more sensitive to cytotoxic therapies than quiescent cells. It has been shown that at least a subset of leukemia stem cells are quiescent and resistant to chemotherapy. A similar phenomenon may exist in malignant epithelial stem cells, although this has not been conclusively documented. For

example, cell cycle profiles of human and murine breast CSC and non-tumorigenic cells were found to be identical, arguing against large difference in cell cycle status between these populations [1, 6].

Other mechanisms of resistance to cytotoxic agents that have been documented in CSCs include the expression of multiple drug resistance transporters [7], residing in hypoxic niches, enhanced DNA repair capacity [5], enhanced free radical scavenging [6, 8], expression of specific drug detoxifying enzymes [3], and activation of signaling pathways such as Wnt/beta-catenin, sonic hedgehog, and Notch [8-10]. It is likely that in many case CSCs employ a combination of mechanisms that make them relatively resistant to cytotoxic therapies compared to their progeny. Furthermore, the mechanisms that predominate likely vary between tumor types and even within tumors arising from the same organ. The CSC hypothesis implies that identification of the specific resistance mechanisms at work in these cells will allow rational design of chemo- and radio-sensitization protocols that could lead to higher tumor control and cure rates.

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Questions to Consider

Basic properties of cancer stem cells (CSCs) include:

- **A.** CSCs arise in the bone marrow
- **B.** CSCs are pluripotent and give rise to mesoderm, endoderm, and ectoderm
- **C.** CSCs can self-renew
- **D.** CSCs must be a minority population of cancer cells

The gold standard assay for proving the existence of cancer stem cells in a purified population of cancer cells is:

- **A.** Sphere formation under non-adherent culture conditions
- **B.** Tumor formation in xenotransplantation assays
- **C.** Colony formation under adherent culture conditions
- **D.** Expression of surface markers found on normal stem cells

Properties of CSCs that contribute to chemotherapy and/or radiotherapy resistance include:

- **A.** Enhanced DNA repair capacity
- **B.** Elevated expression of free radical scavengers
- **C.** Activation of signaling pathways such as Wnt and Notch
- **D.** All of the above

Biology and Use of Hematopoietic Cell Transplantation

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Introduction

Over the past 60 years the field of hematopoietic cell transplantation (HCT) has evolved from experimental animal models of bone marrow transplantation to curative therapy for tens of thousands of people yearly who are affected by a wide variety of marrow failure states, myeloid and lymphoid malignant diseases, immune deficiencies and inborn errors of metabolism. Advances in transplantation immune biology combined with improvements in supportive care have made this evolution possible and have ushered in the modern era of HCT. In this lecture the biologic principles and clinical applications of HCT will be discussed along with its future applications. Selected results demonstrating important principles will be highlighted.

History

The field of transplantation was born out of the political climate that followed WWII and concern for continued atomic warfare and Western governments funneled resources to investigators studying the effects of irradiation. Jacobson showed that mice survived otherwise lethal irradiation exposure if the spleen (a hematopoietic organ in mice) was protected by lead foil. Lorenz observed that mice were protected from lethal irradiation by the administration of syngeneic bone marrow, demonstrating therapeutic efficacy of allogeneic bone marrow suspensions. It was initially thought that components from the shielded spleen or infused marrow stimulated endogenous hematopoietic cell recovery. In 1954, Barnes demonstrated that hematopoietic recovery was from cell transplantation repopulation and not humeral factors.

Clinical application came in 1956 when Barnes described the treatment of murine leukemia by lethal irradiation followed by marrow grafting. He argued that irradiation alone did not kill all leukemia cells, but that residual leukemia was eliminated through immunologic mechanisms of colonizing cells and coined the term “adoptive immune therapy”.

In 1957 Thomas reported the first human studies in terminal leukemia patients treated with irradiation and intravenous infusion of marrow from healthy donors. The first attempts at autologous marrow transplantation appeared during this time in patients with metastatic cancer whose marrow was collected and stored by freezing. Following intensive radiation therapy the marrow was thawed and infused intravenously. Yet support for transplantation waned as a compendium of 203 human allogeneic marrow grafts carried out throughout the 1950s and 1960s showed that none were considered successful. The first positive results came in 1968 from studies of children with severe combined immunodeficiency; the lymphoid elements from the donor graft corrected the immunodeficiency. In 1975 Thomas described the recipe for successful allogeneic transplantation; he stressed the importance of histocompatibility, proper preparation of the patient before transplantation, detailed the technique of marrow transplantation, emphasized the need for post transplantation immune suppression, supportive care, and raised the possibility of using unrelated donors. This report ushered in the modern era of HCT. By the year 2007, more than 700,000 patients worldwide were transplanted and more than 125,000 survived five years or beyond after transplantation.

Sources of Cells

Bone marrow is the traditional source of cells for allogeneic and autologous transplantation. The marrow is aspirated by repeated placement of large bore needles into the posterior iliac crest, generally 50-100 aspirations simultaneously on both sides, while under general anesthesia. The lowest cell dose to ensure

stable long-term engraftment is not defined; a typical collection contains more than 2 x10^8 nucleated cells/kg recipient body weight.

Hematopoietic progenitor cells are present in the blood at very low levels; however a number of different stimuli including chemotherapy, various hematopoietic growth factors and inhibitors of certain chemokine receptors, result in the mobilization of cells from marrow to blood. Once in the blood these cells can be collected by apheresis, and this product has been termed peripheral blood progenitor cells (PBPC) to differentiate from the over used term blood stem cells, which should be reserved for instances where the hematopoietic stem cell population itself has been isolated. Agents used for mobilization include G-CSF, granulocyte-monocyte colony stimulating factor (GM-CSF), interleukin (IL)-3, thrombopoietin, and more recently the chemokine receptor (CXCR4) antagonist AMD3100. The infusion of >2 x10^6 $CD34^+$ cells/kg was suggested as the desired dose for acceptable engraftment kinetics following transplantation.

The use of mobilized PBPC has had a major impact on autologous HCT. In the allogeneic setting, the situation is considerably more complex. The approximately 10x greater number of T cells contained in PBPC grafts led to the concern that higher number of T cells may result in increased incidence and severity of graft-versus-host disease (GVHD). Yet this may not be the case as G-CSF can induce functional immune tolerance *in vivo* in healthy individuals with upregulation of genes related to T-cell helper type 2 (Th2) and regulatory T (Tregs) cells, and downregulation of genes associated with Th1 cells, cytotoxicity, and antigen presentation.

Umbilical cord blood (UCB) collected from the umbilical vessels in the placenta at the time of delivery is a rich source of hematopoietic cells. Because these cells are relatively naïve, recipients may have satisfactory outcomes, even when crossing major histocompatibility barriers. A major limitation is the relatively small number of cells available in the cord blood unit, which limits transplantation mainly for pediatric patients. Studies in adults confirmed prolonged engraftment times. Double-unit UCB transplantation may provide better engraftment by providing a higher $CD34^+$ and $CD3^+$ cell dose. Efforts to expand hematopoietic cells from cord blood to accelerate engraftment and immune reconstitution are ongoing.

Concepts of Curative Therapy - Autologous HCT

The relationship between the dose of chemotherapy and/or radiation and the number of tumor cells killed has been extensively studied *in vitro* and in preclinical animal models, and forms the basis for the decision to pursue autologous HCT. Lymphoma and leukemia are tumors considered to exhibit a rising dose-response curve; meaning as the dose of the cytoxic agent is increased the number of tumor cells killed is increased. The possibility for cure following autologous HCT, therefore, is derived from the beneficial effects of the high-doses of chemotherapy and/or radiation therapy administered during transplant conditioning that enhance tumor cell kill and overcome drug resistance. Dose escalation is possible because the dose limiting toxicities on hematopoiesis are circumvented by the infusion of the autologous hematopoietic cell graft that was harvested beforehand.

Autologous HCT is associated with relatively low non-relapse mortality (NRM). The use of mobilized PBPC has decreased the duration of neutropenia and when combined with improved supportive care the NRM of approximately 10% with marrow in earlier studies has been reduced to 3% with mobilized PBPC.

Tumor Contamination in the Autograft

A concern in autologous HCT is the possibility that residual clonogenic tumor cells may contaminate the product and contribute to tumor relapse. The relative contributions of tumor cell contamination from the cell product and residual disease in the patient are difficult, if not impossible, to discern. One approach to determine if residual malignant cells contribute to relapse is to mark the cells in product at the time of harvest and then assay if the marker-gene is present in malignant cells at the time of a subsequent relapse. Studies using this approach have been performed in patients with leukemia, lymphoma and myeloma. In these studies, cells in the graft were marked with a retroviral vector encoding the neomycin resistance gene. This marker-gene can subsequently be detected in transduced cells either phenotypically, because it confers resistance to the neomycin analogue G418, or genotypically by polymerase chain reaction (PCR). Data from these studies indicated that marrow harvested from patients whose disease was in "apparent" complete remission likely contained residual tumorigenic cells,

because these cells contributed to disease recurrence at medullary and extramedullary sites. The implication is that effective purging may be one requirement for improving outcomes following autologous HCT.

Concepts of Curative Therapy - Allogeneic HCT

Allogeneic HCT is considerably more complicated a procedure than autologous HCT; it involves more pretransplantation preparation, poses a greater risk for complications to the patient, is associated with a significantly higher non-relapse mortality rate, and the period of intensive post transplantation follow up is considerably longer. The decision to pursue allogeneic HCT is based on the type, prognosis, and remission status of the underlying disease, the performance status of the patient, and the availability of an appropriate donor. HLA matched siblings, and matched unrelated donors obtained through international registries are frequently used for allogeneic HCT. To establish donor-derived hematopoiesis, there must be a source of HSC and most commonly this is accomplished using unmanipulated marrow or G-CSF mobilized PBPC.

A major obstacle in allogeneic HCT is overcoming the immune competence of the recipient. The potential to reject infused donor cells is mediated predominantly through regimen resistant host T- and NK cells. Strategies to reduce host immunity and promote donor cell engraftment include the choice of chemotherapy and radiation used in the transplant preparative regimen, and post transplantation immune suppression medications. Donor T-cells in the allograft help promote hematopoietic engraftment as depletion of donor T lymphocytes from the graft before transplantation resulted in a substantial increase in the graft rejection rate. Investigational strategies to enhance donor cell engraftment include the use of monoclonal antibodies directed against T-cell determinants or HSC niches, antibodies conjugated with radioactive isotopes and infusions of subsets of donor immune cells that facilitate engraftment without promoting GVHD.

In contrast to autologous HCT, allogeneic transplantation is associated with NRM rates of 20-40%, and this number largely depends on recipient age, donor type and whether full dose or reduced intensity conditioning is used.

Graft-versus-tumor Effect

An important mechanism of cancer eradication following allogeneic HCT is the immunological-based recognition of residual host tumor cells by donor derived immune cells contained in the donor graft. This phenomenon is termed the graft-versus-tumor (GVT) effect and the lines of evidence supporting this concept are highlighted (Table 1).

TABLE 1. EVIDENCE FOR GRAFT-VERSUS-TUMOR EFFECT IN CLINICAL ALLOGENEIC HCT

Temporal association between immune suppression drug withdrawal and disease remission.
Leukemia relapse rate is lower after allogeneic HCT than syngeneic HCT.
Leukemia relapse is lower in patients who develop GVHD than those who do not.
Leukemia relapse rate is lower after allogeneic HCT without GVHD than syngeneic HCT.
Leukemia relapse rate is higher after TCD allogeneic HCT than after unmodified HCT.
DLI induce remission of leukemia that recurred after allogeneic HCT.1
Allogeneic HCT after RIC induce long lasting remission of leukemia and lymphoma.

DLI = donor lymphocyte infusion
GVHD = graft-versus-host disease
HCT = hematopoietic cell transplantation
RIC = reduced intensity conditioning
TCD = T-cell depleted

Tumor relapse is lower after allogeneic than after syngeneic HCT: Recipients of genetically identical (syngeneic) grafts have significantly higher rates of disease relapse compared to patients who receive grafts from HLA matched siblings. Studies revealed that the alloreactive GVT effect varied based upon disease type. CML appeared to have the greatest allogeneic effect because the risk of relapse was significantly higher among recipients of a syngeneic graft compared to patients who received their graft from an HLA matched sibling. There is less of a GVT effect with ALL as recipients of a syngeneic graft had relapse rates similar to recipients of an HLA matched sibling graft. These observations led to the concept that not all diseases are immunologically identical and that some cancers are better recognized by the donor immune cells than others.

Tumor relapse is higher in recipients of T-cell depleted grafts: The motivation for T-cell depletion was based on the expectation that removal of donor-derived T-cells would reduce the risk of GVHD. The finding that GVHD could be almost completely eliminated by successful T-cell depletion even in the absence of post transplantation immunosuppressive drugs was a major finding. The later observation that these patients had a much higher risk of disease recurrence as well as graft rejection was unanticipated. In these studies overall survival was not improved. These results linked GVHD with GVT effects, and supported the concept that patients who developed alloreactivity as manifested by clinically apparent GVHD, especially chronic GVHD, had a reduced risk of disease relapse.

Donor lymphocyte infusions can induce remissions: The most direct evidence for the GVT effect came from the application of donor lymphocyte infusions (DLI). The observation that patients who had disease relapse after allogeneic HCT could be returned to complete remission by the infusion of donor-derived lymphocytes was an exciting finding. Long-term follow up of patients successfully treated with DLI revealed that patients, who responded, had remarkably durable remissions and excellent outcomes. These observations established the GVT effect as a biologic entity capable of controlling an otherwise lethal condition such as leukemia.

Targets and effector cells in clinical GVT effect: The biologic basis for the GVT effect remains incompletely defined. The target structures which are immunologically recognized by the donor's immune effector cells have been proposed to be alloantigens, such as major and minor histocompatibility antigens, lineage specific antigens, and unique structures on the malignant cells, such as products of chromosomal translocations, or other specific markers of the malignant phenotype. The cells responsible for the GVT effect clearly include T-cells and there is emerging evidence that NK cells are also responsible for tumor cell control, especially following haploidentical transplantation. Humoral immunity has been implicated as playing a role in the GVT effect. A major question is whether the subset of T-cells that induce GVHD is the same T-cell population responsible for the beneficial GVT effect.

Transplant Preparative Regimens

Since the majority of autologous and allogeneic transplant procedures are performed in individuals with cancer, the transplant preparative regimen was designed, at least initially, to provide tumor cytoreduction and disease eradication. In the case of allogeneic HCT, the regimen needed to be sufficiently immune suppressive to overcome host rejection of the graft. A long-term goal remains the development of transplant procedures with reduced toxicities.

Total body irradiation (TBI) has been a primary therapeutic modality in autologous and allogeneic HCT regimens. TBI has excellent activity against a variety of hemato-lymphoid malignancies, has sufficient immunosuppressive properties and is able to treat sanctuary sites like the testicles and the central nervous system (CNS). Concern with TBI is its immediate toxicity on the gastrointestinal tract, liver and lungs and its long-term effects on impairment of growth and development in children, cardiovascular toxicity and secondary malignancies.

Patients with lymphoma may have received prior dose-limiting radiotherapy, often to the mediastinum, which resulted in a high incidence of fatal interstitial pneumonitis following TBI. Therefore, non-TBI containing transplant regimens were developed. The choices of drugs include agents that can be dose-escalated and have improved tumor cell killing at higher doses yet have non-overlapping toxicities.

Reduced Intensity Conditioning (RIC)

The demonstration that immunologic mediated mechanisms can control residual disease challenged the concept that relatively toxic full-dose chemoradiation is required for cure following allogeneic HCT. Thus, reduced intensity regimens were developed and these shifted the burden of tumor eradication to GVT effects. RIC is better tolerated than traditional full-dose regimens and is useful in older patients or in patients who have co-morbid medical conditions that preclude aggressive myeloablative regimens. Patients with relatively indolent disease that is in partial or complete remission may not require the immediate cytoreductive capacity of an aggressive preparative regimen and may be suitable candidates for transplantation using RIC. Less toxic regimens could also be extremely useful in patients with non-malignant diseases where the goal is strictly the establishment of donor hematopoiesis as the treatment effect; examples include genetic disorders, autoimmune diseases, or for the induction of tolerance in combined solid-organ and same donor marrow transplantation.

Despite RIC, clinical outcomes have remained limited by the risks of GVHD and non-relapse mortality. In an effort to reduce GVHD without compromising efficacy of GVT effects, investigators at Stanford developed a murine RIC model of bone marrow transplantation using fractionated total lymphoid irradiation (TLI) and anti-thymocyte globulin (ATG). In these models, TLI and ATS altered residual host T cell subsets to favor regulatory natural killer (NK) T cells that suppress GVHD by polarizing donor T cells toward secretion of non-inflammatory cytokines such as interleukin-4 and by promoting expansion of donor $CD4^+CD25^+FoxP3^+$ regulatory T cells. This skewing of host T cell subsets was a result of NK T cell resistance to radiation-induced apoptosis due to increased expression of anti-apoptotic genes. The favorable ratio of host NK T cells did not affect donor $CD8^+$ T cell cytolytic function and graft anti-tumor activity was preserved. This murine model was translated to clinical transplantation where cancer patients who received TLI and ATG were infused with grafts from HLA matched related and unrelated donors. The majority of patients achieved donor-derived hematopoiesis and TLI and ATG was associated with a very low incidence of GVHD and non-relapse mortality. GVT effects appeared maintained as many patients with residual disease at the start of HCT had similar outcomes to patients in complete remission (Figure 1).

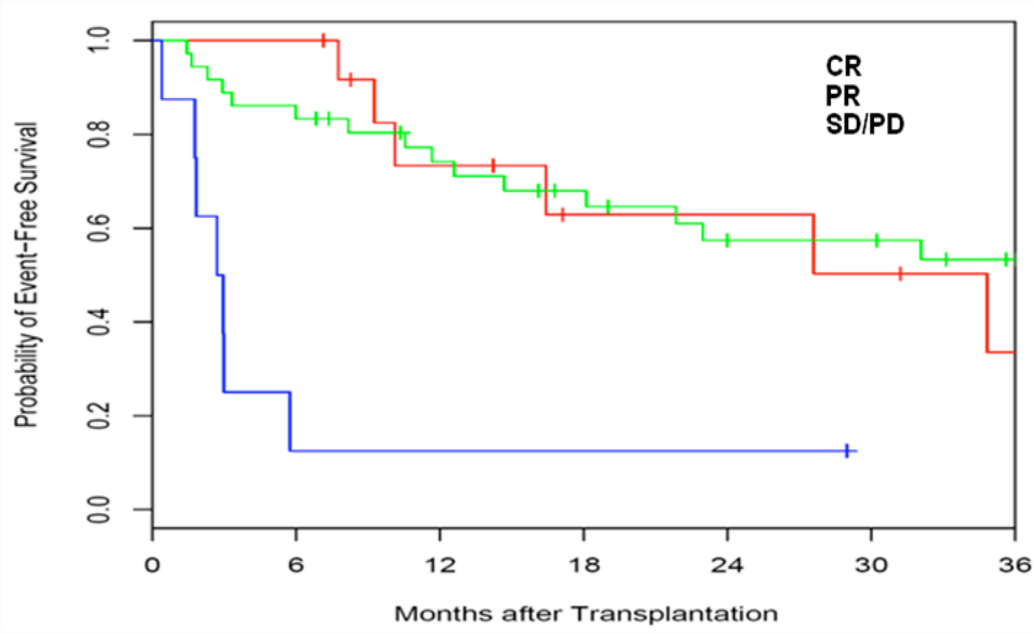

Figure 1: Probability of event-free survival following TLI and ATG conditioning and allogeneic HCT from matched related and unrelated donors in 64 patients with lymphoid malignant diseases stratified according to disease status at the start of HCT: CR = complete remission, PR = partial remission and SD/PD = stable or progressive disease.

Clinical Transplantation

Table 2 is a list of the diseases treated with HCT. The results obtained after transplantation depend on many factors; at times patients undergo transplantation with the expectation of <10% likelihood for a positive outcome, yet in other circumstances a >85% likelihood of a good outcome can be anticipated.

TABLE 2. LIST OF DISEASES TREATED BY HEMATOPOIETIC CELL TRANSPLANTATION

Disease/Condition	Allogeneic HCT	Autologous HCT
Malignant Disease		
Acute myelogenous leukemia	+	+
Acute lympoblastic leukemia	+	-
Chronic myelogenous leukemia	+	+
Chronic lmyphocytic leukemia	+	+
Myelodysplatic syndromes	+	-
Myeloproliferative syndromes	+	-
Non-hodgkin lymphoma	+	+
Hodgkin lymphoma	+	+
Myeloma	+	+
Amyloidosis	-	+
Waldenstrom's macroglobulinemia	+	+
Hairy cell leukemia	+	-
Selected solid tumors	-	+
(testicular cancer, pediatric tumors)	-	-
Neuroblastoma	-	+
Non-malignant Diseases		
Acquired aplastic anemia	+	-
Congenital pure red cell aplasia	+	-
Fanconi anemia	+	-
Thalessemia	+	-
Sickle cell anemia	+	-
PNH	+	-
SCID	+	-
Wiskott-Aldrich	+	-
Congenita leukocyte dysfunction	+	-
Osteopetrosis	+	-
FEL	+	-
Glanzmann disease	+	-
Hereditary storage diseases	+	-
Selected autoimmune diseases	+	+

FEL = Familial erythrophagocytic lymphohistiocytosis
PNH = paroxsysmal nocturnal hemoglobinuria
SCID = severe combined immunodeficiency

A list of the transplant related complications is shown in Table 3. The first 100 days following the cell infusion is typically the time of greatest risk for recipients of autologous and allogeneic HCT. Care by a team skilled in the management of patients undergoing these procedures is of critical importance. Research and progress in overcoming the transplant related complications is critically important in improving overall outcomes.

TABLE 3. COMPLICATIONS OF HEMATOPOIETIC CELL TRANSPLANTATION

Vascular access complications
Graft failure
Blood group incompatibilities and hemolytic complications
Acute GVHD
Chronic GVHD
Infectious Complications
Bacterial Infections
Fungal Infections
Cytomegalovirus Infection
Herpes Simplex Virus Infections
Varicella-zoster Virus Infections
Epstein-Barr virus Infections
Adenovirus, Respiratory Viruses, HHV-6,-7,-8 and other viruses
Gastrointestinal Complications
Mucosal ulceration/bleeding
Nutritional support
Hepatic Complications
Sinusoidal obstructive syndrome
Hepatitis: infectious versus non-infectious
Lung Injury
Interstitial pneumonitis: infectious versus non-infectious
Diffuse alveolar hemorrhage
Engraftment syndrome
Bronchiolitis Obliterans
Kidney and Bladder Complications
Endocrine Complications
Drug-Drug Interactions
Growth and Development
Late Onset Nonmalignant Complications
Osteoporosis/osteopenia, avascular necrosis, dental problems, cataracts, chronic fatigue, psychosocial effects and rehabilitation
Secondary Malignancies
Neurologic Complications
Infectious, transplant conditioning and immune suppression medication toxicities

HHV = human herpes virus subtypes

Disease relapse following autologous and allogeneic HCT is an ominous clinical event. Every effort is made to carefully document relapse as it is common for patients to have residual radiographic abnormalities following transplantation, especially patients with lymphoma. Patients with multiple myeloma have a gradual reduction in the paraprotein level that may take several months after transplant to reach maximal response. And following RIC, the allogeneic GVT effects may take months to result in tumor eradication, and it may be difficult to distinguish slowly regressing disease from slowly progressive disease. Therefore, because patients are sensitized to the possibility of disease relapse, clear and unequivocal documentation is required prior to declaring a patient has in fact relapsed. Areas of research are the development of sensitive markers of tumor burden and strategies to enhance immune mediated anti-tumor reactions in the autologous and allogeneic HCT setting.

Future Directions

HCT represents the best example of the successful use of cell-based therapeutics applied to clinical medicine to treat human diseases.

Understanding the mechanisms and targets of GVT reactions continues to be an area of intense interest. These immunologic reactions are complex; not only are T cell populations responsible for the effector phase, but also other T cell populations regulate these responses. The concept of using regulatory T cells to control adverse effects such as GVHD has been successful in murine models and holds significant promise in its application to the treatment of patients.

Other defined cellular populations, cytokines, and vaccination strategies show promise in reducing the risk of disease relapse following autologous and allogeneic HCT. In addition, improved strategies to reduce the risk of acute and chronic GVHD could enable extension of allogeneic HCT beyond the treatment of patients with hematologic malignancies. For example, animal models of autoimmune diseases demonstrated that these disorders can be effectively treated with HCT and preliminary evidence in patients is supportive. In addition, animal studies demonstrated that combined same donor organ and marrow transplantation resulted in tolerance to the transplanted organ. Early reports of this work in humans suggest that extension of allogeneic HCT to these and other disease settings could provide new therapeutic concepts to improve outcomes for patients with diverse clinical problems.

The near future holds that rather than transplant all the harvested cells, clinicians and researchers in the field will learn to isolate the cell type(s) needed for optimal results which will differ depending on the disease and the outcome that is being sought.

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Questions to Consider

Which is true of alloreactive graft-versus-tumor reactions?

- **A.** the intensity of the GVT effect is dependent somewhat on the tumor type
- **B.** is mediated by donor derived T cells but not NK or B cells
- **C.** is the main mechanism of cure following RIC transplantation
- **D.** answer A and C are true
- **E.** all of the above are true

Cure of disease following autologous HCT:

- **A.** is mediated by immune cells in the graft
- **B.** is largely dependent on the tumor having a rising dose-response curve
- **C.** is likely improved if effective tumor purging strategies are developed
- **D.** answer B and D are true
- **E.** all of the above are true

Graft source for HCT:

- **A.** G-mobilized PBPC grafts contain about 10 fold more T cells than a BM graft
- **B.** G-mobilized PBPC is the preferred product for autologous HCT
- **C.** future products will likely involve sorting for selected effector cell populations
- **D.** answer A and B are true
- **E.** all of the above are true

Adoptive Cellular Therapy

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Introduction

Allogeneic, or donor-derived, hematopoietic cell transplantation (allo HCT) is a potentially curative therapy for hematolymphoid malignancies, including leukemia and lymphoma. HCT was originally envisioned as a means to rescue patients from the most significant toxicity of high-dose chemotherapy and radiotherapy, namely bone marrow aplasia. It was subsequently appreciated that donor immune cells are capable of recognizing and destroying residual malignant, a phenomenon termed the graft-versus-tumor (GVT) effect. This observation, originally made in preclinical studies, is supported by various lines of clinical evidence. First, graft versus host disease (GVHD), the major toxicity of allo HCT, is mediated by donor lymphocytes and is associated with a reduced risk of relapse. A corollary is the reduced rate of GVHD and higher rate of relapse observed in patients with T-cell depleted grafts. For relapse after allo HCT, remission may sometimes be induced by withdrawal of immune suppression. Interestingly patients who receive transplants from syngeneic (i.e. identical twin) donors experience higher relapse rates than patients with HLA-matched, non-syngeneic sibling donors. Finally, and perhaps most persuasively, relapse after HCT may be treated with donor lymphocyte infusion. The main challenge in clinical transplantation is harnessing GVT while minimizing GVHD.

Advances in the phenotypic characterization of immune effector cells, combined with increasingly facile approaches to cell separation by multiparametric flow cytometry and immunomagnetic bead sorting, has enabled the isolation of defined subpopulation of lymphocytes for the purpose of adoptive transfer. Successful adoptive immunotherapy requires methods to generate sufficient numbers of cells without sacrificing effector function. Treatment, or conditioning, of the recipient is also necessary for immunosuppression to prevent rejection and to empty a niche in which transferred lymphocytes may engraft and expand. Numerous potential applications exist for adoptive immunotherapy. For the purpose of this discussion, I will focus on the treatment of hematolymphoid malignancies and the prevention of GVHD.

Donor Lymphocyte Infusions (DLI)

As noted above, relapse of leukemia after allogeneic HCT may be treated by DLI. This approach is most effective for patients with Chronic Myelogenous Leukemia (CML) who have relapsed in chronic phase after HCT where up to 60-70% of patients respond. For patients with Acute Myeloid Leukemia (AML) or Acute Lymphoblastic Leukemia (ALL), the success rate for DLI is less than 20%. DLI is associated with a risk of GVHD approaching 60%. Given this high risk/benefit ratio, there is ample opportunity to improve DLI.

T cells mediate both GVT and GVHD effects. T cells express clonotypic antigen receptors (T cell receptor, TCR) that recognize peptides bound by Major Histocompatibility Class (MHC) antigens. $CD8^+$ T cells are restricted to antigens bound by Class I antigens and are cytotoxic whereas $CD4^+$ cells are restricted by Class II antigens and influence immune responses by the elaboration of cytokines. The immune response observed in MHC matched allo HCT is stimulated by mismatched minor histocompatibility antigens (MiHA). MiHA presentation by recipient, and to a lesser extent donor, antigen presenting cells (APC), is key to the initiation of GVHD.

Subsets of T cells may have differential importance for the initiation and maintenance of GVT and GVHD. In mouse models, both $CD4^+$ and $CD8^+$ are necessary for GVHD development, but CD4 cells alone are sufficient to mediate a GVT effect. Clinical trials have confirmed that CD8 depleted grafts are associated with lower rates of acute GVHD with equivalent relapse rates as non-T cell depleted grafts. In some trials, CD8-depleted DLI was equally efficacious as unmanipulated DLI and conferred a lower risk of GVHD.

One strategy to control GVHD after DLI is the introduction of suicide genes into donor lymphocytes. Suicide genes, (eg the HSV thymidine kinase), encode proteins that render a transduced cell susceptible to the toxic effects of a drug (eg ganciclovir) that is otherwise non-toxic to the recipient. This approach has shown clinical efficacy but suffers from significant limitations, including the immunogenicity of the exogenous protein as well as the need for high purity of transduced lymphocytes.

Knowledge of immunogenic epitopes on target cells is limited, but growing. It is assumed that the frequency of antitumor T lymphocytes is low in unmanipulated donor lymphocyte collections. Viral transduction of tumor-specific T cell receptors and chimeric antigen receptors is feasible and is a promising approach to increasing the specific activity of DLI. Numerous clinical trials have demonstrated the safety of genetically-modified lymphocytes, but there remains an appropriate concern about insertional mutagenesis that limits its clinical applicability.

Haploidentical Transplantation and NK Cells

Many patients in need of transplantation lack a suitably HLA-matched donor. Most of these patients have a sibling, parent, or child who share one HLA haplotype. Transplantation of unmanipulated grafts from such haploidentical donors carries an unacceptable high rate of GVHD and thus are typically depleted of T cells. In HLA-matched transplantation, T-cell depletion is associated with a higher risk of relapse. However, in the setting of haploidentical transplantation, a GVT effect may be mediated by cytotoxic $CD3^{-}CD56^{+}$ Natural Killer (NK) cells. Unlike T and B cells, NK cells do not rearrange antigen receptor genes. Rather, they express a number of activating receptors, including the immunoglobulin Fc receptor CD16, the mediator of Antibody-Mediated Cellular Cytotoxicity (ADCC). Another well-studied NK activating receptor is NKG2D. This non-polymorphic lectin recognizes ligands that are upregulated by ionizing radiation, chemotherapy, and infection.

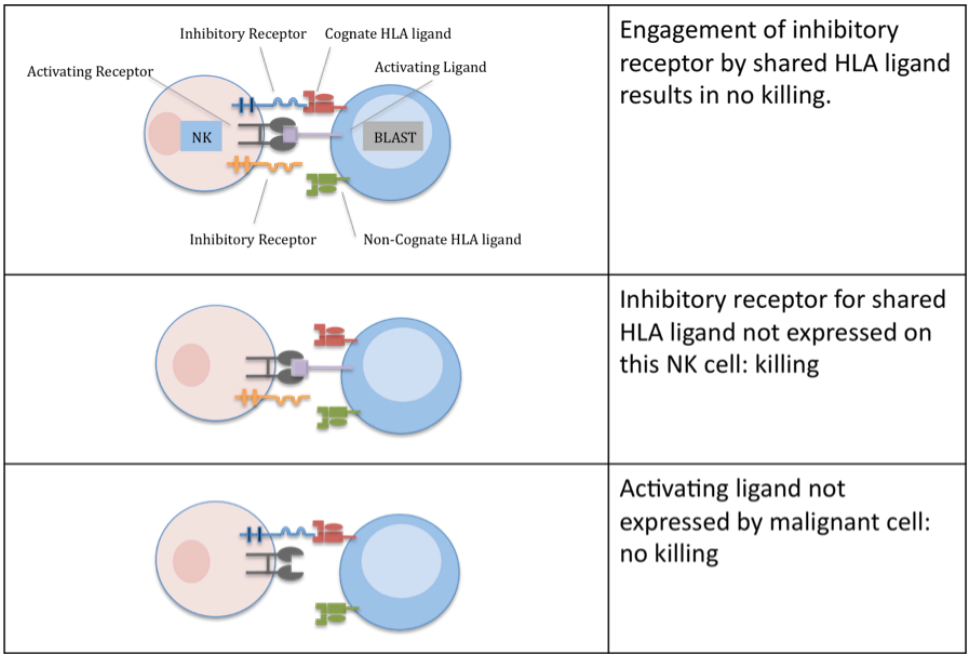

Figure 1: NK cell target recognition of haploidentical leukemic blasts is negatively regulated by inhibitory receptors for self-HLA molecules. Expression of ligands for activating NK cell receptors renders the blast susceptible to NK cytolysis.

NK cell target lysis is not, strictly speaking, restricted by target MHC antigens. Rather, NK cells express inhibitory receptors for self-MHC molecules. Triggering these receptors, known in human as inhibitory killer immunoglobulin receptors (KIRs), counteracts the signals from activating receptors. Multiple KIR genes exist, each with different ligand specificities. Moreover, KIR genes are expressed stochastically, generating a diverse NK cell repertoire. With certain haploidentical donor-recipient pairs, donor NK lack inhibitory KIRs for the recipient's mismatched Class I molecules, resulting in cytolysis. This type of

alloreactivity is termed "missing self recognition." Pioneering data from the group from the University of Perugia, Italy has demonstrated a potent GVT effect when patients with myeloid malignancies receive T-cell depleted grafts from KIR-ligand mismatched donors. Lymphoid malignancies were less susceptible to this effect, for unknown reasons. Rates of acute GVHD were low, but a great number of patients suffered from uncontrolled infections due to the absence of T cell reconstitution.

Many groups have attempted to harness "missing self recognition" by the adoptive transfer of purified NK cells. NK cells constitute 5-10% of peripheral blood lymphocytes and may be isolated from donor apheresis samples using magnetic bead sorting under GMP conditions and infused either with minimal ex vivo manipulation or else expanded and activated with cytokines or feeder cells. As with adoptive transfer therapies with other lymphocyte subsets, post-infusion expansion depends on recipient lymphopenia and may also require high levels of cytokines such as Interleukin-15. Durable persistence of adoptively transferred NK cells has not been demonstrated. Interestingly NK cells do not cause GVHD.

The antitumor activity of adoptively transferred NK cells may be augmented by pretreating recipients with agents that upregulate ligands for activating NK cell receptors such as NKG2D. FDA-approved agents with such activity include histone deacetylase inhibitors, proteosome inhibitors, and hypomethylating agents.

Cytokine Induced Killer (CIK) Cells

CIK cells are a subset of $CD3^{+}CD56^{+}$ cells that circulate at low frequency but may be expanded in vitro with mitogenic antibodies and cytokines. Like NK cells, they kill diverse tumor targets without MHC restriction, often in an NKG2D-dependent mechanism. KIR inhibition does not appear to be operative in CIK allorecognition. Both in vitro and in vivo models have demonstrated that adoptively transferred CIK cells can eliminate malignant leukemias and carcinomas. CIK cells do not appear to cause GVHD and thus are being tested as a treatment and prevention of leukemic relapse.

Regulatory T Cells as a Means to Control GVHD

The mammalian immune system has evolved regulatory networks to prevent pathological autoimmunity. Notably, a subpopulation of CD4+ T cells that constitutively express CD25 and the transcription factor FOXP3 can suppress the activation of conventional T cells (Tcon). Suppressive activity of these regulatory T cells (Tregs) has been attributed to secreted as well as contact-dependent factors. Tregs do not cause GVHD when transplanted across MHC barriers and, in preclinical models, depletion of Tregs may exacerbate GVHD. In the clinical context, poor reconstitution of Tregs after allo HCT is associated with increased risk of GVHD. In mice, cotransplantation of Tregs with Tcons suppresses GVHD without attenuating the GVT effect. Ongoing clinical trials involve infusion of Tregs with Tcon after T-cell depleted haploidentical transplantation in order to restore anti-microbial as well as anti-tumor immunity while minimizing GVHD.

Cell population	Phenotype	Frequency in Human PBMC
T	CD3+	50-80%
	CD3+CD4+	30-60%
	CD3+CD8+	10-40%
NK	CD3-CD56+	5-20%
CIK	CD3+CD56+	<1%
Treg	CD4+CD25+FOXP3+	<2%

Conclusions

It is anticipated that allogeneic HCT will be a safer and more effective therapy when grafts are routinely enriched for antitumor lymphocytes and purged of broadly alloreactive cells. Advances in cell culture techniques and genetic manipulation will likely compensate for the loss of cell yield that occurs with more elaborate cell purification strategies. Among the near-term priorities in this field is the development of sensitive assays in humans to monitor donor cell persistence and trafficking.

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Questions to Consider

Graft versus Host Disease:

- **A.** Occurs only when donor and recipients are HLA mismatched
- **B.** Is initiated by presentation of recipient antigens by donor APC
- **C.** Is dependent on Class II restricted donor NK cells
- **D.** Requires the presence of a functional thymus in the recipient

NK Cells recognize target cells via:

- **A.** Clonotypic MHC-restricted receptors
- **B.** IgD receptor
- **C.** Combinations of inhibitory and activating receptors
- **D.** C5a

Relapse after allo HCT is treated by all of the following EXCEPT:

- **A.** Chemotherapy
- **B.** Withdrawal of immune suppression
- **C.** Infusion of unmanipulated donor lymphocytes
- **D.** Infusion of purified Treg

When compared with unmanipulated grafts, T-Cell Depleted grafts are associated with all of the following EXCEPT:

- **A.** Lower incidence of acute GVHD
- **B.** Delayed immune reconstitution
- **C.** Higher incidence of relapse
- **D.** Lower incidence of graft rejection

Challenges and Opportunities of Dendritic Cell Therapy

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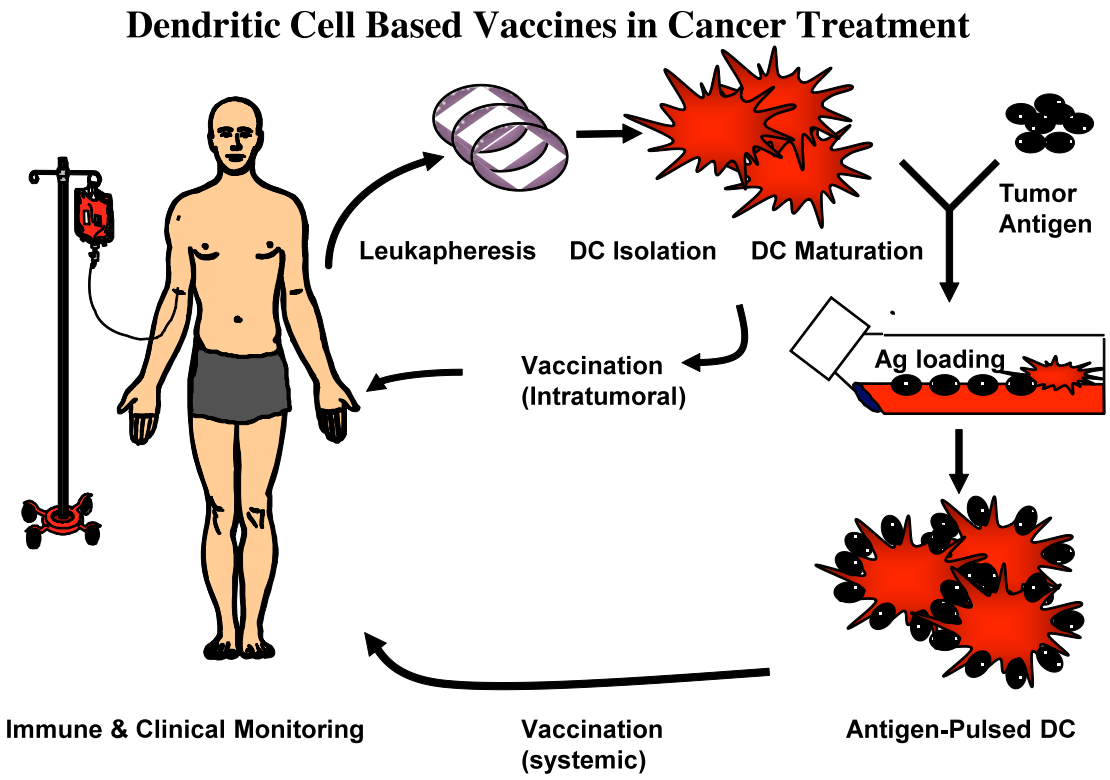
The underlying rationale for active immunotherapy, or vaccination, for the treatment of cancer includes evidence that T cells in tumor-bearing patients can recognize and respond to tumor-associated antigens (TAA) (1), and infiltration of certain human cancers with cytotoxic T cells correlates with improved survival (2). Tumors, however, employ a variety of mechanisms to evade 'immune surveillance' such as the accumulation in the tumor itself of T regulatory cells and soluble factors that prevent T cell and dendritic cell (DC) activation (reviewed in 3 and 4).

