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FROM THE DIRECTOR'S OFFICE

Vision for Clinical Research at the CCR

The close association between basic and clinical research that exists within the CCR enables our clinical program to benefit from a dynamic, collaborative environment that drives our advances in translational research. Distinct from a comprehensive cancer center, our clinical program is a low-volume, high-intensity clinical/translational research enterprise focused intensely upon a small number of patients with cancer or AIDS-related illnesses. Patient volunteers who participate in our clinical trials receive state-of-the-art therapies and approaches to diagnosis and treatment. Genomic profiling is becoming a very important addition to these cutting-edge approaches.

The desire to obtain maximal information from every clinical protocol offered at the Clinical Center drives our investment in genomic profiling. Analysis of every biospecimen donated by patients enrolled in our clinical trials is a major part of our information gathering. Our clinical researchers use a wealth of technologies and analysis platforms to obtain sophisticated data from each biospecimen collected. Aware that such molecular information may soon be critically relevant to establishing markers of drug response or patient stratification—information needed to usher new drugs through clinical trials more efficiently—we have established a centralized facility of human biospecimens for clinical research. We collect material using standardized methods and approaches to ensure that samples are of the highest quality. This care at the outset makes subsequent analysis both possible and reliable.

Our newly established Clinical Molecular Profiling Core is at the heart of our commitment to biospecimen collection and our capacity to perform sophisticated analysis. This core, headed by Paul Meltzer, MD, PhD, will coordinate a complex series of genetic and genomic analyses on human samples collected during a patient's participation in clinical trials. The samples, with patient consent, will be procured under NCI's new guidelines for biospecimens and will be subjected to the most advanced technologies to interrogate the underlying disease using as many approaches as are feasible, based on the

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amount and type of tissue available. We will analyze and mine our acquired data to advance our understanding of the underlying mechanisms and process of cancer, and we will correlate clinical results with molecular targets and pathways where possible.

With much optimism, I welcome this addition to our clinical infrastructure. With this powerful technology, we will accelerate our ability to move discoveries made at the laboratory bench to the clinical setting and benefit many cancer patients.

■ **Lee J. Helman, MD**
Scientific Director for Clinical Research
NCI-Bethesda, Bldg. 31/Rm. 3A11
Tel: 301-496-4257
Fax: 301-480-4318
helmanl@nih.gov

■ FROM THE DIRECTOR'S OFFICE

Introduction to the Clinical Molecular Profiling Core

Oncologists caring for patients are acutely aware that each patient is an individual and that each tumor has its characteristic biological properties. These differences may not be important if there are only a few options for therapy, but now, in the age of targeted therapies with numerous new agents appearing in the clinic and many more on the horizon, matching the right treatment to the right patient appears increasingly important. Identifying the biological differences between tumors and defining the mechanisms by which theses features affect clinical outcomes are key components of contemporary clinical research. Biological data linked to clinical trials can enhance the value of those studies as CCR investigators work toward the rational implementation of targeted therapy. The Clinical Molecular Profiling Core has been created to facilitate biological data collection on every tumor entered into a CCR trial.

Fortunately, technologies for the molecular profiling of cancer have advanced substantially. It is now possible to obtain a tremendous amount of information from clinical specimens. The goal of the Clinical Molecular Profiling Core will be to provide every CCR clinical investigator with access to a suite of technologies for the characterization of biospecimens collected in the course of clinical trials. Because genetic and epigenetic changes are fundamental to cancer progression, the Core will focus primarily on genomic technologies: gene expression profiling, comparative genomic hybridization, high-density single nucleotide polymorphism (SNP) analysis, DNA sequencing, and related assays. Access to the Core will spare clinical investigators the need to develop technical expertise in these areas. Efforts are being made to offer a range of technologies to accommodate the realities of specimen collection in a variety of clinical situations. Specimen tracking, handling, and assays will follow standard procedures to maximize data reliability and maintain compliance with NCI specimen-collection guidelines. Core scientific staff will be available to consult with clinical investigators about assay selection, study design, and specimen requirements. Core staff will also support data analysis and interpretation.

This is an exciting period in the history of cancer research, in large part because there is a sense that advances in cancer biology are leading to real progress in cancer therapy. We trust that the Clinical Molecular Profiling Core will create opportunities for CCR clinical investigators to bring these technologies to bear on their efforts to develop more effective therapies for their patients.

■ **Paul Meltzer, MD, PhD**
Chief, Genetics Branch
NCI-Bethesda, Bldg. 37/Rm. 6138B
Tel: 301-496-5266
Fax: 301-402-3241
pmeltzer@mail.nih.gov

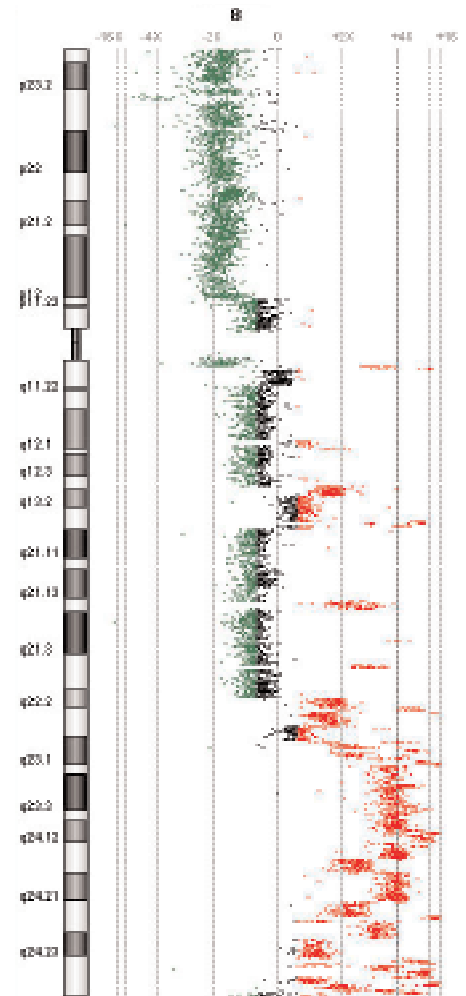

Figure 1. High-resolution array-based comparative genomic hybridization (array-CGH) of a breast cancer. Chromosome 8 is illustrated with gains on the right and losses on the left. Note the complex pattern with precisely defined boundaries of copy number change. This type of data, which can be generated rapidly from small, clinically practical samples, might be useful for tumor classification and gene discovery.

Application of Integrative Functional Genomics To Decode Cancer Signatures

Lee J-S, Chu I-S, Mikaelyan A, Calvisi DF, Heo J, Reddy JK, and Thorgeirsson SS. Application of comparative functional genomics to identify best-fit mouse models to study human cancer. *Nat Genet* 36: 1306–11, 2004.

Lee J-S, Heo J, Libbrecht L, Chu IS, Kaposi-Novak P, Calvisi DF, Mikaelyan A, Roberts LR, Demetris AJ, Sun Z, Nevens F, Roskams T, and Thorgeirsson SS. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. *Nat Med* 12: 410–16, 2006.

