Genetic Interaction between Rb and N-ras: Differentiation Control and Metastasis

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Abstract

The retinoblastoma tumor suppressor gene, Rb, and the ras proto-oncogenes regulate various cellular processes, including differentiation and proliferation. Rb and ras genetically interact to positively influence differentiation in the mouse. This genetic interaction between Rb and ras also affects tumor development, either positively or negatively depending on cell type. Loss of one or two N-ras alleles allows medullary thyroid (C cell) adenomas occurring in Rb heterozygous mice to progress to metastatic carcinomas, an event associated with C cells displaying a less-differentiated phenotype. Here, we discuss the genetic interaction between Rb and ras and the development of a mouse model of medullary thyroid carcinoma. (Cancer Res 2006; 66(19): 9345-8)

Introduction

A focus of cancer biology is to understand how tumor suppressor genes and proto-oncogenes operate not only in isolation but also together to coordinately regulate differentiation and proliferation in a tissue-specific manner. Understanding how these two classes of genes work in concert to regulate physiologic processes will likely facilitate the more difficult task of elucidating how their genetic alterations operate together to effect pathophysiologic processes, such as tumor formation and metastasis. Illustrating these concepts, we describe how cell culture–based findings pertaining to the retinoblastoma tumor suppressor gene, Rb, and the ras proto-oncogenes were validated using mouse genetics. Then, we explain how observations about the genetic interaction between Rb and ras in certain differentiation programs laid the foundation for the development of a mouse model of neuroendocrine metastasis. Our findings have experimentally linked the differentiation function of pRb to its role as a tumor suppressor and revealed a previously unappreciated participation of a tissue-specific genetic interaction between Rb and N-ras in metastasis.

The retinoblastoma protein, pRb, controls proliferation and participates in several differentiation programs. The available evidence suggests that pRb regulates differentiation (e.g., myogenesis, adipogenesis, and osteogenesis) by positively influencing the activity of several differentiation promoting transcription factors, such as MyoD, CCAAT/enhancer binding protein β, Runx2, and the glucocorticoid receptor (1–4). Although the mechanisms by which pRb regulates the activity of these transcription factors are not well understood, they represent downstream effectors of pRb in the control of differentiation.

Like Rb, the three ras proto-oncogenes, encoding H-Ras, K-Ras4a, K-Ras4b, and N-Ras, participate in the control of differentiation and proliferation. Most studies analyzing the functions of Ras use naturally occurring constitutively active forms of Ras. By contrast, relatively little is known about the biological roles of wild-type (WT) ras. Efforts to bridge this gap have been initiated through the characterization of mice lacking different ras alleles. Mice nullizygous for H-ras or N-ras, or both genes, are developmentally normal (see ref. 5). Although K-ras heterozygotes are phenotypically normal, nullizygosity for K-ras leads to embryonic lethality (6).

pRb Can Act Upstream of Ras to Affect Differentiation In vitro

Soon after we and others had placed pRb downstream of Ras in the control of proliferation (7, 8), we asked whether signaling between these two proteins might be bidirectional. This undertaking was motivated, in part, by the observation that the mid-G1 activation of Ras was growth factor independent (9). This suggested to us the possibility that regulators of G1 cell cycle progression (e.g., pRb) might affect the activity of Ras; this link was made with the finding that Rb-deficient fibroblasts display elevated levels of Ras activity (10). The biological function of this communication between pRb and Ras, with pRb upstream of Ras, was not obvious at the time.

To approach this question, we made use of clinically relevant partially penetrant alleles of Rb (11, 12). The protein products encoded by these mutant Rb alleles display partial loss of function, i.e., they score positive for the differentiation function of pRb, e.g., to promote myogenesis and osteogenesis, but have lost the ability to interact with E2F and regulate cell cycle progression (13). Introduction of these partially penetrant Rb alleles into Rb−/− fibroblasts reduced the aberrant levels of Ras activity (10), suggesting that the ability of pRb to influence Ras activity is linked to the function of pRb in differentiation. Consistent with this interpretation, in established assays for the function of pRb, the activity of MyoD and the glucocorticoid receptor (two transcription factors that require pRb for activation) were restored on reduction of elevated levels of Ras activity in Rb-deficient cells (10). Further, others have shown that pRb-mediated control of Ras activity participates in adipogenesis (14). In addition, communication between pRb (LIN-35) and Ras (LET-60) is required for specification of vulval cell fates in the worm Caenorhabditis elegans (15). The ability of

Note: We apologize to our colleagues whose work could not be cited due to strict space limitations.

