National Cancer Institute

CENTER FOR CANCER RESEARCH

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This new quarterly issue of *CCR Frontiers in Science* highlights selected articles from March through May 2006. The complete issues for these months can be viewed via the newsletter archives at http://ccr.cancer.gov/news/newsletter.asp.

Summer 2006
Volume 5

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FROM THE DIRECTOR

The Center for Cancer Research: Finding Opportunities, Facing Challenges

Robert H. Wiltrout, PhD
Director

In 2001, the NCI intramural Divisions of Basic Sciences and Clinical Sciences were merged to form the Center for Cancer Research (CCR). This reengineering was fueled by the rapid pace of biotechnology advancement and the growing need for multidisciplinary approaches to the complex scientific problems NCI researchers are increasingly tackling. CCR's mission is to reduce the burden of cancer through exploration, discovery, and translation. This integrated structure is intended to promote rapid bench-to-bedside translation of promising cancer therapies. In turn, results from the clinic are informing the work of laboratory investigators to further refine therapies. In CCR, we value high-quality, investigator-initiated research, but we are also challenging the customary ways of thinking and organizing, fostering cross-disciplinary and multi-institutional research to solve complex problems in cancer research.

Within the last year, research initiated and developed at the Center culminated in a number of notable advances, including a vaccine against cervical cancer, a promising new immunotherapy against melanoma and renal carcinoma, a U.S. Food and Drug Administration (FDA)–approved drug to treat oral mucositis, a protective agent to prevent hair loss in cancer patients undergoing radiotherapy, and a cutting-edge cancer-patient molecular profiling technology. These advances are having an impact on the NCI Challenge Goal of eliminating the suffering and death due to cancer by 2015 and improving the quality of lives of cancer survivors. At present, a number of additional therapies are working their way through clinical trials to reach the patients.

Going forward, we are leveraging our strengths to respond to emerging needs and opportunities as well as quickly establishing programs in high-priority areas. We are pursuing an interdisciplinary and multidisciplinary "team-science" approach to address the complexity of cancer research, exemplified by the formation of several Centers of Excellence. One example is the Center

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of Excellence in Immunology (CEI), created to foster discovery, development, and delivery of novel immunologic approaches to prevent and treat cancer and cancer-associated viral diseases. CEI's objectives include defining emerging opportunities, overseeing programs in specific areas in immunology and virology, and fine-tuning immunotherapeutic approaches in cancer treatment. The CEI sponsored a highly successful national conference in immunotherapy September 22–23, 2005, on the NIH campus.

We also are leveraging our significant strengths in the fields of immunology and carcinogenesis to address one of the major causes of cancer: chronic inflammation. In 2005, we launched the Inflammation and Cancer Initiative, which includes four key areas of investigative opportunity: cancer-prone chronic inflammatory diseases, innate and adaptive immunity, stem cells, and inflammation-related molecular targets.

Another guiding principle is the redeployment of existing resources into new and promising areas where CCR can make a distinct contribution. An excellent example of this is the realignment of the Laboratory of Experimental and Computational Biology to support NCI's nanotechnology effort, creating an Intramural Cancer Nanotechnology Program (ICNP). CCR investigators seized the opportunity in NCI's new National Advanced Technologies Initiative for Cancer, redirecting their scientific expertise to develop a research portfolio to complement the NCI Alliance for Nanotechnology in Cancer—especially the Nanotechnology Standards Laboratory and molecular targets/molecular oncology efforts.

While our challenges are many, the staff of CCR will continue to seek innovative solutions to the complex problems of cancer by leveraging our internal strengths, identifying new opportunities, and forging fruitful collaborations.

■ **Robert H. Wiltrout, PhD**
Director

CLINICAL RESEARCH

Radioimmunotherapy of Disseminated Peritoneal Disease Targeting HER2

Milenic DE, Garmestani K, Brady ED, Albert PS, Ma D, Abdulla A, and Brechbiel MW. Targeting of HER2 antigen for the treatment of disseminated peritoneal disease. *Clin Cancer Res* 10: 7834–41, 2004.

Radioimmunotherapy (RIT)—the delivery of therapeutic radionuclides to cancer cells via monoclonal antibodies (MAb)—has reemerged as a viable option for the treatment and management of cancer patients. The cell surface antigen, HER2, provides a molecular target to which site-specific, targeted radiation can be effectively delivered via a well-defined, U.S. Food and Drug Administration (FDA)–approved MAb (Herceptin). Monotherapy with Herceptin has resulted in a response rate of 12% to 20% in metastatic breast cancer patients. A large percentage of eligible patients, however, fail to respond to treatment and/or relapse. In addition to breast cancer, HER2 is overexpressed in ovarian cancers and 35% to 45% of all pancreatic adenocarcinomas. RIT offers an opportunity to complement and enhance Herceptin's intrinsic activity by direct incorporation of radiation into the treatment regimen.

It is hypothesized that α-emitters will be most effective in the therapy of metastatic, small lesion disease, vascular-based disease, and vascular targets of tumors. The energy emissions of α-particle decays (4–9 MeV) are discrete and directly deposited over a short distance in tissue (40–100 μm), resulting in a high linear energy transfer. The lethality of α-particle radiation may be at a dose rate as low as 1 cGy/h, and direct cell killing may be executed with as few as 3–7 ^{213}Bi molecules localized to the surface of a tumor cell. The short path length of the emission could also be advantageous in limiting toxicity to normal tissues adjacent to tumor.

The hypothesis for our study was that Herceptin radiolabeled with ^{213}Bi would be therapeutic in two ways. First, Herceptin-targeted ^{213}Bi treatment of disseminated peritoneal disease would be efficacious. Second, as a result of this demonstrated efficacy, Herceptin therapy targeting HER2 could be extended to the treatment of malignancies with low HER2 expression.

A series of *in vitro* and *in vivo* studies were conducted to validate Herceptin as a viable targeting vehicle of α-radiation. The integrity and immunoreactivity of the MAb were maintained following radiolabeling. *In vivo* studies confirmed that radiolabeled Herceptin was effective in targeting the HER2 molecule. When mice bearing 3 d tumor burdens intraperitoneally (i.p.) were administered therapeutic doses of ^{213}Bi-Herceptin (i.p.), a specific dose-dependent response of increased survival was observed (Figure 1). Consistent with the hypothesized merits of α- versus β^--emitting radionuclides, ^{213}Bi-Herceptin lacked efficacy against a larger 5 d tumor burden. The α-emitters are postulated to be ideal for the treatment of smaller tumors/tumor burdens, disseminated disease, and micrometastatic disease, whereas a β^--emitting radionuclide such as ^{90}Y is more appropriate for tumor lesions of about 1 cm or more. Determination of an obvious or real maximum tolerated dose of ^{213}Bi-Herceptin was elusive. None of the animals succumbed to radiation death at the maximum doses administered. Using animal weights as a harbinger of toxicity, mice that received 1 mCi of ^{213}Bi-Herceptin experienced the greatest weight loss. Based on these results, an effective dose of 500 to 750 μCi was established for use in future experiments. This decision was also based on

the desire to combine RIT with other modalities such as chemotherapeutics that would alter tumor sensitivity to the radiation. In the two i.p. tumor models used, the Herceptin vehicle alone failed to elicit any effect on the survival of the animals, a persuasive argument for the treatment of patients with α-particle RIT who are unresponsive to treatment with the unarmed MAb.

