Instituto Juan March
de Estudios e Investigaciones

109 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Tumor Suppressor Networks

Organized by

J. Massagué and M. Serrano

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The lectures summarized in this publication were presented by their authors at a workshop held on the 8th through the 10th of May, 2000, at the Instituto Juan March.

Depósito legal: M-24.472/2000
Impresión: Ediciones Peninsular. Tomelloso, 27. 28026 Madrid.

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Introduction

M. Serrano

Tumor suppressor genes (TSGs) are the exact opposites to oncogenes. TSGs are "guardian genes" that watch over the proper proliferation and differentiation of cells, whereas oncogenes could be seen in this metaphor as "delinquents" that sabotage the normality of these processes favoring an unrestrained proliferation. Cancerous cells only arise when the function of these "guardians" is canceled by mutations, thus escaping their vigilance. To understand the normal functions of TSGs and the consequences of their inactivation is a scientific priority and a necessity to improve cancer therapies.

The first tumor suppressor gene (TSG) to be identified was the retinoblastoma susceptibility gene, Rb, in 1986. Since then, approximately 15 TSGs have been isolated. The normal function of all TSGs is to prevent uncontrolled cell proliferation, and they do this by acting on a variety of processes. Some TSGs directly regulate the cell division rate (Rb, NF-1), while others participate in cellular survival (PTEN), cell differentiation (APC, SMAD4), or in preventing proliferation under stressful conditions (ARF, p16, p53). The study of some TSGs has already moved into the stage of defining pathways. Indeed, it has been very gratifying to see that these pathways involve both TSGs and oncogenes in a kind of guardian-delinquent relation, or cat and mouse game, as it has been referred to by other researchers. This may seem obvious in retrospect, but it was not so when TSGs begun to be characterized. There are now several well-established "TSG-oncogene" antagonistic relations, for example (TSGs are underlined, oncogenes are not): p16/CDK4-cycD1/Rb; ARF/MDM2/p53; PTEN/PI3K; Wnt/APC/β-catenin; NF-1/Ras. These are embryonic pathways that are increasing in complexity and subtlety in these very moments thanks to the work of many laboratories and particularly in those of the participants to this workshop. This is a direction of the research that is now in its early days, and that has been the main focus of the Workshop "Tumor Suppressor Networks".

One of the leitmotivs of the workshop has been the use of the mouse as experimentation system. The manipulation of the mouse genome is increasing in sophistication and power, and these technologies are being used very actively for testing the role of TSGs and for developing mouse cancer models. As a good model system, the mouse occasionally reproduces the pathology of human cancers, but this is not always the case and perhaps it is in these occasions when the

mouse turns out more informative. These "breaks of equivalence" between human and mouse cancers are indeed the motivation for further research and reveal aspects that otherwise may remain hidden. Also, mutations in two or more TSGs are being combined in the same mouse strains, thus testing TSGs networks directly in a mammalian organism.

This workshop has been privileged in many regards, first of all by the quality of the speakers, but also by other factors such as the opportunity of the topic which is in a very exciting and active moment, and finally by the constructive and collaborative attitude of all the participants. In keeping with the mark of the house, the Juan March Institute provided a professional organization and a warm hospitality.

Manuel Serrano

Session 1: Mitogenic and anti-mitogenic signaling
Chair: Luis F. Parada

Smad3, NF1, & p53 cooperate in the promotion of neural tumors

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TGFβ family proteins regulate proliferation and differentiation in a variety of cell types including neural crest cells. The TGFβ signaling pathway is mediated by intracellular proteins, Smads, that complex with Smad4 upon phosphorylation. The Smad protein complex translocates to the nucleus and modulates transcription. Previously, we have shown that Smad3 homozygous mice develop a full spectrum of colorectal cancers from benign adenomas, to metastatic adenocarcinomas, and Smad3 heterozygous mice are cancer free. In addition, we and others have generated a mouse strain that harbors both NF1 (Neurofibromatosis type 1) and p53 mutations in the cis configuration. The cisNF1/p53 mutants develop malignant triton tumors (MTTs) and malignant peripheral nerve sheath tumors (MPNSTs). Here, we show that introduction of the Smad3 mutation into the cisNF1/p53 background not only accelerates tumorigenesis, but also alters the tumor spectrum. First, a cohort of Smad3; NF1/p53 triple mutant mice develop MPNSTs and MTTs that are found associated with peripheral nerves. Second, over 50% of the MPNSTs and MTTs from Smad3; NF1/p53 triple mutant mice do not lose either the NF1 or p53 wild type alleles, in contrast to similar tumors derived from NF1/p53 double mutants. Finally, a high percentage of the triple mutants develop primitive neuroectodermal tumors (PNETs), for which there is no current reliable animal model. We never observe PNETs in the NF1/p53 double mutants or Smad3 homozygous mice. These brain tumors all lose the wild type Smad3 allele and activate Cdk4. Thus, our results provide genetic evidence that Smad3 mediated TGFβ signaling pathway cooperates with NF1/p53 tumor suppressors in the development of both PNS and CNS tumors. To our knowledge, this provides the first genetic model for PNETs in mammals.

In the PNS, neurofibrosarcomas are attributed to loss of NF1 in Scwann cell precursors. To gain insight into the role of NF1 during glial development and in tumorigenesis in the CNS, we have targeted the NF1 mutation to astrocytes by using GFAP-Cre transgenic mice. The astrocyte-specific NF1 mutant mice (NF1GFAPKO) display severe neurological defects including mild ataxia, tremor, and a bizarre behavior-"handstand". These abnormal behaviors suggest deficits associated with the central nervous system (CNS). Histological analysis reveals several developmental defects in the NF1GFAPKO mutant brains. Several adult glia including astrocytes express embryonic markers such as nestin and BLBP (Brain Lipid Binding Protein). Consistent with immature phenotype, the mutant brains remain proliferative during late developmental stages and into adulthood. A subset of the NF1-GFAP mutant mice develops astrocytomas. Taken together, these data not only demonstrate that NF1 plays an important role in the differentiation and proliferation of astrocytic glial cells, but also provide a mouse model for human glial tumors.

Pathways involved in the regulation of cell survival by oncogenes

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Detachment of untransformed adherent cells from extracellular matrix commonly triggers programmed cell death, a phenomenon termed anoikis. This is prevented by transformation of cells by a number of oncogenes, including Ras. In normal cells, matrix adhesion stimulates PI 3-kinase and hence the downstream kinase Akt/PKB to provide a suppression of death signal function. The protective effect of oncogenic Ras is transduced principally through its ability to activate PI 3-kinase and Akt.

The mechanism by which matrix adhesion activates PI 3-kinase activity has been explored by the use of fusion proteins of the pleckstrin homology domain of Akt with green fluorescent protein (GFP). GFP-PH Akt can be used to determine the sites of PI 3-kinase activation in the cell. On new adhesion of epithelial cells to matrix, PI 3-kinase activation occurs at actin and β-catenin rich structures known as podosomes. Focal contacts are not major sites of PI 3-kinase activation, suggesting that p125 FAK may not be involved in the activation of PI 3-kinase in this system. In addition, PI 3-kinase is strikingly activated at sites of cell-cell interaction, suggesting that these contacts also provide an anti-apoptotic signal.

Death is induced following detachment as a result of loss of survival signals to the cell. This allows a constitutive death inducing signal to predominate in the detached cells: this signal appears to emanate from death receptors such as Fas and DR4/5, and can be blocked by dominant negative FADD or over-expression of SODD, both inhibitors of death domain signalling. The detachment-induced death signal is manifested by rapid disturbance of pro-apoptotic Bcl-2 family protein function, followed by loss of cytochrome c from the mitochondria. Oncogene induced survival pathways impact at the earliest stage of this process.

There is a significant transcriptional component to the ability of Akt, and also other Ras effectors such as Raf, to influence cell death. In order to understand more about the mechanism whereby Akt and Raf can protect cells from apoptosis under different circumstances, we have used conditionally active forms of these kinases expressed in normal epithelial cells. Gene Chip technology has been used to study the ability of these kinases to regulate transcription of several thousand. This has revealed novel mechanisms for the regulation of apoptosis downstream of Ras. In particular, the ability of the Raf/MAP kinase pathway to induce autocrine expression of growth factors may account for the Akt-mediated protection of cells from apoptosis following strong activation of this pathway.

In addition to causing the production of survival inducing autocrine factors, activation of the Raf/MAP kinase pathway also causes cells to secrete TGFβ, a factor that causes growth arrest or apoptosis when applied to normal epithelial cells. In cells with activated Raf, TGFβ loses its ability to cause apoptosis, through a mechanism that is not specific to TGFβ signalling or SMAD function. Under these conditions autocrine TGFβ promotes the transformed phenotype of the cells, causing increased invasiveness.

Increased phosphoinositide 3-kinase activity induces a lymphoproliferative disorder and contributes to tumor generation *in vivo*

Ana C. Carrera

Alterations in the cell division:cell death ratio induce multiple autoimmune and transformation processes. Phosphoinositide 3-kinase (PI3K) controls cell division and cell death *in vitro*, but its effect on the function of the cellular immune system and on tumor formation in mammals is poorly characterized. We show that transgenic mice expressing in T lymphocytes an active form of PI3K derived from a thymic lymphoma, $p65^{PI3K}$, developed an infiltrating lymphoproliferative disorder and autoimmune renal disease with increased number of T lymphocytes exhibiting a memory phenotype and reduced apoptosis. This pathology was strikingly similar to that described in mice exhibiting heterozygous loss of the tumor suppressor PTEN, a lipid and protein phosphatase. We show that overexpression of PTEN selectively blocks $p65^{PI3K}$-induced 3T3-fibroblast transformation. Moreover, the early development of T cell lymphomas in $p65^{PI3K}$ Tg $p53^{-/-}$ mice indicated that PI3K contributes to tumor development. These observations demonstrate that constitutive activation of PI3K extends T cell survival *in vivo*, affects T cell homeostasis and contributes to tumor generation, supporting a model in which selective increases in one type of PTEN substrate, the PI3K-derived lipid products, induce tumorogenesis. PI3K thus emerges as a potential target in autoimmune disease and cancer therapy.

The tumor suppressor PTEN

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PTEN is genetically altered in a large number of human cancers. PTEN is a phosphatase that regulates the level of the second messengers phosphatidylinositol-3,4-bisphosphate (PIP2) and phosphatidylinositol-3,4,5-triphosphate (PIP3) by removing the D-3 phosphate from these molecules. PIP2 and PIP3 regulate a large number of proteins in the cell that contain pleckstrin homology (PH) domains. Proteins containing PH domains regulate diverse functions including mitogenesis, apoptosis, and migration. PIP2 and PIP3 are generated by PI-3 kinase, which is activated by a large number of extracellular stimuli. Therefore the loss of PTEN can affect a multitude of signaling pathways.

