



Modeling malignant melanoma in mice: pathogenesis and maintenance

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Malignant melanoma, current challenges

The significance and impact of melanoma as a disease entity cannot be understated. As a cancer type, incidence of melanoma is rising at a rate second only to lung cancer in women and, on its present course, the lifetime risk will reach 1 in 75 among Caucasians in the US by the year 2000 (Rigel *et al.*, 1996). Furthermore, in contrast to most malignancies, melanoma affects a much younger population, metastasizes early in the course of the disease, and fails to respond to current therapeutic regimens (Herlyn, 1993). Presently, clinicians rely solely upon histopathologic criteria to stage melanomas, guide therapeutic decisions, and predict disease outcomes. One system, described by Breslow, measures tumor thickness from the uppermost nucleated layer of the epidermis (i.e., granular cell layer) to the greatest depth of tumor invasion. Another system, known as Clark's classification, correlates anatomic level of invasion, coupled with mitotic index, to prognosis. Such systems serve as useful prognosticators but fall short as definitive predictors of future clinical behavior of melanoma, particularly early melanocytic lesions – an ongoing problem fueled in part by the lack of diagnostic reproducibility among dermatopathologists evaluating such lesions (for review, see Barnhill *et al.*, 1993). The inherent shortcomings of pathology-based parameters have stimulated the search for molecular markers that can predict more accurately the impending tumor phenotype, forecast disease prognosis, and ultimately guide therapeutic decisions.

Despite the long history of clinical and molecular efforts directed towards this disease, surprisingly little is known about the precise genetic lesions leading to melanoma and even more vestigial is our understanding of how the few recognized genetic lesions relate to disease classification or progression. The identification and validation of molecular markers has been hampered by practical issues such as human tissue acquisition and would undoubtedly be accelerated by the availability of a mouse model that faithfully reproduces melanoma progression on the pathological and molecular levels. Furthermore, many of the genetic lesions identified to date, with few exceptions, have

come from the cytogenetic analysis of far-advanced human melanomas, particularly metastatic disease. This fact, coupled with the inherent genomic instability of malignant melanoma, emphasizes the need to utilize primary, early stage disease samples. Moreover, a better genetic roadmap of tumor progression from normal melanocyte to metastatic melanoma will be necessary in order to correlate molecular events with tumor stages. On another level, the melanoma field has a need for *in vivo* systems to aid in the discovery, verification and functional analysis of the growing collection of melanoma susceptibility genes.

In human studies, cytogenetic, linkage, and molecular analyses of familial and sporadic melanomas have implicated many genes and genomic loci in the genesis and progression of melanoma, although very few of these candidate genes have been verified as true etiologic lesions in a formal genetic sense. In surveying the putative melanoma genes/loci for which reasonable experimental support exists, two consistent themes emerge – loss of 9p21 locus, and activation of a receptor tyrosine kinase (RTK), such as EGFR, or mutations in its signaling surrogates such as RAS or PTEN. In this review, we will discuss our ongoing efforts to establish an accurate mouse model based on these known genetic profiles of human melanomas.

Genetics of human melanomas

The familial melanoma locus at 9p21: p16^{INK4a}, p19^{ARF} and p15^{INK4b}

The 9p21 locus in man, and the syntenic region on mouse chromosome 4, contains the closely linked and highly homologous INK4a and INK4b genes, encoding for *p16^{INK4a}* and *p15^{INK4b}*, respectively. Both products of these genes inhibit CDK4/6-directed phosphorylation of pRB. Loss of INK4 function can directly drive CDK4/6 towards a more highly activated state resulting in pRB hyperphosphorylation with associated de-repression of pRB-regulated genes (reviewed in DePino, 1998), as well as enhanced activity of CDK2/cyclin E by indirectly promoting sequestration of the Cip/Kip proteins (p21 and p27) from CDK2/cyclin E (reviewed in Sherr and Roberts, 1999) leading to S phase progression. The INK4a gene has the capacity to encode a second unrelated non-CKI product by alternative exon usage (Quelle *et al.*, 1995). This additional open reading frame initiates in a different first exon (exon 1 β) and continues in an alternative reading frame in the shared exon 2 (Figure 1). The novel protein was named 'p19^{ARF}' for *Alternative Reading Frame* protein. ARF functions as a potent growth suppressor (Quelle *et al.*, 1995, 1997),

