Networks of tumor suppressors

Workshop: Tumor suppressor networks

Sponsored by the Juan March Foundation, and organized by J. Massagué and M. Serrano. Madrid, Spain, May 8–10, 2000.

Introduction

The concept of tumor suppressor networks comes as a logical consequence of the increasing understanding of the biology of tumor suppressor genes and oncogenes. Indeed, it has been gratifying to realize during the past decade that the products of tumor suppressor genes and oncogenes are often involved in the kind of guardian–delinquent relationship, or cat-and-mouse game suggested by the genetic evidence. Thus, some oncogenes directly cancel the protective activities of tumor suppressors and, reciprocally, there are some tumor suppressors that detect the presence of oncogenes and counteract their effects. Our understanding of the signaling network built by these gene products is still sketchy but rapidly growing (Figure 1), and this was the focus of the workshop 'Tumor Suppressor Networks'.

Oncogenes that inactivate tumor suppressors

The Ras oncogene plays a central role in the transmission of external mitogenic signals to the cell. For this, Ras uses a number of effector pathways that can counteract the activity of certain tumor suppressors. Discussions on this topic at the Workshop are summarized under the following two subheadings.

The Ras–Raf pathway. The activation of the Raf–Mek–Erk cascade of kinases by Ras is the main event responsible for the

transmission of proliferative signals from the cell membrane to the nucleus. However, the transcriptional consequences of activating this cascade are still poorly defined. J. Downward (London, UK) reported preliminary data on the short term transcriptional effects of inducing Raf in a mammary epithelial cell line. Data from microarrayed chips revealed a substantial number of autocrine mitogens, survival factors and angiogenic factors that are upregulated upon Raf activation. Among these autocrine factors is the heparin-binding epidermal growth factor (HB-EGF), which in turn binds to the EGF receptor and activates Ras, suggesting a positive feedback loop.

One of the surprises of the meeting came from F. McCormick (San Francisco, CA), who presented data indicating that the activation of the Raf–Mek–Erk pathway results in transcriptional upregulation of the p53–destabilizing oncogene MDM2. Previously, MDM2 was known to be transcriptionally induced by p53 itself, in a negative feedback loop. McCormick, together with M. Oren (Rehovot, Israel), showed that the upregulation of MDM2 by the Ras–Raf pathway occurs independently of p53 and, as expected, results in an inhibition of p53 activity. Building on this, McCormick went on to explain the cellular contexts that allow replication of the modified adenovirus ONYX 015. This virus was originally designed to be cytopathic only in p53-deficient cells. However, colon cancer cells containing activated Ras and, as a consequence, high levels of MDM2, are also susceptible to the cytopathic effects of ONYX 015, presumably because p53 is inhibited by MDM2 in these cells. These results indicate that a p53-deficient cellular context can be created by oncogenic mutation of Ras. In addition, this work exemplifies the importance of classifying cancer cells according to functional criteria rather than on the occurrence of a particular genetic alteration.

The Ras–Raf pathway inactivates not only p53, but also the tumor suppressor NF1 implicated in human neurofibromatosis, as shown by T. Jacks (Cambridge, MA). NF1 binds directly to Ras, triggering its conversion to an inactive Ras conformation. Jacks reported that stimulation of normal primary cells with Ras agonists results in rapid degradation of NF1 protein in a manner that depends on the activity of protein kinase C and the ubiquitin/proteasome pathway. Interestingly, this downregulation of NF1 is transient, and this may help the cell to exit quiescence and enter proliferation.

The Ras–PI3K pathway. In another branch of the Ras effector pathways, Ras activates the phosphatidylinositol 3'-kinases (PI3Ks). The activity of PI3Ks is antagonized by the phosphatase

Fig. 1. A roadmap to the tumor suppressor network. A scheme of the different pathways discussed at the workshop and their interconnectivities.

PTEN, and the balance between these two activities determines the amount of phosphatidyl-inositol triphosphates in the plasma membrane. These lipids, in turn, act as positive regulators of cellular survival through their activation of the Akt kinase. A. Carrera (Madrid, Spain) reported the oncogenic activity of a constitutively active PI3K expressed in the T lymphocytes of transgenic mice. These mice develop a number of lymphoproliferative disorders, including massive lymphocyte infiltration in organs, such as the lung. Interestingly, this lymphoproliferation is polyclonal, and the lymphocytes obtained from these mice have a decreased propensity to undergo spontaneous apoptosis. Continuing with this topic, R. Parsons (New York, NY) described the phenotype of PTEN-deficient mice. The homozygous PTEN^{-/-} genotype is embryonic lethal, but heterozygous PTEN^{+/-} animals develop several lymphoproliferative disorders, including intestinal polyps formed by aggregates of lymphocytes. Again, this lymphoproliferation is polyclonal and is not associated with the loss of the wild-type PTEN allele. The similarity in the phenotypes of the PI3K-transgenic mice (Carrera) and the PTEN^{+/-} mice (Parsons) is in agreement with the evidence indicating that these two proteins antagonistically control the same biochemical process.

