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Review

Animal models of melanoma: a somatic cell gene delivery mouse model allows rapid evaluation of genes implicated in human melanoma

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Abstract

The increasing incidence and mortality associated with advanced stages of melanoma are cause for concern. Few treatment options are available for advanced melanoma and the 5-year survival rate is less than 15%. Targeted therapies may revolutionize melanoma treatment by providing less toxic and more effective strategies. However, maximizing effectiveness requires further understanding of the molecular alterations that drive tumor formation, progression, and maintenance, as well as elucidating the mechanisms of resistance. Several different genetic alterations identified in human melanoma have been recapitulated in mice. This review outlines recent progress made in the development of mouse models of melanoma and summarizes what these findings reveal about the human disease. We begin with a discussion of traditional models and conclude with the recently developed RCAS/TVA somatic cell gene delivery mouse model of melanoma.

Key words Melanoma, animal models, RCAS/TVA

The incidence of melanoma has been increasing at an alarming rate over the past 20 years. About 68 130 new cases are expected this year with nearly 8700 resulting in death [1]. Melanoma, the most rapidly increasing malignancy among young people in the United States and the most common cancer for young adults of 25 to 29 years old [2], accounts for the majority of deaths attributed to skin cancer and has a poor prognosis for advanced stages of the disease[3]. Patients with metastatic melanoma have limited treatment options and median survival ranges from 6 to 12 months in clinical trials [4]. Currently, Interleukin-2 and dacarbazine are two FDA-approved drugs for advanced melanoma, but only a small percentage of patients respond to them [4]. Results from clinical studies with small molecule inhibitors of mutant serine/threonine-protein kinase (BRAF) have been very encouraging and promise to yield a much needed breakthrough in the treatment of tumors with this alteration; however, initial responses are not durable and relapse occurs after a median time of 9 months[5]. Further advances in the management of melanoma require model systems aiding in the understanding of disease behavior and assisting in the development and testing of novel therapeutic strategies. Animal models of melanoma have contributed greatly to the biological understanding of melanoma and also serve as a useful tool for testing potential new therapeutic approaches.

Several animal models of melanoma have been developed, including *Xiphophorus* fish, guinea pig, opossum, and mouse models [6]. The most relevant and successful model has been the mouse due in part to our broad knowledge of mouse genetics. Numerous types of mouse models have been developed that allow researchers to study different components of the disease. Transplantation models, including xenografts of human tumor tissue into immune-deficient mice and syngeneic mouse melanoma cells, have been used for some time. More recently, the development of genetically engineered mouse models (GEMMs) has expanded our knowledge of gene function and elucidated possible targets for treatment of melanoma. Finally, the development of retroviral-vector delivery systems, such as the RCAS/TVA system, has allowed more rapid assessment of the effect of single or multiple genes on tumor initiation, progression, and maintenance. Each model has advantages and disadvantages. However, using each of these models is necessary to further our

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understanding of the disease and improve treatment.

Xenograft Transplantation Models

Xenograft models allow the transplantation of human melanoma cells into immune-deficient mice and are useful for studying metastasis, a pattern often retained after xenotransplantation[7]. The major advantage of these models is their ease of implementation and rapidity for results. They have also been useful in determining mutations required for melanocyte transformation and melanoma cell invasion. Chudnovsky *et al.*[8] expressed several genes implicated in melanogenesis in human melanocytes, integrated these cells into human skin reconstructs, and grafted them onto immune-compromised mice. Their results demonstrated that activation of the Ras pathway, inhibition of the Rb and p53 pathways, and expression of telomerase reverse transcriptase (hTERT) were required to generate invasive melanoma. The ability to study human cells directly is advantageous when considering gene mutation and function, because human skin melanocytes are found throughout the basal layer of the epidermis, whereas mouse melanocytes are predominantly located deeper in hair follicles within the dermis. Mouse melanocytes are only found in the epidermis of hairless areas such as the paws, tails, and ears[6]. Human tissue circumvents this issue when studying the biological transition from radial growth phase (RGP) to vertical growth phase (VGP), which is clinically relevant. A major drawback to the xenograft model is the necessity for immune-compromised mice. However, these mice can accept human immune cells, allowing the interaction between human melanoma cells and specific human immune cells to be assessed[9]. Another disadvantage of the xenograft model is failure to fully replicate the interaction between tumor cells and host stromal cells. This limitation can be overcome by the use of spontaneous melanoma models.

