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CENTER FOR CANCER RESEARCH
frontiers
IN SCIENCE

ANNOUNCEMENT

Moving Ahead to a New Communications Frontier

It is with sincere gratitude to the CCR research community for the rich content you have provided *Frontiers* over the past 5 years that I now announce its termination. Although this will be the last issue of *Frontiers,* it will not be your last opportunity to share your findings with others. In fact, we hope to expand the size and composition of your audience.

Beginning in late June, we will roll out a new magazine called *CCR Connections,* which will once again provide a platform to showcase your work. It will also report your awards, important journal articles, successes in the clinic, and more. In addition to sharing your results with fellow scientists, we will also reach out to interested readers in academe, industry, government, and the public sector. And we will proactively distribute this new publication to CCR alumni and to other key stakeholders who support the intramural research program at NCI.

I ask for your support for *CCR Connections* as it rolls off the presses to report our progress and demonstrate our scientific connectivity to a broader audience.

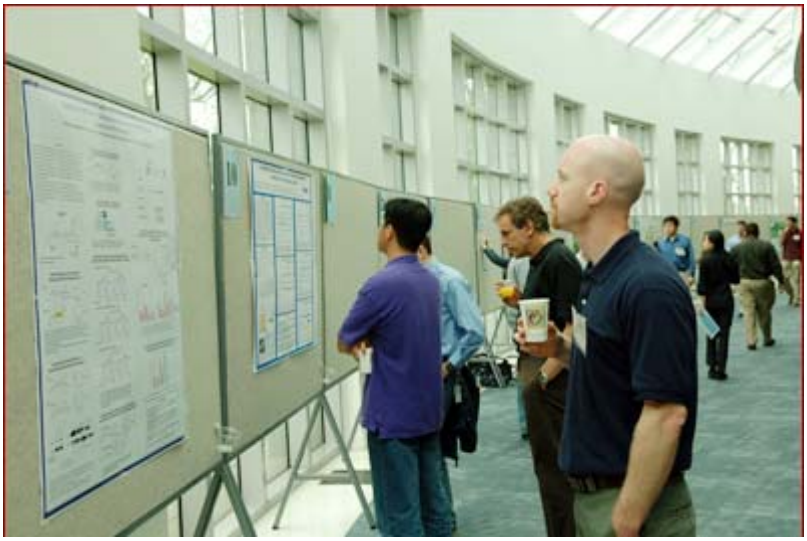
FROM THE DIRECTOR

Cancer and Chromosomes: The 2007 NCI Symposium on Chromosome Biology

Chromosomes have historically been at the center of cancer biology. Chromosomal mutations, such as translocations, deletions, duplications, and aneuploidy, have long been implicated in certain cancers. The NCI has a strong and proud tradition of cutting-edge, innovative research in this field, and NCI scientists have made key

contributions to the elucidation of basic mechanisms in chromosome biology and in the application of these findings to diagnosis and therapy. In recognition of its strength in this area, the CCR has recently established the Center of Excellence in Chromosome Biology (CECB, http://ccr.cancer.gov/initiatives/CECB). Its goals are to integrate the CCR's intellectual and physical resources to promote and lead new initiatives, projects, and collaborations with intramural and extramural scientists from various disciplines to achieve a comprehensive understanding of the mechanisms involved in chromosome biology and to accelerate the translation of laboratory findings into diagnostic and therapeutic applications for patients. As part of these efforts, the CECB organized the NCI Symposium on Chromosome Biology, held on April 26 and 27 in the Natcher Conference Center on the Bethesda campus. This meeting, chaired by Tom Misteli, PhD, of CCR's Laboratory of Receptor Biology and Gene Expression, was highly attended by researchers from around the country and featured outstanding presentations from CCR investigators and leaders in chromosome research from the extramural community.

NCI Director John E. Niederhuber, MD, opened the symposium, expressing his enthusiasm for the meeting, the importance of the work being done, and his thanks to the presenters and organizers. I followed with a brief description of the CCR, including the CECB, explaining how the CCR overall is an integral part of the NCI and elucidating our mission of informing and empowering the entire cancer research community by making breakthrough discoveries in basic and clinical cancer research and by developing them into novel therapeutic interventions for adults and children afflicted with cancer or infected with HIV.



The symposium spanned a wide range of topics, from basic gene control to diagnostic applications of chromosome analysis relevant to cancer. In the first session, the importance of understanding basic genome regulation via biochemical, cell biological, and structural studies was emphasized. Robert Roeder, PhD, of Rockefeller University, described his ongoing pioneering biochemical studies of transcription factors, and Robert Tjian, PhD, of the Howard Hughes Medical Institute at the University of California, Berkeley, emphasized the need for extending these studies into physiological systems, such as differentiation and development. Mikhail Kashlev, PhD, of CCR's Gene Regulation and Chromosome Biology Laboratory, discussed his advances in understanding the mechanisms controlling the fidelity of transcription by RNA polymerase II. Carl Wu, PhD, and Yawen Bai, PhD, both of CCR's Laboratory of Biochemistry and Molecular Biology, highlighted the power of combined biochemical and high-resolution structural studies, which they used to

elucidate the molecular mechanism of histone incorporation into the nucleosome. David L. Levens, MD, PhD, of the CCR Laboratory of Pathology, summarized his efforts to uncover the interplay between conventional transcription factors, dynamic supercoils, and DNA topology-sensing proteins in the control of c-*Myc* expression levels. Gordon Hager, PhD, of the CCR Laboratory of Receptor Biology and Gene Expression, discussed the tremendous contribution cellular imaging methods have made recently to the field, making it possible for the first time to probe the dynamic properties of chromatin proteins in living cells.

One of the most fascinating and important areas in chromosome biology has been the emergence of epigenetics, the theme of the second session. Yi Zhang, PhD, of the University of North Carolina at Chapel Hill, highlighted his work in understanding the activity of demethylases and their biological significance. Shiv Grewal, PhD, of CCR's Laboratory of Biochemistry and Molecular Biology, described the recent progress he has made in elucidating the role of RNAi- and heterochromatin-mediated epigenetic control of the genome. Susan Gottesman, PhD, who works in the Laboratory of Molecular Biology at the CCR, discussed the mechanism of action and several regulatory outcomes of small RNAs in bacteria, and Carlo Croce, MD, of Ohio State University, emphasized the benefits of the microRNA expression profiling of human tumors.

The third session focused on cellular organization of gene expression. David Spector, PhD, from Cold Spring Harbor Laboratory, discussed his work in understanding the dynamics of a certain class of proteins essential in epigenetic silencing mechanisms—called polycomb group proteins—and how they contribute to the inherited epigenetic state of a gene. Steven Kosak, PhD, of Fred Hutchinson Cancer Research Center, described the progress he has made in determining whether gene regulation is related to a general pattern of chromosome organization, and Jeannie Lee, MD, PhD, of the Howard Hughes Medical Institute at Harvard Medical School, spoke about the studies she is performing to better understand the process of X-chromosome inactivation.

DNA damage is a leading cause of tumor formation. The fourth session examined factors leading to such damage and several mechanisms of repair. Frederick Alt, PhD, of the Howard Hughes Medical Institute at Children's Hospital in Boston, described his study comparing classical nonhomologous end-joining and an alternative nonclassical pathway in the repair of DNA double-strand breaks. André Nussenzweig, PhD, of CCR's Experimental Immunology Branch, discussed his work in understanding the maintenance of genomic stability in lymphocytes. Dr. Misteli examined the role of genome spatial organization in the formation of chromosomal translocations, and Geneviève Almouzni, PhD, from Institut Curie, discussed the efforts she is making to better understand the function of chromatin assembly factors *in vivo* and also in connection with replication, repair, and control of histone pools. Michael Bustin, PhD, of CCR's Laboratory of Metabolism, described recent findings regarding the cellular response to DNA damage.

In the last session, which focused on genomic instability, Titia de Lange,

PhD, of Rockefeller University, highlighted some of her recent findings on the molecular mechanisms by which human and mouse telomeres hide chromosome ends from the DNA-damage response. Thea Tlsty, PhD, of the University of California, San Francisco, discussed the importance of understanding the earliest molecular changes in cancer formation. David Pellman, MD, of Dana-Farber Cancer Institute, described mechanisms by which polyploidy might compromise genetic instability, and Thomas Ried, MD, of CCR's Genetics Branch, discussed his investigations of the relationship between chromosomal aneuploidy, nuclear structure, and gene expression in cancer.