Cells lacking PTEN have a several phenotypes that may explain PTEN's tumor suppressor function. Loss of PTEN reduces the apoptotic response of fibroblasts to TNF-alpha, sorbitol, and ultraviolet light. Haploinsufficiency of PTEN in splenocytes leads to a reduction in FAS-mediated apoptosis. In embryonic stem cells, loss of PTEN affects the cell cycle. Reintroduction of PTEN into tumor cell lines induces apoptosis and G1 arrest, blocks migration, and inhibits the expression of VEGF. Biochemically, loss of PTEN results in the elevation of PIP3 and the activation of the AKT oncogene through its PH domain.

PTEN +/- mice develop tumors in multiple organ systems. These include tumors of the breast, colon, and thyroid--lesions that occur in humans with germline PTEN mutations. Large malignant tumors in the mice undergo loss of heterozygosity. Cells were identified with alterations in the cell cycle and apoptosis.

Because of the diverse effects of PTEN, we decided to examine cells for changes in gene expression induced by PTEN. We hoped that this approach would aid in the identification of signaling pathways over which PTEN has the greatest influence. Out of 40,000 genes examined, one candidate was the most induced gene by both chip and membrane hybridization. The level of induction was an order of magnitude greater than the level of induction seen with any of the other candidates. Characterization of this candidate will be discussed.

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Keratin K10, from cell structure to cell cycle through signal transduction

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The members of the large keratin family of cytoskeletal proteins are expressed in a precisely regulated tissue- and differentiation stage-specific manner. Although these proteins are thought to be involved in conferring mechanical integrity to epithelial cells, the functional significance of their complex differential expression is still unclear. In epidermis, basal proliferative keratinocytes express K14; when they terminally differentiate, keratinocytes switch off K14 and start K10 expression, whereas in response to hyperproliferative stimuli, K16 replaces K10.

We have recently demonstrated that the ectopic expression of K10 inhibits proliferation of human keratinocytes in culture, whilst K16 expression appears to promote proliferation of these cells. In addition, K10-induced inhibition can be reversed by co-expression of K16, but not K14. The mechanism by which K10 inhibits cell proliferation is linked to the retinoblastoma pathway. We found that this effect on cell cycle progression can be attributed to alterations in the PI-3 kinase signal transduction pathway mediated by the physical interaction between the non α-helical aminoterminal domain of K10 and Akt/PKB and aPKCζ. This interaction prevents the translocation of these signalling molecules, thus impairing their activation. Finally, to confirm these data *in vivo*, we generated transgenic mice in which K10 expression was ectopically targeted to the basal layer of the epidermis. These mice showed striking alterations in epidermal proliferation and differentiation, and displayed a highly decreased sensitivity to skin chemical carcinogenesis. The biochemical analysis of this phenotype was in agreement with the *in vitro* results.

Session 2: Tumor suppressor networks in development & differentiation
Chair: Anton Berns

TGF-β and oncogenesis

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The TGF-β family of secretory polypeptides is a major source of signals controlling the deployment of a cell's genetic program in metazoan organisms. Unlike classical hormones, whose actions are few and concrete, the members of the TGF-β family have many different effects depending on the type and state of the cell. TGF-β family members are multifunctional hormones, the nature of their effects depending on what has been called "the cellular context".

In spite of such complex response pattern, a disarmingly simple system has been recently elucidated that mediates many diverse TGF-β responses. This system involves a family of membrane receptor protein kinases and a family of receptor substrates –the Smad proteins– that march into the nucleus where they act as transcription factors. The ligand TGF-β assembles a receptor complex that activates Smads, and the Smads assemble multisubunit complexes that regulate transcription. Two general steps thus suffice to carry the hormonal stimulus to target genes.

How can such a simple system mediate a variety of cell-specific responses? We find that an incoming Smad complex is met in the nucleus by a set of partner proteins that are specific to a particular cell type in a particular set of conditions. These partners determine the DNA sequences that the Smad complex will bind, the transcriptional co-activators or co-repressors it will recruit, the other transcription factors it will cooperate with, and how long all this will last. The mix of Smad partners and regulators present in a given cell at the time of TGF-β stimulation thus decides the outcome of the response and defines, in molecular terms, the "cellular context". We have developed methodology to identify the cellular factors that confer the ability to mediate a particular gene response to TGF-β.

We are applying this methodology to the study of TGF-β in cancer. The TGF-β pathway plays a dual role in tumorigenesis. On one hand, the ability of TGF-β to inhibit epithelial cell proliferation is diminished or lost in many carcinoma cells. We have defined mechanisms of cell cycle arrest by TGF-β, and how these mechanisms are inactivated by loss-of-function mutations in the TGF-β pathway in colon cancer or by an inhibitory effect of a hyperactive Ras pathway in breast cancer. On the other hand, TGF-β can exacerbate the malignant phenotype at later stages of tumorigenesis by fostering tumor invasion and metastasis. This is manifest in breast cancer cells that retain TGF-β signaling components but have lost the antimitogenic response, as is the case in cells harboring a hyperactive ErbB-2/EGFR-Ras pathway. In these cells, TGF-β signaling has been reprogrammed, leading to gene responses that support the invasive or metastatic behavior of the cell. Building on our current understanding of the principles that govern signaling specificity by the TGF-β/Smad pathway, we are seeking to delineate the mechanisms of TGF-β stimulation of tumorigenesis.

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Regulation of TGFβ / BMP signaling by ZEB proteins

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The TGFβ family of signaling proteins plays a crucial role in differentiation of a variety of tissues during development (1). One role of these proteins is to arrest cells in the G1 phase of the cell cycle, which is necessary for the cells to launch a differentiation program (1). One target of TGFβ is the retinoblastoma protein (Rb). This protein acts as a transcriptional repressor, blocking expression of S phase genes, and thereby prevent cells from moving from G1 to S phase (2,3). Rb function is blocked by hyperphosphorylation, which we have shown to occurs sequentially by different cyclin dependent kinases during G1 (4). Treatment of cells with TGFβ leads to accumulation of hypophosphorylated Rb and G1 arrest. Conversely, a block in Rb function prevents the G1 arrest imposed by TGFβ indicating that Rb is a critical target for TGFβ-mediated growth suppression (5). Rb binds to a family of cell cycle transcription factors know as E2Fs, blocking their ability to activate transcription (2). However, while tethered to promoters through this interaction with E2F, Rb also recruits chromatin remodeling enzymes such as histone deacetylases and the ATP-dependent nucleosome remodeler SWI/SNF (6,7). We have recently provided evidence that it is this recruitment of chromatin remodeling enzymes, which allows Rb to assemble chromatin on promoters and actively repress transcription, that is essential for Rb to arrest cells in response to TGFβ (6,7).

Following their binding to cell surface receptors, signaling by TGFβ family members involves a cascade of phosphorylations that ultimately leads to formation of a

heterodimeric complex between the transcription factor Smad4 and other Smad family members (1). These complexes bind to promoters of target genes and activate transcription. One linkage between TGFb and Rb phosphorylation is the cyclin dependent kinase inhibitor p15, which blocks cyclin dependent kinase activity leading to accumulation of hypophosphorylated Rb and growth arrest (8). Several different proteins have been identified that regulate Smad activity (TGIF, ski and SnoN). Interestingly, one of these proteins, the ski oncogene, inhibits both Smad activity and Rb repressor activity (9,10). This prevents TGFβ-mediated growth arrest and differentiation and deregulates the cell cycle (11).

We have identified two zinc finger/homeodomain family members, ZEB-1 and ZEB-2 (12-15), that also bind to Smad proteins. These proteins appear to function at least in part in skeletal formation. Mice knocked out for the ZEB-1 gene showed skeletal defects similar to those observed in GDF-5 and other BMP-related molecules, sugesting a role for ZEB in BMP signaling. Targeted deletion of ZEB-2 is currently underway. Both ZEB-1 and ZEB-2 not only bind to Smads but also affect their ability to activate transcription in response to BMPs (and TGFb). Interestingly, it appears that ZEB-1 and ZEB-2 have reverse effects with one augmenting Smad activity and the other inhibiting. We suggest a mechanism where by the expression of ZEB-1 and ZEB-2 in differentiating cartilage/bone regulates responses to BMPs and thus skeletal formation.

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TUMORIGENESIS IN CONDITIONAL TUMOR SUPPRESSOR GENE KNOCKOUT MICE

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Gene inactivation studies are invaluable in assessing the function of oncogenes and tumor suppressor genes in development and malignant growth. However, detailed analysis of the role of tumor suppressor genes in these processes using the conventional knockout mouse models is often hampered by embryonic lethality or developmental aberrations. To circumvent these complicating factors associated with loss-of-tumor suppressor gene function we have generated a series of conditional tumor suppressor gene knockout mice. We have explored methods to switch the genes in a time-controlled and tissue specific fashion. Both transgenesis and somatic gene transfer was used to express Cre recombinase in the desired tissues. This technology permits us to induce specific tumors, to correlate specific genetic lesions with phenotypic characteristics, and hopefully to generate better models for testing intervention protocols. In addition, these mice are valuable source of cell lines that can be tested with respect to parameters that can be better studied in vitro such as growth, cell cycle regulation, response to irradiation, resistance to apoptosis, and genomic instability.

Some of the points mentioned will be illustrated on the basis of studies performed with compound conditional mutants. Genes studied in various combinations include pRb, p107, p16, p19Arf, p53 and Brca1 and Brca2. Inactivation was directed to specific tissues using tissue-specific or regulatable expression of Cre recombinase. Study of the effects of the inactivation of various tumor suppressor genes in specific tissues in vivo and in cell lines in vitro will be described.

Role of beta-catenin/armadillo in cell adhesion and signal transduction

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Beta-Catenin/armadillo is a component of both the cadherin cell adhesion system and the wnt signaling pathway. Wnt signaling increases the levels of cytosolic beta-catenin by preventing its ubiquination and degradation via proteasomes. This allows direct interaction of beta-catenin with transcription factors of the LEF/TCF family and modulation of gene expression. We have shown previously that the protein conductin forms a complex with beta-catenin, the tumor suppressor gene product APC, and GSK3 beta. Conductin induces beta-catenin degradation, whereas mutants of conductin that are deficient in complex formation stabilize beta-catenin. Fragments of APC that contain a conductin-binding domain also block beta-catenin degradation. Thus conductin and the related protein axin are components of the multiprotein complex that direct beta-catenin to degradation.

In beta-catenin, we have now identified amino acids that distinctly affect APC, conduction or LEF-1/TCF binding. These residues form separate clusters in the superhelix built by armadillo repeats. Point mutations in one, APC or conductin binding sites, do not stabilize beta-catenin. Mutants unable to interact with both APC and conductin are fully stabilized. For degradation, it is thus sufficient if conductin or APC are recruited indirectly to beta-catenin and thus to the degradation complex.

We also generated beta-catenin-deficient mouse embryos and observed a defect in anterior-posterior axis formation at E5.5, as visualized by the absence of Hex and Hesx and the mislocation of Cerberus-like and Lim-1 expression in the visceral endoderm. Subsequently, no mesoderm and head structures are generated, but beta-catenin-deficient cells can contribute to these tissues in chimeric embryos. Intercellular adhesion is maintained since plakoglobin substitutes for beta-catenin. Our data suggest that beta-catenin-mediated signalling is essential in anterior-posterior axis formation of the pre-gastrula mouse embryo.

