Meeting Report

Cyclins, Cdks, E2f, Skp2, and More at the First International RB Tumor Suppressor Meeting

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Abstract

The RB1 gene was cloned because its inactivation causes the childhood ocular tumor, retinoblastoma. It is widely expressed, inactivated in most human malignancies, and present in diverse organisms from mammals to plants. Initially, retinoblastoma protein (pRB) was linked to cell cycle regulation, but it also regulates senescence, apoptosis, autophagy, differentiation, genome stability, immunity, telomere function, stem cell biology, and embryonic development. In the 23 years since the gene was cloned, a formal international symposium focused on the RB pathway has not been held. The "First International RB Tumor Suppressor Meeting" (Toronto, Canada, November 19-21, 2009) established a biennial event to bring experts in the field together to discuss how the RB family (“pocket proteins”), as well as its regulators and effectors, influence biology and human disease. We summarize major new breakthroughs and emerging trends presented at the meeting. Cancer Res; 70(15); OF1–5. ©2010 AACR.

Introduction

Twenty speakers gave presentations on an astonishingly diverse array of topics, underscoring the central role of retinoblastoma protein (pRB) in human biology (1). We have grouped these topics into the general areas of cancer, E2f functions, other pRB effectors, and pRB regulators. References are limited to work published in the past year.

RB1 in Cancer

Retinoblastoma tumors carry homozygous RB1 mutations (see below for an intriguing exception), and can be modeled in mice by deleting Rb1 and one of its relatives p107 or p130 (2). pRB mediates cell cycle exit in differentiating retinal neurons, and in its absence E2f1 delays cell cycle exit. Which neuron is tumor-prone, and why, is debated. David Cobrinik (Memorial Sloan-Kettering Cancer Center, New York, New York) showed that human retinoblastoma exploits cone photoreceptor features, including high expression (not amplification) of the proto-oncogenes MYCN and MDM2, and the nuclear receptors TRB2 and RXRG (3). All four factors are critical for growth, thus their prominent expression could sensitize human cone precursors to RB1 mutation and underlie a cone precursor origin of retinoblastoma (3). Studies to date do not rule out an alternative possibility, that tumors might convert to a cone-like epigenetic state during neoplastic transformation (2). Irrespective, the data raise exciting new avenues for treatment. The role of nuclear receptors or Mdm2 in mouse retinoblastoma is unknown, but these tumors arise from amacrine cells (or horizontal cells that convert to amacrine cells during neoplastic transformation), which exhibit naturally high resistance to apoptosis. Mouse data exist on Mycn: David Macpherson (Carnegie Institution, Baltimore, Maryland) reported gain in ∼30 to 50% of tumors from Rb/p107 or Rb/p130 null retina, respectively, and focal, high-level amplifications in a smaller subset, suggesting natural Mycn levels in mouse retina are insufficient for transformation. Brenda Gallie (Ontario Cancer Institute, Toronto, Canada) has found that 1.5% of retinoblastoma tumors lack RB1 mutations, but show high MYCN amplification. Thus, RB1 loss plus naturally high MYCN, or high MYCN alone (plus as yet unknown other changes), are oncogenic in the human retina, and Rb1 plus Rb1-relative loss and Mym gain are oncogenic in mouse retina.

Retinoblastoma exhibits common post-RB1 genetic events. Gallie found that retinoma, a senescent benign tumor replete with p16ink4a and p130 protein and evident in ∼15% of retinoblastoma samples, has a subset of post-RB1 events present in malignant tumors, arguing that the latter derives from the former. A common region of gain on human 1q contains KIF14 and MDM4, and Macpherson showed that the syntenic region, also on mouse chromosome 1q, undergoes copy number increase in retinoblastoma models. Removing one allele of the Mdm2 inhibitor p19Arf accelerated mouse retinoblastoma, and approximately half of these malignancies showed loss of heterozygosity. These and other studies suggest an important
role for the MDM2-p53 pathway in retinoblastoma progression, even though p53 itself is never mutated. In summary, MYCN, the p14ARF−MDM2/MDM4-p53 axis, and KIF14 are key players after RB1 loss in both mouse and human retinoblastoma.

Typically, RB1+ individuals develop multiple tumors in both eyes, but low penetrance mutations that partially disable pRB cause fewer tumors. R661W is the best studied mutation, and David Goodrich (Roswell Park Cancer Institute, Buffalo, New York) is employing the knock-in equivalent he developed (R654W) to model cancer in mice. For example, the RB664W−/−p53+/− prostate is better protected against tumor progression than the RB−/−p53+/− tissue. Some E2f targets are equally deregulated in both genotypes, thus protection must be through distinct E2f targets and/or E2f-independent mechanisms. Goodrich has also developed an epitope-tagged knock-in RB1T allele that will be useful in mapping the protein and genome-wide chromatin targets of pRB in vivo (4).