The symposium, which also included a poster session and reception sponsored by the Foundation for the National Institutes of Health, was a tremendous success. As was clear during the event, the teamwork that the CECB makes possible and the leadership it provides are helping many intramural and extramural scientists make great strides in identifying the role of chromosome biology in cancer and other diseases. The symposium was one of a series of meetings and workshops currently planned by the CECB, including a technical workshop on chromatin immunoprecipitation cosponsored by the Systems Biology Faculty that will be held on July 9, 2007, in the Masur Auditorium on the NIH campus in Bethesda, and a CCR postdoc retreat on chromatin biology on January 28, 2008. For more information on these events, visit the CECB Web site (http://ccr.cancer.gov/initiatives/CECB). Chromosome biology is alive and well at the NCI and will be a core component of our basic discovery portfolio and a promising new direction in our efforts to develop novel therapeutic strategies for many years to come.

Robert H. Wiltrout, PhD
Director

CANCER AND CELL BIOLOGY

Unique MicroRNA Molecular Profiles in Lung Cancer Diagnosis and Prognosis

Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, Stephens RM, Okamoto A, Yokota J, Tanaka T, Calin GA, Liu CG, Croce CM, and Harris CC. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis *Cancer Cell* 9: 189–98, 2006.

Lung cancer is the leading cause of cancer deaths in the world, indicating the obvious need for a better understanding of the mechanisms that underlie carcinogenesis in the lung. Although systematic analysis of mRNA and protein expressions has contributed

to defining the molecular network of lung carcinogenesis, previously unknown markers such as noncoding RNA gene products may also lend insight into the biology of lung cancer.

MicroRNAs (miRNAs) are small noncoding RNA gene products that are found in diverse organisms and play key roles in regulating the translation and degradation of mRNAs. miRNAs have been implicated in various biological processes, including cell proliferation, cell death, stress resistance, and fat metabolism, through the regulation of mRNA stability and/or translation of multiple target genes. Our understanding of miRNA expression patterns and function in normal or neoplastic human cells is just emerging. Although the precise mechanisms regulating miRNA expression are not yet fully understood, several mechanisms, including genetic and epigenetic alteration, might affect its expression and might lead to alterations in the target genes' expression in cancers. To investigate miRNA involvement in lung carcinogenesis, we examined its expression profiles for lung cancers by using miRNA microarray technology.

The miRNA expression profiles of 104 pairs of primary lung cancers and corresponding noncancerous lung tissues were analyzed. Each pair was obtained from the same patient to eliminate genetic differences between tumor and normal tissues. miRNA microarray analysis identified statistically unique profiles that could discriminate lung cancers from noncancerous lung tissues. When we compared miRNA expression among lung cancer tissues with that of corresponding noncancerous lung tissues, 43 miRNAs showed statistical differences in expression. Several of the miRNAs are located inside fragile sites and/or in the cancer-associated genomic regions, such as frequently deleted or amplified regions in several malignancies. This finding, and the fact that more than 50% of miRNAs are located in cancer-related chromosomal regions, supports the hypothesis that miRNAs play a role as a novel class of oncogenes or tumor suppressor genes. We next asked whether the microarray data revealed specific molecular signatures for lung cancer subsets that differ in clinical behavior. We identified six miRNAs that were expressed differently in the two most common histological types of non–small-cell lung cancer (NSCLC), adenocarcinoma and squamous cell carcinoma. No miRNAs were identified as differently expressed when classified by age, sex, or race in our data set.

Our next question was, Do the miRNA molecular profiles of lung cancer correlate with patient survival? We found that the miRNA molecular profile of lung adenocarcinoma correlates with patient survival. Furthermore, the miRNA molecular signature of lung adenocarcinomas, including those without evidence of metastasis, also correlates with patient survival. A univariate Cox proportional hazard regression model with a global permutation test indicated that expression of the miRNAs *hsa-mir-155* and *hsa-let-7a-2* was related to adenocarcinoma patient outcome. Kaplan-Meier survival analysis showed that the lung adenocarcinoma patients with either high *hsa-mir-155* (Figure 1) or reduced *hsa-let-7a-2* expression had poor survival. The difference in the prognosis of these two groups was statistically significant for *hsa-mir-155* ($P = 0.006$; log-rank test) and marginally significant for *hsa-let-7a-2* ($P = 0.033$; log-rank test). Subsequently, a multivariate Cox proportional hazard regression analysis indicated that high *hsa-mir-155* expression correlated significantly

with an unfavorable prognosis independent of other clinicopathological factors ($P = 0.027$; risk ratio 3.03; 95% CI, 1.13–8.14). The miRNA expression signature associated with outcome was confirmed by real-time RT-PCR analysis of precursor forms for the same miRNAs. Furthermore, we were able to cross-validate the clinical importance of outcome-predictive miRNAs using another independent case of adenocarcinoma. These results indicate that miRNA expression profiles are diagnostic and prognostic markers of lung cancer.

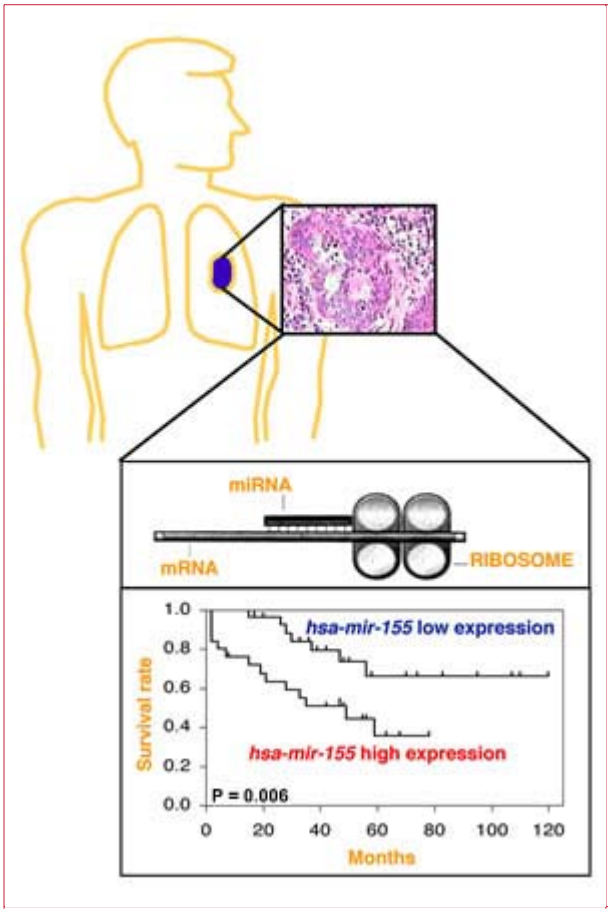


Figure 1. Kaplan-Meier survival analysis showing that the lung adenocarcinoma patients with high *hsa-mir-155* miRNA expression had poor survival.

Although curative resections of patients with early-stage NSCLC are performed, the risk of relapse remains substantial. This may indicate that there are micrometastases that have not been detected by general imaging and/or pathological examinations. Although additional studies confirming our results need to be performed, we anticipate that the miRNA expression signature with other biomarkers will allow the selection of lung cancer patients who may need more aggressive screening and treatment.