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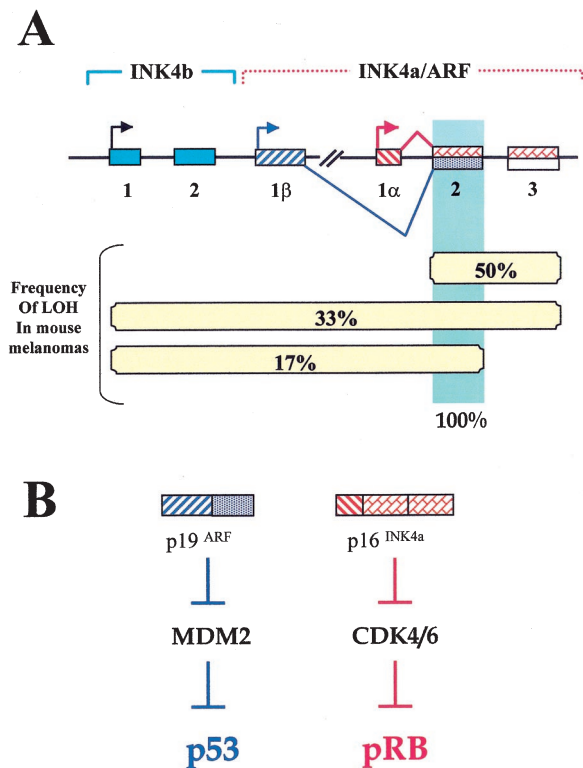


Figure 1 (a) A schematic of the genomic organization at the 9p21 locus and the frequency of LOH observed in melanomas arisen in Tyr-RAS transgenic mice on INK4a^{Δ2/3} background. Note, in a 100% of the cases, exon 2 of INK4a/ARF is deleted. (b) The INK4a/ARF gene is functionally linked to two tumor suppression pathways by virtue of its ability to encode for two distinct products, p16^{INK4a} and p19^{ARF}

blocks oncogenic transformation and sustains p53-dependent apoptosis in RB null cells that have re-entered the cell cycle *in vivo* (Pomerantz *et al.*, 1998) or in the setting of hyperproliferative oncogenic signals (de Stanchina *et al.*, 1998; Zindy *et al.*, 1998; Radfar *et al.*, 1998). That p19^{ARF} may reside in a p53 pathway was first proposed by genetic studies in the INK4a^{Δ2/3} (p16^{INK4a} and p19^{ARF}) null mouse showing an absence of p53 mutations in melanomas (Chin *et al.*, 1997). Analogous genetic observations were demonstrated in p19^{ARF}-deficient MEFs that retained wildtype p53 configuration upon immortalization (Kamijo *et al.*, 1997). These findings, coupled with numerous assays showing that the anti-growth and anti-oncogenic activities of ARF function in a p53-dependent manner (Kamijo *et al.*, 1998; Pomerantz *et al.*, 1998; Stott *et al.*, 1998; Zhang *et al.*, 1998), have forged a direct link between the 'INK4a/ARF' locus and the RB (p16^{INK4a}) and p53 (p19^{ARF}) (Figure 1b). Thus, tumor-associated mutations at the INK4a/ARF locus have the potential of crippling the two most important tumor suppression pathways governing neoplasia, an arrangement that likely provides a basis for the prominent involvement of INK4a in human cancers.