Loss of heterozygosity and RB1 down-regulation are common in basal-like breast cancer, a highly aggressive subtype, as well as in luminal tumors. Eldad Zacksenhaus (Toronto General Research Institute, Toronto, Canada) described a new mouse model for basal-like breast cancer, based on conditional RB1 inactivation in mammary epithelium.7 Interestingly, loss of RB1 alone (without loss of RB1 relatives) is sufficient to induce mammary tumors, and p53 status dictates tumor subtype.

RB1 is also inactivated in hepatocellular carcinoma (HCC). Erik Knudsen (Kimmel Cancer Center, Philadelphia, Pennsylvania) discussed the unexpected finding that conditional RB1 deletion does not perturb quiescence in normal or regenerating mouse hepatocytes (even adding p107 or p130 absence; ref. 5). Although RB1 loss also does not cause HCC, it enhances chemically induced tumorigenesis (see ref. 5 and references therein). The expression profile of pRB target genes predicts disease outcome in human liver cancer, and animal models are revealing the impact of cell cycle inhibitory compounds on HCC.8 pRB blocks aflatoxin-induced HCC through a unique role in limiting E2f1 levels and ectopic DNA synthesis. Aflatoxin has no effect on apoptosis, whether RB1 is intact or not. In some other cell types, for example, gut villi, pRB potentiates activation of proapoptotic E2f targets and cell death in response to genotoxic agents (6).

Although RB1/p107 double knockout (DKO) liver does not develop spontaneous tumors (5), Julien Sage (Stanford University School of Medicine, Stanford, California) showed that RB1/p107/p130 triple knockout (TKO) causes HCC within 4 to 6 months. Molecular analysis connected pocket protein loss with Notch signaling, extending observations made in neural progenitors. Pocket protein loss causes a myeloproliferative disorder in the hematopoietic system, but not leukemia. TKO mouse embryonic stem cells could differentiate into embryoid bodies, and only 5 to 10% of cells showed BrdU incorporation. In these differentiated TKO cells, some E2f targets remained elevated, but others and Myc genes were down-regulated as effectively as in wild-type cells. TKO embryos survived until midgestation in the presence of a wild-type placenta. Thus, multiple TKO cell types have potent backup mechanisms, likely explaining why oncogenic viral proteins bind many factors other than pocket proteins.

Laura Butterita (Fred Hutchinson Cancer Research Center, Seattle, Washington) also discussed extensive feedback controls that prevent cell cycle abnormalities. Robust ectopic division in differentiating Drosophila wing or eye cells requires perturbation of both Rb-E2f and Cdk2 pathways. Removing inhibitors or expressing activators from either side of the equation has minimal effect, which is explained by feedback mechanisms, for example, whereas E2f induces cyclin A, it also induces APc/C, which degrades cyclin A.

**Surprising In vivo Functions of Activating E2fs in Dividing Cells**

Rod Bremner (Toronto Western Research Institute, Toronto, Canada) and Gustavo Leone (Ohio State University, Columbus, Ohio) discussed in vivo functions of the three activating E2fs (E2f1-3), downstream targets of pRB. Typically, E2fs are viewed as dispensable for cell division. Surprisingly, however, many cell types divide in vivo without E2f1-3 (7, 8). In retina, this finding was explained by the ability of Mycn to maintain expression of E2f targets and repress Cdkn1a (p21Cip1; ref. 7).

Unexpectedly, E2f1-3 have a prosurvival role in dividing cells, which seems counterintuitive given that high E2f-3 levels drive apoptosis through p53 and p53-independent means. In retina, survival is linked to E2f-2-dependent expression of Sirt1 and other deacetylases that constrain p53 (7). Death of E2f1-3 null crypt progenitors is p53-independent (8). In addition to promoting survival, E2f1-3 also prevent DNA damage in dividing cells (8), consistent with their induction of genes that support nucleotide synthesis, replication, and repair. Thus, E2f activity must be balanced carefully. Conceivably, low E2f activity could be oncogenic in cells in which Myc is sufficient for division.

“Activating” E2fs are also linked to gene repression, but in vivo relevance is unclear. Leone revealed that E2f1-3 mediate repression in postmitotic differentiating cells (8). In dividing small intestine crypt cells, pRB is phosphorylated and not E2f-bound. Conversely, in differentiating postmitotic villi, pRB is dephosphorylated and forms pRB-E2f repressor complexes. In E2f1-3 TKO, differentiating villi derepression increases E2f targets, and Rb1+/− null villi show E2f1-3-dependent superactivation of these targets and ectopic division.