Efforts to overcome immune "tolerance" to tumors have focused in part on DCs, based on their unmatched ability to stimulate both the innate and cognate arms of the immune system (5). These antigen (Ag) presenting cells normally reside in peripheral tissues where they continuously sample the environment in search of Ags or organisms that are perceived as threats. When stimulated in the presence of TAA and an appropriate "danger signal" (6), DCs not only take up and process these Ags but also undergo maturation and then migrate to the draining lymph nodes where they present the processed Ags to T cells. This results in activation and clonal expansion of Ag specific T helper and cytolytic cells, believed to be key effectors in immune mediated tumor destruction.

On the basis of their unique ability to present Ags and stimulate naïve T cells, DCs are often referred to as "nature's adjuvant" and once it became practical to isolate these cells from peripheral blood precursors, we and others decided to explore their potential utility in tumor immunotherapy. The first DC vaccine trial was performed at Stanford in patients with B cell lymphoma who had failed conventional treatment. In that trial, immature DCs were obtained from the circulation of cancer patients, loaded with tumor specific immunoglobulin idiotype protein (Id) and keyhole limpet hemocyanin as a helper stimulus, and matured *in vitro* (thereby avoiding tumor associated immunosuppressive factors that inhibit DC activation) before being returned to the patients. Despite their advanced disease and the relatively small number ($<5x10^6$) of DCs used per infusion, all four vaccinated patients developed T cell responses against tumor Id and two of the patients went into complete clinical remission (7). Since that first pilot trial we have studied DC vaccination in several types of cancer, including prostate cancer, multiple myeloma, and colorectal cancer, with varying degrees of success. In one of the most interesting of these studies recombinant human Flt3-Ligand (FL) was used to generate large numbers of circulating DCs in patients with metastatic colon cancer. The DCs were then isolated, pulsed with a synthetic peptide derived from carcinoembryonic antigen (CEA), and activated *in vitro* prior to their return to the patients. Remarkably, vaccination with these cells resulted in a significant expansion of CEA specific CD8+ T cells in about half of the patients, and the extent of expansion correlated with clinical responses, including two patients with dramatic tumor regression (8).

Following these early trials, DC vaccines have been evaluated in a wide range of cancers by investigators around the world. To date all of these trials have involved generation and/or manipulation of DCs *in vitro*, followed by injection of the autologous, Ag loaded cells into patients. The diagram below outlines the general steps involved in the preparation of DC vaccines. Unfortunately, the cell isolation, loading and activation methodologies, choice of TAA and route and frequency of administration, have differed widely, which may explain the variable clinical results seen in these trials. In addition, it is now known that DCs prepared using conventional methods include cells that induce immune tolerance as well as cells that induce pro-inflammatory immunity. In addition, the injected DCs may undergo phenotypic and functional modification in vivo. Nonetheless, a DC vaccine for metastatic prostate cancer recently completed phase 3 clinical trials and the data from these trials indicate a significant survival benefit in the patients receiving the vaccine compared to control subjects (9). A decision from the US Food and Drug Administration on whether to license this vaccine is expected this year.



A potentially more efficient and practical approach to cancer immunotherapy would be to load and activate DCs, *in vivo*. Our first effort to develop such an *in vivo* targeting approach utilized FL to increase the number of DCs in accessible sites (e.g., skin and subcutaneous tissue) followed by injection into these sites of a mixture of a TAA and a toll-like receptor (TLR) agonist, CpG. This procedure has proven effective in a wide range of murine tumor models (10), and CpG and other TLR agonists are now being studied in clinical trials in a wide range of tumors. We have also begun to explore the use of chemokines as a means of attracting circulating DCs to tumors, *in situ*. Combinations of chemokines and TLR agonists have also been evaluated. Initial results of such studies have been encouraging (11). Another DC targeting approach has been developed by Bonifaz and colleagues who showed that conjugates of a TAA and monoclonal antibody directed at a DC surface receptor (the C-type lectin rich endocytic receptor DEC-205) can result in potent specific immune activation or suppression (12). In combination, these preclinical studies indicate the feasibility of targeting and activating DCs *in vivo*.

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Questions to Consider

Which of the following may explain the inconsistent results seen in different dendritic cell clinical trials?

A. Different methods of DC preparation/activation

B. Choice of tumor-associated antigen

C. Different routes of administration

D. All of the above

Dendritic cells are believed to induce an immune response mainly through which of the following mechanisms?

A. Presentation of antigens to T lymphocytes

B. Inhibition of Treg cells

C. Direct inhibition of tumor cell growth

D. Induction of antigen-specific antibodies

Pharmaco-genomics and Proteomics of Cancer

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Several hundred small molecules that target signaling proteins are currently being tested in preclinical models of cancer and in the clinic. Despite this tremendous effort, the number of new drugs that showed clear survival benefit remains astonishingly low. In 2009, only two new molecules were approved for treatment of solid tumors (everolimus and pazopanib), both for targets and indications that had already been validated (mTOR and VEGF-R,respectively, both in renal cancer) : this reflects the tremendous challenge of treating cancer with single agents, as well as the challenge of developing agents that are both safe and effective. Experience with GleVec, Tarceva, Sorafenib and PLX 4032 illustrate some of the issues that have been encountered in targeted therapy.

Glevec (Imatinib) targets the BCR-ABL oncogene and is highly effective in the early stage of CML. This is because it prevents production of progenitor leukemia cells from leukemic stem cells. However, it is far less effective in late stages of the disease (blast crisis), because leukemic cells emerge that contain drug-resistant mutations, usually in the BCR-ABL gene. Leukemic cells remain dependent on BCR-ABL, but can no longer bind drug (1). It is remarkable that in CML blast crisis, leukemic cells still depend strongly on BCR-ABL for survival, despite multiple additional mutations and gene copy number changes. These cells are indeed addicted to the oncogenic driver, even at late stage of the disease.

Treatment of patients with CML and analysis of drug resistance has led to new a paradigm in kinase-targeted drug development: the importance of class I and class II kinase inhibitors. Class I inhibitors are ATP-competitive and interact with the active configuration of the kinase. Class II inhibitors stabilize the inactive form. Both block kinase activity effectively. GleVec is a type II inhibitor. Study of mutations that confer drug-resistance to GleVec have identified as many as 20 point mutations have been identified that prevent drug binding inhibition, yet allow the BCR-ABL protein to function effectively as a kinase. In contrast, very few mutations in BCR-ABL confer resistance to a class I inhibitor such as desatinib. Clearly, the closer a compound resembles ATP, the fewer mutations can emerge that fail to bind the drug, yet retain ATP-binding and kinase activity. For these reasons, ATP-competitive compounds may be preferable, since drug-resistance is a major downfall of targeted therapy.

Tarceva targets EGF-R, and non-small cell lung cancers expressing an activated mutant form of EGF-R show dramatic clinical responses. However, as in CML blast crisis, drug-resistant clones quickly emerge and patient benefit is short lived. In this situation, as in blast crisis CML, a second drug that hits the same target is likely to be effective, as most tumors remain EGF-R dependent. Lung cancer patients with high copy numbers of wild type EGF-R appear to show survival benefit, though few short-term clinical responses are observed (2). Tumors from these patients seem able to re-wire their signaling networks and adapt to the presence of drug. This form of resistance is distinct from classical drug-resistance and shows that these tumors are not addicted to the target, and have the flexibility and heterogeneity to survive when the target is shut down. Lung cancer patients with mutant K-Ras show little response to EGF-R inhibitors, and, in the presence of chemotherapy, progress more quickly following treatment. Similarly, in glioblastoma, loss of the tumor suppressor PTEN leads to up-regulation of the PI kinase, and loss of clinical activity of EGF-R inhibitors. Attempts to develop drugs that block the PI kinase pathway are underway. The first of these targets mTOR, a downstream protein complex in the PI kinase pathway. Blocking mTOR paradoxically increases activity of the upstream kinase AKT, through loss of a negative feedback loop. Two drugs may therefore be needed to shut this pathway down effectively.

The Ras/MAPK pathway is activated in many tumors, by mutations in Ras itself or by activation of BRAF. Sorafenib, a Raf kinase/VEGF-R2 inhibitor, has been approved for treatment of advanced hepatocellular carcinoma (HCC), as well as renal cancer. In HCC, sorafenib is more effective against tumors with high levels of P-ERK, consistent with its ability to inhibit Raf kinase activity (3). While

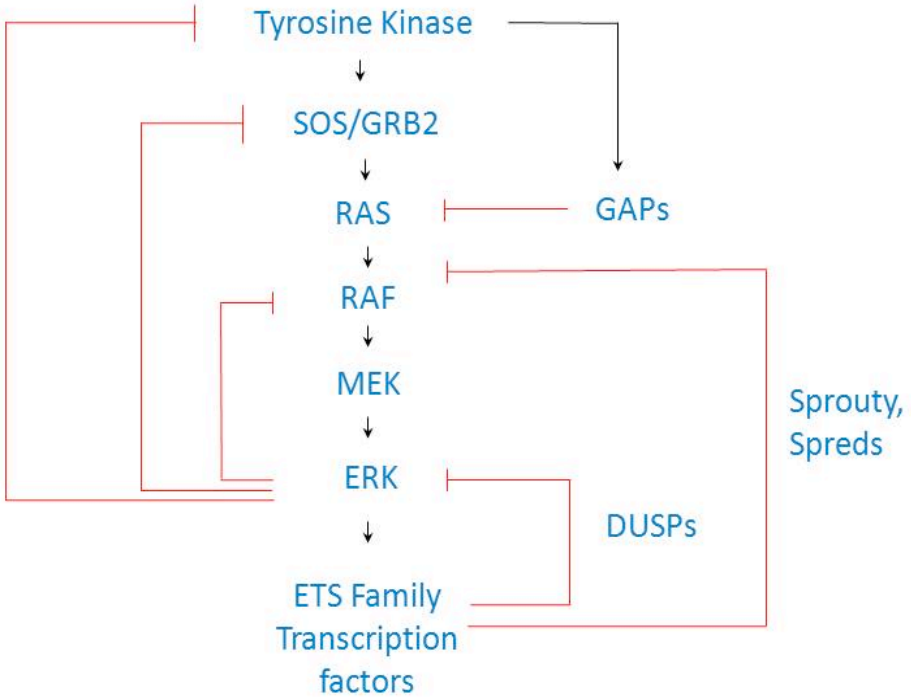

Figure 1. Feedback loops in the Ras MAPK pathway. Blocking MEK activity leads to hyper-activation of upstream elements, including Raf kinase, Sos and EGF-R, with important consequences for therapy. HYperactivation of the pathway leads to induction of MAPK phosphatases (DUSPs) and Sprout/Spred proteins that modulate signaling from Ras to Raf.

HCC do not typically contain activating mutations in RAS or RAF genes, high levels of Raf kinase signaling may be due to loss of function of Sprouty-related proteins that normally provide negative feedback inhibition to the pathway (4). On the other hand, tumors with activated RAS genes have not shown any clinical response to sorafenib. In cell culture, MEK inhibitors seemed like a more effective approach to shutting down this pathway. However, these compounds have, so far, proven ineffective in human cancers as single agents. Feedback loops that regulate the Ras/Raf/MAPK pathway at multiple levels have been identified: ERK phosphorylates receptor tyrosine kinases, Sos, and Raf kinases, for example (Figure 1). When MEK activity is blocked, loss of feedback hyper-activates upstream elements, with important consequences for therapy. Thus, inhibition of MEK activity leads to hyper-activation of Raf kinase and EGF-receptor signaling. These feedback effects are likely responsible for the loss of efficacy of MEK inhibitors in vivo, despite promising preclinical data based on cell lines and mouse models.

The discovery of activated alleles of BRAF in malignant melanoma has changed the prognosis of this disease dramatically. Plexxikon's PLX 4032 is class I BRAF inhibitor, that is selective for the active V600E allele, provides significantly improved clinical outcomes for the majority of melanoma sufferers (5), though progression usually occurs within a year. In addition, complications have arisen, such as the emergence of keratoacanthomas and squamous cell carcinoma in response to drug. This appears to be an on-target effect, because similar lesions have appeared in patients treated with other Raf kinase inhibitors, such as sorafenib (6). This surprising and unwanted side effect may be due to the observation that, paradoxically, RAF inhibitors can lead to hyper-activate RAF kinase activity, suggesting the presence of a feedback system driven by RAF itself (Hall-Jackson et al, 1999). This is because of cross-talk between CRAF and BRAF. One the on hand, CRAF is an inhibitor of BRAF, (7). RAF inhibitors like sorafenib, which are more potent inhibitors of CRAF than BRAF, can block CRAF effectively, but this leads to BRAF activation and increased signaling downstream. Likewise, BRAF-specific inhibitors like PLX 4032, leads to CRAF activation, allowing pathway activation in normal cells (Figure 2).

While CRAF inhibition activates BRAF in normal cells, and vice versa, MEK inhibition can shut down signaling from both kinases. This difference may explain why PLX 4032 appears to be more effective in treating melanoma than MEK inhibitors. High doses of PLX 4032 and, presumably, are well tolerated because the MAPK pathway is not shut down in normal cells. MEK inhibitors, on the other hand, shut the pathway down in normal cells and may therefore lead to on-target side effects that limit dosing (Neal Rosen, personal communication). This provides another rationale for targeting Raf rather than MEK, and tells us that it is essential to understand fully the biochemical properties of proteins being targeted for therapy, as well as the pathways and feedback loops that they regulate.

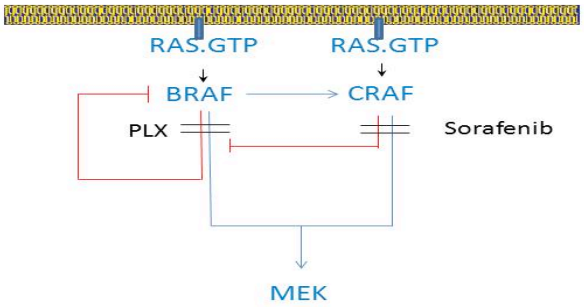

Figure 2. Cross-talk between BRAF and CRAF. Heterodimers of BRAF and CRAF are induced by active Ras in the plasma membrane. CRAF inhibits BRAF, and vice versa, so that drugs selective for one of the two isoforms, leads to hyper-activation of the other (Ref 7 and 8).

In conclusion, genetic alterations have been widely used to pinpoint pathways that are essential to cancer cells and to identify therapeutic targets. Mutant proteins can escape from targeted therapy through second-site mutations that prevent drug binding. Furthermore, the pathways that these mutant proteins regulate involve complex feedback loops and cross talk: therapeutic intervention changes these relationships and creates new networks that can escape therapy: in many cases, a second drug that hits a different target may be necessary to prevent these adaptive responses.

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Questions to Consider

Patients with late-stage CML fail on imatanib because:

- **A.** leukemic cells become less dependent on BCR-ABL
- **B.** second-site mutations in BCR-ABL prevent imatinib binding
- **C.** imatinib induces new mutations that make cells less responsive

MEK inhibitors have failed (so far) in BRAF driven melanoma because:

- **A.** doses that shut down MEK in tumor cells are too toxic to normal cells
- **B.** feedback loops overcome their effects by activating upstream pathways
- **C.** these tumors are MEK-independent

EGF-R inhibitors are most effective:

- **A.** In tumors with amplified EGF-R
- **B.** In tumors with mutant K-Ras
- **C.** In tumors with activated EGF-R

Cancer Pharmacogenomics

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Stanford University

Pharmacogenomics is the study of how variation in drug response is related to variation in genetics. Cancer pharmacogenomics is widely considered to be a major initial application of pharmacogenomics because the prescribing decisions about cancer are high-stakes. However, cancer pharmacogenomics is particularly difficult because there are at least two genomes involved: the baseline patient genome and the genome of the cancer (which itself may be a protean mixture of genomes).

In this talk, I will highlight the basic principles of pharmacogenomics, and provide an introduction to the PharmGKB (http://www.pharmgkb.org/, summarized in Nat. Genetic. 2007 39(4) p 426). We will then examine a few critical examples of cancer pharmacogenomics in order to illustrate the principles of the field. The examples will include:

1) Use of 6-mercaptopurine in leukemia and autoimmune diseases and variation in its metabolism based on polymorphisms in the thiopurinemethyltransferase (TPMT) gene. The resulting drug response may include aplastic anema in cases where patients have inactive TPMT variants. The polymorphisms are primarily in the coding regions of the gene.

2) Use of irinotecan in gastric and other tumors and variation in side effects base on polymorphisms in UDP glucuronosyltransferase 1A1 (UGT1A1). The resulting drug adverse effect is life-threatening diarrhea, and the polymorphism is in the promotor region of the gene.

3) Use of trastuzumab (Herceptin) in HER2-positive breast cancers. Some breast cancers express the HER2 protein and are rendered sensitive to blocking HER2 with antibody and small-molecule based interventions. These cancers show increased responsiveness to treatment. HER2 genotyping was one of the first approved genetic tests associated with approval of a drug by the FDA.

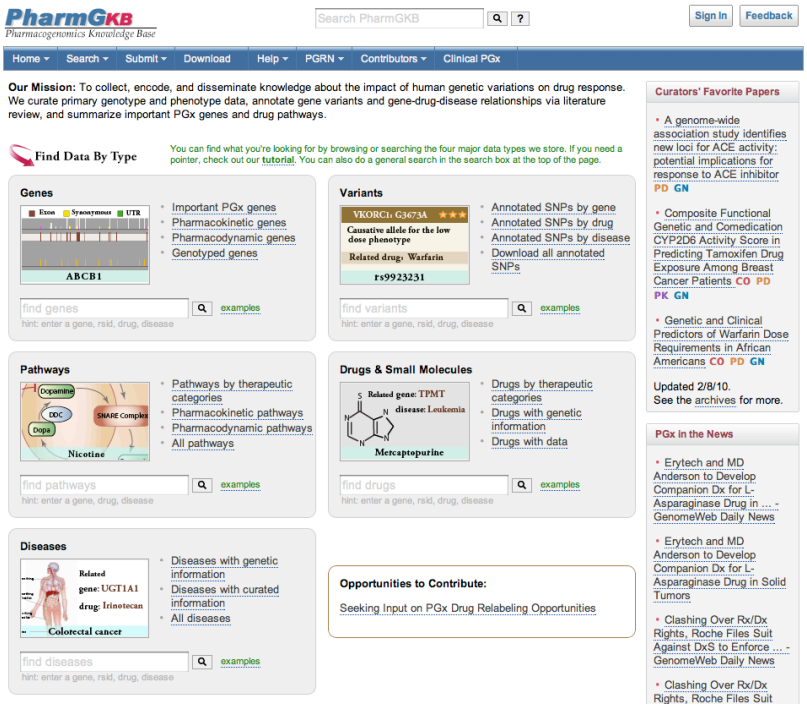
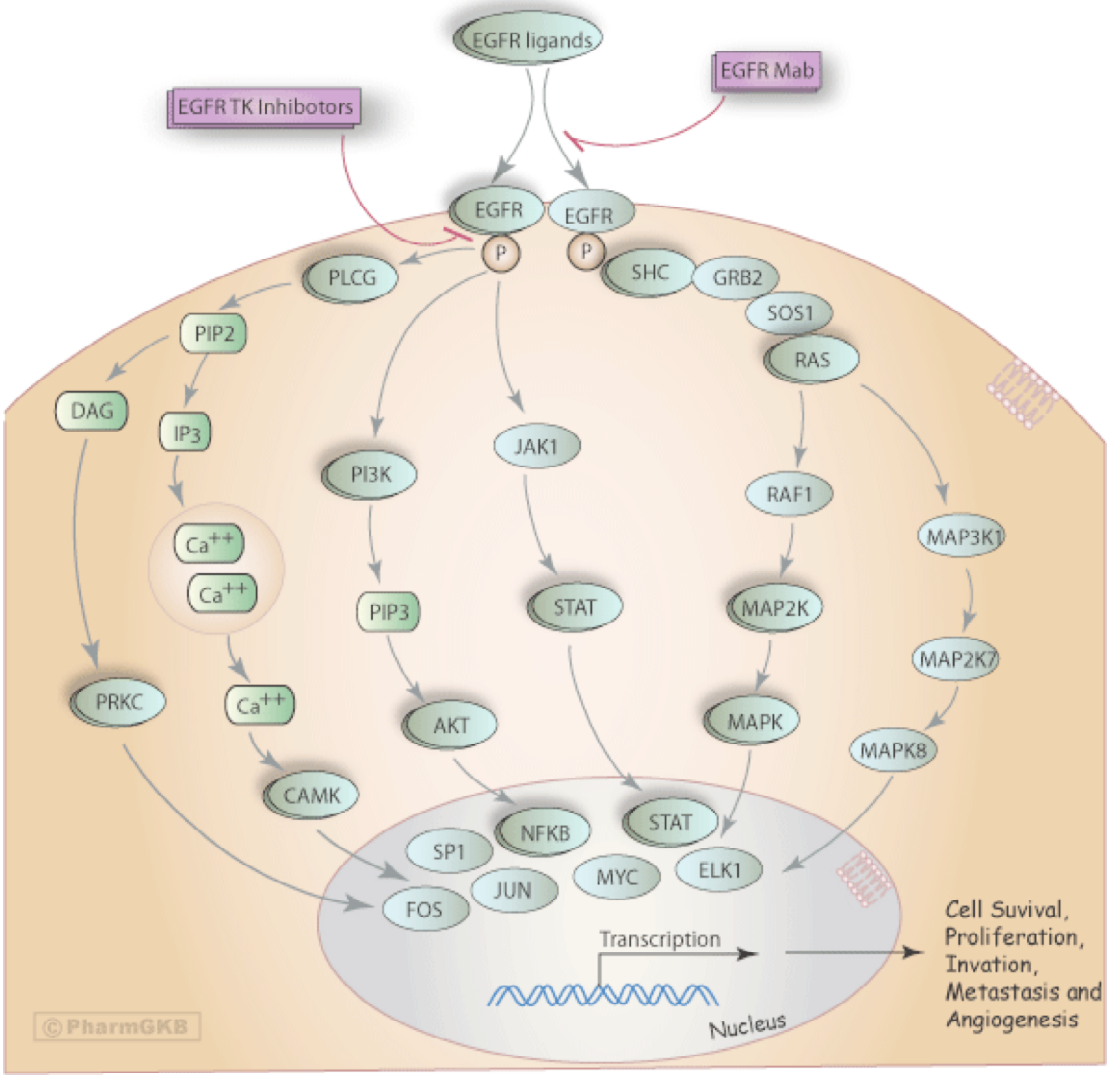


Figure 1.
The PharmGKB home page (http://www.pharmgkb.org/). There are five main (gray) boxes that allow users to type any text in search of (1) genes, (2) gene variants, (3) pathways, (4) drugs, and (5) diseases. The right side shows a news feed and the curators' favorite papers. There is a general search box at the very top, and tabs in the blue bar for general browsing. PharmGKB gets about 40,000 unique IP address visitors per month.

Finally, we will discuss some issues related to FDA approval of drugs for pharmacogenomics and co-approval of genetic tests associated with drug-prescribing decisions.



From PharmGKB, this is the EGFR Inhibitor Pathway Diagram, available at: http://www.pharmgkb.org/search/pathway/egfr-inhibitor/egfr-pd.jsp#

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Questions to Consider

Match the cancer treatment with the relevant gene:

A. trastuzumab	1. UGT1A1 (UDP glucoronyltransferase 1, polypeptide A)
B. 6-mercaptopurine	2. TPMT (thiopurine s-methyltransferase)
C. irinotecan	3. ERBB2 (herstatin, erythroblastic lekuemia viral oncogene homolog 2)

Match the research field of study with the correct description:

A. Pharmacodynamics	1. The study of absorption, distribution, metabolism and excretion of a drug.
B. Pharmacokinetics	2. The study of how DNA variation impacts drug response phenotypes
C. Pharmacogenomics	3. The study of the mechanism of action for a drug

Anti-angiogenic Therapies

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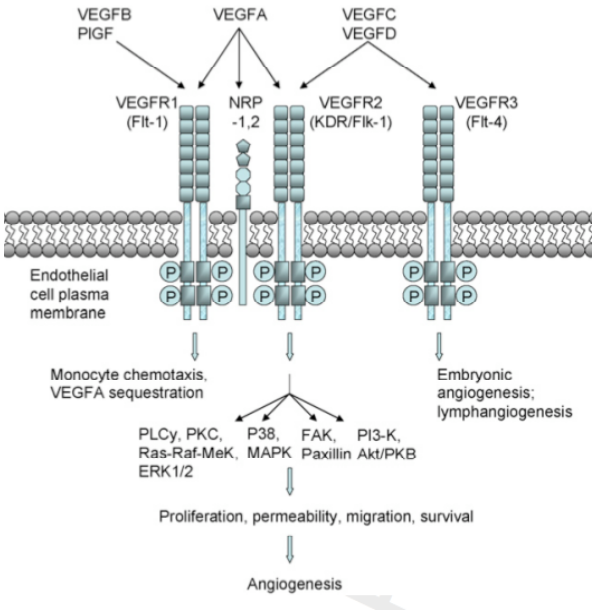
The concept of tumor angiogenesis originated as early as a century ago with the observation of tumors as well-vascularized entities. Judah Folkman hypothesized in 1971 that tumor growth is dependent on angiogenesis, the process by which new blood vessels form from preexisting vasculature. The concept of angiogenesis inhibition as a therapeutic anti-cancer strategy postulates that targeting a tumor's blood supply inhibits neoplastic growth and progression through deprivation of oxygen and nutrients.

Based on observations that tumor growth does not occur continuously but rather as an abrupt and rapid growth occurring after extended periods (up to years) of non-neovascularized tumor dormancy, an 'angiogenic switch' was postulated wherein, neovascularization proceeds and is required for tumor growth beyond the pre-angiogenic dimensions of a few millimeters. The discovery of key molecular modulators of angiogenesis, notably Vascular Endothelial Growth Factor (VEGF), has catalyzed the development of numerous neutralizing therapeutic agents. The validity of VEGF inhibition as a therapeutic strategy has been well supported in randomized phase III clinical trials, as well as U.S. Food and Drug Administration approval of numerous VEGF antagonists for cancer therapy.

Biology of VEGF and Its Receptors

The VEGF gene family consists of numerous members including VEGF-A, -B, -C, -D and PlGF. The most well-characterized member, and the one targeted most frequently for tumor angiogenesis, is VEGF-A, which exists as a homodimeric glycoprotein comprised of two identical 23 kDa subunits. The longer isoforms of VEGF-A are highly extracellular matrix-associated, while the shorter isoforms lack basic residues and tend to be more freely diffusible.



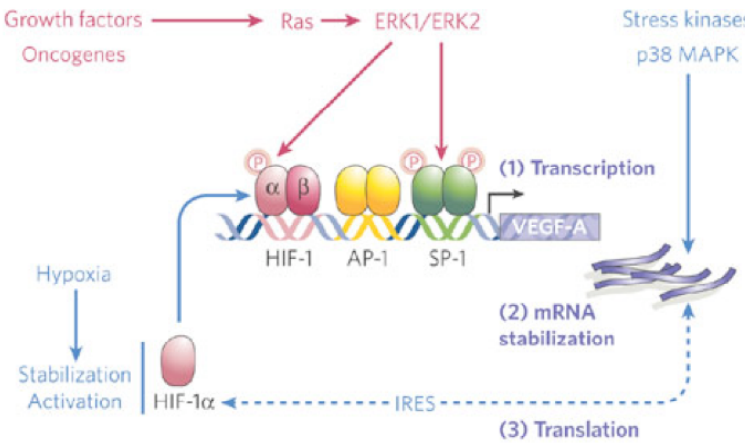
There is substantial heterogeneity amongst VEGF receptors (VEGFRs), which are transmembrane receptor tyrosine kinases, designated VEGFR1/Flt1, VEGFR2/KDR/Flk1, and VEGFR3. The pro-angiogenic actions of VEGF-A are thought to primarily occur through activation of VEGFR2. Although VEGF binds to VEGFR1 with greater affinity (Kd ~ 10-20pmol/L) than VEGFR2 (Kd ~ 75-125pmol/L). VEGFR2 is responsible for most VEGF angiogenic activity including, vascular endothelial cell permeability, proliferation, migration, and survival. VEGF stimulation of VEGFR1 has produces weaker tyrosine kinase activity in comparison to VEGFR2, and genetic deletion of VEGR1 leads to vascular overgrowth and embryonic lethality; hence VEGFR1 has been proposed to negatively regulate VEGFR2 activity by serving as a decoy receptor to VEGF. Surprisingly, mice bearing genetic deletion of the VEGFR1 tyrosine kinase domain do not exhibit embryonic lethality, indicating the potentially dispensible nature of this enzymatic activity. Nonetheless, VEGFR1 participates in monocyte/macrophage migration and chemotaxis, matrix metalloproteinase (MMP)-9 expression, and hematopoiesis. VEGFR3, intriguingly, mediates lymphangiogenesis and is expressed on endothelial "tip" cells. Additionally, Neuropilin-1, a cell surface glycoprotein originally implicated as a semaphorin receptor controlling developmental axon guidance, also functions as a nonclassical VEGF receptor. It binds to a VEGF C-terminal motiff as opposed to the

more N-terminal motifs recognized by the classical VEGF receptors Flk1 and Flt1. Neuropilin-1 has been proposed to be a VEGFR-2/Flk1/KDR co-receptor that enhances VEGF action on endothelial cells.

The individual VEGFs have distinct specificities for VEGFRs, where VEGF-A, -B and PlGF bind VEGFR1, VEGF-A, -C and –D bind VEGFR2, and VEGF-C and –D bind VEGFR3. Relevant to tumor angiogenesis, tumor-derived VEGF-A binds to VEGFR2 on tumor endothelium, leading to VEGFR2 dimerization, tyrosine autophosphorylation and activation of various signaling cascades. VEGF-mediated DNA replication and cell proliferation are thought to be mediated by both the Ras-Raf-MEK-ERK and MAPK pathways; cell survival, through PI3-Kinase and Akt/PKB activation; and cell migration through either FAK and Paxillin, PI3Kinase/Akt, or MAPK activation.

Mechanisms of VEGF Production in Tumors

1. Hypoxia. Tumors in general are rich sources of VEGF, stimulating the recruitment of nourishing neoangiogenic vessels. Tumor cells themselves express VEGF-A in response to several mechanisms including hypoxia, oncogene and tumor suppressor dysregulation, transcription factors, inflammatory mediators, and mechanical forces of shear stress and cell stretch. The VEGF-A gene is a classical hypoxia-regulated gene. Under normoxic conditions, the hypoxia-inducible factor-1α (HIF-1α) transcription factor undergoes prolyl hydroxylation and von Hippel-Lindau (VHL) protein-dependent ubiquitination and proteosomal degradation. In contrast, under normoxia, HIF-1α hydroxylation at proline 402 and 564 by three specific oxygen-dependent proline hydroxylases (PHD 1-3) promotes HIF-1α interaction with the von Hippel-Lindau (VHL) protein, a component of an E3 ubiquitin-protein ligase, targeting HIF-1α for proteosomal degradation.

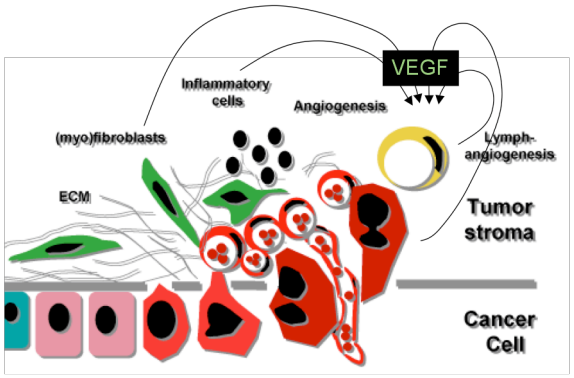


Pouysségur et al., Nature 441, 437-443 (2006)

2. Oncogenes. The role of HIF signaling in VEGF regulation is further demonstrated in renal clear-cell carcinoma (RCC) wherein loss-of-function mutations in the VHL tumor suppressor produce impaired ubiquitination and proteosomal degradation of HIF-1α, engendering marked increases in VEGF-A transcription. Classical oncogenes also regulate VEGF transcription in cancer. The ras oncogene participates in a signaling pathway that ultimately targets a consensus AP-1 sequence existing within the VEGF-A promoter to activate transcription in tumorigenesis. The human VEGF gene also contains seven consensus promoter binding sites for β-catenin/TCF, facilitating VEGF expression in colon cancer.

3. Stromal sources of VEGF. Tumors in general are composed of neoplastic cells, as well as a rich cellular stroma. While VEGF production by tumor cells is well documented, it has become increasingly appreciated that VEGF is abundantly produced by various cell types in the tumor stroma, including fibroblasts, monocyte/ macrophages and endothelial cells themselves. Moreover, tumor endothelial cells express several-fold higher levels of VEGFR2 than normal vasculature. Hence, the expression of VEGF in ischemic areas,

VEGF production by tumor cells and all components of tumor stroma



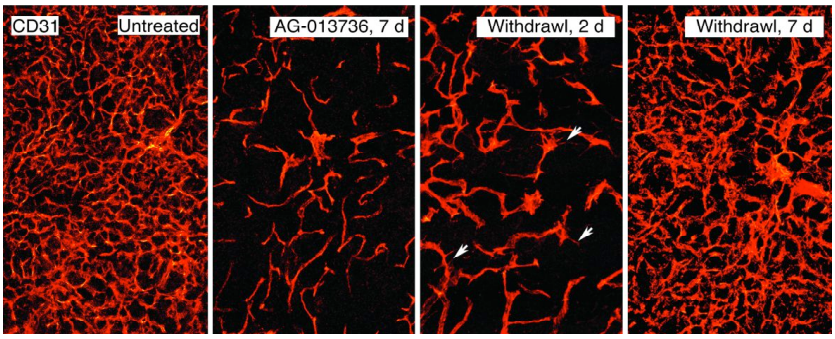
Adapted from www2.unil.ch/cepo_ research/images/fig2.gif

combined with the upregulation of VEGFR2 in tumor vascular endothelial cells may contribute to the specificity and relatively benign side-effect profile associated with VEGF antagonists.

Mechanisms by Which VEGF Inhibition Elicits Clinical Benefit

Numerous mechanisms have been proposed for the action of VEGF inhibitors on tumors, all of which may be simultaneously active:

VEGF inhibition regresses tumor vasculature in a reversible fashion

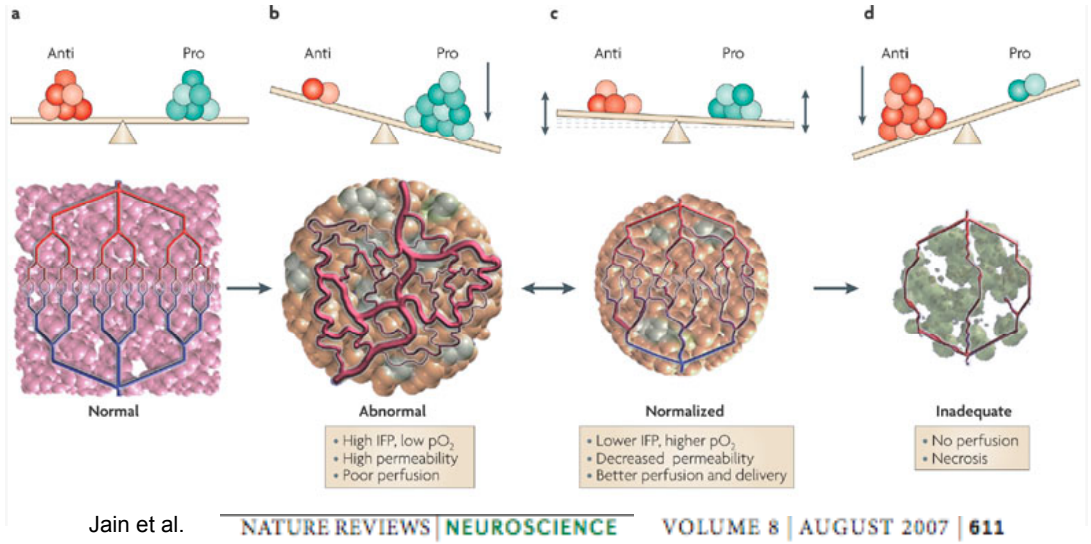


Mancuso et al., J. Clin. Invest. 116:10

1. Inhibition of tumor angiogenesis. The most conventional mechanism for VEGF inhibition would be to decrease endothelial proliferation within tumors. Preclinical modeling has indicated that VEGF inhibition actually induces a rapid regression of tumor vasculature within days. Notably, regressing vessels follow the path of the pre-existing tumor vasculature basement sleeve. This regression is not fixed, as cessation of VEGF inhibition is followed by rapid vessel regrowth.

2. Normalization of tumor vasculature, improvement of chemotherapy delivery. An additional mechanism is that VEGF inhibition "normalizes" the tumor vasculature, reversing the pathophysiologic detachment of tumor pericytes from tumor endothelium (Inai et al., 2004). Such vascular normalization decreases tumor vascular permeability, allowing more effective chemotherapy delivery to the interior of the tumor. Additionally, this vascular normalization decreases tumor interstitial fluid pressure (IFP). Such decreased tumor IFP decreases the pressure gradient against which chemotherapy diffuses into tumors, thus improving intra-tumoral chemotherapy delivery, and providing mechanism for the successful addition of anti-angiogenic therapy to chemotherapy observed in clinical trials (reviewed below).