In melanoma, the 9p21 region commonly sustains homozygous deletions, often eliminating both the neighboring INK4b and INK4a/ARF (reviewed in Ruas and Peters, 1998; Haluska and Hodi, 1998). The high incidence of deletion has fueled an ongoing debate centered on whether each of these genes

contributes to the tumor suppressor activity encoded within 9p21. Regardless, the existence of germline p16^{INK4a} specific exon 1α mutations in familial melanoma kindreds has conclusively established that p16^{INK4a} is a bona fide tumor suppressor in humans (Hussussian *et al.*, 1994; FitzGerald *et al.*, 1996). In contrast, data linking p15^{INK4b} to melanoma is weak since there is no report of mutations exclusively targeting the p15^{INK4b} open reading frame in human melanomas.

The evidence supporting a bona fide tumor suppressor role for p19^{ARF} in human cancers has been mounting and derives from several independent observations. First, mutations or deletions impacting exclusively on the p19^{ARF} 1β exon have been identified in a subset of T cell acute lymphocytic leukemias (Gardie *et al.*, 1998). Second, several melanoma prone kindreds (Soufir *et al.*, 1998) and many primary sporadic melanomas contain mutations that alter or eliminate p19^{ARF} albeit almost always in the setting of accompanying p16^{INK4a} mutations (reviewed in Ruas and Peters, 1998; Sharpless and DePinho, 1999). In the case of human ARF, some of these mutations impact on functionally relevant C-terminal nucleolar import domain sequences (Zhang and Xiong, 1999), although the biological significance of such mutations in melanomagenesis has not yet been established. Third, the E1β promoter can be subjected to methylation-induced repression (Robertson and Jones, 1998) raising the possibility that tumor harboring exclusively p16^{INK4a} mutations may still eliminate ARF via an epigenetic mechanism. Finally, p19^{ARF}-specific knockout mice possess a cancer-prone phenotype (Kamijo *et al.*, 1997) and p19^{ARF} overexpression can suppress Myc/RAS transformation in cell culture (Pomerantz *et al.*, 1998). These organismal and cell culture studies indicate that p19^{ARF} possesses anti-oncogenic activity, thus elevating the likelihood that its ortholog serves a similar role in human tumors.

RTK-RAS-MAPK pathway in pathogenesis of melanomas

Melanocytes originate from a subpopulation of neural crest cells that undergo epithelial/mesenchymal conversion, migrate laterally below the epidermis and ultimately give rise to skin and ear melanocytes (Le Douarin, 1998; Bennett, 1993). These tightly regulated developmental processes are affected through the coordinated activation of multiple and perhaps sequential receptor tyrosine kinases (RTKs). The dependence of melanocytes on complex RTK signaling for proper orchestration of basic cellular functions essential for development and homeostasis likely provides a basis for the frequent involvement of deregulated RTKs and their signaling components (e.g., PTEN or RAS) in human melanomas. The RTKs such as epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor (FGFR) have been particularly illustrative in this regard. The EGFR is an established proto-oncogene which maps to chromosome 7p12–13 (Wang *et al.*, 1993) and is frequently over-expressed (Koprowski *et al.*, 1985; de Wit *et al.*, 1992; Ellis *et al.*, 1992) in association with amplified copies of chromosome 7 in late stage melanomas (Trent, 1991; Bastian *et al.*,

1998). FGFR also appears to play a role in melanoma through the creation of an autocrine loop. Contrary to normal melanocytes, which require supplementation of various growth factors in culture, virtually all melanoma cells can produce bFGF and grow autonomously (Halaban *et al.*, 1988; Becker *et al.*, 1989, 1992). This leads to the establishment of a bFGF-FGFR autocrine loop that has been shown to be essential for melanoma growth *in vivo* (Yayon *et al.*, 1997; Wang and Becker, 1997). In addition to autocrine growth promoting effects, a number of RTK-activating growth factors characteristically produced by melanoma cells can also exert profound influences on the immediate microenvironment through paracrine signaling mechanisms (reviewed by Rodeck and Herlyn, 1991; Shih and Herlyn, 1994). These include promotion of angiogenesis, extracellular matrix degradation and adhesive interactions, and suppression of immunologic response and antiproteolytic activity. These biological endpoints of RTK signaling have been proposed to account for the apparent association of RTK activation with metastatic progression in human melanoma.

