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The Twenty-Second Aspen Cancer Conference: Mechanisms of Toxicity, Carcinogenesis, Cancer Prevention, and Cancer Therapy, 2007

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INTRODUCTION

The 22nd Aspen Cancer Conference on Mechanisms of Toxicity, Carcinogenesis, Cancer Prevention, and Cancer Therapy was held on July 15–18, 2007 in Aspen, Colorado. The goals of the Aspen Cancer Conference are to understand the mechanisms of toxicity and carcinogenesis, to understand and minimize cancer risk, to assess the public-health impact of cancer risk, and to develop the scientific basis for improving the diagnosis, prevention and treatment of toxic, and neoplastic disease. The Aspen Cancer Conference fosters intense interaction, discussion, and collaboration among key stakeholders in cancer research. One of the strengths of the Conference is the sustained participation in and support of the Conference by leaders in cancer research from government, academic, and industry sectors. Because of its unusual qualities, the Aspen Cancer Conference provides an environment that is uniquely conducive to generating novel insights related to current issues in cancer prevention, intervention, and therapy.

The scientific program for the 22nd Aspen Cancer Conference, developed by a Scientific Advisory Committee co-chaired by Benjamin F. Trump (AMC Cancer Center) and Curtis C. Harris (National Cancer Institute, NIH), included eight Conference Sessions, a Poster Session by Aspen Cancer Conference Fellows with a presentation of the Theodore T. Puck Award, a special public Conference Session at the Aspen Institute, and ample opportunity for informal discussion. Session topics included Stromal–Tumor Interactions, Increasing Success Rates of Oncology Drug Development, Tumor Stem Cells, MicroRNA, Background and Induced Mutations, Epigenetics, Novel Targets for Chemotherapy and Imaging in vivo, and Clinical Biomarkers. At the close of the Conference, the Scientific Advisory Committee met to select topics for the 23rd Aspen Cancer Conference. This meeting summary concisely describes the content of each of the Conference Sessions.

SESSION 1: STROMAL–TUMOR INTERACTIONS

Session chair: Stuart Yuspa (National Cancer Institute, Bethesda, Maryland)

Histopathological studies of tumors provide evidence that cancer progression is associated with continuous dynamic changes in tumor–stromal cell interactions and with ongoing changes in

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the tumor microenvironment. Carcinogenesis-associated changes in the tumor microenvironment include increased density of fibroblasts, increased vascularization because of tumor-specific angiogenesis, and increased numbers of invading inflammatory cells. The critical role played by microenvironment in cancer progression is demonstrated by the fact that cancer cells can be “reprogrammed” when they are injected into a wild-type host embryo/blastocyst, and this reprogramming causes them to lose their tumorigenic properties. Furthermore, cancer cells can lay dormant for years before progressing to form visible tumors. It is currently thought that stromal activation plays a significant role in promoting growth of previously dormant cancer cells.

A conceptual framework for tumor stromal interactions includes three key concepts: (1) tumor–stromal cell interactions are dynamic, (2) activated stromal cells have differential effects on tumor and normal cells, and (3) resident or “visiting” stromal cells can have different effects on target tumor cells. Relevant examples include the following: (1) Expression of cyclooxygenase 2 progressively decreases in the stroma and progressively increases in tumor cells as skin lesions progress from benign to squamous cell carcinoma (SCC). Conversely, expression of chloride intracellular channel 4 (CLIC4) is progressively downregulated in colon cancer cells and upregulated in colon cancer-associated stromal cells during colon cancer progression. The differential effects of stromal cells on normal and tumor cells is shown by the fact that an activated stromal cell line LF24 stimulates growth of co-grafted tumorigenic SP-1 cells in a host animal, but does not stimulate growth of co-grafted normal mouse or human keratinocytes. The role of invading inflammatory cells in cancer progression is illustrated in a mouse model for skin carcinogenesis. In this model, selective expression of PKC α in skin epidermis leads to massive invasion of the epidermis by neutrophils, which strongly enhances low dose carcinogen-induced formation of skin papillomas. Lastly, gene expression profiling showed that many immune function genes are strongly downregulated in “high-risk” skin papillomas and SCC, but not in “low-risk” skin papillomas.

The Conference Session on Stromal–Tumor Interactions included presentations by Lisa Coussens (University of California, San Francisco), Thea Tlsty (University of California, San Francisco), and Leland W. K. Chung (Emory University School of Medicine).

Inflammation and Cancer

Lisa Coussens (University of California, San Francisco)—Inflammatory cells and components of the inflammatory response are normal residents of all tissues. However, recent studies suggest that inflammatory cells or signaling factors can strongly promote or suppress cancer progression in multiple tissue and tumor types. In general, T-cell-mediated adaptive immune components are thought to be anti-tumorigenic and the innate immune cell-mediated response is thought to be pro-tumorigenic, but this is far from a clear-cut black and white distinction.

Transgenic mouse models of skin and mammary carcinogenesis have been particularly useful in analyzing the role of inflammatory components in cancer development. Epithelial carcinogenesis is readily studied in a transgenic model in which the human papilloma virus type 16 (HPV16) E6/E7 proteins are expressed from the keratin 14 (K14) promoter. Flow cytometric analysis of skin tumor infiltrates in the transgenic mice showed significant enrichment of specific CD45⁺ immune cell subpopulations at different cancer stages (i.e., hyperplasia, dysplasia, squamous carcinoma). In particular, immune cell infiltrates of hyperplastic, dysplastic, and squamous carcinoma tissues became increasingly enriched for mast cells and immature CD11b⁺GR1⁺ myeloid cells, but not for mature neutrophils, macrophages or T and B lymphocytes. Furthermore, the rate of transgene-induced skin tumor progression and the number of CD45⁺ infiltrating immune cells were dramatically reduced in HPV16/RAG1^{−/−} mice, which are deficient for B and T lymphocytes. This was probably

because of the B cell defect in RAG1^{-/-} mice, because while similar changes were not observed in CD4^{-/-}, CD8^{-/-} T cell deficient mice, immune cell infiltration, and cancer progression was similarly inhibited in B cell-deficient HPV16/JH^{-/-} mice, and in addition, HPV16-induced keratinocyte proliferation and skin cancer progression was restored when “educated” B cells were adoptively transferred back into HPV16/RAG1^{-/-} mice. These data suggest that B cells may play a direct pro-tumorigenic role in HPV16 transgenic mice; however, these data could also indicate that serum factors secreted by “educated” B cells are pro-tumorigenic in this mouse model. This idea was tested by giving HPV16/RAG1^{-/-} mice chronic injections of purified serum from wild-type or HPV16 mice and examining immune cell infiltration and keratinocyte proliferation in 5-month-old-treated animals. In fact, serum from HPV16 mice but not from wild-type mice was sufficient to restore HPV16-induced immune cell infiltration and keratinocyte proliferation in HPV16/RAG1^{-/-} mice. Additional experiments also demonstrated that HPV16-induced skin cancer progression is strongly attenuated when immunoglobulin receptor activation was blocked, suggesting that the putative B cell-derived serum factors that rescue carcinogenesis in HPV16/RAG1^{-/-} mice include immunoglobulins. The nature of these serum factors is under investigation.

In summary, both adaptive immune and innate immune responses strongly influence tumor formation and progression in transgenic mouse models of skin and mammary gland carcinogenesis. However, different immune system components play differential roles in different types of cancer. Additional studies are required to define the roles of different immune system components in different human cancers.

What Is Breast Density and How Does it Contribute to Risk

Thea Tlsty (University of California, San Francisco)—Recent studies suggest that carcinogenesis is the result of a collaboration between the stromal milieu and malignant cells, and that changes in stromal elements may precede cancer onset. One way to examine this phenomenon is to focus on precancerous tissues with higher than normal cancer risk and to try to understand the elements that contribute to the increased cancer risk in these tissues. With regard to breast cancer, it has long been recognized that high mammographic density correlates with a significant increase in breast cancer risk. For example, it has been estimated that a 1% increase in breast density correlates with a 2% increase in cancer risk, and women with the highest observed mammographic density have an approximately 22-fold higher risk for breast cancer than age-matched women with normal mammographic density. Mammographic density is predictive of breast cancer risk as early as 10 yr prior to cancer onset and high mammographic density is observed in up to 44% of breast radiographs.

What biological factors lead to high mammographic density? Molecular studies of breast mastectomy samples showed that high mammographic density correlates with higher expression of collagen A1, increased levels of collagen activation and remodeling, low fat content, and low expression of CD36. CD36 is a class B scavenger receptor and a cell surface marker for adipocyte differentiation, which is involved in cross-talk between thrombospondin 1 and transforming growth factor beta (TGF-β). Because activated TGF-β suppresses expression of CD36, it is possible that high mammographic density also correlates with higher levels of TGF-β, and that TGF-β and thrombospondin 1 are involved in a feedback loop that suppresses expression of CD36, decreases adipocyte differentiation, and increases stromal deposition of collagen. These ideas were tested by conducting in vitro analyses of fibroblasts from human breast tissue with high or low mammographic density. The results showed that exposure to exogenous TGF-β suppresses expression of CD36 to a similar extent in fibroblasts from low density breast tissue (LDAF) or high density breast tissue (HDAF), but that inhibition of TGF-β stimulates CD36 expression to a much greater extent in HDAF than in LDAF. Furthermore, HDAF do not readily differentiate into adipocytes in vitro, while LDAF

differentiate readily into adipocytes in vitro. These data strongly suggest that dysregulation of adipocyte differentiation via altered TGF- β and CD36-mediated cellular signaling may play a role in human breast cancer risk. Increased mammographic density may be a marker for such dysregulation.

β 2-Microglobulin-Mediated Signaling Drives Prostate Cancer Bone Metastasis

Leland Chung (Emory University School of Medicine)—Approximately two-thirds of all cancer deaths per year in the U.S. are attributable to metastasis of the primary cancer to bone. One of the factors that promotes metastasis to bone is “osteomimicry,” the ability of cancer cells to express proteins normally restricted to highly differentiated bone cells. Osteomimicry is particularly pronounced in prostate cancer cell lines and primary and bone metastatic prostate cancer tissues. Many prostate cancer cell lines or prostate tumor tissue samples express osteopontin (OPN), osteonectin (ON) bone sialoprotein (BSP) and/or osteocalcin (OC), and the level of expression of these proteins increases with increasing disease severity/grade, metastatic capacity, and proliferative potential.