The success of comparative sequence analysis in identifying and characterizing genomic regulatory regions with important functional roles is well recognized. Indeed, the neutral theory of molecular evolution provides a framework for the identification of functional DNA sequences in genomes of different species. The theory predicts that functionally important elements in genome sequences tend to evolve at a slower rate than do less important elements. This difference has permitted the identification of both protein-coding and functional non-coding sequences in the genome. Although many of the functional genomic elements are protein-coding sequences, a large number of conserved sequences are regulatory elements known to modulate gene expression. These observations constituted the basis for our hypothesis that, if regulatory elements of evolutionarily related species are conserved, then gene expression signatures reflecting similar phenotypes in the species would also be conserved. In our first study, we tested this hypothesis by comparing global expression patterns of orthologous genes in human and mouse hepatocellular carcinomas (HCCs) and asked whether similar tumor phenotypes could be recognized and, thus, allow the identification of the best-fit mouse models for human HCC. We considered it important to use the mouse cancer models because the extent to which these models reproduce features observed in the corresponding human conditions is uncertain.

Human HCC

	Subclass A	Subclass B
Proliferation	High	Low
Apoptosis	Low	High
Ubiquitination	High	Low

Mouse HCC

	Myc/Tgfa	Myc/E2f1	Myc	E2f1
β-Catenin activation	Low	H	L	M
Genomic stability	High	Low		
Ubiquitination	High	Low		
Apoptosis	Low		High	
Proliferation	High	Low		

Figure 1. Phenotypic similarities between hepatocellular carcinomas (HCCs) generated in transgenic mouse models and subclasses A and B of human HCC. The best-fit HCC mouse models can be used to test hypotheses on tumor progression that are generated by analysis of cross-species gene expression patterns or from other experimental data. These models should also be extremely valuable for testing both potential therapeutic targets identified in human studies and preclinical trials of drugs. H, high; L, low; M, medium.

Orthologous human and mouse genes from both data sets were selected, and the gene expression data were integrated after standardizing the relative expression levels for both species. In hierarchical clustering analysis of integrated data, gene expression patterns of HCC from *Myc*, *E2f1*, and *Myc/E2f1* mice were most similar to those of the better survival group of human HCC, whereas the expression patterns of *Myc/Tgfa* and diethylnitrosamine (DENA)–induced mouse HCC were most similar to those of the poorer survival group of human HCC. These results suggest that these two classes of mouse models might closely recapitulate the molecular patterns of the two subclasses of human HCC. The similarity of gene expression profiles between human and mouse models are in good agreement with the phenotypic characteristics of the tumors (Figure 1). The human tumors with increased proliferation, decreased apoptosis, and worse prognosis are paired with the mouse models that have the same characteristics. The gene expression–based prediction of mouse models is highly concordant with the phenotypes of mice. *Myc/Tgfa* mice have a typically poor prognosis phenotype, such as an earlier and higher incident rate of HCC development and higher mortality, genomic instability, and expression of poor prognostic marker. *Myc* and *Myc/E2f1* mice have a relatively higher mutation frequency regarding β-catenin as well as a higher nuclear accumulation of the protein, which in human HCC are indicative of lower genomic instability and better prognosis.

The fact that these findings were first uncovered by using unsupervised methods and validated later using supervised methods indicates that the underlying principles in gene expression changes are conserved between mouse and human HCC.

In our second study, we extended this comparative functional genomic approach to address the issue pertaining to the cell(s) of origin for tumors. It is axiomatic that cancer cells evolve from normal cells after accumulation of genetic and epigenetic alterations. Also, it has been shown that the gene expression patterns in cancer cells reflect these alterations. Nevertheless, a considerable fraction of the gene expression program of cancer cells is characteristic of the non-transformed cellular lineages from which they originated. Furthermore, analysis of gene expression profiles of cancer cell lines indicated that neither physiological adaptation *in vivo* nor experimental adaptation *in vitro* is sufficient to abolish the gene expression programs acquired during development. These data suggest that the global gene expression profiles of tumors might provide critical information on the cellular origin of tumors.

Because HCC could originate from both adult hepatocytes and hepatic progenitor cells, we decided to test whether global gene expression analysis of human HCC could identify subtypes of HCC derived from these different cell types. The experimental strategy involved the generation of gene expression data from multiple species suitable for integration and cross-comparison. We integrated, using only orthologous genes, gene expression data from rat fetal hepatoblasts and adult rat hepatocytes with HCC gene expression data from human and mouse models. By applying hierarchical clustering analysis of gene expression patterns from human HCC, mouse HCC, rat fetal hepatoblasts, and adult rat hepatocytes, we identified a new prognostic subtype of HCC that shares gene expression patterns with fetal hepatoblasts. The hepatoblast (HB) subtype is distinguished from other types of HCC by the differential expression of hundreds of genes, and the robustness of this gene expression signature in the HB subtype was validated in an independent cohort of HCC patients.

HCC patients who shared a gene expression pattern with fetal hepatoblasts had a poor prognosis. The gene expression program that distinguished this subtype from other types of HCC included markers of hepatic oval cells, suggesting that HCC of this subtype arises from hepatic progenitor cells. Application of network-based pathway analyses of gene expression provided important insights into the pathogenesis of the HB subtype of HCC