To summarize, although abnormal proliferation in Rb1-null-differentiating cells is E2f-dependent, in normal proliferating cells, activating E2fs promote survival and genome integrity rather than division. In postmitotic differentiating cells, E2f1-3 form essential repressor complexes with pRB.
pRB Effectors: E2f and Beyond

pRB binds >100 proteins through various surfaces. Sibylle Mittnacht (Chester Beatty Laboratories, London, UK) showed that the N-terminal domain is structurally related to the A-B pocket domain, and they interact. Peptides that bind to the LxClX cleft of the A-B pocket disrupt N-terminal-A-B pocket interaction. Mittnacht also deduced the effect of tumorigenic mutations on pRB function and distinguished these defects from functionally silent codon variants.

Srikanth Talluri, from Fred Dick’s group (University of Western Ontario, London, Canada), discussed their work with from functionally silent codon variants. Mittnacht also deduced the effect of tumorigenic mutations on pRB function and distinguished these defects from functionally silent codon variants.

Hein te Biele (Netherlands Cancer Institute, Amsterdam, The Netherlands) also described DNA damage in Rb1/p107/p130 TKO MEFs. These cells lack G1 control and are refractory to Bas(V12)-induced senescence. Serum-starved TKO MEFs ignore the serum-dependent G1-S restriction point and enter S-phase, but die at G2/M. Bcl2 expression blocks apoptosis and exposes G2/M arrest mediated by p21/p27-inhibition of cyclin A/B-Cdk1. Readding serum down-regulates p21/p27, and mitotic entry occurs after ~24 hours. Mitogen must be added for at least 6 hours, exposing a second serum-dependent "restriction point" ("R2"). Although reentry is possible, chromosomal breaks and "railroad chromosomes" lack centromeric cohesion. This damage might be related to E2f-dependent and/or -independent chromatin regulation mentioned above. Kay Macleod (University of Chicago, Chicago, Illinois) has shown that erythroblasts lacking E2f2, the major activating E2f in these cells, also have chromosome condensation defects, potentially related to deregulation of the E2f targets Aurora B, caspase-3, and Rad21.

Prior studies proposed that pRB facilitates differentiation by enhancing the function of lineage-specific transcription factors. However, more recent work attributes several differentiation defects to deregulated E2f activity; for example, deleting E2f1 blocks ectopic division in B cell −/− retina, rescuing all but one differentiation defect, and even this cell-cycle-independent abnormality is E2f3a-dependent. Ruth Slack (University of Ottawa, Ottawa, Canada) and Macleod discussed E2f-dependent defects in Rb1−/− cortical neurons and erythroblasts. E2f1, a repressive family member, does not bind pRB and is induced by activating E2f6. Slack showed E2f7 induction in the Rb1−/− cortex and repression of Dlx, a homeobox gene that regulates migration. Macleod showed that Rb1−/− erythrocyte defects are E2f2-dependent. In normal erythrocytes, pRB/E2f2 complexes repress E2f7 and other E2f-target genes, ensuring G1 arrest and enucleation. In Rb1−/− erythrocytes, deregulated E2f2 drives S-phase, increasing reactive oxygen species, single-strand damage, and DNA-damage-induced arrest in G2 (also discussed above), explaining why these cells fail to enucleate. The Rb-E2f pathway also regulates muscle development (12). Indeed, Rb1−/− muscle cells exhibit endoreduplication, incomplete differentiation, and degradation. Apoptosis in unfused myoblasts is mediated by noncanonical cell death. Zacksenhaus showed that induction of several survival pathways allows Rb1-deficient myocytes to fuse and form functional myotubes despite continued DNA synthesis. Thus, Rb1 promotes survival of differentiated muscle and may not be critical to stimulate activity of differentiation factors.

How Is pRB Regulated?

Several posttranslational modifications regulate pRB. Cyclin/Cdk complexes negatively regulate RB and are often

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8 Y. Zhou, A.M. Dirlam, K.F. Macleod, unpublished observations.
overexpressed and/or activated in cancer. In some contexts, active pRB is used in cancer cells, likely to block E2f-induced apoptosis. David Park (University of Ottawa, Ottawa, Canada) discussed the role of pRB phosphorylation in postmitotic neurons after stroke. Cdk4 activity is upregulated following stroke and is critical for death following ischemia. Cdk4 acts upstream of conserved death pathways in vitro and in vivo. Pim1 and Chk1 kinases act reciprocally to regulate the phosphatase Cdc25, which in turn regulates Cdk4 activation after neuronal injury.