Relevance of the RTK signaling pathway in melanoma development is further fortified by the recognition of involvement of its signaling components in processes of cellular transformation including RAS, PI3-K and PTEN. In the case of RAS, activation can be mediated indirectly through different RTKs, such as EGFR, or through activating oncogenic mutations. Activating mutations in N- and H-RAS has been observed in close to 30% of a subset of melanoma, the nodular amelanotic type (Jafari *et al.*, 1995). Although germline heterozygous mutations of PTEN do not confer a melanoma phenotype, they do lead to a cancer-prone state in the mouse (DiCristofano *et al.*, 1998; Stambolic *et al.*, 1998; Podsypanina *et al.*, 1999). Furthermore, allelic loss or mutation of PTEN have been described in uncultured melanoma specimens, metastasis as well as established melanoma cell lines (Guldberg *et al.*, 1997; Teng *et al.*, 1997; Tsao *et al.*, 1998). In addition, re-introduction of PTEN into PTEN-deficient melanoma cells has been shown to suppress growth (Robertson and Jones, 1998). However, the existence of conflicting data in the cases of RAS (references within Chin *et al.*, 1997) and PTEN (Boni *et al.*, 1998) has fueled debate surrounding the pathogenetic relevance of these genetic changes in melanoma development *in vivo*. Thus, there exists a strong need to provide genetic evidence that the mutations described above serve a critical role in transformation and malignant progression of melanocytes and are not simply a correlate of these processes. This need has prompted us and others to exploit the power of mouse genetics-genomics to systematically test the disease relevance of tumor-associated alterations.

Building a mouse model for malignant melanoma

Spontaneous malignant melanomas are a rare occurrence in rodents. Melanomas have been induced by carcinogenesis protocols but arise with low penetrance, long latencies and limited metastatic potential (Berkelhammer and Oxenhandler, 1987). Mintz and

Silvers (1993) have produced a transgenic mouse melanoma model by the melanocyte-specific tyrosinase-directed expression of the SV40 early region (small and large T antigens). These mice develop ocular melanomas and, to a lesser extent, cutaneous melanomas. This transgenic model offers important opportunities for the study of the cancer biology of malignant melanoma *in vivo* but its use in the identification of cooperating lesions is somewhat limited owing to the potent effects of T antigens on multiple and diverse cancer control pathways. On the other hand, this model has been informative in that it suggested that pathways inactivated by T antigen, which include Rb and p53, are highly relevant to the development of malignant melanoma. Thus, one would speculate that inactivation of the p16^{INK4a} gene or the RB pathway alone would be insufficient to bring about malignant melanoma and that disruption of the p53 pathway would be necessary. However, it is intriguing that p53 itself is found to be mutated in only a small percentage of melanomas (Saenz-Santamaria *et al.*, 1995). Thus, it is possible that disruption of the p53 pathway involves an upstream component other than p53 itself. This hypothesis is supported by the frequent deletion of the common exon 2 of INK4a/ARF gene (an exon that dually encodes for p16^{INK4a} and p19^{ARF}) in both familial and sporadic melanomas, perhaps representing an efficient strategy to target both the RB and p53 pathways.