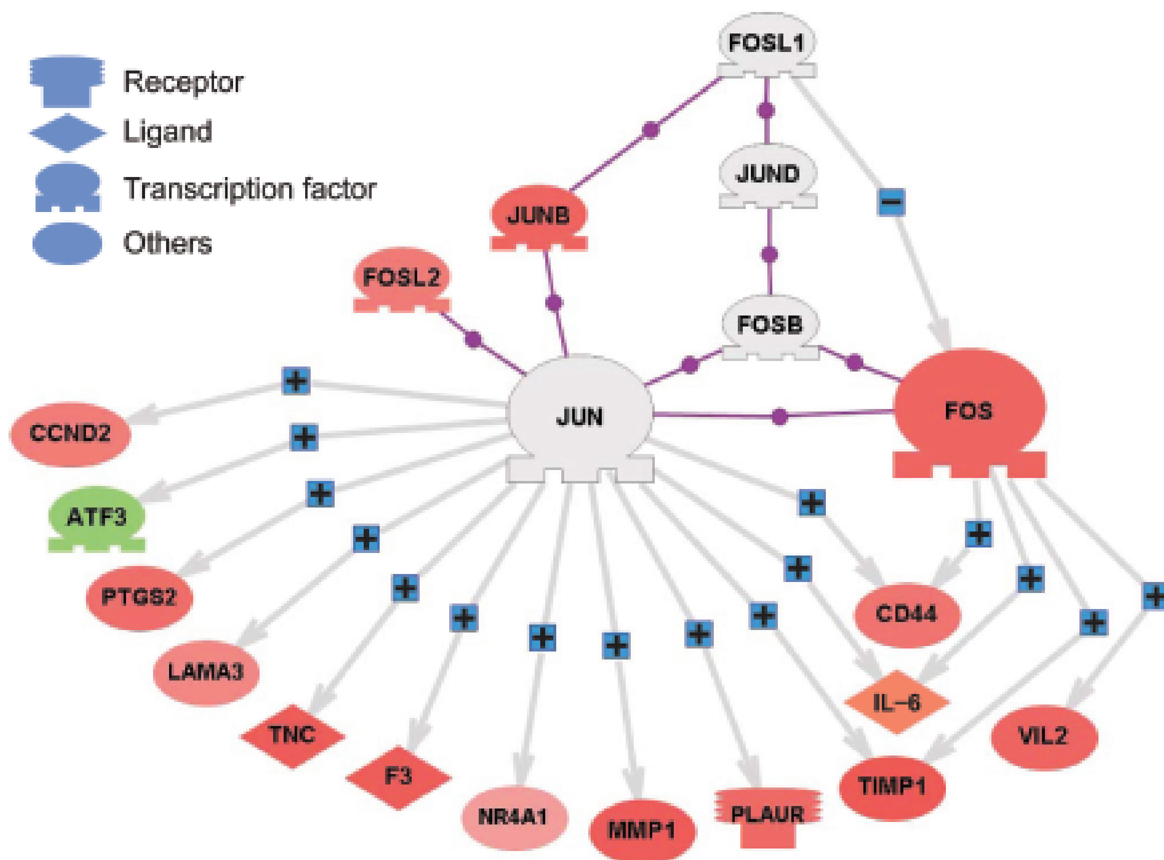

Figure 2. Gene networks of activator protein 1 (AP-1) transcription factors in the HCC subtype with liver progenitor cell signature. Upregulated and downregulated genes in the HCC subtype, with progenitor signature, are indicated in red and green, respectively. Genes in gray color are not on the list but are associated with the regulated genes. Gray lines and arrows represent the direction of transcriptional regulation, and plus and minus signs indicate positive and negative regulation of gene expression. Purple lines represent known physical interactions between connected genes.

(Figure 2). Enrichment of predicted JUN and FOS activity in the HB subtype led us to hypothesize that the activator protein 1 (AP-1) complex was the major driving force in tumorigenesis of the HB subtype. Previous studies have shown that Jun is essential for normal liver development, and it could also be crucial for the initiation of HCC development in mice. Furthermore, higher expression of JUN target genes involved in invasive phenotypes (e.g., MMP1, PLAUR, TIMP1, CD44, and VIL2) was observed in the HB subtype of HCC, indicating the cellular origin of these tumors and accounting for the poor prognosis of the affected individuals.

The success of the new experimental and analytical approaches presented here strongly suggests that more integration of independent data sets will enhance our ability to identify key regulatory elements in cancer development. It is, therefore, reasonable to expect that the clinical inference from transcriptome analyses will be significantly strengthened when gene expression data are integrated with diverse genomics information obtained from DNA sequence, array-based comparative genomic hybridization, and non-coding gene (i.e., microRNA) expression analyses.

Ju-Seog Lee, PhD
Research Fellow
Laboratory of Experimental Carcinogenesis
leeju@mail.nih.gov

Snorri S. Thorgeirsson, MD, PhD
Senior Investigator
Laboratory of Experimental Carcinogenesis
NCI-Bethesda, Bldg. 37/Rm. 4146
Tel: 301-496-1935
Fax: 301-496-0734
snorri_thorgeirsson@nih.gov

IMMUNOLOGY

CD4-CD8 Differentiation in the Thymus: The cKrox of the Matter

Sun G, Liu X, Mercado P, Jenkinson SR, Kypriotou M, Feigenbaum L, Galera P, and Bosselut R. The zinc finger protein cKrox directs CD4 lineage differentiation during intrathymic T cell positive selection. *Nat Immunol* 6: 373–81, 2005.

CD4 and CD8 T cells are essential components of the adaptive immune system and are critical for defense against infection. CD4 T cells recognize peptides of extracellular origin in complex with class II major histocompatibility complex (MHC-II) molecules, and generally provide help to other immune-competent cells. In contrast, CD8 T cells recognize peptides of intracellular origin in complex with MHC-I molecules and differentiate into cytotoxic effectors. Besides their role in infection defense, CD8 T cells have attracted the attention of tumor immunologists, who seek to harness their killing power to eliminate cancer cells. CD4- and CD8-lineage T cells arise in the thymus from nonfunctional precursors upon interaction with MHC-II or MHC-I peptide complexes expressed on the thymic stroma, respectively. How this choice of lineage occurs has puzzled immunologists and others for years and is the main focus of our laboratory. Although signals initiated by the binding of T-cell receptors (TCR) to intrathymic MHC molecules are now largely accepted as critical for lineage choice, the intracellular effectors of this process have remained mysterious. To gain insight into this question, we hypothesized that genes encoding such effectors would be upregulated during lineage choice, and we conducted a microarray screen to compare gene expression in T-cell precursors during and immediately before lineage differentiation. One top candidate that emerged from this search encodes a zinc finger DNA binding protein known as cKrox, a member of a large family of transcription factors involved in many differentiation processes. We found that cKrox was specifically upregulated during the differentiation of CD4 but not of CD8 T cells in the thymus, and that its expression remained CD4 specific in mature T cells.

Besides their role in infection defense, CD8 T cells have attracted the attention of tumor immunologists, who seek to harness their killing power to eliminate cancer cells.

To examine cKrox function during T-cell development, we generated transgenic mice in which this protein is expressed in all developing and mature T cells. Remarkably, these mice had CD4 but not CD8 T cells. This raised the possibility that cKrox might impose CD4 choice to MHC-I–signaled precursors that are normally CD8-bound and, thus, might be one of the long sought-after effectors of CD4-CD8 differentiation. Although this was an appealing perspective, it was also possible that the cKrox transgene simply prevented the differentiation of CD8 T cells without affecting their lineage direction. To distinguish between these possibilities, we generated cKrox transgenic mice whose T cells all carry the same TCR specificity for a defined MHC-I–peptide complex. Normally, T-cell precursors in such mice fail to express cKrox and develop into CD8 cells. In the presence of the cKrox transgene, however, these precursors were redirected into CD4 cells, indicating that cKrox promoted CD4 choice at the expense of CD8 choice. Importantly, cKrox also imposed the functional helper differentiation characteristics

of CD4 cells: Whereas MHC-I–specific CD8 T cells normally are cytotoxic, the MHC-I–specific CD4 T cells that developed in cKrox transgenic mice lacked cytotoxic properties (such as expression of the enzyme perforin) and had gained attributes of helper function. These findings indicate that cKrox is a master developmental regulator that imposes CD4 differentiation to developing thymocytes. In parallel to this work, the laboratory of Dietmar Kappes, PhD, Fox Chase Cancer Center, independently showed that a spontaneous point mutation in the gene encoding cKrox (which these authors called Thpok and that is now officially referred to as Zbtb7b) resulted in a phenotype mirroring the one observed in the cKrox transgene: Mice carrying this mutation lacked CD4 T cells and had MHC-II–specific cytotoxic CD8 T cells.