Vascular normalization, decreased IFP and increased chemotherapy delivery



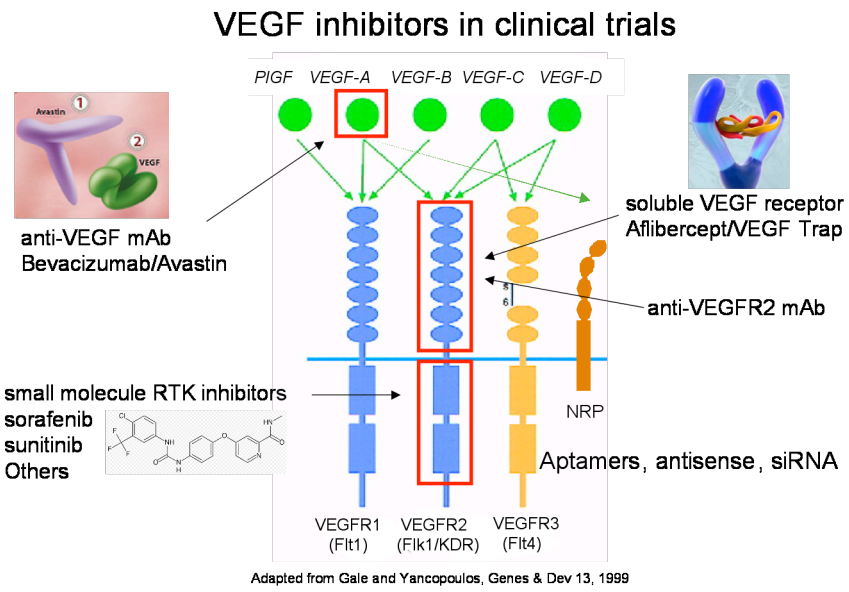
Jain et al. NATURE REVIEWS | NEUROSCIENCE VOLUME 8 | AUGUST 2007 | 611

3. Reduction of edema. In brain tumors, such as glioblastoma, vessel leak with subsequent edema can elevate intracranial pressures to dangerous levels, causing damage to the brain by herniation and significantly increasing patient mortality. The treatment of brain tumors with bevacizumab/Avastin

(discussed below) clearly reduces vascular leak and edema in glioblastoma patients as indicated by strong reduction in extravasated gadolinium signal on MRI, which can be accompanied by improved neurologic status. Such MRI findings actually overestimate the reduction in tumor vascularity from VEGF inhibitors, since comparatively smaller reductions in vascular content are measured when larger intravascular tracers such as iron-based nanoparticles are used for imaging.

Clinical Trials of VEGF Inhibitors

The recent success of a growing number of VEGF antagonists in randomized phase III clinical trials underscores the critical role of VEGF in cancer and the efficacy of targeting VEGF as a therapeutic strategy. In general, the therapeutic strategies have included direct inactivation of VEGF-A by monoclonal antibodies or soluble receptors, small molecule antagonists of the VEGFR tyrosine kinase domain, and antibodies directly targeting VEGF rceptors.



1. Bevacizumab (Avastin) is an intravenously administered, recombinant humanized monoclonal antibody targeting VEGF-A and all of its isoforms. In February 2004, a randomized double-blind placebo-controlled phase III trial was published in which colon cancer patients were treated with chemotherapy (irinotecan/5-FU/leucovorin) alone vs. chemotherapy plus bevacizumab. The bevacizumab/chemotherapy regimen improved median survival from 15.6 to 20.3 months ($p < 0.001$), progression free survival (6.2 to 10.6 months), and time to progression (6.7 to 8.8 months) in parallel (Hurwitz, 2004). This trial was the first randomized phase III cancer trial to demonstrate a survival advantage for an anti-angiogenic agent, and led to FDA approval for colon cancer. In lung cancer, the combination of bevacizumab and chemotherapy has provided an approximately 2-month survival advantage versus chemotherapy alone. In other solid tumor types, such as breast and renal cell carcinoma, bevacizumab has not yielded survival advantages, but rather an increase in the "time to progression", i.e. the amount of time before tumor regrowth is observed on therapy. Overall, bevacizumab has received FDA approval for the treatment of colon, lung, breast and renal cell carcinoma.

2. Small molecule VEGF inhibitors (sunitinib, sorafenib, etc). Small molecule antagonists of VEGF receptors, including sorafenib (Nexavar), sunitinib (Sutent) and pazopanib (Votrient), have also recently demonstrated efficacy in randomized cancer trials. The archetypal VEGF receptors (VEGFR1-3) are tyrosine kinases that transfer a phosphate group from ATP to protein substrates. These agents are orally bioavailable small molecule compounds that are ATP-mimetic and act as competitive inhibitors of ATP for the intrinsic VEGFR tyrosine kinase activity. In renal cell carcinoma (RCC) patients, the small molecule VEGF inhibitors have elicited approximately 5-6 months delay in time to progression, and trends towards 2-5 month increases in overall survival, although the latter measure has not reached statistical significance. In hepatocellular carcinoma, sorafenib elicited a 2.8 month survival increase in a phase III trial ($p=0.01$). Sorafenib, sunitinib and pazopanib have all received FDA approval for RCC, while sorafenib is the only VEGF inhibitor of any class that is FDA approved for hepatocellular carcinoma.

3. Other types of VEGF inhibitors. Additional strategies are being pursued for pharmacologic VEGF inhibition, including soluble VEGF receptors (aflibercept/VEGF Trap), anti-VEGFR2 neutralizing antibodies and anti-VEGF-A aptamers.

4. Side effects of VEGF inhibitors. In general VEGF inhibitors have been fairly well tolerated with known toxicities including hypertension, proteinuria, bleeding and thrombosis. Additionally, reports exist of increased hematocrit. An emerging concept is that chronic VEGF inhibition leads to tumor adaptation and resistance, with increased synthesis of alternative angiogenic factors, such as Fibroblast Growth Factor (FGF) and Hepatocyte Growth Factor (HGF). Further, recent preclinical animal data indicates that VEGF inhibition may result in development of tumors with increased metastatic potential, questioning the utility of chronic therapy.

5. Non-VEGF approaches to angiogenesis inhibition. Myriad other endothelial pathways and strategies are being pursued for angiogenesis inhibition. These include other endothelial receptor tyrosine kinase/ligand families (c.f. Tie/angiopoietin, Eph/ephrin), endothelial junctional molecules (VE-Cadherin), G-protein coupled receptors, Notch/Delta (DLL4) and neutralization of angiogenic growth factors such as FGF and HGF. Further, so-called vascular disrupting agents that target endothelial microtubules are under development.

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Questions to Consider

The maximal survival benefit from VEGF inhibitors observed in phase III randomized cancer trials has been on the order of:

- **A.** Days
- **B.** Months
- **C.** Years

VEGF inhibition may benefit patients by all of the following mechanisms except:

- **A.** Increasing tumor interstitial fluid pressure
- **B.** Inhibiting tumor endothelial growth
- **C.** Increasing intratumoral chemotherapy delivery
- **D.** Decreasing cerebral edema

Proposed mechanisms of resistance to VEGF inhibitors include:

- **A.** Increased intratumoral metabolism of VEGF antagonists
- **B.** Neutralizing immune responses against VEGF inhibitors
- **C.** Tumor synthesis of alternative pro-angiogenic factors
- **D.** Down-regulation of endothelial VEGF receptors

Molecular Therapies Targeting Oncogenes

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One of the most important discoveries in Cancer Research was the discovery of oncogenes. There discovery made it clear that cancer is a multi-step process whereby an individual cell acquires a series of mutant proto-oncogenes and tumor suppressors collectively culminating in pathological phenotype of relentless cellular proliferation and growth, blocked differentiation, the inappropriate induction of angiogenesis, widespread tissue invasion and often the loss of genomic stability. Given the genetic complexity of tumorigenesis, it is perhaps surprising that there are circumstances when cancer can be reversed through the repair or inactivation of individual mutant oncogenes. However, recent experiments in mouse models and clinical results using new pharmacological agents demonstrate that cancer can be treated through the targeted repair and/or inactivation of oncoproteins. Hence, cancers appear to be dependent upon particular oncogenes to maintain their neoplastic properties, thus exhibiting the phenomena of "oncogene addiction." (Joe and Weinstein, 2008). Hence, some oncogenes mediate signaling processes that underlie the etiology of cancer, and these mutant oncogenes are likely to represent the best targets for the treatment of cancer.

There are at least four major oncogenic signaling pathways that have been implicated in the pathogenesis of cancer: surface receptors, receptor/signaling molecules, protein kinases, GTPases and transcription factors (Figure 1).

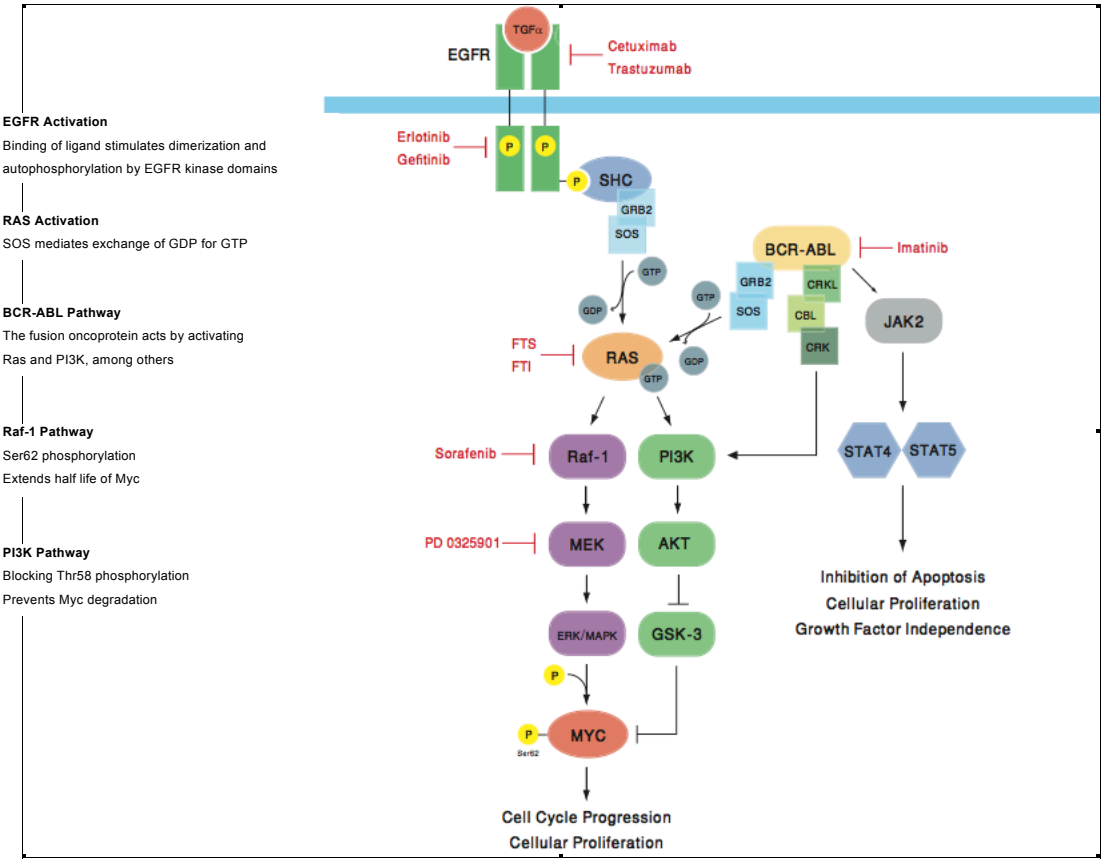

Figure 1. Many oncogenic signaling pathways are good targets for the treatment of cancer.

Cell surface receptors are the starting point for all signaling cascades, so it is not surprising that receptors for growth factors were some of the first proto-oncogenes discovered (Olayioye et al. 2000). A multitude of cell surface receptors have been implicated in tumorigenesis including the epithelial growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR) and insulin-like growth factor receptor (IGFR). A proto-typical example of receptors is the ErbB family of transmembrane tyrosine kinase receptors that includes four family members: EGFR (ErbB1), HER2/NEU. Activation of these receptor pathways induces cellular proliferation, survival and motility (Bianco et al. 2006). Each of these receptors exhibits tissue-specific expression. Correspondingly, mutated receptors have been implicated in particular types of human

Receptor signaling is frequently mediated through small GTPases. A characteristic feature of GTPases is that they must first be modified to localize to the plasma membrane where they are active (Downward 1996). The RAS family represents a prototypical example of small GTPases consisting of three members: H-RAS, K-RAS, and N-RAS. RAS family members are some of the most commonly mutated genes associated with human cancers. At least 25% of human tumors exhibit activating point mutations of a RAS gene. RAS proteins are known to regulate many cellular processes including cellular growth, proliferation, apoptosis and angiogenesis.

Kinases are among the most abundant signaling molecules with approximately 500 members, many of whom when mutated, function as oncogenes. The ability to readily pharmacologically target the ATP binding domains of kinases has made these gene products attractive drug targets. The most well known example of a kinase associated with neoplasia is the BCR-ABL fusion protein, which results from a chromosomal translocation between the ABL proto-oncogene and the BCR locus. BCR-ABL overexpression has been implicated in the pathogenesis of CML and ALL. The targeted inactivation of BCR-ABL through the drug imatanib mesylate, is the most regarded example of a successful targeted therapeutic (Gleevec, STI-571).

Nuclear transcription factors are some of the most frequently implicated proteins in cancer. The family of MYC proto-oncogenes (c-, n-, l-MYC) are overexpressed in up to half of all human cancers. MYC has been shown to regulate the transcription of thousands of target genes suggesting that these gene products function as grand coordinators of gene expression programs.

c-MYC was the first family member to be discovered. c-MYC is expressed in most cell types and is found to be overexpressed in most types of human cancers. In particular, c-MYC was found to be activated through chromosomal translocation in Burkitt's lymphoma. n-MYC is expressed in neuronal cells and is often amplified in neuroblastoma. l-MYC was first identified in lung tissue and is associated with small cell lung carcinoma. To date, transcription factors have yet to be successfully targeted using small molecules.

Conditional transgenic mouse model systems have emerged as a very streategy for defining when and how the inactivation of oncogenes will be useful to induce tumor regression. Many reviews have extensively described the application of conventional transgenic mouse models for the development of therapeutics for cancer (Van Dyke and Jacks 2002; Weiss and Shannon 2003; Gutmann et al. 2006). Conditional transgenic models have been used to evaluate the consequences of oncogene inactivation in vivo (Figure 2).

From these studies, several general themes emerge regarding the role of oncogenes in the initiation and maintenance of tumorigenesis, as we have described (Felsher 2003, 2004a, 2004b; Giuriato et al. 2004; Bachireddy et al. 2005; Shachaf and Felsher 2005a, 2005b). Oncogene inactivation can reverse tumorigenesis by inducing sustained tumor regression through differentiation, proliferative arrest, apoptosis, cellular senescence and the inhibition of angiogenesis (Felsher, 2008). The specific consequences of the inactivation of an oncogene depend upon the type of tumor. In some cases, even briefly inactivating an oncogene may be sufficient to induce sustained tumor regression, but in other cases, this has not been observed (Boxer et al. 2004). Oncogene inactivation may uncover the stem cell properties of tumor cells and induce a state of tumor dormancy. Finally, the genetic context can affect whether inactivation of an oncogene will induce sustained regression, or if the tumors can relapse acquiring additional genetic events.

Collectively these results illustrate these experimental results illustrate that cancers can become "addicted" to specific oncogenes. Hence, the inactivation of these oncogenes is sufficient to induce the

sustained regression of some tumors. Defining the mechanisms by which cancers are addicted to oncogenes will be important not only for gaining insight into the nature of tumorigenesis but also for designing the best therapies for cancer.

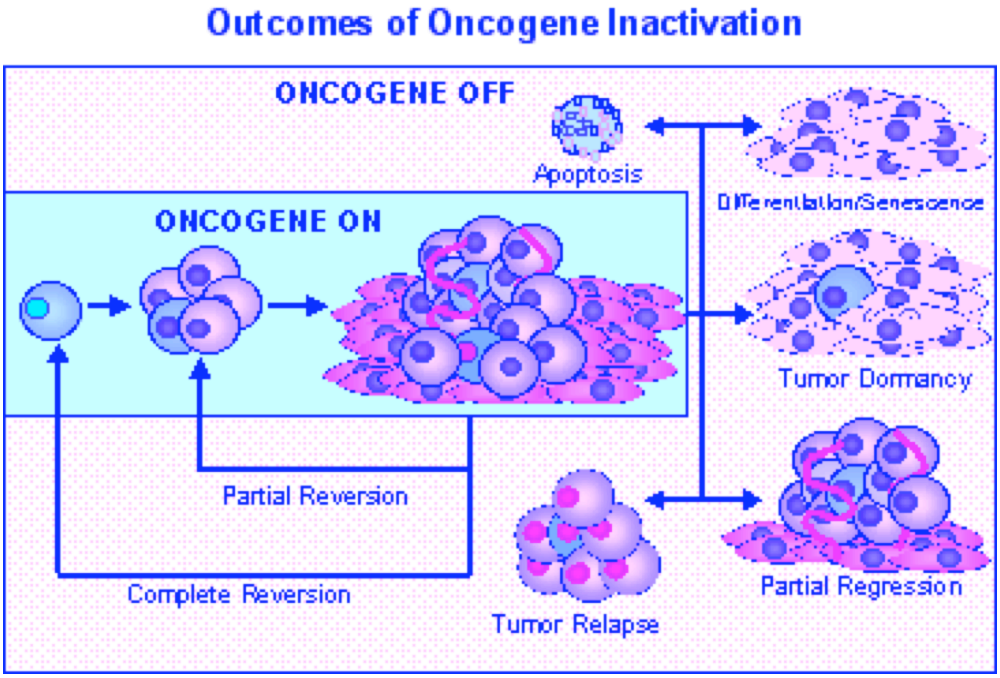

Figure 2. Different outcomes observed upon oncogene inactivation.

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Questions to Consider

Oncogene addiction has been described as an explanation for why inactivation of an oncogene can result in tumor regression.

Which of the following mechanisms has been suggested to be associated with oncogene addiction?

A. proliferative arrest

B. differentiation

C. senescence

D. angiogenesis

E. all of the above

Targeted therapies have been developed for the treatment of cancer that inhibit the function of which of the following class of gene products except?

A. kinases

B. surface receptors

C. transcription factors

D. apoptosis proteins

E. G proteins

Oncogene inactivation has been associated with the following phenotypic consequences in tumors in animal models:

A. tumor elimination

B. tumor dormancy

C. multipotent differentiation

D. all of the above

E. none of the above

Wnt Signaling in Cancer

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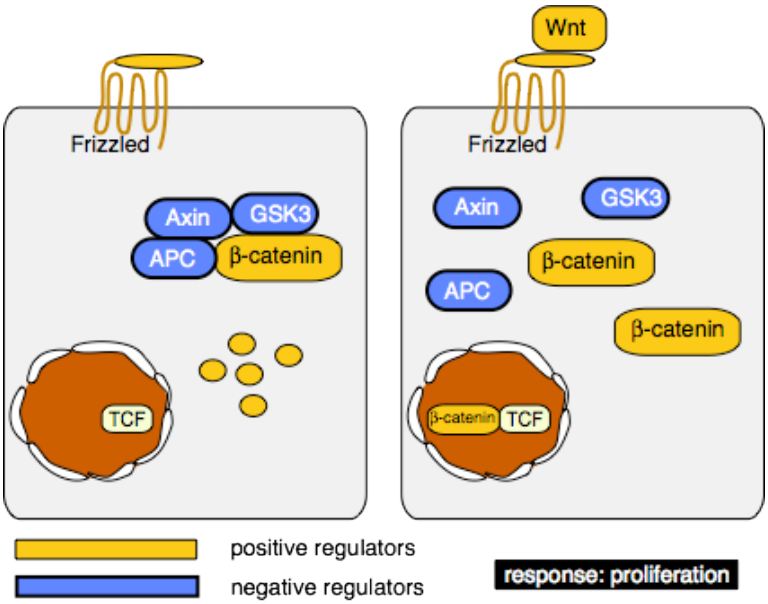
Wnt proteins comprise a large family of highly conserved growth factors that control important developmental and homeostatic processes. Their implication in a wide array of developmental events and human diseases has made Wnts and their signaling pathways the subject of intense investigation over the last two decades. Recently, association between Wnt signaling and stem cell fate has added fuel to an already active field. Through biochemical, genetic, and genomic approaches, our knowledge of Wnt proteins, their production and signaling properties, and their evolutionary history has expanded rapidly.

Membership in the Wnt family is defined by amino acid sequence rather than functional properties. It is therefore not too surprising that Wnts have been associated with a number of different activities and downstream signaling pathways. The defining event in canonical Wnt signaling is the cytoplasmic accumulation of β-catenin and its subsequent nuclear translocation and activity. Under non stimulated conditions, a β-catenin destruction complex formed by proteins that include Axin, Adenomatous Polyposis Coli (APC) and glycogen synthase kinase-3b (GSK-3) keeps cytoplasmic levels of β-catenin low through phosphorylation by GSK-3 (Figure 1). Phosphorylated β-catenin becomes ubiquitylated and is targeted for degradation by the proteasome.

Following Wnt binding to a receptor complex composed of members of the Frizzled (Fz) family of seven transmembrane, serpentine receptors and low density lipoprotein (LDL) receptor-related protein (LRP), the Axin/APC/GSK-3 complex is inhibited, leading to a block in β-catenin phosphorylation by GSK-3. Hypophosphorylated β-catenin accumulates in the cytoplasm and is translocated to the nucleus where it regulates target gene expression through partnerships with the TCF/LEF family of transcription factors (Figure 1).

Figure 1 Wnt signaling without (left) and with Wnt (right)

Negative regulators can be found to be mutated in cancer, leading to activation of the Wnt pathway.



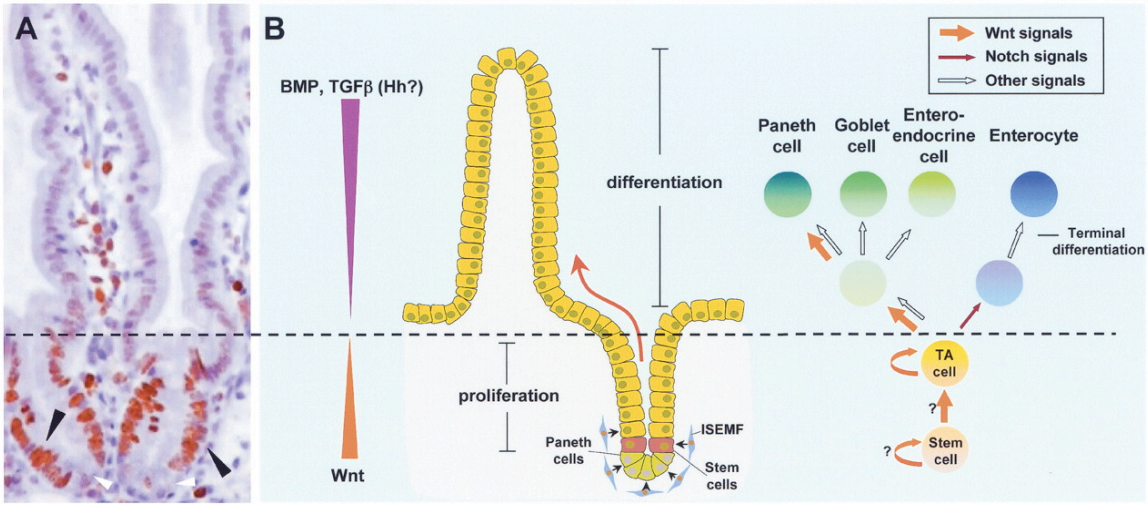
Several components f the Wnt signaling pathway have been implicated in human tumors or experimental cancer models. Wnt-1 was found as an oncogene activated by the Mouse Mammary Tumor Virus in murine breast cancer. APC was first isolated as a tumor suppressor gene in human colon cancer. After establishing that APC and β-catenin bind to each other, activating mutations in the human β-catenin gene were found in human colon cancer and melanomas. These mutations alter specific β-catenin residues important for GSK3 phosphorylation and stability. APC mutations in the germ line predispose to colon

cancer in families. In tumors, the second allele is found to be deleted. Interestingly, mutations in the human AXIN1 gene were reported in human hepatocellular carcinomas.

These discoveries underscore the important predictive value of these pathways: by establishing how genes control signaling (e.g. whether they act as repressors or activators) one can make educated guesses how these gene might contribute to human cancer. A repressor can be a tumor suppressor gene and an activator a dominant oncogene. The same principle applies to other pathways, such as the Hedgehog-Patched pathway and the TGF beta pathway.

In normal physiology, the Wnt signaling pathway has frequently been found to regulate the self-renewal of stem cells. Examples include stem cells in the intestine (Figure 2), in the mammary gland, the hair follicle and in the blood (HSC). In the contexts of stem cell self-renewal, expression of Wnt genes from

Figure 2 Stem cells in the intestine (From Clevers et. al.)



cells close to the stem cells (the niche) provides an external and restrained signal for a limited number of stem cells. In early stages of cancer, mutations in Wnt components such as APC and Axin leads to an unrestrained and perpetual state of self-renewal. After additional mutations in other pathways (ras, P53), fully malignant tumor cells emerge.

Because of the importance of Wnt signaling in cancer, there is significant interest in finding molecules interfering with the pathway. Some of these are promising and are listed on the Wnt homepage. http://www.stanford.edu/~rnusse/wntwindow.html

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Questions to Consider

Cancer can be the consequence of:

- **A.** loss of Wnt gene expression
- **B.** hyperactivation of APC
- **C.** loss of function mutations in Axin
- **D.** loss of function mutations in beta-catenin

Blocking Wnt signaling may be beneficial in the treatment of cancers caused by a mutation in APC. Which component of the Wnt pathway would you target?

- **A.** Wnt
- **B.** Frizzled
- **C.** TCF
- **D.** LRP

Genome Regulation in Cancer

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Large-scale Transcriptional Signatures in Cancer

Global gene expression patterns can provide comprehensive molecular portraits of biologic diversity and complex disease states. Cancer samples in particular show large-scale changes–in comparison to normal tissue of the same histologic type and amongst cancer samples that carry the same traditional diagnoses. Unsupervised analysis where similarities in the overall gene expression pattern is allowed to drive the grouping of tumor specimens has revealed the molecular heterogeneity of cancer. Many human cancers that carry the same pathologic diagnosis—e.g. breast cancer—are actually comprised of several distinct molecular subtypes that are distinguished by the variation in expression of dozens or hundreds of genes. These subtypes are stable through time in individual patients, show different propensities for disease progression, and are now known to be driven by different oncogenic pathways.

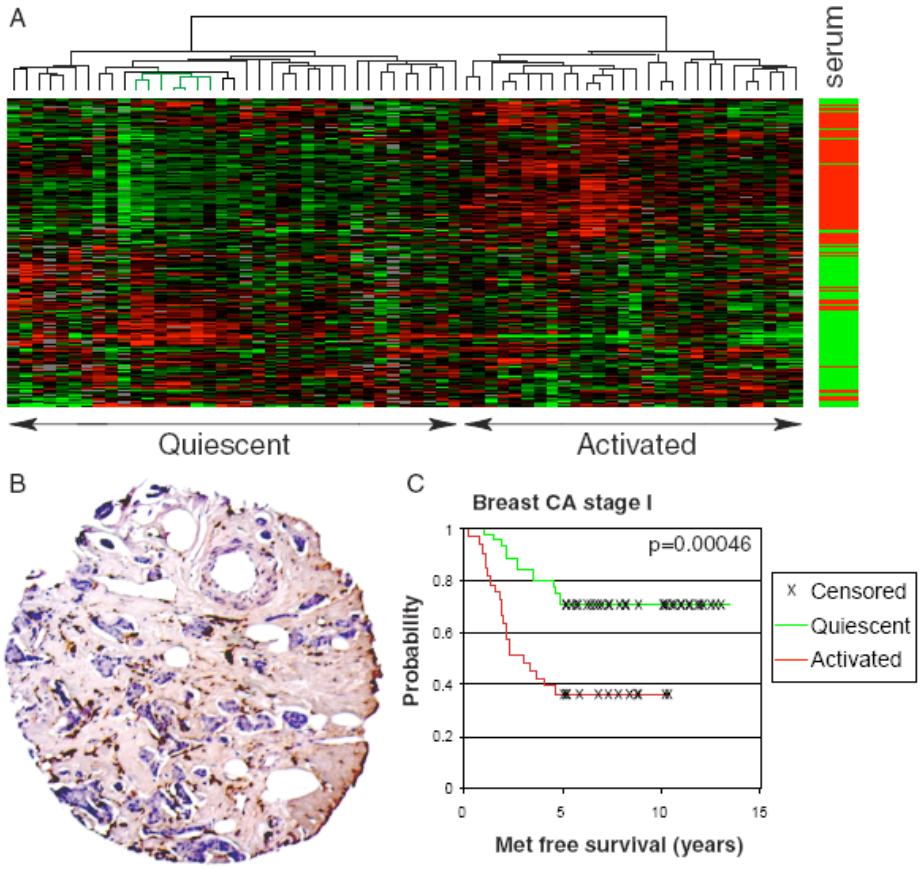

Figure 1. A gene signature predicts breast cancer progression. (A) Expression of genes induced by serum—as a model of wound healing—occurs in a biphasic pattern among human breast tumors. The activated group coordinately up regulates the serum-induced genes whereas the quiescent group expresses serum-repressed genes. The 7 benign samples are indicated by green branches. The status of serum regulation of each gene is shown on the right bar: Red indicates serum-induced; green indicates serum-repressed. (B) Validation of gene expression by in situ hybridization on tissue microarrays. The gene in question is expressed in stromal fibroblasts (stained brown) but not in tumor cells (counterstained purple by hematoxylin). (C) Prognostic value of fibroblast serum response. Kaplan-Meier survival curves of 78 breast cancer patients prospectively classified as having activated or quiescent stroma. Patients were matched for age, histologic diagnosis, and tumor stage and underwent the same treatment protocol. Tumors with activated phenotype had significantly increased risk of metastasis and mortality.

Top Down and Bottom up Approaches to Gene Expression Analysis

Understanding the physiologic meaning and genetic basis of the myriad gene expression changes have been a challenge. In the "top-down" approach, known clinical variables, such as tumor grade or eventual outcome, can guide the identification of specific gene signatures that act as biomarkers for cancer diagnosis and prognosis. In the "bottom-up" approach, investigators turn to nature for guidance about which gene expression patterns should be prioritized: By generating extensive compendia of gene signatures of different types of cells, cells engaged by different signaling pathways, and cells treated with different agents, investigators can then ask whether those signatures show up in the gene expression programs of authentic human tumors. The presence of specific gene signatures can thus suggest the presence specific cell types and signaling events in those tumors. Various methods that formalize this concept include *gene module map*, and *gene set enrichment analysis* (GSEA).

Several new analytic strategies have now been developed to improve the interpretation of gene expression data. Because genes work together in groups to carry out specific functions, another strategy to refine gene set based analysis is to use knowledge about biological pathways-so called bibliomic approach. Here, the gene set is not defined by coordinate change in expression in an alternative experiment, but by knowledge of the function. Such pathways can even be organized into networks, where the genes are nodes and functional relationships define the edges between the nodes. Regulatory relationships can also be directly learned from cancer samples. For instance, by integration of DNA copy number variation (CNV) and gene expression, one can gain insight into how gene amplifications or deletions affect gene expression. Such relationships can be in cis, where the CNV affects genes within or nearby the CNV, or in trans, where the CNV affects a factor that then impacts gene expression on a global level.

Epigenomic Profiling of Cancer

Large-scale measurements of chromatin states can provide additional views of cancer. Instead of measurement of genes that are expressed, chromatin state measurements suggest mechanisms for gene control and reveal future potential (or lack thereof) of specific transcriptional programs. Epigenomic profiling include measurements of DNA cytosine methylation, nucleosome occupancy, histone modifications, and occupancies of chromatin modification enzymes. Although features of chromatin states, rather than mRNA levels, are measured, the strategies to associate chromatin states with behaviors of cancer or the use of gene set level analysis are very similar to mRNA analysis.

Questions to Consider

What is the meaning of "bottom-up" approach to analyze gene expression patterns in cancer?

- **A.** Genes are prioritized by the magnitude of change in their level of expression.
- **B.** Genes are prioritized by their genomic coordinates.
- **C.** Genes are prioritized by their association with known clinical parameters in the samples.
- **D.** Genes are organized based on the similarity of their patterns of expression to other known biological states.

The main gene expression differences in breast cancers from different patients reflect:

- **A.** Temporal phases of the disease
- **B.** Response to different prior therapies
- **C.** Distinct molecular subtypes of disease
- **D.** Intrinsic ethnic differences among the human population

The gene expression programs in cancer stem cells is related to that in:

- **A.** Rapidly dividing cells
- **B.** Embryonic stem cells
- **C.** Progenitor cells of the lineage that give rise to the cancer
- **D.** Renal tubule cells that efficiently efflux drugs

Engineering Protein-based Cancer Therapeutics and Diagnostics

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Protein Engineering Using High-throughput Library Screening Methods

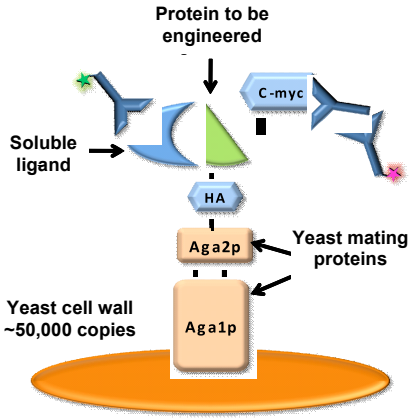

Figure 1. Yeast surface display system. Protein expression on the yeast cell surface can be measured by flow cytometry using antibodies against the C-terminal c-myc tag, and protein-protein interactions can be measured with antibodies against a ligand of interest, or through fluorescent-labeled ligands.

Yeast surface display is a powerful directed evolution technology that has been used to engineer proteins for enhanced binding affinity [1, 2], proper folding [3, 4], and improved stability [5, 6]. For example, we have applied this technology to rapidly characterize protein-protein interactions [7], to engineer epidermal growth factor receptor for soluble expression in yeast [4], to engineer growth factor ligands with enhanced potency for wound healing applications [8], and to engineer knottin peptides against tumor-associated integrin receptors (see below) [9, 10]. The yeast surface display system takes advantage of the chaperone-assisted folding, disulfide bond formation, and the quality control mechanisms of the eukaryotic secretory pathway [11]. A schematic of the yeast display platform is shown in Figure 1. Proteins are displayed on the yeast surface through genetic fusion to the yeast mating agglutinin protein Aga2p [12]. Aga2p is disulfide bonded to Aga1p, which is covalently linked to the yeast cell wall. The protein of interest is flanked by N-terminal hemagglutinin (HA) and C-terminal c-myc epitope tags, which are used to confirm expression of the construct on the yeast cell surface and to quantitate surface expression levels [12].

High-throughput screening of tens of millions of yeast-displayed mutants allows for the rapid isolation of proteins with altered properties. Combinatorial libraries of up to 108 transformants can routinely be created, with each yeast cell displaying approximately 50,000 identical copies of a particular mutant protein on its surface. Yeast-displayed protein libraries are then stained with fluorescently-labeled ligand or receptor (or primary and secondary antibodies). Fluorescence activated cell sorting (FACS) is used to screen the yeast libraries for mutants with a desired phenotype, usually increased binding affinity to a target protein. The sorted yeast are propagated in culture, and the screening process is repeated several times to obtain an enriched yeast population consisting of a small number of unique clones.

One of the benefits of yeast display over other protein engineering technologies such as phage or mRNA display is that multi-color FACS can be used to quantitatively discriminate clones that differ by as little as 2-fold in binding affinity to the desired target [13]. This is accomplished by normalizing yeast expression levels (through detection of the c-myc tag) with binding levels, such that a clone that is expressing a high amount of protein but possesses low ligand binding affinity can easily be distinguished from a clone that is expressing a lower amount of protein but actually has higher ligand binding affinity.

Knottins as scaffolds for protein engineering and tumor targeting applications. Non-antibody protein scaffolds have attracted attention recently because of the limitations of antibodies, which include expensive recombinant production in mammalian cells, poor penetration of solid tumors, inability to site-specifically incorporate chemical modalities, and large intellectual property barriers for development. As such, several alternative scaffolds have been developed and commercialized over the past decade

including ankyrin repeats, affibodies, fibronectin domains, lipocalins, and avimers [14-16]. In addition, there are many platforms available for creating mono- and bi-specific antibody fragments [17]. A desirable scaffold should be non-immunogenic, and should have high thermal and proteolytic stability, high expression in microbial systems, and the ability to tolerate mutations while retaining its fold.

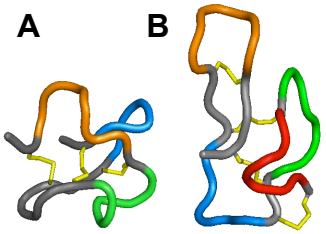

Figure 2. A) EETI-II (PDB 2ETI) and B) AgRP (PDB 1MRO) knottin proteins. Colors indicate loops that can be mutated to engineer new binding proteins.

Members of the knottin family (which includes protease inhibitors, toxins, and antimicrobials) attracted our interest as scaffolds for engineering tumor-targeting proteins for several reasons. Knottins are small, compact polypeptides (typically 20-50 amino acids) that consist of a core of at least 3 disulfide bonds that are interwoven into a "knot" conformation [18] (Figure 2). As a result, they possess high thermal and proteolytic stability and appear to be non-immunogenic [19-21]. There is great sequence diversity among knottin family members, as the loops connecting the conserved cysteine residues are highly tolerant to substitution or incorporation of additional amino acids. Due to their small size, knottins can easily be produced by microbial expression or by chemical synthesis [9, 10]; the latter allows for site-specific conjugation of imaging probes, radioisotopes, or chemotherapeutic agents for direct delivery to cancer cells. In addition, these peptides can be chemically modified to tailor their *in vivo* pharmacokinetic properties for diverse clinical applications. **One major goal of the Cochran lab is to exploit these attributes to engineer knottin peptides as novel targeted cancer therapeutics and diagnostics.** Towards this goal, we have taken two knottin peptides with vastly different biological functions and engineered them to bind to tumor-associated integrin receptors with antibody-like affinities [9, 10]. We prepared fluorescent and radiolabeled versions of these engineered knottin peptides, and in collaboration with faculty from the Molecular Imaging Program at Stanford, showed that they have desirable biodistribution and pharmacokinetics in murine tumor models (high tumor uptake, low uptake and retention in the kidneys and liver) for tumor diagnostic applications or as radiotherapy or chemotherapy delivery agents [22]. This work is described in detail below.

In contrast to disulfide-bonded cyclic peptides, which have been extensively identified from phage-display libraries against a wide range of tumor targets, knottin peptides are stable in serum for several days [9]. In addition, binding epitopes contained within a knottin scaffold possess conformational constraints that may result in less entropic penalties upon binding compared to cyclic peptides. This is because the loops of a knottin are not simply connected through a single direct disulfide bond, but are instead anchored to the peptide backbone through flanking disulfide bonds (Figure 1). These epitopes could be evolved for high affinity target binding through a variety of strategies using directed evolution. The larger surface area provided by a knottin scaffold compared to a cyclic peptide may also be advantageous for generating high affinity binders against a desired target.