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©2006 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-06-1250
pRb to affect Ras activity seems therefore to be linked to the control of differentiation.

**Genetic Interaction between Rb and ras In vivo: Embryogenesis**

To assess the physiologic relevance of the cell culture–based observations described above, we asked whether Rb and ras genetically interact in the mouse. Rb-deficient embryos die midgestation with defects in several tissues, affecting proliferation, differentiation, and survival in both a cell autonomous and nonautonomous manner (16, 17). We determined whether loss of ras might influence any of the phenotypes that characterize Rb−/− embryos. Much of our effort was focused on skeletal muscle differentiation because this is the best-characterized cell autonomous defect effected by Rb deficiency (18). Loss of K-ras or N-ras resulted in near normal skeletal muscle differentiation in Rb−/− embryos (19, 20). Further, the expression of muscle creatine kinase, a marker of differentiation and transcriptional target of MyoD, was restored in Rb−/−;K-ras+/− and Rb−/−;N-ras+/− mice. By contrast, analyses of the proliferative defects that characterize Rb-deficient skeletal muscle as well as other tissues revealed that these were not ameliorated on concomitant loss of ras, consistent with the notion that pRb regulates differentiation and proliferation through distinct pathways. These findings extended our *in vitro* findings, establishing that a communication between Rb and ras exists *in vivo* to effect differentiation (Fig. 1).

**Genetic Interaction between Rb and ras In vivo: Tumorigenesis**

Careful analysis of the genetics of retinoblastoma provided an inroad into elucidating the functions of pRb that contribute to its tumor suppressor function. Classic retinoblastoma is a highly penetrant disease with >90% of carriers developing tumors. By contrast, patients harboring partially penetrant alleles (discussed above) are either asymptomatic or develop a significantly less severe form of the disease. This, together with the characterization of the protein products encoded by partially penetrant alleles of Rb (see above), suggests that the differentiation function of pRb participates in tumor suppression. To test this possibility, we asked whether the tumor phenotypes of Rb heterozygous mice (e.g., status of differentiation) were altered by loss of ras in such a way as to affect life span of the mice.

Mice heterozygous for Rb succumb to tumors of the pituitary gland and also develop medullary thyroid (C cell) adenomas. Loss of a single K-ras allele prolonged the survival of Rb+/− mice by >40% (20). Pituitary tumors arising in Rb+/−;K-ras−/− were

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**Figure 1.** Genetic interaction between Rb and N-ras during embryogenesis and tumorigenesis. The genetic interaction between Rb and N-ras affects various differentiation processes. During embryogenesis, the genetic interaction between Rb and N-ras positively affect the activity of MyoD, and this explains, in part, how loss of N-ras restores muscle differentiation in Rb-deficient embryos. Similarly, the genetic interaction between Rb and N-ras is associated with pituitary tumors displaying a more differentiated phenotype. In contrast, the genetic interaction between Rb and N-ras is associated with C-cell thyroid adenomas progressing to metastatic carcinomas, a process associated with their acquiring a less-differentiated phenotype.
more differentiated and less invasive. Similar results were observed in the analyses of Rb N-ras compound mice (19).

Collectively, the findings described above suggest that the ability of pRb to affect differentiation in a Ras-dependent manner contributes to its tumor suppressor function. What they do not tell us is how. Specifically, in contrast to our studies on myogenesis where we had prior knowledge that both pRb and Ras affect MyoD function, we do not know what target of the genetic interaction between Rb and ras effects tumor suppression (Fig. 1). Further, we do not know whether these results might be generalized to other tumor types. In this regard, we do know that there is a degree of tissue specificity dictating the outcome of the genetic interaction between Rb and ras (see below).