These studies demonstrated the feasibility of locoregional administration of a MAb to target a short-lived radionuclide for the treatment of disseminated peritoneal disease. The effectiveness of Herceptin radiolabeled with an α-emitting radionuclide is attributed to both the nature of the disease and accessibility of the tumor. RIT targeting of the HER2 molecule is appealing in that it may prove beneficial even for those patients with a lower expression of the receptor who would not normally be eligible for immunotherapy. Patients with a scoring of 2^+ or 3^+ are typically selected for treatment with Herceptin; as a consequence, a low percentage of patients are actually eligible to receive it. RIT with Herceptin would greatly expand the population eligible for treatment. α-Particle RIT offers the opportunity of complementing the intrinsic cytostatic therapeutic efficacy of Herceptin with high linear energy transfer radiation. Studies are currently under way in our labs examining the potential of combining modalities such as targeted radiation therapy with chemotherapeutics and radiosensitizers.

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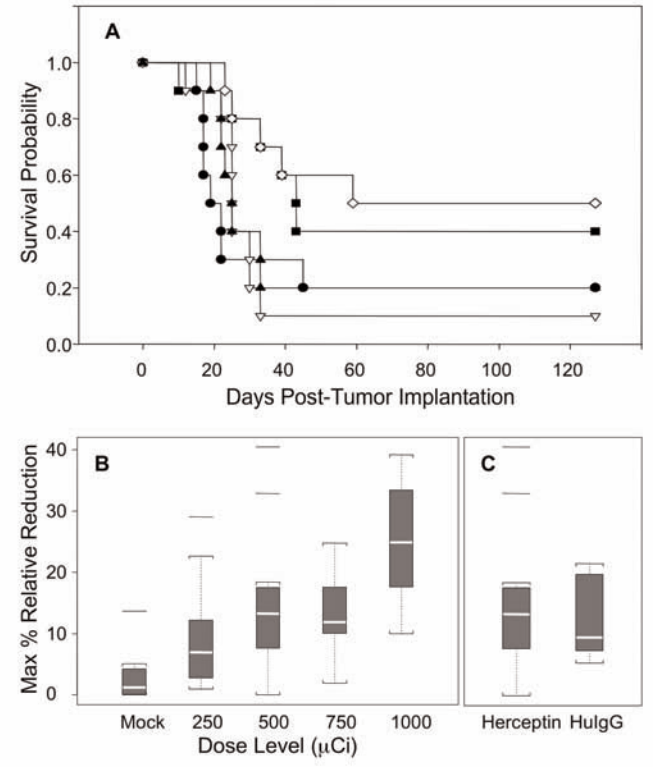

Figure 1. Increasing μCi doses of ^{213}Bi-CHX-A″-Herceptin (^{213}Bi-Herceptin) were administered intraperitoneally (i.p.) to mice bearing 3 d LS-174T i.p. xenografts. (Panel A: ●, mock-treated; ▽, 250 μCi; ■, 500 μCi; and ◇, 750 μCi ^{213}Bi-Herceptin. ▲, 500 μCi ^{213}Bi-HuIgG was used as a non-specific control.) Toxicity of radioimmunotherapy with ^{213}Bi-Herceptin was determined by monitoring the animal weights for 2–3 weeks following radioimmunotherapy (RIT). The maximum relative weight reduction was calculated for each of the treatment groups and presented as box plots (Panel B). Specificity of the effect of the radioimmunotherapy is illustrated with a comparison between the mice that received either 500 μCi ^{213}Bi-Herceptin or 500 μCi ^{213}Bi-HuIgG (Panel C). The light line is the median. The upper region of the box represents the third quartile. The lower portion is the first quartile. The brackets delineate 1.5 times the interquartile range, and the lines outside of the brackets represent outlying observations.

Keratinocyte Growth Factor Decreases Oral Mucositis Resulting from Intensive Therapy for Hematologic Malignancies

Spielberger R, Stiff P, Bensinger W, Gentile T, Weisdorf D, Kewalramani T, Shea T, Yanovich S, Hansen K, Noga S, McCarty J, LeMaistre CF, Sung EC, Blazar BR, Elhardt D, Chen M-G, and Emmanouilides C. Palifermin for oral mucositis after intensive therapy for hematologic cancers. *N Engl J Med* 351: 2590–8, 2004.

Keratinocyte growth factor (KGF) was identified and cloned in the Laboratory of Cellular and Molecular Biology, Division of Cancer Etiology, NCI, in the late 1980s. It was purified from fibroblast culture fluid based on its ability to stimulate DNA synthesis in a keratinocyte cell line and was subsequently shown to be active on a variety of epithelial cells, but not other cell types. KGF is a member of the fibroblast growth factor (FGF) family (FGF-7) and acts through a subset of FGF receptor isoforms (FGFR2b) that are expressed almost exclusively by epithelial cells.

KGF functions as a mesenchymally derived, paracrine mediator of epithelial homeostasis, with remarkable cytoprotective effects. The upregulation of KGF after epithelial injury suggests that it has an important role in tissue regeneration. In addition to stimulating repair, other studies demonstrated that the timely administration of recombinant KGF could prevent or reduce damage from a variety of toxic agents, including chemotherapy and radiation. In 1992, KGF technology was licensed to Amgen, Inc., for the development of therapeutic products. Among several potential applications, the decision was made to initially focus on the reduction of damage to the oral cavity that results from high-dose chemo/radiotherapy.

Oral mucositis is a major debilitating side effect of intensive cancer treatments. Severe oral mucositis is associated with pain, difficulty eating and speaking, and gastrointestinal bleeding. It has a negative effect on patients' quality of life and often results in a delay or reduction in cancer therapy. Until now, there has been no effective way to prevent or limit this condition. Encouraging results were obtained with KGF in a series of clinical trials, leading to a pivotal phase 3 trial reported in the *New England Journal of Medicine* (referenced above).

Patients in this study received autologous peripheral blood progenitor cell transplants for hematologic malignancies. Prior to the transplants, they were treated with a standard combination of fractionated total body irradiation for 3 or 4 days, followed by VP-16 and cyclophosphamide. Patients received either the vehicle control or KGF (60 micrograms/kg/day) in three daily intravenous injections both before the start of radiation and after chemotherapy. Clinical staff monitored the appearance of the patients' mouths on a daily basis. Severe mucositis was characterized by widespread erythema and ulceration in the oral cavity, and the ability to eat either only a liquid diet or nothing at all. Additional information was gathered from hospital records and from patients' diaries about their health.