To understand the mechanism by which bone proteins are upregulated in prostate cancer cells, conditioned medium from a highly metastatic human prostate cancer cell line, ARCaP (ARCaP CM), was collected and tested for its ability to induce expression of OC and BSP in various human prostate cancer cell lines. The results showed that exposure to 15 μ g/mL ARCaP CM induced expression of OC and BSP fourfold to eightfold in LNCaP and C4-2B human prostate cancer cells, but had no effect on the expression of glyceraldehyde-3-phosphate dehydrogenase in these cell lines. Additional studies identified β 2-microglobulin (β 2-M) as the component of ARCaP CM that activates the OC and BSP promoters and demonstrated that CRE elements in the promoter region in the human OC and BSP promoters are required for ARCaP CM-induced expression of target genes.

β 2-M binds to MHC class I HLA antigens, stimulates proliferation of prostate, breast and myeloma cancer cells, promotes secretion of IL-6, IL-8, and IL-10 by cancer cells, osteoblasts, and dendritic cells, and is secreted by inflammatory, stromal, and epithelial cells. Intracellular β 2-M expression is often low in cancer cells, but higher concentrations of β 2-M are secreted by more malignant prostate cancer cells. Furthermore, C4-2 human prostate cancer cells that overexpress β 2-M expressed higher levels of BSP and OC, had higher capacity for intraosseous growth and formed much larger xenograft tumors in nude mice than control C4-2 cells. Conversely, injection of β 2-M-targeted short-interfering RNA (siRNA) caused regression of xenograft tumors, induced apoptosis of prostate cancer cells, and downregulated expression of the androgen receptor (AR) and prostate-specific antigen (PSA). Antibody to β 2-M also induced apoptosis and downregulated expression of AR and PSA in prostate cancer cells.

It is probably that β 2-M exerts its biological effects via its influence on several major signaling pathways, including p13K-Akt, MAPK, AR and their downstream effectors. Thus, in cancer cells, antibody to β 2-M suppressed the activation of Akt, MAPK and CREB, and activated JNK. Although antibody to β 2-M induced apoptosis in prostate cancer cells, it did not adversely affect survival of normal cells.

Exposure to β 2-M also induced a pattern of gene expression changes in ARCaP cells known as the “epithelial to mesenchymal transition,” which is characterized by lower expression of E-cadherin and cytokeratins, and higher expression of N-cadherin, vimentin and RANK-ligand (RANK-L). After exposure to β 2-M, ARCaP cells have greater mobility and invasiveness, and induce rapid and invasive metastases to bone and adrenal gland, as well as high rates of lethality when injected into nude mice.

It should be emphasized that exposure to exogenous β 2-M or overexpression of endogenous β 2-M had similar effects on the proliferative and metastatic potential of human breast, lung, and renal cancer cells, suggesting that β 2-M has potential as a therapeutic target for multiple cancers. Moreover, the above results suggest that β 2-M could be an especially potent and valuable therapeutic target, because it has pleiotropic biological effects on cancer cells through its effects on RANK-L, AR-, and CREB-mediated signaling pathways, while being relatively innocuous to normal cells.

SESSION 2: TUMOR STEM CELLS

Session Chair: Cheryl Walker (University of Texas, MD Anderson Cancer Center)

The defining property of stem cells is their ability to self-renew, either in an asymmetrical or symmetrical manner. Stem cells are also relatively rare, tend to be sequestered in a protective niche, and are pluripotent. After asymmetrical division, one of the two progeny cells is a pluripotent stem cell with unlimited self-renewal potential, while the other has lower capacity for self-renewal and/or differentiation and usually gives rise to one or more lineages of terminally differentiated cells.

Several techniques are available to identify stem cells. Multiple distinct hematopoietic lineages can be readily identified with cell surface markers and fluorescence-activated cell sorting (FACS). Dye retention capacity also distinguishes stem cells from non-stem cells, and is probably a reflection of increased density of membrane transporters in stem cells. Lastly, stem cells tend to cycle much more slowly than other proliferating cells. Thus, when DNA or protein is pulse-labeled in a heterogeneous population of cells, stem cells retain the label much longer than non-stem cells.

The quantitative and qualitative properties of the stem cell population in a tumor are likely to strongly influence the outcome of chemotherapy. According to the conventional model, drug resistance occurs when a clonal population of drug-resistant tumor cells survives and proliferates, even if a chemotherapeutic agent is cytotoxic to the majority of the tumor cells. In contrast, according to the cancer stem cell model, drug resistance occurs when tumor stem cells are intrinsically drug-resistant or acquire drug resistance. In this case, the drug-resistant tumor stem cells retain an unlimited capacity to replenish the population of proliferating tumor cells. Thus, the only way to eliminate the tumor, is to eliminate the rare tumor stem cells within the tumor.

The Conference Session on Tumor Stem Cells included presentations from Sean Morrison (University of Michigan, Center for Stem Cell Biology) and Kornelia Polyak (Dana Farber Cancer Center, Harvard Medical School).

Neural Crest Stem Cells and Carcinogenesis in NF-1-Deficient Mice

Sean Morrison (University of Michigan, Center for Stem Cell Biology)—The Neurofibromatosis 1 gene (NF1) encodes neurofibromin, a Ras-Gap protein that negatively regulates Ras signaling in mammalian cells. Defects in NF1 cause benign dermal neurofibromas and plexiform neurofibromas, which progress into malignant peripheral nerve sheath tumors (MPNSTs) in humans. In mice, conditional mutation of NF1 in fetal development results in a higher frequency of neurofibromas than conditional mutation of NF1 at later developmental stages, suggesting that early neural progenitors, possibly neural crest stem cells (NCSCs), are the source of neural malignancies in NF1-deficient mice.

NCSCs, which form in the neural crest during fetal development, have the capacity to self-renew and to generate neurons, glia, and myofibroblasts. These differentiated neural cells populate various compartments of the adult peripheral nervous system, functioning as

melanocytes, Schwann cells, and sensory, sympathetic, parasympathetic or enteric neurons, and glia. Expression of the cell surface markers $p75^+ \alpha_4^+$ is diagnostic of NCSCs, allowing them to be isolated by flow cytometry. When purified $p75^+ \alpha_4^+$ rat cells are injected into chick embryos, they differentiate into glia and neurons and populate diverse neural compartments in the chick.

NF1-deficient mice die at E13.5. In early fetal development prior to E13.5, a significant overproliferation of NCSCs was observed in dorsal root ganglia (DRG), sympathetic chain (SC) and sympathetic nerves, but not in the gut of NF1-deficient mice. Clonal analysis of isolated NCSCs from these compartments showed that NF1-deficient NCSCs from overpopulated compartments had a greater self-renewal potential and greater capacity to proliferate in vitro in the presence of low concentrations of growth factors than wild-type NCSCs. This is consistent with the hypothesis that the absence of NF1 might upregulate Ras signaling and increase sensitivity to growth factors. This result also suggested that NF1-deficient NCSCs might persist into later stages of gestation or postnatally, while wild-type NCSCs do not. This could not be tested in NF1-deficient mice, because they die at E13.5. However, this question was addressed in a mouse strain that conditionally deletes NF1 at later stages of development. Interestingly, NCSCs were not observed in DRG, sciatic nerves or SC during E17 to E19 or P30 to P60 after conditional late stage deletion of NF1 in neural lineages. Thus, NF1-deficiency results in transient early fetal overproliferation of NCSCs, but does not produce a detectable late stage cellular phenotype in NCSCs in mice.

Expression of $p16^{\text{Ink4a}}$, $p19^{\text{Arf}}$, and $p21^{\text{Cip1}}$ is higher in NF1-deficient neurons than in wild-type neurons, suggesting that the activation of these tumor suppressors might prevent overgrowth of NCSCs in NF1-deficient mice and prevent tumor formation from NF1-deficient NCSCs in adult animals. This idea was tested by comparing tumor formation in $\text{NF1}^{+/-}$, $\text{NF1}^{+/-} p16^{\text{Ink4a-/-}}$, and $\text{NF1}^{+/-} p16^{\text{Ink4a-/-}} p19^{\text{Arf-/-}}$ mice. The results showed that $\text{NF1}^{+/-} p16^{\text{Ink4a-/-}}$ mice do not develop neurofibromas or MPNSTs but $\text{NF1}^{+/-} p16^{\text{Ink4a-/-}} p19^{\text{Arf-/-}}$ mice develop large numbers of lethal MPNSTs as well as hematopoietic malignancies. Nevertheless, NCSCs do not persist in these animals, and clonal outgrowths from the MPNSTs in tumor suppressor-deficient animals do not resemble outgrowths from NCSCs. Thus, MPNSTs must derive from differentiated NF1-deficient neural cells and not from NF1-deficient NCSCs. These observations provide a clear example of adult stage tumors that do not develop from adult stem cells. However, they do not disprove in a general manner the cancer stem cell hypothesis.

Stem Cells and Cancer in the Breast

Kornelia Polyak (Dana-Farber Cancer Institute, Harvard Medical School)—

Several human breast tumor subtypes, classified as luminal A, luminal B, HER2-positive, and basal-like, have been identified by gene expression profiling. The clonogenic origin of these different breast tumor subtypes is at present not clear, but it is possible that they derive from a bipotential breast stem or progenitor cell or from a luminal or myoepithelial progenitor cell. Commonly expressed luminal cell markers include Muc1, ESA, and cytokeratins 8 and 18, while commonly expressed basal/myoepithelial cell markers include cytokeratins 5, 6, 14, and 17, p63, CD10, SMA, and vimentin.

Recent studies show that individual tumors contain diverse cell subtypes, and these cell subtypes can be fractionated and purified based on specific expression markers. In general, $\text{CD24}^- \text{CD44}^+$ cells are less differentiated and express more stem cell markers, while $\text{CD24}^+ \text{CD44}^-$ cells are more differentiated and express fewer stem cell markers. CD24^+ cells express transporters and genes involved in carbohydrate synthesis and catabolism more abundantly, while CD44^+ cells express genes involved in cell motility, angiogenesis and cell proliferation more abundantly.