Of the many proteins that are activated by their association with phosphatidyl-inositol triphosphate, the serine/threonine-kinase Akt has received the most attention because of its key role in executing the survival effects promoted by PI3K. J. Downward has applied microarray technology to analyze the short-term transcriptional consequences of activating Akt in an inducible manner. Downward drew two main conclusions: Akt has little impact on the induction of NF-κB transcriptional targets, and Akt downregulates the transcriptional targets activated by members

of the Forkhead family. A novel player in the regulation of Akt activity was presented by J.L. Jorcano (Madrid, Spain) who showed that Akt itself is sequestered by keratin 10 intermediate filaments in keratinocytes. This effect on Akt localization may explain why keratin 10 prevents cell proliferation. The cytostatic role of keratin 10 was dramatically illustrated by the phenotype of transgenic mice expressing keratin 10 in the germinal layer of the skin. These mice die at an early age with an epidermis that is no longer stratified but formed by a single layer of cells, and are resistant to skin carcinogenesis, probably due to the proliferative impairment produced by the expression of keratin 10.

Tumor suppressors that are activated by oncogenes

Some tumor suppressors are wired so that they detect and respond to the presence of oncogenic signals, thus acting as safeguards against oncogenes. This is the case for the tumor suppressor genes INK4a and ARF, which are partly encoded by alternative reading frames of a common genetic locus. In spite of this, INK4a and ARF have separate promoters and biochemical activities that confer quite distinct personalities on them. The tumor suppressor ARF binds and inhibits MDM2, thus stabilizing p53. ARF is transcriptionally upregulated in response to a number of oncogenes, notably oncogenic Ras and Myc, thus signaling the presence of these oncogenes to p53. S. Lowe (Cold Spring Harbor, NY) demonstrated that lymphomas arising in Myc transgenic mice have significantly different responses to chemotherapy depending on the status of the ARF-p53 pathway. In particular, Myc-lymphoma cells deficient in p53 or ARF have

an increased resistance to chemotherapy and relapse with a shorter latency than cells with an intact ARF-p53 response. Also, Lowe presented preliminary evidence suggesting that the Caspase 9 cofactor Apaf-1, a downstream effector of the apoptotic program, could be a bona fide tumor suppressor.

The transcription factors that regulate ARF have so far remained elusive, but C. Sherr (Memphis, TN) presented new data suggesting that DMP1, which was previously isolated in his laboratory as a cyclin D2-interacting protein, plays such a role. DMP1 binds to and transactivates the ARF promoter, leading to ARF-dependent cell-cycle arrest. Furthermore, embryonic fibroblasts derived from DMP1-null mice do not induce ARF either in response to Ras, or during the normal onset of replicative senescence. These observations reveal that DMP1 could be an important component of an anti-oncogenic pathway mediated by ARF.

Regarding INK4a, its expression is upregulated in response to oncogenic Ras and also in association with replicative senescence. An intriguing hint toward the understanding of the regulation of INK4a was provided by M. De Luca (Rome, Italy) who described the involvement of the 14-3-3 σ protein in the regulation of INK4a. Proteins of the 14-3-3 family have multiple functions, some of them associated with cytoplasmic retention of specific serine/threonine-phosphorylated proteins (including the above-mentioned Forkhead transcription factors, which are thereby inhibited). De Luca has now found that artificial downregulation of 14-3-3 σ confers an extended lifespan on human keratinocytes, apparently by preventing the upregulation of INK4a. These effects on INK4a expression could be due to a hypothetical INK4a transcriptional inhibitor normally retained in the cytoplasm by 14-3-3 σ .

The cell-cycle arrest elicited by oncogenic Ras and mediated by ARF and INK4a is reminiscent of replicative senescence. M. Serrano (Madrid, Spain) reported the identification of new markers specific to Ras-induced senescence, which are not upregulated in association with other forms of cell-cycle arrest including replicative senescence and organismal aging. These markers, identified using DNA microarrays, include genes of unknown function that may contribute, together with INK4a and ARF, to the cell-cycle arrest induced by Ras.