The identification of cKrox as a master switch of CD4-CD8 lineage differentiation raises many questions. One key issue will be to investigate how cKrox is upregulated during the development of T cells recognizing MHC-II–peptide but not MHC-I–peptide complexes. The search for cKrox target genes should provide insight into the mechanism of lineage differentiation in the thymus. It is interesting that some cKrox homologs repress gene expression by recruiting enzymes (histone deacetylases) that promote the closure of chromatin to transcription. The possibility that cKrox affects lineage differentiation by altering chromatin is intriguing. Indeed, whereas many differentiation processes mediated by changes in chromatin organization are intimately associated with cell division (during which chromatin reorganization occurs), this is not the case with CD4-CD8 lineage differentiation.

Remy Bosselut, MD, PhD
Investigator
Laboratory of Immune Cell Biology
NCI-Bethesda, Bldg. 37/Rm. 3032A
Tel: 301-402-4849
Fax: 301-402-4844
remy@helix.nih.gov

CANCER AND CELL BIOLOGY

Targeting Cancer Cells by Exploiting Karyotypic Complexity and Chromosomal Instability

Roschke AV, Lababidi S, Tonon G, Gehlhaus KS, Bussey K, Weinstein JN, Kirsch IR. Karyotypic "state" as a potential determinant for anticancer drug discovery. *Proc Natl Acad Sci U S A* 102: 2964–9, 2005.

Most cancers have an abnormal chromosomal content, called aneuploidy, characterized by changes in chromosomal structure and number. Chromosomal aberrations tend to be more numerous in malignant tumors than in benign ones, and karyotypic complexity is associated with poorer prognoses and aggressive clinical and distinctive histopathologic features. Therefore, quantitative or qualitative changes in the karyotypic state of malignancy could represent determinants for anticancer therapies and might ultimately allow targeting of the most aggressive and incurable cancers.

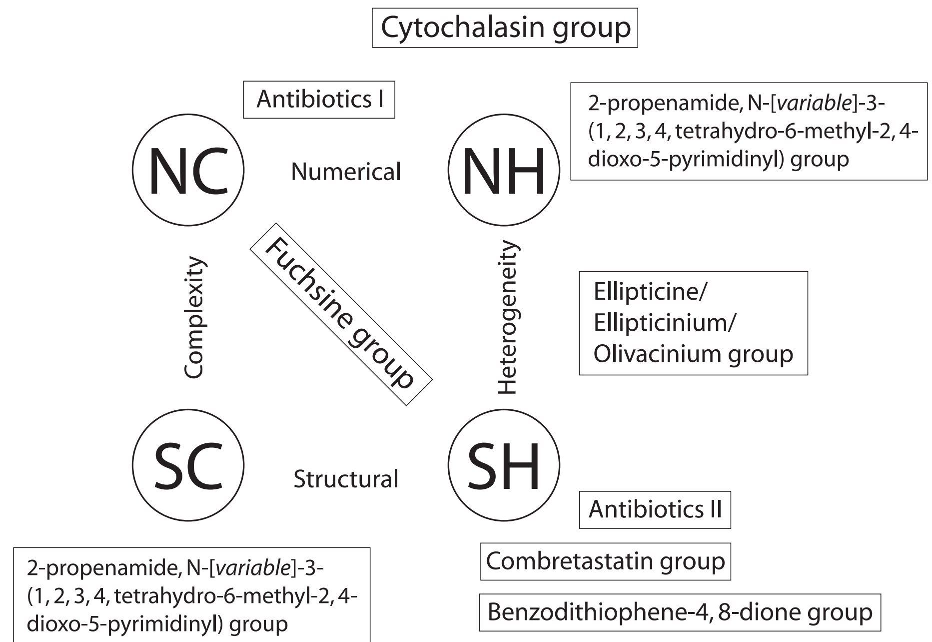

Figure 1. Stratification of compound groups based on activity associated with a particular karyotypic parameter. NC, numerical complexity; NH, numerical heterogeneity; SC, structural complexity; SH, structural heterogeneity.

We have completed a detailed analysis of the chromosomal aberrations present in the drug-discovery panel of 60 human cancer cell lines (the NCI-60), used by the NCI Developmental Therapeutics Program (DTP) to screen compounds for anticancer activity (Roschke AV et al. *Cancer Res* 63: 8634–47, 2003). Measures of karyotypic complexity include the number of clonal chromosomal rearrangements present in a cell line (structural complexity), the number of chromosome deviations from the ploidy level (numerical complexity), and modal chromosome number. Measures of cell-to-cell

chromosomal variability, which reflect the degree of ongoing instability, include numerical and structural heterogeneity. The NCI-60 cancer cell lines show wide variation in these parameters. Karyotypes of these cell lines have been made publicly available on two Web sites (http://www.ncbi.nlm.nih.gov/sky/skyweb.cgi and http://home.ncifcrf.gov/CCR/60SKY/new/demo1.asp).

We then looked for relationships between markers of the chromosomal state and drug resistance or sensitivity. As a first snapshot, we used a 1,429-drug subset of the more than 100,000 compounds tested against the cell lines in a short-term cytotoxicity assay. This subset was selected because each agent had been tested at least four times on all or most of the NCI-60. It includes most of the drugs currently used clinically for cancer treatment, along with many candidates that have reached clinical trials. A correlation analysis was performed comparing sensitivity data (expressed as the negative logarithm of GI50 [growth inhibition of 50%]) and each of the karyotypic parameters. A positive correlation between sensitivity to a given compound and an increased level of a given karyotypic parameter means that cell lines with higher values for that specific parameter would be more sensitive to the growth inhibitory action of that agent. The positive correlations between drug sensitivity and karyotypic complexity and heterogeneity found in this analysis (122 statistically significant positive correlations, $P < 0.05$) allowed us to identify agents that are more active against karyotypically complex and chromosomally unstable cancer cells. Grouping of selected agents based on their functional classification or chemical structure yielded seven distinct groups of chemical compounds. Relationships between karyotypic parameters and sensitivity of cancer cells to identified classes of agents are diagrammed in Figure 1.

To explore the possibility that an agent targeted a particular cell lineage that just happened to be more karyotypically complex or that other cellular "states," such as mismatch repair status or p53 gene status, might be the critical factors acting as determinants of sensitivity or resistance to these compounds, we reanalyzed the data for selected compounds from each group. We did this sequentially, leaving out one and then another of each of the nine lineages in the panel, or leaving out the six mismatch repair-defective cell lines or the 18 p53 wild-type cell lines present in the panel. The essential features of the correlations that we described and the groups of compounds that we identified were not changed by these additional tests.