The frequent deletion of sequences encoding both p16^{INK4a} and p19^{ARF} served as a basis for the construction of a mouse harboring a germline deletion of the exons 2/3 region of the INK4a gene (Serrano *et al.*, 1996). This INK4a^{Δ2/3} allele eliminates coding sequences for both p16^{INK4a} and p19^{ARF}, thus rendering INK4a^{Δ2/3} mice dysfunctional for both RB and p53 (Pomerantz *et al.*, 1998; Sharpless and DePinho, unpublished results). These INK4a^{Δ2/3} mice were found to be highly cancer-prone and derivative cells exhibited an immortalized phenotype, providing genetic proof that this locus encodes tumor suppressor activity and that INK4a/ARF functions as a critical mediator of senescence (Serrano *et al.*, 1996). Interestingly, despite the strong link between INK4a/ARF and melanoma in humans, the INK4a^{Δ2/3} mice failed to develop melanoma. Rather, the most common malignancies encountered in this model were fibrosarcomas and B-cell lymphomas (Serrano *et al.*, 1996).

Given the known resistance of rodents to melanoma development, we suspected that the lack of melanoma in INK4a^{Δ2/3} null mice related in part to species-specific differences in the melanocyte microenvironment. In humans, most melanomas arise within the epidermal microenvironment as *in situ* lesions in radial growth phase. Progression towards the vertical growth phase (downward invasion into the dermis) is thought to require additional genetic alternations that may confer a survival advantage in the less 'supportive' dermal milieu. This supposition is based upon the observation that epidermal melanoma cells do not readily survive and proliferate when transplanted into a dermal microenvironment (Nesbit *et al.*, 1999). Since melanocytes of the adult mice reside in the dermis, it is, therefore, reasonable to assume that multiple pro-survival/growth stimulatory signals are needed in order to reach a critical transformation threshold in the

mouse, providing a basis for observed resistance of rodents to melanoma. With this hypothesis in mind, we speculated that the addition of oncogenic stimuli in an INK4a null setting may promote the transformation of these murine dermal melanocytes. The candidate oncogenic lesion considered early in the course of these studies was oncogenic RAS, based upon several lines of experimental evidence suggesting potent cooperative interaction between activated H-RAS^{V12G} and INK4a/ARF deficiency in cultured fibroblasts (Serrano *et al.*, 1997), and the presence of activating N- and H-RAS mutations in human nodular melanoma (Jafari *et al.*, 1995).

Guided by this rationale, we generated mice where activated H-RAS^{V12G} was expressed in melanocytes with the aid of the tyrosinase promoter and a newly identified upstream enhancer element (Ganss *et al.*, 1994). Against the wildtype background, spontaneous cutaneous melanomas emerged at a very low incidence and with a long latency (Chin *et al.*, 1997). Significantly, these rare melanomas showed spontaneous deletion of both *INK4a* alleles. Correspondingly, INK4a^{Δ2/3} mice of mixed genetic background developed spontaneous cutaneous melanomas with high penetrance after a short latency (Chin *et al.*, 1997), providing unequivocal evidence that *ink4a* deficiency can cooperate with oncogenic RAS to accelerate the genesis of melanoma *in vivo*. These tumors resembled human nodular melanomas in that they were amelanotic and highly vascular, and they exhibited strong immunoreactivity to tyrosinase related protein I (TRP1), thus confirming their melanocytic origin.

This model now provided an opportunity to dissect the relative contributions of INK4a/b locus gene products in the development of melanoma. First, by generating melanoma susceptible RAS transgenic mice on INK4a^{Δ2/3} heterozygous background, we examined the status of the remaining wildtype allele of *ink4a/b* in ensuing melanomas. By Southern blot analyses and multiplex PCR, we found that, in 100% of the cases, there was LOH involving the exon 2 region of *ink4a*, a region dually encoding for both p16^{INK4a} and p19^{ARF} (Figure 1a). These LOH studies in the murine melanomas, coupled with the known genetic profile of human melanoma, unequivocally demonstrated that elimination of the INK4a tumor suppressor gene function is a *sine qua non* of melanomagenesis. However, since INK4a^{Δ2/3} mutant mouse lacks both functional p16^{INK4a} and p19^{ARF}, these experimental results could not address the relative contribution of either product to *ink4a* tumor suppressor function. In the case of p16^{INK4a}, the existence of germ line E1α specific point mutation in melanoma susceptible individuals (Hussussian *et al.*, 1994; FitzGerald *et al.*, 1996) provides the unequivocal genetic proof for its role in melanoma genesis. Equivalent genetic evidence has not yet been obtained for p19^{ARF} in human melanomas (see above). Although p19^{ARF} specific knockout mice are prone to the development of lymphomas and fibrosarcomas (Kamijo *et al.*, 1997), the role of p19^{ARF} in melanoma has not been established.