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A POTENTIAL SHUTTLING ROLE FOR E-APC, A NEW *DROSOPHILA* ADENOMATOUS POLYPOSIS COLI HOMOLOGUE.

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Adenomatous polyposis coli (APC) is an important tumour suppressor protein with a critical role in preventing colon cancer. APC functions in a complex with glycogen synthase kinase 3 and axin to promote the degradation of β-catenin, an effector of the Wnt/Wingless signal transduction pathway; however its precise molecular function in the destabilisation of β-catenin is currently unknown.

E-APC is a recently discovered *Drosophila* APC homologue. Intriguingly, E-APC is concentrated in the adherens junctions of *Drosophila* epithelial cells where it associates with E-cadherin and Armadillo (the *Drosophila* homologue of β-catenin). Conditions that delocalise E-APC from the adhesive zones result in high levels of free Armadillo and ectopic Wingless signalling, which suggests that the junctional association of E-APC is critical for its function in promoting Armadillo degradation[1]. APC is also found in the cytoplasm and nucleus of *Drosophila* and mammalian cells. We have recently proposed a model whereby APC functions to concentrate β-catenin at the plasma membrane based on its ability to shuttle between the various subcellular compartments[2]. We have set out to test this model by examining the subcellular localisation of E-APC under conditions that disrupt the actin and microtubule cytoskeletal networks. Preliminary results indicate that E-APC is delocalised from the adherens junctions in embryos treated with drugs that disrupt the actin cytoskeleton, but not in embryos treated with drugs that depolymerise microtubules. Furthermore E-APC localisation is disrupted in *Drosophila* ovaries which are mutant in the actin binding protein profilin (*chickadee* mutants). We are currently testing the subcellular localisation of E-APC under conditions that disrupt nuclear transport.

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TCF factors, mediators of Wnt signaling

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Factors of the TCF/LEF HMG domain family (TCFs) exist throughout the animal kingdom. It has become evident that TCFs interact with the vertebrate WNT effector beta-catenin to mediate axis formation in Xenopus. Likewise, Armadillo (the Drosophila ortholog of beta-catenin) is genetically upstream of Drosophila TCF in the Wingless pathway. Upon Wingless/Wnt signaling, Armadillo/ beta-catenin associate with nuclear TCFs and contribute a trans-activation domain to the resulting bipartite transcription factor. In the absence of Wnt signaling, Tcf factors associate with proteins of the Groucho family of transcriptional repressors to strongly repress target gene transcription.. The cytoplasmic tumor suppressor protein APC or its homolog APC2 bind to beta-catenin causing its destruction. In APC-deficient colon carcinoma cells, beta-catenin accumulates and is constitutively complexed with the TCF family member Tcf-4. In APC-positive colon carcinomas and melanomas, dominant mutations in beta-catenin render it indestructable, providing an alternative mechanism to inappropriately activate transcription of TCF target genes. Tcf-4 gene disruption leads to the abolition of stem cells in the crypts of the small intestine, while Tcf-1 gene knockout severely disables the stem cell compartment of the thymus.

Session 3: Around p53
Chair: Tyler Jacks

The NF1 tumor suppressor: biochemical studies and mouse modeling

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Individuals with neurofibromatosis type I (NF1) develop multiple benign peripheral nerve sheath tumors and as well as other tumor types. Because loss of function mutations in the NF1 gene appear to be responsible for the disease, NF1 has been classified as a tumor suppressor. Its encoded protein, neurofibromin, exhibits homology to the catalytic domain of mammalian and yeast Ras-GAP proteins and has been shown to stimulate the GTPase activity of Ras in vitro; however, little is known about how its activity is regulated or in what contexts it affects the Ras-MAP kinase pathway. We have found that neurofibromin is degraded in response to a variety of growth factors. This process is rapid and transient; degradation initiates in less that 2 minutes and protein levels are re-elevated by 30 minutes. Neurofibromin is a direct target of the proteasome, as it is ubiquitinated in vivo and its degradation is prevented by proteasome inhibitors. Interestingly, PKC inhibitors also block its degradation while expression of activated PKC isoforms promote it, suggesting that this regulation is dependent on PKC. To gain insight as to how neurofibromin affects the Ras pathway, we examined MAP kinase activity and the growth properties of wild-type versus Nf1 - deficient mouse embryonic fibroblasts (MEFs). Exposure to sub-threshold concentrations of serum or growth factors resulted in a transient activation of MAP kinase and did not induce DNA synthesis in wild-type MEFs. However in Nf1-/- MEFs, the same stimuli elicited a dramatically sustained activation of MAP kinase and was sufficient to induce proliferation. Notably, the attenuation of the MAP kinase signal in the wild-type MEFs temporally correlated with the reappearance of neurofibromin. Thus, neurofibromin can act as a critical regulator of the duration of MAP kinase activity and in this way may play a pivotal role in preventing inappropriate proliferative responses.

A mouse model of astrocytoma development involving the mutation of Nf1 and p53 will also be described. Humans with germline mutations in NF1 have an increased risk of optic pathway gliomas, malignant astrocytomas, and glioblastomas. The p53 tumor suppressor is often mutated in a subset of astrocytomas that develop at a young age and progress slowly to glioblastoma (termed secondary glioblastomas, in contrast to primary glioblastomas that develop rapidly de novo). This mouse model shows a full range of astrocytoma progression, from low-grade astrocytoma to high-grade glioblastoma multiforme, and may be an accurate mouse model of human secondary glioblastoma involving p53 loss. This is the first reported mouse model of astrocytoma initiated by loss of tumor suppressor genes, rather than overexpression of transgenic oncogenes.

Regulation of cellular p53 activity

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The p53 tumor suppressor protein is usually latent in non-stressed cells. The cellular levels and activity of p53 increase in response to a wide array of stress-induced signals, resulting in the triggering of a p53-mediated cascade of events. To a large extent, p53-activating stress signals operate through modulating the p53-mdm2 autoregulatory loop. Attempts are being made to elucidate the molecular mechanisms that affect particular components of this loop, as well as the nature of the signals that impinge on p53.

p53 undergoes phosphorylation on multiple sites in response to DNA damage, in a way that contributes to its activation. One of the sites whose phosphorylation is regulated by DNA damage is serine 20 of p53 (1). Using variants of p53 mutated at position 20, we found that substitution of serine by aspartic acid renders the mutant p53 more resistant to inhibition by Mdm2, whereas alanine at position 20 has the opposite effect (2). In parallel, Mdm2 also undergoes altered phosphorylation upon exposure to DNA damage. One such phosphorylation event affects the reactivity of Mdm2 with the 2A10 antibody (3). The rapid kinetics of induction of this modification by DNA damage, as well as its dependence on ATM, support a role in p53 activation (3). Phage display analysis reveals that Mdm2 contains two epitopes for 2A10, both within the C terminal part of the protein. Mutational analysis is being employed to investigate how phosphorylation at these sites affects the functionality of Mdm2.[(a)]

Mutations in the WRN helicase give rise to the Werner premature aging syndrome. Werner patients possess an elevated cancer risk, and their cells exhibit enhanced genomic instability. These features also characterize cells with defective p53 function. We therefore investigated a possible relationship between p53 and WRN. We and others found that WRN and p53 can engage in direct physical association (4,5). Excess WRN induces p53 accumulation and augments p53-mediated transactivation and apoptosis. Of particular note, cells of Werner patients exhibit a defect in the induction of p53 by various DNA damaging agents, including UV radiation and the topoisomerase I inhibitor camptothecin. WRN may therefore be another molecule that, upon detection of genomic stress, can signal through p53.[(b)]
Another protein recently found to affect p53 activity is beta catenin. Excess deregulated beta catenin activity is a hallmark of colorectal cancer, as well as a variety of other epithelial tumors. In colorectal cancer, deregulation of beta catenin activity is an early event in tumor progression. At a late stage in the process, p53 mutations are encountered at a very high frequency. We found that excess beta catenin can increase the levels and activity of p53 (6). By and large, this is attained through stabilization of the p53 protein, in part through inhibition of Mdm2-mediated degradation (6). In the cells overproducing modest amounts of beta-catenin, stabilization of p53 appears to occur primarily through induction of the p19ARF tumor suppressor protein. On the other hand, very high levels of beta catenin can stabilize p53 also through ARF-independent pathways, and partially also through Mdm2-independent mechanisms [(c)]. Activation of p53 by deregulated beta catenin may give rise to a strong

selective pressure against the continued expression of functional p53 in cells where beta catenin has become aberrantly active. This may provide a partial explanation for the prevalence of p53 mutations in tumor cells that carry deregulated beta catenin. However, p53 mutations occur much later that the mutations that cause beta catenin deregulation. This suggests that additional events may be needed before p53 function becomes truly rate limiting. Such putative events and their contribution to p53 activation will be discussed

[a] In collaboration with D. Shkedy, R. Khosravi and Y. Shiloh, Tel Aviv University
[b] In collaboration with C-E Yu, and G.D. Schellenberg, VA Medical Center, Seattle
[c] In collaboration with A. Ben-Ze'ev, J. Zhurinsky and B. Geiger, The Weizmann Institute

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Response and roles of p53 in DNA damage, replication and repair

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P53 responds to multiple intracellular signaling pathways. Upon (irradiation of human cells p53 is phosphorylated at a number of sites within its N-terminus including S15, S20 and S33. Of particular interest is S20 since it is located directly within the Mdm2 interaction region. To identify an S20 kinase Hela extracts were fractionated and it was discovered that the activity that phosphorylates S20 co-purifies with the human homologue of Chk1 (hChk1). We then demonstrated that both recombinant hChk1 and the human homologue of Cds1 (hChk2) can phosphorylate p53 not only at S20 but at a number of other sites within p53 including S15 and S37. Since modulation of Chk kinase *in vivo* has a significant effect on p53 protein levels, it is likely that Chk kinases are an integral part of at least a subset of the cellular DNA damage signaling pathways to p53.

Since p53 can respond to multiple cellular stresses it was of interest to examine possible cross-regulation between upstream signaling pathways. To test this cells were arrested in early S phase by either hydroxyurea or aphidicolin. Under such conditions p53 is phosphorylated at sites including S20 and S15. Interestingly, however, not only does p53 appear to be transcriptionally inert, but it fails to become activated upon treatment of cells with (IR. Our results suggest that when cells experience stalled DNA synthesis, p53 is held in a partial state of repression which cannot be reversed by one of its normal activation signals. We are currently investigating the mechanism of this unexpected response.

We previously discovered that Ref-1 is a potent activator of p53 in vitro and *in vivo*. Since Ref-1 is an A/P endonuclease we tested whether p53 might affect the process of base excision repair (BER). It was discovered that p53 strongly stimulates BER *in vitro*, possibly through its ability to facilitate Ref-1 loading of DNA polymerase ∃ onto DNA. Tujmor-derived mutant forms of p53 are very inefficient at stimulating BER in vitro. Several lines of evidence support an impact of p53 on BER *in vivo* suggesting a novel direct role for p53 in DNA damage-induced base excision repair processes.