KGF markedly reduced the duration of severe oral mucositis: the placebo group averaged 9.0 days, whereas the KGF cohort averaged only 3.0 days ($P < 0.001$). The incidence of severe oral mucositis also was significantly lower in the KGF group, 63% versus 98% for the placebo. This effect was due to a decline in the most debilitating form of mucositis, associated with an inability to eat, that corresponded to 62% of the placebo population but only 20% of the KGF group. Consistent with the decline in mucositis, there was a substantial reduction in the amount of analgesic medicine required by patients treated with KGF ($P < 0.001$), and a decrease in the use of total parenteral nutrition to supplement oral intake ($P < 0.001$). These favorable results were corroborated by the patients' reports of mouth/throat soreness and functional status (e.g., ability to drink, eat, talk, and sleep). Furthermore, patients treated with KGF were less likely to experience episodes of febrile neutropenia, reinforcing the idea that a decrease in damage to the mucosa would reduce infection. Side effects of KGF were mild to moderate in severity, transient, and attributable to its pharmacologic action on skin and oral epithelium (e.g., rash, pruritis, erythema, and taste alteration).

Based on these results, the U.S. Food and Drug Administration (FDA) approved KGF[1] to reduce severe oral mucositis in patients with hematologic malignancies who were receiving chemotherapy and radiation prior to autologous bone marrow/peripheral blood progenitor cell transplants. Approximately 10,000 adults in the United States undergo transplantation each year. Additional clinical trials have been initiated to test the safety and efficacy of KGF in the solid tumor setting, particularly head/neck, lung, and colorectal carcinomas. Positive results in these populations could lead to a substantial increase in the number of patients treated with KGF. By decreasing the toxicity of therapeutic agents, KGF might also foster the development of more potent and effective cancer treatments.

[1] Palifermin is the generic name for KGF in the clinic, and Kepivance™ is the trade name of the product from Amgen that went on the market in January 2005.

Note: As a co-inventor on patents pertaining to KGF, the author acknowledges that he has a financial interest in its commercial development.

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Metastasis Susceptibility

Park YG, Zhao X, Lesueur F, Lowy DR, Lancaster M, Pharoah P, Qian X, and Hunter KW. Sipa1 is a candidate for underlying the metastasis efficiency modifier locus Mtes1. *Nat Genet* 37: 1055–62, 2005.

Metastasis, the final stage of cancer progression and the source of most cancer-related mortality, is usually thought to be the result of oncogenic mutation and somatic evolution of tumor cells, either within the primary tumor mass or at distant sites. This hypothesis, while consistent with much of the data, does not entirely explain all experimental and clinical observations. Additional variables that contribute to metastatic progression therefore need to be identified and investigated to develop a more comprehensive model of the terminal stages of cancer progression.

One variable that may contribute to the complexity of this process is genetic background. Germline polymorphism has long been associated with human cancer risk. Much of human molecular epidemiology is based on the premise that certain constitutional polymorphisms are associated with different susceptibilities. Numerous examples of this type of cancer-associated variation also exist in experimental organisms, and hundreds of modifier or quantitative trait loci have been mapped in the mouse and rat that influence a wide variety of pathological conditions common in human populations, including disorders such as cancer, diabetes, and hypertension. These data suggest that many, probably most, phenotypes have a significant genetic contribution, even traits as complex as tumor dissemination.

Evidence supporting the role of germline polymorphism in metastasis has come from the recent identification of a candidate for the metastasis efficiency modifier locus, *Mtes1*. Previous genetic studies demonstrated the presence of

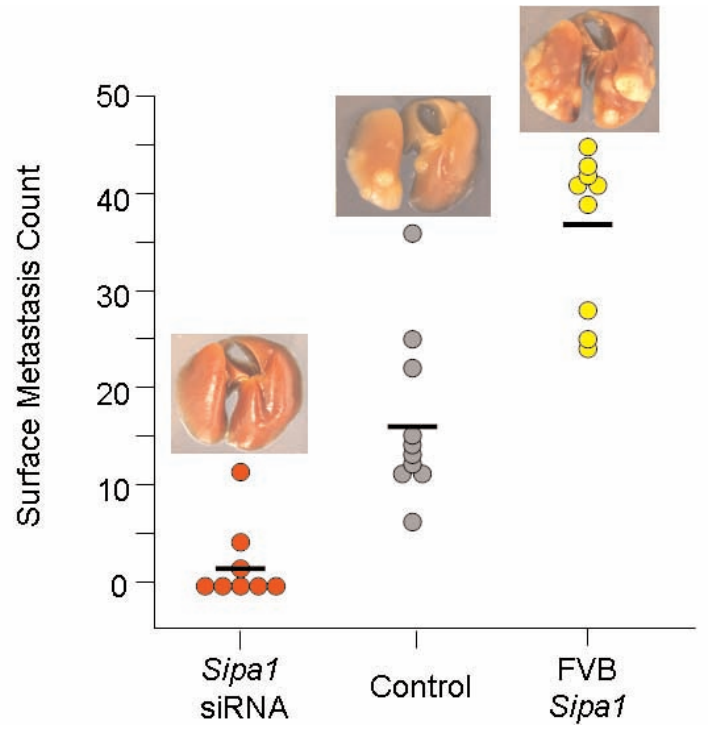

Figure 1. Modulation of *Sipa1* levels significantly influences the metastatic capacity of a highly metastatic mouse mammary tumor cell line. Wild-type cells (center), cells overexpressing (right; FVB *Sipa1*), or cells silenced (left; siRNA) for the *Sipa1* gene were implanted into the flanks of mice and allowed to develop into tumors. The metastatic capacity was then determined by counting lung surface nodules. Representative images of the lungs from each group are shown above the scatterplots. The black bars represent the median value for each experimental group.

polymorphic genes in the mouse genome that suppressed the ability of a highly aggressive transgene-induced mammary tumor to metastasize to the lung. Genetic mapping studies revealed that one of these metastasis-suppressing genes, designated *Mtes1,* was located on proximal mouse chromosome 1, in a region orthologous to human chromosome 11q13. Genomic analysis of the region identified several interesting polymorphic genes, and subsequent *in vitro* and *in vivo* experiments identified a polymorphism in the negative regulator of Rap1 GTPase, *Sipa1,* which significantly influenced its protein-protein interactions and enzymatic function. In tumor cell lines, the rate of metastases was increased by *Sipa1* overexpression and decreased by knocking down its expression in spontaneous metastasis assays in mice (Figure 1). The potential role of *SIPA1* in human metastasis was investigated by examination of publicly available gene-expression profiles, which revealed higher expression of *SIPA1* in metastatic prostate cancer compared with localized tumor, in agreement with the mouse data. *In toto,* the mouse results strongly support the concept of naturally occurring genetic variants playing an important role in the final, lethal stages of cancer, and the human data implicate *SIPA1* in the metastatic process in human cancer.