In collaboration with David G. Covell, PhD, Anders Wallqvist, PhD, and Ruili Huang, PhD (Screening Technologies Branch, NCI), we performed a much larger-scale correlation analysis of karyotypic parameters using data obtained from approximately 30,000 chemical compounds tested on the NCI-60 cell lines, and identified additional classes of chemical agents associated with karyotypic parameters (Wallqvist A et al. *Mol Cancer Ther* 4: 1559–68, 2005). As an aid to this analysis, we employed computational tools based on methods of self-organizing maps (SOMs) used by the Covell lab to cluster the NCI's database of GI50 measurements of these 30,000 compounds across the panel of NCI-60 cancer cell lines (http://spheroid.ncifcrf.gov). When we made projections on these maps of the compounds that have been identified as positively and significantly correlated with karyotypic parameters, they mainly hit a relatively unexplored region in the SOM, where standard anticancer drugs are not, for the most part, present, and where mechanisms of action of chemical compounds are among the least elucidated. These findings suggest that these "lead" compounds identified as active against karyotypically complex and/or chromosomally unstable cancer cells may, indeed, represent new classes and mechanisms of action for potential anticancer agents.

The karyotypic parameters associated with the activities of these compounds may well be markers for underlying genes or pathways that are the true targets of these agents. It is equally important, however, to recognize that certain agents may be active against the "state" of complexity or instability itself rather than against any specific gene product or pathway. It is plausible that the assessment of the chromosomal state of a cancer cell population could serve as a future guide for the selection of drugs active against aggressive and intractable cancers.

- **Anna V. Roschke, PhD**
  Expert
  Genetics Branch
  roschkea@mail.nih.gov

- **Kristen S. Gehlhaus, MHS**
  Biologist
  Genetics Branch
  gehlhauk@mail.nih.gov

- **Ilan R. Kirsch, MD**
  Senior Director, Oncology Research
  Amgen
  1201 Amgen Court West
  AW1-J 4144
  Seattle, Washington 98119-3105
  Tel: 206-265-7316
  lkirsch@amgen.com

***CCR Frontiers in Science*—Staff**

Center for Cancer Research
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FGF8 Takes Center Stage During Kidney Development

Perantoni AO, Timofeeva O, Naillat F, Richman C, Pajni-Underwood S, Wilson C, Vainio S, Dove LF, and Lewandoski M. Inactivation of FGF8 in early mesoderm reveals an essential role in kidney development. *Development* 132: 3859–71, 2005.

The family of human and mouse fibroblast growth factors (FGFs) is large, numbering 22. Originally named for their effect on cultured cells, they regulate a wide variety of cellular and morphogenetic processes. Arguably, FGF8 is the busiest family member. It was isolated from mammary tumor cells and has since been implicated in the oncogenesis of sex hormone–related cancers of the breast and prostate. Most of our knowledge of how FGF8 controls morphogenesis comes from studying its various roles during mouse development. FGF8 is required for normal gastrulation, the embryonic stage when the three germ layers—mesoderm, ectoderm, and endoderm—are formed. Thanks to techniques of conditional mutagenesis, we also know that FGF8 is required for left/right asymmetry and regulates the development of different brain regions, the eyes, heart, limbs, and face. Equally diverse are the cellular processes that FGF8 regulates; depending on the embryonic stage, it controls cell growth, apoptosis, migration, and gene expression.

Vertebrates are segmented, as demonstrated by somite formation (Figure 1, part A)—blocks of mesoderm lining the anterior-posterior embryonic axis and giving rise to muscle, dermis, and vertebrae. Manipulation of the chick embryo suggested that FGF8 regulates somitogenesis by keeping the presomitic mesoderm unsegmented until the appropriate cue induces the next somite in the embryo's tail end. However, this idea could not be tested genetically for lack of an appropriate Cre mouse that would inactivate *Fgf8* expression in forming somites and yet allow normal gastrulation. In our recent publication cited above, we characterize such a mouse line, called T-Cre, because Cre is controlled by regulatory elements of the *T* (or *Brachyury*) gene and hence is expressed prior to somitogenesis in the early mesoderm as it forms during gastrulation. T-Cre–mediated *Fgf8* inactivation yielded embryos that gastrulated normally and generated presomitic mesoderm devoid of *Fgf8* gene product (Figure 1, part A). These embryos gave us a surprising result: FGF8 is not required for somitogenesis because the somites and their derivatives were normal (Figure 1, part A). Our current unpublished work addresses this conundrum by demonstrating that the role of FGF8 in this process is partially redundant with a subset of five other FGF genes coexpressed in this region.

Although somitogenesis was unaffected in these mutants, neonates died because they lacked functional kidneys (Figure 1, part B). A central event during kidney development is a reciprocal induction between two lineages: the ureteric bud (UB) and metanephric mesenchyme (MM). As a result, the MM condenses and converts to an epithelium that undergoes a series of morphogenetic changes to form the structures of the nephron. In turn, the UB branches outward toward the periphery of the growing kidney where this mutual induction event repeats as the kidney grows. The end result is a functioning kidney consisting of a large number of nephrons connected by the UB-derived collecting ducts.

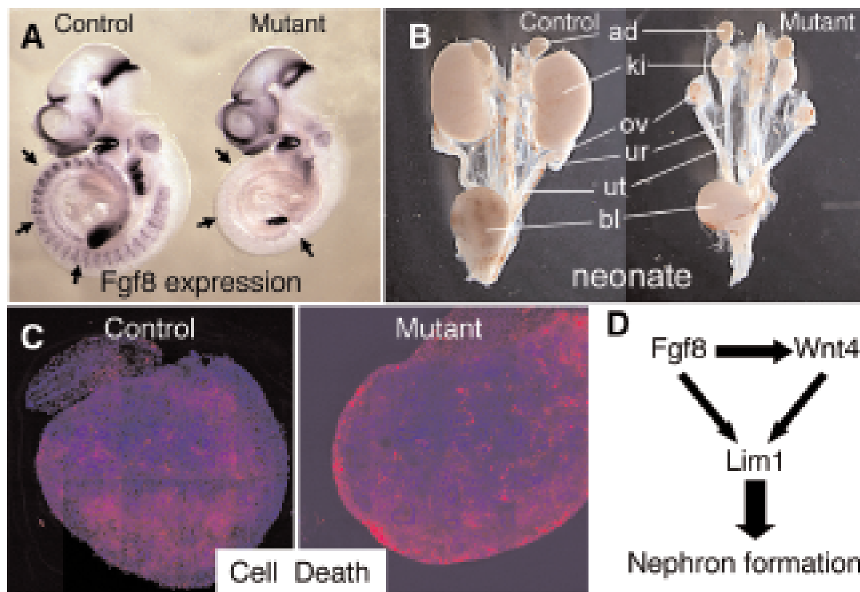

Figure 1. *A)* Somites (arrows) of mutant embryos at 9.5 days of gestation display no *Fgf8* expression. *B)* Kidneys of mutant neonates are hypoplastic and nonfunctional. *C)* A ring of aberrant cell death (red) occurs in mutant kidneys at 14.5 days of gestation, where nephrogenesis would normally be taking place. *D)* Data from mutant analysis and *in vitro* explant experiments were used to generate a model wherein FGF8 induces *Wnt4* gene expression and then both FGF8 and WNT4 are required for *Lim1* gene expression and nephrogenesis. ad, adrenal gland; bl, bladder; ki, kidney; ov, ovary; ur, ureter; ut, uterus.