Interestingly, deletion of the *ink4b* exons were seen as well, but always in accompaniment of large deletion of the region encompassing exon 2 of *ink4a* (Figure 1a), a profile similar to that described in human 9p21 associated tumors. In these cases, there was still an

intact allele of *ink4b* that was in *cis* with the *ink4a* mutant allele. Furthermore, examination of melanomas arisen in INK4a^{-/-} animal revealed no INK4b deletion, suggesting that once INK4a is eliminated, there was no longer a genetic pressure to delete sequences at the 9p21 locus (Chin *et al.*, 1997). Together, these results would suggest, but could not rigorously prove, that *Ink4b* loss in melanomas simply reflects an innocent bystander effect. To definitively address the possible pathogenetic role of *Ink4b* in melanoma development, future studies will need to examine the melanoma susceptibility of Tyr-RAS transgene against a germline p15^{INK4b} mutation. Moreover, should tumor arise in these mice, it will be important to catalog whether mutations in the p19^{ARF} open reading frame have resulted.

Role of activated RAS in melanoma maintenance

With a goal toward constructing a more faithful model for human cancer, we engineered a mouse in which expression of the dominantly acting oncogene can be regulated *in vivo*. This approach was prompted in part by the well-known features of human cancer, namely that most mutations are somatically acquired and occur well after immunologic tolerance to self antigen has been established. As most tissue-specific promoters (including tyrosinase) are activated prior to immunodifferentiation and thymic maturation, tumor immunity studies that employ constitutively active transgenes may not properly model the *de novo* presentation of tumor antigens in adult human cancers. On a more practical level, the constitutive expression of activated oncogenes in the developing melanocytes can impact adversely on melanocyte differentiation, hence affect viability of the founder and/or foster 'negative selection' against transgene expression. This problem was encountered in our production of the constitutive Tyr-RAS transgenic in which fivefold more pronuclear injections were required to achieve the usual number of founders and these founders were all low transgene expressors. Several Tyr-RAS founders expressing moderate levels of RAS were found to be runted, suffered from inner ear defects, and died early in life.

In addition to more faithful recapitulation of cancer genesis, a conditional system also permits an assessment of the function of an oncogene in an established tumor by controlling its expression in the fully-formed tumor. Advanced malignancy represents the phenotypic end-point of many successive genetic lesions that impact on the function and regulation of oncogenes and tumor suppressor genes. The established tumor is maintained and sustained through complex and poorly understood host-tumor interactions that guide such processes as angiogenesis and immune sequestration. The numerous and diverse genetic changes that accompany tumor development raises questions as to whether causal genetic alterations remain relevant to maintenance of the transformed phenotype. An *in vivo* conditional system would allow one to begin to address these genetic changes and their relevance to tumor maintenance, and to study the heterotypic host-tumor cell interactions that sustain the tumor organ and ultimately point to therapeutic target that disturb this symbiotic relationship.