The existence of these polymorphic metastasis-susceptibility genes may have a significant impact on clinical prognosis. At present, evidence of lymph node metastasis is one of the most powerful prognostics for disease course in breast cancer. However, about 30% of women who are node negative at diagnosis develop metastatic disease, whereas 30% of women who are node positive are disease free a decade after local

therapy. As a result, many women who do not benefit from aggressive systemic treatment may be receiving adjuvant therapy, with its associated side effects and morbidity. Conversely, there may be individuals who would benefit from systemic adjuvant therapy but are not treated due to the apparent localized nature of the tumor. Identification and screening of allelic variants of metastasis-susceptibility genes may therefore significantly improve patient stratification based on inherited risk assessment instead of lymph node status. This may ultimately enable more accurate tailoring of treatment, and thereby reduce the overall morbidity and mortality of cancer.

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■ TUMOR BIOLOGY

Studying Tumor-host Interactions Reveals a Novel Mechanism for the Activity of TIMP-2

Feldman AL, Stetler-Stevenson WG, Costouros NG, Knezevic V, Baibakov G, Alexander HR Jr, Lorang D, Hewitt SM, Seo DW, Miller MS, O'Connor S, and Libutti SK. Modulation of tumor-host interactions, angiogenesis, and tumor growth by tissue inhibitor of metalloproteinase 2 via a novel mechanism. *Cancer Res* 64: 4481–6, 2004.

Tumor growth, invasion, and metastasis are the results of a complex series of interactions between tumor cells and the cells that make up the host microenvironment. Each of the cell types involved in this process has the potential to influence the other cell types through secreted cytokines and through alterations of the environment, such as changes in pH and oxygen content. This complex interplay is extremely difficult to model in an *in vitro* system. This difficulty led us to develop an *in vivo* model system that would allow these interactions to be studied at both a genomic and proteomic level.

The model is based on altering the expression of a single factor by the tumor cell using retroviral transduction and studying the effects of this change on the surrounding host environment and on the tumor cells themselves growing *in vivo*. The effects seen *in vivo* can then be compared to the differences seen between the altered cell line and the wild-type parental cell line *in vitro;* those changes unique to the *in vivo* observations can be ascribed to a relationship between the tumor and the host.

To test this model system, we chose to study tissue inhibitor of metalloproteinase 2 (TIMP-2). TIMP-2 is an endogenous protein present in a variety of tissues and characterized by its ability to both block metalloproteinase activation in the extracellular matrix and to inhibit the development of blood vessels. Previous work has demonstrated that these two activities may be attributable to separate domains of the TIMP-2 protein (i.e., truncated forms of the protein have different activities, with one portion inhibiting metalloproteinase activity and an alternative portion inhibiting angiogenesis). To identify the pathways through which TIMP-2 mediates its antiangiogenic activity *in vivo,* we applied the following experimental design.

A murine colon cancer line, MC38, was chosen because of its ability to form significant tumor neovasculature when grown as subcutaneous tumors in syngeneic BL/6 mice. The *TIMP-2* gene was cloned into a retroviral vector, and MC38 cells were transduced with either a TIMP-2–expressing retrovirus or a null retrovirus control. Clones were selected, and a high-expressing TIMP-2 clone was chosen for further study, which was identified as MET-11. MET-11 and the null retrovirus–transduced tumor line, MEX, demonstrated no difference in their *in vitro* growth characteristics. MET-11 and MEX cells were then injected subcutaneously into BL/6 mice and were allowed to grow for 18 days. Between day 6 and day 18, MET-11 tumors were significantly smaller than their MEX or wild-type counterparts and had significantly less vascularity as determined by immunohistochemical staining of the tumors with CD31 antibody and vessel counts. This observation was consistent with the known antiangiogenic activity of TIMP-2. Tumors were harvested at day 6 and day 12. RNA was extracted from both MET-11 and MEX tumors, and cDNA microarray analysis was performed. A comparison was also made between MET-11 and MEX cells grown *in vitro*. Figure 1 depicts the array analysis schema.

To identify differentially expressed genes between MET-11 and MEX tumors grown *in vivo,* we chose day 6 for analysis, as this was a time point in which both MET-11 and MEX tumors were of similar size. We hypothesized that the gene differences seen here have a cause-effect relationship on the change in growth characteristics seen between day 6 and day 18. Gene expression changes were also compared at day 12 to look for those genes that were persistently altered in expression between MET-11 and MEX tumors. Those genes that were altered between MET-11 and MEX at day 6 and persisted through day 12 *in vivo* but were not altered *in vitro* were selected for further study.

We found 13 genes to be differentially expressed between MET-11 and MEX

tumors that fulfilled our criteria of greater than 2-fold up- or downregulation at both day 6 and day 12 *in vivo* and no differential expression *in vitro*. Among these genes, *PTPN16 (MKP1)* was found to be upregulated to the greatest degree in MET-11 tumors compared to MEX tumors at day 6. PTPN16 is a protein-tyrosine-phosphatase that dephosphorylates p38 MAP kinase, thus inactivating it. p38 MAP kinase is known to play an important role in both vascular endothelial grow factor (VEGF) and basic fibroblast growth factor (bFGF) signaling, and therefore, its modulation may be important with respect to TIMP-2's angiogenic inhibitory activity.

We sectioned MET-11 and MEX tumors at day 6 and analyzed the levels of protein expression for PTPN16, p38 MAP kinase, and phosphorylated p38 MAP kinase. We found, in concordance with the RNA data, that PTPN16 expression was significantly elevated in TIMP-2–expressing tumors (MET-11) compared with their null-transduced counterparts (MEX). In addition, whereas total p38 levels were similar in both tumors, the proportion of phosphorylated p38 was significantly reduced in the MET-11 TIMP-2 overexpressers. This observation fit with the increased levels of PTPN16. To test whether increased expression of PTPN16 and therefore decreased phosphorylation of p38 led to the impaired growth we saw in TIMP-2 overexpressing tumors, we inoculated BL/6 mice with 1×10^6 MET-11 tumor cells in their flank. Tumors were allowed to grow for 14 days, at which time mice were divided into two groups: one group received systemic phosphate-buffered saline (PBS) injections from day 14 until day 25, whereas the other group received systemic injections of orthovanadate (a phosphatase inhibitor) over the same time period. Tumors growing in the mice receiving orthovanadate grew significantly larger than did those in the mice receiving the PBS control. Tumors harvested from mice receiving orthovanadate compared with tumors harvested from mice receiving PBS showed increased phosphorylation of p38 MAP kinase consistent with an inhibition of PTPN16 activity.