Molecular Imaging of Biomarkers Expressed on Tumors and the Tumor Vasculature

There is a critical need for molecular imaging probes that will specifically target vasculature receptors and allow non-invasive characterization of tumors for patient-specific cancer treatment and disease management [23-25]. For example, integrins are a family of cell surface adhesion receptors that non-covalently associate into α/β heterodimers with distinct ligand binding specificities and cell signaling properties [26]. Several integrins (including $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$), have generated clinical interest due to their expression on the surface of many types of cancer cells and/or the tumor neovasculature, as well as their proposed role in mediating angiogenesis, tumor growth, and metastasis [27-29]. Rational drug design and phage display have identified small peptides and peptidomimetics that target $\alpha_v\beta_3$ (and $\alpha_v\beta_5$) integrins or $\alpha_5\beta_1$ integrin [30-32]. However, chemical modifications that can be made to these small peptides to improve their receptor binding affinity, tumor uptake, and *in vivo* pharmacokinetics are limited. Moreover, covalent attachment of imaging probes have affected their integrin binding properties [24]. Despite the prevalence of integrin-binding peptides and peptidomimetics in the literature, suboptimal tumor targeting efficacy and pharmacokinetics have limited their clinical translation as molecular imaging agents [33]. Only one compound, a glycosylated cyclic RGD pentapeptide (^{18}F-

Galacto-c(RGDfK), has advanced to the clinical level for molecular imaging in human subjects [34, 35]. While this agent was able to identify integrin-positive lesions in human subjects and image intensity correlated with $\alpha_v\beta_3$ integrin expression, its relatively poor tumor uptake and high background (e.g., in the liver) indicates there is room for substantial improvements. In a human melanoma mouse xenograft model, ^{18}F-Galacto-c(RGDfK) also exhibited low tumor uptake values [36], suggesting that imaging agents that have weak tumor contrast in small animal models will also correlate to weak imaging signals in humans. To address these issues, the Cochran lab was interested in developing knottin peptides as a new class of tumor-targeting agents, particularly for cancer therapy and diagnostic applications. The first example in this area of research is described in detail below for engineered knottin peptides that bind to tumor-related integrin receptors.

Engineered Knottin Peptides that Bind Tumor-associated Integrins with Antibody-like Affinities

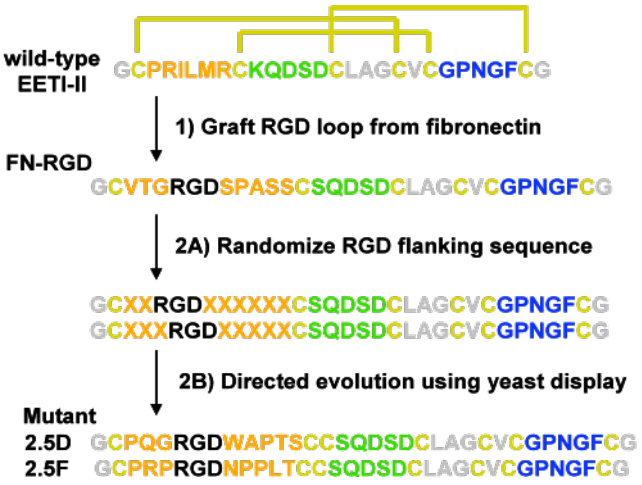

Figure 3. Strategy for EETI-II engineering. First, the trypsin binding loop (orange) was substituted with an integrin binding sequence from fibronectin to create FN-RGD. Second, yeast-displayed libraries were created by randomizing the residues flanking the RGD sequence, and were screened to identify mutants with high affinity binding to αvβ3 integrins. Ref [9]. Colors correspond to EETI-II structure in Figure 1A.

We demonstrated that knottin peptides could be engineered to present non-native epitopes to bind to tumor-specific integrin receptors with high affinity [9, 10]. We used the *Ecballium elaterium* trypsin inhibitor II (EETI-II), a knottin from the squash family of protease inhibitors [37] (Figure 1A), and a truncated form of the Agouti-related protein (AgRP) [38] (Figure 1B), a knottin from human that regulates melatonin, as molecular scaffolds for engineering integrin-binding peptides with antibody-like affinities through directed evolution. First, yeast surface display was used to engineer EETI-II mutants that bound to αvβ3 and αvβ5 integrin receptors with low nanomolar affinity (Table 1 and Figure 3) [9]. In the process, we also discovered the first known peptide that binds with high affinity to αvβ3, αvβ5, and α5β1 integrins (Table 1). Since all three of these integrins can be co-expressed on tumors and contribute to angiogenesis, this knottin peptide has potential as a broad spectrum cancer therapeutic or imaging agent. Second, yeast display was used to engineer AgRP mutants that specifically bound to αvβ3 integrins with picomolar to nanomolar affinities [10].

Importantly, none of these engineered knottin peptides bind with appreciable affinity to αiibβ3 integrin, the platelet receptor. We produced these mutants either by chemical synthesis and *in vitro* folding or by recombinant expression in yeast at yields of greater than 10 mg/L. Next, we showed that the engineered EETI-II and AgRP knottin peptides strongly inhibited tumor cell adhesion to the extracellular matrix protein vitronectin, and in some cases

	EETI-II Loop 1 Sequence	IC_{50} (nM) U87MG	Binding specificity
Echistatin	N/A	5 ± 1	αvβ3, αvβ5, α5β1, αiibβ3
c(RGDyK)	N/A	860 ± 400	αvβ3, αvβ5
FN-RGD	CVTGRGDSPASSC	370 ± 150	αvβ3, αvβ5
FN-RDG	CVTGRDGSPASSC	N/D	N/D
Mutant 2.5D	CPQGRGDWAPTSC	19 ± 6	αvβ3, αvβ5
Mutant 2.5F	CPRPRGDNPPLTC	26 ± 5	αvβ3, αvβ5 α5β1

Table 1. Integrin binding affinity and specificity. Column 2: Sequences represent amino acids 2-14 of the engineered EETI-II proteins. Column 3: Competition binding of ^{125}I-echistatin to U87MG tumor cells. Column 4: Integrin binding specificity measured by competition binding of ^{125}I-echistatin to integrins coated on microtiter plates. IC_{50} = Half-maximal inhibitory concentrations. N/D = not determinable due to weak binding.

fibronectin, depending on their integrin binding specificity (data not shown). This work is described in detail in manuscripts published in *Journal of Molecular Biology* [10], and in *Proteins: Structure, Function, and Bioinformatics* [9].

Engineered EETI-II Knottin Peptides as a New Class of *in vivo* PET Imaging Probes

In collaboration with Prof Sanjiv Sam Gambhir, MD, PhD and Prof Zhen Cheng, PhD in the Molecular Imaging Program at Stanford, we established knottin peptides as a new class of agents for imaging integrin expression *in vivo* [22]. We used optical imaging and positron emission tomography to demonstrate that integrin binding affinity strongly influenced tumor uptake of knottin proteins in living organisms (Fig. 4 and data not shown). We also demonstrated that knottin peptides elicit desirable pharmacokinetic and biodistribution profiles for molecular imaging applications in living subjects. The N-terminal amine of the knottin peptides was chemically conjugated to a 1,4,7,10-tetraazacyclododecane-N,N',N",N"'-tetraacetic acid (DOTA) chelating agent and the radionuclide ^{64}Cu. ^{64}Cu-DOTA-labeled knottin peptides rapidly cleared from all tissues via renal excretion and exhibited no significant liver or muscle uptake. In contrast, ^{64}Cu-DOTA-c(RGDyK), a cyclic peptidomimetic under development for *in vivo* molecular imaging applications, showed substantial non-specific uptake the liver. MicroPET imaging in U87MG tumor-bearing mouse xenografts showed that higher tumor uptake was exhibited by ^{64}Cu-DOTA-2.5D and 2.5F knottins relative to ^{64}Cu-DOTA-FN-RGD and ^{64}Cu-DOTA-c(RGDyK), which bind to integrins with weak affinity (Fig. 4). Similar results were seen with *in vivo* biodistribution studies (data not shown). Tumor uptake was significantly blocked by co-injection of ^{64}Cu-DOTA-2.5D with a molar excess (0.5 μmol) of unlabeled c(RGDyK) (Fig. 4). Furthermore, ^{64}Cu-DOTA-conjugated knottin peptides were shown to be stable upon incubation in mouse serum, and also exhibited minimal metabolic breakdown in the blood and tumor after murine injection (data not shown). Thus, engineered knottin peptides show great potential as clinical diagnostics for a variety of cancers, and as radiotherapy and targeted drug delivery agents. This work is described in detail in a manuscript published in *Cancer Research* [22].

Summary

We engineered knottin peptides to bind to tumor-related integrin receptors with low nanomolar affinity [9, 10]. We showed that radiolabeled knottin peptides exhibit desirable pharmacokinetics and biodistribution in living subjects, namely high tumor uptake and low uptake and retention in the kidneys and liver, for potential use as tumor diagnostic agents or for targeted tumor therapy [22]. In addition, we observed a strong correlation between tumor uptake and integrin binding affinity [22]. Small proteins, like antibody fragments and the alternative scaffolds discussed above, exhibit rapid clearance in the body, allowing low background signals to be obtained for imaging applications. However, when conjugated to radiometals these scaffolds show extremely high renal uptake and retention [39, 40]. Radiolabeled EETI-II-based knottin peptides pass through the kidneys without significant metal retention (Fig. 4), making them extremely promising for targeted drug delivery or radiotherapy applications where toxicity is a concern.

In terms of therapy, $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$ integrin receptors are overexpressed in tumors and the tumor neovasculature and are involved in mediating angiogenesis and metastasis. We showed that knottin peptides induce cell death, like other integrin-targeted agents; however, we believe these effects will be much greater when knottin peptides are conjugated to a chemotherapeutic agent such as paclitaxel. In future studies, we will test the ability of engineered knottin peptides and knottin-chemotherapeutic conjugates to inhibit tumor growth, angiogenesis, and metastasis. In addition, we have expanded this technology and have now engineered knottin peptides that bind with high affinity to other cancer targets besides integrins, and are currently testing their potential as tumor-targeting agents.

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Questions to Consider

Which attribute of protein-based therapeutics cannot be easily modulated using directed evolution?

- **A.** Affinity
- **B.** Pharmacokinetics
- **C.** Stability
- **D.** Expression

Why are natural knottin peptides not good cancer drug candidates?

- **A.** They are immunogenic
- **B.** They are not stable to body temperature
- **C.** They do not bind to tumor biomarkers
- **D.** They are not stable to serum proteases

Why are engineered knottin peptides promising targeted drug-delivery agents?

- **A.** They have a long circulation half-life
- **B.** They exhibit high tumor and bone uptake
- **C.** They exhibit low non-specific uptake in the kidneys
- **D.** They are broken down (proteolyzed) rapidly in the body

How a Cancer Center Can Support Your Research

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Director, Stanford Cancer Center

Role of a Cancer Center

Cancer Centers developed out of the Nixon Administrations "war on cancer" that led to the development of the National Cancer Institute as a semi-autonomous arm of the National Institutes of Health. The goal of a cancer center is to promote the research of its members through providing opportunities for interdisciplinary collaborations and providing core resources that will enable basic, clinical, and population-based research activities. Stanford's Cancer Center has been funded for three years from the National Cancer Center to carry out these activities. We currently have about 315 members from across the Stanford campus, extending to four Schools and 33 Departments. The Cancer Prevention Institute of California (CPIC), located in Fremont, is our partner in developing opportunities for integrating cancer-relevant population studies into our basic and clinical research programs. CPIC is responsible for obtaining data on cancer incidence from cancer registries throughout a nine county region in the Bay area that is used in surveillance research to better understand patterns of cancer occurrence, treatment and survival in the population.

Mechanisms to Improve Collaborations

The mission of the Center is to promote interdisciplinary research, recognizing that many of the most important advances in cancer research and care come out of individual ideas and initiatives. However, in the current era, no one person is capable of integrating all aspects of cancer biology, clinical medicine, and epidemiology. The Stanford Cancer Center is committed to taking advantage of our outstanding basic research and technology development to advance the care of cancer patients and to promote cancer prevention. We do this through the following mechanisms:

Research Programs

We currently have 10 research programs, each of which has a leader and co-leader who use cancer center resources to hold meetings, provide individual incentives, and promote collaborations within the group and among groups. Our program and leaders are:

Cancer Biology	Michael Cleary and Linda Boxer
Radiation Biology	Amato Giaccia and Quynh Le
Cancer Stem Cells	Michael Clarke and Roel Nusse
Cancer Imaging and Early Detection	Sam Gambhir and Chris Contag
Molecular Therapeutics	Dean Felsher and Brandy Sikic
Cancer Immunology and Immunotherapy	Ed Engleman and Mark Davis
Lymphoma and Hodgkins Disease	Ron Levy and Richard Hoppe
Hematopoietic Cell Transplantation and Immunoreconstitution	Rob Negrin and Ken Weinberg
Cancer Epidemiology	Alice Whittemore and Ester John
Cancer Prevention and Control	Marcia Stefanick and David Spiegel

Developmental Cancer Research Awards

The Cancer Center awards several $50k and one or two $100k awards annually to develop new ideas that need funding to get preliminary data. Awards are for translational research and research in the

population sciences. Some preference is given to collaborative efforts and to specific areas that we hope to incentivize. Information on these awards, due in May, can be obtained from the Stanford Cancer Center website http://cancer.stanford.edu/research/devresawds/2009devresrfa.html

Recruitment

The Cancer Center facilitates the recruitment of faculty who have cancer-specific research interests that extend from clinical investigation through outcomes and health policy research to the basic and population sciences. Over the past four years, more than 55 faculty have been recruited to Stanford who are new cancer center members.

Communication

The Cancer Center puts out a monthly Bulletin that contains opportunities for grant submissions, new awards and papers of interest, and a note on ongoing initiatives. There is also a newsletter that goes out quarterly and features articles of general interest for the membership. To subscribe to the e-mail bulletin visit: https://mailman.stanford.edu/mailman/listinfo/stanford_cancer_center_news

Where is the Cancer Center?

The administrative center will be newly located on the second floor of the Lokey Stem Cell Building that has a number of laboratories focused on cancer and cancer stem cell research. It has close ties to the Institute for Stem Cell Biology and Regenerative Medicine, located on the third floor. The Cancer Center is associated with the Clinical Cancer Center that has outpatient disease-specific clinics that go across departments. A new building, scheduled to open in 2012 at the 800 Welch Road location, will house the infrastructure for clinical research. Core facilities are located in Beckman, Lokey, CCSR and off-site on California Avenue. All core facilities and mechanisms for access can be located on the Cancer Center website: http://cancer.stanford.edu/research/core/.

Membership

Membership is open to all faculty at Stanford who have highly cancer-relevant interests. Trainees who are working with Cancer Center members can apply for developmental support with their mentor. Associate Members may not yet have achieved independent funding but have full access to core facilities.

Has the "war on cancer" succeeded? Have cancer centers played a role?

Gina Kolata: "Is cancer just an impossibly hard problem? Or is the United States, the only country to invest so much in cancer research, making fundamental mistakes in the way it fights the cancer war?" NY Times, April 2009.

Recommended Reading

Stanford Cancer Center website: http://cancer.stanford.edu

NCI website: http://www.cancer.gov

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Subjects for Discussion

What is the best way to facilitate cancer research and the careers of young researchers, given a $4 billion dollar annual investment by the NCI?

How do participants in the CCRTP define the impediments to pursuing and contributing to cancer research activities at Stanford?

Cancer Genomics and Cancer Gene Discovery

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Cancer genomics entails the application of genomic-scale approaches to study cancer at the level of genome, rather than individual genes. Over the past decade, DNA microarrays have revolutionized cancer genomics research, and now "next generation" DNA sequencing promises even greater return. An immediate goal of such studies is to catalog the spectrum of genome alterations, including DNA mutations and structural alterations, as well as the resultant changes in gene expression and pathway activity. The broader goals include furthering our understanding of cancer development and progression, and identifying strategies for improved cancer diagnosis, prognostication and treatment.

One area in particular where cancer genomics has made significant inroads is in breast cancer research. Microarray-based gene-expression profiling has refined the molecular classification of breast tumors, identifying estrogen receptor (ER)-positive subtypes (luminal A and B) with distinct clinical behavior, and highlighting a triple-negative (ER/PR/HER2-negative) subtype with "basal-like" expression patterns and unfavorable prognosis. Prognostic and predictive gene-expression signatures have even entered into clinical practice.

Transcriptional profiling has also informed key facets of breast cancer biology. For example, epithelial-mesenchymal transition (EMT) is a normal process in development, thought to be co-opted by cancer cells to effect invasion and metastasis. In one study, comparison of epithelial-like and mesenchymal-like breast cancer lines defined an EMT signature with prognostic relevance. A top signature gene, LYN (a Src-family kinase), was itself prognostic and associated with basal-like cancers. LYN was further characterized as a novel mediator of EMT, and a potential "theranostic" marker of response to Src-family kinase inhibitors (e.g. dasatinib), with particular relevance to basal-like breast cancers.

Genomic profiling by array-based comparative genomic hybridization (CGH and SNP arrays) has also defined the spectrum of genomic DNA aberrations in breast cancer. Notably, most breast cancer genomes fall into one of three distinct patterns of DNA copy number alteration (Figure 1). "Simple"

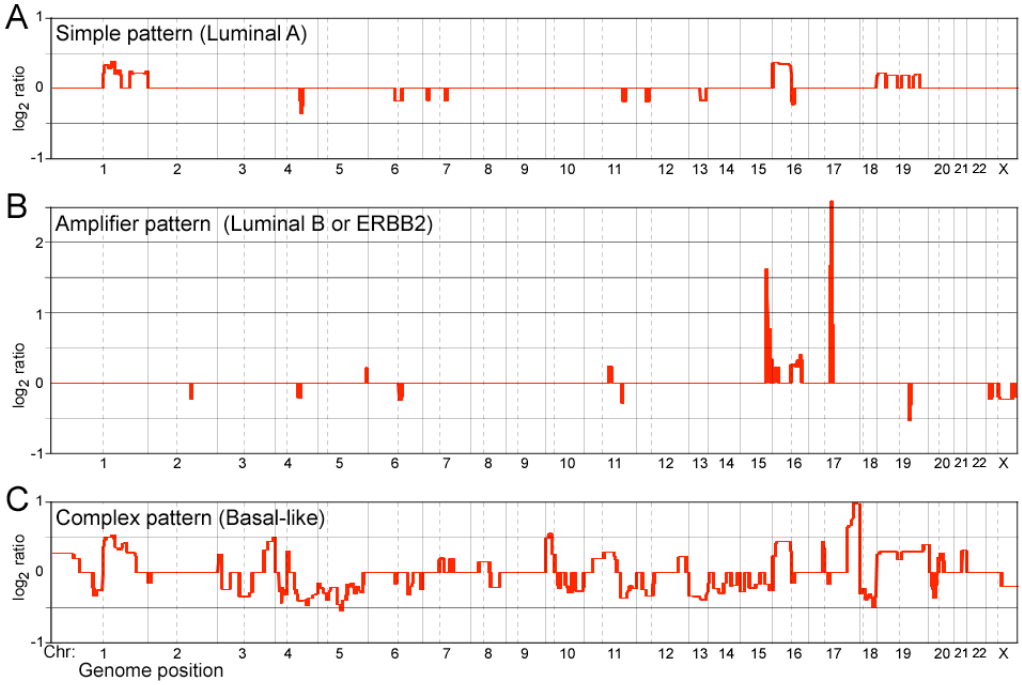

Figure 1. Genomic profiling reveals distinct patterns of DNA copy number alteration among breast cancers. Exemplary genomic profiles are shown here for individual breast tumors representing (A) "Simple" pattern, characteristic of luminal A tumors; (B) "Amplifier" pattern, typical of luminal B and ERBB2 subtype tumors; (C) "Complex" pattern, characteristic of basal-like tumors. For each of the above graphs, the red trace represents the segmented CGH profile (tumor/normal fluorescence $\log_2$ ratio *vs.* genome map position).

pattern genomes have few aberrations, tending to occur as gains or losses of whole chromosome arms (often 1q+, 16p+, 16q-), and are associated with luminal A tumors. "Amplifier" pattern genomes exhibit focal, high-level DNA amplifications, including at 8p12 (*FGFR1*), 8q24 (*MYC*), 11q13 (*CCND1*), and 17q12 (*ERBB2*), and are characteristic of luminal B and *ERBB2* (HER2)-positive tumors. Lastly, "complex" (or "sawtooth") pattern genomes exhibit numerous copy number transitions (commonly including 4q-, 5q-, 10p+), and are associated with basal-like tumors. The three patterns target distinct loci, indicating the involvement of distinct cancer genes. The different patterns also imply distinct underlying mechanisms of genomic instability. In particularly, the complex pattern, also evident in *BRCA1*-mutant tumors, suggests a defect in the repair of DNA double-strand breaks, with implications for therapy, e.g. using poly (ADP-ribose) polymerase inhibitors.

By pinpointing DNA amplifications and deletions, genomic profiling has also sped the discovery of novel cancer genes. A particularly good example is in the emerging area of "lineage-dependent" oncogenes. Such genes are typically master transcriptional regulators of a specific cell-lineage, that become aberrantly expressed (often by DNA amplification) in tumors derived from that lineage. Recent discoveries include *MITF* in melanoma, *TTF1* (*NKX2-1*) in lung cancer (Figure 2), and *GATA6* in pancreatic cancer. Additional studies should clarify how such genes control normal lineage differentiation in one cell context, while are oncogenic in another. Because such genes exhibit oncogene "addiction", they also provide new opportunities for therapeutic intervention.

Next-generation DNA sequencing now provides a new tool to profile DNA mutations and structural alterations in cancer genomes. In particular, paired-end sequencing of genomic DNA fragments reveals the architecture of structural rearrangements, including balanced events not visible by CGH. Transcript sequencing (including paired-end RNAseq) can also now define the spectrum of transcript variants. One such notable class is fusion genes, like the prototype *BCR-ABL* in chronic myeloid leukemia, which can provide useful diagnostic markers and therapeutic targets. While frequent in leukemias, whether such fusion genes are prevalent among the common epithelial cancers remains unclear, though the recent finding of *TMPRSS2-ETS* fusions in prostate cancer has reenergized the search.

In summary, cancer genomics investigates the genomic-scale landscapes of cancer, to unravel pathogenetic mechanisms, and to define better strategies for diagnosis and therapy. Advancing technologies, combined with innovative analysis, should help usher in the new era of personalized cancer medicine.

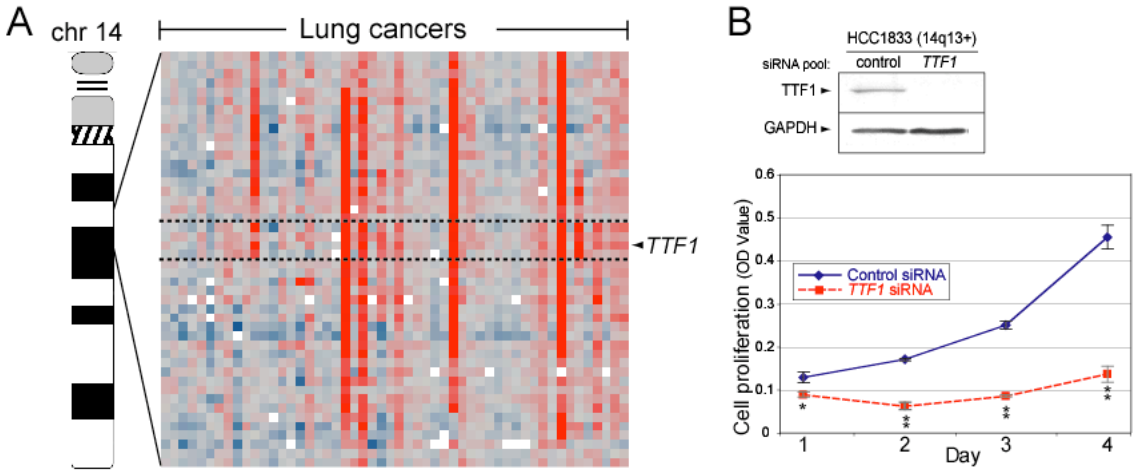

Figure 2. Genomic profiling identifies *TTF1* as a lineage-dependent oncogene in lung cancer. (A) Heatmap of array CGH data, where columns are lung cancer samples and rows are genes ordered by their position on 14q. Red indicates DNA amplification, with the amplicon core (spanning *TTF1*) bracketed. (B) RNAi knockdown of TTF1 (confirmed by Western blot, *above*) inhibits cell proliferation (WST-1 assay, *below*) in a lung cancer cell line with 14q13 amplification, indicative of oncogene dependency.

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Questions to Consider

All of the following are true of "complex pattern" breast cancer genomes, <u>except</u>:

- **A.** they are characteristic of basal-like breast cancers
- **B.** they are characteristic of breast tumors of *BRCA1* mutation carriers
- **C.** they are comprised of numerous high-level DNA amplifications
- **D.** their patterns are consistent with a possible dysfunction of DNA double-strand break repair

All of the following are true of "lineage-dependent" oncogenes, <u>except</u>:

- **A.** they are often master transcriptional regulators of a specific cell lineage
- **B.** their deregulated expression in cancer often results from DNA amplification
- **C.** cancer cells are often "dependent" on their expression for cell proliferation or survival
- **D.** prototypical examples include MYC, RAS and TP53

All of the following are true of oncogenic fusion genes, <u>except</u>:

A. they are commonly described in leukemias

B. they are rare events in prostate cancer

C. they often provide excellent diagnostic markers and therapeutic targets

D. next-generation DNA sequencing provides a new tool for their discovery

High-throughput Gene Sequencing and Cancer

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Introduction

Mutations and genomic aberrations in cancer genomes contribute to the development of cancer (Kinzler, Nilbert et al. 1991) and tumor behavior such as metastatic spread (Vogelstein and Kinzler 1992). These same mutations and aberrations may be useful as genetic prognostic and predictive biomarkers with clinical application. Full genome sequences of a number of tumors as well as surveys of the coding regions of cancer genomes have been recently been published, pointing to a new way of understanding the genetic and genomic basis of cancer (Pleasance, Cheetham et al. ; Pleasance, Stephens et al. ; Mardis, Ding et al. 2009).. These systematic, large-scale DNA sequencing surveys have revealed that the genetic spectrum of mutations in cancers appears to be highly complex with numerous low frequency bystander somatic variations, and a limited number of common, frequently mutated genes (Stratton, Campbell et al. 2009).

Examples

Citing some examples, the earliest surveys of cancer genomes by sequencing involved analysis of the exome, the compilation of the exons in the human genome. These early studies used traditional Sanger DNA sequencing. For example, Wood et al. (2008) sequenced exons of 20,857 transcripts, representing 18,191 genes from 11 breast and 11 colorectal cancers and reported 1,718 genes with somatic non-silent mutations (Wood, Parsons et al. 2007). By evaluating the mutation data they identified 140 candidate cancer genes in both tumor types that are significantly represented above background mutations (Wood, Parsons et al. 2007). Their study revealed only few frequently mutated genes among abundance of rare variants and the identified gene mutations clustered key biological pathways such as PI3K-mediated signal transduction (Wood, Parsons et al. 2007). Likewise, Jones et al. (2008) sequenced 20,661 protein-coding genes in 24 pancreatic cancers and identified 1,327 genes with one mutation and 148 genes with two or more mutations (Jones, Zhang et al. 2008). An average of 63 genetic alterations was identified in the 24 pancreatic cancers surveyed that clustered in 12 generally defined cellular signaling pathways and biological processes. Similarly, Parsons et al. (2008) sequenced 20,661 protein coding genes in 22 glioblastoma multiforme samples (Parsons, Jones et al. 2008). Importantly, they identified frequent (12%) mutations in the *IDH1* (isocitrate dehydrogenase enzyme) gene that were associated with young patients with secondary disease and increased overall survival. Similarly, the Cancer Genome Atlas consortium, a National of Institute project geared towards the genetic characterization of cancer, sequenced 601 genes in 91 glioblastoma multiforme samples. Out of the total, 224 of these genes contained mutations in at least one sample. However, only eight genes and their mutations were identified to be statistically overrepresented compared to background mutations (2008). Noteworthy, the *IDH1* gene was not among the list of candidates in the Cancer Genome Atlas effort - underlining the importance of the comprehensive genome sequencing.

Sequencing Cancer Genomes with Next Generation Technology

Currently, next generation DNA sequencing technology is the platform of choice for characterizing the DNA of cancer genomes. These sequencers produce Gigabases worth of DNA sequence data and have enabled the comprehensive resequencing of cancer genomes. All of these new next generation sequencing methods generally require that the matched normal genomic DNA from the same individual be sequenced simultaneously to discriminate germline variants from somatic mutations. With multi-dimensional genomic characterization involving transcriptome and copy number variation surveys carried through DNA sequencing, new biological and clinical relevant genetic and genomic changes can be revealed (Network 2008).

To sequence a cancer genome, many research groups use "shotgun" methods. This typically involves the random fragmentation using physical or chemical breakage of cancer genomic DNA from a given sample with subsequent ligation of the adaptor oligonucleotide sequences to the ends of the DNA fragments. This preparation is frequently referred to as a "sequencing library". Sonication, physical interruption, chemical or enzyme-based methods have been utilized in fragmentation of the genomic DNA sample. Furthermore, next generation systems can sequence paired ends of a DNA fragment of known size with next-generation platforms (Korbel, Urban et al. 2007; Campbell, Stephens et al. 2008). This paired-end approach results in mate pairs of sequences derived from the same DNA molecule. As a result, one can detect a variety of structural variation and rearrangements. This includes insertions, deletions, and translocations from a cancer genome.

An example of this whole genome approach and the entailed cost is the recent publication of a cancer genome from an acute myelogeneous leukemia (AML) sample with normal cytogenetics (e.g. diploid chromosome complement without aneuploidy) (Ley, Mardis et al. 2008). For this cancer genome, the leukemia sample required 32.7-fold sequencing coverage, equivalent to 98 billion bases, and 13.9-fold coverage (41.8 billion bases) for the normal skin sample. The majority of mutations (5) were bystanders and not confirmed in other AML samples. Two mutations were known AML mutations that had previously been discovered. The cost was described as being over $150,000 for the two samples, which obviously limits the application of this type of sequencing approach to large clinical populations.

Other methods to sequence cancer genomes involves using RNA from cancer samples and sequencing the transcriptome to obtain information about the expressed subset of a cancer genome to find mutations and rearrangements. Large sample sizes and deeper resequencing are much needed in resolving clinical and biological relevance of the mutations as well as in detecting somatic variants in heterogeneous samples and cancer cell sub-populations. However, even with the next generation sequencing technologies, the overwhelming size of the human genome and need for very high fold coverage represents a major challenge for up-scaling cancer genome sequencing projects. Assays to target, capture, enrich or partition disease-specific regions of the genome are also being employed for reducing the complexity of sequencing cancer genomes.

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Questions to Consider

For short read sequences, what are the most straightforward methods to identify an inversion from a cancer genome?

- **A.** Confirmation of a breakpoint junction via a single sequence read
- **B.** Alterations in fold coverage of the tumor compared to the normal genome
- **C.** Unique mapping of two mate pair sequences on the same chromosome but falling outside of the predicted fragment range
- **D.** Strobe sequencing of large fragments

What is the key indicator of adequate sequencing of a cancer genome?

- **A.** Phred-like quality scores
- **B.** Mappable base pairs
- **C.** Fold sequencing coverage
- **D.** Concordance of single nucleotide variants with known SNPs

How can one overcome issues of highly repetitive motifs that complicate cancer genome targeted resequencing?

- **A.** Deep fold coverage exceeding 100
- **B.** Utilizing only very high Phred-like scores greater than 40
- **C.** Assembly of sequence to recreate long regions instead of alignment
- **D.** Repeat masking

Cancer Genes and Clinical Diseases

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Stanford University

The rapid advances in our understanding of the genetic basis for many common as well as rare cancers, together with technical strides allowing for rapid and affordable diagnostic genotyping and DNA sequencing, have resulted in the ability to identify and diagnose a number of cancer susceptibility syndromes, and provide comprehensive cancer susceptibility assessment for unaffected family members. Identifying individuals at elevated risk for developing cancer provides the opportunity for more focused and intensive efforts for prevention, screening and early detection of cancer. Approximately one-third of common cancers appear to have a familial basis, and up to 10% are clearly hereditary. Families exhibiting multiple cancers occurring at a relatively young age, often multiple times within single individuals, and following an autosomal dominant pattern of inheritance are most likely to have a highly penetrant cancer susceptibility syndrome appropriate for clinical genetic testing. A number of genes resulting in dominant cancer susceptibility syndromes that confer high relative risks of cancers among carriers were identified through classic genetic linkage analyses over the past several decades (Table 1). Most of these hereditary cancer susceptibility genes fall into the class of tumor suppressor genes or DNA repair genes, and result in genetic instability when both copies are mutated or lost from a cell. However, when a single mutant copy is passed on through an individual's germline DNA, all cells of the child are heterozygous for this mutation, and thus only one additional "hit" is required for functional loss. Therefore, an individual with a germline cancer gene mutation not only has an elevated risk of developing cancer, but has a 50% chance of passing on the mutant allele to each child, resulting in an autosomal dominant pattern of cancer risk transmission, with on average half of each generation inheriting the gene. Most cancer susceptibility genes are less than 100% penetrant, meaning that not every individual inheriting the altered gene will develop cancer. Therefore, to provide adequate genetic counseling, careful family histories, knowledge of mendelian genetics, and information regarding the types of cancer and their age-specific penetrance are necessary.

Table 1. Autosomal Dominant Hereditary Cancer Syndromes and Causative Genes

Breast and Ovarian Cancer	
	BRCA1&2
Colon Cancer and Polyposis	
HNPCC	MMR
FAP	APC
Polyposis	MYH
Cowdens	PTEN
Peutz-Jehgers	STK11
Juvenile Polyposis	SMAD4
	BMPR1A
Other GI Cancers	
Gastric	CDH1
Pancreas	p16
MEN2/MTC	
	RET
Von Hippel Lindau	
	VHL
Li-Fraumeni	
	p53

Despite the success in identifying these highly penetrant cancer susceptibility genes (e.g. BRCA1, BRCA2, MSH2, MLH1), they are relatively rare, and do not account for most familial cancers. Presumably, other lower risk, less penetrant genes contribute to most familial cancer risk, likely in a multigenic fashion not always showing a Mendelian pattern of inheritance. Detecting these genetic alterations requires different strategies than linkage analysis, several of which have become more common in recent years. One approach compares the frequency of alleles of genes or associated single-nucleotide polymorphisms (SNPs) in cases and controls across the entire genome. Recently, large-scale consortia examining thousands of cases and controls have identified 30 or more susceptibility loci for lung, prostate, breast, and colorectal cancer. The large number of subjects and the huge number of SNPs (>500,000) permit the detection of very small, but highly significant associations between any one SNP and disease. The type of genes or genomic loci detected by these "genome-wide association studies" (GWAS) are very different than the candidate genes found by linkage; they are more common (usually found in well over 5% and sometimes up to 50% of the population) and confer far lower levels of cancer

risk (often with relative risks of only 1.1 – 1.3) (Figure 1). The biologic relationship of many of these genetic markers to the pathogenesis of a tumor is not yet understood. For instance, one region of chromosome 8q24 harbors several (possibly five) independent loci, each of which is associated with an increased risk of breast, prostate, or colorectal cancer or all of these cancers, but no candidate genes have

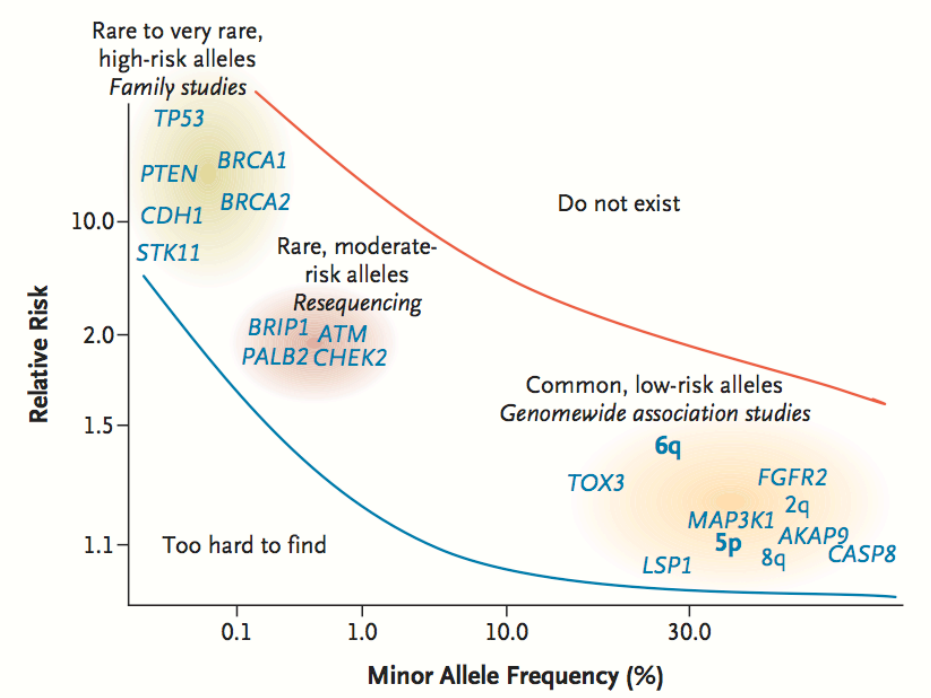

Figure 1. Breast-Cancer Susceptibility Loci and Genes (from WD Foulkes, N Engl J Med 2008; 359:2143)

been identified in this "gene desert." Although several commercial entities have begun to offer genotyping of individuals for alterations in these low-penetrance alleles, the clinical utility and translation of these results to patient management, screening and risk reduction remains unclear. Likely more relevant for clinical use is the emergence of massive parallel "next generation" DNA sequencing technologies, that allow for resequencing of candidate genes (or more recently, of all exons or even the whole genome) in affected persons in families with familial cancer syndromes. This approach will likely identify rare variants conferring a higher relative risk (>2), particularly when multiple, additive risk alleles segregate in an individual. This high-throughput approach to germline sequencing will rapidly transform risk assessment and presents a major challenge to bringing "personalized" medicine to the clinic.

Indications for clinical genetic testing for cancer susceptibility include when 1) an individual has a personal or family history features suggestive of a genetic cancer susceptibility condition, 2) the genetic test can be adequately interpreted, and 3) the test results will aid in diagnosis or influence the medical or surgical management of the patient or family members at hereditary risk of cancer. Genetic testing should only be done in the setting of pre- and post-test counseling, which should include discussion of possible risks and benefits of cancer early detection and prevention modalities. None of the cancer susceptibility tests currently available are as yet appropriate for screening of asymptomatic individuals in the general population. Genetic testing usually begins in an individual affected with cancer; if a germline mutation is identified, unaffected first degree relatives are at 50% risk for carriage and can consider undergoing testing. Unaffected family members who are identified to be genetic mutation carriers must then discuss with a clinical cancer geneticist individualized options for cancer screening, using radiographic, biochemical, endoscopic, or direct physical examination approaches. Appropriate treatment options may include risk-reducing surgery and chemopreventive strategies, in individuals with a known mutation of a cancer predisposition gene. A concerted effort is required to ensure that the information conveyed by a genetic test result is properly understood by the patient. Issues of concern in this regard include the meaning of a negative test in a family lacking a known deleterious mutation in

the gene being analyzed, the implications of detecting a mutation of uncertain significance, and the probabilistic nature of a positive test result.

Future directions for clinical and genetic research in this field include identifying additional cancer susceptibility genes and genetic modifiers of cancer risk, improved diagnostic imaging and biomarkers for screening high-risk individuals, discovery of chemopreventative agents, a better understanding of cancer genetics in various ethnic populations, and using DNA sequence and molecular signatures to improve cancer diagnosis and treatment.