To this end, a tet-regulated RAS transgenic mouse (Tyr/Tet-Ras) was generated that possessed both the activator (Tyr-rtTA) and the reporter (Tet-Ras) transgenes on the *INK4a*^{A2/3} null background (Chin *et al.*, 1999). Activated RAS expression can be regulated by doxycycline in the media or in drinking water *in vitro* or *in vivo*, respectively (Figure 2). Moreover, these mice developed spontaneous cutaneous melanomas in a strictly doxycycline-dependent manner, i.e. melanomagenesis occurred only in doxycycline-treated Tyr/Tet-RAS *INK4a*^{-/-} mice. These doxycycline-induced tumors shared all of the features of the constitutive Tyr-RAS *INK4a*^{-/-} melanomas (Chin *et al.*, 1997). Specifically, they presented as amelanotic, invasive and highly vascular tumors which upon histological examination exhibited a spindle morphology with anaplastic and pleomorphic cytology, strong immunoreactivity to the early melanocyte-specific marker tyrosinase-related protein-1 (TRP-1) (Thomson *et al.*, 1988), and robust H-RAS^{V12G} expression and activity.

In cell culture, the presence or absence of doxycycline did not significantly influence the subconfluent growth rates of multiple independently derived melanoma lines in both high and low serum conditions. In contrast, regulation of transgene expression *in vivo* is associated with striking phenotypic changes in estab-

lished tumors. When these doxycycline-treated Tyr/Tet-RAS *INK4a*^{-/-} mice bearing one or multiple independent primary melanomas were withdrawn from doxycycline administration, established melanomas (0.3–1.5 cm in diameter) rapidly regressed to barely detectable or undetectable masses with only residual scattered tumor foci on microscopic examination within 10–14 days (Figure 2c). In other words, removal of activated RAS in an established melanoma resulted in tumor regression, a remarkable finding that establishes an essential role for activated RAS in tumor maintenance. Furthermore, upon re-induction with doxycycline, the remaining minimal residual disease recurs to form a large tumor in a predictable manner at the same site, making this model potentially useful for study and therapy of minimal residual disease.

With the capacity to regulate H-RAS^{val12} activity in established primary or explanted melanomas *in vivo*, this model system now provides an unprecedented opportunity to determine what role activated RAS plays in tumor genesis and maintenance and to investigate the mechanisms underlying tumor regression following down-regulation of RAS. One hypothesis for tumor regression following doxycycline withdrawal is that continued expression of RAS confers the tumor cells the capacity to evade host immune response (Doherty *et al.*, 1994), thus tumor regression occurs due to onset of immune rejection by the host. However, this does not appear to be operative in our model since the regression kinetics of SCID tumor explants derived from purified Tyr/Tet-RAS *INK4a*^{-/-} melanoma cell lines were similar from that of the primary tumors in immune competent hosts. Although they maintain functional natural killer cells and macrophages, SCID mice lack B and T lymphocytes, indicating that a fully functional immune system does not play a principal role during the initial phases of regression during which the bulk of the tumor burden is eliminated. Notwithstanding these observations, it will be important to assess whether minimal residual disease is completely eliminated in these SCID explant studies.

Another well known aspect of host-tumor interaction involves angiogenic support. Analysis of regressing Tyr/Tet-RAS *INK4a*^{-/-} tumors was highlighted by dramatic activation of apoptosis in the tumor cell compartment as well as host-derived endothelial cells. The finding of endothelial cell apoptosis would suggest that vascular integrity of the tumor is compromised when RAS expression is downregulated, and that sustained activated RAS expression may be required for the critical host-tumor symbiotic interaction that sustains stable tumor vasculature. Previous cell culture based studies have shown that oncogenic K- and H-RAS can stimulate expression of VEGF (Rak *et al.*, 1995; Arbeiser *et al.*, 1997; Okada *et al.*, 1998), one of the most potent endothelial cell survival factors. These *in vitro* data would imply that activated RAS might maintain the tumor vasculature through enhanced VEGF gene expression. However, preliminary data from this *in vivo* model underscores the complexity of the RAS-VEGF angiogenesis link. Specifically, although a decline in VEGF mRNA and protein was associated with a decrease in RAS activity following doxycycline withdrawal in cell culture, an initial increase in VEGF was observed *in vivo* following