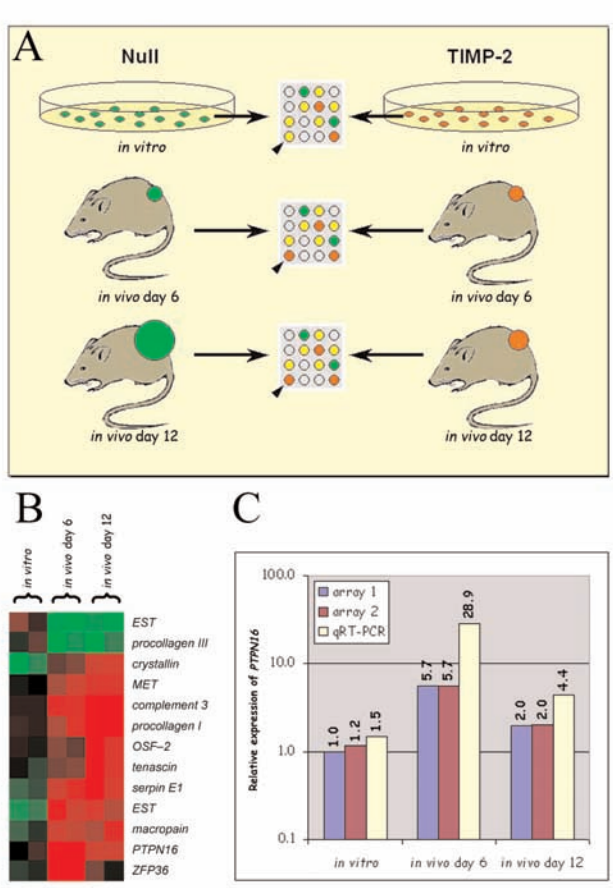

Figure 1. Identification of genes associated with the host response to tissue inhibitor of metalloproteinase 2 (TIMP-2). *A)* Strategy for comparing gene expression patterns of MC38/null and MC38/TIMP-2 tumor cells *in vitro* and *in vivo*. Using cDNA microarrays, MC38/null (green) and MC38 TIMP-2 (red) were compared *in vitro* and *in vivo* after 6 or 12 days of growth. Genes associated with tumor-host interactions due to TIMP-2 might be similarly expressed *in vitro* (e.g., yellow spot in lower left corner of top array, arrowhead), but differentially expressed *in vivo* (red spot in lower left corner of bottom two arrays, arrowheads). *B)* cDNA microarray analysis identified 13 such genes. Each pixel represents the expression ratio on one array. Red indicates upregulation in the MC38/TIMP-2 sample, and green indicates downregulation. Color intensity is proportional to expression ratio. Black represents ratios close to 1.0. *C)* Microarray and qRT-PCR data for *PTPN16*, the murine gene for mitogen-activated protein (MAP) kinase phosphatase-1 (MKP-1). Expression ratios were close to 1.0 *in vitro* but showed upregulation in MC38/TIMP-2 tumors *in vivo*.

This model system, which allowed us to study tumor host-interactions, led us to hypothesize a new mechanism of action for TIMP-2 with respect to its effects on tumor blood vessel growth. TIMP-2 upregulates the expression of PTPN16, resulting in a decrease in the phosphorylation status of p38 MAP kinase. Inactivation of p38 MAP kinase inhibits the ability of VEGF and bFGF to signal through their receptors. Since VEGF and bFGF are important mitogens for endothelial cell proliferation, this inhibition would be expected to impair the ability of a tumor to develop a blood supply. This model system can be used to study other genes to identify their *in vivo* mechanisms of action and represents a technique for applying both genomic and proteomic approaches to the study of tumor-host interactions.

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The Slinky as a Ubiquitous Pathogen Recognition Structure

Bell JK, Botos I, Hall PR, Askins J, Shiloach J, Segal DM, and Davies DR. The molecular structure of the Toll-like receptor 3 ligand-binding domain. *Proc Natl Acad Sci U S A* 102: 10976–80, 2005.

When considering antigen recognition, antibodies and T-cell receptors, the receptors of the adaptive immune system, typically come to mind. However, immunologists have known for some time that other, innate forms of antigen recognition must exist, since infectious agents are held in check prior to the development of adaptive immune responses. The most dramatic demonstration of the innate response is the ability of immunodeficient mice that lack antibodies or T-cell receptors to survive in non-sterile environments. In both immunodeficient and normal mice, pathogen invasion results in the immediate recruitment of phagocytes and other immune cells that ingest the pathogen, produce toxic substances that kill it, or both. So how, in the absence of T cells and antibodies, are these pathogens recognized? Recently, a family of homologous proteins known as the Toll-like receptors (TLRs) was shown to serve just such a pathogen-recognition function. The TLRs were discovered as homologs of the *Drosophila* receptor Toll, an essential component of the immune response to fungi in flies, and it is now known that similar molecules serve immune functions throughout the animal and plant kingdoms. In humans, 10 TLR paralogs recognize a wide variety of "pathogen-associated molecular patterns" (PAMPs), including lipids, proteins, carbohydrates, and nucleic acids from bacteria, parasites, and viruses. We asked how only 10 germline-encoded molecules are able to recognize such a wide variety of structures at the molecular level.

The TLRs are type I integral membrane receptors, each consisting of an N-terminal extracellular PAMP-binding domain, a single transmembrane helix, and a C-terminal, cytoplasmic signaling domain. Our approach was to express large amounts of the extracellular domains (ECDs) of each TLR for X-ray crystallographic analysis and ligand-binding studies. In the paper cited above, we presented the first crystal structure of a TLR ECD, the unliganded form of TLR3-ECD. TLR3 responds to dsRNA from viruses, and we found that purified TLR3-ECD protein binds pI:pC (a dsRNA surrogate) in solution.

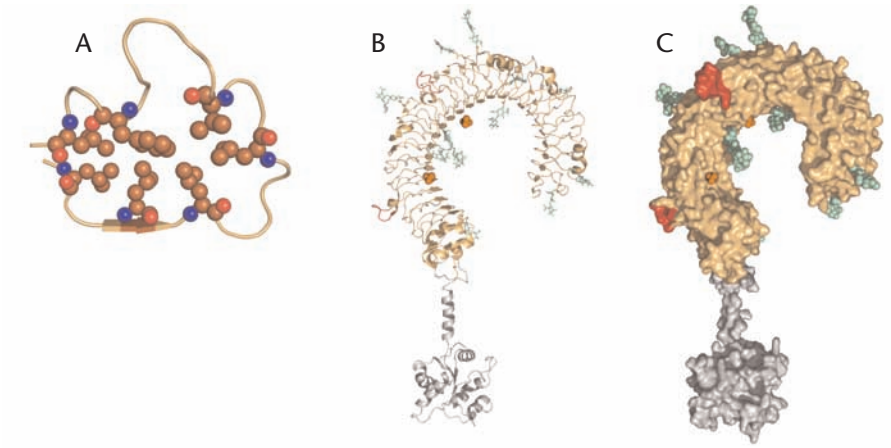

Figure 1. Structure of the Toll-like receptor 3 (TLR3) extracellular domain (ECD) and model of the full-length receptor. *A)* A single leucine-rich repeat (LRR) loop highlighting conserved hydrophobic side chains (brown spheres) that form the core of the solenoid. *B)* A cartoon trace showing the curved solenoid, or "slinky" shape of the ECD. β-strands are shown as arrows on the concave surface of the ECD. *C)* A surface rendering of TLR3. In *B* and *C*, glycans are shown in green, sulfate ions in orange, and insertions in LRRs 12 and 20 in red. Transmembrane and cytoplasmic domains, based on previously reported structures, are shown in gray.