RNA interference in *Drosophila* cells

Greg Hannon

As ever-increasing amounts of genomic information become available, there is an acute need for facile methods for linking sequence to function. This has led a number of investigators to use genetically tractable model systems for functional studies of oncogenes and tumor suppressor proteins that lie in evolutionarily conserved biological pathways. Two systems that have proven particularly fruitful have been C. elegans and Drosophila. These models allow both forward and reverse genetic approaches. In both cases, a strong impact has been made by the discovery that double-stranded RNA can trigger gene silencing in a highly effective and highly specific manner. This effect is commonly known as RNA interference or RNAi.

Unfortunately, there is often a barrier, both in terms of facilities and expertise, which must be overcome before the use of model organisms by those that do not traditionally work with these systems. This has limited the utility of tools such as RNA-interference for those that traditionally work on mammalian systems. In an effort to democratize this approach, we asked whether RNAi could be effective in cultured Drosophila cells.

Introduction into Schneider S2 cells of double stranded RNA causes specific silencing of genes that are homologous to the input dsRNA. RNA can be introduced by transfection, or indeed by simply adding dsRNA to the media in the absence of serum. Both of these procedures elicit a response in nearly 100% of the cell population.

We have shown that RNAi can be used to create informative phenotypes in cultured cells, and are moving toward the use of this approach in our efforts to understand the biological roles of human oncogenes, such as myc, that are conserved in Drosophila. However, we have also begun a major effort toward understanding the mechanistic basis of RNAi.

We have developed an *in vitro* system from cultured S2 cells in which the process of dsRNA-induced gene silencing can be studied at a biochemical level. This system has revealed that RNAi is ultimately carried out by a sequence specific nuclease that carries a fragment of the input dsRNA as a likely guide to substrate selection. Our ongoing efforts to understand the multiple steps of this process may eventually permit the realization of our ultimate goal : to establish RNAi in mammalian systems.

Session 4: Defense responses to oncogenic stresses
Chair: Charles J. Sherr

In vivo role of the cyclin D-dependent kinases and their regulators

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Normal cell growth and differentiation requires precise control of the mechanisms that regulate the entry, progression and exit from the cell cycle. Entry of quiescent cells into the cell cycle is driven by the cyclin D-dependent kinases Cdk4 and Cdk6. Proper regulation of the activity of these kinases appears to be critical for normal cell proliferation since the majority of human tumors carry mutations that result in deregulation of these kinases. In spite of the wealth of information regarding the activity of these kinases and their regulators (CAK, INK4 inhibitors, Cdc25 phosphatases, etc.) little is known regarding the role that the different Cyclin D–dependent Cdk4 or Cdk6 complexes play in vivo.

During the last few years, several laboratories, including ours, have begun a systematic effort to generate mice carrying targeted mutations in these genes. For instance, mice defective for cyclin D1, cyclin D2 and Cyclin A2 have already been described. Moreover, genes encoding each of the known members of the Cip/Kip and INK4 families of cell cycle inhibitors have also been ablated in mice.

In our laboratory, we have generated mice lacking P57Kip2, P15INK4b and P18INK4c. P57Kip2 defective mice have already been published and will no be discussed here (Yan *et al.* Genes & Dev. **11**, 973, 1997; Zhang *et al.* Nature, **387**, 151, 1997). Ablation of P15INK4b and P18INK4c (Franklin *et al.* Genes & Dev. **12**, 2899, 1998) genes results in similar lymphoproliferative disorders and tumor formation, albeit P15INK4b mutant mice display a significantly lower incidence of neoplasias. In addition, mice lacking P18INK4c have deregulated epithelial cell growth that leads to the formation of cysts, mostly in the cortical region of the kidneys and the mammary epithelium. Concomitant loss of both P15INK4b and P18INKc does not result in significantly distinct phenotypic manifestations except for the appearance of cysts in additional tissues. These results indicate that P15INK4b and P18IKN4c are tumor suppressor proteins that act in different cellular lineages with few, if any, compensatory roles.

We have also begun the systematic manipulation of the three Cdk loci involved in G1/S progression. To date, we have generated two strains of mice that either do not express Cdk4 [Cdk4 (neo/neo) mice] or express a mutant Cdk4 protein (Cdk4 R24C) that cannot bind the INK4 family of cell cycle inhibitors (Rane *et al.*, Nat Genet., **22**, 44, 1999). This mutation had been previously found to be associated with human hereditary melanoma.

Mice devoid of Cdk4 expression are viable, but small in size and mostly infertile. The partial male sterility in cdk4 (neo/neo) mice is due to a block in late spermatogenesis due to the absence of Leydig cells. Female sterility is due to a defect in the formation of the corpus luteum. Cdk4 (neo/neo) mice also have decrease cellularity in the adenohypophysis which may contribute to the small size and sterility. Susprisingly, Cdk4 (neo/neo) mice develop insulin-dependent diabetes due to a dramatic reduction in beta islet pancreatic cells. Mice expressing the mutant Cdk4 R24C protein are viable and fertile. These mice display hyperproliferative abnormalities in most of the cell types affected in the Cdk4-deficient mice, including testicular Leydig cells, adenohypophysis and pancreatic beta cells. Indeed, most of these mice develop multiple tumors after 10 to 16 months of age, in a variety of cell types and tissues. These results illustrate the distinct roles that cell cycle regulators play in vivo and how their mutation results in abnormal proliferation that leads, in most cases, to tumor development.

The p53 pathway in tumor suppression and cancer therapy

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The p53 tumor suppressor is activated by a variety of stresses, including DNA damaging agents and mitogenic oncogenes. Once activated, p53 can produce several outcomes, including transient arrest, senescence, or apoptosis. Our laboratory has studied primary and early passage fibroblasts in an attempt to understand p53 function in cells with a well-defined genetic background. Using this approach, we have shown that the ARF tumor suppressor is required for oncogene signaling to p53 and, while not inducible by DNA damage, can potentiate a DNA damage response (de Stanchina et al., 1998). We also have used normal fibroblasts to show that p53 can promote apoptotic cell death through the Apaf-1/Caspase-9 death effector complex (Soengas et al., 1999). These data demonstrate that Apaf-1 and Casp-9, like p53, have tumor suppressor properties. More recently, we have begun to extend observations from these simple fibroblast systems to epithelial cells, animal models, and primary human tumors (e.g. (Schmitt et al., 1999)). First, we have shown that oncogene signaling to p53 can operate through ARF in epithelial cells, where this pathway acts to limit proliferation and tumorigenic potential. Second, we have shown that effector components of p53 in apoptosis are altered in human tumor cells. Finally, we have developed powerful new methods for studying the impact of the p53 pathway on therapy-induced cell death in vivo, and are investigating the impact of p53 pathway components on this response. Our results further define the p53 tumor suppressor pathway, and establish physiological systems for testing novel anticancer therapies targeted against mutations in p53 or the p53 pathway.

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The ARF tumor suppressor

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Mitogen-dependent progression through the G1 phase of the cell cycle depends upon cyclin-dependent kinases (CDKs) whose activities are required for chromosomal DNA synthesis. Entry into the cell cycle from quiescence and progression into S phase require phosphorylation of key substrates, including (i) the retinoblastoma protein (Rb), thereby activating an E2F-dependent transcriptional program; (ii) the CDK inhibitor p27, triggering its proteolytic degradation and facilitating further CDK activation; and (iii) most probably proteins at replication origins whose modifications are required for the initiation of DNA synthesis *per se*. Dismantling the "Rb pathway" through either cyclin D or CDK4 overexpression, or loss of the tumor suppressors, the CDK inhibitor $p16^{INK4a}$ or Rb itself, occurs so frequently in cancer, irrespective of tumor type or patient age, that such events may be necessary in the life history of many, if not all, tumor cells.

Inappropriate signaling through the cyclin D/CDK4 – Rb – E2F circuit can trigger a checkpoint that restrains cells from progressing through the cell cycle by invoking either growth arrest or apoptosis. The checkpoint depends upon the product of the alternative reading frame of the *INK4a/ARF* locus ($p19^{ARF}$ in mouse, $p14^{ARF}$ in humans), which antagonizes Mdm2 to activate p53. ARF is induced by overexpression of S phase-promoting proteins or oncogenes, such as E2F-1, Myc, adenovirus E1A, activated Ras, and v-Abl. Conversely, ARF inactivation disables tumor surveillance by allowing the latter proteins to function as pure growth promoters. Although conclusions regarding the mechanism of ARF action initially derived from work with cultured primary cells, several animal models for cancer progression have now reinforced the view that ARF acts in tumor surveillance in a number of different biologic settings.

Mouse $p19^{ARF}$ and human $p14^{ARF}$ are nucleolar proteins, and their binding relocalizes Mdm2 to the nucleolus enabling p53 accumulation in the nucleoplasm. Although the architecture of the mouse and human ARF polypeptides is somewhat different, both ARF proteins contain two binding sites for Mdm2 separated by spacer sequences. Paradoxically, signals required for ARF nucleolar localization overlap one of the Mdm2 binding sites. The N-terminal 37 amino acid segment of mouse ARF contains all essential sequences required for Mdm2 binding, nucleolar localization, and p53-dependent cell cycle arrest. ARF binds to a central acidic domain of Mdm2 located downstream of the Mdm2 nuclear localization and nuclear export sequences (a.a. 210-304). This region of Mdm2 is not required for p53 binding (conferred by the Mdm2 N-terminus) or for E3 ubiquitin ligase activity (restricted to the RING domain at the Mdm2 C-terminus). ARF binding to Mdm2 is cooperative, inducing a conformational change that reveals a cryptic nucleolar localization signal located within the C-terminal Mdm2 RING finger domain. The integrity of the latter sequence is required for localization of the ARF-Mdm2 complex to the nucleolus. Relocalization of Mdm2 to the nucleolus by ARF illustrates a novel mechanism for preventing p53 turnover and facilitates our understanding of how stress signals that induce DNA damage can synergize with ARF to regulate p53 function.

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Characterization of oncogene-induced cell senescence

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The *INK4a-ARF* locus encodes two tumor suppressors, $p16^{INK4a}$ and $p19^{ARF}$, each regulating a different tumor suppressor pathway. Namely, $p16^{INK4a}$ activates Rb by inhibiting the CDK4 and CDK6 kinases; and $p19^{ARF}$ activates p53 by blocking the destabilizing effects of MDM2.