We determined that *Fgf8* was expressed in the condensing mesenchyme and that mutants suffered aberrant apoptosis in the MM of the kidney cortex, preventing new nephron formation (Figure 1, part C). Besides this role as a survival factor for this progenitor population, we also found that FGF8 regulates the expression of specific genes crucial for normal kidney development. Microarray analysis of microdissected kidneys at 12.5 days gestation, when mutant and control kidneys cannot be distinguished grossly,

revealed a number of genes misregulated in the mutant tissue. Follow-up work led us to focus on two of these genes that proved to be pivotal to understanding the *Fgf8* kidney phenotype. One of these genes encodes the secreted signaling molecule WNT4 and the other the transcription factor LIM1. MM-specific loss of either gene causes an arrest in kidney development that resembles the T-Cre–mediated inactivation of *Fgf8.* By determining the expression pattern of each gene in mouse mutants lacking one of the other two genes, the epistatic relationship was determined. This information, along with *in vitro* explant experiments, indicated that both FGF8 and WNT sources are required in parallel for normal development, resulting in the model shown in Figure 1, part D.

It is intriguing that the FGF/WNT nexus we uncovered occurs in such processes as brain and limb development as well as during oncogenesis. Therefore, the task before us is to determine how the molecular interactions of these signaling pathways regulate normal development and how they cause disease when they go awry.

Alan O. Perantoni, PhD
Senior Investigator
Laboratory of Comparative Carcinogenesis
NCI-Frederick, Bldg. 538/Rm. 224
Tel: 301-846-6529
Fax: 301-846-5946
peranton@ncifcrf.gov

Mark B. Lewandoski, PhD
Investigator
Cancer and Developmental Biology Laboratory
NCI-Frederick, Bldg. 539/Rm. 105
Tel: 301-846-5510
Fax: 301-846-7117
mlewandoski@mail.ncifcrf.gov

TUMOR BIOLOGY

Controlling Angiogenesis Through Thrombospondin-1 Regulation of Nitric Oxide Signaling

Isenberg JS, Ridnour LA, Perruccio EM, Espey MG, Wink DA, and Roberts DD. Thrombospondin-1 inhibits endothelial cell responses to nitric oxide in a cGMP-dependent manner. *Proc Natl Acad Sci U S A* 102: 13141–6, 2005.

Preventing angiogenesis—the recruitment of new blood vessels—has become a major focus for cancer treatment and prevention. Angiogenesis is tightly regulated by a balance between pro- and antiangiogenic factors. The gaseous redox molecule nitric oxide (NO) is known to play a crucial role in blood pressure control, but it was also recently found to promote angiogenesis at physiological levels. Although the latter activity of NO is beneficial in wound-healing responses, it may also promote angiogenesis in tumors. Several pro- and two antiangiogenic factors have been shown to modulate the endothelial form of an enzyme that generates NO. Now, ongoing studies by our group and that of David Wink, PhD (Radiation Biology Branch), reveal that additional molecular targets involved in redox signaling are convergent nodes for signaling by a variety of antiangiogenic agents. One of these is the potent endogenous angiogenesis inhibitor thrombospondin-1, a drug mimetic of which (ABT-510) is currently in phase II clinical trials for cancer treatment. Expression of thrombospondin-1 is commonly diminished or absent in pathology specimens from several major cancers, and studies in mice showed that approximately 0.1 nM levels of circulating thrombospondin-1 can limit tumor growth and angiogenesis. Yet, previous studies using cultured vascular endothelial cells required 1 to 10 nM concentrations of thrombospondin-1 to inhibit their growth or movement.

Our collaborative studies have identified crosstalk between NO and thrombospondin-1 in endothelial cells that can explain this discrepancy. In the above, and in an accompanying paper (Ridnour LA et al. *Proc Natl Acad Sci U S A* 102: 13147–52, 2005), we describe novel mechanisms by which thrombospondin-1 inhibits angiogenesis stimulated by NO. In Isenberg et al., we show that low-dose NO increases the efficacy of thrombospondin-1 to inhibit endothelial cell growth, movement, and adhesion by a factor of 100 to 1,000. We show that this activity is shared by antibodies that recognize the thrombospondin-1 receptor CD36 and by recombinant parts of the thrombospondin-1 molecule known to interact with this receptor on endothelial cells. This inhibition is mediated by way of thrombospondin-1 blocking the NO-mediated activation of soluble guanylyl cyclase (sGC). This enzyme mediates the synthesis of cyclic-GMP (cGMP) in cells, an important molecule in signaling pathways leading to tumor angiogenesis (Figure 1, part A). By blocking the NO-mediated activation of sGC, thrombospondin-1 also blocks the ability of an angiogenic molecule produced by many tumors, vascular endothelial growth factor, to stimulate cGMP production in endothelial cells. Finally, using transgenic mice, we show that levels of cGMP in vascular endothelial cells are elevated in the absence of endogenous thrombospondin-1 and are more sensitive to further elevation in response to NO donors. Therefore, endogenous levels of thrombospondin-1 clearly limit NO signaling through this pathway in vascular cells.

In Ridnour et al., we show that NO and thrombospondin-1 form a feedback loop, whereby NO downregulates