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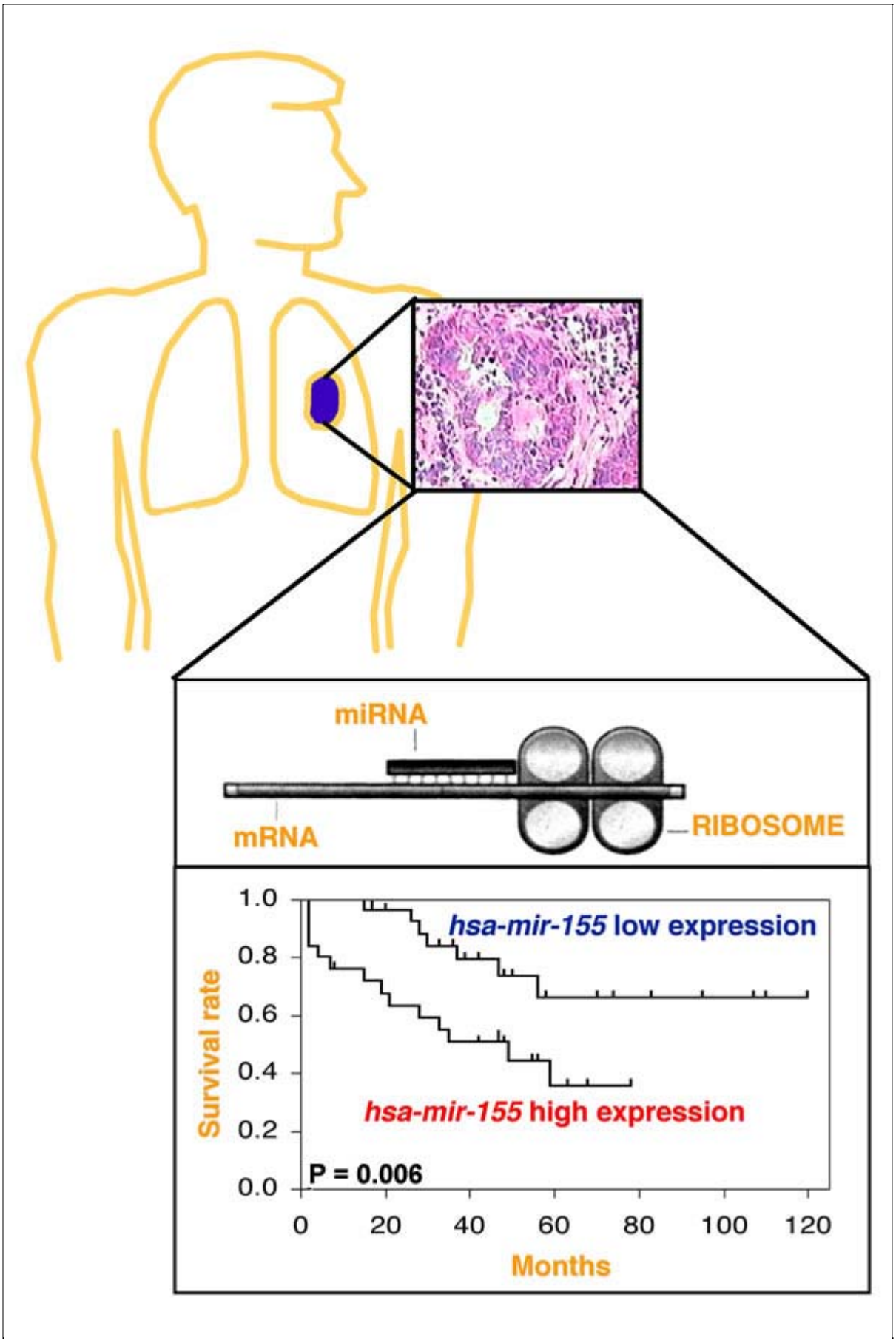
miRNA
mRNA
RIBOSOME
hsa-mir-155 low expression
hsa-mir-155 high expression
P = 0.006
Survival rate
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
Months

Figure 1. Kaplan-Meier survival analysis showing that the lung adenocarcinoma patients with high *hsa-mir-155* miRNA expression had poor survival.

CARCINOGENESIS

Breast Cancer Risk May Be Linked to Genetic Variants of the Mannose-binding Lectin 2 Gene

Bernig T, Boersma BJ, Howe TM, Welch R, Yadavalli S, Staats B, Mechanic LE, Chanock SJ, and Ambs S. The mannose-binding lectin (*MBL2*) haplotype and breast cancer: An association study in African-American and Caucasian women. *Carcinogenesis* 28: 828–36, 2007.

Early observations of cancer patients who had fully recovered from an acute bacterial infection suggested that innate immunity has antitumor activity. Today, we know that activation of innate immunity can lead to the elimination of cancer cells through cellular mechanisms such as complement-activated lysis and C3b-mediated phagocytosis.

Innate immunity depends on both pattern-recognition receptors and the complement system for target recognition. Innate pattern recognition receptors are ubiquitously expressed by immune and non-immune cells and recognize pathogen-associated molecular patterns. Among these receptors, toll-like receptors are currently of interest in cancer biology because of their altered expression in tumors and their ability to activate NF-κB and inflammatory responses. The major role of the complement system is to promote clearance of invaders and altered host cells. In this function, complement aids the tumor-specific T-cell response in the elimination of cancer cells.

Complement activation leads to the liberation of pro-inflammatory factors and the activation of inflammatory cells, which may have pro-carcinogenic effects. This mechanism could have significant implications for breast cancer because tumor-infiltrating phagocytes and pro-inflammatory cytokines have been found to augment angiogenesis and breast tumor invasiveness. Proteomic studies have identified complement component 3 (C3)–derived peptides as candidate breast cancer serum markers, and both C3 and natural killer cells are regulated by estrogen receptor α. Cell surface deposition of C3 in breast tumors has been observed, and cell membrane proteins that prevent complement-mediated cell toxicity, such as CD46, are expressed in breast tumors. It is surprising how little attention has been paid to the analysis of complement resistance in tumor cells or to ways that this phenomenon might be targeted in cancer therapy.

Complement activation proceeds through three different pathways that converge in the activation of C3. Activation of complement by lectin is crucial for innate immunity and is

driven by the mannose-binding lectin (MBL) protein. This relationship has been revealed by analysis of common single nucleotide polymorphisms (SNPs) in the *MBL2* gene, which encodes MBL. The frequency of SNP-determined MBL deficiency is significantly higher in patients presenting with various infections and autoimmune disorders than it is in the general population, indicating the importance of MBL in host defense.

MBL is a plasma protein of hepatic origin. SNPs in exon 1 of *MBL2*, known as the B-, C-, and D-alleles, alter the functional properties and circulating levels of MBL protein. They create, together with three linked promoter polymorphisms (known as H/L, Y/X, and P/Q), several well-characterized haplotypes that strongly influence complement activation. The prevalence of *MBL2* variations is associated with race/ethnicity; the variant B allele occurs in approximately one of four Caucasians, whereas the variant C allele is common in the sub-Saharan African populations.

We investigated the association of *MBL2* genotypes with the risk of developing breast cancer and comprehensively analyzed the genotype and haplotype of 26 *MBL2* SNPs in a case-control study of breast cancer. We found that an SNP in the 3´ untranslated region (UTR) of *MBL2* (rs10824792) was associated with an approximate 50% reduction in breast cancer risk in African American women but not Caucasian women. Haplotype analysis of *MBL2* showed that the frequency of the corresponding 3´ haplotype was also significantly lower in breast cancer patients than in controls among African American women. Our study suggests that a common genetic variant in the 3´ UTR of *MBL2* may reduce the risk for breast cancer in African American women, probably through an interaction with the 5´ secretor haplotypes that are associated with high concentrations of MBL.

Because these are preliminary findings, we interpret them with caution. Future studies are required to corroborate the relationship between the 3´ UTR haplotype of *MBL2* and breast cancer. It is plausible, however, that *MBL2* genetic variants modify the risk of breast cancer in one race/ethnic group but not in another. The *MBL2* haplotype structure is very different between African Americans and Caucasians. MBL also may interact with other breast cancer risk factors that are more common in the African American population than in Caucasians. MBL is found in complexes with four structurally related proteins, the MBL-associated serine proteases (MASPs) 1, 2, and 3 and Map19. Functionally, the protein complex between MBL and MASP-2 is the most significant. Because the *MASP-2* gene harbors several allele variants whose frequency varies widely among different race/ethnic groups, the association of *MBL2* variants with breast cancer is possibly influenced by *MASP-2* gene polymorphisms in a race/ethnicity-dependent manner.

Our study was not the first to observe an association between *MBL2* genotypes and human cancer. A recent case-control study of stomach cancer found a significant association between an increased cancer risk and the HYD haplotype of *MBL2*, which encodes a functionally impaired MBL protein and results in lower protein serum levels (Baccarelli A et al. *Int J Cancer* 119: 1970–5, 2006). Thus, additional evidence exists that MBL function contributes to human cancer risk. Although these findings require verification by other studies, future research should investigate the implication of *MBL2* genetic variants in

response to therapy and disease outcome.

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CARCINOGENESIS

Differential Susceptibility of Mice Humanized for Peroxisome Proliferator-activated Receptor α (PPARα) to Wy-14,643–induced Liver Tumorigenesis

Morimura K, Cheung C, Ward JM, Reddy JK, and Gonzalez FJ. Differential susceptibility of mice humanized for peroxisome proliferator-activated receptor α to Wy-14,643–induced liver tumorigenesis. *Carcinogenesis* 27: 1074–80, 2006.