Chemically Induced Models

Spontaneous melanoma is extremely rare in laboratory animals[7]. Chemical carcinogens, such as 7,12-dimethylbenz(a)anthracene (DMBA) and 12-O-tetradecanoylphobol-13-acetate (TPA), have been used to induce melanoma in mice. DMBA is an immuno-suppressing, polycyclic aromatic hydrocarbon[10]. TPA, a phorbol ester, acts as a tumor promoter by activating protein kinase C[11], which in turn phosphorylates some growth factor receptors, including epidermal growth factor receptor[12]. These are often used in combination with other modeling techniques, including ultraviolet (UV) radiation, xenotransplantation, or genetic engineering, to decrease the latency of developing melanoma[13-15]. The most obvious disadvantage to chemical induction is the lack of clinical relevance to the human disease. However, an advantage of this model is that the immune system is fully functional and thus, these mice can be used to test immunotherapeutic strategies including vaccines, cytokines, antibodies, or any combination therein. Another potential advantage to this system is that DMBA alone can induce nevi in pigmented mice[16], whose establishment can be used to study mechanism(s) of malignant transformation. To induce melanoma, an initial dose of DMBA (200 to 500 ng) is administered to the skin of mice, and then the same area is treated two to three times per week with TPA (5 μg)[13,15]. However, the DMBA plus TPA protocol typically yields papillomas and small nevi much more frequently than melanoma. To circumvent this problem, many carcinogen-induced melanomas have been used to develop syngeneic transplantation models.

Syngeneic Transplantation Models

Syngeneic models have been used for more than half a century and include the S91 melanoma in DBA/2 mice[17], Harding-Passey melanoma in BALB/c × DBA/2F1 mice[18] and B16 melanoma in C57BL/6 mice[19,20]. Recently, a syngeneic model in FVB/n mice has also been developed[21]. These models are useful for addressing basic questions, but because they have a functional immune system, they are most suitable for studying the effects of immunotherapy as a treatment for melanoma. The most widely used syngeneic transplantation melanoma model is B16, which was derived from a chemically induced melanoma arising in a C57BL/6J mouse[20]. While these cells express low levels of major histocompatibility complex class I (MHC I) molecules, they express high levels of melanoma-associated antigens, such as gp100 or tyrosinase related protein 2 (TRP2), which are immunotherapeutic targets[22]. We have previously used this model to demonstrate that the inflammatory killing of normal melanocytes activates a potent T-cell response targeted against a specific subset of self-antigens, but can also lead to the immunoselection of resistant variants[23]. A limitation of most syngeneic melanoma models is a lack of understanding regarding the alterations that drive tumor formation and progression.

Genetically Engineered Models

Molecular analysis of familial and sporadic melanomas has identified several genomic loci