Seth Rubin (University of California, Santa Cruz, California) discussed structural aspects of how phosphorylation affects pRB-E2f interaction. pRB forms two contacts with E2f: the pRB C-terminus binds E2f/Dp1 heterodimer, and the pRB pocket binds the E2f trans-activation domain (TD). Phosphorylation of Ser/Thr sites in the pRB spacer or N-terminus inhibits Ib-pocket/E2f-TD interaction. Moreover, the phosphatase PP1 binds C-terminal residues 870 to 882, and not only dephosphorylates pRB, but prevents cyclin-Cdk binding. Thus, catalytically dead PP1 could block Cdk2-mediated phosphorylation of pRB.

pRB is phosphorylated sequentially by cyclin D1/2/3-Cdk4/6 and cyclin E1/2-Cdk2 during G1/S transition, and then cyclin A1/2-Cdk2/1. Peter Sicinski (Dana-Farber Cancer Institute, Boston, Massachusetts) described redundancy in this system. Cyclin D1/D2/D3 TKO embryos survive to midgestation and exhibit normal proliferation in most compartments. TKO MEFs proliferate relatively normally, but are hypersensitive to Cdk2-inhibition. When TKO cells exit quiescence, pRB and p107 phosphorylation, presumably via Cdk2-cyclinE, is slightly delayed. Likewise, MEFs lacking cyclin E1/E2 proliferate, although they cannot reenter the cell cycle from quiescence. Thus, dividing cells do not require sequential D-E-A kinase pRB phosphorylation, but adapt and use different cyclin/Cdk combinations. Indeed, combined ablation of all A- and E-type cyclins in fibroblasts causes arrest, which is not cell cycle phase-specific (13). D+E or D+A knockout studies will also be interesting. Parallel studies by others show that CDK1 is the only essential CDK, whereas CDK2-6 are dispensable for cell cycle progression.

In addition to posttranslational modifications, Rb1 is also regulated transcriptionally. This finding is most evident during embryogenesis, in which Rb1 is abundant in the nervous system, muscle, liver, and the tissues affected in Rb1−/− or placental-rescued Rb1−/− mutant embryos. Zacksenhaus showed previously that 6 kb of upstream sequence is sufficient for expression in the nervous system, but not skeletal muscle. Using a green fluorescent protein (GFP) reporter knocked-in into a 200-kb bacterial artificial chromosome encompassing most of Rb1, Sage observes expression in several tissues including nervous tissue and skeletal muscle, but not liver, thus Rb1 regulation involves multiple remote enhancers (14). Vectors that permit appropriate expression will facilitate structure-function analysis of pRB in vivo.

Sicinski also revealed Cdk-independent cyclin D1 functions. Previously, Hinds showed that knock-in mice expressing D1K112E, which does not bind Cdk4/6, retain D1−/− phenotypes related to tumorigenicity, but lack D1−/− developmental defects. Using new Flag-HA-cyclin-D1 knock-in mice, proteomics, and chromatin immunoprecipitation coupled to DNA microarray (ChIP-chip) Sicinski’s laboratory showed that cyclin D1 binds to promoter regions of several genes (15). In the retina, this activity is required for D1-mediated transcription of Notch1. In D1−/− retina, Notch1 is down-regulated, thus causing premature cell cycle exit. Remarkably, transduction of activated Notch partially rescues the division defects. Thus, hypocellularity of the D1−/− retina is due to both reduced division rate and early cell cycle exit linked to lower Cdk4/6 and Notch1 activity, respectively.

Future Perspectives

Despite the more than two decades that have passed since its molecular secrets began to unfold, the RB pathway continues to surprise. Retinoblastoma is still yielding fundamental insights into cancer, emphasizing the value of studying this rare but, relatively speaking, simpler class of neoplasia. New animal models underscore the importance of the RB family in other cancers, but also raise questions about how cells cope with Rb1 loss, why some cell types are more susceptible than others, and when and where RB family action is most critical: Is it primarily in the cell cycle and cell survival? Or are its effects on genome stability and other aspects of cellular metabolism just as critical? And if they are, how is the pathway controlling these key determinants? Many questions also remain about where and when RB effectors are critical for its action in vivo. Clearly, E2f and Skp2 are major RB targets, and key goals are: (a) to revisit the plethora of phenotypes linked with Rb family loss, and define those that are E2f- and/or Skp2-dependent or -independent; and (b) to define the relevance of other RB protein-protein interactions (for example, LxCxE partners). At the atomic level, we still need a fuller understanding of the details of how RB is modified, and how, exactly, this affects its partners. Surprising new functions for activating E2fs in promoting survival and DNA integrity, and for cyclin D1 in supporting Notch action, suggest that similar genetic and biochemical studies with these and other members of the RB pathway will yield more exciting discoveries. It will also be important to define whether the links between low E2f and DNA damage, and between cyclin D1 and Notch signaling, are important in human cancer and other disorders in which the RB pathway is commonly perturbed. Where the field will take us in the next few decades is impossible to predict, but we are certain it will continue to have a broad impact on our understanding of biology and human health.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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