The peroxisome proliferator-activated receptor (PPAR) family of ligand-activated nuclear receptors consists of three members, PPARα, PPARβ/δ, and PPARγ. Collectively, these receptors are involved in the control of lipid homeostasis and have been shown to be promising targets for drugs used in the treatment of dyslipidemia, type 2 diabetes, and syndrome X. PPARα is the target of lipid-lowering fibrate drugs, and PPARγ is the target of thiozolidinedione anti–type 2 diabetes drugs. PPARα was the first member of the family to be cloned and was named based on its ability to be activated by peroxisome proliferators. Studies using PPARα ligands and *Pparα*-null mice revealed that the physiological role of PPARα is to stimulate fatty-acid catabolism. During starvation, PPARα activates target genes encoding peroxisomal and mitochondrial enzymes involved in fatty acid transport and β-oxidation. A large number of synthetic chemicals, collectively termed peroxisome proliferators, activate PPARα. These include the lipid-lowering fibrate drugs fenofibrate (TriCor), gemfibrozil (Gemcor, Lopid), and clofibrate (Atromid-S), widely prescribed to lower plasma triglycerides and LDL-cholesterol levels, and phthalate esters, which are used in the production of pliable plastics. In rats and mice, the response to peroxisome proliferators is particularly robust, with a massive induction of both peroxisomal and mitochondrial fatty acid–metabolizing enzymes accompanied by peroxisome proliferation. Chronic treatment with a PPARα agonist results in an increased incidence of liver tumors through a PPARα-mediated mechanism, as revealed by the resistance of *Pparα*-null mice to liver cancer induced by the potent experimental peroxisome proliferator/PPARα ligand Wy-14,643. Peroxisome proliferators were subsequently found to be classic non-genotoxic carcinogens that, in contrast to those that are genotoxic, are not activated to electrophilic derivatives that can bind DNA and directly mutate genes; their mechanism of action in causing hepatocarcinogenesis is largely unknown. The dual ability of these chemicals to induce cell proliferation and oxidative stress is generally thought to cause

cell transformation and cancer in target tissues such as liver. Of great interest to human health, epidemiology studies on patients receiving fibrate drugs for the treatment of hyperlipidemia suggest that humans are resistant to the carcinogenic effects of fibrate drugs even though they produce a 100% incidence of liver tumors in rats and mice after 1 year of ingesting the chemical through their diets. The mechanism for the difference in the toxicity and carcinogenic effects of peroxisome proliferators between species is not known. To investigate the molecular basis for the species differences in response to peroxisome proliferators, a PPARα-humanized mouse model was developed.

A mouse line was generated in which the human PPARα (hPPARα) was expressed in liver in a *Pparα*-null background. The hPPARα and wild-type (murine PPARα, or mPPARα) mice response to treatment with the potent PPARα ligand Wy-14,643 was revealed by the induction of genes encoding mitochondrial and peroxisomal lipid-metabolizing enzymes, fatty acid transporters, and other PPARα target genes. hPPARα-expressing mice treated with Wy-14,643 had low levels of fasting serum total triglycerides, similar to the mPPARα-expressing mice (Figure 1, part A). No difference was noted from controls in drug-treated *Pparα*-null mice, although they had a lower constitutive level of serum triglycerides. mPPARα-expressing mice treated with Wy-14,643 showed a marked hepatomegaly (Figure 1, part B) due to increased cell proliferation, as well as cell hypertrophy as a result of peroxisome proliferation. Wy-14,643–treated mPPARα-expressing mice also exhibited hepatocellular proliferation, revealed by the extent of hepatomegaly, the incorporation of bromodeoxyuridine, and the induction of numerous cell-cycle control genes (Figure 1, part C). In contrast, the hPPARα mice exhibited no hepatocellular proliferation. In addition, cell-cycle control genes were not induced in Wy-14,643–treated hPPARα mice, in contrast to Wy-14,643–treated mPPARα mice, which had a significant increase in the mRNAs that encode proliferating cell nuclear antigen (PCNA), cMYC, cJUN, cyclin-dependent kinases 1 and 4 (CDK1, CDK4), and several cyclins. hPPARα mice were also found to be resistant to Wy-14,643–induced hepatocarcinogenesis; of the 20 mice treated, only 1 exhibited a carcinoma after 1 year of Wy-14,643 treatment, in contrast to a 100% incidence in the mPPARα-expressing mice. These findings suggest that the species-specific effects of fibrates are likely due to differences in the profile of genes activated by mPPARα versus hPPARα following fibrate treatment. Both receptors activate genes involved in fatty-acid transport and β-oxidation, but only mPPARα activates genes involved in the control of cell proliferation (Figure 1, part D). Thus, although both receptors induce genes encoding fatty acid metabolism and increased reactive oxygen species (ROS), only mPPARα activates cell proliferation. This species-specific regulation of gene expression will dictate whether a fibrate drug or other PPARα ligand exhibits a carcinogenic effect on the liver. The mechanisms of species differences in response to hepatocarcinogenesis and the identity of the differentially regulated PPARα target genes are currently under investigation.

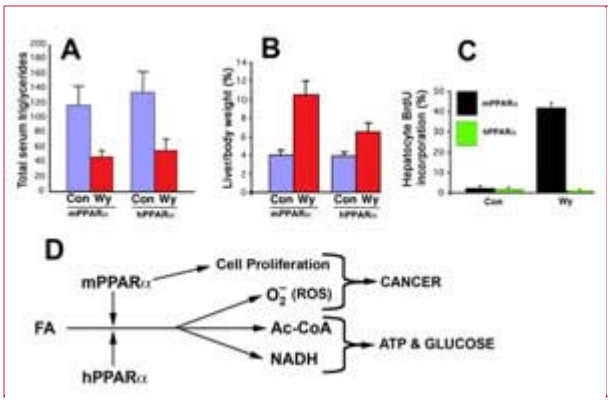

Figure 1. Summary of species differences in response to non-genotoxic carcinogen peroxisome proliferators. Murine peroxisome proliferator–activated receptor (mPPARα)–expressing and human PPARα (hPPARα)–expressing mice were fed a diet containing Wy-14,643 for 2 weeks, and fasting serum triglyceride levels *(A)*, hepatomegaly *(B)*, and hepatocyte proliferation *(C)* were measured. *(D)* A model for the difference between species in fatty-acid (FA) transport in response to PPARα ligand. Ac-CoA, Acetyl CoA; BrdU, 5´-bromodeoxyuridine; Con, control; NADH, oxidized nicotinamide adenine dinucleotide; ROS, reactive oxygen species; Wy, Wy-14,643–treated.

In conclusion, the development of hPPARα-expressing mice revealed that PPARα is responsible for the species differences in response to fibrate drugs. These mice are not only of value to study mechanisms of hepatocarcinogenesis but can be used by the pharmaceutical industry to test the safety of drugs being developed to treat hyperlipidemia, type 2 diabetes, and syndrome X.

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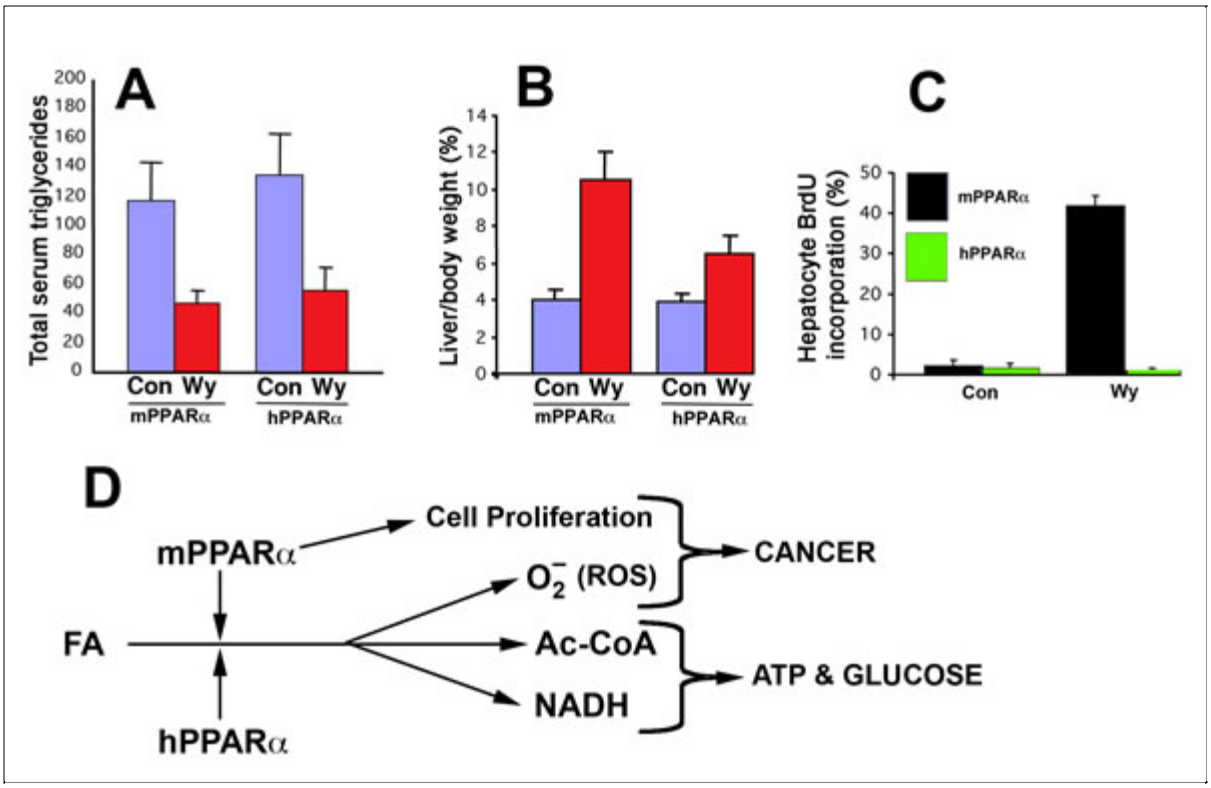

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MOLECULAR BIOLOGY

Achilles-Heel Genetic Screens for Cancer Targets

Ngo VN, Davis RE, Lamy L, Yu X, Zhao H, Lenz G, Lam LT, Dave S, Yang L, Powell J, and Staudt LM. A loss-of-function RNA interference screen for molecular targets in cancer. *Nature* 441: 106–10, 2006.