implicated in the genesis and progression of melanoma [6,24,25] (Table 1), with several of these alterations being reproduced in mice (Table 2)[13,14,21,26-50]. Linkage studies in melanoma-prone families have implicated the tumor suppressor locus *CDKN2A (cyclin-dependent kinase inhibitor 2A*), located at 9p21, as a melanoma susceptibility locus [51]. This locus is functionally inactivated in a significant percentage of sporadic melanoma [24] and encodes two independent protein products, $p16^{INK4a}$ and $p14^{ARF}$ [52,53]. $p16^{INK4a}$, also known as multiple tumor suppressor 1 (MTS1) or CDKN2A, is a specific inhibitor of Cyclin D/CDK4 or CDK6 complexes [54]. By inhibiting the kinase activity of CDK4 and CDK6, $p16^{INK4a}$ blocks pRB phosphorylation and prevents G_1/S cell cycle progression[52]. $P14^{ARF}$ ($p19^{ARF}$ in mice) [55] stabilizes p53 [52], and promotes cell cycle arrest in G_1 and G_2 in response to oncogenic stimuli[56,57]. In familial and sporadic melanomas, exon 2, which is common to both p16 and p14, is frequently deleted [24], ablating tumor suppression by the RB and TP53 pathways, possibly explaining why TP53 is found mutated in only a small percentage of human melanoma[58].

Activated *RAS* oncogenes, which constitutively stimulate mitogen-activated protein kinase (MAPK) signaling[59], have been detected in approximately 20% of human melanomas [60]. Recently, mutations in *BRAF*, which activate MAPK signaling, have also been found in a high percentage (> 65%) of malignant melanoma [61]. With mutually exclusive mutations taking place in *RAS* and *BRAF* [61], the MAPK signaling pathway is constitutively activated in over 85% of malignant melanoma cases, indicating the importance of the MAPK pathway in melanomagenesis.

A role for HRas and NRas in melanoma formation was confirmed by tyrosinase-driven expression of mutant $HRas^{G12V}$ and $NRas^{Q61K}$ in the melanocytes of *Ink4a/Arf*-deficient mice, resulting in the formation of melanoma in 50% or 90% of the mice, respectively, by 6 months [30,39]. An inducible $HRas^{G12V}$ melanoma mouse model null for the tumor suppressors *Ink4a/Arf* also showed the importance of $HRas^{G12V}$ expression in the maintenance of melanoma [62]. Inducible tyrosinase-driven expression of $BRaf^{V600E}$ in mouse melanocytes leads to benign melanocytic hyperplasia[26,38] and tumor formation in about 50% of the mice with a median latency of 12 months [44]. Loss of *Ink4a* or *Ink4a/Arf* decreased the latency and increased the penetrance of tumor formation in this model[38,44]. $BRaf^{V600E}$ expression in combination with conditional *Pten* gene silencing in melanocytes leads to metastatic melanoma with a median latency of 50 days[26]. This model was also used to test the therapeutic efficacy of combined MEK and mTOR inhibition using PD0325901 and rapamycin, respectively. The mice treated with either agent had stable disease, whereas the mice treated with the combination therapy demonstrated significant tumor regression[26].

Exposure to UV radiation is thought to be a causal

Table 1. Summary of putative loci and their involvement in familial or sporadic melanoma

Locus: gene	Familial or sporadic	Alteration in familial melanoma	Alteration in sporadic melanoma	Melanoma samples analyzed
9p21: CDKN2A ($p16^{INK4a}$ $p14^{ARF}$)	Both	Point mutation	Point mutations, deletions, promoter methylation	Cell lines, melanoma, metastases
12q14: CDK4	Both	Point mutation	Point mutations	Cell lines
1p36	Familial	Linkage	–	–
6p24: TFAP2A	Both	Linkage	Decreased expression	Cell lines
7p11–13: EGFR	Sporadic	–	Amplification	Cell lines
7q33: MET	Sporadic	–	Amplification	Melanoma
3p21: CTNNB1 (β-catenin)	Sporadic	–	Point mutations	Cell lines
13q14: RB1	Sporadic	–	Point mutations	Cell lines
17p13: TP53	Sporadic	–	Point mutations	Cell lines
16q22: CDH1 (E-cadherin)	Sporadic		Decreased expression	Cell lines, melanoma, metastases
12p13: CDKN1B (p27)	Sporadic		Decreased expression	Melanoma, metastases
6q	Sporadic	–	LOH and cytogenetic alterations	Cell lines
10q23: PTEN	Sporadic	–	LOH and point mutations	Cell lines, melanoma
11q22–23	Sporadic	–	LOH	Cell lines
1p13: NRAS	Sporadic	–	Point mutations	Melanoma
8q24: MYC	Sporadic	–	Overexpression	Cell lines, melanoma, & metastases
7q34: BRAF	Sporadic	–	Point mutations	Cell lines, melanoma
2q34: ERBB4	Sporadic	–	Point mutations	Cell lines, melanoma
16q24: MC1R (melanocortin receptor)	Sporadic	–	Point mutations	Blood cells