TGF- β cytostatic signaling

TGF- β has antimitogenic activity that is partly mediated through induction of INK4b (which cooperates with the Cdk2 inhibitor p27^{Kip1}) and partly through downregulation of Myc. These effects have long been of interest because of their effectiveness at causing cell-cycle arrest and because of the proclivity of tumor cells to lose this response. TGF- β receptors signal through Smad transcription factors and several of these components are mutated in colon and pancreatic cancers. However, the response to this pathway largely depends on the cell type. J. Massagué (New York, NY) presented an experimental approach recently used to identify cell-specific Smad-interacting factors that determine DNA binding specificity. Moreover, Massagué obtained evidence for two separate arms of a mechanism for transcriptional activation of INK4b. One arm involves recognition of the INK4b promoter by a Smad complex and the other involves alleviation of INK4b repression by Myc. Down-

regulation of Myc and induction of INK4b therefore appear to be tightly linked events in the cell-cycle arrest response to TGF- β . Importantly, Ras oncogenes silence the cytostatic effect of Smads, and endow breast cancer cells with the ability to form bone metastasis in response to TGF- β . The search is on for mediators of this aberrant response to TGF- β .

The many talents of β -catenin

β -catenin is a multifaceted protein. β -catenin is part of the intracellular scaffold that supports the cell-cell junctions by associating with the cytoplasmic tail of the cell adhesion receptor E-cadherin. β -catenin also functions as co-activator for transcription factors of the LEF (or TCF) family. In the latter guise, β -catenin can have oncogenic activity. The levels of β -catenin are kept low in normal cells because of the destabilizing effect of a complex that involves at least two tumor suppressors: axin (or the related protein conductin) and adenomatous polyposis coli (APC). In response to Wnt growth factors, axin/conductin and APC are inactivated, leading to an increase in β -catenin-LEF transcriptional activity. All of the above-mentioned components are mutated in colorectal cancers, albeit with varying frequency. W. Birchmeier (Berlin, Germany) presented recent data on the characterization of the interaction surfaces between β -catenin and its negative regulators conductin and APC, and between β -catenin and LEF. An elegant demonstration of the power of these studies is the success of Birchmeier's laboratory in designing a small drug that can block the β -catenin-LEF interaction. If effective *in vivo*, this drug (or its improved derivatives) might cancel the oncogenic activity of the entire β -catenin pathway.

H. Clevers (Utrecht, Netherlands) pointed to the existence of further complexities based on the characterization of the three *Caenorhabditis elegans* β -catenin homologs, each of which has a different role in this organism. Only the β -catenin homolog BAR1 has the classical behavior of activating the LEF transcription factor. The homolog WRM-1 instead acts as a repressor of LEF, and the homolog HMP2 seems to be exclusively devoted to cellular adhesion. Speaking about yet another model organism—the fruit fly *Drosophila melanogaster*—F. Townsley (with Mariann Bienz, Cambridge, UK) provided elegant insights into the subcellular localization of E-APC. This new member of the *Drosophila* APC family colocalizes with β -catenin at adherens junctions, and this requires an intact actin cytoskeleton. These properties suggest that APC regulates β -catenin not only by prompting its degradation, but also by determining its localization to specific sites within the cytoplasm.

A long-standing question in this field has been the identity of the transcriptional targets of β -catenin-LEF that mediate oncogenicity. In this regard, F. McCormick described the upregulation of cyclin D1 by β -catenin-LEF, and M. Oren reported that β -catenin-LEF can activate p53 by both ARF-dependent and ARF-independent mechanisms. These findings further reveal the intricate connectivity of the proto-oncogenic and tumor suppressor networks.

p53 and DNA damage

The tumor suppressor p53 is the most frequently mutated gene in human cancers. The function of p53 is not restricted to

'guarding' against oncogenic stresses (as discussed above), but also, and perhaps more importantly, p53 can 'guard' against the presence of DNA damage. Ionizing radiation rapidly triggers the phosphorylation of p53 at residue Ser20, rendering p53 insensitive to the inhibitory actions of MDM2. C. Prives (New York, NY) took a brave approach and purified the activity responsible for this phosphorylation. The effort turned up the protein kinases Chk1 and Chk2, whose homologs in the yeast *Schizosaccharomyces pombe* were previously known to participate in the DNA damage response. Prives' laboratory has found that modulation of Chk kinase *in vivo* and *in vitro* affects p53 protein levels and activity. These results agree nicely with the recent finding that the *Chk2* gene is mutated in some Li-Fraumeni patients who have wild-type p53 alleles. Li-Fraumeni syndrome is a hereditary tumor-predisposing disease that is frequently (but not always) associated with p53-mutant alleles. All together, these results suggest that Chk2 mutations may be equivalent to p53 mutations in their tumor susceptibility effects.