Expression of oncogenic Ras in primary cells is initially mitogenic but, after a period of a few days, triggers an anti-proliferative response mediated by both $p16^{INK4a}$ and $p19^{ARF}$ [1,2]. Cells arrested by introduction of oncogenic Ras are vey similar to senescent cells and, consequently, this phenomenon has been designated "premature senescence" [1]. We regard this response as an anti-tumoral mechanism that prevents the propagation of oncogenically-driven cells. Importanly, rodent cells genetically deficient in either $p16^{INK4a}$, $p19^{ARF}$ or p53 do not enter "premature senescence" upon introduction of oncogenic Ras but, on the contrary, they are efficiently transformed. This permissivity to Ras transformation was not observed with cells deficient in the cell-cycle regulators p21 [3] or p27 (unpublished observations).

We have performed a large-scale screening aimed at identifying other tumor-suppressor pathways that could be activated in response to the aberrant activity of oncogenic Ras. For this, we have used filters of high-density cDNA arrays (containing 18.000 ESTs) and we have compared the expression profile of cells arrested by serum deprivation with the expression profile of cells arrested by Ras-induced premature senescence. We have identified and validated a total of five genes that are highly upregulated during Ras-induced senescence. We will discuss the significance of these genes in senescence and in the response to oncogenic stresses.

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Down regulation of 14-3-3σ prevents clonal evolution and leads to immortalization of primary human keratinocytes

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In human epidermal keratinocytes, replicative senescence is determined by a progressive decline of clonogenic and dividing cells, and its timing is controlled by clonal evolution, that is by the continuous transition from stem cells to transient amplifying cells. We now report that down regulation of 14-3-3σ, which is specifically expressed in human stratified epithelia, prevents keratinocyte clonal evolution thereby forcing keratinocytes into the stem cell compartment. This allows primary human keratinocytes to readily escape replicative senescence. 14-3-3σ-dependent bypass of senescence is accompanied by maintenance of telomerase activity and by down-regulation of the $p16^{INK4a}$ tumour suppressor gene, hallmarks of keratinocyte immortalization.

Taken together, these data therefore suggest that inhibition of a single endogeneous gene product fosters immortalization of primary human epithelial cells without the need of exogeneous oncogenes and/or oncoviruses.

Interactions between Ras, beta-catenin and p53 pathways in human cancer

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Activation of Ras and beta-catenin signalling pathways occurs frequently in many types of cancer. Inactivation of the p53 pathway is also a frequent event. In metastatic colon cancer, all three pathways are altered in the majority of cases. Ras activates at least two signalling pathways, the Raf-MAP kinase cascade and the PI 3' kinase pathway. Cyclin D1 is a transcriptional target of the MAP kinase pathway, and the PI 3'kinase affects cyclin D1 stability. We have identified cyclin D1 as a transcriptional target of the beta-catenin pathway in colon cancer cells. We are now investigating which of these pathways is the major contributor to cyclin D1 expression in these cells, and whether interactions between these pathways are synergistic.

We have also identified mdm-2 as a major target of the Raf-MAP kinase pathway. Induction of mdm-2 transcription is p53-independent, and occurs through Ets and Ap-1 sites in the P2 promoter, just upstream from the p53-responsive element. In tumor cells that retain wild type p53, Ras may contribute to suppression of p53 activity through induction of mdm-2. In Ras transformed cells, resistance to apoptosis following radiation is a result of increased mdm-2. Furthermore, high levels of mdm-2 enable the oncolytic adenovirus ONYX-015 to replicate efficiently, despite the presence of wild-type p53. Loss of p14ARF, a protein that inhibits mdm-2, also facilitates replication of this agent.

The Ras pathway also activates p53, through the p14ARF pathway. We believe that Ras induces expression of mdm-2 early in G1 following stimulation of receptor tyrosine kinases. This mdm-2 keeps p53 at a basal level and allows progression to S-phase. E2F is activated, partly through ras-dependent transcription of cyclin D1, and E2F turns on expression of p14ARF. This protein can inhibit mdm-2 and allow accumulation of p53. This regulates entry into S-phase.

Inhibition of the MAP kinase pathway results in G1 arrest in colon cancer cells. This is the result of loss of cyclin D1 and cdk4, and subsequent redistribution of p27 to cdk2. Of these two effects, inhibition of cdk4 seems more important, as forced expression of high levels of p27 do not cause growth arrest in these cells. High levels of cdk4 activity may therefore make colon cancer cells relatively independent of cdk2 activity.

Session 5: DNA damage and DNA stability
Chair: David M. Livingston

Telomeres, cancer and DNA damage

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Telomere length has been proposed to limit the proliferative capacity of cells. Introduction of constitutively expressed telomerase catalytic subunit into cells with a limited life span is sufficient to stabilize their telomeres and, in some cases, to extend their life span indefinitely (Bodnar et al., 1998). Telomerase can also cooperate with oncogenes to transform primary human cells in culture, suggesting that telomere maintenance by telomerase contributes to malignant transformation (Hahn et al., 1999). These findings have led scientists to propose that telomerase inhibition in human cancers could lead to telomere shortening eventually compromising tumor growth.

The characterization of mice genetically deficient for telomerase activity, is an important step toward understanding the role of telomerase in telomere maintenance and tumorigenesis (Blasco et al., 1997). As telomeres shorten and chromosome fusions accumulate with increasing generations, $mTR^{-/-}$ mice show defects in proliferative tissues. We will describe skin tumor susceptibility of wild-type and different generation telomerase-deficient $mTR^{-/-}$ mice. Our results indicate that telomerase inhibition and short telomeres have a dramatic effect in carcinogen-induced skin tumorigenesis.

Recently, it has been proposed that telomeres may have a role in DNA repair in mamals. To address this we have studied radiation sensitivity of late generation $mTR^{-/-}$ mice which lack telomerase activity and show short telomeres.

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Functional analysis of the von Hippel-Lindau and retinoblastoma tumor suppressor proteins

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Von Hippel-Lindau disease is a hereditary cancer syndrome characterized by the development of hemangioblastomas of the central nervous system and retina, pheochromocytomas, and clear cell carcinomas of the kidney. This disease is caused by germline mutations of the VHL tumor suppressor gene. Tumors develop when the remaining wild-type allele is inactivated. The product of the VHL gene, pVHL, forms multimeric complexes that contain elongin B, elongin C, Cul2, and Rbx1. These complexes resemble so-called SCF complexes (Skp1- Cdc53-F-box protein), which function as E3 ubiquitin ligases. Cells lacking pVHL overproduce hypoxia-inducible mRNAs under well-oxygenated conditions. Recent work from Peter Ratcliffe's group showed that cells lacking pVHL are unable to degrade members of the HIF (hypoxia-inducible factor) transcription factor family. We have gone on to show that a frequently mutated region of pVHL binds directly to HIF. Furthermore, pVHL binds to a region of HIF called the oxygen-dependent degradation domain which is necessary and sufficient to render HIF unstable in the presence of oxygen . Finally , we provide the first direct evidence that the pVHL complex does, indeed, function as an E3 ubiquitin ligase and that HIF is one of its targets.

pRB is a prototypical tumor suppressor protein that has been intensively studied for the past 10 years. The majority of human tumors harbor mutations that directly or indirectly inactivate pRB. Inactivation of pRB leads to derepression of E2F-responsive promoters including the promoter for ARF. ARF, in turn, neutralizes MDM2 and thereby stabilizes p53. Thus, pRB and a second tumor suppressor protein, p53, are in a regulatory network. Adding complexity to this network, we have now discovered that the p53 homolog, p73, it itself transcribed from an E2F-responsive promoter. This finding may account for the earlier observation that E2F can induce apoptosis in p53-defective tumor cells and has implication for treating cancer cells with E2F agonists.

Genomic integrity in mammalian cells: a key role for homologous recombination

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DNA repair processes play an essential role in maintaining chromosome structure and genetic integrity. The significance of these processes is emphasized as defects in DNA repair pathways are linked to human disease and malignancy. A DNA double-strand break (DSB) is one type of lesion that can compromise genomic integrity. Two major pathways are responsible for the repair of DSBs in mammalian cells, nonhomologous end-joining (NHEJ) and homologous recombination (HR) (Liang et al. 1998). A role for NHEJ is well established in mammalian cells whereas a role for HR in DNA repair is just emerging.

We have recently established that HR with a sister-chromatid is a prominent pathway for DSB repair in mammalian cells (Johnson and Jasin, submitted). Considering the lethality associated with disruption of some genes implicated in HR, sister-chromatid repair of spontaneously arising lesions, perhaps from replication, may be essential for cellular viabilty. HR between homologous chromosomes or homologous sequence repeats on heterologous chromosomes can also be used to repair a DSB, albeit less efficiently than between sister-chromatids (Richardson et al. 1998). Interestingly, translocations are not recovered from HR between heterologous chromosomes, although they are readily recovered from NHEJ and homologous annealing events (Richardson and Jasin, in press).

An essential component of HR pathways is the evolutionarily conserved strand transferase, RecA in bacteria or Rad51 in yeast. Several mammalian proteins have been implicated in HR based on their sequence homology to yeast Rad51, including a Rad51 homolog which is 68% identical. In addition, there are the distantly related XRCC2 and XRCC3 proteins, which share about 20% identity with human Rad51 (Liu et al., 1998). Chromosome aberrations are frequent in hamster cell lines deficient in the XRCC2 and XRCC3 proteins, apparently due to reduced HR (Johnson et al. 1999; Pierce et al. 1999). NHEJ, however, is normal. The products of the hereditary breast cancer genes *BRCA1* and *BRCA2* are also implicated in HR as they have been shown to associate with Rad51 (Chen et al., 1998). Mouse ES cells with a hypomorphic *BRCA1* allele exhibit reduced levels of HR, but normal NHEJ (Moynahan et al., 1999). These studies emphasize the importance of homologous repair in maintaining genomic integrity and guarding against tumorigenesis.

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Functional analysis of the BRCA1 gene product

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BRCA1 is a large, nuclear protein dedicated, in part, to the suppression of breast and ovarian cancer. BRCA1 participates in certain DNA damage responses and is a significant contributor to genome integrity control. It is an ATM substrate, a contributor to mitotic checkpoint control, and a participant in the transcription- coupled repair of oxidative DNA damage. Its biochemical partners include proteins dedicated to ds break repair [DSBR], such as Mre11, Rad 50, and Nbs1 [the MRN complex] and Rad 51. It also has the potential to affect certain transcription regulation events. The evolutionary preservation of key elements of BRCA1 sequence extends through mammals to related gene products of at least one plant species (Arabidopsis Thaliana) and Xenopus laevis, implying that BRCA1 exerts certain basic survival functions vital for a diverse collection of organisms. Genome integrity control may well be one such function.