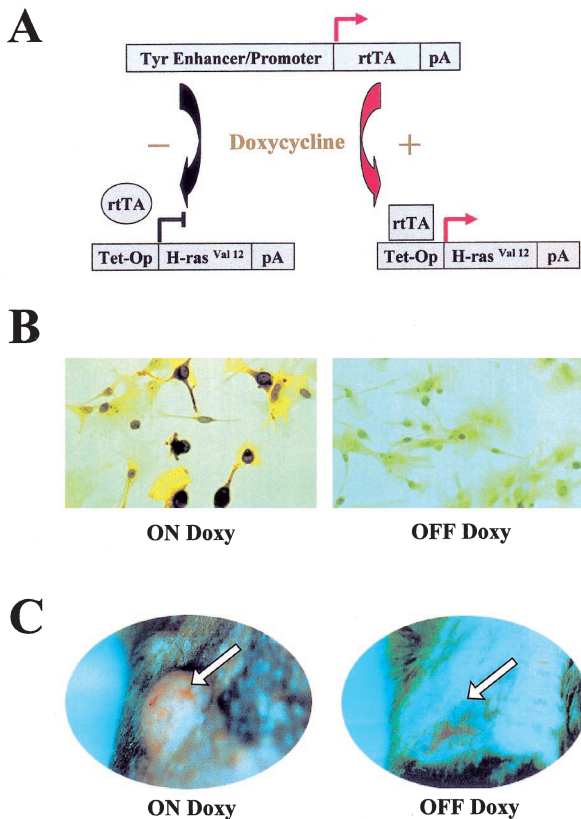


Figure 2 Expression of inducible H-RAS^{V12G} transgene is strictly regulated *in vitro* and *in vivo*. (a) Only in the presence of doxycycline, the tetracycline transactivator (rtTA) can bind to the multimerized tet-operons and activate the H-RAS^{V12G} transgene. (b) Immunohistochemistry staining for RAS in cells adapted to culture from Tyr/Tet-RAS melanomas with or without doxycycline supplementation. (c) Regression of primary cutaneous melanomas after withdrawal of doxycycline from drinking water of Tyr/Tet-RAS mice

doxycycline withdrawal (Chin *et al.*, 1999). These rising levels of VEGF are coincident with the initial stages of regression and tumor collapse and thus point to RAS-independent mechanisms of VEGF stimulation, such as hypoxia-induced VEGF upregulation (Schweiki *et al.*, 1992; Goldberg and Schneider, 1994; Mukhopadhyay *et al.*, 1995; Mazure *et al.*, 1996). Furthermore, to determine whether sustained VEGF expression can rescue the tumor regression phenotype, we have generated retrovirally transduced-VEGF expressing doxycycline-responsive melanoma cell line. Although these VEGF-transduced cells exhibited a more rapid tumor growth in SCID mice, doxycycline withdrawal still resulted in regression of these tumors despite high level of enforced VEGF expression. Furthermore, as in primary tumors and SCID tumors derived from the parental untransduced melanoma lines, this tumor regression was associated with marked activation of apoptosis in both tumor cells and host endothelial cells. The failure of enforced VEGF expression to 'rescue'/reverse the tumor regression phenotype and associated vascular collapse after doxycycline withdrawal conclusively establishes that VEGF is not sufficient for tumor maintenance and that the role of

activated RAS in tumor maintenance extends beyond the regulation of VEGF gene expression.

Given the pleiotropic effects of activated RAS and its importance in such a wide variety of human cancers, it is not surprising that its role in maintenance of established melanomas extends beyond VEGF and likely extends beyond the process of tumor angiogenesis. This *in vivo* inducible model can serve as an ideal system in which additional aspects of host-tumor symbiosis can be uncovered. In addition to angiogenesis, these aspects may include immune surveillance, extracellular matrix degradation or other yet to be identified processes that are essential for the maintenance of established melanomas or other solid tumors in their natural settings.

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