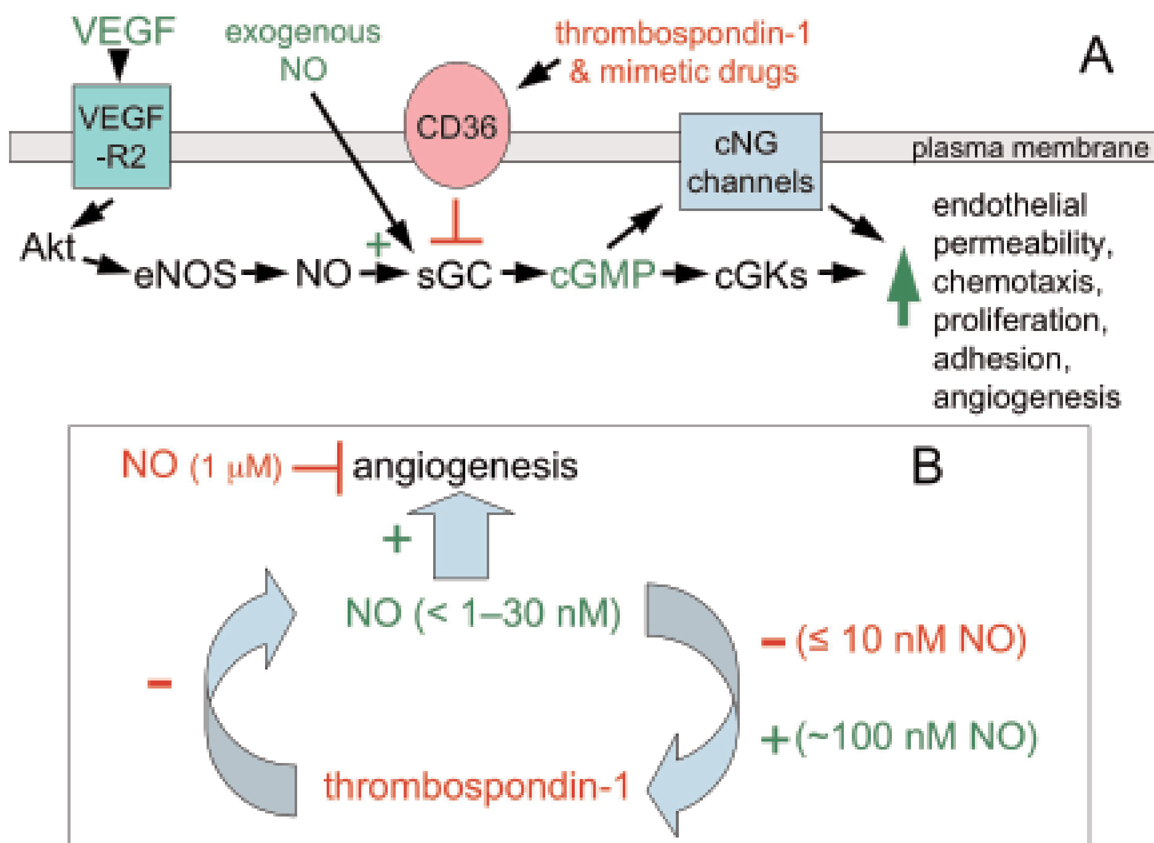

Figure 1. Crosstalk between thrombospondin-1 and nitric oxide (NO) controls angiogenesis. *A)* Angiogenic signaling induced by vascular endothelial growth factor (VEGF) through its receptor activates Akt, which in turn phosphorylates and activates endothelial nitric oxide synthase (eNOS). The resulting NO binds to and activates soluble guanylyl cyclase (sGC), leading to accumulation of intracellular cyclic-GMP (cGMP). cGMP binds to and activates kinases (cGKs) and cGMP-gated channels (cNG) to stimulate endothelial cell responses required for angiogenesis. Thrombospondin-1 inhibits sGC activation and thereby prevents angiogenic signaling. *B)* Complementing the blocking of NO signaling by thrombospondin-1, low pro-angiogenic doses of NO suppress thrombospondin-1 expression to remove this inhibitor and facilitate angiogenesis. At higher levels of NO, this feedback is reversed by induction of additional signals that restore expression of inhibitory thrombospondin-1 as well as direct inhibition of angiogenesis by NO-derived reactive nitrogen species.

thrombospondin-1 and thrombospondin-1 inhibits NO-stimulated pathways that induce angiogenesis (Figure 1, part B). At low levels of NO (1 nM), thrombospondin-1 expression is blocked at the mRNA and protein levels, facilitating the pro-angiogenic activity of NO. This inhibition is reversed at higher levels of NO via induction of the phosphatase MKP-1, engaging inhibitory feedback to limit the angiogenic response to NO. Finally, at high NO levels such as would be produced by activated macrophages (1 μM), angiogenesis is directly inhibited by NO via phosphorylation of p53. This finely tuned feedback mechanism appears to be critical to control both wound healing and tumor angiogenesis.

Our ongoing studies suggest that both tumor growth and wound healing processes, such as those secondary to surgical treatment of solid tumors, can be controlled by peptides derived from thrombospondin-1 that target NO signaling mechanisms. Our data and those from other recent publications show that nitric oxide synthase inhibitors can increase the efficacy of radiation and chemotherapy. Similarly, ABT-510, the drug mimetic of thrombospondin-1 mentioned earlier, binds to CD36 and enhances tumor responses to radiation and chemotherapy. The identification of this novel relationship between thrombospondin-1 and NO and the molecular mechanisms involved reveals new molecular targets for controlling angiogenic responses and could lead to novel treatment strategies combining these agents to increase cancer survival.

■ **David A. Wink, PhD**
Senior Investigator
Radiation Biology Branch
wink@mail.nih.gov

■ **David D. Roberts, PhD**
Senior Investigator
Laboratory of Pathology
NCI-Bethesda, Bldg. 10/Rm. 2A33
Tel: 301-496-6264
Fax: 301-402-0043
droberts@helix.nih.gov

TNF Produced by Distinct Types of Leukocytes: The Good and the Bad

Grivennikov SI, Tumanov AV, Liepinsh DJ, Kruglov AA, Marakusha BI, Shakhov AN, Murakami T, Drutskaya LN, Förster I, Clausen BE, Tessarollo L, Ryffel B, Kuprash DV, and Nedospasov SA. Distinct and nonredundant *in vivo* functions of TNF produced by T cells and macrophages/neutrophils: protective and deleterious effects. *Immunity* 22: 93–104, 2005.

Tumor necrosis factor-α (TNF-α) is a pleiotropic immunoregulatory cytokine initially brought to prominence because of its antitumor effects. It was later demonstrated that TNF is a critical mediator of host defense to bacterial infections, in particular, because of its role in granuloma formation. The "dark side" of TNF is best illustrated by its involvement in sepsis and in several autoimmune diseases with an inflammatory component. TNF is also involved in skin carcinogenesis and could be a critical player in other inflammation-induced cancers. Systemic TNF blockade represents a highly efficient therapy for patients with rheumatoid arthritis and Crohn's disease, and trials are under way to evaluate the efficacy of TNF blockers in psoriasis and in cancer.

Mice with complete or partial TNF ablation may serve as useful models to evaluate the consequences of TNF blockade. In particular, studies in mice have suggested the possibility of deleterious side effects to anti-TNF therapy, which has held true for a fraction of patients who have indeed developed various bacterial infections, including tuberculosis. We used Cre-loxP technology to generate a panel of novel mice with conditional TNF ablation in distinct types of immune cells. One possibility we wanted to evaluate was whether beneficial TNF could predominantly be coming from one cell type and harmful TNF from another.

In collaboration with Lino Tessarollo, PhD (Mouse Cancer Genetics Program, NCI-Frederick), and our sister lab at the Engelhardt Institute of Molecular Biology in Moscow, we generated mice with highly efficient and specific TNF ablation in cells of the innate immune system, such as macrophages and neutrophils (M-TNF mice), as well as in both major types of lymphocytes (T-TNF and B-TNF mice). All these mice have shown distinct phenotypes, indicating important and non-redundant functions *in vivo* for TNF produced by macrophages, T cells, and B cells (Figure 1).