What is presently unclear are the answers to two prime questions: how does BRCA1 exert its tumor suppressing function(s) and why are BRCA1 tumors manifest primarily in the breast and ovary? In an effort to address the first question, we have established a system for performing genetic analyses of certain BRCA1 functions. The approach is based on the observation that a BRCA1 -/- cell line is hypersensitive to gamma irradiation (IR), as manifest in two ways. The DNA is hypernickable after standardized doses of IR, and double strand break repair (DSBR) is inefficient. The method allows one to probe the behavior of selected mutant alleles expressed in BRCA1 -/- cells at physiological levels. When such an analysis was performed in a BRCA1 -/- breast tumor cell line, it became clear that, unlike the wt BRCA1 protein, synthesis of BRCA1 species containing single, clinically relevant missense mutations which, in the aggregate, affect a diverse set of functional domains each failed to correct either the hypernickability or the DSBR defect. Thus, one can hypothesize that multiple BRCA1 domains cooperate to exert a common function(s). One view of such a coordinated function(s) is that BRCA1 operates, at least in part, as a scaffold, bringing the diverse functions of multiple proteins (e.g. MRN and Rad 51) to bear on limiting nickability and promoting DSBR. In this regard, we have recently discovered and cloned a new BRCA1-associated protein (p130) which, although unique, contains a large, DNA-dependent helicase motif with significant homology to XP-D. BRCA1/p130 binding depends upon the integrity of the BRCA1 C-terminal BRCT motifs, and a genetic analysis in BRCA1 -/- cells strongly suggests that a physiological interaction with p130 contributes to BRCA1- mediated DSBR. This result suggests that the role of BRCA1 in DSBR could include an indirect influence on DNA mechanics. Given these and the above-noted genetic data, one might hypothesize that at least part of the BRCA1 contribution to tumor suppression involves proper limitation of DNA nickability and regulation of the ds break repair process.

POSTERS

Calmodulin binds to p21^{cip1} and is involved in the regulation of its nuclear localization

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P21^{cip1}, first described as an inhibitor of cyclin-dependent kinases, has recently been shown to have a function in the formation of cyclin D/cdk4 complexes and in their nuclear translocation. The dual behavior of p21^{cip1} may be due to its association with other proteins. Different evidence presented here indicate an *in vitro* and *in vivo* interaction of p21^{cip1} with calmodulin: 1) purified p21^{cip1} is able to bind to calmodulin-Sepharose in a Ca^{2+}-dependent manner and this binding is inhibited by the calmodulin-binding domain of calmodulin-dependent kinase II; 2) both molecules co-immunoprecipitate when extracted from cellular lysates; and 3) colocalization of CaM and p21^{cip1} can be detected *in vivo* by electron microscopy immunogold analysis. The carboxy-terminal domain of p21^{cip1} is responsible for the calmodulin interaction, since p21$^{145-164}$ peptide is also able to bind calmodulin and to compete with full-length p21^{cip1} for the calmodulin binding. Because treatment of cells with anti-calmodulin drugs decreases the nuclear accumulation of p21^{cip1}, we hypothesize that calmodulin interaction with p21^{cip1} is important for p21^{cip1}, and in consequence for cyclin D/cdk4, translocation into the cell nucleus.

The Protein SET Regulates the Inhibitory Effect of $p21^{Cip1}$ on Cyclin E-CDK2 Activity

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The cyclin-dependent kinase inhibitor $p21^{Cip1}$ has a dual role in the regulation of the cell cycle: it is an activator of cyclin D1-CDK4 complexes and an inhibitor of cyclins E/A-CDK2 activity. By affinity chromatography with $p21^{Cip1}$-sepharose 4B columns we purified a 39 kDa protein, which was identified by microsequence analysis as the oncoprotein SET. Complexes containing SET and $p21^{Cip1}$ were detected *in vivo* by immunoprecipitation of Namalwa cell extracts using specific anti-$p21^{Cip1}$ antibodies. We found that SET bound directly to $p21^{Cip1}$ *in vitro* by the carboxy-terminal region of $p21^{Cip1}$. SET had no direct effect on cyclin E/A-CDK2 activity although it reversed the inhibition of cyclin E-CDK2, but not of cyclin A-CDK2, induced by $p21^{Cip1}$. This result is specific for $p21^{Cip1}$ since SET neither bound to $p27^{Kip1}$ not reversed its inhibitory effect on cyclin E-CDK2 or cyclin A-CDK2. Thus, SET appears to be a modulator of $p21^{Cip1}$ inhibitory function. These results suggest that SET can regulate G1/S transition by modulating the activity of cyclin E-CDK2.

Transcriptional regulation of human p16

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The tumor suppressor p16 regulates the cell-cycle by binding and inhibiting the CDK4/cyclin D kinase. The protein levels of p16 are mainly determined by the transcriptional activity of the INK4a gene. Therefore, it is interesting to study the transcriptional regulation of p16. So far there are only three stimuli that regulate p16 levels: the status of Rb, the accumulation of cell doublings and oncogenic ras (1).

Previously, a region at the INK4a promoter has been defined sufficient for basal promoter activity (2). Based on this, we have further analyzed the region responsible for this activity by using electrophoretic mobility shift assays (EMSA). In particular, we have used nuclear extracts from cells that either overexpress p16 (Hela, 293, VA13) or that express low or moderate levels of p16 (IMR90, WI38). These extracts have been tested in EMSA using DNA fragments from the above-mentioned region. We have found two specific complexes that bind to this region, and the abundance of one of them perfectly correlates with the levels of p16 expression. We are now attempting to identify which are the proteins that form this complex. In addition, we have identified critical nucleotides required for the formation of these complexes. We are also studying the relevance of these nucleotides in the transcriptional activity of the INK4a gene.

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Disruption of the apoptosis suppression function of the retinoblastoma protein by coexpression of its c-terminal fragment

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Programmed cell death plays an important role in physiological processes, such as immune- and nervous-system development, and contributes to defense mechanisms important for the prevention of cancer. Apoptosis can also be triggered by various external stimuli including DNA-damaging agents such as chemotherapeutic drugs and irradiation. Several lines of evidence indicate that the tumor suppressor protein Retinoblastoma (pRb) actively regulates cell death. Firstly, pRb nullizygous mouse embryos exhibit massive cell death in the central and peripheral nervous systems. Secondly, pRb has been shown to inhibit apoptosis induced by several stimuli, such as ionizing radiation, IFN-γ, Ceramide, and p53 overexpression. Although it has been proposed that this process may involve the binding of pRb with apoptosis-related proteins, such as E2F, Cyclin D, Mdm2, Id proteins, and c-Abl, the identity of the pRb partner in this process has yet to be established. To this end, we have tested the ability of several pRb mutants to inhibit ionizing radiation-induced apoptosis and found that wild-type pRb and pRb 379-928 (which binds all the proteins mentioned above) similarly inhibited cell death, while pRb 1-792 (which binds cyclin D and Id family members but does not interact with E2F, c-Abl or Mdm2) and pRb 768-928 (which efficiently binds to c-Abl and Mdm2, but not to E2F, Cyclin D, or Id proteins), failed to inhibit apoptosis. Ectopic expression of another pRb family member, p107, also inhibits ionizing radiation-induced cell death and this function also correlates with the ability of p107 to interact with E2F. These data would suggest that the interaction of pRb family members with E2F suffices to promote cell survival. We have found, however, that coexpression of a mutant pRb C-terminal fragment, pRb SEΔ, which disrupts the interaction of Mdm2 and c-Abl, but not that of E2F, with full-length pRb, overcomes the apoptosis inhibition function of full-length pRb, indicating that the formation of a multimeric complex involving E2F, pRb, and either Mdm2 or c-Abl is required for the apoptosis suppression function of pRb. Experiments to address the role of c-Abl and Mdm2 in this process are currently underway.

Beta-catenin independent induction of p53 activity by Dishevelled

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The Wnt signaling pathway has been implicated in many biological processes, especially tumorogenesis. Dishevelled (Dvl) is a major component of the Wnt signaling pathway, upstream of beta-catenin. We found that overexpression of exogenous Dvl strongly induced p53 activity in human embryonic kidney epithelial 293 cells, as measured by elevated p21 protein level or activity of luciferase reporter driven by p53 responsive promoters. Beta-catenin did not induce p53 under the same experimental condition, although it strongly activats TCF dependent responses. APC, Axin/Conductin, GSK3 and dominant-negative TCF4 can efficiently block the ability of Dvl to upregulate beta-catenin, yet they all failed to block the induction of p53 by Dvl. Currently, we are generating a cell line expressing inducible forms of Dvl and investigating the mechanism of this Dvl induced p53 activity. Our results not only present a new regulation of p53 but also will shed light on how Wnt signaling pathway links to oncogenesis.

Inactivation of *Pten* and *Cd95* (*Fas*) is crucial to the development of γ-radiation-induced mouse thymic lymphomas

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Loss of heterozygosity (LOH) analysis performed in 68 γ-radiation-induced primary thymic lymphomas of F1 hybrid mice, provided evidence of significant LOH on chromosome 19 in a region that contains the *Cd95* and *Pten* genes. Here, we demonstrate that both genes are inactivated in 13 of 31 (41.9%) early lymphomas and, more frequently, in frank lymphomas (58/68, 85.3%, for *Cd95*; 54/68, 79.4%, for *Pten*). These data suggest that inactivation of *Cd95* and *Pten* is required for γ-radiation-induced thymic lymphoma progression.

Structure of an E6AP-UbcH7 complex which ubiquitinates p53 in cervical cancer

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The E6AP ubiquitin-protein ligase (E3) mediates the human papillomavirus-induced degradation of the p53 tumor suppressor in cervical cancer and is mutated in Angelman syndrome, a neurological disorder. The crystal structure of the catalytic hect domain of E6AP reveals a bilobal structure with a broad catalytic cleft at the junction of the two lobes. The cleft consists of conserved residues whose mutation interferes with ubiquitin-thioester bond formation, and is the site of Angelman syndrome mutations. The crystal structure of the E6AP hect domain bound to the UbcH7 ubiquitin-conjugating enzyme (E2) reveals the determinants of E2-E3 specificity, and provides insights into the transfer of ubiquitin from the E2 to the E3.

Inhibition of PI-3 kinase pathway induces a senescent-like arrest mediated by $p27^{Kip1}$

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Abstract

A senescent-like growth arrest is induced in mouse primary embryo fibroblasts by inhibitors of P-I3 kinase. We observed that the senescent-like growth arrest is correlated with an increase in $p27^{Kip1}$ but down-regulation of other CKI including $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, $p19^{INK4D}$ and $p21^{CIP1}$ as well as negative cell cycle regulators such as p53, p19ARF, implying that this senescence related growth arrest is independent of the activity of p53, p19ARF, p16 and p21, which are associated with replicative senescence. The increase in $p27^{Kip1}$ was a consequence of an increase in stability of $p27^{Kip1}$ protein following treatment with specific P-I3 kinase inhibitors. The $p27^{Kip1}$ binds to the cyclin/CDK2 complexes and causes a decrease in CDK2 kinase activity. We also demonstrated that ectopic expression of $p27^{Kip1}$ can induce permanent cell cycle arrest and a senescence phenotype in wild-type mouse embryo fibroblasts. In summary, these observations taken together suggest that $p27^{Kip1}$ could be an important mediator of the permanent cell cycle arrest induced by PI-3 kinase inhibitors. Our data suggest that repression of CDK2 activity by $p27^{Kip1}$ is required for the P-I3 kinase induced senescense, yet mouse embryo fibroblasts derived from $p27^{Kip1-/-}$ mice entered cell cycle arrest after treatment with LY294002. We show that this is due to a compensatory mechanism by which p130, but not other CIP/KIP or INK4 family of CKIs, functionally substitutes for the loss of $p27^{Kip1}$. This is the first description that p130 may have a role in inhibiting CDK activity during senescence.

c-Myc antagonizes p53 in myeloid leukemia cells

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Overexpression of c-*myc* is found in many tumors, including chronic myeloid leukemia in blast crisis. We used the K562 human myeloid leukemia cell line as a model to study the functional interaction between c-Myc and p53. Using two pathways methods, we generated double K562 transfectant cell lines with conditional expression of either c-Myc or p53. The cells expressed the $p53^{Val135}$ mutant, which adopts a wild-type conformation at 32°C, while c-Myc induction was achieved with a zinc-inducible expression vector. We found that p53 in wild-type conformation resulted in growth arrest and apoptosis of K562. However, c-Myc partially rescued the cells from apoptosis, as assessed by cell morphology, annexin binding and DNA laddering. This effect was accompanied by an inhibition of the induction of $p21^{WAF1}$ gene mediated by 53, while the induction of the *bax* mRNA was unaffected. Consistent with its effect on $p21^{WAF1}$ expression, Myc induction resulted in increased Cdk2 activity in p53 expressing cells.