Genetic Interaction between Rb and ras In vivo: Metastasis

A completely different scenario emerged in our analysis of thyroid C-cell adenomas. In Rb heterozygotes, these lesions are typically only visible on histologic examination. By contrast, loss of N-ras resulted in the development of palpable tumors of the thyroid in Rb+/− mice, this occurring with high penetrance in Rb+/−;N-ras+/− mice and in about half of the Rb+/−;N-ras+/− animals. The contribution of N-ras loss to growth of the primary tumor was underscored by the observation that a fraction of the tumors arising in Rb+/−;N-ras+/− mice had lost the remaining WT N-ras allele (21). However, this genetic event occurred in about a third of the mice, suggesting that N-ras was also haploinsufficient for suppression of primary tumor formation.

C-cell tumors arising in Rb+/−;N-ras+/− and Rb+/−;N-ras−/− mice metastasized to the liver, lung, and kidney as well as to other organs, an event not observed in Rb+/− animals (21). In metastases occurring in Rb+/−;N-ras−/− mice, loss of the remaining N-ras allele was observed, suggesting that this genetic event contributes materially to their metastatic behavior.

To begin to understand how Rb N-ras nullizygous C cells acquire the capability to metastasize, we took advantage of our ability to suppress this behavior by ectopic expression of N-Ras (21). N-ras nullizygous C cells displayed invasive behavior attributable, at least in part, to elevated RhoA activity, two properties of these cells that could be reversed by introduction of N-Ras. Ectopic expression of K-Ras did not affect RhoA activity, suggesting that this was a unique property of N-Ras. This interpretation is consistent with our observation that K-ras heterozygosity has no effect on the development of C-cell adenomas in Rb+/− mice (20). Whether there exists a specific signaling pathway linking N-Ras to RhoA or whether loss of N-ras allows for elevated RhoA activity to be selected for remains to be determined. Further, Rho signaling has been shown to affect a plethora of cellular activities and phenotypes, such as transcription, formation of actin-myosin stress fibers and focal adhesions, stability of adherens junctions, differentiation, and cell fate determination and proliferation (22–25). The signaling pathways engaged following loss of Rb and N-ras to effect metastasis await to be elucidated.

The observation that loss of a proto-oncogene contributes to tumor progression and malignancy, although at first blush may seem counterintuitive, is not all that surprising. For more than 20 years now, we have known that introduction of constitutively active, oncogenic Ras into the neuroendocrine cell line PC12, derived from a pheochromocytoma, promotes its differentiation rather than transformation (26, 27). The same is true of lines derived from C-cell tumors and small cell lung carcinomas, also of neuroendocrine origin (28, 29). Consistent with these findings, neuroendocrine tumors rarely harbor oncogenic mutations in any of the ras alleles (30, 31). Thus, there seems to be a degree of consonance between these observations and our own finding that the genetic interaction between Rb and ras affects differentiation.

The observations described above prompted us to hypothesize that the loss of N-ras in Rb-deficient C cells is associated with a less-differentiated phenotype, the opposite of what we observed in the pituitary gland and the opposite of what occurs when oncogenic Ras is introduced into C-cell tumor lines. Human C-cell tumors can be graded based on their differentiation status through the analyses of nuclear to cytoplasmic ratio and nuclear features. In addition, the expression of carcinoembryonic antigen can be used to distinguish human C-cell adenomas and carcinomas histologically. Using these criteria in our mouse model, neoplasms arising in Rb heterozygous mice were classified as differentiated adenomas and those in Rb+/−;N-ras−/− and Rb+/−;N-ras−/− animals as carcinomas (21). Thus, Rb heterozygotes develop C-cell adenomas and loss of WT N-ras allows for their malignant progression, a process that is associated with the acquisition of a less-differentiated phenotype (Fig. 1).