Cancer is the consequence of genetic damage to a susceptible cell, which often deregulates signaling pathways and causes unchecked proliferation and survival. In many cases, the cancer cell becomes “addicted” to the deregulated pathway so that interference with it abrogates the transformed phenotype. Successful therapeutic targeting of

a specific genetic abnormality in cancer is exemplified by Gleevec, a kinase inhibitor that is used in the treatment of chronic myelogenous leukemia. Finding molecular components of essential signaling pathways in cancer cells is therefore a rational algorithm for the development of effective cancer therapies. Recent understanding of RNA interference (RNAi)—a sequence-specific, posttranscriptional gene inactivation process—has quickly transformed this conserved cellular mechanism into a powerful laboratory tool to probe gene function. Genetic screens using RNAi are feasible because of its exquisite specificity and the relative ease with which it can be applied on a large scale. Here we describe an inducible RNAi genetic screen that can reveal genes essential for cancer cell proliferation or survival and identify molecular targets in cancer.

We constructed a retroviral vector that enabled the doxycyline-inducible expression of short-hairpin RNAs (shRNAs), which can mediate RNAi. We then created a library of shRNA vectors targeting 2,500 human genes. Each vector contained a unique 60-base-pair "barcode," enabling the abundance of each shRNA vector to be monitored in a population of transduced cells using DNA microarray technology. Retroviral pools from this library were used to infect cell lines representing two distinct molecular subgroups of diffuse large B-cell lymphoma (DLBCL), named activated B cell–like (ABC) DLBCL and germinal-center B cell–like (GCB) DLBCL. Infected cells were divided into two groups that were either induced to express shRNA or left untreated. After allowing the induced cells to grow for 3 weeks, genomic DNA was harvested and the barcode sequences were amplified. Fluorescently labeled barcodes from uninduced and induced groups were co-hybridized to a DNA microarray containing complementary barcode sequences. The microarray fluorescent signals indicated relative abundance—depletion or enrichment—of individual shRNA vectors within the induced and uninduced populations, reflecting the effect of each shRNA on the proliferation or survival of cancer cells (Figure 1).

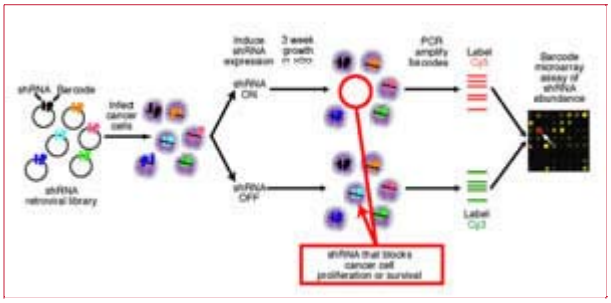

Figure 1. Inducible, barcode short-hairpin RNA (shRNA) library screen strategy for genes controlling cancer cell proliferation and survival. Infection of an shRNA retroviral library into a cancer cell line produces a "cellular library" with each cell carrying one or more shRNAs. Each shRNA is tagged by a known, unique barcode. Infected cells are divided into two subpopulations, one induced for expression of shRNAs and the other serving as the control. The inducibility of the shRNA library is important to prevent the loss of shRNA species that are acutely deleterious to infected cells. A time-dependent selective pressure is applied and genomic DNA fragments carrying the barcode representing each shRNA from each subpopulation are amplified by PCR, labeled with different fluorescent dyes, and cohybridized to a microarray containing complementary barcode oligonucleotides. The microarray is scanned and the relative abundance in the two subpopulations of an shRNA targeting a gene that influences cell proliferation or survival can be quantified.

The screen that we performed was aimed at uncovering shRNAs that are selectively toxic to

one lymphoma type but not the other, presumably due to the underlying molecular differences between the two lymphoma types. Remarkably, we discovered that the shRNAs that targeted genes in the NF-κB pathway were toxic to ABC DLBCL but not GCB DLBCL cell lines. This finding was in keeping with our previous demonstration that ABC DLBCLs depend on constitutive NF-κB signaling for survival. Unexpectedly, these shRNAs targeted three genes, *CARD11, MALT1,* and *BCL10,* which lie in a signaling pathway upstream of IκB kinase, the key regulator of the NF-κB pathway. Thus, our genetic screen has begun to unravel the mystery of constitutive IκB kinase activity in ABC DLBCL.

We are continuing similar screens of other forms of cancer and are consistently identifying new cancer type–specific pathways that control cell proliferation and survival. These signaling pathways could be activated by gene mutations or alterations in gene copy number that are present in particular cancer types; therapies targeting these pathways would be predicted to have a large therapeutic index. Alternatively, the pathways uncovered by our genetic screens may not be directly activated by oncogenic events but might be features of the normal cells from which the cancer develops. In this scenario, therapeutic targeting of the pathway might eliminate normal cells as well as cancer cells. For certain cancers such as lymphomas, however, the normal cellular counterparts, B lymphocytes, are dispensable for short periods of time and can be renewed.

We envision a new taxonomy of cancer centered around a cancer's dependence on particular regulatory pathways. The Achilles-heel genetic screen that we have employed is a powerful method to achieve this end and will likely hasten the development of pathway-specific therapies.

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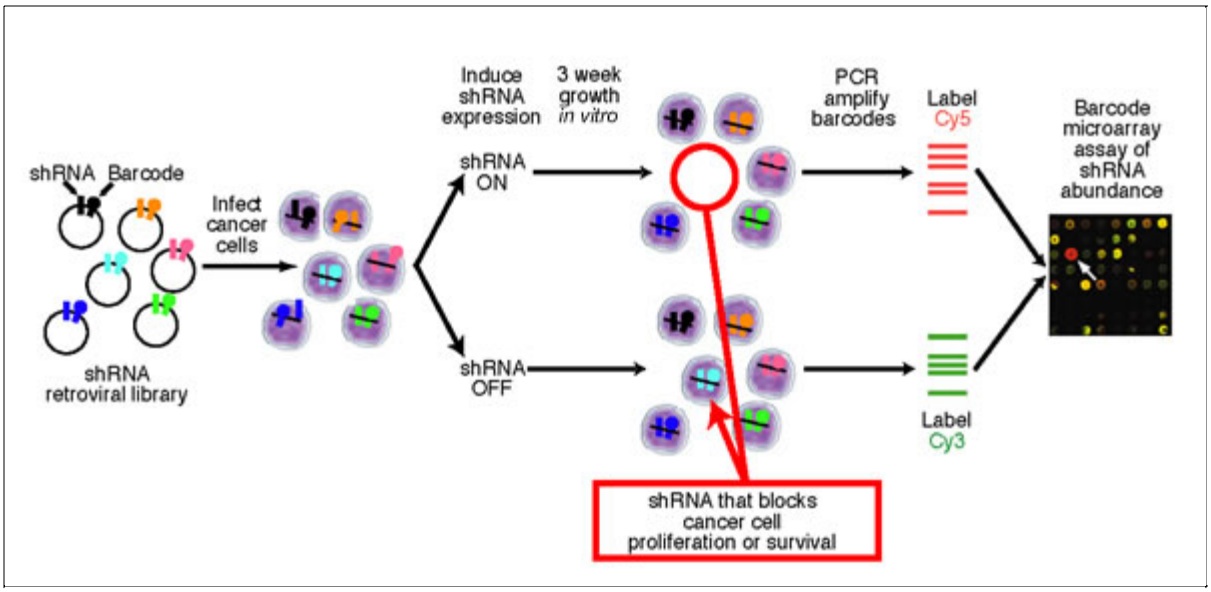

Figure 1. Inducible, barcode short-hairpin RNA (shRNA) library screen strategy for genes controlling cancer cell proliferation and survival. Infection of an shRNA retroviral library into a cancer cell line produces a "cellular library" with each cell carrying one or more shRNAs. Each shRNA is tagged by a known, unique barcode. Infected cells are divided into two subpopulations, one induced for expression of shRNAs and the other serving as the control. The inducibility of the shRNA library is important to prevent the loss of shRNA species that are acutely deleterious to infected cells. A time-dependent selective pressure is applied and genomic DNA fragments carrying the barcode representing each shRNA from each subpopulation are amplified by PCR, labeled with different fluorescent dyes, and cohybridized to a microarray containing complementary barcode oligonucleotides. The microarray is scanned and the relative abundance in the two subpopulations of an shRNA targeting a gene that influences cell proliferation or survival can be quantified.