Modified from reference [24]. "–" indicates not applicable.

Table 2. Summary of genetically engineered melanoma mouse models

Genetic change	Background	Latency/Penetrance	Promoter	Reference(s)
$NRas^{Q61R}$-IRES-Cre	$Ink4a/Arf^{f/f}$	8 weeks (median survival)/63%	DCT-TVA	[21]
$BRaf^{CA/wt}$ $Pten^{f/f}$		10 weeks/100%	Tyr::CreERT2	[26]
LSL-$KRas^{G12D}$		17 weeks/100%	Tyr::CreERT2	[27]
	$p53^{f/f}$	31 weeks median/45%		[28]
	$p16^{f/f}$	24 weeks median/73%		
	$p53^{f/f}$ $p16^{f/f}$	9 weeks median/100%		
$HRas^{V12G}$	$Ink4a/Arf^{-/-}$, Pten ±	~19 weeks/75%	Tyr	[29]
tetO::$HRas^{G12V}$	$Ink4a/Arf^{-/-}$	26 weeks/60%	Tyr::rtTA	[30]
LSL-$KRas^{G12D}$, LSL-$BRaf^{D549A}$		26 weeks/100%	Tyr::CreERT2	[31]
β-$catenin^{sta}$, $NRas^{N61K}$		27.6 weeks median/85%	Tyr	[32]
$NRas^{Q61K}$ Tyr::CreERT2/$p53^{f/f}$		28.5 weeks/100%	Tyr	[33]
HGF/SF	$Cdk4^{R24C/R24C}$	12 weeks/100% with DMBA/TPA	MT	[34]
	$p16^{-/-}$	< 30 weeks/100% with neonatal UVR		
	$p19^{-/-}$	15 weeks median/70% with neonatal UVR		[35]
	$p16^{-/-}$ $p19^{-/-}$	7 weeks/100% with neonatal UVR		
		30 weeks/50% with DMBA/TPA		[13]
$Pten^{f/f}$ ± β-$catenin^{loxex3/wt}$	$Ink4a/Arf^{f/f}$	40 weeks/100%	Tyr::CreERT2	[36]
MIP-2	P16 ± p19 ±	~28 weeks median/18.5%	Tyr	[37]
$BRaf^{V600E}$	Ink4a/Arf ±	7–58 weeks median/90%–100%	Tyr	[38]
	p53 ±	15–65 weeks median/30%–100%		
		42–85 weeks/<10%		
$NRas^{Q61K}$	$Cdk4^{R24C/R24C}$	~43 weeks/100%	Tyr	[33]
	$Arf^{-/-}$	25%		
	$p16^{-/-}$	26 weeks median/>90%		[39]
Ret		28 weeks/80% with UVR	MT	[40]
	Ednrb ±	70 weeks/~40%		[41]
	$IL6^{-/-}$	65 weeks/47%		[42]
$Xpc^{-/-}$	$Ink4a/Arf^{-/-}$	50 weeks/~70% with neonatal UVR		[43]
LSL-$BRaf^{V600E}$		52 weeks median/64%	Tyr::CreERT2	[44]
	$p16^{-/-}$	13 weeks median/80%	Tyr	
$HRas^{G12V}$		52 weeks/57% with UVR		[45]
	$Cdk4^{R24C/R24C}$	65 weeks/33% and 52 weeks/100%		[14,46]
	$p19^{-/-}$	81 weeks/52% and 50 weeks/50%		[47,48]
	$p16^{-/-}$	89 weeks/33% and 50 weeks/50%		[47,48]
	$p53^{-/-}$	17 weeks median/26%		[48,49]
Grm1		Up to 87 weeks/100%	DCT	[50]

f/f, flox/flox; CA, cre-activated; UVR, UV radiation; LSL, lox-stop-lox; sta, stable; Tyr, tyrosinase; MT, metallothionein; DCT, dopachrome tautomerase.