The TLR3-ECD consists of 23 tandem repeats of a motif known as the leucine-rich repeat (LRR). In three dimensions, each LRR forms a loop, with consensus hydrophobic residues pointing inward, forming a stabilizing hydrophobic core (Figure 1, part A). When hooked together, the LRRs create a large solenoid in the shape of a horseshoe; overall, the TLR3-ECD can be described as a 23 turn "slinky" (Figure 1, part B). The concave inner surface consists of a large parallel β-sheet, with each β-strand roughly perpendicular to the solenoid axis and linked to the next strand by an irregular loop. LRR12 and LRR20 contain large insertions (Figure 1, parts B and C, highlighted in red). Since these insertions are unique to TLR3 and are conserved in all known mammalian TLR3 orthologs, they likely play important roles in TLR3 function, perhaps in ligand binding. The molecular surface of the TLR3-ECD is abundantly and unevenly populated with N-linked carbohydrates. However, one surface of the ECD is devoid of carbohydrate and free to interact with either ligand or another protein molecule (Figure 1, part C). In the absence of a TLR3-dsRNA complex structure, we can only speculate where ligand binding occurs. However, the presence of bound sulfate molecules from the crystallization medium (Figure 1, parts B and C) provides clues. The sulfate ions mimic the phosphate residues from the nucleotide backbone of a dsRNA molecule, indicating areas of the receptor that are capable of recognizing ligand.

The binding of PAMPs by TLRs triggers inflammatory processes that can have either beneficial or detrimental consequences. Understanding how the recognition of pathogens by TLRs occurs should aid in the development of TLR agonists and antagonists for use as adjuvants in vaccine development, or as anti-inflammatory drugs.

This project is a collaboration between the laboratories of David Segal, PhD, Experimental Immunology Branch/ National Cancer Institute (NCI), and David Davies, PhD, Laboratory of Molecular Biology/National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), with help from an intramural biodefense award from the National Institute of Allergy and Infectious Diseases (NIAID).

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■ MOLECULAR BIOLOGY

How Selenium Makes Its Way into Protein as Selenocysteine, the 21st Amino Acid in the Genetic Code

Carlson BA, Xu X-M, Kryukov GV, Rao M, Berry MJ, Gladyshev VN, and Hatfield DL. Identification and characterization of phosphoseryl-tRNA$^{[Ser]Sec}$ kinase. *Proc Natl Acad Sci U S A* 101: 12848–53, 2004.

In 1970, a kinase activity that phosphorylated a minor species of seryl-tRNA to form phosphoseryl-tRNA was observed in rooster liver (Maenpaa PH and Bernfield MR. *Proc Natl Acad Sci U S A* 67: 688–94, 1970), and a minor species of seryl-tRNA that decoded the termination codon UGA was observed in bovine and chicken livers (Hatfield D and Portugal FH. *Proc Natl Acad Sci U S A* 67: 1200–06, 1970). The seryl-tRNA in both cases was subsequently identified by us as selenocysteine (Sec) tRNA$^{[Ser]Sec}$, but despite many efforts, the kinase activity remained elusive. Sec is now regarded as the 21st amino acid in the genetic code, marking the first expansion to the code since it was deciphered by Marshall Nirenberg and collaborators at the NIH in the 1960s.

The biosynthesis of Sec, unlike the 20 other amino acids in the genetic code, occurs on its tRNA, and it is the pathway by which the element selenium makes its way into protein. A stem-loop structure in the 3′-untranslated region of selenium-containing (selenoprotein) genes is responsible for recoding UGA for Sec, which circumvents the normal function of UGA as a stop codon in protein synthesis. The stem-loop structure in the selenoprotein genes is recognized by a specific factor, designated SBP2, that forms a complex with Sec tRNA$^{[Ser]Sec}$ and its specific elongation factor and inserts

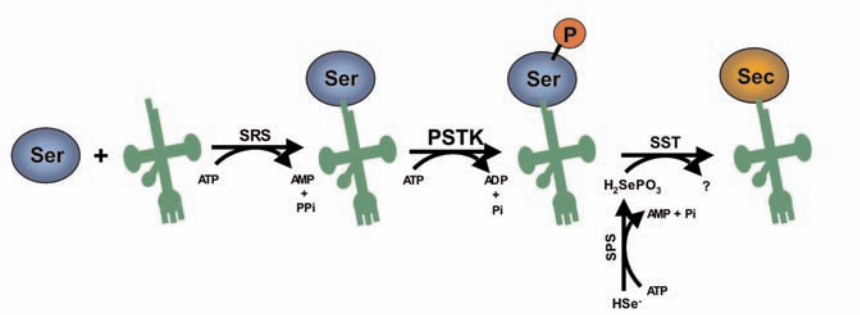

Figure 1. Proposed pathway of Sec biosynthesis on its tRNA in mammalian cells. Serine (Ser, shown in blue as an oblong circle) is attached to tRNA$^{[Ser]Sec}$ (shown in green as a cloverleaf structure) by seryl-tRNA synthetase (SRS) to form seryl-tRNA$^{[Ser]Sec}$ (shown in blue as serine attached to tRNA$^{[Ser]Sec}$) and is then phosphorylated by phosphoseryl-tRNA kinase (PSTK) to form the intermediate phosphoseryl-tRNA$^{[Ser]Sec}$ (P, shown in red as a circle attached to serine). The phosphate on phosphoseryl-tRNA$^{[Ser]Sec}$ is then replaced by the selenium donor that is likely activated by selenophosphate synthetase (SPS), and the compound is converted to selenocysteyl-tRNA$^{[Ser]Sec}$ (Sec, shown in gold as an oblong circle attached to tRNA$^{[Ser]Sec}$) by Sec synthase (SST).

Sec into protein in response to UGA. Although many factors dedicated to the insertion of selenium into protein as Sec have been identified, the biosynthesis of Sec in eukaryotes and the role of phosphoseryl-tRNA$^{[Ser]Sec}$ have not been resolved.