SNP analysis suggested that CD24⁺ CD44⁻ and CD24⁻CD44⁺ cells from the same tumor are clonally related, but can be both genetically and epigenetically distinct. For example, CD44⁺ cells have an activated TGFβ-signaling pathway, while TGFβ-signaling specifically decreases in CD24⁺ cells because of the increased TGFβR2 promoter methylation. This is consistent with the observation that CD44⁺ cells respond strongly to an inhibitor of TGFβR, acquire more differentiated epithelial cellular morphology and membrane localization of e-Cadherin and β-catenin, while CD24⁺ cells do not respond to the inhibitor of TGFβR. Furthermore, gene expression profiling identified breast tumors with a higher or lower fraction of CD44⁺ cells and revealed that lymph node-negative breast cancer patients whose tumors were enriched for CD44⁺ cells had shorter overall survival as well as shorter distant metastasis-free survival. Interestingly, CD24⁺ cells appeared to be more prevalent in metastases to liver, bone, or mesenterium, even when the primary breast tumor was enriched for CD44⁺ cells. This suggests that the tumor cells may be altered during the metastatic process, or that CD24⁺ breast cancer cells are intrinsically more competent for metastasis. In summary, these studies demonstrate that cancer cell phenotype is subject to dynamic regulation by genetic and epigenetic mechanisms as well as by the tumor microenvironment. Thus, tumor progression is a dynamic and complex process that is influenced strongly by the intrinsic level of genetic instability in a given tumor at a given time and location.

SESSION 3: INCREASING SUCCESS RATES OF ONCOLOGY DRUG DEVELOPMENT

Session Chair: Cecil Pickett (Biogen Idec)

Sixty-eight oncology and 434 non-oncology drugs were approved by the U.S. Food and Drug Administration (FDA) during 1990–2005. Seventy-nine percent of approved oncology drugs were small molecule compounds and 18% were biologics; however, the fraction of biologics increased to 29% during 2002–2005. A significantly higher fraction of oncology drugs received priority status (i.e., expedited access, orphan drug designation, FDA priority rating) from the U.S. FDA than non-oncology drugs. Despite this, the overall time-to-approval was approximately 1 yr longer for oncology drugs than for non-oncology drugs. Furthermore, transition probability decreased significantly at later stages of clinical testing of oncology drugs and Phase II to Phase III transition probability was significantly lower for oncology drugs than for non-oncology drugs. These observations suggest that better prioritization of drug candidates is needed, especially in Phase II and Phase III stages of drug development and that there is a need for better target selection, target validation and patient recruitment and stratification, and/or selection.

The Conference Session on Increasing Success Rates of Oncology Drug Development included presentations by David Parkinson (Biogen Idec) and Richard Gaynor (Eli Lilly and Company).

New Approaches to Cancer Therapeutics Development: Biologically Driven Drug Development

David Parkinson (Biogen Idec)—Cancer research is at the leading edge of biomedicine and is a major focus of the drug development industry. Oncology drugs represent 15% of all drugs currently in development, followed next by therapeutics for neurological and infectious diseases, each representing 10% of all drugs under development. However, 89% of all oncology drugs are in preclinical stages of development, and most of these will never progress to clinical development stages. In addition to early failure, oncology drugs have a higher late failure rate (i.e., failure at Phase III or beyond) than other types of drugs. Thus, there remains a large unmet need for effective oncology drugs. The failure to produce large numbers of effective oncology drugs may be largely because of the extremely complex biological mechanisms that regulate

cell growth and differentiation, and unanticipated difficulty in understanding and manipulating appropriate targets and/or pathways by pharmacological approaches.

Approaches to developing oncology therapeutics have evolved through several stages in the past 30 yr. Conventional chemotherapeutic approaches based on cytotoxic drug treatments (i.e., cis-platinum, taxane, alkylating agents, topoisomerase inhibitors) were first developed in the 1980s. These drugs are “non-targeted” and relatively non-specific, selectively acting on replicating cells. First generation targeted drugs including protein tyrosine kinase inhibitors (i.e., herceptin, gleevec, erbitux, iressa, rituxan, avastin) were developed in the 1990s, and 2nd generation multi-targeted drugs (i.e., sunitinab, sorafenib, lapatinib etc.) began to be developed around 2000. Novel approaches currently under investigation include inhibition of histone deacetylase (HDAC), Akt/mTOR, or heat shock proteins, and various types of combination therapies. However, in general, drug development strategy is still based on a “single-target,” “magic bullet” type of thinking, even though recent studies show that cells, organisms, and diseases are better analyzed and understood with a holistic, pathway-oriented, systems level approach.

Systems level analysis of cancer biology has demonstrated that dysregulation or dysfunction in key biological pathways is critical to cancer progression. These pathways include apoptosis, cell cycle arrest, DNA repair, senescence, acetylation, adhesion, or growth factor/hormone dependence. One might call this type of biological analysis a “pathophysiological” approach. Thus, if the challenge for the future is “How can we more efficiently generate effective oncology drugs?” ...Then, we might also ask “How can we do systematic drug development with a pathophysiological approach?”

One of the biggest impediments to such an approach is patient characterization, which will be required to stratify patients for prognostic purposes, and to match patients with biologically appropriate therapeutics. This will require assessment of heterogeneity within tumor types and across cell populations as well as an understanding of how tumor progression varies in the context of the individual patient. In other words, oncology research must deal with the reality of biological complexity at the level of the individual patient.

At the clinical level, there are serious pragmatic barriers to overcome as well. These include development and acceptance of novel clinical endpoints or novel clinical trial designs, difficulties recruiting appropriate patients in sufficient number, lack of access to tissue samples, patient privacy issues, and lack of global harmonization.

Oncology research has had several success stories to date, including herceptin for Her2-positive breast cancer, gleevec for GIST, and understanding the role of EGFR mutation in the response to EGFR inhibitors. In addition, failures in oncology drug development can be instructive and can potentially lead to more successful outcomes in the future. Future successes will probably depend on improved target selection and the ability to characterize and monitor individual patients on a physiological level. It may also be necessary to accept conversion to chronic disease as an acceptable outcome of cancer therapeutic interventions.

Specific Targets for New Anticancer Drugs

Richard Gaynor (Eli Lilly and Company)—The phosphoinositide-3-kinase (PI3K)/Akt pathway is a survival pathway that is often activated in human breast, prostate, brain, ovarian, and non-small cell lung cancer (NSCLC). Multiple mechanisms of activation of this pathway have been observed including inactivating mutation of PTEN, amplification or activating mutation of PI3K, and amplification of Akt 1, 2, or 3. Activated Akt stimulates cell growth and proliferation and inhibits apoptosis. Because the genetics of the PI3K/Akt pathway are

well understood, components of this pathway are considered good candidates for oncology therapeutics.

Mutations in Akt are infrequently observed in cancer cell lines, but an E17K-activating mutation was observed in Akt1 at relatively high frequency in primary tumor cells. The E17K mutation lies in the N-terminal pleckstrin homology domain (PHD) of Akt1, which is involved in PIP3 lipid binding and membrane anchoring. E17K Akt1 is constitutively phosphorylated on T308 and to a lesser extent on S473, and is also constitutively bound to the plasma membrane in cultured cells in the presence or absence of exogenous serum. E17K Akt1 also stimulates growth of transduced Rat1 fibroblasts in soft agar and is more leukemogenic than wild-type Akt1 when injected into irradiated mice. These observations demonstrate that E17K is an activating oncogenic variant of Akt1, and suggest that somatic mutation of Akt1 can play a role in human cancer.

It is predicted that inhibitors of Akt1 or other components of the Akt1 pathway will induce apoptosis, reduce phosphorylation of downstream targets of Akt, and suppress growth of Akt-dependent tumors. Recent screens identified two ATP competitive inhibitors of Akt, LY 2-A, and LY 2-C, which are derivatives of an isoquinoline sulfonamide-based scaffold, and which are equally active against Akt 1, 2, and 3. LY 2-A and LY 2-C inhibited Akt1 kinase activity with an IC₅₀ of approximately 20 nM and were equally effective against wild-type and E17K Akt1. LY 2-A and LY 2-C rapidly induced apoptosis in PTEN-proficient and PTEN-deficient tumor cells in vitro, and induced caspase 3 expression and PARP cleavage in a concentration-dependent manner. These drugs also decreased cell viability and strongly enhanced the cytotoxicity of chemotherapeutics such as camptothecin. LY 2-A was effective as a single agent or in combination with TemodarTM in inhibiting growth of U87MG glioblastoma xenograft tumors in vivo.

In summary, the PI3K/Akt pathway has been validated as a target for oncology therapeutics. Rapid development of targeted therapeutics for this pathway is expected in the near future, some of which may be useful in combination with other targeted or traditional chemotherapeutic oncology drugs.

SESSION 4: MICRO RNA

Session Chair: Curtis Harris (National Cancer Institute)

MicroRNAs (miRNAs) are 20–22 nucleotide non-coding RNA molecules transcribed by RNA polymerase II. miRNAs form short stem loop structures that bind to and usually destabilize or inhibit translation of target mRNAs in the cytoplasm. It has been predicted that the human genome encodes more than 1000 miRNAs, but few of the predicted miRNAs have been experimentally verified. However, miRNAs are known to play a role in regulating many biological processes including apoptosis, DNA repair, senescence, cell proliferation, and differentiation. miRNAs also play a role in carcinogenesis: miRNAs that downregulate tumor suppressor genes are procarcinogenic, and miRNAs that downregulate oncogenes are anti-carcinogenic.

The analysis of miRNA function and expression involves miRNA expression profiling, quantification of specific miRNAs, identification of miRNA target genes, and characterization of functional effects on miRNA target genes. Prediction of miRNA expression and/or function by computational analysis is feasible, but not yet extremely reliable.