We asked whether c-Myc impaired the transactivation activity of p53. We found that c-Myc inhibited the transactivation of $p21^{WAF1}$, Bax and Mdm2 promoters mediated by p53. This was demonstrated by co-transfection of promoter-luciferase reporter with a c-*myc* constitutive expression vector, a c-Myc-estrogen receptor fusion protein and by induction of c-*myc* by zinc in transfected cells. These results suggest that c-Myc overexpression may antagonize the pro-apoptotic function of p53, thus providing a putative molecular mechanism for the c-*myc* deregulation observed in many human tumores.

Physical and functional interactions between ARF and MDM2

Susana Llanos, Paula Clark and Gordon Peters

The *CDKN2A* locus on human chromosome 9p21 encodes two structurally unrelated proteins that both function in tumour suppression. The product of the alpha transcript, $p16^{INK4a}$, induces a G1 cell cycle arrest by inhibiting phosphorylation of the retinoblastoma protein (pRb) by the cyclin-dependent kinases, Cdk4 and Cdk6. In contrast, the product of the beta transcript, $p14^{ARF}$, activates a p53 response resulting in elevated levels of MDM2 and $p21^{CIP1}$ and a cell cycle arrest in both G1 and G2/M. Interestingly, the expression of ARF is positively regulated by E2F1 and negatively regulated by p53, suggesting that ARF plays a central role in linking oncogenic signals that impinge on pRb and cell cycle arrest or apoptosis mediated via p53.

Activation of p53 by ARF depends on a direct interaction between ARF and MDM2, a multi-functional protein that blocks the transcriptional activity of p53, promotes the nuclear export of p53 by nucleo-cytoplasmic shuttling, and acts as an E3 ubiquitin ligase for p53, thereby promoting its destruction by cytoplasmic proteasomes. ARF appears capable of inhibiting the MDM2-mediated ubiquitination of p53 in vitro and of retaining p53 in the nucleus by interfering with nucleo-cytoplasmic shuttling. However, the precise mechanisms remain unclear since ARF is predominantly nucleolar whereas MDM2 and p53 are normally nucleoplasmic. Two conflicting models have emerged, one of which contends that ARF sequesters MDM2 in the nucleolus whereas the other claims that MDM2 relocates ARF in so-called nuclear bodies. To investigate such issues, we have exploited a human cell line (NARF2) in which ARF expression is controlled by an inducible promoter. In these cells, relatively modest induction of ARF leads to increased levels of endogenous MDM2, p53 and $p21^{CIP1}$. Immunofluorescence analyses indicate that under these conditions, in which ARF elicits a cell cycle arrest, the endogenous MDM2 remains nucleoplasmic and is not co-localised in the nucleolus with ARF. This presents an obvious paradox: how can ARF prevent MDM2-mediated destruction of p53 if the proteins are not co-localised in the cell. One possibility is that permanent association with MDM2 may not be required for ARF function. The other is that under conditions in which we can detect ARF by immunofluorescence, it is present in vast excess over the endogenous MDM2. Thus, a small proportion of the induced ARF may be bound to MDM2 in the nucleoplasm whereas the excess is deposited in the nucleolus. We are currently trying to generate active forms of ARF that do not localise in the nucleolus. In contradiction to published data, we find that the amino terminal domain of human ARF does associate with nucleoli, albeit less efficiently than the full length protein, and is able to elicit the full panoply of ARF-mediated responses.

A Common Polymorphism Acts as An

Intragenic Modifier of Mutant p53 Behavior

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The p53 tumor suppressor protein homolog p73 can activate p53-responsive promoters and induce apoptosis in p53 nullizygous cells. We have found that some tumor-derived p53 mutants can bind to, and inactivate, p73. Furthermore, the binding of such mutants is influenced by whether p53 codon 72, by virtue of a common polymorphism in the human population, encodes Arg or Pro. The ability of mutant p53 to bind to p73, to neutralize p73-induced apoptosis, and to transform cells in cooperation with EJ-Ras, was enhanced when codon 72 encoded Arg. Squamous cells produce p73 and we found that the Arg allele was preferentially mutated and retained in various squamous cell tumors arising in Arg/Pro germline heterozygotes. Thus, inactivation of p53 family members may contribute to the biological properties of selected p53 mutants. Furthermore, a polymorphic residue within p53 is an intragenic modifier of mutant p53 behavior.

Status of $p19^{ARF}$ in mice lacking exons 2 and 3 of the *INK4a/ARF* locus

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The *INK4a/ARF* locus encodes two tumor suppressors, $p16^{INK4a}$ and $p19^{ARF}$. The INK4a and ARF genes have their own separate promoters, each of which produces a different transcript. The INK4a transcript is formed by exons 1α, 2 and 3; whereas the ARF transcript is formed by exons 1β, 2 and 3. Previously, a mutation consisting on the elimination of exons 2 and 3 has been introduced into the mouse germline (1). These mutant mice are tumor prone, and this phenotype has been attributed to the combined effects of eliminating both $p16^{INK4a}$ and $p19^{ARF}$. However, it is well established that all the functional domains of murine $p19^{ARF}$ are encoded by exon 1β (2), and the INK4a/$ARF^{\Delta ex2,3}$ mutation does not alter exon 1β. For these reasons, we have analyzed in detail the functional status of $p19^{ARF}$ in homozygous INK4a/$ARF^{\Delta ex2,3}$ cells.

We have found that INK4a/$ARF^{\Delta ex2,3}$ cells express a chimeric transcript containing exon 1β fused to exons from the gene immediately downstream of $p16^{INK4a}$. Regarding the regulation of the chimeric transcript, we have seen an increase of its expression with the accumulation of cell doublings, and in response to oncogenic Ras. We have preliminary results indicating that this chimeric transcript is functional in producing cell cycle arrest when overexpressed in p53-positive cells.

Together, these data suggest that the INK4a/$ARF^{\Delta 2,3}$ mutation does not completely inactivate $p19^{ARF}$ function.

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TGF-β1 signaling mechanisms involved in tumor progression of squamous cell carcinomas.

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TGF-β1 acts as a suppressor of tumor formation by virtue of its role of inhibiting proliferation of epithelial cells, but it also stimulates malignant progression of later stages of tumorigenesis by inducing a transition from squamous to spindle cell carcinomas associated with increased invasive and metastatic abilities (1, 2). Stimulation of malignancy by TGF-β1 appears to require cooperation with a Ras oncogene (3).

We are studying the transduction mechanisms triggered by TGF-β1 in keratinocytes to regulate genes either involved in growth inhibition, as the cell cycle inhibitor $p21^{Cip1}$, or implicated in malignant progression, such as the urokinase-type plasminogen activator (uPA), its inhibitor PAI-1, and the metalloproteinase MMP-9. Our results show that TGF-β1 activates both the Ras-MAP kinase and the Smad signaling pathways in keratinocytes, and that one or another transduction pathway regulates TGF-β1-induced expression of specific genes, depending on the cell status (mainly, the presence of a Ras oncogene). Thus, in transformed PDV keratinocytes which contain a mutated Ras gene, stimulation by TGF-β1 of uPA expression/secretion requires signaling through Ras/MEK/Erk, while TGF-β1 regulation of PAI-1 promoter activity and MMP-9 secreted levels depend on Smad. Smad signaling activity appears to mediate TGF-β1-induced up-regulation of $p21^{Cip1}$ and growth arrest in immortalized MCA3D keratinocytes (which contain normal Ras genes). However, in PDV cells, the Smad pathway is only partially involved in TGF-β1-induced growth inhibiton, and does not mediate TGF-β1 enhancement of $p21^{Cip1}$ levels. PD098059, a specific inhibitor of MEK, disminishes TGF-β1-induced $p21^{Cip1}$ levels in PDV but not in MCA3D cells, suggesting an involvement of Erk in up-regulation of $p21^{Cip1}$ by TGF-β1 in transformed PDV keratinocytes.

Interestingly, PDV dominant-negative Smad4 cell transfectants, but not MCA3D dominant-negative transfectants, showed constitutive hyperactivation of Ras/Erk signaling activity,

increased production of uPA, higher motility properties, and a change to a fibroblastoid cell morphology associated *in vivo* with progression from well differentiated to poorly differentiated tumors. These results indicate that, in Ras-transformed keratinocytes, Smad4 (or the Smad pathway) acts inhibiting Ras-dependent Erk signaling activity. Loss of Smad4 function in these cells results in hyperactivation of Erk signaling and progression to undifferentiated carcinomas.

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The *Rb* gene family and the control of cell cycle: characterization of Rb-/-;p107-/-;p130-/- triple knock-out cells.

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The *RB* gene is mutated in retinoblastoma and in many other types of human tumors, suggesting an important role in tumor suppression. The normal gene product, pRB, acts as a central cell cycle regulator, and blocks cells in the G1 phase of their cycle, by complexing with E2F transcriptional activators. *RB* belongs to a family of three genes encoding related proteins, pRB, p107 and p130. Each family member interacts with specific E2F factors, and the three genes are differentially expressed during mouse development, suggesting that each protein has some distinct functions. All three *RB* family members have been mutated in mice by gene targeting, and intercrossing experiments have provided strong evidence that the three genes have overlapping functions in some tissues of the developing mouse.

We have generated mouse embryonic stem (ES) cells and mouse embryonic fibroblasts (MEFs) deficient for the three family members (triple knock-out, or 'TKO' cells). We are using these cells to study the effect of this triple mutation in cell cycle, cell differentiation and cell death, *in vivo* and *in vitro*. Also, by comparing the TKO cells with single and double mutant cells, we are determining the precise roles of each family member in different aspects of cell regulation. Results will be presented indicating that even though the three family members have been previously implicated in differentiation, the TKO cells are still able to differentiate into various cell types *in vivo* and *in vitro*. We will also present evidence that absence of the three family members leads to various cell cycle defects and confers several characteristics of transformed cells to MEFs.