Using a comparative genomics approach that searched completely sequenced archaeal genomes for a kinase-like protein with the pattern of occurrence similar to that of components of the Sec insertion machinery, we detected a candidate gene for mammalian phosphoseryl-tRNA$^{[Ser]Sec}$ kinase *(pstk)*. Mouse *pstk* was cloned, and the gene product (PSTK) was expressed and characterized. PSTK specifically phosphorylated the seryl moiety on seryl-tRNA$^{[Ser]Sec}$ and in addition had a requirement for ATP and Mg^{++}. Proteins with homology to mammalian PSTK occur in the fruit fly, *Drosophila,* the nematode, *Caenorhabditis elegans,* and in the archaea, *Methanopyrus kandleri* and *Methanococcus jannaschii.* This suggests a conservation of its function across archaea and eukaryotes that synthesize selenoproteins, and the absence of this function in bacteria, plants, and yeast. The fact that PSTK has been highly conserved in evolution suggests that it plays an important role in selenoprotein biosynthesis and/or regulation.

The recent identification of the means by which cysteine (Cys) is synthesized on its tRNA in some archaea provides an excellent model of how Sec is biosynthesized on its tRNA. Cys RNA^{Cys} is aminoacylated by phosphoserine to form phosphoseryl-tRNA^{Cys} that in turn is converted to cysteyl-tRNA^{Cys} by an enzyme that replaces the phosphate on serine with an activated form of sulfur (Sauerwald A et al. *Science* 307: 1969–72, 2005). Since phosphoserine is attached

to $tRNA^{[Ser]Sec}$, it would seem to be the most likely intermediate in Sec biosynthesis wherein selenium would be activated by selenophosphate synthetase, an enzyme previously identified in mammals. This pathway of Sec biosynthesis is shown in Figure 1. Interestingly, Sec $tRNA^{[Ser]Sec}$ has a dual role of serving as the carrier molecule for the biosynthesis of Sec and as the adaptor molecule for decoding UGA for the insertion of Sec into protein.

It should also be noted that selenium is an essential micronutrient in the diet of mammals. Numerous health benefits have been associated with selenium, such as preventing cancer and heart disease, delaying the aging process, and delaying the onset of AIDS in HIV-positive patients, as well as beneficial roles in male reproduction, immune function, and development. Most, if not all, of these health benefits are due to selenoproteins. Thus, it is of paramount importance to determine how this element makes its way into protein. The identification and characterization of PSTK provides a major step in establishing the pathway of Sec biosynthesis.

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■ CELL BIOLOGY

Modifying Chromatin to Protect the Genome

Shroff R, Arbel-Eden A, Pilch D, Ira G, Bonner WM, Petrini JH, Haber JE, and Lichten M. Distribution and dynamics of chromatin modification induced by a defined DNA double-strand break. *Curr Biol* 14, 1703–11, 2004.

Cells respond to a double-strand break (DSB) in their DNA by phosphorylating chromatin in a large region surrounding the break site. In post-replicative cells, this modification promotes the *de novo* deposition of cohesin, a multiprotein complex that is normally loaded onto chromosomes during replication. DSB-induced cohesin loading is likely to tether break ends close to the sister chromatid, facilitating repair and helping the cell to maintain genome integrity.

DSBs induce the rapid phosphorylation of the H2AX isoform of histone H2A to form γH2AX (Rogakou EP et al. *J Biol Chem* 273: 5858–68, 1998), which is thought to play an important role in break repair (Fernandez-Capetillo O et al. *DNA Repair* 3: 959–67, 2004). The study cited at the top of this article (Shroff R et al. *Curr Biol* 14, 1703–11, 2004) and a second study (Ünal E et al. *Mol Cell* 16: 991–1002, 2004) provide a picture of one way that γH2AX protects the genome from damage.

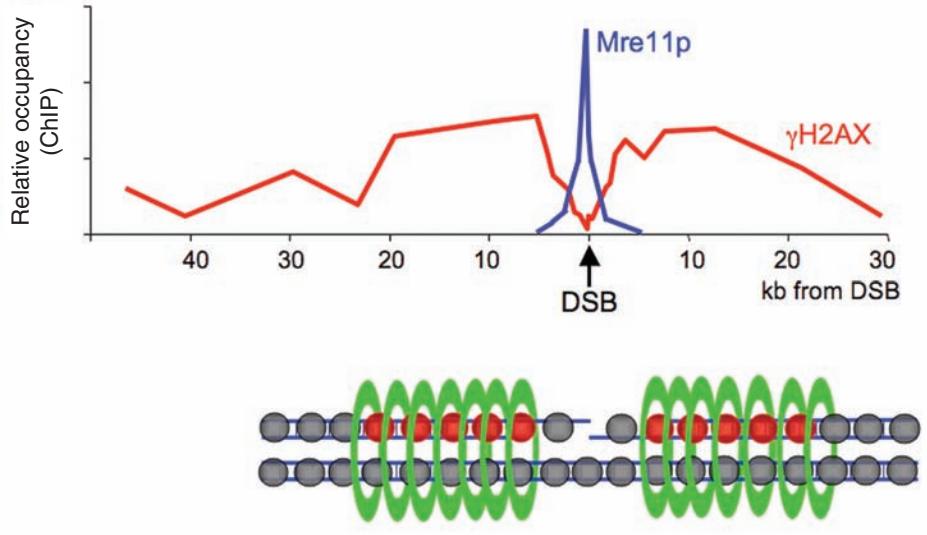

Figure 1. γH2AX recruits cohesin to damage sites. Top—experimental data showing the broad region where γH2AX (red) forms; in contrast, repair proteins (here Mre11p, blue) bind in a narrow region. Bottom—nucleosomes containing γH2AX (red) recruit cohesin complexes (green rings), which use the unbroken sister chromatid to "splint" broken ends together, while leaving the ends themselves free for repair. ChIP, chromatin immunoprecipitation; DSB, DNA double-strand break.

Shroff R et al. used chromatin immunoprecipitation (ChIP) to probe γH2AX formation and recruitment of the repair protein Mre11p at a site in the budding yeast genome where breaks can be formed in a controlled manner. The relative contribution of the two yeast damage-response kinases, Tel1p (ATM homolog) and Mec1p (ATR homolog), to H2AX phosphorylation was also determined.

A panel of mutants in DNA damage response/repair genes was used to show that both Tel1p and Mec1p phosphorylate H2AX. Mutants blocking steps further down the DNA response/repair pathway had no effect on γH2AX formation, confirming that γH2AX formation is part of the initial DNA damage response. In the G_1 phase of the cell cycle, Tel1p was responsible for most γH2AX formation, a finding similar to those obtained in studies of mammalian cells.

ChIP analysis showed that γH2AX and Mre11p occupy distinct regions around the induced DSB. Mre11p, like other repair proteins, bound to sites directly adjacent to the DSB (within 1–2 kb). Conversely, γH2AX was present in a 40–50 kb region surrounding the break site. γH2AX was most abundant in a 3–5 kb band on either side of the break, with

significant levels up to 25 kb from the peak site. Remarkably, very little γH2AX was detected at sites within 1–2 kb of the DSB, although ChIP analysis showed that histones were still present in this interval (Figure 1).