There is a great deal of interest in defining the role played by miRNAs in cancer, and the possible utility of miRNAs as diagnostic/prognostic tools or therapeutic targets in cancer cells. miRNA expression profiling demonstrated that unique miRNA signatures are associated with

different types of tumors, and that multiple miRNAs are differentially expressed in cancer cells. In some cases, specific miRNA signatures correlate with improved patient survival, and thus, miRNA expression profiles may have potential prognostic value for some cancer patients. The regulation of miRNA expression is complex, and may involve gene amplification and/or deletion, specific cell-signaling pathways (i.e., p53, *c-myc*, hypoxia), and epigenetic mechanisms.

Additional studies of miRNA expression and function will probably lead to novel insights into mechanisms of human carcinogenesis. In the future, it is expected that miRNA profiling may have both diagnostic and prognostic value and that some miRNAs may be valuable therapeutic targets in human cancer patients.

The Conference Session on MicroRNA included presentations by Scott Hammond (University of North Carolina, Chapel Hill), Carlo Croce (Ohio State University), and Scott Lowe (Cold Spring Harbor Laboratory).

Processing and Biology of Micro RNA

Scott Hammond (University of North Carolina, Chapel Hill)—Global analysis of miRNA expression during mouse embryogenesis revealed that miRNAs fall into one of two developmental expression patterns: either they are expressed during early embryonic stages and decline to low or absent expression in later embryonic stages (i.e., miR17-92, miR290–295) or they are absent during early embryonic stages and their expression increases in later embryonic stages (i.e., let-7, miR-125). The latter group of miRNAs are generally restricted to differentiated cell types. This suggests that miRNAs expressed in early embryogenesis may promote and maintain cells in an undifferentiated state, while miRNAs expressed later in embryogenesis may promote or maintain specific pathways of cellular differentiation. Interestingly, cancer cells tend to overexpress miRNAs that belong to the former group and repress miRNAs that belong to the latter group.

miRNA17-92 is a cluster of five miRs co-expressed on a single polycistronic primary transcript encoded by ORF25 on human chromosome 13. In the mouse, miR17-92 is expressed early in development and then declines during development. To determine if miR17-92 promotes cell proliferation and cancer, it was overexpressed in Eμ-*myc* hematopoietic stem cells, which were then injected in recipient mice. The results showed that expression of miR17-92 in this leukemia model strongly decreased overall and leukemia-free survival in recipient mice. These data confirm that miR17-92 is oncogenic when expressed in hematopoietic stem cells in the adult mouse.

The let-7 group of miRNAs are not expressed in early mouse development, but are induced later in development by an unknown mechanism. Let-7 miRs are often repressed in human cancer cells, suggesting that they may target oncogenes and be functionally equivalent to a tumor suppressor. In fact, recent studies indicate that let-7 miRs suppress expression of Ras and other oncogenic proteins. This suggests that re-expression of miRs that down-regulate oncogenic proteins could be a useful therapeutic approach for human cancer.

Previous studies showed that mature let-7 miRNAs are induced at mid-gestation. However, the primary transcript for let-7 is constitutively expressed throughout mouse embryogenesis, even though little or no precursor transcript was detectable at any mouse developmental stage. A similar pattern is observed for other miRNAs that are induced during mid-gestation or later during mouse development. Thus, these miRNAs are not regulated at the level of transcription. One possible explanation for these data is that an inhibitor or activator regulates DROSHA-dependent processing of specific miRNA primary transcripts. This idea was confirmed with an in vitro DROSHA processing assay with extracts from P19 cells, HeLa cells, or mixed

extracts. The results showed that extracts of P19 cells process exogenous miR-17 but not exogenous let-7 g, extracts from HeLa cells process both miR-17 and let-7 g, and that mixed extracts process exogenous miR-17, but not exogenous let-7 g. Thus, the phenotype of P19 cells is dominant, suggesting that p19 cells express a diffusible inhibitor that specifically prevents processing of let-7 g.

A metadata analysis of miRNA and mRNA expression profiles suggested that dysregulation of miRNA processing is common in human cancers. Regardless of the mechanism by which they are dysregulated, it appears that the downregulation of anti-carcinogenic miRNAs and upregulation of oncogenic miRNA may play a role in cancer. Thus, mechanisms that restore normal miRNA expression in cancer cells may be a useful therapeutic approach for human cancer.

The Role of miRNA in Cancer

Carlo Croce (Ohio State University)—Chronic lymphocytic leukemia (CLL) is strongly associated with chromosomal aberrations (CAs), including deletions, and translocations on human chromosome 13. Extensive genetic analysis failed to associate a specific gene coding region with CLL, even when the chromosomal interval had been narrowed to a 34 kb region containing informative translocation breakpoints. However, the CLL-linked region of human chromosome 13 encodes two miRNAs, miR15, and miR16, which could potentially play a role in the etiology of this disease. This idea was consistent with the observations that many miRs in the human genome map to fragile sites or cluster in regions associated with human cancers, and more specifically that miR15 and miR16 are downregulated in more than 60% all CLL cases and in 100% of the indolent (less-aggressive) form of CLL.

Global miRNA expression analysis by microarray showed that human normal and tumor tissues have distinct miRNA expression signatures, in the same manner as human normal and tumor tissues and cancer cell types have distinct global gene expression signatures. Bioinformatic analysis identified 13 miRNAs that were specifically up-or downregulated in CLL, and mutations in some of these miRNAs, including miR15a/16-1, were more frequent in CLL patients than in normal controls.

Bioinformatic analysis predicted that Bcl-2 was a possible target of miR15/16. Bcl2 was confirmed as a target of miR15/16 by comparing Bcl-2 expression in CLL cell lines. This experiment showed that Bcl-2 expression was inversely correlated with miR15/16 expression in a panel of CLL cell lines. In addition, a cell line that did not express miR15 or miR16 expressed a very high level of Bcl-2, which is consistent with Bcl-2 as a target for downregulation by miR15/16. Bcl-2 expression decreased when these cells were transfected with wild-type, but not with the mutant miR15a or miR16-1 precursor. These results suggest that miR15/16 acts as a natural antisense RNA for Bcl-2, and that it is capable of strong downregulation of Bcl-2 expression in vivo.

To test the idea that miRNAs play a direct role in driving carcinogenesis, a transgenic mouse was generated for miR155, a miRNA that is overexpressed in the aggressive form of CLL and in human B cell lymphomas including Burkitt's lymphoma and Hodgkin's lymphomas. The miR155 transgenic mouse, which was engineered to express a high level of miR155 in B cells, displayed a very strong cancer susceptibility, developing an aggressive B cell lymphoma as early as 4 wk and dying by 6–7 months. This result strongly supports the hypothesis that dysregulation of miRNAs can play a significant role in human cancer.

miRNA expression profiling in solid tumor tissues revealed that 21 miRNAs are commonly upregulated in three or more common solid tumor (i.e., breast, lung, colon, pancreas, stomach, and prostate). The most commonly upregulated miRs were miR21, miR17-92, and miR191.

Dysregulation of miR191 is associated with chromosomal translocations in patients with acute lymphocytic or acute myelogenous leukemia (ALL/AML), suggesting that miR191 may play a role in the pathogenesis of this disease. This idea is being tested in transgenic mice that overexpress miR191.

The observation that some miRs are overexpressed in several different solid tumor types suggests that they may be downstream targets of pathways that are activated in cancer. These and other data suggest that these miRs might be excellent targets for oncology therapeutics.

Modulating Cancer Phenotypes With RNA Interference: Characterization of Tumor Suppressor Genes In Vivo

Scott Lowe (Cold Spring Harbor Laboratory)—Re-engineered shRNAs or miRNAs expressed from RNA polymerase II promoters can be used in sophisticated in vivo model systems to manipulate and examine the in vivo effects of tumor suppressors or oncogenes. For example, wild-type or p53-deficient green fluorescent protein (GFP)-tagged fetal hepatoblasts can be engineered to express targeted shRNAs or miRNAs and transplanted into the spleen of recipient mice. The engineered hepatoblasts home to the liver of the recipient mouse, and the chimeric mice develop GFP-labeled liver carcinomas at characteristic rates, depending on the genotype of the transplanted hepatoblasts. In this model, cells that constitutively express p53 shRNA and activated Ras promoted liver carcinomas with essentially the same efficiency as p53-deficient cells expressing activated Ras. With a similar approach, candidate tumor suppressor genes associated with liver cancer can be validated. For example, constitutive expression of shRNAs targeting DLC1, a region on human chromosome 8p that is frequently deleted in liver cancer, enhanced *c-myc*-dependent liver carcinogenesis in the same mouse model.

Novel tumor suppressors, or gene combinations that act as tumor suppressors, can be identified by transfecting hematopoietic stem cells from *Eμ-myc* mice with pools of shRNAs from a comprehensive shRNA library, and transplanting the engineered cells into recipient mice. Multiple “hits,” (i.e., combinations of uncharacterized shRNAs that promote lymphomagenesis) have been identified with this type of screen for novel tumor suppressors. A similar approach was used to identify genes that enhance or decrease susceptibility to chemotherapeutic agents such as doxorubicin or camptothecin. As expected, shRNA-targeted to p53, Chk2, and top2a were identified in this screen, and the expression of shTop2a, which downregulates Top2a, increases the resistance of tumor cells to doxorubicin, but not to camptothecin. In a similar manner, shTop1 increases resistance to camptothecin. However, it was somewhat unexpected that tumor-free survival was greater in doxorubicin-treated mice bearing lymphoma cells expressing shTop1. This result indicates that the downregulation of Top1 sensitizes cells to topoisomerase II-targeted chemotherapeutic drugs.

Conditional p53-targeted shRNAs have also been expressed in a tissue-specific manner in mouse. In these systems, p53-targeted shRNA can be conditionally turned on (or off) in the presence of doxycycline. Thus, p53 expression can be turned on or off in the livers of chimeric mice, and the growth or regression of GFP or luciferase-labeled tumors monitored over time. Similar experiments have been performed in mice expressing a conditional p53-shRNA gene in the germline. When p53-shRNA was specifically induced in B cells, the rate of lymphoma onset was dramatically increased; however, lymphomas regressed when the expression of p53-shRNA was suppressed. These data demonstrate tissue-specific temporally regulated and reversible carcinogenesis in hematopoietic cells of the mouse. In principle, any target tumor suppressor or oncogene could be manipulated in a similar manner.