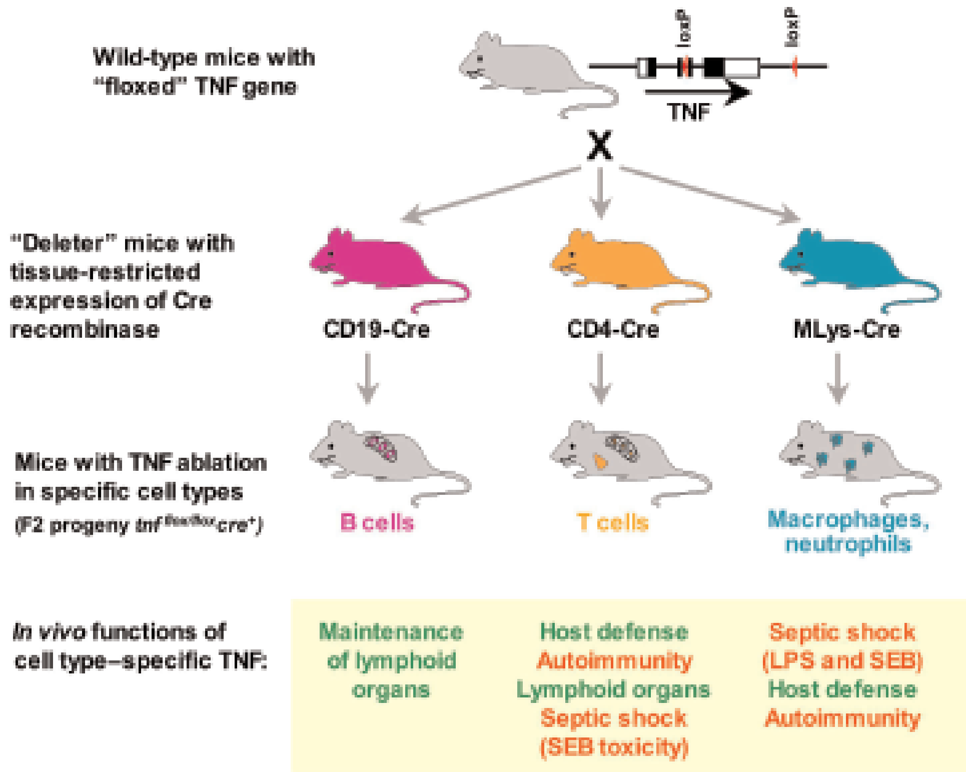

Figure 1. Tumor necrosis factor (TNF)–"floxed" mice were generated by homologous recombination in embryonic stem (ES) cells with subsequent removal of neo-cassette. Three different cell type–specific deleter mice were used to generate the experimental panel in the study. Beneficial (green) and detrimental (red) *in vivo* effects of TNF produced by various types of leukocytes are listed. LPS, lipopolysaccharide; SEB, staphylococcal enterotoxin B; MLys, macrophage lysozyme; loxP, target sequences for the site-specific Cre-recombinase.

Mice with TNF ablation in macrophages and neutrophils produced almost no detectable systemic TNF in response to lipopolysaccharide (LPS) and were protected from LPS–D-galactosamine (Dgal) liver toxicity. Under these challenges, both B-TNF and T-TNF mice had the wild-type phenotype. However, in models of toxicity in which T cells were activated by staphylococcal enterotoxin B (SEB) or concanavalin A (ConA), T-TNF mice showed protection from TNF-mediated toxicities. Thus, different toxic agents induce either macrophage/neutrophil or T cell–derived TNF.

Macrophage/neutrophil-derived TNF also turned out to be critical in resistance to the intracellular pathogen *Listeria monocytogenes*. Surprisingly, however, mice with TNF ablation only in T cells also showed defects in host defense against high doses of *L. monocytogenes*. Importantly, macrophages and neutrophils in T-TNF mice retained full ability to produce high levels of systemic TNF, as indicated by challenge experiments with LPS and other bacterial products. Why couldn't this abundant TNF compensate for the lack of TNF produced by T cells? What is the intrinsic non-redundant role of T cell–derived TNF? These questions remain to be answered. We hypothesize that T cells produce TNF in such a way that it either remains membrane bound or is released within the space of cell-to-cell contacts. A possible alternative is that in different *in vivo* situations, macrophages are desensitized and TNF may be produced only by T cells.

Although B-TNF mice had a wild-type phenotype in these challenge models, TNF produced by B cells is critically involved in providing maintenance signals for the organized lymphoid tissues, such as in the spleen (Endres R et al. *J Exp Med* 189: 159–68, 1999) or Peyer's patches (Tumanov AV et al. *J Immunol* 173: 86–91, 2004).

Thus, TNF produced by each type of immune cell analyzed in our study may be both good and bad, depending on the pathophysiological model. It is conceivable that the thresholds for protective and deleterious TNF functions may differ, and this could be exploited in future protocols of therapeutic TNF ablation.

Sergei I. Grivennikov, PhD
Visiting Fellow
Basic Research Laboratory
NCI-Frederick, Bldg. 567/Rm. 207
Tel: 301-846-6682
Fax: 301-846-7268
grivennikovs@ncifcrf.gov

Sergei A. Nedospasov, PhD, DSci
Investigator
Basic Research Laboratory

Present address:
Engelhardt Institute of Molecular Biology
Russian Academy of Sciences
Vavilov Street 32
Moscow 119991, Russia
Tel: +7-495-135-9964
Fax: +7-495-135-1405
snedos@online.ru

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dm368n@nih.gov
Tel: 301-846-1697

Carcinogenesis, Cancer and Cell Biology, Tumor Biology

Joseph A. DiPaolo, PhD
jd81a@nih.gov
Tel: 301-496-6441

Stuart H. Yuspa, MD
sy12j@nih.gov
Tel: 301-496-2162

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Frank M. Balis, MD
fb2y@nih.gov
Tel: 301-496-0085

Caryn Steakley, RN, MSW
cs397r@nih.gov
Tel: 301-435-3685

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Jonathan D. Ashwell, MD
ja9s@nih.gov
Tel: 301-496-4931

Jay A. Berzofsky, MD, PhD
jb4q@nih.gov
Tel: 301-496-6874

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levensd@mail.nih.gov
Tel: 301-496-2176

Carl Wu, PhD
cw1m@nih.gov
Tel: 301-496-3029

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Larry K. Keefer, PhD
keefer@ncifcrf.gov
Tel: 301-846-1467

Christopher J. Michejda, PhD
cm304t@nih.gov
Tel: 301-846-1216

Sriram Subramaniam, PhD
ss512h@nih.gov
Tel: 301-594-2062

Translational Research

Elise C. Kohn, MD
ek1b@nih.gov
Tel: 301-402-2726

Leonard M. Neckers, PhD
neckersl@mail.nih.gov
Tel: 301-496-5899

Virology

Vinay K. Pathak, PhD
vp63m@nih.gov
Tel: 301-846-1710

John T. Schiller, PhD
js153g@nih.gov
Tel: 301-496-6539

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