event in at least 80% of the malignant melanoma cases [63,64]. Epidemiological data suggest that malignant melanoma results from an exposure to intense UV light, especially during childhood [65]. Experiments in mice corroborate these findings. Exposure to UV radiation in neonatal hepatocyte growth factor/scatter factor (HGF/SF)–transgenic mice induced melanoma in 50% of the mice by 1 year [66], with the loss of *Ink4a/Arf* significantly promoting melanomagenesis, as 50% of the mice developed melanoma by 50 days [35]. In transgenic mice expressing HGF, the location of mouse melanocytes more closely resembles that of human skin as they are aberrantly distributed throughout the epidermis, including the dermoepidermal junction [67]. Thus, this model is well suited to elucidate the role of environmental damage versus genetic predisposition.

Gene knockout and transgenic technology has facilitated the development of mouse strains that can be used as model systems to assess tumor development and treatment. However, despite the great use of such models, there are several limitations. Most human tumors contain multiple genetic changes required for tumor initiation, progression, and metastasis [68]. To accurately model a particular human tumor, many mouse strains generally have to be interbred in a costly and labor intensive process. In addition, many model systems are based on animals that express oncogenes under the control of tissue-specific promoters and/or contain germline inactivating mutations in tumor suppressor genes. In many cases, these alterations have

deleterious effects on development and reproductive fitness [69], making it very difficult to obtain animals carrying the desired combination of genotypes. Moreover, tumors in other tissues can potentially arise. For example, tyrosinase-driven expression of activated HRas or NRas in the melanocytes of *Ink4a/Arf*-deficient mice results in the formation of melanoma in 50% or 90% of the mice, respectively, by 6 months [30,39,62]. However, nearly 70% of these mice develop lymphomas and sarcomas by 4 months due to the germline deficiency of *Ink4a/Arf* [54]. Although these models have shown the importance of Ras in melanoma formation and maintenance, they are not ideal to study metastatic melanoma because most of the animals expire rapidly and it is difficult to discern if tumors found in other tissues are of primary or metastatic origin.

The development of inducible gene expression systems has helped to overcome some of the aforementioned limitations [62,70]. By expressing the gene in a temporally restricted manner, inducible systems avoid the deleterious effects of the oncogene on development and fitness. Embryonic lethality associated with the germline deficiency of many tumor suppressor genes can be overcome by the cre/lox system, allowing temporal and spatial control of tumor suppressor gene inactivation [71-73]. Before the development of this technology, analysis of tumor formation in mice with inactivating mutations was limited to mice heterozygous for the specific mutation [74] or chimeric mice partially derived from embryonic stem cells homozygous for a particular mutation [75]. Although these conditional approaches overcome the adverse effects on development, they still require the generation of multiple transgenic or conditionally-inactivated strains to accurately model tumors. In addition, the induction techniques tend to activate a host of melanocytes simultaneously rather than individual cells, which differs from the natural occurrence of human disease.

RCAS/TVA Mouse Models

A retrovirally-based gene transfer method has been designed to overcome many of the aforementioned limitations. This system uses a viral vector, RCAS, derived from the avian leukosis virus (ALV) [76]. The receptor for subgroup A-ALV is encoded by the *tv-a* gene and is normally expressed in avian cells as a transmembranous (*tva*950) or glycosylphosphatidylinositol (GPI)-membrane linked form (*tva*800) as a result of alternative splicing [77,78]. Expression of either form in cells resistant to infection by ALV has been shown to confer susceptibility to infection by RCAS [77-79]. In addition, transgenic mice expressing *tva*950 or *tva*800 have been generated and are susceptible to RCAS infection *in vivo* [79-81].