SESSION 5: BACKGROUND AND INDUCED MUTATIONS

Session Chair: William Farland (Colorado State University, Fort Collins)

To understand carcinogenesis, it is also necessary to understand the basic biological processes that regulate cell growth and genomic stability, and the mechanism(s) by which DNA damage generates mutations. Although many previous analyses emphasize exogenous sources of DNA damage that cause mutations, endogenous agents also damage DNA and generate mutations at a relatively low, but significant basal rate. Endogenous sources of DNA damage include reactive oxygen and nitrogen species, alkylating agents, estrogen, chlorinating compounds, metabolic intermediates, and enzyme-induced errors of normal DNA metabolism. The basal or “background” mutation rate is estimated to be 2×10^{-10} single base substitutions/nucleotide/cell division or 10^{-7} mutations/gene/cell division in cell culture. Although induced mutations are expected to be linearly related to dose, such mutations accrue in addition to basal mutations, and thus the shape of the dose–response curve may be complex.

Genetic risk factors can modulate the response to both endogenous and exogenous sources of DNA damage, and thus, variations in the basal rate of mutagenesis are expected in a population of cells, organisms, or individuals. Such genetic factors might modulate DNA repair capacity, the fidelity of DNA replication, cell proliferation or the processing of endogenous reactive metabolites or exogenous genotoxic carcinogens. Because of these factors, it will be necessary to address two key questions concerning induced and background mutagenesis. First, the relationship (additive, synergistic, or antagonistic) of background and induced mutations must be determined. And second, regarding carcinogenesis, it must be determined whether and when cancer is associated with a mutator phenotype, where the background mutation rate is higher than normal. The answers to these questions will inform our understanding of carcinogenic mechanisms and facilitate more accurate predictions of human risk. Ultimately, this understanding will be important for guiding rational and reasonable regulatory policy decisions that protect human health.

The Conference Session on Background and Induced Mutations included presentations by James Swenberg (University of North Carolina, Chapel Hill), Ron Cannon (National Institute of Environmental Health Sciences), and Martyn Smith (University of California, Berkeley).

Low Dose Mutagenesis and Risk Assessment

James Swenberg (University of North Carolina, Chapel Hill)—Regulatory risk assessment policy in the U.S. usually employs linear extrapolation, which is based on the assumption that linearity pertains at low doses, even if it is only observed experimentally at high doses. This approach leads to conservative assessment of risk, but more importantly, it is subject to a high degree of uncertainty. In many cases, this approach is adopted either because genotoxicity is the presumed mechanism of action (MOA) of a compound of interest, or because information about MOA is unavailable.

The 2005 EPA Guidelines for Carcinogen Risk Assessment permit linear, non-linear or both linear and non-linear extrapolations of cancer risk, and recommend use of linear extrapolation for direct genotoxic mutagens. The Guidelines also suggest using “Framework Analysis” to support or rule out a specific MOA. Framework Analysis involves postulating key events, a dose–response relationship and temporal association of key events, followed by evaluation of strength, consistency and specificity, biological plausibility and coherence, other modes of action, as well as uncertainties, inconsistencies, and data gaps.

A compound is categorized as “genotoxic” if it scores positive in one or more diagnostic in vitro assays (i.e., Ames assay, forward mutation assay, micronucleus assay). For genotoxic compounds, the following key events are proposed: genotoxicity, DNA adducts, mutations in

surrogate genes, mutations in cancer genes, and cancer. DNA adducts are expected to be linear at low doses when the adduct is formed solely from exogenous sources. However, endogenous sources confound this analysis, and can cause non-linearity at low doses.

Mutations in surrogate genes are notably nonlinear at low doses, and the specific dose–response curve for a given agent is driven by the biology of the agent and the biological system: in general, endogenous sources of DNA damage and endogenous DNA repair processes drive low-level mutagenesis (background mutation rate), while exogenous sources of DNA damage drive mutagenesis at higher levels (induced mutation rate). Many *in vivo* dose–response curves have an inflection point, which is the point at which exogenous DNA damage begins to drive mutagenesis. Background rates of mutagenesis at the thymidine kinase (TK) or hypoxanthine-guanine phosphoribosyltransferase (HPRT) loci have been measured many times in many experimental systems. These rates vary and can increase with the amount of time cells are maintained in culture. Furthermore, exposure to sequential low dose (≥ 0.125 μ M MNU and 1.0 μ M EMS) exposures to methylnitrosourea (MNU) and EMS appear to be additive with respect HPRT or TK mutagenesis.

Because most mutagenesis studies are performed at relatively high doses, there is a paucity of low dose mutagenesis data. More low dose mutagenesis experiments are needed to determine “no observed effect levels” for mutations in surrogate genes and in other types of mutagenesis assays. These studies will also help identify the background mutagenesis rate in relevant biological systems. Further studies are needed to infer mutation rates in target cancer genes from mutagenesis rates in surrogate genes, to determine if mutations are or are not linear at low doses in biologically relevant systems, and to determine a Point of Departure for setting acceptable exposure levels to potentially genotoxic compounds.

The Tg.AC Mouse Model: From “Promotional to Mutational”

Ronald Cannon (National Institute of Environmental Health Sciences)—The Tg.AC transgenic mouse model, which carries activated v-Ha-Ras under direction of a ζ -globin promoter, was designed as a model for leukemia. Accordingly, Tg.AC transgenic mice express the transgene in erythrocytes, bone marrow, liver, and spleen, develop dramatically enlarged livers and spleens and demonstrate a high rate of leukemia as adult mice. Tg.AC transgenic mice are also highly susceptible to induced skin papillomas and this strain has been used extensively as a model for chemopromoter-dependent skin carcinogenesis.

During long-term maintenance as a breeding colony, “non-responder” mice that lack susceptibility to skin papillomas appeared in the Tg.AC line. Molecular studies revealed that a palindromic promoter region (PPR) near the transgene insertion site on mouse chromosome 11 was required for ectopic transgene activation and expression, but was absent in transgene-expressing tumors. Furthermore, deletions including the PPR occur spontaneously at a frequency of approximately 3% in the Tg.AC germline, and mice that lose the PPR have a “non-responder” phenotype. One possible explanation for these data is that the PPR facilitates transgene-dependent skin carcinogenesis by a recombination-dependent mechanism that deletes the PPR and activates transgene transcription from an upstream promoter. However, in some cases, deletion of the PPR and the upstream promoter suppresses subsequent transgene activation and produces the “non-responder” phenotype.

To test this idea, the chromosome 11 region into which the Tg.AC transgene had inserted was cloned and characterized. Unexpectedly, this region encodes an ABC transporter protein with three promoters. When the transgene inserted into the 10th intron, exons 11–15 of the *Abc13* gene were deleted, making it functionally null. Expression of *Abc13* protein was undetectable in Tg.AC or “non-responder” homozygotes, but was detectable in heterozygous or wild-type mice. It is currently postulated that *Abc13* plays a role in binding and transport of serum lipids.

These observations are consistent with the phenotype of Tg.AC mice, which shows some defects in fat metabolism.

Mutational and Cytogenetic Biomarkers of Cancer Risk

Martyn Smith (University of California, Berkeley)—CAs in cultured blood lymphocytes are predictive of many types of human cancer. Further, for several types of human leukemia and lymphoma, molecularly characterized translocations are known to play a causal role in disease pathology (i.e., AML, $t(8;21)$; CML, $t(9;22)$; ALL, $t(14;18)$). However, the available techniques for analyzing CAs and translocations are labor intensive and poorly suited to epidemiological studies. The ability to do low or moderate throughput genetic analysis at the single cell level would facilitate analysis of translocations and other CAs, and would represent a great advance for studies on human cancer and other human genetic diseases. One new microfluidics-based approach to single cell genetics involves capturing individual cells in lipid droplets, which also contain PCR reagents. Combined with efficient and sensitive microfluidics and appropriate use of labeled reagents and/or precursors, this approach could rapidly advance the field of single cell genetics, and would be expected to have many applications in the area of cancer research.

Traditional approaches to analyze CAs include metaphase fluorescent in situ hybridization (FISH), interphase FISH, and quantitative real-time PCR (RT-PCR). Recent FISH studies in a benzene-exposed population showed a strong increase in the frequency of $t(8;21)$ in circulating lymphocytes in individuals exposed to >31 ppm benzene ($P < 0.0001$) as well as moderately increased frequency of $t(14;18)$. The frequency of $t(14;18)$, which has a high prevalence in lymphocytes of healthy unexposed individuals, increases with smoking, aging, hepatitis-C infection, benzene exposure and dioxin exposure, and is strongly correlated with follicular lymphoma, suggesting that it might be a useful biomarker for lymphoma. However, quantification of $t(14;18)$ by RT-PCR showed higher incidence of $t(14;18)$ in unexposed controls than in benzene-exposed individuals. Benzene-exposed individuals also had no detectable $t(8;21)$ translocations by RT-PCR. The discrepancy between FISH and RT-PCR data could indicate that $t(14;18)$ - and $t(8;21)$ -bearing lymphocytes are unstable in benzene-exposed individuals that RT-PCR detects a different subset of genetic rearrangements than FISH, or that G_0 lymphocytes (RT-PCR assay) are molecularly distinguishable from the cycling lymphocytes characterized by FISH. However, PCR was successfully used to backtrack from lymphocytes of childhood leukemia patients to birth blood samples (Guthrie cards), demonstrating that specific translocations present at birth are probably key early events in childhood leukemia occurring mostly before birth of the child. This suggests that exposures in utero could be critical in causing childhood leukemia. Biomarkers of in utero chemical exposure are being developed to determine which exposures may be causal factors.

SESSION 6: EPIGENETICS

Session Chair: Webster Cavenee (University of California, San Diego)

Early epigenetic studies demonstrated that methylation of cytosines in CpG islands is a cellular mechanism that reversibly modifies expression of downstream genes. Although many other epigenetic mechanisms have been identified, hypermethylation of CpG islands in the promoters of tumor suppressor genes, or hypomethylation of the promoters of proliferation genes remains a mechanism for dysregulating gene expression in cancer cells.