MOLECULAR BIOLOGY

Hsp90 Keeps the Activity of the Oncogenic ErbB2 Kinase at Bay

Xu W, Yuan X, Beebe K, Xiang Z, and Neckers L. Loss of Hsp90 association up-regulates Src-dependent ErbB2 activity. *Mol Cell Biol* 27: 220–8, 2007.

The oncogenic receptor tyrosine kinase ErbB2, also called HER2, has high kinase activity and is a preferred dimeric partner of other members of the family, which includes the epidermal growth factor receptor (EGFR or ErbB1), ErbB3, and ErbB4. Interaction of wild-type ErbB2 with the molecular chaperone Hsp90 is necessary for protein stability. In the current study, we demonstrated an additional function for Hsp90

association—namely to serve as a break on ErbB2 kinase activity.

Hsp90 inhibition by geldanamycin or its derivative 17AAG, which is currently in phase II clinical trials, induces rapid and profound ErbB2 degradation. We have previously shown that this requires a direct interaction between Hsp90 and the ErbB2 kinase domain. Point mutations within the kinase domain of ErbB2 that disrupt Hsp90 association (ErbB2-5M) confer resistance to Hsp90 inhibitors. Interestingly, ErbB2-5M displayed significantly elevated steady state kinase activity compared with the wild-type protein, and it was better able to transform NIH3T3 cells. These data suggested that Hsp90 association represses ErbB2 activity.

We sought to identify the molecular mechanism underlying the elevated activity of ErbB2-5M. One way to regulate the activity of a receptor tyrosine kinase is via phosphorylation of its activation loop (A-loop). Phosphorylation stabilizes the A-loop in a conformation that is permissive for substrate binding. Indeed, Western blotting with site-specific antibodies showed increased tyrosine (Y)877 phosphorylation in the A-loop of ErbB2-5M (compared with the wild-type protein). Phosphorylation of the A-loop is often mediated by intermolecular action between the protomers of a receptor dimer, but we showed that phosphorylation of Y877 on ErbB2 is carried out by Src kinase. There are ten members of the Src family. Although many of them are expressed primarily in hematopoietic cells, three of them, Src, Fyn, and Yes, are expressed in cells that also express ErbB2. We investigated the involvement of these three kinases in phosphorylating ErbB2 on Y877 by knocking down their expression with specific siRNAs and found that all three contribute to Y877 phosphorylation. Consistent with this finding, simultaneous knockdown of all three kinases reduced Y877 phosphorylation to a lower level than did individual knockdowns. Further, Y877 on ErbB2-5M was not hyper-phosphorylated in SFY cells, which are deficient in these three kinases, but its phosphorylation was restored when Src expression was restored in these cells.

By using molecular and pharmacological techniques, we showed that Y877 phosphorylation markedly elevates ErbB2 kinase activity. In contrast, even though Src mediates phosphorylation of the analogous residue in the A-loop of EGFR (ErbB1), EGFR kinase activity is not affected. To explore why these highly homologous kinases respond differently to Src-mediated A-loop phosphorylation, we compared the sequences and 3-dimensional structures of EGFR and ErbB2 kinase domains. We observed some sequence differences in the A-loops (Figure 1, part A) and in the topology of surrounding regions. Energetic analysis indicated that, in EGFR, an unphosphorylated A-loop adopts an activated configuration stabilized by both intramolecular interactions and interactions with solvent. Phosphorylation of the loop further stabilizes the configuration but does not introduce additional conformational changes. In contrast, the unphosphorylated A-loop of ErbB2 cannot adopt an activated configuration due to lack of favorable intra-molecular and solvent interactions. It flips away from the ATP-binding cleft, and is incapable of aligning ATP and substrate. Upon Y877 phosphorylation, the phosphoryl group establishes strong salt bridges that induce a conformational change in the A-loop and enable it to attain the active conformation, as shown

in Figure 1, part B.

Figure 1. The A-loop of the ErbB2 kinase domain adopts a different conformation from that of epidermal growth factor receptor (EGFR or ErbB1). *A)* Sequence alignment of the A-loops of EGFR and ErbB2. Identical residues are indicated with a vertical bar, similarity by a dot. Asterisks indicate the phosphorylated tyrosine residues. *B)* Superimposition of the modeled structures of the ErbB2 kinase domain with the A-loop in phosphorylated or unphosphorylated states. Red denotes the unphosphorylated and blue denotes the phosphorylated A-loop.

Our data thus uncovered a novel function of Hsp90 as a repressor of Src-dependent ErbB2 activation. These results also revealed the molecular mechanism by which Src kinases activate ErbB2, and they suggest that Src is a viable molecular target in tumors expressing elevated ErbB2 activity.

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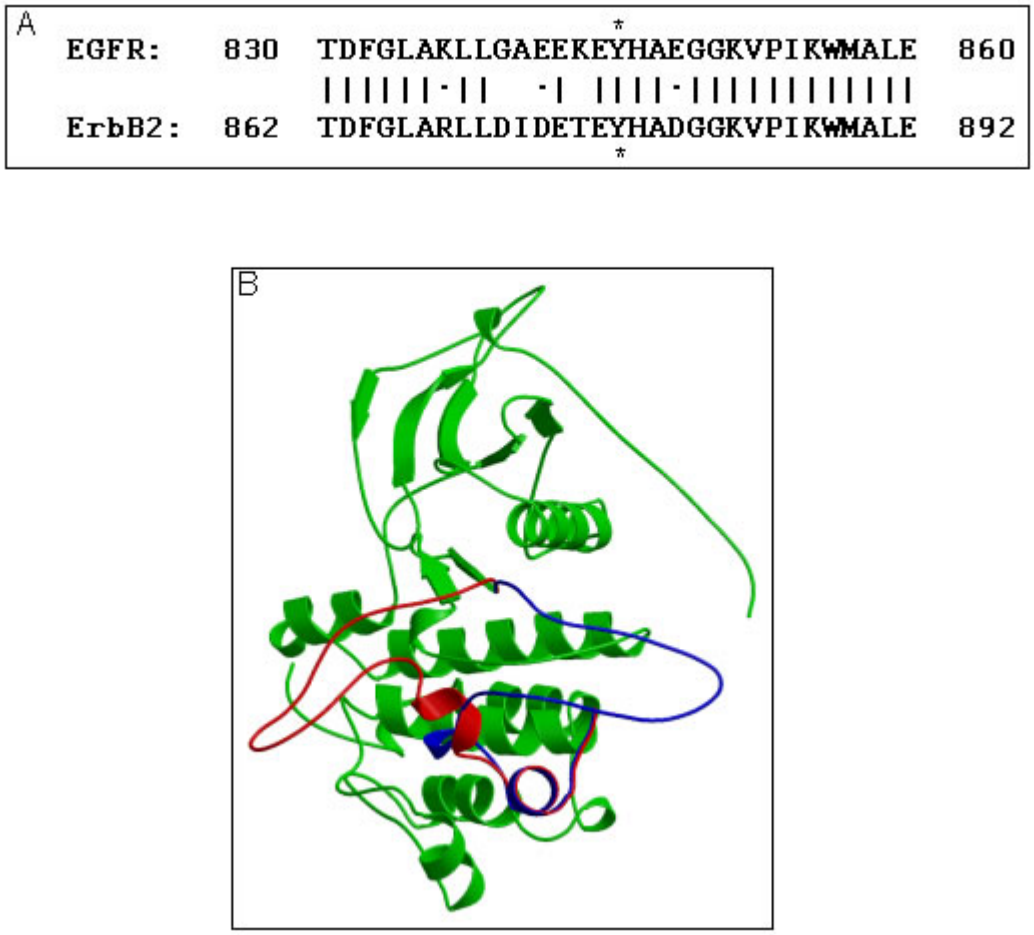

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MOLECULAR BIOLOGY

Regulation of Skin Pigmentation via Modification of Tyrosinase Function

Ando H, Kondoh H, Ichihashi M, and Hearing VJ. Approaches to identify inhibitors of melanin biosynthesis via the quality control of tyrosinase. *J Invest Dermatol* 127:751–61, 2007.