Several features of the RCAS/TVA retroviral vector system offer unique advantages, particularly the ability to deliver genes in an efficient and stable manner. Infection with RCAS results in the stable integration of the viral DNA into the genome of replicating cells. In avian cells, the viral vector is replication-competent and can deliver experimental genes of reasonably useful size (up to about 3 kb). Defective vectors, which lack the envelope gene, can deliver genes up to about 4 kb when the envelope protein is supplied in *trans* [82]. High titer viral vector stocks can be generated in the DF-1 cell line, derived from immortalized chicken fibroblasts [83,84]. In replicating mammalian cells expressing TVA, the viral vector can stably integrate into the DNA and express the inserted experimental gene at high levels, but the virus is replication-defective since viral RNA and proteins are inefficiently produced [79]. Therefore, the viral vectors cannot spread in the target animals. In addition, since the envelope protein is poorly produced, there is no interference to superinfection. Theoretically, there is no limit to the number of experimental genes that can be introduced. The ability of TVA-expressing mammalian cells to be infected by multiple ALV-derived viruses allows efficient modeling of human melanoma because multiple oncogenic alterations can be introduced into the same cell or animal without the expense associated with mating multiple strains of mice.

An RCAS retroviral vector containing the gene for *Cre recombinase* has also been generated to allow targeted inactivation of tumor suppressor genes flanked by *loxP* recombination sites [85]. Although it will still be necessary to breed mice containing the specific allele flanked by *loxP* sites with mice that express TVA in the desired cell type, this system eliminates the need to express Cre from a tissue-specific promoter and allows a more selective inactivation of the tumor suppressor gene. In addition, a knock-in mouse line was recently generated that allows expression of TVA in a Cre-recombinase-dependent manner. This strain greatly enhances this system by making it easier to generate mice that express TVA in a tissue-specific fashion [86].

The RCAS family of retroviral vectors has been successfully used in the development of several cancer models in mice [87,88]. In these models, tumors evolve from gene mutations in developmentally normal somatic cells in the context of an unaltered microenvironment, thereby closely mimicking the human disease. Using this system, newly identified genes can be rapidly validated for their roles in tumor formation, progression, maintenance, and resistance to therapy. To model human melanoma using the RCAS/TVA system (Figure 1), transgenic mice expressing TVA from the dopachrome tautomerase (DCT) promoter, also known as tyrosinase-related protein 2 (TRP2), were generated [89]. This promoter was chosen to drive the expression of the

EBV VCA-IgA, Epstain-Barr virus viral capsid antigen-IgA antibody.