Several methods have been developed to examine gene-specific or global patterns of DNA methylation. These include use of methylation sensitive restriction enzymes, PCR-based methods, differential hybridization, immunological methods, and DNA chip-based

approaches. With these methods, many hyper-or hypo-methylated genes have been identified in cancer cells.

Histones are subject to numerous epigenetic modifications, including acetylation and mono-, di- and trimethylation on specific lysine residues, chromatin immunoprecipitation (ChIP)-based methods have been used to characterize patterns of histone modification in different chromosome regions, also called “the histone code,” in normal and cancer cells. In many cases, dysregulation of DNA methyl transferases, histone acetyltransferases, HDACs, or histone methyl transferases correlates with changes in the histone code. Recent studies demonstrate that therapeutic approaches that modulate epigenetic mechanisms have potential for treating human cancer.

The Conference Session on Epigenetics included presentations by James Herman (Johns Hopkins University), Steven Belinsky (Lovelace Research Institute), and Dimitri Loukinov (National Institute of Allergy and Infectious Diseases).

Epigenetic Progression of Human Lung Cancer

James Herman (Johns Hopkins University)—Hypermethylation of promoter regions is a common mechanism for silencing tumor suppressor genes in human cancer. The adenomatous polyposis of the colon (APC) gene is one of the best known examples of a gene that is turned off by promoter hypermethylation in cancer. In normal colon cells, the APC promoter region is completely unmethylated, while in cells from most, but not all, adjacent colon tumors, the CpG island in the APC promoter is heavily methylated or the APC gene is mutated. APC promoter methylation correlates with loss of APC expression in colon tumors.

The correlation between tumor suppressor methylation and cancer progression was examined in tissue samples from patients with esophageal cancer, which progresses through three distinct dysplastic stages (ED1, ED2, and ED3/CIS) to squamous cancer. In 119 tumor samples, 8 of 14 tumor suppressor genes (i.e., p16, MGMT, MLH1, APC, BRCA1, RAR β , CDH1, DAP-K etc.) were methylated in more than 10% of the tumor samples, and the fraction of tumors with methylation was higher in later cancer stages. Late stage tumor samples also had more methylated promoters than early stage tumor samples from the same patient. Similarly, p16 methylation increased with increasing disease severity in staged squamous lung cancer.

Progression of adenocarcinoma (AC) in the lung is less well characterized than progression of squamous lung cancer. However, small foci of cellular atypia, known as atypical adenomatous hyperplasia (AAH), occur in a large fraction of patients with primary lung AC and only rarely in non-AC patients, suggesting that AAH may be precursor lesions for AC. This idea was tested by examining methylation-dependent silencing of 18 genes in diverse cellular pathways in normal, AAH, and AC samples with nested methylation-specific PCR (MSP). The results showed that the percentage of tumors with methylation of a specific promoter tended to be higher in AAH than in normal tissue and higher in AC than in AAH. In addition, the mean number of methylated promoters was 0.67, 1.3, 2.6, and 3.72 in normal, low grade AAH, high grade AAH, and AC, respectively, indicating that promoter methylation increases in these tissue types. This supports the hypothesis that AAH is a precursor lesion for AC in the lung.

Similar experiments were conducted with a focus on the Wnt signaling pathway, whose role in lung cancer progression is still unclear. These experiments showed increased methylation of promoters of Wnt pathway antagonists with progression from normal lung, low grade AAH, high grade AAH to lung AC (mean number methylated promoters was 1.6, 1.7, 3.29 and 6.42, respectively). These results suggest that the Wnt pathway may be activated by methylation-dependent silencing of Wnt antagonists as lung AC progresses.

In summary, these results indicate that promoter methylation is relatively common in lung cancer, and affects multiple pathways that regulate cell proliferation. Methylation as a mechanism for gene silencing may be more common in cancer than gene mutation. This may reflect the fact that methylation (and other epigenetic mechanisms) is a common and biologically relevant mechanism for regulating cell growth and differentiation in normal cells, while mutation is not.

Role of the Cytosine DNA-Methyltransferases in Gene Promoter Hypermethylation and Transformation

Steven Belinsky (Lovelace Research Institute)—More than 60 genes have been identified in lung cancer cells that are silenced by promoter hypermethylation. Some promoter methylation events in lung tumors occur in the earliest cancer stages that can be detected histologically. Some studies also suggest that promoter hypermethylation might correlate with DNA damage leading to carcinogenesis. This idea was tested by examining the relationship between p16 promoter methylation, DNA damage, and cancer risk. Initial studies were performed in a plutonium-exposed human population, in which the exposed workers had an approximately fourfold higher risk of lung cancer than the controls. The results indicated that a statistically significant increase in p16 promoter methylation occurred in workers with the highest plutonium exposure dose, and that the frequency of p16 methylation increased with increasing exposure at three different plutonium doses. The percentage of tumors with multiple methylated promoters was also significantly higher in exposed than in unexposed workers.

Molecular studies in cell culture were also performed with two hTERT/CDK4-immortalized bronchial epithelial cell lines, one from a smoker without lung cancer (HBEC1) and one from a smoker with lung cancer (HBEC2). HBEC1 and HBEC2 cells were exposed to very low doses of the direct-acting carcinogens MNU or benzo(a)pyrenediolepoxide (BPDE1) as single agents or in combination for up to 12 wk. Time to transformation, gene specific promoter hypermethylation, and effects on cytosine DNA methyltransferases are being assessed.

Regulation of Demethylation/Activation of Germ/Stem Cell Genes by BORIS

Dmitri Loukinov (National Institute of Allergy and Infectious Diseases)—CTCF and its germ cell-specific paralogue BORIS are 11 zinc-finger transcriptional regulatory proteins that bind to a common set of binding sites in eukaryotic genomes. BORIS appeared in evolution after the duplication of the DNA binding domain of CTCF in mammals. This domain has an identical exon/intron structure in mammalian CTCF and in BORIS, but a different structure in humans and chickens. It is worth noting that the amino acid sequence of the DNA binding domain of human and chicken CTCF is 100% conserved, despite the presence of several new introns, suggesting tremendous evolutionary pressure on the CTCF DNA recognition domain through 300 million yr of evolution. The DNA binding domain of CTCF and BORIS are closely related, but the N- and C-terminal regions of BORIS are unrelated to CTCF. Although they bind to the same DNA target sites, their binding to those sites is expected to have different consequences.

CTCF target sequences are highly diverged. CTCF binding footprints are unusually long, averaging around 50 bp. Binding to such diverged sites is facilitated by alternative zinc-finger usage. Depending on target site sequences, CTCF has diverse regulatory roles, and is capable of functioning as an either activator, repressor, insulator, enhancer, or as a part of a composite hormone-dependent gene silencer. Overexpression of CTCF leads to the arrest of cell proliferation, but is not immediately cytotoxic. Knockout of CTCF results in apoptosis of cells in culture and causes early embryonic lethality in the mouse. CTCF is located at 16q22, a locus showing frequent loss of heterozygosity in human cancer, while BORIS is at 20q13, a locus that is frequently amplified in human cancer. These data suggest that aberrant activation of

BORIS in cancer cells might act as a dominant-negative with respect to CTCF. BORIS, indeed, is frequently activated in a wide spectrum of cancers including, but not limited to breast, prostate, ovary, uterine, gastric, liver, lung, endometrial, glial, colon, and esophagus.

Many tumor suppressor genes are silenced by promoter methylation in human cancer. The promoter regions of 70 genes reported to be silenced by aberrant DNA methylation were analyzed for the presence of CTCF binding sites by EMSA. All selected promoters contained one or more CTCF target site, suggesting that CTCF may protect against aberrant methylation.

CTCF is thought to be a methylation-dependent insulator involved in imprinting control regions. However, it is not true that all CpG-containing CTCF sites are methylation-sensitive. In fact, many CTCF-binding sites that act as repressors of gene expression are methylation-insensitive. Some recently published examples include the promoter regions of cancer testes genes MAGE-A1, NY-ESO, and SPAN-X. Such genes are silenced in normal somatic cells, but are co-expressed with BORIS in germ cells.

For the MAGE-A1 promoter region, CTCF-binding sites are occupied by CTCF in normal somatic cells. CTCF is bound to methylated sequence in heterochromatin. When BORIS was expressed either ectopically or upon 5' azacytidine treatment, it displaced CTCF from its binding site in the MAGE-A1 promoter, resulting in partial demethylation of the promoter and the activation of MAGE-A1 expression. Treatment with BORIS-targeted siRNA before 5'-azacytidine blocks this effect. Similar results were observed for the master germ cell transcriptional regulator, Oct-4. These data suggest that displacement of CTCF by BORIS at target binding sites correlates with the demethylation and derepression of some cancer-testis and of germ cell-specific genes like Oct-4. This effect has been called "site-specific epigenetic reprogramming," and it occurs via a CTCF-to-BORIS switch.

SESSION 7: NOVEL TARGETS FOR CHEMOTHERAPY

Session Chair: Robert Radinsky (Amgen, Inc.)

A significant focus of oncology research is identification of novel cancer-specific targets. Suitable oncology targets must be quantitatively or qualitatively different in cancer cells than in normal cells, or must modulate the interaction between tumor and stromal cells or between the tumor and the immune system. Preferred target pathways include apoptosis, cell signaling, angiogenesis, replication, senescence, metabolism, and cell cycle arrest. Some of the more recently developed modalities target protein degradation, epigenetic mechanisms of gene regulation, and protein acetylation.

The ubiquitin-proteasome-degradation system was recently validated as a suitable oncology target, when the U.S. FDA approved Velcade™ for treatment of multiple myeloma and mantle cell lymphoma. The ubiquitin-proteasome-degradation system regulates the turnover of cellular proteins by a complex multistep pathway involving three enzyme subclasses: E1 ubiquitin activating enzymes activate ubiquitin or ubiquitin-like proteins (UbIs); E2 ubiquitin conjugating proteins form a conjugate with ubiquitin or UbIs; and E3 ubiquitin ligases transfer the activated ubiquitin or Ubl to lysine residues on the target protein. Polyubiquitinated substrates are rapidly unfolded and degraded by the proteasome. Monoubiquitinated proteins can retain normal stability, but have altered functional properties. UbIs, such as Nedd8 and SUMO, have a similar protein fold as ubiquitin and act in functionally similar manner. As a whole, the ubiquitin-proteasome-degradation system offers multiple viable oncogenic targets with different levels of specificity, depending on the chosen target. Some E3 enzymes have very high specificity (i.e., MDM2 is a ring-finger ubiquitin E3 ligase that promotes specific degradation of p53).