Although much of the emphasis regarding DNA damage and cancer has been put on p53, M. Oren shifted the protagonism to MDM2. He reported that MDM2 is phosphorylated upon ionizing irradiation, partially losing its ability to degrade p53. This phosphorylation is known to be mediated by the serine/threonine kinase ATM. Interestingly, a mutant MDM2 with an amino acid change that mimics the phosphorylation by ATM is less efficient at destabilizing p53. Collectively, Prives and Oren put Chk and ATM proteins on center stage in the transmission of DNA damage signals to the MDM2/p53 inhibitor/agonist pair.

Genetic stability

Loss of genetic stability is perhaps the best defining property of cancer cells in solid tumors, and many tumoral properties are likely to be secondary to this loss. Genetic stability can be lost in many ways, including impairment in mismatch repair, improper chromosome segregation, chromosomal rearrangements and telomere loss. With the exception of mismatch repair proteins, little is known about the molecular components that are responsible for the other aspects of genetic instability. M. Jasin (New York, NY) reported that cells deficient in the breast tumor suppressor BRCA1 or BRCA2 are impaired in double-strand DNA break repair by homologous recombination. Moreover, Jasin reported that XRCC2 and XRCC3, which hypersensitize cells to ionizing radiation and DNA cross-linking agents when mutated, are also involved in double-strand break repair, probably in cooperation with Rad51 (which is the central protein in the homologous recombination process). D. Livingston (Boston, MA) discussed the identification of a novel BRCA1-associated protein, p130, which belongs to the family of helicases. Importantly, mutation of the active site of this new helicase also results in decreased DNA break repair activity. Together, these studies strengthen the notion that one way in which BRCA genes suppress tumorigenesis is by limiting the accumulation of double-strand breaks in a cell's genome.

Although genetic instability is a driving force in the evolution of tumors, there are certain chromosomal elements that must be retained for cell survival, and these include the telomeres at the ends of chromosomes. Perhaps because of the importance of telomeres in regulating the expansion of cell populations, organ-

isms have set the dose of the telomere rejuvenating enzyme telomerase sparingly. Conversely, most tumor cells aberrantly activate telomerase, thus ensuring a renewal of telomere length. M. Blasco (Madrid, Spain) described the consequences of telomere loss for skin tumorigenesis using mice genetically deficient in telomerase activity. In agreement with the essential role of telomeres for continued proliferation, mice with critically short telomeres are refractory to skin tumorigenesis.

Hypoxia and angiogenesis

Tumor suppressors can protect from tumor development by means other than constraining cell proliferation and repairing genomic damage. This was illustrated nicely by the work of W. Kaelin (Boston, MA) on the tumor suppressor VHL. Inherited loss-of-function alleles of this gene are responsible for the Von Hippel-Lindau syndrome, which is characterized by the development of hemangioblastomas (blood vessel tumors), pheochromocytomas, and clear cell carcinomas of the kidney. Interestingly, the cell type that gives rise to the hemangioblastomas is not an endothelial cell, but probably a mesenchymal cell that triggers uncontrolled vascularization by hyperstimulating neighbor endothelial cells. VHL protein is known to be part of a SCF-type ubiquitin ligase complex responsible for the short half-life of the transcription factor HIF (hypoxia-inducible factor). HIF is an activator of the angiogenic switch that triggers expression of hypoxia-responsive genes including vascular endothelial growth factor (VEGF), which does what its name indicates. Kaelin presented recent work mapping the VHL-HIF interaction, and also dissecting the defects of the mutant alleles of VHL. Remarkably, some VHL mutations diminish the interaction with HIF, while other mutations diminish the interaction of VHL with the other components of the ubiquitin ligase complex.

Animal models based on tumor suppressor networks

Genetically manipulated mice are essential for the study of tumor suppressors and oncogenes. However, reproducing a particular human cancer in mice is an unpredictable adventure. More often than not, deficiency of a tumor suppressor gene results in different cancer types in mice compared with humans. This phenomenon has the invaluable importance of revealing complexities that would otherwise go unnoticed.