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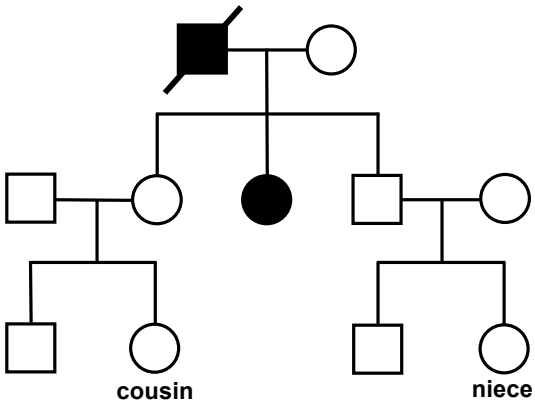
Questions to Consider

Question 1

The father of this family died of male breast cancer, and one of his daughters was diagnosed with breast cancer at the age of 48. She and her niece seek genetic counseling, and find that they have a mutation in the BRCA2 gene.



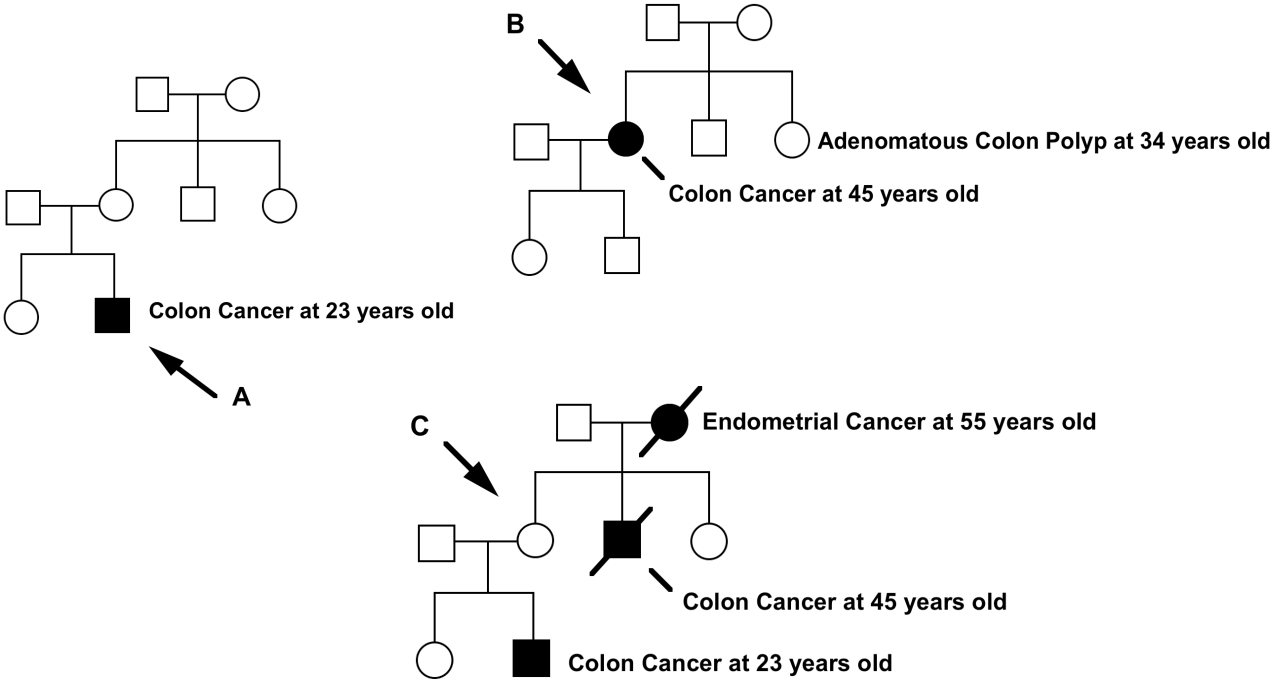
A. What is the probability that the niece's female cousin has inherited the familial mutation?

B. If penetrance of a familial BRCA2 allele is 10% in men, what is the probability that the cousin's brother will be diagnosed with breast cancer?

C. One person in this pedigree is known to be a carrier of the BRCA2 mutation, but is unaffected. Who is this person, and why is he/she known to be a carrier?

Question 2

Which of the following *individuals* (A, B or C) has the highest likelihood of having a germline mutation in either the MLH1 or MSH2 DNA mismatch repair genes, resulting in the inherited cancer syndrome Hereditary Non-Polyposis Colorectal Cancer (HNPCC)?



Question 3

A 32 year old woman comes to your primary care medical clinical with the results of a genotyping study performed by a direct-to-consumer company on her saliva DNA, which she won through an office raffle. It states she has a polymorphic change at a locus that in several genome-wide association studies is statistically associated with an increased risk for breast cancer, with a relative risk of 1.1. She is quite alarmed, and seeks your advice on what her breast cancer risk really is. You suggest the following:

A. You agree that she has a genetic mutation in a breast cancer gene, and is at substantially elevated risk and should consider bilateral prophylactic mastectomies.

B. You tell her the test suggests that she is at over 100% increased risk for breast cancer, and should consider mastectomies or regular and intensive screening using mammagrophy and MRI scans.

C. You obtain a family history, and upon hearing that she has no known relatives with breast cancer, but does have a maternal aunt with colon cancer diagnosed at age 45, counsel that she has a 10% increased risk of breast cancer and should start regular mammography screening.

D. You tell her that such tests are not validated, and are impossible to interpret with regard to her personal risk of breast cancer.

The Impact of Radiation Biology Research on Cancer Radiotherapy

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Examination of the role of radiotherapy in the initial treatment of cancer patients indicates that approximately one-third of all patients are treated with either radiotherapy alone or in combination with chemotherapy and surgery. Even more significant is the fact that most radiotherapy is only used to control local or regional tumor growth, and the treatment of metastatic disease has been largely untouched until recently. The use of radiotherapy to treat metastatic disease, which is the underlying reason for the death of ninety percent of cancer patients, brings a new window of opportunity for radiotherapy and also new thinking about the biology of hypofractionated radiotherapy. While tumors can acquire resistance to different forms of chemotherapy, there are no tumors that are inherently resistant to radiation. This important difference between radiation and chemotherapy is underappreciated as the development of most new anti-cancer therapies are not targeted for combination with radiation, and have very low curative potential by themselves. The major factor that influences the effectiveness of radiotherapy is the tumor microenvironment, in particular hypoxia. To date, the development of drugs to target tumor hypoxia has been unsuccessful, in large part due to the failure to stratify patients in trials based on whether or not they possessed hypoxic tumors. Hopefully, future trials will benefit from the mistakes of the past.

"Seeds" Influencing the "Soil"

Metastasis is a remarkably inefficient, multi-step process. The determinants of "successful" metastatic growth in a given organ are poorly understood, but there is substantial evidence to suggest that tumor cells and host tissue both play important roles in metastasis. A great deal of work has been devoted to understanding the genetic and phenotypic characteristics of metastatic tumor cells, and tumor cells clearly must have (or develop) the ability to metastasize before dissemination from a primary tumor can occur. However, the majority of tumor cells that escape from a primary tumor mass either die in the

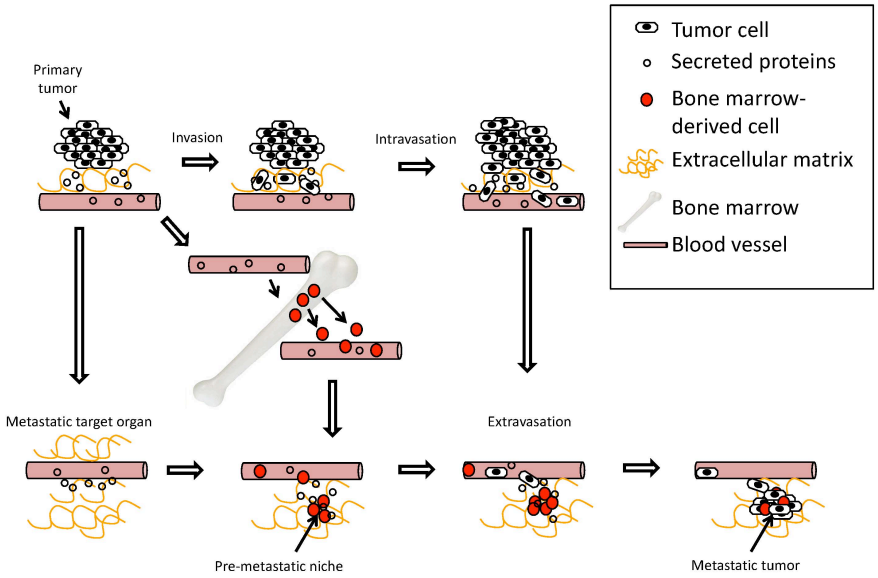

Figure 1: The multiple steps of metastasis including formation of the pre-metastatic niche.

circulation or fail to invade into distant organs. Furthermore, tumor cells that enter metastatic target organs can die or lie dormant for extended periods of time; only a small proportion survive and proliferate to form micrometastatic tumor foci. The continued growth of micrometastatic tumor foci into macrometastatic tumors requires a switch to active angiogenesis, representing a collaboration between the metastatic tumor cells and surrounding host tissue.

The role of host tissues in allowing (or even promoting) metastatic tumor growth was somewhat controversial in the past. Work by Stephen Paget in the late 1800's led to his "seed-and-soil" hypothesis, suggesting that metastatic tumor cells (seeds) must localize in suitable host tissues (soil) in order to produce metastatic tumor foci. Thus, the propensity of a given tumor cell type to preferentially metastasize and grow in a distant target organ is related to the inherent (or acquired) properties of the tumor cells and the target organ itself. Some tissues are more receptive to a given metastasizing tumor cell type, which can explain the tendency of some tumor cells to metastasize to some organs more often than other organs in a way that cannot be explained by differences in blood flow. In addition to target organ-specific growth of metastases, metastatic tumor foci seem to grow preferentially in specific areas of some tissues. These observations imply that differences in the regional tissue microenvironment may influence the survival and proliferation of metastatic tumor cells.

Recent evidence indicates that primary tumors actively modify the metastatic soil to promote subsequent metastatic tumor growth (*Figure 1). An important step forward came when Kaplan *et al.* (2005) observed significant accumulation and aggregation of bone marrow-derived cells (BMDCs) in metastatic target organs after implantation of metastatic tumors. The BMDCs aggregated into specific microregions of tissues before the arrival of metastatic tumor cells, and the presence of BMDC aggregates correlated with subsequently increased metastatic growth in these tissues. It was in this seminal paper that the term "premetastatic niche" was first coined. The premetastatic niche is made up of both cellular and extracellular matrix components, and is mediated by signals provided by the primary tumor (Figure 2).

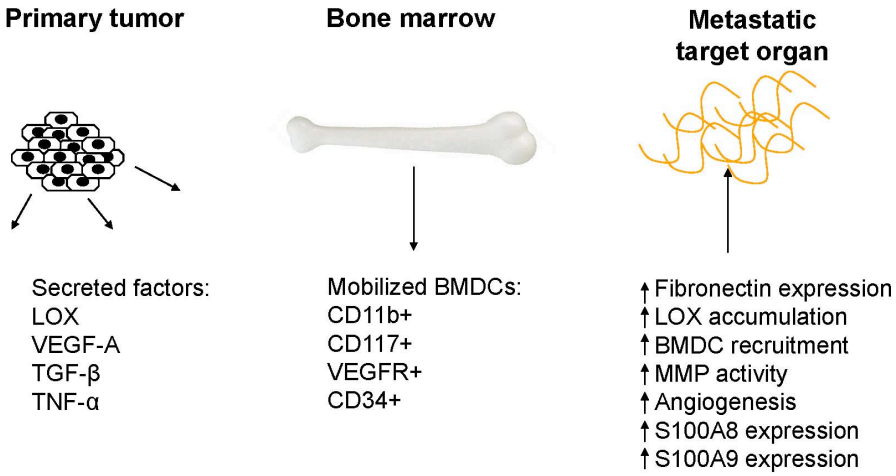

Figure 2: Pre-metastatic niche formation requires collaboration between the primary tumor, bone marrow-derived cells, and the extracellular matrix in metastatic target organs. LOX is the only tumour-secreted protein identified to date that is directly involved in formation of pre-metastatic niches.

Premetastatic Niche Formation Precedes Metastatic Growth

The presence of metastatic tumor cells is not required for formation of the premetastatic niche. Indeed, accumulation of finronectin (FN), lysyl oxidase (LOX), and aggregation of BMDCs in premetastatic niches occurs prior to the arrival of detectable numbers of disseminated tumor cells. Furthermore, formation of premetastatic niches can be induced in tumor-free mice after intraperitoneal injection of serum-free conditioned media preincubated with metastatic tumor cells in vitro. We have found that conditioned media derived from human and murine mammary tumor cells incubated in hypoxia induces

BMDC accumulation in metastatic target organs that can be disrupted when LOX is inhibited with a small molecule inhibitor (β-aminoproprionitrile; BAPN) or inhibitory antibody. In addition, BMDC accumulation did not occur after daily injections of conditioned media derived from tumor cells stably expressing LOX shRNA. Interestingly, conditioned media obtained from hypoxic tumor cells that contained high levels of enzymatically active LOX was a more potent inducer of the premetastatic niche than conditioned media derived from normoxic tumor cells that contained significantly lower levels of enzymatically active LOX. Thus, premetastatic niche formation was dependent on the availability of enzymatically active LOX secreted from hypoxic metastatic tumor cells.

BMDCs may affect the local cytokine milieu within metastatic target organs, independent of the presence or absence of metastatic tumor cells. It is therefore possible that BMDC accumulation induced by metastatic tumors may "precondition" target organs to promote subsequent metastatic growth. Indeed, the induction of pulmonary premetastatic niches by injecting LOX-containing conditioned media stimulated the metastatic growth of largely nonmetastatic mammary tumors expressing LOX shRNA. Enhanced CD11b+ cell accumulation and pulmonary metastasis of LOX shRNA tumors occurred when exogenous LOX was injected daily during primary tumor growth, and even when LOX was provided only for 2 weeks prior to tumor implantation. Thus, lung tissue could be preconditioned by LOX resulting in BMDC accumulation, premetastatic niche formation, and improved metastatic growth of typically nonmetastatic tumors.

Evidence that the site-specificity of metastasis is at least partly mediated by proteins secreted by tumor cells comes from a set of experiments in which conditioned media derived from B16 cells was used to modify the metastatic site-specificity of LLC cells. Mice were injected with conditioned media derived from B16 cells in vitro to initiate BMDC accumulation in B16 metastatic target organs. LLC cells were then implanted subcutaneously, and injection of B16 conditioned media was continued during tumor growth. Interestingly, metastatic foci of LLC cells were observed in B16 metastatic target organs, indicating that the proteins secreted from B16 tumor cells influenced the subsequent sites of metastatic growth of LLC tumor cells. Whether or not the B16 media was also affecting the LLC cells in the primary tumor is an open question, and the identity of the secreted protein(s) responsible for site-specific metastasis is unknown. Nevertheless, these data underscore the importance of tumor-derived secreted proteins in formation of premetastatic niches and enhancing metastatic growth independent from the presence of metastatic tumor cells in the organ.

BMDCs and Hypoxia

Preferential accumulation of CD11b+ cells is observed in hypoxic regions of primary tumors, and recruitment of CD45+ myeloid cells (including CD11b+ cells) to primary tumors is dependent on the transcriptional activity of hypoxia-inducible factor-1 (HIF-1). BMDCs are recruited to injured or ischemic normal tissue, and primitive hematopoietic cells are found preferentially in hypoxic "niches" in the bone marrow. Thus BMDCs accumulate in hypoxic normal and neoplastic tissue, suggesting that oxygenation levels in premetastatic sites may influence BMDC accumulation. However, despite evidence of severe hypoxia in micrometastases, the role of regional oxygenation status within the premetastatic niche itself is unknown.

Therapeutic Targeting of the Premetastatic Niche

By improving our understanding of how premetastatic niches develop and the role of premetastatic niches in enhancing metastatic tumor growth, a variety of therapeutic strategies to therapeutically target premetastatic niches can be developed. Immunological inhibition of VEGFR-1+ cells or of the FN receptor VLA-4 has been shown to reduce BMDC accumulation in metastatic target organs and inhibit metastatic growth. Similarly, inhibition of enzymatically active LOX with the small molecule inhibitor BAPN or with a LOX antibody prevented premetastatic niche formation and metastatic growth of human and murine mammary tumors. LOX secreted by hypoxic tumor cells is an excellent therapeutic target to decrease premetastatic niche formation and metastatic growth. LOX is essential for premetastatic niche formation, and LOX also influences metastatic dissemination of cells from primary tumors. Furthermore, inhibition of LOX is less likely to induce pleiotropic side effects compared to targeting BMDCs based on immunological inhibition of cell surface markers.

While the mechanisms underlying the formation of premetastatic niches are being uncovered, very little is known about the maintenance of premetastatic niches after they have formed. Proteins such as LOX secreted by hypoxic tumor cells are required to initiate premetastatic niches, but we do not know if continual secretion of LOX by primary tumors or continued activity of LOX within premetastatic niches are required to maintain premetastatic niches over time. Presumably, after metastatic tumor cells have arrived in premetastatic niches, paracrine secretion of LOX and other factors could act locally even after removal of the primary tumor to maintain the niche. However, what if premetastatic niches have formed in metastatic target organs, but metastatic tumor cells have not yet arrived? The stability of the premetastatic niche in the absence of metastasizing tumor cells is unknown.

The timing of therapeutic intervention may be essential when targeting the premetastatic niche. Since premetastatic niches develop prior to the arrival of metastatic tumor cells, the value of targeting premetastatic niches after metastatic disease is present must be considered. It is conceivable that maximal therapeutic benefit may be realized by targeting formation of the premetastatic niche in patients with localized primary tumors at risk of developing metastatic disease, in order to prevent metastatic growth before gross metastatic disease is detectable. However, in order to select patients that have premetastatic niches in metastatic target organs and are therefore at risk for developing metastatic disease, we must develop reliable methods to detect components of premetastatic niches in metastatic target organs and/or blood.

Evidence for Premetastatic Niches in the Clinic

Clinical data is emerging to indicate that BMDC accumulation and premetastatic niche formation may occur in cancer patients. VEGFR-1+ cells have been identified in some clinical tissues, both associated with metastatic tumor cells and in regions of tissue that do not contain detectible tumor cells. MMP-9 levels are elevated in the lungs of patients with a range of solid primary tumors. LOX and CD11b+ cells have also been observed in a variety of metastatic tissues by staining of tissue microarrays. Thus, many of the important players involved in premetastatic niches have been observed in clinical biopsies of metastatic tissue. However, metastatic tissues are seldom biopsied on a routine basis, particularly before evidence of disseminated metastatic disease in the tissue. Thus, alternative methods of detection are required to identify patients that have developed premetastatic niches with undetectable metastatic disease, who are at high risk of growing metastatic tumors.

Many components of the premetastatic niche are transported to metastatic target organs through the bloodstream, providing an easily accessible source for detecting circulating proteins and/or cells. Enzymatically active LOX can be detected in the plasma, and we have found that circulating levels of LOX are related to the presence of metastatic disease in patients with prostate cancer or head and neck cancer. Elevated levels of CD11b+ cells are found in the peripheral blood of patients with cancer of the head and neck, lung, or breast, and in patients with renal cell carcinoma. Moreover, increased numbers of circulating CD11b+ cells have recently been correlated with clinical stage and metastatic tumor burden in a mixed group of patients with a variety of tumor types. These findings illustrate the feasibility of measuring premetastatic niche components in the circulation of cancer patients, although further correlation of these data with identification of patients that have developed premetastatic niches (and with therapeutic outcome) is required before blood samples can be used to direct therapeutic targeting of the premetastatic niche.

Concluding Remarks

Emerging evidence supports the idea that the primary tumor microenvironment can influence the microenvironment within distant metastatic target organs. The identification of BMDCs in premetastatic niches has opened up new avenues of research into the role that host tissues may (inadvertently) play in promoting the survival and growth of metastatic tumor cells. While a number of BMDC types are found in varying amounts in metastatic target organs, the common thread seems to be the relationship of BMDC accumulation with enhanced metastatic growth. The involvement of BMDCs in promoting metastatic tumor growth has enormous potential to improve our understanding of the metastatic process and to design novel therapies to target metastatic disease. By studying the determinants of "successful" metastatic growth, we will be better equipped to design more effective strategies to treat or prevent metastatic cancer.

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Questions to Consider

How does "the seed" condition "the soil" for metastatic tumor growth?

- **A.** Secretion of anti-angiogenic growth factors
- **B.** Recruitment of bone marrow derived cells
- **C.** Inhibiting extracellular matrix production

How would you be able to assess whether metastasis was dependent on or independent of the formation of a premetastatic niche?

- **A.** Assess patterns of metastasis with blood flow from the primary tumor
- **B.** Attempt to co-localize metastatic cells with markers of a premetastatic niche
- **C.** Test whether metastasis occurs in the lung after cells are directly injected into the tail vein of a mouse

Bone marrow derived cells play important roles in tumor progression. How would you determine what type of bone marrow derived cells are needed for metastatic growth and their function?

- **A.** Attempt to isolate bone marrow derived cells and screen for cell surface antigens
- **B.** Culture cells and study their morphology
- **C.** Functionally demonstrate that a unique type of bone marrow derived cells can increase metatstatic spread

Describe how you might therapeutically target bone marrow derived cells in the clinic?

- **A.** Inject antibodies directed against cell surface antigens of bone marrow derived cells
- **B.** Inhibit the signaling pathway needed to recruit the bone marrow derived cells
- **C.** Perform a bone marrow transplant from a cancer free donor

Molecular Imaging in Radiation Therapy

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Noninvasive biological imaging has been developed and enhanced for over a half century since the first medical ultrasonic (US) measurement in 1954, the invention of a clinical x-ray computed tomography (CT) scanner in 1972, and the acquisition of the first magnetic resonance image (MRI) in 1973. These methods have evolved to offer high imaging performance in terms of scanning time, spatial resolution, and image quality, and now are commonplace in medical settings. In general, the source of contrast in these imaging modalities is gross tissue anatomy or structure. For example, x-ray CT differentiates tissues on the basis of their density and x-ray absorption, which vary greatly between bone, air, and water but minimally between different soft tissue types. MRI detects differences in proton density and magnetic relaxation, which can vary substantially between soft tissues. Many of these properties have only tangential relationships to the physiologic or functional aspects of the tissue being imaged, however. A tumor may be difficult to localize on CT because its x-ray absorption properties are not significantly different than the tissue from which it arose. This shortcoming of conventional anatomic imaging prompted the development of molecular imaging, loosely defined as the noninvasive detection, localization, and quantitation of specific molecular entities or physiologic processes in a living organism.

The field of molecular imaging has undergone tremendous expansion in the last 20 years. Molecular imaging modalities have been developed to localize both endogenous imaging signals, such as the resonances of atoms within specific metabolites detected with magnetic resonance spectroscopy (MRS), as well as to image exogenous imaging agents with specific biological activities, such as measuring glycolytic activity with [^{18}F]-fluorodeoxyglucose (FDG) positron emission tomography (PET) or quantifying vascular parameters through dynamic imaging of a blood pool contrast agent with MRI. Many of these molecular modalities have reached a state of development where they are routinely used in the diagnosis, staging, and restaging of a variety of cancers.

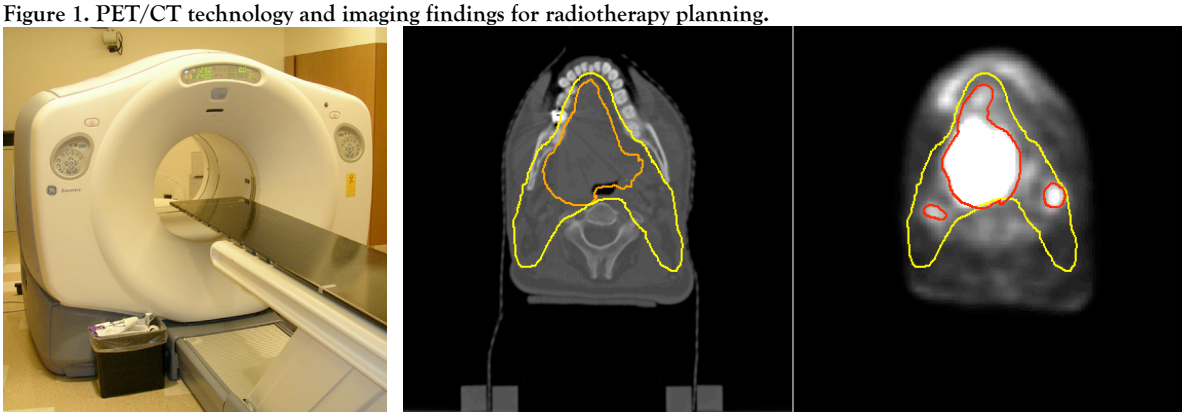
With the export of imaging methods from radiology divisions into associated departments as well as the advent of image-guided radiation therapy, radiation oncologists have begun to incorporate molecular imaging into the planning of radiation treatments as well as the monitoring of the outcome of these treatments. While preliminary results have supported the hypothesis that many of these modalities have roles to play in improving the practice of radiation oncology, the use of these methods in the context of radiotherapy presents new challenges for molecular imaging. Identification of the imaging methods with the most utility for radiation therapy, establishment of quantitative clinical parameters that can be extracted from imaging data, and segmentation of cancer-associated regions from molecular imaging datasets for radiotherapy planning are but a few of the hurdles that must be overcome to fully exploit the potential of these new technologies for radiation oncology. In this lecture the current status of molecular imaging in radiation therapy will be reviewed. We will focus on two molecular imaging techniques that have advanced the furthest in their deployment in radiation oncology departments, positron emission tomography (PET) and magnetic resonance spectroscopy (MRS). The basic physics of these modalities will be summarized, and preliminary results of studies assessing their use in tumor diagnosis, staging, treatment planning, and followup will be reviewed.

Positron Emission Tomography (PET)

Positron emission tomography (PET) is an imaging modality derived from the unique physics of radioactive decay of positron-emitting radionuclides. Such non-natural isotopes, including ^{11}C, ^{13}N, ^{18}F, ^{64}Cu, and ^{124}I, are unstable and decay through the emission of a positron, which subsequently annihilates with an electron to produce two high energy photons emitted in opposite directions. The geometry of these photons allows inference of the location of the positron emitting probe by detecting two simultaneous photons in a detector array and following the line joining them. Modern clinical PET scanners achieve spatial resolutions of 4-6 mm, and are commonly integrated with x-ray CT systems to produce so-called PET/CT scanners capable of the acquisition of anatomic CT and functional PET data

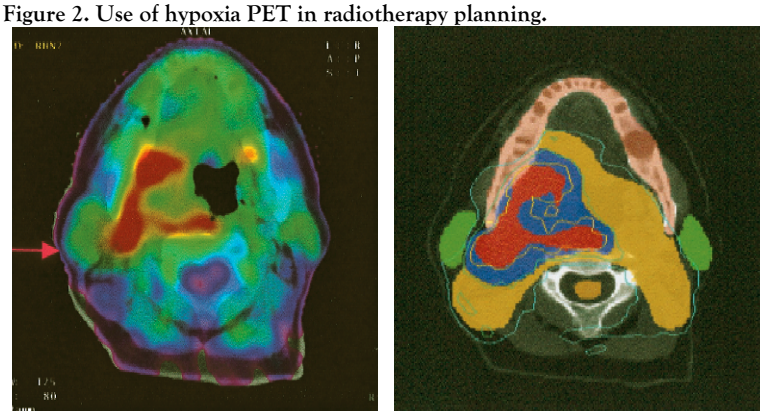
in a single examination. The development of hybrid PET/CT scanners has accelerated the inclusion of PET data in radiation treatment planning, as it is possible using these devices to acquire coregistered PET data at the time of CT simulation required for radiotherapy planning as shown in **Figure 1**. A variety of groups have evaluated the use of PET for radiation therapy planning as an adjunct to conventional CT, and have compared the tumor margins identified with PET to those defined on CT. While image processing mechanisms for segmenting pathologic tissue from normal tissue for radiation target delineation have been developed, at present research continues into the optimal strategy for inclusion of functional imaging information in this process.

Figure 1. PET/CT technology and imaging findings for radiotherapy planning.



One of the strengths of PET is that the scanner technology is not specific to any radionuclide or compound; it can be used to map the distribution of any positron-emitting radionuclide-containing compound with a subject. The prevalent PET imaging agent or "radiotracer" in use is 2-deoxy-2-[^{18}F]fluoro-glucose (FDG), an analog of glucose that cannot be fully metabolized and is trapped in glycolytic cells. While FDG PET now has proven clinical applications in a variety of oncologic sites, it has several shortcomings. Observation of elevated FDG uptake on a PET scan of a cancer patient could be associated with 1) increased tumor mass and/or cellularity, 2) upregulation of glucose transporters, 3) upregulation of hexokinase, 4) inflammatory responses, and/or 5) muscle activity. Therefore while FDG PET can provide valuable clinical information, there have been subsequent efforts to develop more specific PET radiotracers that can report on specific aspects of an individual patient's cancer and more fully characterize it so as to select and monitor the most effective course of treatment. [^{18}F]fluorothymidine (FLT) is a thymidine analog that is taken up into cells and phosphorylated by thymidine kinases, resulting in FLT accumulation being a surrogate of cellular proliferation. An area of PET tracer development with great relevance to radiation oncology is that of agents targeting hypoxic cells. Radiotracers of this type that have been evaluated in the clinic include [^{18}F]fluoromisonidazole (FMISO), [^{18}F]fluoroazomycin arabinoside, [^{18}F]EF5, and [^{64}Cu]ATSM. Efforts to apply these imaging methods towards identifying and treating regions of therapy-resistant, poorly oxygenated cells within tumors are currently in development, as shown in **Figure 2**, where a [^{64}Cu]-ATSM image (left) is used to direct radiation therapy to regions of hypoxia in a tumor (right).

Figure 2. Use of hypoxia PET in radiotherapy planning.



In addition to radiotracers that become trapped within cells, a second class of PET agents are those that bind to specific proteins expressed on the surface of cells. An emerging radiotracer of this type are arginine-glycine-aspartic acid (RGD) peptides labeled with [^{18}F] or [^{64}Cu], which bind to $\alpha_V\beta_3$ integrins expressed on endothelial cells of angiogenic vessels,

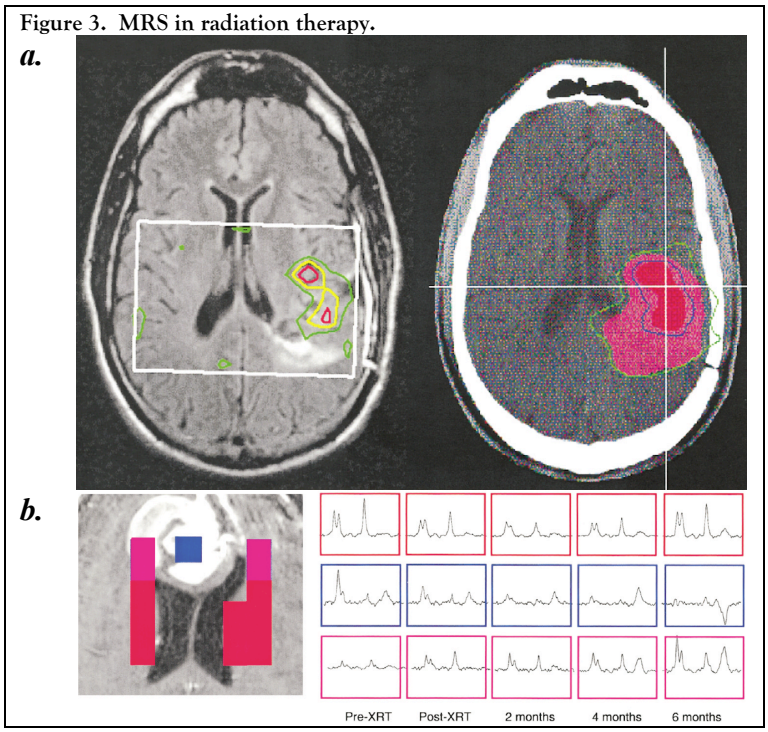
allowing imaging of the molecular aspects of angiogenesis using PET. In addition to small molecule probes, labeled antibodies against a variety of targets have been developed as PET radiotracers, providing a mechanism for translating antibodies developed for molecular biology applications into imaging agents.

Magnetic Resonance Spectroscopy (MRS)

While magnetic resonance imaging (MRI) uses the large concentration of protons in water in biological tissues to produce a magnetic signal for imaging, magnetic resonance spectroscopy exploits the variation of magnetic signal based on the chemical environment to identify specific molecules. The proton (^{1}H) has the highest intrinsic sensitivity for nuclear magnetic resonance measurement and has been studied in the most detail *in vivo*. While single voxel spectroscopic acquisitions remain useful for a variety of diagnostic purposes, application of MRS in radiation oncology has been contingent on the development of methods for imaging spectroscopic signals. This procedure, termed chemical shift imaging (CSI) or magnetic resonance spectroscopic imaging (MRSI), has been applied to acquire MR spectra from two- and three-dimensional grids of voxels. Current MRSI technology allows acquisition of volumetric data of spatial resolutions from 5 to 10 mm in acquisition times of as little as a minute for "fast" or "spiral" MRSI to as long as 20 minutes for conventional pulse sequences. An advantage of MRS is that it does not require any specialized hardware beyond a standard clinical MR scanner.

Unlike PET, MRS relies on detection of endogenous molecules within a subject, and is therefore dependent on whatever metabolites give detectable signals, as opposed to PET where probes can be designed to quantify a broad spectrum of molecular and physiologic signals. The tumor types that are amenable to interrogation with MRS currently include brain, prostate, and breast. MRS at a field strength of 1.5T can quantify relative levels of choline, a cell membrane component that is upregulated in cancer as tumor cells rapidly divide and assemble membranes. In brain, MRS also can detect N-acetyl aspartate (NAA), a marker of normal neuronal metabolism, while in prostate, quantification of citrate, a marker of normal ductal metabolism, is possible. MRS of these two tumor sites therefore offers simultaneous measurement of both a tumor-associated and a normal-associated marker, enhancing the specificity of this technique for diagnosing cancer. In breast, an MRS-visible normal tissue marker has not yet been established.

Figure 3. MRS in radiation therapy.



MRS of brain and prostate tumors has been incorporated into cancer staging, treatment planning, and followup. **Figure 3a** demonstrates the use of MRS images in the treatment planning of a recurrent glioma undergoing radiosurgery, with the MRI with the MRS-derived tumor contours shown at left and the CT and radiosurgical target volume at right. In **Figure 3b**, the spectroscopic response of glioblastoma multiforme treated with external beam radiotherapy (XRT) is observed through serial MRS measurements over the six months following treatment. A reduction in all metabolites is seen in the high dose region (blue), while surrounding regions exhibit a progressive increase in choline:NAA ratio indicating tumor recurrence (magenta).

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Questions to Consider

Why is computed tomography used to plan radiation treatments?

- **A.** Because it does not involve any additional radiation dose to the patient
- **B.** Because it offers the best contrast between tumor and normal tissue
- **C.** Because it is the most cost effective imaging modality for this purpose
- **D.** Because it can be used to calculate the dose delivered by a beam of radiation

Which of these is NOT a benefit of incorporating fluorodeoxyglucose-positron emission tomography (FDG-PET) into radiation treatment planning?

- **A.** Improved spatial resolution
- **B.** Improved contrast between tumor and normal tissue
- **C.** Improved sensitivity for detecting lymph node involvement and tumor spread
- **D.** Allows measurement of tumor response based on the difference between pre-treatment FDG uptake and subsequent FDG-PET examinations

Which of these is NOT a limiting factor in the use of proton magnetic resonance spectroscopy in studying a particular tumor type?

- **A.** The magnetic field homogeneity and susceptibility of the anatomic location
- **B.** The efficacy of fat and water suppression methods for the anatomic location
- **C.** The availability of a transmit-receive apparatus specific to the anatomic site
- **D.** The presence of useful MRS-visible metabolites in the tumor and surrounding normal tissue

Radiation Therapy Trials in Pediatric Malignancies

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The cures in childhood cancer have come from multi-disciplinary, risk-adapted therapy involving Pediatric Oncologists, Pediatric Surgeons, and, importantly, Pediatric Radiation Oncologists. The greatest improvements in outcome of childhood cancer have been demonstrated with evidence generated from prospective, randomized clinical trials, involving large numbers of children, and large numbers of clinician-scientists and other health care workers pursuing a common goal – "Cure without Late Effects". The key elements in reaching this goal, and the reason those in the field relish the opportunity to tell this tale, is that the clinical research in pediatric cancer invites collaboration, is gratifying, provides life long continuity. In this field, the large majority of children arrive very sick, but go home well, and stay well. Current data now show that 80% of children with cancer are cured of their cancer! What other intellectual pursuit offers a similar outcome as a measure of success?

To provide the best oversight of a rather complex field, I focus on a story of one disease that has served as a model for the field in general. This story is of Pediatric Hodgkin's Lymphoma as I have seen evolve over a 50 year perspective.

Evolution of Pediatric Hodgkin's Therapy

Prior to 1960 there was no curative treatment for patients with Hodgkin's disease. The treatment was palliative, with a goal to relieve pain and reduce suffering. In the 1960s, with the advent of high-energy radiation therapy, gradually high dose and extended field radiation was tried as the sole treatment of those afflicted with Hodgkin's disease. The treatment with radiation required precise staging and the concept of laparotomy with splenectomy was introduced. By the late 1960s chemotherapy agents were devised with new drug combinations investigated, most notably MOPP chemotherapy. A series of well designed clinical trials were initiated at Stanford by Drs. Henry Kaplan and Saul Rosenberg in an attempt to improve event-free survival (EFS) and overall survival (OS) for patients with Hodgkin's disease (now termed Hodgkin's lymphoma); adults and children alike were eligible for these studies. The standard radiation dose was (35-44 Gy) given to extended radiotherapy volumes and it achieved excellent long-term disease-free survival. However with sufficient follow-up time, growth abnormalities were noted particularly in children and adolescents who were not yet fully developed when irradiated. They exhibited shortened sitting height, small neck and collar size, and atrophy of soft tissues and muscles. Concern for these effects led the Stanford investigators to a series of protocols exclusively for children and adolescents which modified treatment strategies to address their specific needs, most notably to reduce the growth disturbances observed with high dose, extended field radiation in children.

The first Stanford Pediatric trial, initiated in the early 1970s "Low dose, involved field radiation + MOPP", and subsequently its successor study, initiated in the early 1980s, "Low dose, involved field radiation + MOPP/ABVD" resulted in cure rates of 90% and better demonstrated that multi-agent chemotherapy could be used to treat subclinical disease and thus reduce the required dose of radiation delivered to involved sites. These studies revolutionized the approach to management and treatment of Hodgkin's Lymphoma for children and adolescents: cure rates were higher than had ever before been observed, and growth alterations from radiation to the growing musculo-skeletal system had been avoided. However, with the high cure rates and emerging follow-up, new sequelae became apparent, this time from chemotherapy, most notably infertility and secondary leukemia from MOPP, and cardiac and pulmonary dysfunction from ABVD. Gradually routine staging changed from surgical staging with exploratory laparotomy with splenectomy to clinical staging, so to avoid surgical complications such as overwhelming infections in the asplenic patient, and bowel obstruction.

By the 1990s the emphasis focused on avoiding complications from staging and treatment whenever possible. New "risk-adapted" protocols were designed to tailor treatment to the child's risk group, based upon initial stage and extent of disease, so to minimize acute and late complications. The concept of low-

dose radiation used with chemotherapy was accepted world wide, and investigators around the world all had opinions about how best to treat children with Hodgkin's lymphoma. Many multi-agent chemotherapy programs were developed, all combined with low dose radiation as pioneered by the Stanford group. No longer was attention focused only on growth and development, for soon long-term follow-up studies in the survivors emerged reporting a decline in event-free survival due to events unrelated to Hodgkin's disease, but attributed to complications from the specific treatment and protocol used. Over the subsequent years, the concerns first observed in children with Hodgkin Lymphoma, brought attention and a general recognition of late effects from radiation therapy and chemotherapy that apply to all patients of all ages, and with all diagnoses.