The PI3K-Akt-mTor pathway has also recently been developed as an oncology target pathway. For full activation, Akt must be phosphorylated on T308 and S473 and levels of phospho-Akt are increased in many cancer cells. Activated Akt stimulates protein synthesis, transcription, proliferation, and cell survival. PTEN, which negatively regulates this pathway via PI3K, is often mutated in cancer cell lines. Rapamycin downregulates the Akt pathway via direct interaction with mTor. However, prolonged exposure to rapamycin occasionally activates Akt. This pathway also affords multiple oncology targets.

Mammalian HDACs play important roles in chromatin remodeling and the regulation of gene expression. The acetylation of histones relaxes chromatin structure and activates the expression of a specific subset of genes; thus, inhibition of histone deacetylation promotes the expression of these genes, which tend to have proapoptotic and anti-angiogenic effects on target cells. Some non-histone proteins, such as HSP90, are also regulated by acetylation. HSP90 may be a suitable oncology target, because it may stabilize oncogenic proteins. Many HDAC inhibitors are currently being tested in preclinical or clinical trials. An HDAC inhibitor has been approved by the U.S. FDA for treatment of non-Hodgkins lymphoma and has produced both complete and partial responses in patients with this disease.

The Conference Session on Novel Targets for Chemotherapy included presentations by Teresa Soucy (Millenium Pharmaceuticals, Inc.), Phillip Dennis (National Cancer Institute), and Lorraine Gudas (Weill Cornell Medical College).

Beyond Bortezomib: Inhibiting Nedd8-Activating Enzyme as a Novel Anti-Cancer Strategy

Teresa Soucy (Millenium Pharmaceuticals, Inc.)—Nedd8 is a ubiquitin-like protein that participates in a protein degradation subpathway that requires E2 enzyme Ubc12 and cullin-dependent E3 ligases. The Nedd8 pathway regulates the activity of cullin-dependent ubiquitin ligases and thereby modulates the stability of a diverse group of targets including p27, *c-Jun*, I κ B, Hif1 α , and Cdt1. These proteins play important roles in cell proliferation; thus inhibition of the Nedd8 pathway was predicted to be a potentially useful mechanism to inhibit cancer cell proliferation. Nedd8-activating enzyme (NAE), which activates Nedd8 prior to its conjugation to Ubc12, was selected and characterized as a novel oncology target in the Nedd8 pathway. NAE was validated as an oncology target by showing that siRNA-mediated downregulation of NAE β inhibits proliferation and induces apoptosis in multiple cancer cell lines, and that NAE is overexpressed in primary human colon, lung, and ovarian tumors and secondary human liver tumors, but is expressed at a low level in normal human cells. These data validate NAE as an oncology target.

Small molecule inhibitor libraries were screened and inhibitors with a high specificity for NAE were identified. The selected compounds did not inhibit ubiquitin- or SUMO-activating enzymes. The compounds blocked the formation of the Ubc12-Nedd8 and Nedd8/cullin conjugates and stabilized targets of NAE-dependent degradation including Hif1 α , *c-Jun*, and Cdt1 in human HCT116 colon cancer. Similar observations were made in other human cancer cells. NAE inhibitors and NAE-targeted siRNA also altered cell cycle progression and caused cells with high DNA content to accumulate. This was probably because of the stabilization of the replication licensing factor Cdt1 which must be degraded during late S phase to prevent DNA re-replication. Previous studies showed that DNA re-replication because of the overexpression of Cdt1 activates a DNA damage response and cell cycle arrest. Consistent with these observations, NAE inhibitors also induced cell cycle arrest and a DNA damage response.

NAE inhibitors were characterized in vivo in several mouse xenograft models. Initial pharmacodynamic (PD) experiments showed time and dose-dependent inhibition of the Nedd8 pathway in HCT116 xenograft tumors following the administration of a single dose of NAE

inhibitor. Furthermore, repeated dosing at 30 or 60 mg/kg once or twice daily strongly inhibited growth of HCT116 xenograft tumors. Similar results were observed with OCI-Ly10 xenograft tumors, which require the activation of the NF- κ B pathway for survival and growth.

In summary, these results demonstrate that the Nedd8 pathway is a suitable oncology target. The inhibition of NAE leads to re-replication, cell cycle arrest, and/or apoptosis in human cancer cells. Efficacy studies showed that chronic dosing with NAE inhibitors inhibits the growth of HCT116 and OCI-Ly10 xenograft tumors in nude mice. Additional preclinical and clinical studies are needed to evaluate the potential of NAE inhibition as a therapeutic approach for human cancer.

AKT and mTOR as Molecular Targets in Lung Tumorigenesis

Phillip Dennis (National Cancer Institute)—Lung cancer is the leading cause of cancer death in the United States, and is expected to kill more than 161 000 Americans in 2007. Tobacco use is associated with a risk of lung cancer and accounts for approximately 30% of all cancer deaths. NSCLC, for which no successful therapy is available, accounts for the majority of lung cancer cases (87%).

Akt/mTOR is activated in many human cancers including breast, prostate, ovarian, cervical, colorectal, bladder, endometrial, and brain cancer. The Akt pathway is also activated by multiple molecular mechanisms including the mutation or epigenetic silencing of PTEN, amplification or mutation of PI3K, amplification of Akt1, 2 or 3, as well as by alterations in Ras or EGFR signaling. Experimental mouse transgenic models demonstrate that the overexpression of Akt or underexpression of PTEN increases susceptibility to brain, skin, prostate, ovary, uterine, adrenal, hematopoietic, and mammary gland tumors. Thus, targeted inhibition of the Akt/mTOR pathway has been suggested as a potential therapeutic approach for lung and other human cancers.

Recent studies explored whether phospho-Akt is a suitable biomarker for lung cancer risk. High levels of S473 Akt phosphorylation have been observed in multiple human tumors. However, full activation of Akt requires both S473 and T308 phosphorylation. Tissue microarray studies showed that S473 T308 phosphorylated Akt could be used as a marker to successfully discriminate cancerous lung tissue from adjacent normal lung tissue; singly phosphorylated Akt could also be used, but provided a lower level of discrimination. Furthermore, T308 phosphorylation of Akt was highly predictive of a poor prognosis and was often present in early cancer stages (i.e., dysplastic lesions).

The A/J mouse model of 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK)-induced carcinogenesis was used to evaluate rapamycin as a therapeutic approach for lung cancer. These studies showed that continuous dosing with 1.5 mg/kg rapamycin every other day decreased NNK-induced tumor multiplicity by 90% and decreased NNK-induced tumor volume by 80%. Other studies showed similar efficacy of mTor inhibitors in treating mutant k-Ras-dependent lung cancer, activated Akt-dependent prostate cancer and Her2/VEGF-dependent breast cancer.

Despite promising results in using inhibitors of Akt/mTOR as oncology therapeutics, there have been concerns over whether rapamycin, the best characterized inhibitor of the Akt/mTOR pathway, might have adverse immunomodulatory effects in human patients. This concern stems primarily from two studies reviewed by the U.S. FDA, which suggested that rapamycin is T_H2 polarizing and increases the number of Foxp3⁺T-regulatory cells (Tregs). These assertions were tested with the mouse model of NNK-induced lung cancer described above. Contrary to previous studies, the present study showed that rapamycin did not deplete B cells, granulocytes or macrophages, produced a 50% decrease in splenic T cells, no change in natural

killer cells and a 30% decrease in lung tumor-associated Foxp3⁺ Tregs, and no change in T_H1-T_H2 balance. These mouse studies suggest that rapamycin is very modestly immunosuppressive, and indicate that inhibitors of Akt/mTOR may be safe and clinically useful for the treatment of human cancers, including NSCLC.

Retinoids, Stem Cells, and Transcriptional Regulation

Lorraine Gudas (Weill Cornell Medical College)—Retinoids such as retinoic acid (RA) are signaling molecules that regulate cell proliferation and differentiation. Retinoids have been used clinically to treat skin diseases and emphysema and to prevent and treat some types of cancer. Novel clinical uses for retinoids are under investigation, such as use of retinoids to treat disorders of memory and learning and diabetes.

There are three RA receptors (RARs): RAR α , RAR β , and RAR γ . RAR β negatively regulates cell proliferation and RAR α and RAR γ regulate cellular differentiation. ChIP analysis showed that RAR γ is constitutively bound to the RA response element (RARE) in the 3'-flanking DNA of homeobox gene *h1* (*Hoxa1*), but is not constitutively bound to the proximal promoter of *Hox1a*. Although RAR γ binds to the *Hoxa1* RARE in the absence of RA, RA is required to recruit coactivators p300 and pCIP to the RAR γ /RARE complex.

HOX genes are positively regulated by RA in embryonic stem cells and negatively regulated by polycomb group (PcG) proteins, which form two distinct PcG repressive complexes, PRC1 and PRC2. PRC2 contains Suz12 and the histone methyltransferase EZH2. ChIP analysis showed that Suz12/PRC2 binds constitutively to the *Hoxa1* RARE and the *Hoxa1* proximal promoter, and that RA dissociates Suz12 from both binding sites. Furthermore, Suz12/PRC2 was also bound to RAREs in *Cyp26* and RAR β promoters in the absence, but not the presence of RA. Although the mechanisms by which PcGs are recruited to RAREs and are released from RAREs by RA are not known, these observations suggest that RA and PcGs may cooperatively regulate expression of RA target genes in stem cells and possibly other types of cells.

RA negatively regulates expression of the transcription factor Rex1 (zfp42), a zinc-finger protein that is overexpressed in human and mouse stem cells. Rex1 is also expressed in normal human kidney cells, but is absent from most renal clear cell carcinoma cells and other human cancer cells. The functional role of Rex1 is not understood. However, yeast two hybrid data suggest that Rex1 interacts with a component of PRC2. Thus, loss of Rex1 expression in renal carcinoma or other cancer cells might lead to dysregulation of PRC2 and its target genes.