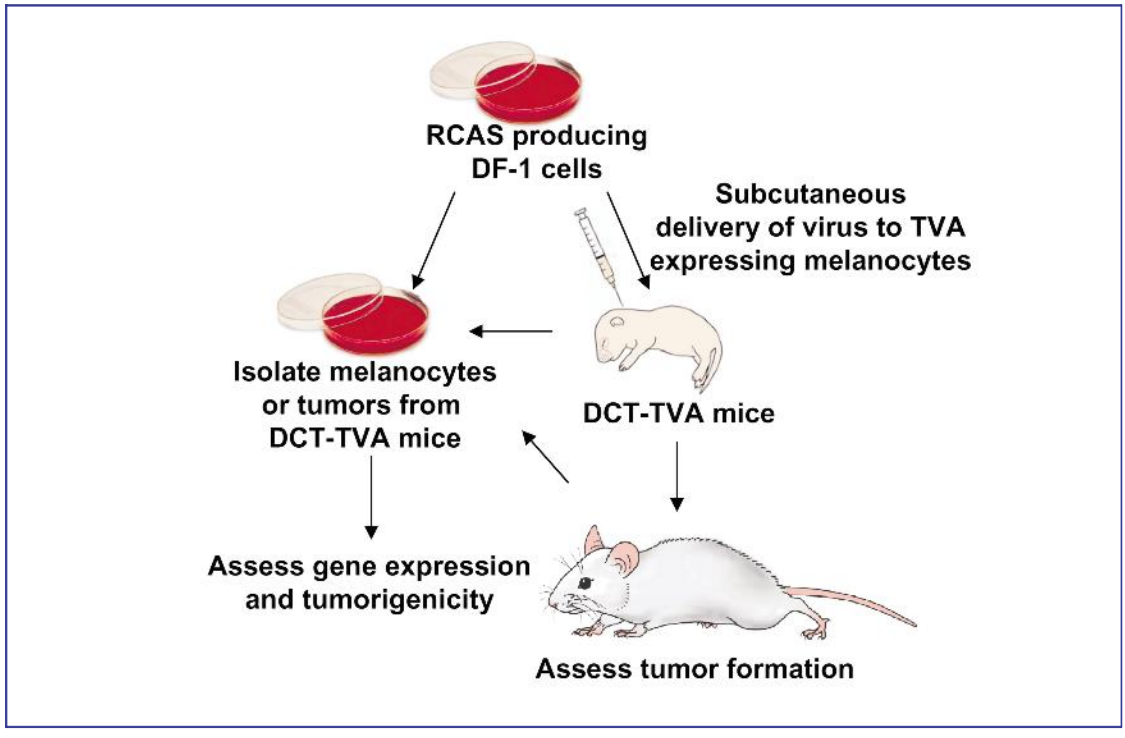

Figure 1. Diagram of the RCAS/TVA melanoma mouse model and associated procedures. Expression of the TVA viral receptor is driven by the DCT promoter, which is expressed early in melanocyte development when cells are mitotically active. DCT-TVA mice are crossed with *Ink4a/Arf*$^{lox/lox}$ mice to generate DCT-TVA–*Ink4a/Arf*$^{lox/lox}$ mice. TVA negative mice are used as a control. Newborn mice are injected subcutaneously with viral producing cells, which are cleared by the host immune system within 1 week. Animals are monitored for tumor development, which is first observed after a latency of about 4 weeks. Melanocytes and tumors can be isolated and established in culture for further analysis. Images were produced by MediaLab at the Department of Biochemistry, University of Wisconsin at Madison.

viral receptor, since this gene is expressed early in melanocyte development when the cells are mitotically active [89-92]. Because a significant percentage of familial and sporadic melanomas have mutations that functionally inactivate *INK4a* and *ARF*, DCT-TVA mice were crossed to *Ink4a/Arf*$^{lox/lox}$ mice to generate DCT-TVA–*Ink4a/Arf*$^{lox/lox}$ mice. As proof-of-principle, newborn mice were injected subcutaneously with RCAS viruses containing Cre-recombinase and NRAS^{Q61R}. Whereas no tumors were detected in TVA-negative mice, melanomas were visible in DCT-TVA–*Ink4a/Arf*$^{lox/lox}$ mice as early as 3 weeks. Within 12 weeks, more than one-third of DCT-TVA–*Ink4a/Arf*$^{lox/lox}$ mice developed melanoma histologically similar to the human disease. Delivery of a virus in which *NRAS*^{Q61R} and *Cre* expression was linked by an internal ribosomal entry site (IRES) resulted in tumor formation in more than two-thirds of TVA-positive mice. Short-term cultures from the primary tumors were established and these cells were syngeneic with the DCT-TVA–*Ink4a/Arf*$^{lox/lox}$ strain, forming tumors in 100% of recipient mice. Passage of these cells *in vivo* resulted in the development of spontaneous metastases [21].