Today the standard of care of children with Hodgkin's lymphoma is risk adapted, response driven therapy. Patients are divided into risk groups: low risk, favorable; intermediate risk; and high risk, unfavorable. Treatment is designed so the patients in the low risk group are given the least amount of therapy resulting in few or no risk of complications or late effects from the disease or its therapy. By contrast, patients in the high risk group are given the most aggressive therapy needed for cure of the disease. All children receive multi-agent chemotherapy; radiotherapy is also used, often with higher doses administered to those who have an incomplete (or partial) response to induction chemotherapy, with lower doses or no radiation used for those with low risk disease. Patients with low risk disease have a likelihood of 90-95% EFS; those with intermediate risk disease approximately 85-90%, and those with high risk disease, approximately 80%. The Stanford V chemotherapy protocol combined with radiation is currently in use by many groups, and appears to be yield the most promising results.

The most commonly observed late effects seen in children with Hodgkin's disease

Second Malignancies: One of the earliest reports regarding second malignant neoplasms (SMN) in survivors of Hodgkin's disease when diagnosed in childhood came from the Stanford group reporting on 694 children and adolescents. They reported 56 patients who developed 59 secondary malignancies: 48 solid tumors, 8 leukemias, and 3 non-Hodgkin's lymphomas, with follow-up out to 31 years. The relative risk of developing a second cancer was 15.4 for females and 10.6 for males. Breast cancer and sarcoma were the most common solid tumors. The actuarial risk at 20 years follow-up was 9.7% for males, 16.8% for females, and 9.2% for breast cancer.

Table 1. SMN in Pediatric Hodgkin's Lymphoma Cohorts

				ANY SMN			
Author, Group	No. of Patients	Median Age at Diagnosis (years)	Median Follow-Up (years)	20 Year	30 Year	SIR	AER
Bhatia	1,380	11.7	17	9.3	23.7	18.5	65
CCSS	1,815	14	18	7.6		9.7	51.3
Green	182	15.3 (mean)	17.1	12.7	26.3	Male: 9.4 Female: 10.2	
O'Brien, Stanford	110	11.3	20.6	17	29.4	22.9	93.7

	Solid SMN		Breast Carcinoma		Thyroid Carcinoma	
Reference	SIR	AER	SIR	AER	SIR	AER
Bhatia	18.5	51	55.5	53	36.4	9
CCSS			26.3		18.3	
Green			7.8		Male: 158.8 Female: 38	
O'Brien, Stanford	19.8	75.3	72.3	83.6	53.2	22.9

Table 1 displays data from 3 large pediatric series with median follow-up >15 years. The Late Effects Study Group (LESG) is a self-report retrospective study with matched sibling controls. Patients reported by the LESG were largely treated in the 1970-80s, with high radiation doses to extended radiation fields. These data are shown from Bhatia et al. 2003, and from other investigators (shown as CCSS) reporting >1800 patients (references: Neglia, Kenney, Sklar). The most common solid tumors observed are breast, thyroid, bone, brain, and GI cancers. Data are shown as: cumulative incidence in %, SIR (standardized

incidence ratio calculated as the ratio of observed to expected cases), and AER (absolute excess risk per 10,000 person-years calculated as number of observed cases minus expected cases divided by person-years of follow-up multiplied by 10,000. The cumulative incidence was in the range of 7-9% at 20 years, with SIR of any tumor 10-18, and AER of 51-65. These data alerted investigators to the need to modify therapy.

It was thought that perhaps newer treatment approaches would minimize this risk. The Stanford report of 110 patients represents children treated with current standard of therapy, low-dose radiation and chemotherapy. In this group, all patients received either 6 cycles of MOPP or 6 cycles of MOPP/ABVD and 15-25.5 Gy of radiation. However the results were remarkably similar with a cumulative incidence of first SMN of 17% at 20 years after diagnosis, approaching 30% at 30 years of follow-up. The SIR for any SMN was 17 with an AER of 74.3 cases per 10,000 person-years. The most common tumors were: leukemia, thyroid carcinoma, breast cancer, and sarcoma. Thus the risk of a SMN following successful treatment of Hodgkin's disease is elevated and remains so, despite modification of dose of radiation. The impact of genetic predisposition, specific chemotherapy agents and of hormone status is unclear.

The observation of serious late effects in the pediatric survivors of Hodgkin Lymphoma has called attention to the larger problem of chronic disease in all survivors of childhood cancer. Many of these chronic conditions do not become apparent until 20-30 years after the treatment of the childhood cancer. The growing list of these conditions include: neurologic; infertility; hearing, speech and vision; cardiovascular; pulmonary; endocrine; musculo-skeletal; renal; and gastrointestinal problems. Many of these chronic conditions are exaggerated by disorders of body weight including obesity, alterations in pubertal development, osteoporosis and osteonecrosis, risk-taking behavior, and psycho-social adjustment problems.

Problems first observed in children are now being observed in their adult counterparts as increasing attention is being placed upon long-term survival studies, and wellness clinics.

Summary

The success story of Hodgkin's disease in children exemplifies the larger story of success in the treatment of childhood cancer. During one investigator's lifetime, the outlook for afflicted children with Hodgkin Lymphoma has escalated from only a few surviving, to the large majority surviving with a high quality of life. Yet, there remains much research to be done so that one can boast even higher cure rates with fewer late effects.

The gratification to a clinician investigator in the field of Pediatric Radiation Oncology comes from:

- High cure rates in the majority of those afflicted, thus the ability to truly impact a patient's life.
- Long survival for those afflicted, thus many years of future quality life.
- The opportunity for continuity of patient care
- The challenges to be creative and innovative, designing individualized therapeutic plans, which can be used by those who care for adults as well as children.

Collaboration and participation in prospective clinical trials to obtain solid answers to relevant clinical questions, evidence based medicine.

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Questions to Consider

The treatment for Hodgkin's Disease (Hodgkins Lymphoma) in children:

- **A.** is patterned to duplicate the treatment used in adults
- **B.** is designed to consider the late effects in children
- **C.** is less effective than the similar treatment in adults

Second malignancies occurring after successful Hodgkin's treatment in children:

- **A.** are of less importance in children than adults because children are not exposed to naturally appearing carcinogens such as tobacco, and other environmental mutagens
- **B.** are more related to genetics and family history, than to the treatment administered
- **C.** are more common in girls than boys, because of the incidence of breast cancer

The treatment for Hodgkin's Lymphoma in children is:

- **A.** risk-based therapy
- **B.** response based therapy
- **C.** combined modality therapy
- **D.** all of the above

Hodgkin's Disease:
A Paradigm for Clinical Research

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Hodgkin's Disease has attracted the attention of physicians and researchers for more than a century, and to a degree out of proportion to its relative incidence. The very nature of the disease, a true neoplasm, an inflammatory, even infectious disease, an unusual immunologic reaction, or a combination of these pathogeneses has long been and continues to be controversial. But, perhaps the most unique fact about this disease, is that if untreated it is uniformly fatal, and today, more than 90% of patients can be cured, with minimal long term side effects of the successful therapy.

The virtual cure of Hodgkin's Disease is a story of clinical research, a major collaborative program at Stanford, for more than 50 years. Without an animal model, or in vitro cell lines, and no unique genetic, molecular or immunologic markers, Stanford clinical trials, clinical observations, and long term data collection over decades have resulted in one of the most dramatic success stories in cancer management.

The story began at Stanford a half a century ago, when Henry Kaplan, a radiotherapist, and Edward Ginzton, a nuclear physicist, invented a medial linear accelerator. Randomized clinical trials, initiated by Kaplan and Rosenberg in 1962 have continued for the subsequent 45 years, often benefited by the results and observations of others. Scores of investigators at Stanford, and with the informed consent of over 3000 patients, the virtual cure of Hodgkin's Disease is a true paradigm for successful clinical research.

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Questions to Consider

The current diagnostic studies indicated for the majority of new patients with Hodgkin's Disease include:

A. bone marrow aspiration and biopsy

B. exploratory laparotomy with splenectomy

C. bipedal lymphangiography

D. whole body PET/CT scan

E. all of the above

The diagnosis of Hodgkin's Disease is dependent upon a biopsy demonstrating:

A. B-cell surface marker, ie CD-20

B. CD-30 and CD-15 markers

C. T-cell clonal marker

D. Reed-Sternberg giant cells in an appropriate cell background

E. all of the above

Stanford's contribution to improved survival of Hodgkin's Disease has been:

A. invention of the medical linear accelerator

B. improved radiation therapy fields

C. novel chemotherapy

D. combined modality clinical trials

E. all of the above

Therapeutic Targeting of Human Acute Myeloid Leukemia Stem Cells

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Acute Myeloid Leukemia Is Organized as a Hierarchy Initiated by Leukemia Stem Cells

Acute myeloid leukemia (AML) is a clonal malignancy of the bone marrow characterized by the accumulation of immature myeloid cells defective in their maturation and function. AML affects 13,000 adults annually in the United States, most of them over the age of 65. Current standard of care includes treatment with several cycles of high dose chemotherapy and often includes allogeneic bone marrow transplantation. Even with these aggressive treatments, which have significant side effects, five-year overall survival is between 30-40%, and much lower for those over age 65 (Estey and Dohner, 2006). AML is a heterogeneous disorder classified according to the recent World Health Organization (WHO) scheme, which was developed to reflect the molecular understanding of several subtypes of AML and to take into account the prognostic importance of cytogenetic abnormalities. Immunophenotyping by flow cytometry is routinely performed on clinical specimens and shows that most, but not all, AML samples are predominantly CD34 positive.

Much is now known about the biology of AML including: chromosomal translocations resulting in gene fusions, chromosomal and subchromosomal deletions of putative tumor suppressor genes, dysregulation of programmed cell death, single gene mutations correlating with leukemia, and derangements of the telomere/telomerase system. Recent experiments have added to our fundamental knowledge by demonstrating that AML, like normal hematopoiesis, is composed of a hierarchy of cells that is maintained by a small pool of self-renewing leukemia stem cells (LSC) (Figure 1) (Dick, 2008). Both *in*

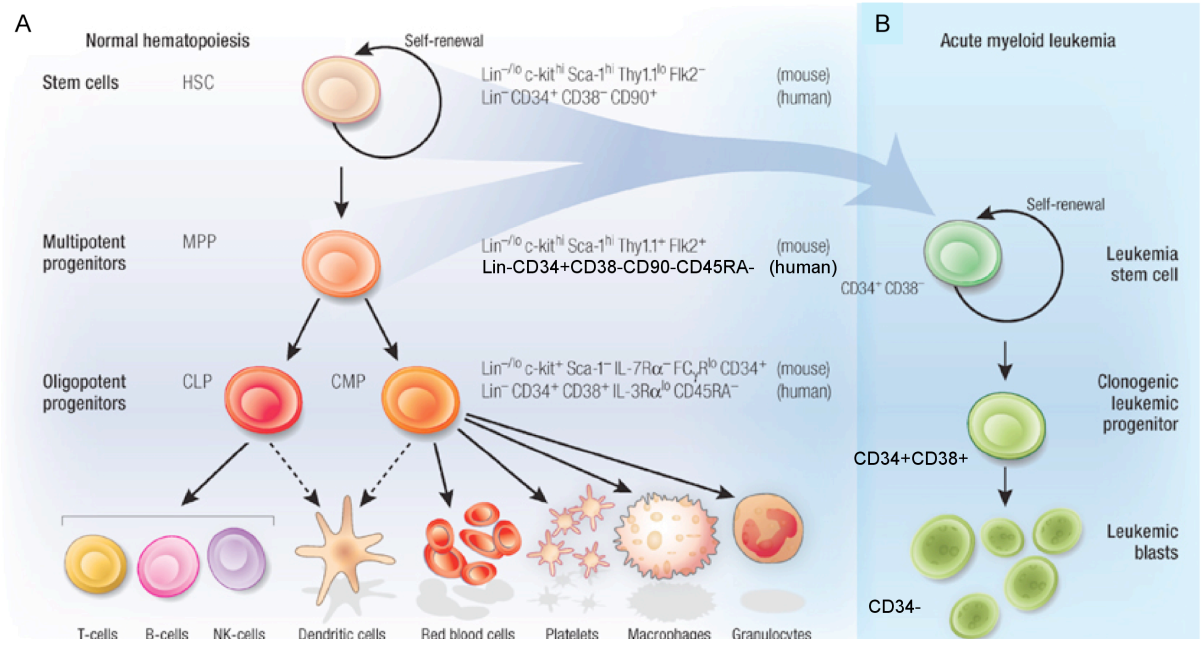

Figure 1. Hierarchical Model of Normal Hematopoiesis and Human Acute Myeloid Leukemia

(A) Self-renewing HSC reside at the top of the hierarchy, giving rise to multipotent progenitors that in turn give rise to lineage-committed progenitors that eventually produce terminally differentiated blood cells. (B) AML is organized as a hierarchy initiated by self-renewing leukemia stem cells that give rise to leukemic progenitors, which in turn give rise to the leukemia blasts.

vitro and in vivo experimental approaches using fluorescence activated cell sorter (FACS)-purified subpopulations of leukemia cells were utilized to identify AML LSC. Dick and colleagues were the first to establish xenotransplantation models for human AML, and used this assay to identify long-term engrafting AML subpopulations. In published reports assaying a variety of clinical and biological subtypes of AML, LSC were found to be positive for expression of CD34 and negative for expression of CD38. However, recent experiments suggest that in some cases, leukemia stem cell (LSC) activity may exist in additional subpopulations. In normal hematopoietic development, this Lin-CD34+CD38- fraction is known to be enriched for hematopoietic stem cells (HSC), leading to a proposal that AML LSC arise from mutational transformation of HSC. Similar to the normal hematopoietic differentiation pathway, these LSC in turn give rise to leukemia progenitor cells with the surface immunophenotype of Lin-CD34+CD38+, which further differentiate into the bulk leukemic blast population defined as Lin-CD34- (Figure 1B).

Clinical Significance of the Cancer Stem Cell Model for Human AML

According to the cancer stem cell model, tumors are organized as a hierarchy maintained by a small pool of self-renewing cancer stem cells (Reya et al., 2001). While some controversy still surrounds this model, there is strong evidence for the existence of cancer stem cells in several human malignancies including acute myeloid leukemia, acute lymphoblastic leukemia, breast cancer, colon cancer, head and neck cancer, and several brain tumors. Within a tumor, it is only these cancer stem cells that are able to transplant the disease and to differentiate into the heterogeneous cells composing the tumor. This model postulates that one reason for the relative ineffectiveness of current cancer chemotherapy regimens is that these therapies target the rapidly proliferating tumor cells, which do not include all the cancer stem cells. Thus, tumors often shrink in response to chemotherapy, but almost universally recur due to sparing of some cancer stem cells. A key implication of this cancer stem cell model is that in order to eradicate the tumor and cure the patient, therapies must target and eliminate the cancer stem cells.

The primary evidence supporting the cancer stem cell model in AML derives from xenotransplantation experiments in immunodeficient mice, but what is the evidence that this model is clinically relevant? The clinical significance of the cancer stem cell model for AML is suggested by one small study that identified an inverse correlation between the frequency of CD34+CD38- cells at the time of diagnosis with the duration of relapse free survival. To further investigate this question, we employed a bioinformatic approach in which we defined a gene expression signature of purified LSC from primary AML patient samples and xenografts, based on a functional definition in transplantation assays. Using previously published gene expression data of bulk AML from four independent cohorts totaling 1047 patients, we evaluated this LSC signature for associations with known predictors of risk including cytogenetic subtype and molecularly-defined mutations, and as an independent prognostic factor. We found that the LSC signature was similarly expressed across most AML subtypes, but was lower in prognostically favorable subgroups. Strikingly, high expression predicted inferior overall (OS), event-free, and relapse-free survival in these independent cohorts, whether considering patients with a normal karyotype [hazard ratio (HR) range for OS 1.8-2.4, $p<0.005$ in all cases], or those with cytogenetic anomalies (HR range for OS 1.5-1.9, $p<0.02$ in all cases). In multivariate analysis, the LSC signature predicted poor outcomes independently of age, prognostic molecular mutations, and cytogenetic risk group.

Therapeutic Targeting of Acute Myeloid Leukemia Stem Cells

Given the significance of leukemia stem cells in the pathogenesis of AML, a great deal of research and development has focused on the therapeutic targeting of these cells with both small molecules and monoclonal antibodies. Several small molecules that appear to have activity against primary AML LSC have been identified using a number of strategies including rational screening and bioinformatic approaches. These compounds include parthenolide, TDZD-8, and celastrol, which are all believed to act via inhibition of NFκB (Guzman et al., 2005). With each of these molecules, *ex vivo* treatment of CD34+CD38- AML cells preferentially inhibited their engraftment in immunodeficient mice compared to normal CD34+CD38- cells. Notably, the efficacy of these compounds on *in vivo* established AML has not been reported. Regardless, parthenolide is currently in phase I clinical trials for AML.

In parallel, a great deal of effort has focused on the identification of cell surface molecules preferentially expressed on AML LSC compared to normal HSC, and the eventual targeting of such molecules with

therapeutic monoclonal antibodies. Several such antigens and antibodies have been identified and developed including CD44 and CD123. Targeting of CD44 with a monoclonal antibody was shown to markedly reduce AML engraftment in mice, with evidence that it acts specifically on LSC to induce differentiation (Jin et al., 2006). A monoclonal antibody directed against CD123 was recently reported to have efficacy in reducing AML LSC engraftment *in vivo*; however, this antibody did not appear to have great efficacy at eliminating established AML (Jin et al., 2009). The anti-CD123 antibody can not only recruit immune effector functions, but it also can block IL3 signaling, which may ultimately be more potent in human subjects. The anti-CD123 antibody has been developed for clinical use and is currently in phase I clinical trials for AML.

CD47 is an Adverse Prognostic Factor and Therapeutic Antibody Target on AML LSC

Our investigation of cell surface molecules expressed on AML LSC led to the identification of increased CD47 expression on LSC compared to normal HSC (Majeti et al., 2009). CD47 is a cell surface molecule that serves as the ligand for SIRPα on the surface of phagocytes, which in turn transmits a dominant inhibitory signal for phagocytosis. In this way, CD47 essentially functions as a "don't eat me" signal. We hypothesized that increased CD47 expression contributes to pathogenesis by inhibiting phagocytosis of AML LSC (Jaiswal et al., 2009). Consistent with this hypothesis, we found that increased *CD47* expression predicted worse overall survival in 3 independent cohorts of adult AML patients. Furthermore, we predicted that disruption of the interaction of CD47 with SIRPα would result in phagocytosis and elimination of AML LSC. We found that blocking monoclonal antibodies directed against CD47 enabled phagocytosis of AML LSC, but not normal CD34+ human bone marrow progenitor cells, by human macrophages *in vitro*. Additionally, coating of human AML LSC with anti-CD47 monoclonal antibodies inhibited their engraftment *in vivo* in a xenotransplantation assay. Finally, analogous to a clinical therapy, treatment of human AML-engrafted mice with anti-CD47 antibody eliminated AML cells in the peripheral blood and bone marrow, and targeted LSC (Figure 2). In a parallel set of experiments, we found that a blocking anti-mouse CD47 antibody also enabled phagocytosis of mouse AML cells, and most importantly, was not toxic to wild-type mice. On the basis of these results, we are currently developing a humanized anti-CD47 antibody as a novel therapeutic for human AML.

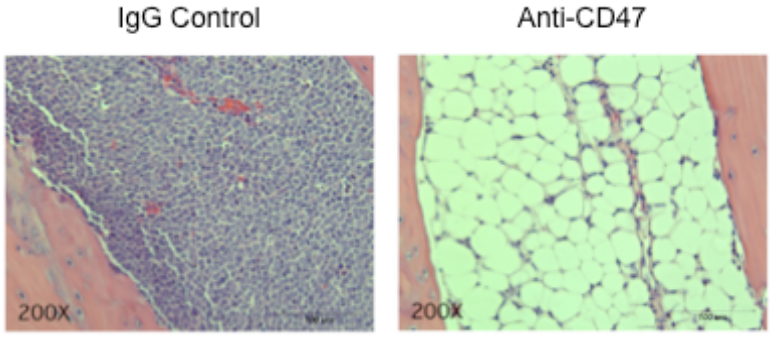

Figure 2: A Blocking Anti-CD47 Antibody Eliminates AML In Vivo

Immunodeficient mice were transplanted with human AML LSC. Several months later, mice were treated with either a control IgG or anti-CD47 antibody for 2 weeks. Histological examination of the bone marrow demonstrated elimination of AML cells in anti-CD47 treated mice.

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Questions to Consider

All of the following are true about AML stem cells <u>except</u>?

- **A.** They sit atop a cellular hierarchy of subpopulations of leukemia cells
- **B.** They are primarily enriched in the CD34+CD38- fraction of leukemia cells
- **C.** They are defined by the ability to engraft in immunodeficient mice
- **D.** They can be specifically targeted by current standard therapies

The clinical significance of AML stem cells is indicated by which of the following?

- **A.** Residual LSC have been demonstrated to be responsible for relapse
- **B.** An LSC gene expression signature is predictive of overall survival in multiple AML cohorts
- **C.** AML LSC have been shown to transplant the disease in humans
- **D.** The allogeneic transplant graft-versus-leukemia effect is targeted against LSC

What is the primary mechanism of action of anti-CD47 antibody against AML LSC?

- **A.** It stimulates NK cell mediated cell killing
- **B.** It causes direct apoptosis
- **C.** It enables phagocytosis by blocking a "don't eat me" signal
- **D.** It enhances anti-leukemia T cell responses

Mechanisms of Human Epithelial Tumorigenesis

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More than 90% of all human tumors arise in epithelia that are bound by an underlying basement membrane (BM). BM invasion is a central determinant of malignancy in a wide array of epithelial cancers, including breast, prostate, lung, kidney and skin. Understanding the mechanisms that mediate epithelial tumor progression holds promise to target key processes responsible for the vast majority of cancer mortality.

Three-dimensional (3D) human tissue model systems are emerging as a powerful platform in which to define mechanisms of key steps in epithelial tumorigenesis, such as invasion. Human skin tissue is extremely plastic and can be genetically engineered and regenerated on immune deficient animals as well as in organotypic settings. Such human tissue model systems were used to generate the first direct malignant transformation of human cells and have been extended to recapitulate faithful human tissue models of invasive neoplasms of the skin. These successfully modeled neoplasms, including basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and malignant melanoma (MM), account for approximately as many cancers annually in the US as all other malignancies combined. These models are being used to systematically elucidate the genes required for cutaneous carcinogenesis and to test their potential role as therapeutic targets.

The minimal genes sufficient for malignant transformation of human skin tissue have been characterized and shed insight into the genesis of specific epithelial maligancies. For BCC, single-gene activation of the Sonic Hedgehog pathway is sufficient to transform normal skin into neoplasia indistinguishable from BCC. In the case of SCC, two genes are sufficient, namely activation of the Ras/Raf/Mek/Erk MAPK pathway combined with a G1 escape mechanism such as sustained Cdk4 expression. In the case of MM, Ras/PI3K signaling, Cdk4 and hTERT co-expression are capable of direct conversion of normal tissue into invasive melanocytic neoplasia with all the cardinal features of locally invasive melanoma. These minimal gene sets have shed light on epithelial tumorigenesis by demonstrating that as few as 1, 2 or 3 genes are sufficient for malignant conversion of intact 3D human tissue and defining the core oncogenic pathways involved. Additionally, they have demonstrated that genomic instability and memory-based immune elements are dispensable for epithelial carcinogenesis.

Finally, the capacity for direct transformation of intact human tissue has enabled identification of key potential therapeutic targets in human epithelial tumor progression. Towards this goal, inducible human epithelial tumor progression models have recently been generated to study human tumor-stroma co-evolution in real-time. Gene expression profiling of both epithelia and stroma at specific time points during tumor progression revealed sequential enrichment of genes mediating discrete biologic functions in each tissue compartment. In epithelia undergoing the transition from normal tissue to invasive cancer, genes mediating cellular biosynthesis peaked first followed by those promoting proliferation; clusters enriched for genes involved in extracellular matrix remodeling and increased cell motility displayed maximum levels at later time points while expression of differentiation-enriched gene sets were progressively suppressed. In stroma, in contrast, angiogenesis genes peaked early followed by progressive reorganization of extracellular matrix, all accompanied by evidence of progressive immune suppression in the local tumor microenvironment. To identify tumor-intrinsic and extrinsic targets, a core cancer progression signature was distilled from both tumor and stroma and subjected to network modeling. Network topology predicted that tumor development depends upon specific extracellular matrix-interacting network hubs, which are being systematically targeted to block epithelial tumor progression.

Taken together, these advances indicate that epithelial tumorigenesis involves alterations in minimal gene sets that control dominantly acting signaling networks to directly transform epithelial tissue in situ. Epithelial cells undergoing tumor progression then interact with surrounding stroma via specific ligands and secreted molecules to progress to malignant invasion through the BM and into surrounding tissue. These models provide new approaches to identify core cancer mechanisms and new therapeutic targets in human epithelial tumorigenesis.

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Questions to Consider

The most common tissue origin for human malignancies is:

- **A.** connective tissue
- **B.** hematopoietic and lymphatic system
- **C.** surface epithelium
- **D.** central and peripheral nervous systems

Ideal features to enhance medical relevance of preclinical cancer models include all of the following except:

- **A.** human primary cells
- **B.** 3-dimensional tissue setting
- **C.** mutant human genes observed in spontaneous cancers
- **D.** transformed murine cancer cell lines

Head and Neck Cancers

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Head and neck squamous cell carcinoma (HNSCC) is an epithelial malignancy of the mucosa lining structures that include the oral cavity, paranasal sinuses, pharynx, and larynx. It is the most common neoplasm of the upper aerodigestive tract. Epidemiological and experimental evidence strongly indicates an association between the incidence of this malignancy and exposure to tobacco carcinogens that come into contact with the mucosa through either inhaled smoke or directly from chewing tobacco. Although there has been a decline in tobacco-related HNSCC in the U.S., there has been a rising incidence in human papilloma virus (HPV) – related HNSCC. While there have been some modest improvements in the treatment of advanced cases, recurrence rates are still as high as 30-50%, and the therapy alone – which includes a combination of surgery, chemotherapy, and radiation – often results in significant morbidity and long-term functional toxicity. Recently, attention has been directed toward exploiting biologic targets that are overexpressed or constitutively activated in these cancers. Specifically, inhibition of a receptor tyrosine kinase, the epidermal growth factor receptor (EGFR) has been examined in HNSCC since these tumors express this receptor highly.

This lecture will discuss three topics of active translational research in HNSCC:

- HPV and p16 as biomarkers of prognosis in HNSCC
- Head and neck cancer stem cells
- Targeted therapy against EGFR

HPV and p16 as Biomarkers of Prognosis in HNSCC

Approximately 25% of HNSCC tumors contain HPV DNA. HPV-associated HNSCC most commonly involves the oropharynx (tonsil, base of tongue). Among oropharyngeal HNSCC, over 50% are HPV-positive. While HNSCC overall has been decreasing, the incidence of *HPV-associated* HNSCC has been increasing (~1% per year). These tumors tend to occur in younger patients and are more common in Caucasians. Unlike HPV-negative HNSCC tumors, they tend to be less associated with tobacco and alcohol use.

Characteristics	HPV-positive SCC	HPV-negative SCC
Subsites	Oropharynx (50% HPV+, 25.9% all sites)	All head and neck sites
Incidence	Increasing (1% per year)	Decreasing (1-2% per year)
Age	Younger	Older
Race	Caucasian >>>African American	Caucasian = African American
Risk factors	Multiple sex partners; oral sex	Smoking, alcohol
Tumor stage	Higher stage (more nodal mets)	Lower stage (higher T-stages)

From James Lewis, M.D., Washington University

HPV is a double-stranded DNA virus of the Papillomaviridae family. While there are over 100 different subtypes, the subtypes that are associated with HNSCC (the "high-risk" subtypes) are primarily HPV-16 and HPV-18, with HPV-16 predominating. HPV infects the basal cells of the epithelium through the interaction of capsid proteins with α6-integrin, followed by endocytosis of the virus. Once in the nucleus, the virus is sustained as multiple copies in an episomal form, but it can also integrate into the host genome.

The HPV genome is approximately 8 kb in size and generally consists of 8 genes. The early (E) genes are expressed in undifferentiated proliferating basal cells, while the late (L) genes (L1 and L2 capsid proteins) are only expressed in the differentiating cells of the suprabasal epithelial layers. Of the early gene products, the E6 and E7 proteins have been of greatest interest since they have been shown to inactivate known tumor suppressor proteins. E6 binds p53 and marks it for proteasome-dependent degradation. The inactivation of p53 prevents activation of programmed cell death. In addition, E6 has been shown to be able to activate telomerase. E7 binds and inactivates members of the retinoblastoma family, including Rb, p130 and p107. E7 binds hypophosphorylated Rb, releasing E2F and promoting cell cycle progression.

In addition to being able to immortalize keratinocytes, E6 and E7 have also been implicated in the transformation of epithelial cells. While these genes are expressed in both the low-risk and high-risk types, E6 and E7 of high-risk HPV bind their target tumor suppressor proteins with greater affinity. Interestingly, progression from dysplasia to carcinoma in cervical cancer appears to require integration of the viral DNA into the host genome. Consistent with this, the majority of premalignant cervical lesions have HPV DNA in an episomal form, whereas 90% of carcinomas have HPV DNA in the integrated form. Although this requirement is not well understood, it does not appear to involve insertional mutagenesis of host genes.

Aside from the epidemiologic differences mentioned earlier, HPV+ HNSCC appears to differ significantly from HPV- HNSCC in many other ways. The gene expression profile of HPV+ tumors is different from that of HPV- tumors. There is more genomic instability observed in HPV- tumors. The majority of HPV- tumors have mutations in p53, whereas mutations in p53 are rarely found in HPV+ tumors. In addition, the cyclin dependent kinase inhibitor p16 is often inactivated in HPV- tumors but is almost always overexpressed in HPV+ tumors. Because Rb negatively regulates the expression of p16, the overexpression of p16 is thought to be the result of inactivation of Rb by the HPV E7 protein. This has important implications clinically.

A number of studies have demonstrated that patients with HPV+ HNSCC tumors tend to have a much better clinical outcome compared to those with HPV- tumors. Overall, the former has an 18% reduced risk of dying and a 38% reduced risk of recurrence compared to patients with HPV- tumors. When subsite analysis was performed, this better outcome was limited to HNSCC of the oropharynx – the site most often associated with HPV infections. In this site, there was a 28% reduced risk of death and a 49% reduced risk of recurrence. Thus, much attention has been directed at detection of HPV in HNSCC to predict prognosis and guide therapy.

Detection of HPV in HNSCC has been approached in a number of ways. PCR amplification of HPV genome DNA has been used to determine presence or absence of HPV, but this does not provide accurate information regarding whether the HPV DNA is in an episomal or integrated form. In situ hybridization (ISH) using complimentary nucleic acid probes can distinguish between episomal and integrated forms of HPV infection; however, the sensitivity is lower than PCR-based assays. Biologically active HPV can be detected using quantitative RT-PCR assessment of E6 and E7 transcripts from fresh tumor samples or by complimentary RNA probes; however, these techniques are not routinely performed due to their more cumbersome procedures.

The most clinically useful marker of HPV infection in HNSCC has been the overexpression of p16, measured by immunohistochemistry. A large number of studies have demonstrated a strong statistically significant correlation between p16 immunostaining and the presence of HPV DNA measured by PCR or ISH. The correlation is not perfect in that some HPV+ tumors do not express p16. In addition, p16 can be found to be expressed in some tumors that are not HPV+ and in some non-malignant HPV- tonsil epithelium samples. Nevertheless, both the robust staining by antibodies to p16 and the strong correlation between p16 staining and HPV infection has led to the use of this marker in clinical practice. Furthermore, data from a recent multicenter trial indicates that p16 staining may be even more predictive of improved survival than HPV infection status. It is likely that this biomarker will be included in future staging systems of oropharyngeal squamous cell carcinoma and consideration of de-intensifying therapy for patients with p16+ tumors is being actively discussed.

Head and Neck Cancer Stem Cells

Recurrence following standard radiation therapy of HNSCC demonstrates an interesting feature of this tumor (and likely all tumors). While the natural tendency is to think that tumors shrink with therapy in a centripetal pattern (Figure 1), histological analysis of recurrent tumors demonstrates that, in fact, the tumors shrink globally but leaving the most resistant cells as microfoci of tumor if not completely eradicated. Thus, salvage surgery requires resection of the same amount of tissue (if not more) than what was represented by the original tumor.

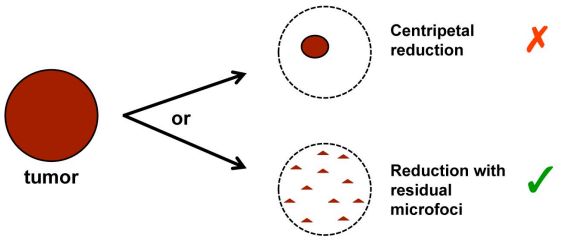

Figure 1. Two possible outcomes after incomplete response to radiation. Histological analysis of recurrent tumors following radiation treatment indicates that reduction with residual microfoci of tumor cells is the way tumors recur. It has been hypothesized that these residual tumor cell nests arise from the more radiation-resistant cancer stem cells.

A biologic explanation for why certain cells are more resistant to chemotherapy and radiation therapy comes from work describing a subpopulation of cancer cells with "stem cell"-like properties (herein, called "cancer stem cells" or "CSCs") in solid malignancies. As with normal adult somatic stem cells, these CSCs represent a minority population within a tumor. What has come to be known as the *cancer stem cell hypothesis* proposes that a tumor can be viewed as an aberrant "organ" that is sustained (in a way that is similar to normal tissues) by this minority CSC population. According to the hypothesis, the CSC population within a tumor has the unique capacity to (1) self-renew to generate additional CSCs and (2) differentiate to generate phenotypically diverse (non-CSC) cancer cells, which make up the majority population within a tumor. This hypothesis is founded on the following observations. When the CSC population is isolated from human tumors and transplanted into immunodeficient mice, a very small number of these cells can form a tumor. This is in contrast to the non-CSC population, which when transplanted in much greater numbers than what was used for the CSC population, cannot form a tumor. Importantly, the tumor that arises from the transplanted CSCs is composed of a heterogeneous population of cells (CSCs and non-CSCs) that reflects the original tumor, indicating that the CSC population can differentiate into non-CSC cells. Current studies in HNSCC have enriched for populations that contain CSCs using the marker CD44.

The observation that a "stem cell"-like population of cells within a tumor has the exclusive capacity to form tumors upon serial passages *in vivo* has led to the notion that therapy should be directed toward the eradication of cancer stem cells in particular. Indeed, in glioblastoma, the CSC population has been shown to be significantly more resistant to radiation therapy than the non-CSC population, offering an explanation for why these tumors often regress with radiation therapy but rapidly recrudesce following treatment. Similarly, in pancreatic cancer, the CSC population appears to have a high degree of resistance to gemcitabine chemotherapy. Recent work has demonstrated that CSCs have a much greater capacity to handle reactive oxygen species and the oxidative stress induced by radiation, providing an explanation for the greater resistance to this type of therapy. As mentioned, treatment of advanced head and neck cancer usually includes chemotherapy and radiation, and our own data at Stanford have demonstrated that head and neck cancers with greater percentages of CSCs are clinically much more aggressive, again indicating the need for targeted therapy against CSCs.

Targeted Therapy Against EGFR

EGFR is a member of the ErbB/HER family of receptor tyrosine kinases. It has been found to be overexpressed in the majority of HNSCC tumors. Elevated levels of mRNA for EGFR and one of its ligands, TGF-α, has been found in 92% and 87%, respectively. Elevated levels of EGFR protein have been demonstrated in 47% of HNSCC. Overexpression of EGFR results from increased mRNA synthesis and decreased downregulation of the protein. Unlike non-small cell lung cancer, activating mutations of EGFR in HNSCC are uncommon. Only one study has reported finding EGFR mutations.

This mutant form called EGFRvIII is characterized by a deletion of exons 2-7. It has a truncated ligand binding domain and as such, is constitutively active.

EGFR binds to a number of different ligands, including EGF, TGF-α, and amphiregulin. Upon ligand binding, the receptor can either homodimerize or heterodimerize with other ErbB receptors (ErbB2, ErbB3 and ErbB4), resulting in autophosphorylation of the receptor. Signaling complexes are recruited to the phosphorylated receptor and can activate the ras/MEK/ERK pathway, the PI-3 kinase/akt pathway, STAT3, and PLCγ. Stimulation of EGFR with TGF-α results in STAT3 activation, promoting tumor growth; whereas, stimulation with EGF results in PLCγ activation, promoting migration of HNSCC cells. In addition to these signaling pathways, there are reports that EGFR is also internalized upon ligand bind and translocated to the nucleus where it can activate transcription of genes. Numerous preclinical studies examining EGFR and these signaling pathways in the biology of HNSCC have provided a rationale for targeting EGFR in HNSCC.

Currently, there is only one targeted therapy approved for clinical use in HNSCC. This agent is a humanized monoclonal antibody to EGFR (known as cetuximab, C225, and Erbitux). The primary indication for its use is in the treatment of locally advanced (stage III and IV, non-metastatic) disease in combination with radiotherapy. In these situations, cetuximab + radiation is considered to be a more easily tolerated alternative to the standard cisplatin + radiation, especially for older patients (>70 yrs) and those with numerous co-morbidities. In a phase III study of cetuximab + radiation, there was a 10% benefit in overall survival at 5 years that was associated with the addition of cetuximab. The second indication for cetuximab is in the setting of recurrent or metastatic disease. When used in these situations, cetuximab by itself has been shown to result in a 13% objective response rate and a 33% rate of maintaining stable disease.

The primary mode of action of cetuximab is thought to be the blocking of ligand binding and inhibition of EGFR signaling. However, because small molecule tyrosine kinase inhibitors of EGFR do not appear to have the same clinical efficacy as cetuximab, some have speculated that cetuximab's mechanism is more complicated. One proposed mechanism of action is "antibody-dependent cellular cytotoxicity" (ADCC), which involves target cell lysis by immune cells with Fcγ receptors (i.e. macorphages and NK cells) that bind the Fc portion of antibody bound to the target (in this case, cetuximab). Indeed, polymorphisms in the genes encoding the Fcγ receptors have been correlated with response to cetuximab.

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Questions to Consider

A good prognostic indicator of HNSCC is:

- **A.** HPV-6 infection
- **B.** p16 positive staining of the tumor cells
- **C.** HPV DNA that has integrated into the host genome
- **D.** CD44 positive staining of the tumor cells

HPV-associated HNSCC is:

- **A.** most common in the oral cavity
- **B.** associated with a poor prognosis
- **C.** associated with more frequent lymph node metastases
- **D.** older patients

Cetuximab (anti-EGFR antibody) therapy:

- **A.** inhibits EGFR signaling through the MEK/ERK pathway
- **B.** inhibits EGFR signaling through the PLCγ pathway
- **C.** induces killing by NK cells
- **D.** all of the above

Translational Research in Prostate Cancer: The Challenge of Screening and Prognostication

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Prostate Cancer as a Model for Cancer Screening

One strategy for reducing cancer deaths is aggressive screening to identify cancers when they are small, localized (non-metastatic), and amenable to local therapies that will eradicate them. Over the past two decades prostate cancer has served as a model for the effects of wide-scale application of a cancer screening method to the population. Prostate Specific Antigen (PSA) is a serine protease made uniquely by the prostate gland and can be detected in the serum. Elevations of serum PSA levels have been used to screen for prostate cancer, and men with high PSA levels are counseled to undergo systematic biopsies to test for the presence of cancer. Although PSA has been used extensively to screen for prostate cancer over the past 25 years, many now question its utility and point out the consequences of large-scale screening. Fueling this debate are two recent randomized trials that suggest that PSA screening has little effect on reducing prostate cancer death rates.