Impact of mutated tumor suppressors genes on chemoresistance in a physiological and genetically controlled mouse lymphoma model

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Tumor suppressor genes controlling programmed cell death are suspected to play a major role in anticancer drug mediated cytotoxicity. If disruption of the apoptotic machinery, acquired under tumor progression or in response to chemotherapy, actually plays a critical role for treatment failure, the genetically encoded cell death program would be an excellent target for new therapeutic strategies including small molecule approaches or gene transfer. However, current experimental procedures testing this hypothesis – clonogenic assays *in vitro* or mutation analysis and immunohistochemistry of patient biopsy material – cannot reflect the complex reality of an actual malignancy *in situ*.

A novel approach to study and control the genetic mechanisms of drug resistance *in vivo* is based on the B cell lymphoma developing *Eμ-myc* transgenic mouse, which recapitulates the clinical behavior of human lymphomas and leukemias. Lymphomas arise in a physiological environment, are transplantable into non-transgenic, syngeneic mice and can be modified by different genetic strategies (cross to other transgenic or 'knock-out' mice; retroviral introduction of genes or 'dominant-negatives' into lymphoma or bone marrow cells). The model allows to address the actual contribution of tumor suppressor genes, related mutants and the tumor microenvironment to lymphomagenesis and drug response. The comparison of essentially the same lymphoma population with or without introduction of a gene of interest and reconstituted in numerous recipient mice can give direct and highly comparable insights into the complex mechanisms of treatment failure.

We previously explored the impact of frequently mutated tumor suppressors like the *p53* gene and the *INK4a/ARF* locus on lymphomagenesis and treatment response in the *Eμ-myc* model using a 'knock-out' cross approach and demonstrated, that mutations of these tumor suppressor loci can actually accelerate lymphomagenesis and simultaneously promote chemoresistance by disabling overlapping proapoptotic functions of p53 and the *INK4a/ARF* gene product $p19^{ARF}$.

We are now interested in genes controlling cell death downstream of p53, hereby focusing on *bcl-2* and *caspase 9*. Myc and the prosurvival oncoprotein Bcl-2 cooperate in lymphomagenesis, but the clinical relevance of Bcl-2, overexpressed in many cancer entities, is controversial. Accurate *in vitro* evaluation of Bcl-2 overexpression in clonogenic requires specific test conditions, imitating survival signals provided by the tumor microenvironment. Thus, our model comparing the same tumor subpopulations with and without Bcl-2 overexpression in their natural environment *in vivo* provides an unbiased novel experimental strategy. Caspase 9, involved in the execution phase of p53-dependent apoptosis, has been identified to act like a tumor suppressor, but its impact on lymphomagenesis and treatment response remained undetermined. By introduction of a dominant-negative *caspase 9* into *Eμ-myc* lymphoma and *Eμ-myc* transgenic bone marrow, reconstituted in non-transgenic recipients, we will test if a gene of the terminal apoptotic cascade can still interfere with lymphoma onset and treatment response.

These novel approaches will be useful to evaluate strategies overcoming drug resistance *in vivo* and provide the tools for mutation-based drug discovery programs.

Polo-like kinase-1 is a target of the DNA damage checkpoint

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The phosphatase Cdc25C is a well established target of the DNA damage checkpoint. However, the exact mechanism by which DNA damage causes inhibition of Cdc25C remains unclear. We show that DNA damage causes inhibition of Polo-like kinase-1 (Plk1)-mediated Cdc25C phosphorylation. We show that activation of Plk1 in G_2 is inhibited by DNA damage. This inhibition of Plk1 was seen with various DNA damaging agents and in various cell lines, demonstrating that DNA damage-induced Plk1 inhibition is a general phenomenon. Also, activated, mitotic Plk1 is inhibited by DNA damage, indicating that the Plk1 inhibition is not secondary to the DNA damage-induced G_2 arrest. In line with this, we can show that DNA damage blocks degradation of cyclin B and mitotic exit, consistent with the notion that Plk1 is required at multiple stages during mitosis. Importantly, overexpression of Plk1 in combination with Cdc25C-S216A, which cannot be phosphorylated by Chk1, can override the G_2 arrest induced by DNA damage. From these data we conclude that Plk1 is an important target of the DNA damage checkpoint, enabling cell cycle arrest at multiple points in G_2/M.

The tumor suppressor PTEN is phosphorylated at serine/threonine residues in the C-terminal regulatory domain.

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Pten (phosphatase and tensin homolog deleted in chromosome ten), also called *Mmac1* (mutated in multiple advanced cancers 1) and *Tep1* (TGF-β regulated and epithelial cell -enriched phosphatase 1), is a tumor suppressor gene recently discovered by reverse genetics (1, 2). *Pten* is frequently mutated in a wide number of tumors, and germ-line mutations in *Pten* gene are associated with syndromes that share a high susceptibility to tumor incidence (3). The PTEN aminoacid sequence is homologous to that of the dual specificity phosphatases, enzymes which dephosphorylate phosphoserine, phosphothreonine and phosphotyrosine residues. Tyrosine dephosphorylation by PTEN of the focal adhesion kinase and the adapter protein Shc, has been described (4). Also, PTEN dephosphorylates the lipid second messenger phosphatidyl-inositol-(3,4,5)-trisphosphate at the 3' position, involving to this tumor suppressor in the signal transduction pathway of the PI3-kinase oncogene (5). However, nothing is known about how the activity of PTEN is regulated *in vivo*. Since phosphorylation is a general mechanism by which the function of a large number of signal transduction molecules is regulated, we have investigated the phosphorylation of PTEN and its relation with PTEN functions. We have found that PTEN is a phosphoprotein *in vivo* under normal conditions of cell growth. The phosphorylation of PTEN takes place at serine and threonine residues in the C-terminal domain of the molecule; such domain is likely to play a regulatory role, as deduced from the PTEN three-dimensional structure (6). The putative role of phosphorylation in the regulation of the PTEN tumor suppressor function will be discussed.

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$p27^{KIP1}$ expression in aggressive B-cell malignancies is a warning signal of malfunction of the p16 and p53 tumor suppressor pathways

Raquel Villuendas

ABSTRACT.

p27/KIP1 over-expression in a group of aggressive B-cell lymphomas with high proliferative index and adverse clinical outcome has been shown in previous studies. This anomalous expression of p27 has been described in cases with inactive p53 protein, and associated with the formation of p27/Cyclin D3 complexes and the absence of p27/CDK2 complexes. In the present study we have performed a molecular study of both p53 and p16 genes simultaneously with the immunohistochemical analysis of p27 expression, in a group of aggressive B-Cell Lymphomas, trying to analyze the relationship between p53 and p16 silencing and p27 anomalous over-expression. Twelve cases with anomalous p27 high expression were identified in a series of 47 patients. In all of them (except one), inactivation of p53/ARF, p16/Rb or both pathways was found. More frequently inactivation of p16 gene was identified (8/12). An analysis of the clinical relevance of the simultaneous inactivation of both pathways, p16/Rb and p53/ARF was also performed, showing that the group with simultaneous p53 and p16 inactivation together with p27 over-expression had an advance increase in proliferative activity (p: 0.002) and, also, had a higher tendency for an adverse clinical outcome. In conclusion, these data show that p27 anomalous expression is associated with inactivation of p16/Rb or/and p53/ARF pathways in aggressive B-cell lymphomas. This finding is consistent with an scenario of CKIs competition for CDK4 binding, where the absence of p16 or p21 allows the redistribution of p27 from complexes containing CDK2, where p27 is active, to other containing CDK4/Cyclin D3, where p27 is inactive, stabilized, and detectable by immunohistochemical techniques. However, not all cases with p53 or p16 silencing show p27 accumulation, which indicates the existence of additional factors to explain this finding.

Rb-independent effects of $p16^{ink4a}$

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The tumor suppressor $p16^{ink4a}$ is an inhibitor of cyclin D-containing complexes. When $p16^{ink4a}$ is expressed in a cell, it disrupts cyclin D/cdk4 complexes, inhibiting their kinase activity, and releasing the protein $p27^{kip1}$. $p27^{kip1}$ then binds to cyclin E/cdk2 complexes and inhibits them. The final consequence is an arrest in cell proliferation. Despite these seemingly pleiotropic effects on cell cycle-related proteins, overexpression of $p16^{ink4a}$ can arrest the proliferation of cells that have a functional pRB protein, but not those where pRB is not present or is otherwise inactivated. This implies that all the effects of $p16^{ink4a}$ that are relevant to cell cycle arrest are meditated by pRB. Nevertheless, it is possible that $p16^{ink4a}$ may have effects on the cell cycle not mediated through pRB, that do not lead to an arrest in cell proliferation but affect the regulation of the cell cycle in more subtle ways. Given that a high percentage of tumor cells show overexpression of $p16^{ink4a}$, and are *Rb*-negative, it is important to study these *Rb*-independent effects of $p16^{ink4a}$.

We have used a cell line derived form *Rb*-/- MEFs (*Rb*-/- 3T3 cells). These cells express endogenous $p16^{ink4a}$, but have detectable cyclin D/cdk4 complexes, and a cell cycle-regulated cdk4 activity. Overexpression of human $p16^{ink4a}$ in these cells leads to disruption of cyclin D/cdk4 complexes, inhibition of cdk4 kinase activity to background levels, displacement of $p27^{kip1}$ to cyclin E/cdk2, and partial inhibition of cyclin E-associated kinase activity. Cells overexpressing $p16^{ink4a}$ also have lower levels of cyclin D1 and cyclin E proteins. This is presumably a translational or postranslational effect, as the mRNAs encoding both proteins are not downregulated when $p16^{ink4a}$ is overexpressed.

The effects of $p16^{ink4a}$ on G1 cyclin-dependent kinases is reflected on an altered phosphorylation of the *Rb*-related proteins p107 and p130. Both proteins have been proposed to be phosphorylated by cyclin D/cdk4, at least when overexpressed. We have found that endogenous p107 and p130 are relatively underphosphorylated in $p16^{ink4a}$-overexpressing cells. Nevertheless, at least p130 retains a cell cycle-regulated phosphorylation, what implies that this protein has a cyclin D-dependent and a cyclin D-independent cell cycle-regulated phosphorylation.

The fact that *Rb*-/- cells do not arrest in G1 when expressing $p16^{ink4a}$, has allowed us to analyze the effects of this situation in later phases of the cell cycle. Significantly, cyclin A kinase activity is lower in $p16^{ink4a}$-overexpressing cells with respect to control *Rb*-/- cells, despite the fact that $p27^{kip1}$ does not seem to bind to cyclin A in this situation. We are presently analyzing this *Rb*-independent effect of $p16^{ink4a}$ in more detail.

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The lectures summarized in this publication were presented by their authors at a workshop held on the 8th through the 10th of May, 2000, at the Instituto Juan March.

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