Human kidney tumors are often vitamin A-deficient and lack lecithin:retinol acyl transferase (LRAT), which is required for uptake and esterification/storage of retinol. Very low levels of LRAT mRNA and/or protein have been observed in human breast and bladder tumors. Interestingly, LRAT and RAR β can be induced by RA in normal human cells but not in most human tumor cells. One possible explanation for these observations is that the promoters of LRAT and/or RAR β undergo epigenetic silencing in tumor cells. This idea was tested by treating RA-resistant human kidney cancer cells with the HDAC inhibitor trichostatin A (TSA). The results showed that the level of RAR β mRNA increased significantly in human kidney cancer cells treated with TSA or TSA/RA. In addition, although TSA alone inhibited proliferation of kidney cancer cells, strong synergistic inhibition of cell proliferation was observed in cells treated with TSA/RA. Liposomal RA plus TSA also inhibited growth of human SK-39 kidney cancer cell xenografts in nude mice, but liposomal RA alone did not. These results suggest that combined therapy with retinoids and HDAC inhibitors may be clinically useful for treating human kidney cancer and possibly other human cancers.

SESSION 8: IN VIVO IMAGING AND CLINICAL BIOMARKERS

Session Chair: Alexander Wood (Novartis Institutes for Biomedical Research, Cambridge)

Drug discovery and development is a challenging process with a long time frame and relatively high attrition rate. New tools, including imaging tools, are needed to improve and expedite/accelerate successful drug development. Imaging tools might be particularly useful in preclinical stages for target validation, PD/PK studies, toxicity and safety testing, and in clinical stages for PD/PK studies, efficacy testing, and patient stratification. Well established imaging modalities with both clinical and preclinical applications include magnetic resonance imaging (MRI), X-ray, XR-CT, ultrasound, positron emission tomography (PET) and single photon emission computed tomography (SPECT). New preclinical modalities include optical methods and mass spectroscopic imaging. These methods have been used to visualize many biological processes and events, including cell proliferation, cell death, vascular permeability, blood flow and hypoxia, as well as specific metabolic events such as uptake or metabolism of glucose.

Imaging modalities clearly have unique strengths and weaknesses; thus, one method may be much more appropriate than another for addressing a specific biological question. PET imaging is useful for assessing metabolic activity in vivo, but it requires use of radiolabeled tracers, and different tracers are more or less useful in different cell types and different tumors. Dynamic contrast enhanced MRI is useful for assessing vascularization, blood flow, and the efficacy of anti-angiogenic agents. For pharmacokinetic (PK) studies, PET is more sensitive than MRI/MRS, but MRI/MRS can be used to follow metabolites, while PET cannot. Nevertheless, when a compound can be analyzed by MRI/MRS (which is true for relatively few oncology drugs), MRI/MRS provides high spatial resolution, which is not possible with PET. When possible, it is best to combine more than one modality, so that the strengths and weaknesses of one method are complemented by the strengths and weaknesses of a second method. This approach can yield high quality functional and structural data about diverse biological systems.

The Conference Session on In Vivo Imaging and Clinical Biomarkers included one presentation by David Piwnica-Worms (Washington University, St. Louis).

Real-time Imaging of Signal Transduction and Targeted Drug Action in Living Animals

David Piwnica-Worms (Washington University, St. Louis)—The tools of molecular imaging can provide spatially and temporally resolved information on biological structures and functions. Because imaging data are obtained non-invasively, scientists can use imaging modalities to interrogate protein and gene expression or flux through metabolic pathways in real-time in living cells and/or organisms. Thus, molecular imaging has become an important tool for biomedical scientists in clinical and basic research settings. Because researchers have increasingly recognized the importance of regulatory mechanisms and emphasize more and more the context of gene and protein expression as well as environmental cues and modulators, they have turned to imaging technologies to advance research on human health and disease.

Biological imaging requires an endogenous gene or exogenous transgene and/or their protein products as signal sources and an imaging probe. Imaging probes can be injectable agents such as radiopharmaceuticals or labeled proteins or they can be produced by genetically encoded reporter genes such as GFP or luciferase. Highly sophisticated imaging systems have been generated to examine specific biological questions with transgenic mice that conditionally express imaging probes or signals. These systems are, at present, used primarily in research applications.

Firefly luciferase (FLuc) has been used extensively to carry out bioluminescence-based real-time molecular imaging of many biological processes. In recent studies, a modified yeast two hybrid assay was developed, so that bioluminescence could be used to monitor and characterize

specific protein–protein interactions in vivo in real-time. In a simple version of this system, the firefly luciferase reporter gene (FLuc) under control of the Gal4 promoter ($5 \times \text{Gal4:FLuc}$) is actively transcribed only when a Gal4-BD/Cdc25c fusion protein interacts with a VP16/14-3-3 ϵ fusion protein. To use this system in living mice, $5 \times \text{Gal4:FLuc}$ transgenic mice are injected with a bicistronic DNA cassette that encodes the two interacting fusion proteins. When delivered by hydrodynamic tail vein injection, the DNA cassette integrates into hepatocytes in the injected animal. This allows rapid screening of different protein fusion partner pairs to assess their ability to interact and promote bioluminescence in vivo.

The FLuc system can also be used to directly monitor protein–protein interactions by using a split-enzyme approach known as “luciferase complementation imaging.” In this system, N- and C-terminal inactive fragments of FLuc are fused to putative protein interacting domains of other proteins of interest. When the two fusion proteins interact, the two inactive luciferase fragments “complement” each other, and regain their ability to produce light. Initial screens showed that the optimal N-terminal luciferase fragment is aa 2–416, and the optimal C-terminal luciferase fragment is aa 398–550. Luciferase complementation has been used to characterize the rapamycin-dependent interaction between FKBP12 and the FKBP rapamycin-binding domain of mTOR (FRB). In cultured cells, luciferase activity is rapamycin-dependent and does not occur when mutant S2035I FRB-NLuc is substituted for wild-type FRB-NLuc. This is consistent with the fact that the S2035I mutation destroys the rapamycin-binding pocket in FRB. When cells expressing FRB-NLuc and CLuc-FKB12 are examined in vivo, this system can be used to generate PK/PD data on rapamycin or novel rapamycin-like compounds.

Luciferase fusion proteins were also used to interrogate the NF- κ B pathway. In this system, a reporter construct was designed to generate a fusion protein in which an intact I κ B domain is separated from an intact luciferase domain by a short protein linker. When co-expressed in cells with endogenous I κ B, I κ B, and the I κ B-luciferase fusion protein were phosphorylated and subsequently degraded with similar kinetics. Thus, bioluminescence of the I κ B-luciferase fusion protein could be used to monitor the activation or suppression of the NF- κ B pathway in vivo. Similar approaches have been used to interrogate Wnt- β -catenin interactions and activation/suppression of Wnt pathway signaling in vivo.

These imaging systems are particularly valuable because they provide real-time readout of biological processes in living cells and animal model systems. More sophisticated systems provide highly detailed spatially and temporally resolved data on specific protein–protein interactions and/or metabolic processes. These approaches are increasingly useful in understanding human metabolism and the pathological features of human diseases including cancer, and can also facilitate the development of effective therapies for clinically important human diseases.

ASPEN CANCER CONFERENCE 2007 SPECIAL SESSION

The Aspen Cancer Conference held a special session on Monday July 16, 2007 at the Given Institute. This session was open to the public and was intended as a community outreach event. The session featured three short lectures and a panel discussion, followed by a reception and silent auction. Raymond DuBois (University of Texas, MD Anderson Cancer Center) provided an overview of “Cancer Prevention: What the Public Needs to Know.” David Parkinson (Biogen Idec) discussed “The Future of Cancer Therapy: Applying the New Cancer Biology Towards Better Treatment,” and H. M. (Bob) Pinedo (VUmc Cancer Center Amsterdam) described “New Advances in Therapy for Colorectal Cancer.” DuBois, Parkinson, and Pinedo were then joined by Ira S. Jaffrey, an oncologist from Glenwood Springs, Colorado, for a panel discussion. The panelists addressed questions from the audience on topics related to cancer susceptibility, diagnosis, and treatment.

PRESENTATION OF THE THEODORE T. PUCK AWARD

The 2007 Theodore T. Puck Award was presented to Mona Gauthier (University of California, San Francisco) for her excellent poster presentation entitled “COX-2 identifies two populations in DCIS with differential risk for subsequent disease.”

Abbreviation

HPV16	human papilloma virus type 16
TGF-β	transforming growth factor β
BSP	bone sialoprotein
OC	osteocalcin
ARCaP CM	conditioned medium from ARCaP cells
β2-M	β 2-microglobulin
siRNA	short-interfering RNA
AR	androgen receptor
NF1	neurofibromatosis 1 gene
MPNSTs	malignant peripheral nerve sheet tumors
NCSCs	neural crest stem cells
U.S. FDA	US Food and Drug Administration
HDAC	histone deacetylase
PI3K	phosphoinositide-3-kinase
NSCLC	non-small cell lung carcinoma
miRNA	microRNA
CLL	chronic lymphocytic leukemia

CAs	chromosomal aberrations
GFP	green fluorescent protein
MOA	mechanism of action
MNU	methylnitrosourea
PPR	palindromic promoter region
FISH	fluorescent in situ hybridization
RT-PCR	real-time PCR
ChIP	chromatin immunoprecipitation
APC	adenomatous polyposis of the colon
AC	adenocarcinoma
AAH	atypical adenomatous hyperplasia
NAE	Nedd8-activating enzyme
PD/PK	pharmacodynamic pharmacokinetic
NNK	4-(<i>N</i> -nitrosome-thylamino)-1-(3-pyridyl)-1-butanone
RA	retinoic acid
RAR	retinoic acid receptor
RARE	retinoic acid response element
PcG	polycomb group
LRAT	lecithin: retinol acyl transferase
TSA	trichostatin A

MRI

magnetic resonance imaging

PET

positron emission tomography

FLuc

firefly luciferase

FRB

FKBP rapamycin-binding domain of mTOR