There are several interrelated issues stemming from our mouse model of metastasis that remain to be addressed. At a very basic level, we need to understand how the genetic interaction between Rb and N-ras effects the progression from thyroid C-cell adenomas to metastatic carcinomas. As discussed above, we think it involves the differentiation status of the cells, but how this “progression” occurs, whether it involves dedifferentiation of a mature C cell or targeting of a C-cell progenitor where loss of Rb and N-ras maintain a less-differentiated state, remains to be determined. Regardless, the metastatic potential of Rb N-ras mutant C cells may reflect the acquisition of the normal migratory and invasive properties C cells possess during embryogenesis. At a functional level, perhaps, loss of Rb resulting in adenomas sets up a cellular context that allows loss of N-ras to facilitate progression to a more malignant state.

At the molecular level, we need to understand how Rb and N-ras genetically interact to effect tumor progression. This will entail identification of participants downstream of the genetic interaction of Rb and N-ras. In this regard, we do not know whether Rb and N-ras work together on a common target, as our cell culture and in vivo work on MyoD and myogenesis would suggest (Fig. 1). It is possible, for example, that Rb and N-ras each have independent downstream effectors and that these operate in parallel to influence tumor progression. The identification of downstream effectors of N-Ras that prevent malignant progression of Rb-deficient C-cell adenomas may help distinguish between these possibilities. The available evidence suggests that Ras engages classic effector pathways (e.g., Raf) in neuroendocrine cells. Perhaps the ultimate, more distal effectors of Ras signaling in neuroendocrine cells are distinct from that in other cell types, and such a finding may tell us why these cells seem to respond differently to Ras compared with other cell types. In this scenario, we might imagine that a signaling event triggered by loss of N-ras engages the cellular context or differentiation status, unique to neuroendocrine cells, set up by nullizygosity for Rb to effect metastasis.

There is also the intimately related issue of N-ras as a tumor suppressor. Loss of N-ras in WT mice does not result in any tumor phenotypes, properties we usually do not associate with tumor suppressors. Perhaps, as discussed above, it is only in the
context of Rb deficiency that the effect of N-ras loss on tumorigenicity is observed. However, in different cellular contexts and in the presence of activating mutations in Ras, tumor suppressor roles have been ascribed to K-ras and N-ras in systems of chemically induced carcinogenesis (32, 33). Further studies will be required to assess the tissue and genetic context in which ras genes operate as tumor suppressors (e.g., N-ras in C-cell tumors; Fig. 1) or tumor promoters (e.g., N-ras in pituitary tumors; Fig. 1).

The mouse model of medullary thyroid carcinoma described is similar to the human disease both phenotypically and histologically. Nevertheless, the utility of this model may rest on whether genetic lesions in Rb and N-ras participate in human medullary thyroid carcinoma. In this regard, loss of pRb protein expression has been observed (34). In addition, losses on chromosome 1 have been reported for human medullary thyroid carcinoma (35). The loss of pRb is the target, as our mouse model would suggest, and whether loss of N-ras and Rb are coincident.

The genetics of familial and sporadic medullary thyroid cancer is poorly characterized. Mutations in the RET proto-oncogene are found in patients with multiple endocrine neoplasia 2, of which medullary thyroid carcinoma is a component, and some sporadic medullary thyroid carcinomas. RET mutations are thought to lead to C-cell hyperplasia, consistent with mouse models, and subsequent genetic alterations allow for the development of carcinomas, which patients usually present with. Thus, a mouse model, in which metastases occur at high frequency, may guide the development of treatments for medullary thyroid cancer. It may be used for preclinical testing. In addition, the identification of components of the pathways downstream of N-ras loss leading to carcinoma formation may suggest targets for therapeutic intervention. Additionally, further characterization of the nature of the link between the genetic interaction between Rb and N-ras and the differentiation status may inform therapeutic strategy for the disease. Specifically, as alluded to above, Rb and N-ras loss may target a stem or progenitor cell that normally gives rise to C cells. Such cells may be resistant to standard chemotherapy, and it may be for this reason that radiation and chemotherapy are ineffective in the treatment of metastatic medullary thyroid carcinoma. Efforts toward combating the problem of tumor stem cells and drug resistance in other cancers then may be applicable to medullary thyroid carcinomas.

Acknowledgments

Received 3/31/2006; revised 6/2/2006; accepted 6/23/2006.  
Grant support: NIH/ National Cancer Institute grant R01 CA60842.  
We thank Chandrayee Das for critical reading of the article.

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