The disparity between the location of repair proteins and of γH2AX indicates that γH2AX most likely does not directly recruit repair proteins to DNA damage sites. Instead, we suggested that this large region of γH2AX creates chromosome structural changes that promote damage repair.

Ünal E et al. (*Mol Cell* 16: 991–1002, 2004) and Ström L et al. (*Mol Cell* 16: 1003–15, 2004) provide support for this idea by examining the distribution of cohesin around a DSB. Cohesin is normally loaded onto chromosomes during replication, and holds sister chromatids together until mitosis. These papers report that DSBs provoke post-replicative cohesin loading in a large region, and that this additional cohesin is important for efficient DSB repair. The distribution of cohesin closely resembles that of γH2AX, suggesting that γH2AX might play a role in this damage-induced cohesin loading. Ünal E et al. showed that this is, in fact, the case. Mutants unable to form γH2AX do not recruit cohesin to DSBs and, consequently, have defects in repairing gamma ray–induced chromosome breaks.

These findings suggest a picture where γH2AX formation and the subsequent recruitment of cohesin stabilize broken chromosomes, using the unbroken sister chromosome as a splint to hold broken ends together while leaving the actual site of damage open for repair proteins (Figure 1). This helps ensure that DSB repair occurs efficiently and with fidelity, maintaining genome integrity in the face of DNA damage and avoiding the genome rearrangements associated with cancer.

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■ DEVELOPMENTAL BIOLOGY

Telomere-associated Protein TIN2 Is Essential for Early Embryonic Development

Chiang YJ, Kim SH, Tessarollo L, Campisi J, and Hodes RJ. Telomere-associated protein TIN2 is essential for early embryonic development through a telomerase-independent pathway. *Mol Cell Biol* 24: 6631–4, 2004.

The ends of linear eukaryotic chromosomes consist of telomeres that contain telomeric DNA repeats, $(TTAGGG)_n$ hexanucleotide repeats in mammalian chromosomes, and a number of associated proteins. This telomeric structure is critical for distinguishing the chromosomal terminus from free ends of damaged DNA, and thus, telomeres prevent the triggering of inappropriate cell cycle arrest and/or apoptotic responses normally elicited by DNA damage. In eukaryotic cells, the mechanism of chromosomal replication during cell division results in incomplete terminal synthesis, so that in the absence of a compensatory mechanism, 50–200 bases of terminal telomeric DNA are lost with each division. Thus, successive cycles of cell proliferation can lead to progressive telomere shortening, until a critically short length is reached at which telomere function is compromised, with consequences that can include replicative senescence, apoptosis, and tumorigenic chromosomal instability. A compensatory mechanism capable of adding terminal telomeric repeats is mediated by the RNA-dependent DNA polymerase, telomerase. This enzyme consists of two essential molecular components, the telomerase RNA (TR) component, which includes a template for telomeric DNA, and the catalytic telomerase reverse transcriptase (TERT), which mediates telomere synthesis. Importantly, recent discoveries have demonstrated that maintenance of telomere function is also dependent on the influence of additional telomere-associated proteins, and elucidating the function of these proteins is, therefore, an area of considerable interest.

TIN2 (TRF1-interacting protein 2) was recently identified as a telomere-associated protein that interacts with TRF1, a molecule that binds directly to telomeric DNA and functions as a negative regulator of telomere length. TIN2 contains N-terminal basic and acidic regions, a central TRF1-binding domain, and a C-terminal region. The basic and acidic regions are required for the regulation of TRF1 activity by TIN2. The TRF1-binding domain associates with the TRF1-homodimerization domain, providing for the recruitment of TIN2 to the telomere. *In vitro* studies have shown that overexpression of TIN2 inhibits telomere elongation in human cell lines, whereas expression of dominant-negative mutants of TIN2 enhances telomere elongation. It has been suggested that the binding of wild-type TIN2 induces changes in TRF1 conformation that in turn favor a telomeric structure that is inaccessible to telomerase, thus preventing telomerase-mediated telomere elongation. The absence of TIN2 would conversely favor telomerase accessibility and telomere elongation.

The physiological role of TIN2 during *in vivo* development and in normal cell function had not previously been assessed. To better understand the *in vivo* function of TIN2, we have, therefore, studied the effect of TIN2 mutation on mouse development, using gene-targeting technology. No homozygous TIN2$^{-/-}$ mice were identified in the offspring of TIN2$^{+/-}$ mouse intercrosses. Furthermore, homozygous TIN2-deficient embryos were absent as early as day 7.5. This finding indicated that TIN2 is essential for mouse development and that homozygous inactivation of TIN2 is lethal before day 7.5 of embryonic development. However, day 3.5 TIN2$^{-/-}$ embryos were obtained in expected frequency (1/4) among offspring of TIN2$^{+/-}$ intercrosses. When day 3.5 TIN2$^{-/-}$ embryonic cells were cultured, it was striking that they were uniformly defective in their differentiation, in comparison to day 3.5 wild-type embryonic cultures. Wild-type embryonic cultures grew to form multilayered cell masses, whereas TIN2$^{-/-}$embryonic cultures were flat and contained few viable cells. A growth and/or survival defect was thus apparent in TIN2$^{-/-}$ cells at an early stage of embryonic development.

The previously identified function of TIN2 was proposed to involve enhancing the activity of TRF1 in downregulating the telomerase elongation of telomeres. We asked whether the embryonic lethality observed in TIN2$^{-/-}$ mice might be telomerase dependent. To explore this possibility, TIN2$^{+/-}$ mice were bred to mTERT$^{-/-}$ mice that lacked telomerase activity. It was striking that no TIN2$^{-/-}$ mTERT$^{-/-}$ offspring were observed, whereas TIN2$^{+/+}$ mTERT$^{-/-}$ and TIN2$^{+/-}$ mTERT$^{-/-}$ mice survived. Thus, embryonic lethality of TIN2$^{-/-}$ mTERT$^{-/-}$ mice indicated that the requirement for TIN2 in mouse development reflects a previously unappreciated telomerase-independent function of this molecule.

Recently, it was reported that inactivation of the mouse *TRF1* gene results in embryonic lethality, and that *TRF1* knockout blastocysts have a cell growth defect and increased apoptosis. The phenotype of *TIN2* knockout mice thus appears to be similar to that of TRF1-deficient mice. These observations imply that, in addition to the telomerase-dependent functions played by TIN2/TRF1 complexes, both TIN2 and TRF1 also function in telomerase-independent roles. To understand the telomerase-independent roles of TIN2 and TRF1 in embryonic development and in adult animals, studies of inducible *TIN2* or *TRF1* conditional knockout mice will be informative. We have in fact generated *TIN2* conditional knockout constructs using cre/loxP techniques and will use these constructs in studies of inducible and tissue-specific *TIN2* inactivation. Additional telomere-associated proteins may be involved in the potentially complex functions of TIN2 and TRF1, and we are currently pursuing genetic approaches to analyze candidate components involved in these functions.

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