In this talk we will review aspects of the PSA screening controversy and address challenges that have arisen as a result of PSA screening. Aggressive and repeated use of PSA as a screening tool has led to a decrease in diagnosis of locally advanced and metastatic prostate cancer from approximately 30% of cases in 1980 to 5% of cases in 2005. Furthermore, prostate cancer deaths have dropped from their peak of 40,000 in 1992 (approximately 5 years after PSA screening began) to an estimated 27,360 in 2010. Whether this drop is due to PSA screening is unknown, although some experts consider PSA screening to be largely responsible. However, 2 recent prospective randomized trials have called into question of PSA screening for reducing prostate cancer deaths. The PLCO trial in the US showed no difference in prostate cancer death rates after ten years of PSA screening. The ERSPC trial based in Europe showed modest improvements in survival by nine years, but at the expense of significant overtreatment of prostate cancer.

Consequences of PSA Screening

The challenges arising from PSA are likely to be germane to most cancers as new screening tools are developed. As a screening test, PSA has relatively poor sensitivity and specificity. The poor specificity of PSA has meant that many men (about 2/3 of the 1 million men biopsied every year) have undergone an uncomfortable biopsy, with potential grave side effects, for no reason. Therefore, the next challenge in prostate cancer is to develop a screening test to improve on the specificity of PSA to reduce unnecessary biopsies. Furthermore, approximately 15% of men harboring prostate cancer are missed by routine PSA screening highlighting the need for a more sensitive test for prostate cancer. Whether early detection of these "low PSA" tumors impacts prostate cancer death rates is unknown and is deserving of further study.

Perhaps the greatest challenged unearthed by PSA screening is over-diagnosis of prostate cancer. Since initiation of PSA screening, the gulf between the number of cases detected and the number of lethal cases has widened such that a man has a 1/6 lifetime risk of being diagnosed with prostate cancer and only a 1/35 chance of dying of his disease. This translates into many men being told they have prostate cancer who will never die of their disease, do not need to be treated, and indeed should never have been detected. Unfortunately, these men are faced with a decision to undergo life-altering treatments in the face of inadequate data on whether such treatments are necessary. Recent data suggests that systematic screening for lung cancer and serendipitous discovery of renal cancers could lead to a similar scenario.

Strategies to Address the Unintended Consequences of Screening

The clinical challenges that arise from PSA screening present researchers with several translational research opportunities, particularly in the development and application of genomic and imaging technologies.

Markers of disease risk: Genome-wide scans have identified high-risk alleles for cancer susceptibility in individuals who can then be either screening more aggressively and at a younger age or treated with prophylactic removal of the organ at risk when possible. In the future, high-risk individuals could be offered cancer preventive interventions.

New biomarkers of prognosis: Given the disparity in the number of cases discovered vs. the number of lethal cases of prostate cancer, considerable effort has been devoted to developing clinical and molecular classification schemes of low- and high-risk cancers. Genomic analysis of tumors can provide new classifications of tumors that correlate with biological (and clinical) differences in the behavior of tumors that had not been distinguishable using traditional clinical classifiers. In prostate cancer and kidney cancer, gene expression subclasses have been identified that that are correlated with treatment efficacy and with survival after therapy. Application of a variety of statistical tools has led to identification of genes that differ between normal tissues and cancer that are candidate diagnostic markers. Gene sets that correlate with clinical outcome (recurrence or death) have helped identify putative prognostic markers that could inform decisions regarding the necessity for and type of therapy. Some markers have already been incorporated into routine clinical care and many more are certain to follow. However, many challenges continue in evaluation and application of these biomarkers in the clinic.

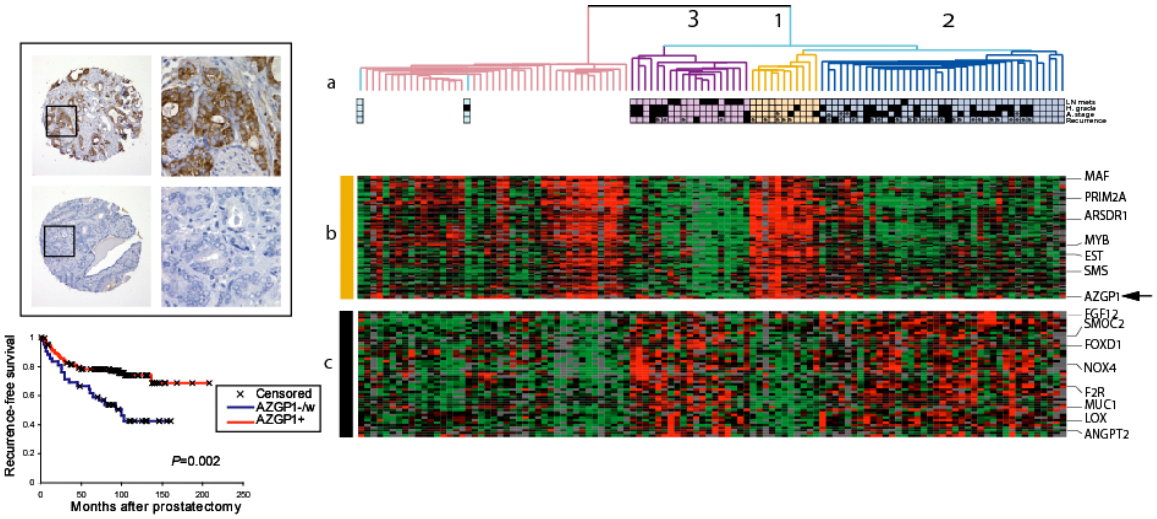

Figure. AZGP1 is differentially expressed between subclasses of prostate cancer and predicts outcome after surgery in an independent cohort of patients.

Clinical/translational research opportunities: The literature is replete with putative prognostic biomarkers for prostate cancer that have been discovered through genomic and hypothesis-based research. However, nearly all of these biomarkers have been tested as prognosticators on relatively small patient datasets derived from single institutions, usually from whatever samples were at hand - including old samples harvested prior to widespread PSA screening. These sample collections are often marred by selection biases, limited follow-up, limited sample sizes, unclear or widely differing definitions of endpoints, and differences in patient populations that make it impossible to compare results from different institutions. Furthermore, very few markers have been validated on independent patient sample sets. Therefore, despite the plethora of candidate prognostic biomarkers, no markers or sets of markers are currently in use for the clinical management of prostate cancer. While the need for rigorously validated markers of prognosis is acute in prostate cancer, the steps toward validation of a

tissue marker are not firmly established. Clinician-scientists will need to assemble bioreagents (blood, tissues, etc.) from affected patients that have been collected to minimize selection biases. These datasets will serve as a foundation for testing the performance of biomarkers in order to demonstrate their usefulness in patient care. These experiments are likely to be expensive and somewhat boring, but are essential to realizing personalized medicine in the context of cancer screening.

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Questions to Consider

What are possible unintended consequences of applying a cancer screening strategy to a population?

- **A.** Discovery indolent tumors that do not require treatment
- **B.** Direct harm from the screening
- **C.** Unnecessary patient anxiety due to false positive tests
- **D.** All of the above

What features of prostate cancer confound development and testing of screening strategies?

- **A.** Prostate cancer progresses rapidly
- **B.** Prostate cancer is relatively uncommon
- **C.** There are no cancer specific biomarkers for prostate cancer
- **D.** There are no effective treatments for prostate cancer

What are possible strategies for remedying problems arising from widespread prostate cancer screening?

- **A.** Development of prognostic schemes or biomarkers
- **B.** Increase the frequency of PSA testing
- **C.** Discontinue prostate cancer screening
- **D.** Devise uniform treatment strategies for prostate cancer

Early Detection of Ovarian Cancer Using Targeted Micro-Bubble-Enhanced Ultrasound

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Ovarian cancer is the leading cause of gynecological cancer deaths and the fifth most common cause of cancer death overall in women, despite accounting for only 3% of all new cancer cases. Causing an estimated 14,600 deaths in the U.S. in 2009, ovarian cancer has the highest mortality rate among the gynecologic malignancies. The vast majority (90-95 %) of cases are sporadic with an increased incidence after age 50. The risk for ovarian cancer is increased in women with first-degree relatives affected by the disease, especially if there are multiple family members with ovarian or breast cancer. In multiple affected families germline mutations in BRCA 1 and 2 genes may be present substantially increasing the lifetime risk for developing ovarian cancer.

Ovarian malignancies comprise a very diverse group of neoplasias with the etiology, natural history and clinical behavior varying significantly by histologic subtype. Ovarian malignancies also include other entities such germ cell tumors, sex-cord stromal tumors, mixed tumor types and rarely sarcomas. The vast majority (85-90%) are epithelial cancers, which have traditionally been thought to derive from epithelium on the ovarian surface or within inclusion cysts. One of the most malignant and feared type of epithelial ovarian cancer is the papillary serous adenocarcinoma. More recently, the origin of the invasive serous carcinoma has been questioned and there is evidence that some or even most serous "ovarian" cancers actually originate in the fallopian tube. The natural history of ovarian cancers is poorly understood, but when diagnosed in an early stage (FIGO stage I), ovarian cancer patients have a high chance to be cured with currently available therapy. Even in advanced stage, aggressive surgical tumor debulking is attempted, since optimal tumor volume reduction prior to the initiation of combination chemotherapy has been shown to increase survival time. Epithelial ovarian cancers are typically chemosensitive and respond well to initial chemotherapy, but tumor recurrence is common and resistance to

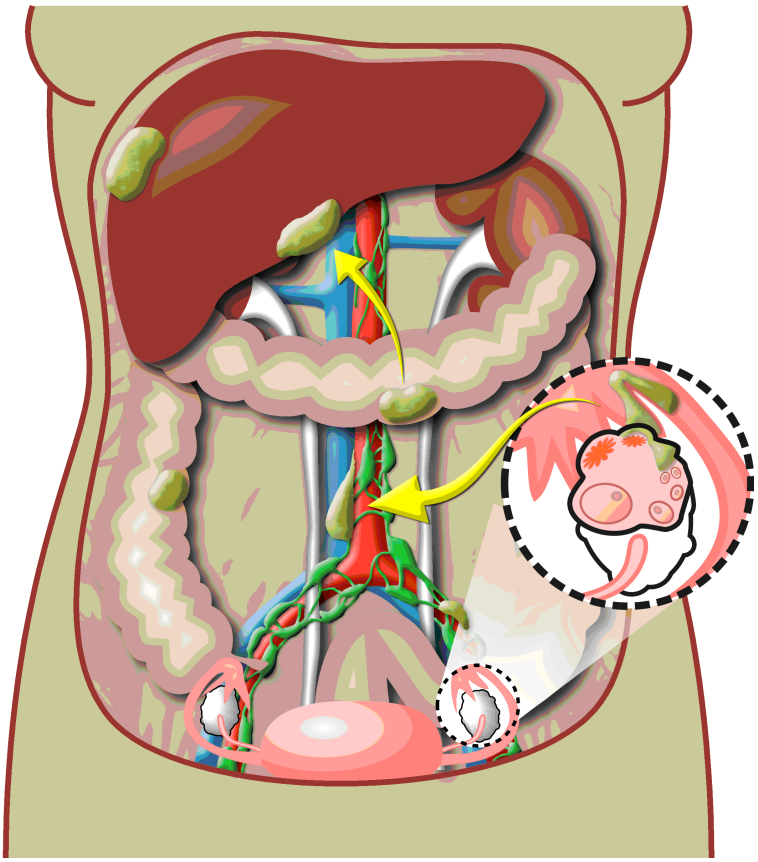

Figure. Anatomic conditions that favor an early spread of "ovarian" cancer:

The primary tumor (in olive green color) develops in the epithelium of the ovarian surface/Fallopian tube. As the ovarian surface epithelium is in direct contact with the peritoneum, early metastatic spread to the peritoneum is facilitated, which is present in the vast majority of patients (yellow arrow; tumor deposits pastel green). Lymphatic spread to pelvic and abdominal lymph nodes is also common (bright green).

further therapy develops in all patients over time. Consequently, patients with advanced ovarian cancer face tremendous morbidity during the course of the disease.

Overall 5-year survival has not really drastically improved over the last few decades, changing from 37% in 1974 to 53% in 1998. Further improvement in patient survival is hampered by the fact that in the vast majority of cases the disease is diagnosed at an advanced stage (FIGO stages III and IV) when extended peritoneal involvement or bulky disease is already present. Earlier detection of ovarian cancer has the potential to improve patient survival, but currently available diagnostic tools lack either sufficient sensitivity, specificity or both and, therefore, are not applicable to widespread patient screening. Other contributing factors for diagnosis in typically advanced stages are non-specific disease symptoms, changing ovarian morphologic appearance depending on age and hormonal status and anatomical conditions of the ovaries that favor an early spread of the disease.

In this talk we will discuss:

- the specific challenges ovarian cancer poses on early detection
- the problems of implementing screening for ovarian cancer to either defined target populations or the general female population and how they potentially may be overcome
- currently available diagnostic tools (i.e. blood tests and ultrasound) for ovarian cancer
- novel blood tests and imaging tests as well as their potential for implementation in diagnostic algorithms
- the principle of molecularly-targeted microbubble-enhanced ultrasound
- ongoing research in the field of molecularly-targeted contrast-enhanced ultrasound with regard to ovarian cancer early detection

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Questions to Consider

The main challenges for earlier detection of ovarian cancer include:

- **A.** Lack of specific symptoms
- **B.** Suboptimal performance of currently available diagnostic tools
- **C.** Anatomical conditions of the ovaries favor an early spread of the disease
- **D.** All of the above

What are suitable imaging targets for functionalized microbubbles in targeted contrast-enhanced ultrasound?

- **A.** Cell surface proteins on vascular endothelial cells
- **B.** Cell surface proteins on tumor cells
- **C.** Protein markers in tumor cells
- **D.** All of the above

What are some of the main advantages of molecularly-targeted contrast-enhanced ultrasound?

- **A.** It can obtain information on blood flow at the microvascular level
- **B.** The ability to characterize the molecular phenotype of microvascular endothelial cells
- **C.** When combined with harmonic imaging it enhances the signal of the surrounding tissue

Molecular Targets in Breast Cancer

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Cancers arising in the breast comprise a diverse group of neoplastic diseases. Attempts to dissect the different subtypes of breast cancer to better formulate treatment algorithms has been an ongoing endeavour spanning several decades. The repertoire of anti-cancer therapies with clinical activity in the treatment of breast cancer has also exploded over the past decade and now include a diverse group of cytotoxic agents, hormonal therapies, non-hormonal targeted therapies, immunotherapies, and other biologic therapies with unknown mechanisms of action. None of these therapies are broadly effective against all breast cancers, making it increasingly necessary to classify and identify breast cancers by the presence of molecular targets or molecular markers that predict response to specific forms of targeted therapies. The most functionally relevant current evidence classifies breast cancers according to one of several gene expression profiles consisting of basal-like, HER2, and luminal A and B subtypes. In general, the luminal subtypes are characterized by a dominant role of estrogen receptor signaling, and the basal subtype is characterized by defects in DNA repair. Abnormalities in receptor tyrosine kinase signaling can be found among all the subtypes, but the HER2 subtype is highly dependent on this signaling mechanism. This has provided a general framework for the development of targeted therapies.

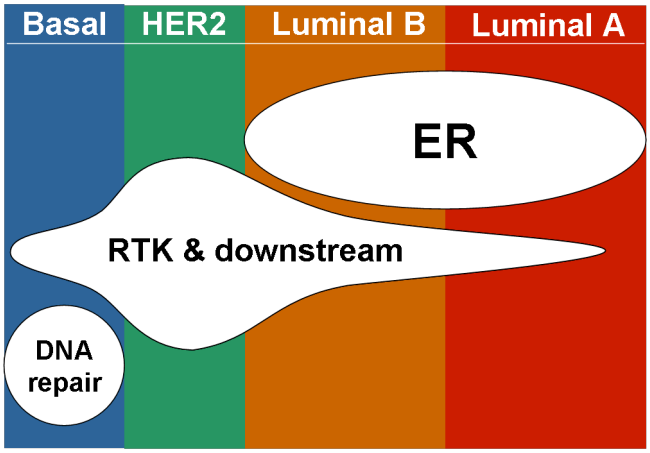

Figure 1: Common breast cancer subtypes and dominant molecular mechanisms within the subtypes that have proven to be fruitful targets for therapy.

Targeting Estrogen Receptor Signaling

A hallmark of the luminal subtypes of breast cancer is the expression and functional relevance of the estrogen receptor alpha (ERα) nuclear transcription factor. The growth and survival of many of these cancers requires the activities of ERα. The activities of ERα consist predominantly of ligand-dependent nuclear transcription functions, although ligand-independent functions may also exist. ERα is not an oncogene and its functions are not known to be pathologically hyperactivated through mutation, overexpression, or other structural alteration. Its necessary role in these cancer cells may be a feature inherited from their ancestral cell type or its cooperative functions may have been engaged during tumorigenesis. Either way, it is clear that modulation of ERα activities in these cancer subtypes frequently has profound effects on their biological behavior. Three targets have been pursued in this endeavour: 1) ovarian estrogen production; 2) tumor ERα; 3) extra-ovarian estrogen biosynthetic pathways. The suppression of ovarian estrogen secretion has been used for decades using surgical removal or radiation ablation of the ovaries, and more recently through the use of pituitary-active peptide hormones that induce a pharmacologic menopause.

Manipulating tumor ER has been the mainstay of pharmacologic approaches for most of the past 3 decades. Most of this effort has concentrated on ERα, while the role of the homologous ERβ in breast tumorigenesis remains yet undefined. In its most simplistic Traditional paradigms considered estradiol as a tumor-promoting ligand and small molecule mimetics of estradiol were developed to bind ERα competitively with estradiol with intent to produce a loss-of-function phenotype. It is now clear that this view is over-simplistic. The family of ERα binding agents, collectively called Selective Estrogen Receptor

Modulators (SERMs), exert potentially opposite effects on ERα within breast cancer cells and within other body tissues. Detailed structures of the ERα ligand-binding domain in conjunction with estradiol or a number of synthetic ligands have been elucidated. Collectively, the evidence shows that ligand binding induces conformation changes within the ligand binding domain that influence its ability to interact with potential transcriptional cofactors. The fact that different ligands induce different conformations in the ligand-binding domain, and the fact that different tissues and different tumors may engage a different repertoire of cofactors, introduces the degree of complexity into the system that most likely accounts for the pleiotropic effects of SERMs in different tumor and different body tissues. Purely inhibitory SERMs have also been developed that induce the cytoplasmic degradation of ERα, producing a true loss-of-function phenotype.

Inhibition of extra-ovarian biosynthesis of estrogen has been pursued for many years. The importance of this is underscored by the fact that the tumor cells and their microenvironment are actively involved in some of the steps in estradiol biosynthesis. A principal challenge has been the specificity of inhibition in the context of the common biochemical pathways employed in the biosynthesis of most steroidal hormones. But inhibitors of aromatase have proven to be well tolerated and highly active in the treatment of hormone-dependent breast cancers and are now at the forefront of the hormal therapy of breast cancer.

While much progress has been made in understanding the synthesis of estrogen and the activation of the estrogen receptor, these advances fail to identify a molecular basis underlying response and resistance to hormonal therapies. Much of the actual biological consequences of hormonal manipulation are due to the transcriptional consequences of manipulating ERα function and our understanding of this field is still in its infancy. Ongoing lines of investigation are attempting to unravel the complex transcriptional program that mediates the hormonal control of breast cancer behavior.

Targeting HER2 Signaling

Amplification and overexpression of the Human Epidermal Growth Factor Receptor (HER) family of receptor tyrosine kinases are another feature of many breast cancers. Amplification of EGFR occurs in approximately 5% of cases, often within the basal-like subtype. However the tumor-driving role of this target is not well established by experimental models and the corresponding lack of efficacy seen with EGFR-targeting therapies in this disease subtype further underscores the need to better understand the role of EGFR in breast cancer tumorigenesis. Amplification of HER2 occurs in another 25% of cases, defining the HER2 subtype but also seen in luminal subtypes. HER2 is a highly transforming oncogene when overexpressed and its amplification is an early event in breast tumorigenesis, frequently seen in many pre-invasive cancers of the breast. An abundance of *in vitro* and *in vivo* experimental models have solidly established HER2 function as the driver of tumor survival and progression in this disease leading to the promising treatment hypothesis that inactivation of HER2 should produce complete remission in this disease. Two decades of targeted therapies have produces several classes of HER2-targeting drugs, but the clinical benefits of these agents continue to be incremental in nature. Many of these approaches emerged from a primitive understanding of the structural and functional aspects of HER2 signaling. Developments in the past few years now provide considerable insight into the structural basis for HER2 activation and the

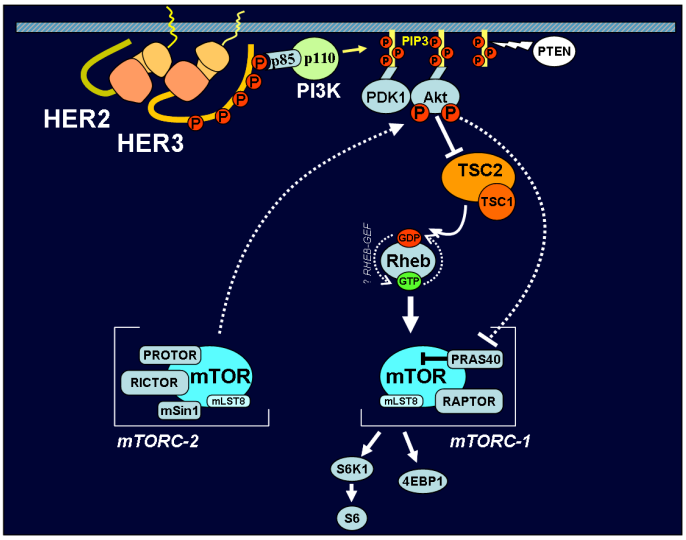

Figure 2: Downstream network topology linked with HER2-HER3 signaling. Many components of this signaling pathway are activated in breast cancers and are high targets for drug development.

critical role of its kinase-inactive partner HER3. HER3 functions as the allosteric activator of the HER2 kinase domain and as the most important signaling substrate of the activate kinase. As such HER3 functions both upstream and downstream of HER2 and the HER2-HER3 complex is best considered one oncogenic unit. Attempts to inhibit it reveal that the HER2-HER3 complex is endowed with a robust signal buffering capacity that protects it against a nearly two-log inhibition of HER2 catalytic activity. This is driven by a HER3-linked downstream network topology that functions to preserve HER2-HER3 signaling and underlies the difficulties in developing highly effective treatments for this disease. The target of this disease has now been functionally redefined as the HER2-HER3 heterodimer and numerous pharmacologic programs are underway to inactive it.

The value of HER2 as a target for drug development exceeds the value ascribed to its tumorigenic functions. Massive HER2 expression on the cell surface provides a unique tumor-identifying antigen for the delivery of various HER2-targeting cytotoxic agents or HER2 -targeting immunotherapies and many such approaches are underway.

Targeting Signaling from RTKs

Many of the proto-oncogene signaling proteins functioning downstream of growth factor receptors which are frequently mutated in many types of cancer are only rarely mutated in breast cancers. These include mutation activation of Ras, Raf, Akt and inactivation of PTEN. However mutational activation of PI3K is seen in about 20-40% of cases and represents the most frequent mutation seen in breast cancers. The value of activated PI3K as a target for drug development continues to be studied and much more evidence will be forthcoming in the next few years regarding the isoform of PI3K that is the most relevant target, the therapeutic index afforded by PI3K as a target, and the driving role of this target in breast cancers.

Targeting DNA Repair

While the expression of high value molecular targets have fueled the development of numerous targeted therapies for the luminal and HER2 subtypes of breast cancers, there have been no credible targets defined yet for the basal-like subtype. As such, there is great need and much effort ongoing to identify novel targets in this disease subtype. This search for targets within this subtype has led beyond the traditional realm of target discovery with much success.

Traditionally, molecular targets that have been considered high value for cancer therapy have been signaling proteins that promote cancer progression through overactive signaling or even through their normal signaling functions. The oncogenic functions of such targets are readily amenable to inhibitory drugs. However, equally important in driving cancer progression are the loss of tumor suppressor functions. Such functions have not traditionally been labelled molecular targets since pharmaceutical technologies to restore their functions are not yet feasible. However the loss of tumor suppressor functions can create susceptibilities that can be exploited for cancer therapy. An example of this has been breast cancers that have loss of function of the BRCA1 or BRCA2 tumor suppressor genes, frequently within the basal-like subtype. These cancers have specific defects in DNA double strand break repair leaving them highly susceptible to agents that induce double strand breaks. Consistent with this, these cancers are particularly sensitive to platinum agents that induce DNA crosslinks and have been commonly used in cancer therapy. Recent mechanistic insights into the specific functions of BRCA1 and BRCA2 in initiating repair and facilitating homologous recombination at sites of double strand breaks has led to a rational targeting approach to capitalize on the defects of this tumor type. One such approach has been to interfere with DNA nick repair by targeting the PARP enzyme, leading to irrepairable double strand breaks during DNA replication with consequent failure of replication specifically in tumor cells and apoptotic death. Early clinical studies of this treatment hypothesis have been highly promising and have fueled efforts to identify even larger subsets of tumors, perhaps with defects functionally related to BRCA1/BRCA2 and that may also be highly susceptible to PARP inhibition. Therefore PARP is a high value molecular target in BRCA1/2 deficient breast cancers. Ongoing studies using synthethic lethality screens in these and other basal-like tumors are likely to identify additional high value molecular targets.

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Questions to Consider

Why do different SERMs have different anti-tumor effects?

- **A.** they have differences in biodistribution
- **B.** they induce different conformational changes in ER
- **C.** they are metabolized differently
- **D.** they have different potencies

Why are HER2-amplified breast cancers not highly sensitive to our current HER2-targeted therapies?

- **A.** because there are other driving pathways in parallel to HER2
- **B.** because HER2 acquires mutations that make it resistant to drugs
- **C.** because HER3 takes over when HER2 is inhibited
- **D.** because HER3 is upregulated to compensate for a drop in HER2 activity

Why do PARP inhibitors selectively kill BRCA1 deficient tumor cells?

- **A.** these tumor cells are unable to repair nicks
- **B.** these tumor cells are unable to repair double strand breaks at the replication fork
- **C.** these tumor cells are more susceptible to double strand breaks
- **D.** these tumor cells have amplification of PARP

The Hedgehog Signaling Pathway as a Target for Anti-cancer Therapeutics

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Introduction

The Hedgehog (Hh) signaling pathway is conserved across animals from insects to mammals. It is one of a handful of developmental pathways (other examples are the Wnt, Notch, and TGF-beta pathways) that controls cell proliferation, differentiation and patterning in a range of tissues during development. Mutations that inactivate the pathway in humans and mice lead to birth defects. In adults, Hh controls the proliferation and function of organ stem or progenitor cells, allowing the repair of organs in the face of cell turnover and injury. Inappropriate activation of the pathway has been implicated in the genesis of human cancers that develop in the skin, brain, lung, prostate, pancreas, and upper gastrointestinal tract. Activation of Hh signaling can be caused by genetic mutations in pathway components or by over-expression of the secreted ligand

Inhibitors of the Hh pathway have entered clinical trials. Based on promising activity against advanced basal cell cancers and medulloblastomas, these inhibitors are being tested in a wide range of malignancies, including colon cancer, small-cell lung cancer, and ovarian cancer.

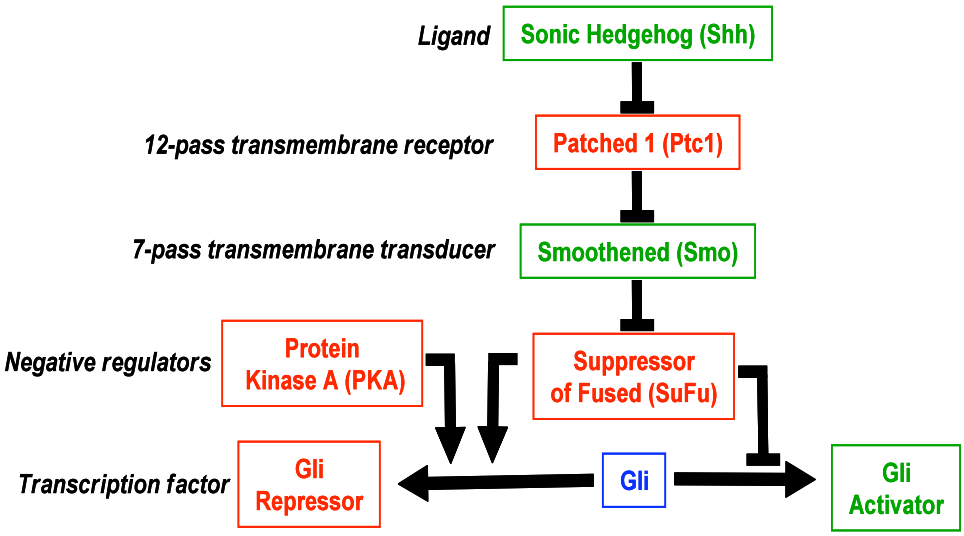

Figure 1. The mammalian Hedgehog signaling pathway. Positive regulators are shown in green boxes and negative regulators in red boxes. Abbreviations for the proteins used throughout the text are given in parentheses.

Overview of Mammalian Hedgehog Signaling

The overall structure of the Hh pathway is composed of a series of repressive interactions (Figure 1). In the absence of signal, the pathway is shut off by a 12-pass transmembrane protein Patched 1 (Ptc1). Ptc1 inhibits the function of a second 7-pass transmembrane protein, Smoothened (Smo). The pathway is activated by one of three secreted proteins, Sonic Hedgehog (Shh), Indian Hh (Ihh), and Desert Hh (Dhh). Shh binds to Ptc1 with the help of co-receptors and somehow inhibits its activity, allowing the de-repression of Smo. Once unleashed from Ptc1, Smo inhibits Supressor of Fused (SuFu), a negative regulator of the pathway, and allows activation of target gene transcription through three Gli (Gli1-3) transcription factors. Smo is thought to initially cause the activation of Gli2, which functions primarily

as a transcriptional activator and induces the expression of Gli1, an early target gene of the pathway. Gli3 exists in two forms, a full-length transcriptional activator (Gli3A) or an N-terminal fragment that functions as a repressor (Gli3R). Protein kinase A (PKA) negatively regulates the pathway at the level of Gli by increasing the repressor to activator ratio. While the relative contributions of the Gli proteins are tissue and context-dependent, Hh generally shapes the transcriptional response in both normal and cancer cells by altering the ratio of activator and repressor functions of the Glis. It is important to note that some of earliest genes induced by Shh include components of the pathway itself, such as *Ptc1 and Gli1*, and assays for pathway activity in tumors often measure the products of these genes. Other genes activated in response to Hh signaling in tumors include Cyclin D, which drives proliferation, Bcl-2, which blocks apoptosis, VEGF and angiopoietins, which promote angiogenesis, and SNAIL, which induces the epithelial-mesenchymal transition.

Mechanisms of Hh Pathway Activation in Tumors

The effective use of anti-Hh drugs, either alone or in combination with chemotherapy, requires an understanding of how the Hh pathway is activated in tumors. Two major mechanisms, ligand-independent and ligand-dependent, have been uncovered (Figure 2).

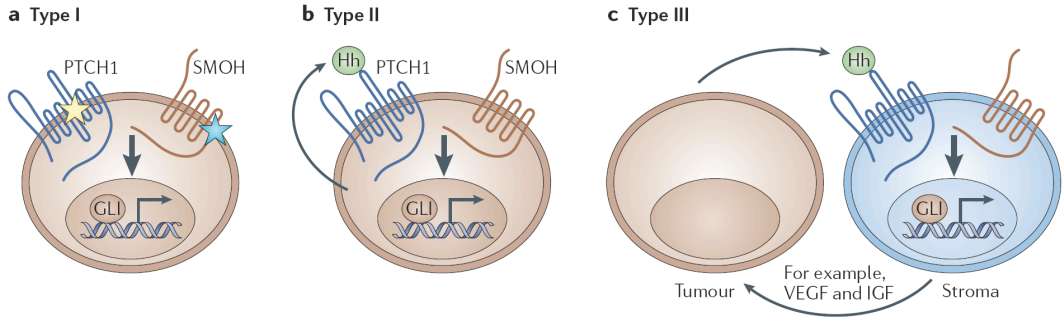

Figure 2. Models of Hh pathway activation in cancer. **a** Mutations in pathway components such as Ptc1 and Smo leads to ligand-independent signaling. **b** and **c** Ligand overexpression activates signaling by an autocrine (**b**) or paracrine (**c**) mechanism. In the autocrine model, tumor cells produce and respond to Hh ligand. Pathway activation my occur in all tumor cells or in a small number of tumor stem cells. In the paracrine model, tumor cells produce Hh ligand and surrounding stromal cells respond by producing additional growth factors to support tumor growth or survival. IGF, insulin-like growth factor; VEGF, vascular endothelial growth factor. Note that this figure and caption are taken from reference 2, Rubin and De Sauvage (2006.)

Ligand-independent Ligand-independent Signaling

The link between activated Hh signaling and cancer was first established with the discovery that human patients with autosomal dominant Gorlin's Syndrome, characterized by a high incidence of basal cell cancer (BCC), medulloblastoma (MB) and sarcomas, carried a heterozygous mutation in *Ptc1*. Consistent with Knudson's two-hit hypothesis, the tumors displayed loss-of-heterozygosity for *Ptc1*, indicating *Ptc1* is a true tumor suppressor and that pathway activation is sufficient to cause tumor formation. In addition to this familial syndrome, sporadic loss-of-function mutations in the negative regulators, Ptc1 and SuFu, and gain-of-function mutations in the positive regulator Smo have been found in ~25% of medulloblastoma and ~85% of BCC. Taken together, this data first established that activation of the pathway can drive tumor formation and that components in the pathway are both proto-oncogenes (Smo) and tumor suppressor genes (Ptc1 and SuFu) in human cancer.

In these cases, since the pathway is activated by mutations in intracellular components, signaling has become independent of ligand. Effective drugs against this type of unrestrained signaling must be directed downstream of the mutated component. Tumors carrying activating mutations in the Hh pathway are uniquely dependent or addicted to this signal and thus single agent Hh inhibitors are likely to be effective, in a manner similar to how erlotinib is effective as a single agent in lung adenocarcinomas bearing mutations in the EGF receptor.

Ligand-dependent Signaling

Hh signaling has also been implicated in a variety of other cancers arising in the lung, pancreas, upper gastrointestinal tract, colon, prostate and breast. It is important to note that in these cases, no mutations are found in any of the Hh pathway components. Instead, the pathway is driven by the unrestrained secretion of one of the ligands, Shh, Ihh, or Dhh, by the tumor cells. The ligands can activate signaling either in the tumor cells themselves (autocrine signaling) or in the surrounding stroma (paracrine signaling). While the precise mechanism is controversial, the paracrine signaling model has recently gained a considerable amount of experimental support. In this scenario, tumor-derived Shh turns the Hh pathway on in stromal cells, which then trigger pathways that facilitate tumor growth, tumor invasion, angiogenenesis, and desmoplasia. The precise signals that feed back from the stroma to facilitate tumor development remain to be identified. In this scenario, the Hh pathway is activated in genetically stable stromal cells and resistance to Hh inhibitors is less likely to develop.

In cases of ligand-dependent signaling, Hh signaling likely plays a more supportive role in tumorigenesis and Hh inhibitors will be most effective in combination with chemotherapy. There is also literature suggesting that such signaling plays a role in the growth of a small population of tumor sustaining or tumor "stem" cells that could be eradicated by combining chemotherapy, directed against the bulk tumor, with Hh inhibitors.

Drugs Against the Hedgehog pathway

The most "druggable" target in the Hedgehog pathway is the 7-pass transmembrane protein Smo. The first Smo inhibitors, Cyclopamine and Jervine, were isolated as the teratogenic components of corn lilies. Cyclopamine was the first Smo inhibitor tested in human patients, applied as a topical formulation for the treatment of BCC. Several second-generation Smo inhibitors with improved pharmacodynamic and pharmacokinetic properties have been developed and have entered clinical trials. GDC-0449, an orally available Smo inhibitor, has shown promising activity in advanced basal cell carcinoma, with 8/9 patients in a Phase I study showing a partial response or stable disease. GDC-0449 also produced a response in a single patient with metastatic medulloblastoma; however, a mutation in Smo that prevented binding of the drug led to the rapid emergence of resistance. Based on these results, GDC-0449 is being tested in a Phase II trial of advanced BCC, in the maintenance therapy for 2nd or 3rd remission ovarian cancer, and for the first line treatment of metastatic colorectal cancer and small cell lung cancer (the latter two in combination with standard chemotherapy).

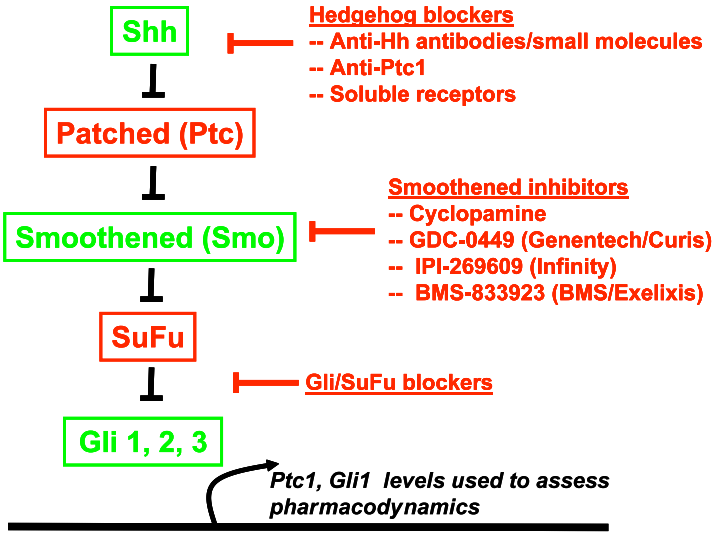

Figure 3. Three strategies for targeting the Hh pathway at the level of Shh, Smo or Gli. Only Smo inhibitors have entered clinical trials.

While Smo inhibitors are the only anti-Hh agents currently in clinical trials, active development programs are looking for drugs that may work either upstream of Smo, at the level of the ligand Shh or the receptor Ptc1, or downstream at the level of the SuFu or Gli proteins (Figure 3).

Challenges

Hh pathway inhibitors are a new frontier in cancer treatment. Trials over the coming years will determine how such inhibitors can be used effectively in combination with chemotherapy or in combination with inhibitors against other cancer pathways. A major goal is to develop predictive biomarkers that can used to identify patients who stand the best chance of benefiting from anti-Hh drugs. The overexpression of Hh ligands in tumor biopsies or in circulating tumor cells is being evaluated in early trials.

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Questions to Consider

Which of the following sporadic cancers is most likely to carry a mutation that leads to activated hedgehog signaling?

- **A.** medulloblastoma
- **B.** basal cell carcinoma
- **C.** small cell lung carcinoma
- **D.** pancreatic carcinoma

Which of the following resistance mechanisms have been established for the Smoothened inhibitor GDC-0449 in human patients?