Melanin synthesis in the skin, hair, and eyes is ultimately regulated by tyrosinase, the critical rate-limiting enzyme produced by melanocytes within those tissues (Figure 1). Following the translation and subsequent processing of tyrosinase in the endoplasmic reticulum (ER) and Golgi, it is trafficked to specialized organelles, termed melanosomes, wherein melanin is synthesized and deposited. In the skin and hair, melanosomes are transferred from melanocytes to neighboring keratinocytes and are distributed in those tissues to produce visible color. Melanin in the skin is not only important for cosmetic appearance, but has other critical functions, such as photoprotection from UV radiation. Excess melanin production or its abnormal distribution can cause irregular hyperpigmentation of the skin. In order to develop therapies or prophylactics that improve or prevent hyperpigmentary disorders, such as melasma and age spots, disruption of tyrosinase activity has usually been targeted.

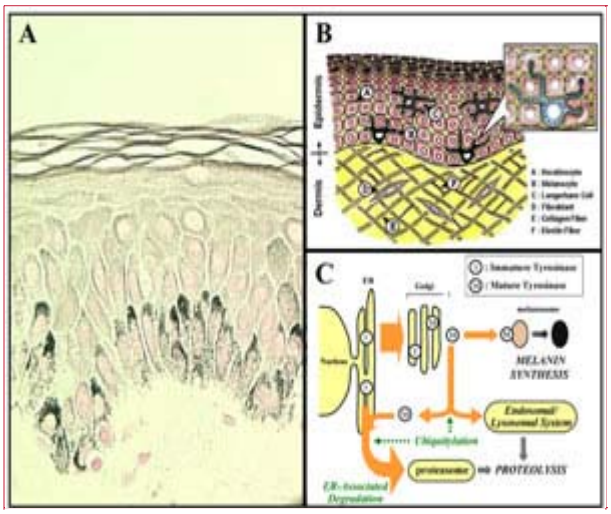

Figure 1. Regulation of skin pigmentation. *A)* Histology of human skin; black melanin pigment is seen just above the dermal-epidermal border. *B)* Schematic showing types of cells present in the epidermis and dermis. *C)* Schematic of tyrosinase processing and degradation within melanocytes. After maturation in the Golgi, tyrosinase is trafficked either to melanosomes for melanin synthesis or to the degradation machinery. ER, endoplasmic reticulum.

Levels of intracellular proteins are regulated by a balance between their synthesis and degradation, which is also true for tyrosinase. However, in contrast to effects on other proteins, reduced stability and function of tyrosinase has dramatic results on ensuing pigmentation. Tyrosinase is degraded endogenously, at least in part, by proteasomes, and several types of inherited hypopigmentary diseases (e.g., oculocutaneous albinism [OCA] and Hermansky Pudlak syndrome) involve the aberrant processing/trafficking of tyrosinase and its degradation or secretion to the extracellular milieu. In this study, we consider the quality control of tyrosinase and its stability and implications for the regulation of skin pigmentation.

Many targets exist for controlling melanin synthesis via the regulation of tyrosinase function. These include the following:

- **Inhibiting tyrosinase transcription.** Decreases of tyrosinase mRNA levels can be elicited by 5-BRDUR (5-bromodeoxyuridine), TPA (12-*O*-tetradecanoylphorbol-13-acetate, TGF-β1 (transforming growth factor-β1), and TNF-α (tumor necrosis factor-α). The transcription factor MITF (microphthalmia-associated transcription factor), termed the master regulator of melanocyte function, directly controls tyrosinase gene expression, and a number of factors that decrease levels of mRNAs encoding tyrosinase and/or MITF have been identified, including agouti signal protein, ceramide, dihydrolipoic acid, sphingosine-1-phosphate, lysophosphatidic acid, and sphingosylphosphorylcholine.
- **Affecting tyrosinase maturation.** Tyrosinase is a glycoprotein with 6 conserved N-linked glycosylation sites. Mutations in the tyrosinase gene are responsible for OCA type 1 and mutations of critical glycosylation sites reduce its catalytic function. Abnormal glycosylation of tyrosinase in the ER/Golgi inhibits its folding and maturation and results in hypopigmentation. Thus glycosylation inhibitors, such as glucosamine and tunicamycin, inhibit melanin synthesis.
- **Inhibiting catalytic function.** Inhibitors of tyrosinase activity include competitive and non-competitive inhibitors. Hydroquinone and azelaic acid, both used as potent therapies for hyperpigmentary disorders, are competitive inhibitors but are cytotoxic to melanocytes. Arbutin, a derivative of hydroquinone, also inhibits tyrosinase activity, as does 4-tertiary butylphenol, aloesin, 4,4′-dihydroxybiphenyl, and 4-n-butylresorcinol. Tyrosinase activity depends on 2 copper atoms bound at its active site, and metal chelators inhibit its activity (e.g., phenylthiourea, kojic acid, and ellagic acid).
- **Accelerating tyrosinase degradation.** Many physiological factors in the skin can regulate tyrosinase degradation (e.g., TGF-β1, TNF-α, and 25-hydroxycholesterol). Linoleic acid, an unsaturated fatty acid, is a major component of cell membranes and is of special interest in this regard. Topical application of linoleic acid decreases UV-induced hyperpigmentation of the skin. Fatty acids can regulate tyrosinase degradation in contrasting manners via their effects on proteasomes—for example, linoleic acid accelerates whereas palmitic acid decelerates its degradation. We have shown that linoleic acid increases levels of ubiquitylated tyrosinase, which leads to its accelerated degradation by proteasomes.

We have shown that tyrosinase destined for degradation in the ER is degraded by proteasomes, via ER-associated protein degradation (ERAD). ERAD is a mechanism for quality control of proteins, which involves their retention in the ER or retro-translocation to the cytosol if misfolded or unassembled. This is followed by their deglycosylation, ubiquitylation, and subsequent proteolysis by proteasomes. Tyrosinase degradation can occur following its maturation in the Golgi, which suggests that it is also subject to post-Golgi–associated protein degradation.

Thus, skin pigmentation is regulated physiologically at many levels that affect the function of tyrosinase. Such regulation has dramatic effects on visible pigmentation and the function of the skin and thus provides an ideal model for the study of such processes. Each of those levels of regulation is a tempting target for affecting pigmentation and thus optimizing skin

morphology and function.