Cancer progression is dynamic and depends on interaction between the tumor and its microenvironment. In this model, only a few cells are modified and thus, cells surrounding the tumor are normal. Because the mechanisms by which the microenvironment facilitates tumor progression are not well understood, this model is ideal for studying the interaction between the tumor and the surrounding stroma as well as further defining the role the microenvironment plays in melanoma progression and metastasis. It has also become evident that the order of genetic changes is critical to the successful formation and progression of the tumors. Genes can easily be delivered sequentially in this model to further study the natural evolution of this disease.

Most cancer models using the RCAS/TVA system have analyzed gain-of-function phenotypes by delivering and overexpressing a particular gene of interest. As others have showed that retroviral vectors can be used to stably express short hairpin RNA (shRNA) under the control of an RNA pol III promoter [93-95] to reduce target gene expression through RNA interference (RNAi), it was a natural progression to apply this technology to the RCAS family of vectors to take advantage of this

replication-competent retroviral system [96]. For this approach, we used the RCAN vector such that the inserted shRNA could be expressed under the control of the human H1 promoter and not the viral long terminal repeat (LTR) promoter and enhancer. Human melanoma cells were engineered to express TVA to allow cell-specific targeted infection by the retroviral vector. We demonstrated that viral-mediated delivery of shRNA specifically reduces target gene expression in melanoma cells *in vitro*. Recently, it has also been demonstrated that these retroviral vectors can also be used to deliver specific micro RNA (miRNA) sequences[97]. This approach extends the use of the RCAS/TVA system to include loss-of-function analyses of specific genes.

We have further modified this model system to allow the regulation of gene expression post-delivery using the tetracycline (tet)-regulated system [98]. A tet-responsive element (TRE) was inserted into the RCAN viral vector to drive the expression of the inserted gene. Expression from the TRE requires the presence of a tetracycline transcriptional activator (tTA) such as Tet-off, or a reverse tTA (rtTA) such as Tet-on. In the context of Tet-on, the Tet-responsive gene is only expressed in the presence of doxycycline (Dox); in the context of Tet-off, the Tet-responsive gene is repressed in the presence of Dox [99]. Using this approach, we demonstrated that the suppression of NRas causes melanoma regression induced by NRas and Cre in DCT-TVA–*Ink4a/Arf*$^{lox/lox}$ mice (unpublished data). This data confirms the importance of Ras signaling for melanoma maintenance in this context. We hope to use this model to further delineate mechanisms of resistance to Ras inhibition such that rational combination therapies can be developed.

As with all models, there are limitations to the use of the RCAS/TVA system. The expression of genes greater than 3 kb is unsuccessful, requiring the use of defective vectors that produce lower titers. Target cells must be actively dividing to allow integration into the host genome and although lentiviral vectors can be used to circumvent this limitation, they are less efficient [100]. The site of integration is thought to be random and has the potential to affect the expression of host genes. In addition, the integration site may affect the level of expression of the virally delivered genes. Fortunately, the advantages of this model greatly outnumber the disadvantages and there are numerous alternative strategies available to circumvent the few limitations to this approach.

Conclusions

Although experiments performed *in vitro* are useful and necessary as a first step to study the effects of gene over-expression or loss and to test potential therapies, it is not possible for *in vitro* systems to fully recapitulate the complexity of the whole organism and the microenvironment in which tumors develop. Therefore, the development of effective and efficient *in vivo* models is important to more closely mimic the complex realities of human melanoma and to increase our understanding of the biology of this disease. Increasingly sophisticated models have been developed that not only allow temporal and spatial control of gene expression or loss but also permit the testing of novel anti-melanoma therapies. All the model systems possess unique advantages and disadvantages, necessitating the use of each melanoma model as appropriate. The combined knowledge obtained from the use of each model described above will ultimately bring us closer to developing more effective treatment modalities for patients with advanced melanoma.

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