Neurofibromatosis type I, as briefly mentioned before, is a hereditary syndrome produced by mutant alleles of the *NF1* gene. The disease is characterized by malignant peripheral nerve sheath tumors (MPNSTs), probably derived from tumoral Schwann cells. *NF1*^{-/-} mice die *in utero* at mid-gestation, but heterozygous *NF1*^{+/-} mice develop pheochromocytomas with low penetrance and therefore do not reproduce the human malignancy. The laboratories of L. Parada (Dallas, TX) and T. Jacks have studied various combinations of *NF1*^{+/-} with other mutations in tumor suppressor genes. Importantly, *NF1*^{+/-}-*p53*^{+/-} mice develop MPNSTs with high frequency and in association with loss of the wild-type *NF1* and *p53* alleles. Jacks reported that in addition to tumors of the peripheral nervous system, *NF1*^{+/-}-*p53*^{+/-} mice also develop tumors in the brain, exhibiting

a complete progression pattern from low-grade astrocytoma to high-grade glioblastoma multiform. Parada further showed that a triple combination of NF1^{+/-}-p53^{+/-}-Smad3^{+/-} results in an acceleration of MPNSTs appearance, and in the development of primitive neuroectodermal tumors (PNETs) in the brain, for which there were no previous animal models. Interestingly, the tumors developed by these triple mutant mice retain the wild-type NF1 and p53 alleles but have lost the wild-type Smad3 allele, drawing further attention to the tumor suppressor role of the TGF- β pathway.

As a major checkpoint that integrates multiple extracellular and intracellular signals, the retinoblastoma gene (Rb) is central to our understanding of the G₁ phase of the cell cycle. A. Berns (Amsterdam, Netherlands) reported on the efforts of his laboratory in trying to reproduce retinoblastomas in mice. In a situation parallel to the NF1 mice, Rb^{-/-} mice die *in utero*, and Rb^{+/-} mice develop pituitary tumors but not retinoblastomas. In one approach, Berns described the phenotype of mice lacking Rb only in cells expressing the interphotoreceptor retinoid binding protein (IRBP)-producing cells, which include the retinal pigmented cells. However, these mice do not develop retinoblastomas, not even in combination with a null mutation in the Rb-homolog p107, in which case mice develop pineal tumors and PNETs.

Among the most important regulators of Rb are the Rb kinase CDK4 (a positive regulator of proliferation) and the CDK4 inhibitors of the INK4 family. Berns provided evidence that, unlike ARF^{-/-} mice, INK4a^{-/-} mice (which retain wild-type ARF) do not develop tumors at an early age. Time will tell whether tumors develop in older mice deficient for INK4a, as is the case in humans carrying mutant alleles of INK4a. M. Barbacid (Madrid, Spain) reported that INK4b^{-/-} and INK4c^{-/-} mice have an increased incidence of diverse spontaneous tumors. Interestingly, double knock-out mice INK4b^{-/-}INK4c^{-/-} do not show synergistic effects or novel phenotypes, suggesting that these highly related proteins are not regulated by compensatory mechanisms. Barbacid also reported that a knock-in mutation that renders CDK4 insensitive to the inhibition by INK4 proteins results in multiple tumors in various organs, at almost 100% penetrance before 1 year of age. Furthermore, treatment of these mice with carcinogens resulted in the rapid onset of metastatic melanomas. These results are particularly gratifying because, in humans, a mutant CDK4 allele carrying the same mutation is associated with predisposition to melanoma.

Before closing this section, we must mention the great insight provided by other animal models that have been briefly mentioned above, such as yeast, *Drosophila* and *C. elegans*. The possibility of achieving faster and simpler functional genetic tests in animal cells was raised by G. Hannon (Cold Spring Harbor, NY) who showed that cultured *Drosophila* cells are susceptible to the phenomenon of RNA interference (RNAi). In RNAi, introduction of double-stranded RNA causes gene silencing by specifically triggering the degradation of homologous mRNA molecules. This opens the possibility of performing functional phenotypic screenings in cultured *Drosophila* cells, with the aim of inactivating known or hypothetical tumor suppressors. Hannon is also attempting to activate this process in mammalian somatic cells.

Joan Massagué

Conclusion

The work presented at this Workshop revisited old signaling pathways, consolidated new ones, and provided new insights into the connectivity of these pathways. These networks are cell specific, and change as a function of the developmental state of the cell and its particular circumstances (environment, mitogenic or anti-mitogenic factors, DNA damage, mutations, and so on). It is essential to define, as thoroughly as possible, all the relevant links and components of these networks to understand (and hopefully manipulate) cellular decisions.

This workshop was special in many regards: the quality of the presentations, the fact that all speakers presented fresh results from ongoing projects, and the friendly and constructive discussions. The Juan March Foundation made this possible by providing efficient organization and a lovely venue.

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Received June 12, 2000; accepted June 28, 2000

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DOI: 10.1093/embo-reports/kvd034