- **A.** overexpression of smoothened
- **B.** loss of SuFu
- **C.** overexpression of Gli
- **D.** mutation of Smo

Smo inhibitors will be ineffective in which of the following tumors?

- **A.** tumors that show high Gli1 levels in the stromal compartment
- **B.** tumors that show a loss of SuFu protein
- **C.** tumors that show a loss of Patched protein
- **D.** tumors that overexpress Shh

Cancer Survivorship Studies

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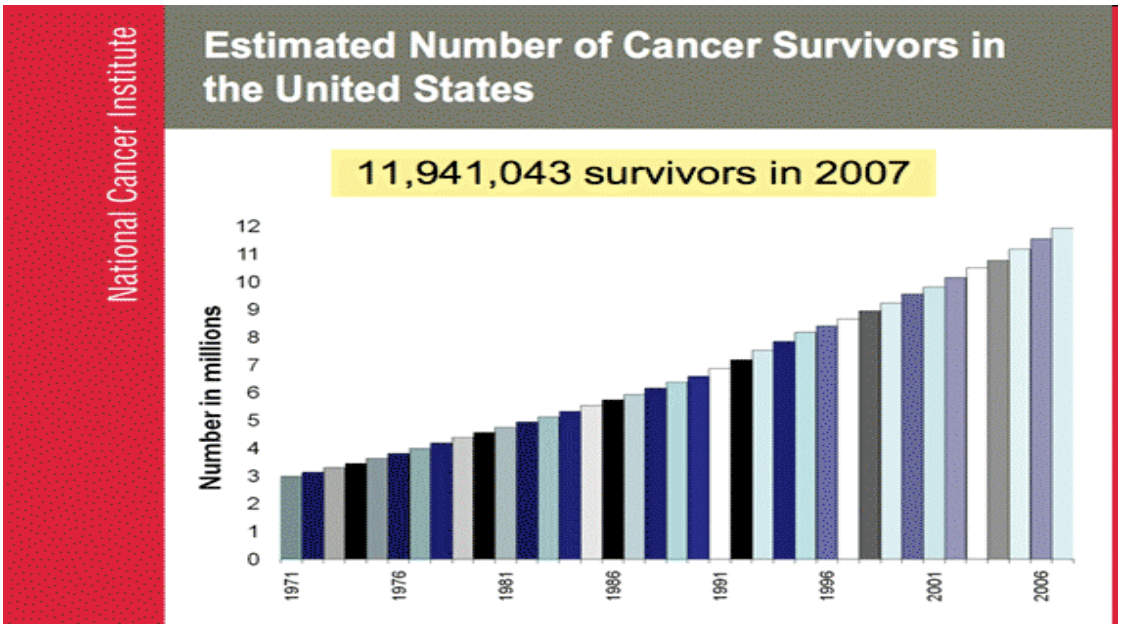
Psychosocial research with cancer patients addresses the nature of stress and emotional distress, coping mechanisms, doctor-patient communication, social support, psychosocial intervention, recurrence risk, and mind-body interactions. Cancer is a stressor in many senses, involving existential concerns, treatment decisions, side effects of surgical and medical treatments, changes in social support and social roles, financial concerns, and the constant threat of recurrence [1]. The ways in which people cope with these stressors, and their resources of emotional support, can have profound effects on the quality and potentially on the quantity of their survivorship. [2] Due to better screening and treatments, the population of cancer survivors has grown from 3 million in the U.S. in 1971 to more than 12 million. Problems they face include late effects of primary treatment, fatigue, cognitive limitations, financial, vocational, emotional, and social difficulties.



Studies of cancer inpatients show that one-quarter report significant depression, and another 25% suffer from anxiety disorders of various types.[3] It is easy to misinterpret the symptoms of depression, including hopelessness, fatigue, sleep disturbance, and loss of appetite, as consequences of cancer and its treatment. Yet many cancer patients suffer unnecessary feelings of hopelessness, helplessness, and worthlessness due to treatable depression. Similarly, a substantial minority of cancer patients experience post-traumatic stress symptoms of intrusive thinking, numbing, and irritability seen among trauma survivors, but in relation to their cancer.

Depression has predicted shorter cancer survival in 19 of 24 recent studies.[4] Recent work in stress response has emphasized the cumulative effect of stressors on physiological response systems, referred to as 'allostatic load.' Exposure to frequent or severe stress over a period of time, especially when it occurs early in life, may lead to sensitization of the hypothalamic-pituitary-adrenal (HPA) axis, resulting in pronounced stress response. The diagnosis of cancer, and coping with all the psychological, physical, and physiological factors that accompany the treatment of cancer, constitutes a major stress burden, especially for those already sensitized by prior stressors. Current research suggests that much of the HPA dysregulation attributed to depression actually has more to do with a history of trauma. [5] Cancer-related fatigue has also been shown to be associated with abnormal diurnal patterns of cortisol, [6] so stress-related HPA dysfunction may account for a number of common problems in coping and survivorship with cancer.

Conversely, there is evidence that resilience to stress, including disease-related distress, is associated with emotional style. [7] Indeed, finding meaning in the midst of experiencing a distressing situation, for example after a cancer diagnosis, has been linked with a positive psychological state. Better adaptation does not involve maintaining a positive attitude, but rather dealing directly with negative affect when it arises. [2] Suppression of negative emotion tends to reduce experience of all emotion, positive and negative, and hamper the ability to identify and cope with stressors.

Principles of Cancer Stress Management

*F*acing rather than fleeing

*A*lter perception of the situation

*C*ope actively

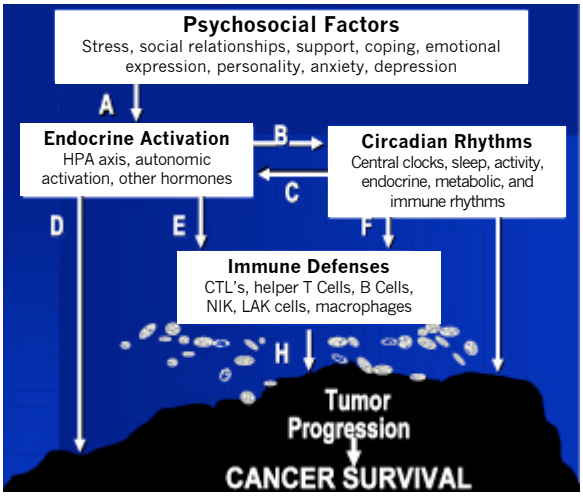
*E*xpress emotion

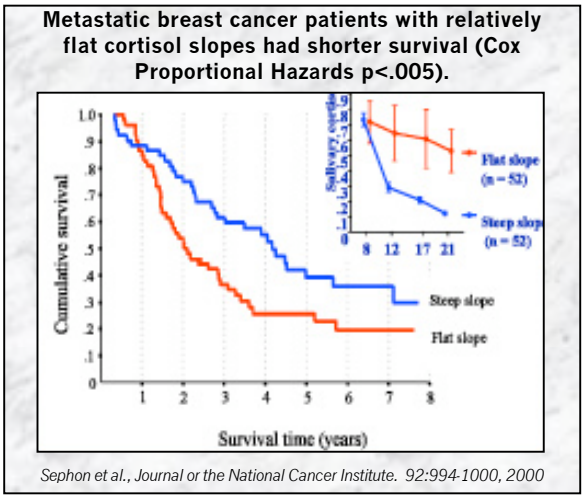
*S*ocial support

A growing body of evidence demonstrates that psychotherapeutic techniques such as group therapy [8] and psychoeducation[9-10] can promote resilience and reduce distress, pain, and social isolation. Therapeutic domains include building new networks of social support, encouraging the expression of emotion related to the stress of illness, detoxifying fears of dying and death, restructuring life priorities, improving relationships with family and friends, and clarifying communication with physicians. [11] In addition, specific stress management techniques such as training in self-hypnosis can effectively alter perception of pain and anxiety and facilitate medical procedures. [12] These mind/body techniques work by altering specific parts of the brain involved in perceptual processing. The modulation of perception, emotion, cognition and social support is a critical element in managing the stress of medical illness.[13] Earlier reports that group therapy might extend survival time for women with metastatic breast cancer [14] have recently been confirmed in some [15] but not all studies.[16-17] Our recent replication trial found such an effect only among ER negative patients.[18] An independent randomized trial among women with primary breast cancer found that a group intervention resulted in significantly lower rates of relapse and mortality. [19] Another 10-year follow-up reported longer survival among solid tumor patients randomized to receive intensive psychotherapy at the time of initial surgery. [20]

Mechanisms have been proposed whereby the neuroendocrine and neuroimmune correlates of stress may promote neoplastic growth [21-22]. Abnormal diurnal patterns of cortisol predict shorter survival with metastatic breast cancer[23]. Stress hormones may suppress immune resistance to tumors since glucocorticoids are potently immunosuppressive. Differential effects of glucocorticoids on gluconeogenesis in healthy versus tumor cells may lead tumor cells to develop a metabolic advantage by becoming resistant to the catabolic action of cortisol, which inhibits the uptake of glucose in numerous cell types. Several studies have found an association between stress-related elevation of glucocorticoids and more rapid tumor growth in animals. There is recent evidence that glucocorticoids, for example dexamethasone, inhibit natural apoptosis and the apoptotic effect of paclitaxel in breast tumors by inactivating a MAP kinase. Glucocorticoid receptor activation can stimulate growth of breast cancer cells through direct transactivation of genes encoding proteins that reduce susceptibility to apoptosis. Cortisol down-regulates the expression of the breast cancer oncogene BRCA1 in a nonmalignant mouse mammary cell line EPH4.[24] Thus there are a variety of means by which depression-related abnormalities in HPA function could affect the rate of cancer progression. Another pathway involves sympathetic catecholamines triggering beta 2 adrenergic receptors on breast and ovarian tumor cells. In a murine model, adrenergic stimulation of a cAMP- protein kinase A pathway led to increased production of VEGF, growth of blood vessels, and larger tumor volume.[25] This enhanced tumor growth was blocked by propranolol, a beta adrenergic blocker, and the use of siRNA to human ADRB1 or ADRB2 tumor cell adrenoreceptors.

Thus the stressors associated with cancer and its treatment complicate the course of cancer. At the same time, they are potentially manageable with appropriate problem identification, improved coping, diagnosis and treatment of anxiety and depression, and provision of psychosocial support. We are developing new techniques for enhancing and better understanding the psychophysiology of cancer survivorship.





Survivorship Planning and Support

Survivorship programs need to address the following issues:

1. **Screening protocols for recurrence**

2. **Surveillance for late effects of cancer treatment**
   - **A.** Cardiotoxicity
   - **B.** Cognitive decline
   - **C.** Second cancers

3. **Genetic Issues**
   - **A.** Implications for patient's progeny
   - **B.** Implications for extended family

4. **General health maintenance**
   - **A.** Tobacco control
   - **B.** Substance abuse
   - **C.** Diet
   - **D.** Exercise

5. **Psychiatric late effects**
   - **A.** Depression
   - **B.** PTSD
   - **C.** Marital disruption
   - **D.** Social isolation

6. **Financial problems**
   - **A.** Insurance discrimination
   - **B.** Disability
   - **C.** Work limitations

"Lost in Transition," A Report of the Institute of Medicine of the National Academies of Science issued in 2006, noted that cancer survivorship is a neglected area. Our success in treating cancer opens new domains and time frames for ongoing support to make the most of our hard-won improvements in survival. Good survivorship planning should begin with initial diagnosis and treatment, and follow patients throughout their course of treatment and follow-up care.

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Questions to Consider

Supportive psychotherapy for those with cancer:

A. should be confined to treating those with major psychiatric diagnoses

B. is rarely indicated

C. has been supplanted by pharmacotherapy

D. should explore uncomfortable affect related to illness

Physiological variables shown to link stress to disease progression include all of the following except:

A. diurnal variation in cortisol

B. natural killer cell activity

C. lipid levels

D. insulin resistance

Community Education

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Director of Community Education
Cancer Prevention Institute of California and Stanford Cancer Center

GOALS

- **Reduce health disparities**
- **Improve the public health**
- **Strengthen ties with community agencies and collaborators**
- **Provide access to trusted information and resources**
- **Help individuals make informed decisions about their health and health care**
- **Facilitate communication between patient and health care provider**

RATIONALE

- **Community/patient education is a missing link between research and treatment; health and illness**

DEFINITION OF COMMUNITY

FUNDAMENTALS FOR SUCCESSFUL COMMUNITY EDUCATION

CANCER AND CULTURE

PRACTICAL ISSUES

STRATEGIES

- **Patient care**
- **Research**
- **Information**
- **Training**
- **Collaboration**
- **Recognition**

RESOURCES

CHALLENGES

RECOMMENDATIONS

SUCCESSFUL/IMPLEMENTED EXAMPLES

SUMMARY

Recommended Reading

1. **eHealth Literacy: Essential Skills for Consumer Health in a Networked World.** Cameron D. Norman, PhD and Harvey A. Skinner, PhD, CPsych; J. Med Internet Res 2006 Apr-Jun; 8 (2); e9 Published online: www.ncbi.nlm.nih.gov/pmc/articles/PMC1550701

2. **Making Health Literacy Real: Adult Literacy and Medical Students Teach Each Other**. Jean Hess, MS; Julia S. Whelan, MS, AHIP; J. Med Libr. Assoc 97 (3); July 2009.

3. **Promoting Health Literacy**. Alexa T. McCray, PhD. J Am Med Inform Assoc. 2005 Mar-Apr; 12(2), 152-163. Published online: www.ncbi.nlm.nih.gov/pmc/articles/PMC551547

4. **Giving Everyone the Health of the Educated: An Examination of Whether Social Change Would Save More Lives Than Medical Advances**. Steven H. Woolf, MD, MPH, Robert E. Johnson, PhD., Robert L. Phillips, Jr. MD, MSPH and Maike Philipsen, PhD. American Journal of Public Health, April 2007, Vol 97, No. 4.

5. **The President's Cancer Panel – Annual Reports for the years 1996 – 2009**
   (Summaries of Findings & Recommendations) http://deainfo.nci.nih.gov/advisory/pcp/pcp.htm

2008 -2009	Reducing Environmental Cancer Risk, What We Can Do
2007 -2008	Maximizing Our Nation's Investment in Cancer
2006 - 2007	Promoting Healthy Lifestyles
2005 - 2006	Assessing Progress, Advancing Change
2004 - 2005	Translating Research Into Cancer Care: Delivering on the Promise
2003 - 2004	Living Beyond Cancer: Finding a New Balance
2002	Facing Cancer in Indian Country
2000 – 2001	Voices of a Broken System: Real People, Real Problems
1999	The National Cancer Program: Assessing the Past, Charting the Future
1997 - 1998	Cancer Care Issues in the US: Quality of Care, Quality of Life
1997	The Meaning of Race in Science--Considerations for Cancer Research
1996	Fighting the War on Cancer in an Evolving Health Care System

Not Yet Released/Completed

2009	America's Demographic and Cultural Transformation: Implications for the Cancer Enterprise

Scheduled

2010 - 2011	The Future of Cancer Research: Accelerating Scientific Innovation

Questions to Consider

The average reading level of an American adult is:

- **A.** 5th grade
- **B.** 8th grade
- **C.** 10th grade
- **D.** 12th grade

What is the single greatest factor affecting comprehension?

- **A.** Reading grade level
- **B.** Sentence length
- **C.** Use of jargon
- **D.** Use of words that have multiple meanings

Health literacy is "the degree to which consumers have the capacity to obtain, process, and understand basic health information and services needed to make appropriate health decisions." (*Healthy People, 2010*)

True or False: Health literacy varies by context and setting and is not necessarily related to years of education or general reading ability.

Psychosocial Impacts of Cancer in Children

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It's not hard to imagine that being diagnosed with cancer as a child can be devastating. Children have important developmental tasks to accomplish in order to stay on track to be fully functional adults. Cancer quickly becomes a barrier to virtually all of these normal childhood tasks. In addition, the necessary structure and safety net provided by parents and familiy within which children can grow and thrive is disrupted in multiple ways. All family members are impacted, sometimes with long lasting effects. The large majority of children diagnosed with cancer will now become long-term survivors. An understanding of the ways in which children and families are affected in the psychosocial and emotional aspects if their lives is important if that survival success is to be reflected also in social functioning and quality of life.

Children define themselves to a great extent according to their perceived position and relationships within the family unit. Parents as well derive a large portion of their self-worth assessment from the strength and success of their family relationships. When the family is seen as strong and successful, then members of the family are confident of their roles and feel competent to face life's challenges. When something alters the family structure and function in a way that leads to feelings of fragmentation and insecurity, then all family members begin to doubt their ability to be successful.

Parents have a strong hard-wired biological need to protect their children, and for most parents their worst fear is to be threatened with the loss of a child. The trauma of a cancer diagnosis begins the first time they hear the word "cancer" used to describe their child's illness, because of the strong association with death in our collective experience. Parents often describe a sense that their mind "turns off" after they hear the word, and they feel numb, a strong sense of unreality, denial, confusion, anger and loss of control.

There is no time to deal with these emotional responses, however, because along with the diagnosis comes the need to comprehend and sign a complex consent document and give permission to start the treatment, which often consists of chemical poisons, damaging radiation, and disfiguring surgery. For many families, there is also a need to travel significant distances to a major medical center in order to find the appropriately skilled clinicians to treat the child. This leads to multiple practical challenges for the family to face throughout the treatment course, which can last for years.

One parent (usually the mother) will need to be separated from the rest of the family for extended periods of time to be with the ill child at the treatment center. This parent will feel at the same time trapped in a foreign environment without the usual support systems, and guilty for having abandoned the rest of the family who are at home struggling to maintain their routines. Travel and food expenses for the displaced parent as well as loss of income in two earner households and medical expenses not covered by insurance can rapidly lead to financial hardships.

The other parent is left to fend for him/herself essentially as a single parent, while also feeling the stress of the separation and an overwhelming sense of helplessness trying to comprehend what's going on from a distance and to figure out how to support the parent who is at the treatment center as well as the ill child. Frequent trips back and forth add to the financial burden, and raise the need for child care arrangements for the well siblings who are usually left at home to try to attain some sense of "normalcy" in their lives in spite of the very abnormal experience which the family is going through. The bills still need to be paid, the house still needs to be cleaned, the laundry still needs to be done, the lawn still needs to be mowed, and food still has to appear on the table. Fathers, who often feel that their role is to "fix" any problems that come along for their family, can be overwhelmed by the feeling of loss of control.

The children who have been diagnosed with cancer have essentially been plucked from their former lives and placed in a frightening and fast moving new environment filled with uncertainty and fear. They sense the enormous distress being experienced by their parents while they are also undergoing painful

procedures and being poked and prodded by a seemingly endless line of strangers. They are separated from their peer group and familiar activities and find themselves in a world where they interact almost exclusively with adults. The topics of conversation are not dolls and videogames, but blood counts and methotrexate. They throw up and get mouth sores, and lose a lot of weight. They have major surgical procedures that leave large scars or even rob them of body parts. They lose their hair and may develop changes in skin pigmentation. When they look in the mirror, they don't recognize the face that looks back at them.

For these children, it seems that the whole world has turned its attention to them. People come to visit and make a big fuss over them. People bring presents. Celebrities stop by to say hello in the hospital. More people bring presents. They get cards and letters in the mail. People send flowers and balloons. There are no rules. People give them whatever they ask for. They no longer have to do their chores. They no longer have to say "please" and "thank you" to people. They get more presents.

While it might seem that this would be every child's dream (and certainly most of these young patients take some pleasure in the attention) at a very basic level having all of the traditional rules and expectations disappear leads to a fundamental disquiet and worry for these children. Kids push back against rules and expectations, but in a secure functional family unit the pushing is mostly to define where the hard line is. Kids feel more secure when they know that someone is watching out for them and will define the borders of acceptable behavior in a way that will keep them safe. Children with cancer often more than anything else want to be treated as "normal" kids.

And then there are the well siblings of our young cancer patients. It wasn't that long ago that our approach to the well siblings was summarized in a comment that a mother made in a video called "Siblings Speak Out" that was filmed at Denver Children's Hospital: "They're well, they're healthy, they can take care of themselves for a while." Luckily, we have learned a lot since then, but the well siblings are still the group that gets the least attention in the family unit, and for whom the fewest services are available either at the treatment center or in the community.

For the siblings, the family unit that defines their place in the world is literally turned upside-down overnight. All of the routines, family relationships, habits and behaviors that have molded them and supported them seem to disappear. The family is separated, and one parent and their ill sibling are gone for long periods of time. The other parent is often consumed with work and trying to maintain the household. They are left with neighbors, friends, or relatives sometimes for weeks at a time, or they may be shuttled around to different places every few days. They may have to fend for themselves for long periods, sometimes even at a relatively young age, or they may be expected to take on a significant parenting role for younger siblings in the household.

Siblings feel like they have suddenly become invisible. Everyone they encounter asks them how their ill sibling is doing, but no one asks how they are doing. At school or in other social situations, they become the kids from the "cancer family" and no one knows what to say or what to do. The siblings feel they have been abandoned, that no one loves them, that they are unimportant. They get jealous or angry with their ill sibling, and then they feel guilty for having had those feelings and conclude that they must be a bad person. They often remember a time when they wished that something bad would happen to their ill sibling in the course of an argument or disagreement, and they feel that it is all somehow their fault. They feel like they are different from their peers because nobody understands what they are going through. They feel stressed, anxious, jealous, angry, guilty, depressed, frightened, and alone.

The siblings express their distress in different ways, and depending on their age and development. Young children often begin to show regressive behaviors, such as enuresis when they had been potty trained for some time, or reverting to baby talk. School age children often develop discipline problems at school or their grades drop suddenly. Adolescents are at risk for a variety of potentially dangerous anti-social behaviors such as experimenting with drugs or alcohol, sexual promiscuity, or other high-risk activities.

All members of a family where a child is diagnosed with cancer are impacted in significant ways in the psychological and emotional arena. Even in the best of circumstances, these issues need attention and a plan of care to minimize the damage. Families with significant stressors or a pattern of dysfunction before the diagnosis are at a much higher risk.

We have made considerable progress in improving the outlook for cure for children diagnosed with cancer. These children and their families will survive the experience, but their level of function and quality of life may be limited because of the psychosocial and emotional trauma that they undergo. To minimize the damage and maximize the potential for full recovery to a high functioning and high quality role in life subsequent to the cancer experience, we must fully assess family function and risk factors at the time of diagnosis and develop with the family a plan for adequate attention to psychosocial stressors complete with support services both at the treatment center and in the community.

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Questions to Consider

When a child is diagnosed with cancer, what members of the nuclear family are likely to have clinically significant psychological and emotional issues?

- **A.** The child with cancer
- **B.** The child with cancer, the parents, and the well siblings
- **C.** The child with cancer and the parents
- **D.** The child with cancer and the well siblings

What is the best predictor of the ability of a family to cope with the psychosocial stressors brought on by the diagnosis of cancer in a child?

- **A.** The age of the child at the time of diagnosis
- **B.** The age of the parents at the time of the diagnosis
- **C.** The family functioning prior to the diagnosis
- **D.** The number of children in the family and their school performance

Well siblings of children with cancer express their distress in different ways. What correlates best with the types of behavior they manifest?

- **A.** How old they are at the time of the cancer diagnosis
- **B.** Their level of social skills and school performance
- **C.** How close in age they are to the ill child
- **D.** How far away they live from the hospital where their sibling is treated

Social Adaptation after Cancer

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More than half of all newly diagnosed cancer patients will become long-term survivors (five or more years) and it is estimated that there are over 10 million long-term cancer survivors in the US. Most long-term cancer survivors score well on quality of life instruments and report levels of psychological well being similar to the general population. However, despite these good indicators of well-being, survivors continuously face physical, psychological and social challenges in their daily lives. As their ranks grow, a body of research focused on 'survivorship' has developed which investigates the continuing challenges facing long-term survivors. ***Cancer survivorship can be defined as the experience of "living, with, through or beyond cancer."*** It is essential to recognize 'survivorship' as a distinct phase in the cancer trajectory.

Psychosocial Consequences of Cancer

Patients frequently discuss physical problems with healthcare professionals but only raise psychosocial concerns when specifically prompted. Most investigators have concluded that emotional quality of life and psychosocial adjustment are good for the majority of relapse-free, long-term cancer survivors. For most survivors, the emotional and psychosocial aspects improve during the first two years after treatment, stabilize, and then by five years, reach levels that are comparable with or better than those of individuals who have not had cancer. However, among the survivors who report overall emotional adjustment, there are areas of continuing disruption and difficulties. Understandably, ***fear of recurrence*** is the most commonly reported ongoing emotional problem. Specific events that trigger feelings of vulnerability and worry continuously occur many years after treatment and include: news of other people's cancer, follow-up appointments and follow-up diagnostic tests. Cancer diagnosis and treatment causes tremendous disruption in patient's lives and the extent of these difficulties depends in part on the degree to which life roles, goals and expectations are disturbed. Thus, *age at the time of diagnosis* is a strong predictor of long-term fallout. In general, younger age in survivors of adult cancers is associated with greater ***emotional disturbances*** such as such as depression and anxiety whereas older cancer survivors are more likely to report difficulties associated with ***functional limitations.*** Additionally, younger survivors must contend with infertility and family planning. Other individual characteristics associated with emotional and psychological fallout include prior psychological disorders, unemployment and less economic resources. Financial/practical concerns facing cancer survivors include ***returning to employment, gaining credit or obtaining health insurance.***

In the survivorship literature, ***access to positive social support has consistently been associated with better emotional and psychosocial adjustment***. Virtually every study that examined the impact of social support or perceived access to support reported a significant advantage in emotional recovery. Low levels of social support predicted feelings of helpless-hopelessness and anxious pre-occupation whereas high levels of social support engendered a "fighting spirit" coping. Thus, various levels of social support influence whether long-term survivors employ maladaptive coping versus adaptive coping.

Post Traumatic Growth after Cancer

Although cancer survivors commonly reports symptoms of traumatic stress, they also report personal growth related to their diagnosis and treatment. Positive change in the aftermath of a crisis is not a new phenomenon. ***Post-traumatic growth*** has been defined as the "positive psychological change experienced as a result of the struggle with highly challenging life circumstances". Cancer survivors commonly report growth in 3 specific life domains: *enhanced social resources, enhanced personal resources and improved coping skills*. See Table 1.

Table 1: Commonly Reported Benefits of Cancer	
Social Resources	**Deeper love for family**
	Improved relationships with family and friends
	Increased time and effort invested into relationships
Personal Resources	**Better outlook on life**
	Greater satisfaction with religion
	Increased compassion, sympathy and concern for others
Coping Skills	**Greater acceptance of circumstances**
	Learning to take things as they come
	More effective coping with stress

Predictors of posttraumatic growth include younger age at diagnosis, greater processing of one's emotions, perceived social support and the use of 'positive reframing' or finding something positive in their diagnosis. ***Younger age at diagnosis appears to be the most robust predictor of growth.***

Thriving after Cancer

Resilience and personal growth after suffering a crisis are innate human characteristics. Four possible consequences to an individual's level of functioning occur following a major adverse event and are depicted in Figure 1:

- ***Continued Decline*** – recovery is impossible and death occurs
- ***Survival with Impairment*** – previous level of functioning is never regained or survival with permanent impairment
- ***Resilience*** – recovery to baseline functioning
- ***Thriving*** – recovery beyond the level of functioning; positive changes can include increases in strength and /or psychological growth

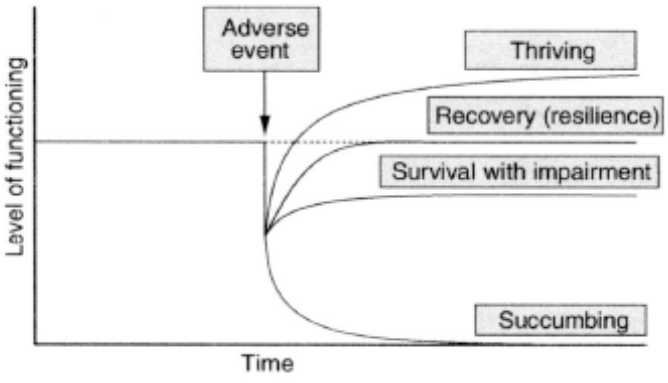

Figure 1. Model of Resilience and Thriving

Coping responses such as *problem-focused coping* and *positive reframing* are important determinants of 'thriving'. Environmental factors that contribute to 'thriving' including social support from family/friends or more formally, from health professionals. In contrast, 'maladaptive behavior' such as 'avoidance coping' and 'disengaging from the struggle' fosters poorer outcomes. Perhaps the most famous example of 'thriving' after cancer is Lance Armstrong, the seven-time winner of the *Tour de France*. In his book, "It's Not About the Bike", he details his personal story of his battle against testicular cancer that included surgeries and chemotherapy, his journey of self-discovery and his period of survivorship which also included his subsequent multiple victories in the *Tour*.

Summary

Clearly, having been treated for cancer continues to affect the lives of survivors even years after treatment has ended. Access to social support is unequivocally the most valuable emotional resource for a patient living with and living after cancer. Other effective means of coping used by survivors include positive thinking helping others, confronting reality and spirituality and confrontive strategies (facing the situation). Cognitive-behavioral stress management also facilitates posttraumatic growth. Providing optimal care for cancer survivors requires an understanding of the psychological, as well as the physical late effects of the disease and treatment.

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Questions to Consider

The most commonly reported ongoing emotional problem after cancer diagnosis and treatment is:

- **A.** Depression
- **B.** Anxiety
- **C.** Fear of recurrence
- **D.** Vulnerability

Effective means of coping used by cancer survivors include all of the following except:

- **A.** Positive reframing
- **B.** Problem-focused coping
- **C.** Social support
- **D.** Avoidance coping

The single most important factor associated with better emotional adjustment related to cancer survivorship is:

- **A.** Optimism
- **B.** Access to positive social support
- **C.** Exercise
- **D.** "Fighting spirit" coping

Sexuality and Cancer

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Introduction

The diagnosis of cancer not only threatens health and life itself but all aspects of the human experience. Sexuality is a broad multidimensional construct with physiological, psychological and social dimensions and dynamic interactions between these dimensions. The diagnosis and treatment of cancer can negatively impact each of these dimensions resulting in altered sexuality and negatively impacting not only the quality of life of the cancer survivor but also the intimate partner(s).

In the hierarchy of human needs, the theorist, Abraham Maslow, recognized sexual activity as a basic human need and placed love and connection to others on a higher tier. All individuals have a life long need for emotional connection to others and are sexual beings regardless of age, sexual orientation, state of health or current relationship status.

Sexuality encompasses our view of ourselves as men or women. Sexuality is expressed in the way we dress, our attitudes, values, roles and relationships. Developmental stage, age, cultural norms, past experiences and the intimate partner(s) all influence sexuality and it's expression. Sexuality is an integration of mind and body and is a vehicle for experiencing and expressing emotions. In order to fully appreciate the affects of cancer and the treatment of cancer on sexuality, one must consider the broad concept of sexuality and not simply sexual activity.

Incidence and Significance

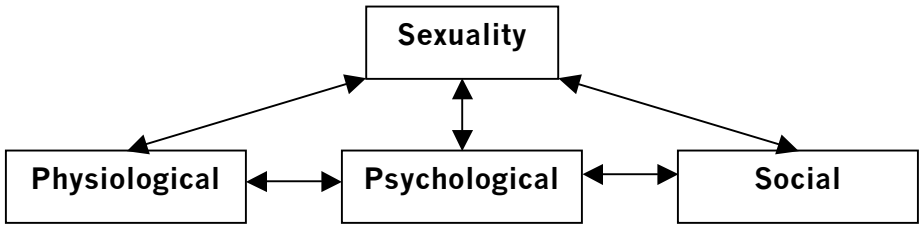
The incidence of altered sexuality in cancer survivors is high, in some reports greater than 90% and often persists for many years following treatment. The incidence and types of alterations vary across diagnoses and therapies. The incidence of sexual dysfunction in female hematopoietic cell transplantation (HCT) survivors ranges from 32-82% and in male HCT survivors the incidence ranges from 23-46% (Syrjala, Kurland, Abrams, Sanders, & Heiman, 2008; Syrjala et al., 1998). Loss of sexual desire was ranked as the sixth most severe side effect of chemotherapy by one group of cancer survivors with a variety of cancer diagnoses (Carelle et al., 2002). In another study, cancer survivors reported that two variables, altered sexuality and family distress, exerted the most negative effect on measures of social well-being (Ferrell & Dow, 1997). A third study reported that 41% of cancer survivors were experiencing sexual dysfunction and in ranking the top 10 overall problems experienced, sexual dysfunction ranked sixth (Baker, Denniston, Smith, & West, 2005).

Etiology

The etiology of altered sexuality may be due to: 1) the cancer, 2) the psychological distress associated with a diagnosis of cancer, 3) the treatment of cancer, with different treatment strategies leading to varied alterations in sexual health, 4) side effects associated with treatment, 5) psychological distress following therapy and 6) alterations in relationships during and following treatment, especially the relationship with the intimate partner(s). For survivors of HCT alterations in sexuality result from the therapy antecedent to transplant, the preparative regimen and the development and treatment of complications associated with HCT. The following table summarizes some of the physiologic, psychological and social dimensions altered by the diagnosis and treatment of cancer.

Alterations in Sexuality for Survivors of HCT	
Physiological Variables	▪ disruption of the neurovasculature of the genitalia ▪ alterations of the hormonal milieu ▪ side effects of treatment (for example, pain, nausea, vomiting, diarrhea, fatigue, skin changes, decreased physical stamina and peripheral neuropathies) ▪ side effects of medications (for example anti-depressants, anti-hypertensives)
Psychological Variables	▪ anxiety ▪ depression ▪ anger ▪ emotional response to infertility ▪ fear of relapse ▪ sense of vulnerability ▪ changes in body image ▪ decreased self-confidence ▪ worries about loved ones ▪ concerns about the reaction of the sexual partner(s)
Social Variables	▪ relationship stress ▪ changes in roles ▪ anxiety, depression and concerns of the sexual partner(s)

Alterations in sexuality and sexual health will rarely be isolated to a single variable, which complicates both the diagnosis and treatment of altered sexual health. The complexity of the interrelationships between physiologic, psychological and social dimensions of sexuality are shown in the following figure.



The Sexual Response Cycle and Diagnosis of Altered Sexual Health

Our understanding of the human sexual response cycle continues to evolve. Kinsey's studies were the first to begin to illuminate the phases of sexual functioning. The work of Masters and Johnson followed defining the sexual response as linear and consisting of four phases; excitement, plateau, orgasm and resolution. In the late 1970's, Kaplan modified the Masters and Johnson model to incorporate the concept of desire and developed a three-stage model consisting of desire, excitement and orgasm. More recent investigations of the female sexual response cycle suggest that these phases are not linear but circular in nature (Basson, 2001).

Disorders of the Sexual Response Cycle Based on the Diagnostic and Statistical Manual of Mental Disorders, 4th edition	
Phases of the Sexual Response Cycle	
Desire	Hypoactive Sexual Desire Disorder
Arousal	Female Sexual Arousal Disorder Male Erectile Disorder (ED)
Orgasm	Orgasmic Disorders
Resolution	Sexual Pain Disorders including dyspareunia and vaginismus

Disorders of the sexual response cycle are characterized by physiologic or psychological changes that negatively influence sexual functioning resulting in psychological distress or stress within relationships (American Psychiatric Association, 2000). The sexual response cycle focuses on sexual activity and provides a standardized framework for assessing and diagnosing sexual dysfunctions but is overly narrow in addressing the broader concept of sexuality.

Assessment and Interventions

Silence on the part of the healthcare professional and the cancer survivor is perhaps the biggest barrier to minimizing altered sexuality. Opening the discussion of altered sexuality during and after treatment combined with education may be the most important interventions that can be initiated by healthcare professionals (Chasle & How, 2003; Gallo-Silver, 2000; McKee & Schover, 2001).

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1. American Psychiatric Association, Task Force on DSM-IV. (2000). *Diagnostic and statistical manual of mental disorders : DSM-IV-TR* (4th , text revision. ed.). Washington, DC: American Psychiatric Association.
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Pelusi, J. (2006). Sexuality and body image. Research on breast cancer survivors documents altered body image and sexuality. Am J Nurs, 106(3 Suppl), 32-38.

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Questions to Consider

The body image scores of cancer survivors generally returns to normal within a few years of diagnosis.

A. true

B. false

The Diagnostic and Statistical Manual of Mental Disorders, 4th edition, characterizes sexual disorders based on physiologic changes that negatively affect sexual functioning.

A. true

B. false

The etiology of erectile dysfunction in cancer survivors is:

A. Secondary to low testosterone levels.

B. Secondary to neurovascular changes to the penis.

C. Secondary to decreased sexual desire.

D. Both a and c

E. All of the above

In several studies altered sexuality and sexual dysfunction did not rank in the top ten concerns of cancer survivors.

A. true

B. false

STANFORD CANCER CENTER

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Palo Alto, CA 94304

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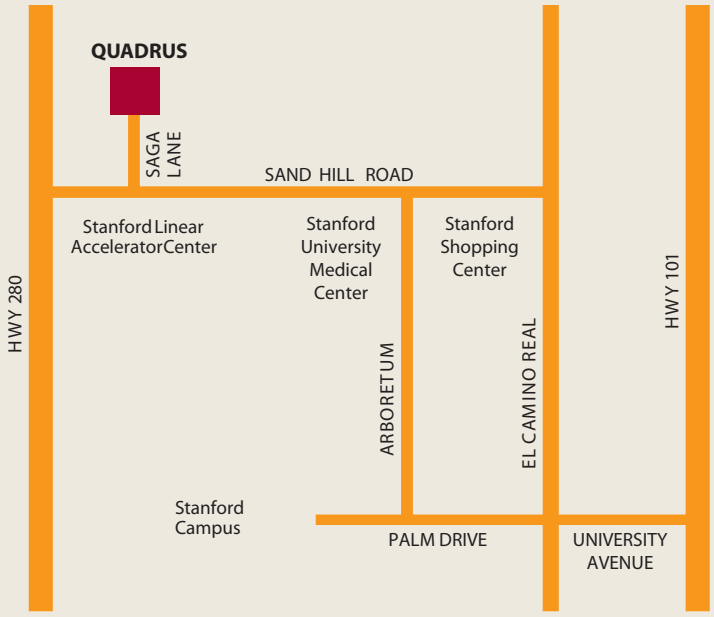
From Palo Alto: From El Camino, turn onto Sand Hill Road. Go west on Sand Hill Road for 2.5 miles, then turn right onto Saga Lane.

From San Francisco: Take 280 south to the Sand Hill Road exit. Go east on Sand Hill Road for 1.5 miles, then turn left at the light at Saga Lane.

From San Jose: Take 280 north to the Sand Hill Road exit. Go east on Sand Hill Road for 1.5 miles, then turn left at the light at Saga Lane.

Parking

Parking for the Quadrus Conference Center is located just down the hill from the Center itself and is marked by low signs along Saga Lane. Very limited parking is available right next to the conference center, for attendees who require closer access. Parking is free; however, space for parking is limited and early arrival is always recommended.



What participants had to say about the 2009 CCRTP . . .

"It was a great week. The quality of speakers and the choice of topics was 'cutting edge' and stimulating."

"The structure of the course, incorporating bench, clinical, and population science presentations in a wide variety of disciplines, mirrored perfectly the translational research model."

"This is a very carefully designed and well-organized course, and I felt lucky to be able to participate."

"This has been the most coherently organized, well-represented, and well-fed course/conference I have ever been to! I am greatly looking forward to returning next year."

"CCRTP was a great reminder that research and hard work can eventually yield effective therapies. It is easy to lose touch of that when bogged down in labwork as a postdoc."