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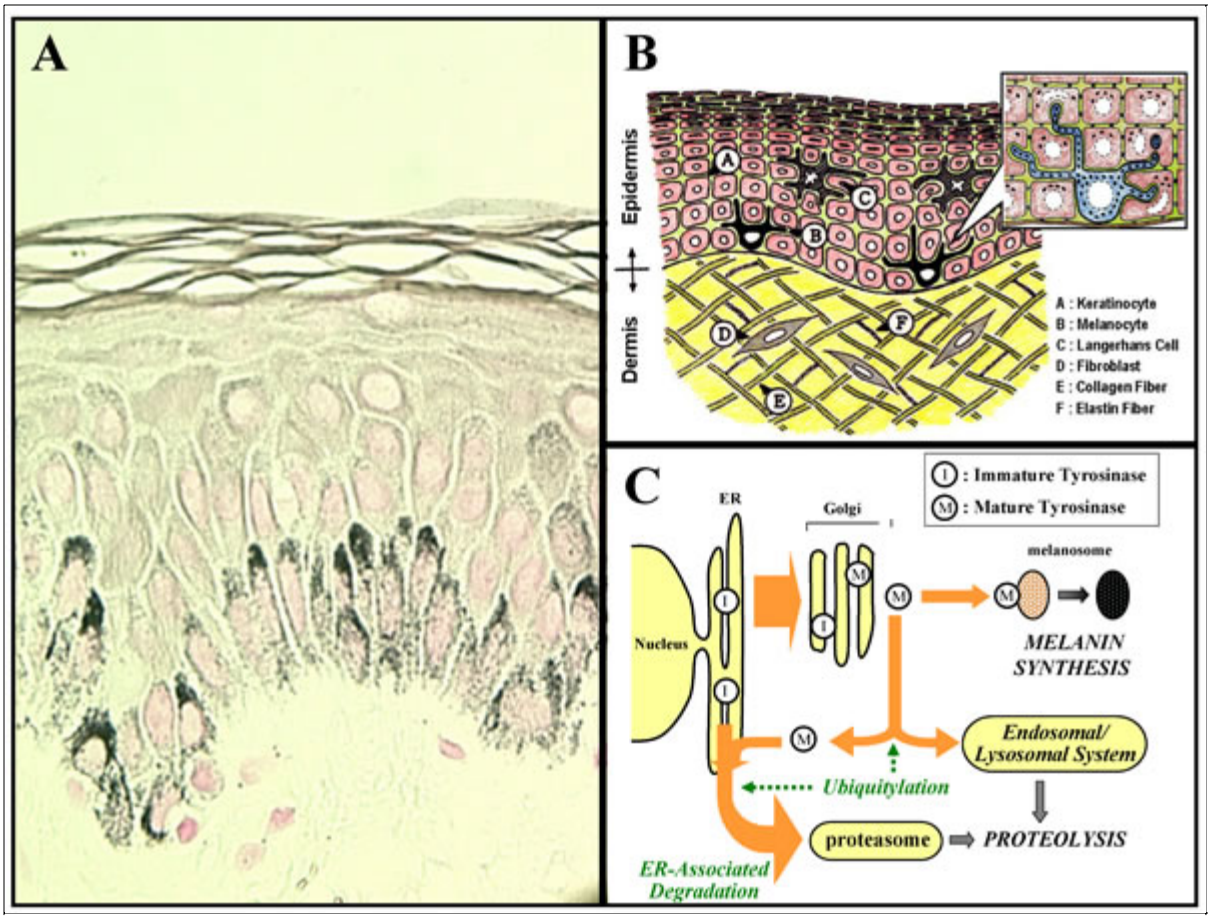

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TUMOR BIOLOGY

Methylation of Genes in Prostate Tumor–Associated Stromal Cells

Hanson JA, Gillespie JW, Grover A, Tangrea MA, Chuaqui RF, Emmert-Buck MR, Tangrea JA, Libutti SK, Linehan WM, and Woodson KG. Gene promoter methylation in prostate tumor–associated stromal cells. *J Natl Cancer Inst* 98: 255–61, 2006.

There is currently much interest in characterizing changes in the cells that compose the tumor microenvironment because it is now thought to be as important as the tumor itself in tumor progression. The prostate gland is composed of glandular epithelial tissue that is supported by stromal compartments predominantly comprising fibroblasts and smooth muscle cells. The stromal compartment of the tissue microenvironment associated with cancer epithelia is fundamentally different from that of normal tissue. Unique characteristics of the tumor microenvironment include an activated cellular phenotype that more readily supports tumor growth, presence of modified extracellular matrix proteins, and increased micro-vessel density. Several studies have noted alterations in gene and protein expression in stromal cells associated with tumors. Genetic modifications such as loss of heterozygosity, p53 mutation, and mutation of the phosphatase and tensin homolog (*PTEN*) gene have been described in stromal cells adjacent to breast carcinomas. Although most DNA-methylation studies have focused on tumor epithelial cells, we and others have more recently shown aberrant DNA-methylation patterns in tumor-associated stromal cells.

Epigenetic alterations such as promoter DNA hypermethylation are one of the hallmarks of carcinogenesis; hypermethylation is one of the most common alterations in human prostate cancer with more than 90% of tumors having aberrantly methylated genes. DNA methylation refers to the covalent bonding of a methyl group to the dinucleotide CpG, catalyzed by a group of enzymes called DNA methyltransferases. The majority of CpG dinucleotides in the genome, which are methylated in normal cells, are dispersed across retrotransposons or are found within the coding regions and introns of genes. About 15% of these dinucleotides are clustered in what are called CpG islands in the promoter regions of genes and are normally unmethylated. In tumors, promoter CpG islands are often methylated (or hypermethylated), a state that facilitates tumorigenesis by the silencing of tumor suppressor or other regulatory genes.

To investigate the presence of epigenetic changes in the tumor microenvironment, we evaluated the methylation of three genes important in prostate carcinogenesis in the tumor epithelium and stromal cells from prostate specimens of prostate cancer patients. For this analysis, we used two separate microdissection techniques (laser capture and expression

microdissection) to validate the selective isolation of epithelium from the stromal compartment. This was important because cross-contamination of cell types (in particular, the presence of tumor epithelium in stromal samples) could produce spurious methylation results. Gene-methylation status was analyzed using quantitative methylation-specific PCR and was confirmed in some samples by a second technique shown to be accurate for quantitative methylation analysis (pyrosequencing). We found that glutathione S-transferase pi 1 (*GSTP1*) and retinoic acid receptor β2 (*RARβ2*) were methylated in the tumor epithelia of all patients (similar to other reports) and in the tumor-associated stroma of 80% of the samples. Methylation of *CD44* was observed in 80% of prostate tumor epithelia samples but not in any of the tumor-associated stroma (Figure 1). The biological significance of the presence of *GSTP1/RARβ2* methylation and the absence of *CD44* methylation in stroma is unclear. Methylation of *GSTP1* and *RARβ2* has been shown to be prevalent in prostate tumors (in more than 90%), and their methylation occurs early in prostate carcinogenesis (seen in approximately 50% of prostate intraepithelial neoplasia, a prostate cancer precursor lesion). Methylation of *CD44*, however, is more likely to be associated with aggressive cancer (more prevalent in high-grade tumors) and is not observed in early pre-neoplastic lesions.

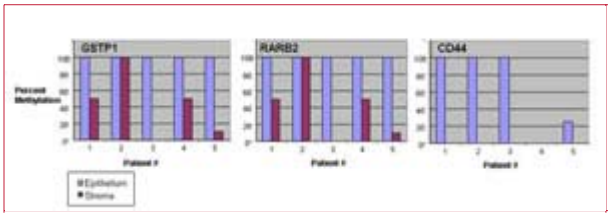

Figure 1. Methylation of *GSTP1, RARβ2,* and *CD44* in epithelial and stromal tissue taken from microdissected whole-mount prostate sections.

These findings raise several questions regarding the mechanism of aberrant promoter methylation in neoplastic and associated stromal cells. At present, it is not known whether tumor and stromal cell methylation are interdependent or if they are independent responses to the microenvironment. Although epigenetic changes in tumor cells have been very well characterized, the cause of changes in DNA methylation are unknown. Some likely sources include response to inflammation and/or infection.

Our findings of gene-specific hypermethylation in prostate tumor epithelia and its associated microenvironment may have implications for cancer prevention, treatment, and diagnosis. Identification of changes in stroma that contribute to tumor progression may provide more effective treatment modalities by altering the "soil" for tumor growth. We are currently pursuing methylation screening techniques to investigate the patterns of gene methylation specific to the stromal compartment of prostate tumors.

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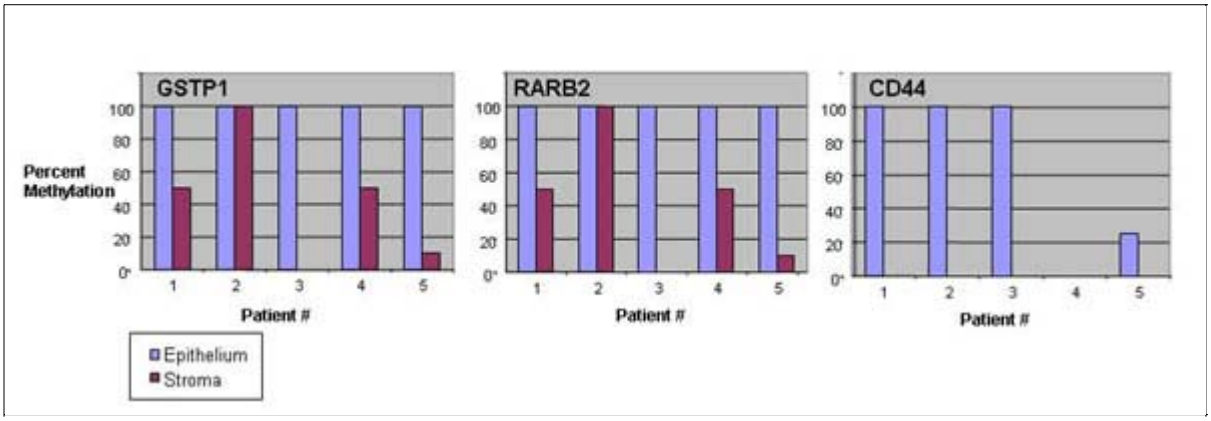

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