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(54) Title: MURINE BRAFV600E MELANOMA CELL LINE

(57) Abstract: A method for producing a BRAFV600E mouse melanoma cell line by sequential in vivo and in vitro growth is provided. The method comprising excising a first melanoma tumor from a mouse carrying a Cre-activated allele of BRAFV600E, dissociating cells of the excised tumor, injecting the dissociated tumor cells into an immune-compromised mouse, allowing the injected tumor cells to develop into a second tumor, and culturing the cells from excised second tumor to produce a BRAFV600E melanoma cell line. Further provided is a BRAFV600E mouse melanoma cell line produced by the method.



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MURINE BRAFV600E MELANOMA CELL LINE

Introduction

[0001] This application claims the benefit of priority of U.S. Provisional Application No. 61/904,623, filed November 15, 2013, the content of which is incorporated herein by reference in its entirety.

[0002] This invention was made with government support under grant numbers P30RR032136, CA009658, AI007363, GM00874, R01 AR-26599, CA77267, R01 CA120777, R01 CA134799 and GM103415 awarded by the National Institutes of Health. The government has certain rights in the invention.

Background

[0003] Rates of melanoma are steadily on the rise, with a current lifetime incidence of 1 in 50 individuals (Balch, et al. (2004) *CA: A Cancer Journal for Clinicians* 54(3):131-49; Smalley, et al. (2005) *Anticancer Therapy* 5(6):1069-780. In over 65% of human malignant melanomas, the *BRAF* oncogene encodes BRAF^{V600E} (Davies, et al. (2002) *Nature* 417(6892):949-54), a protein whose expression increases melanoma progression via activation of signaling cascades and downstream genes (Koya, et al. (2012) *Cancer Res.* 72(16):3928-37; Joseph, et al. (2010) *Proc. Natl. Acad. Sci. USA* 107(33):14903-8; Yang, et al. (2010) *Cancer Res.* 70(13):5518-27; Vultur, et al. (2011) *Clin. Cancer Res.* 17(7):1658-63; Chapman, et al (2011) *New Engl. J. Med.* 364(26):2507-16). Vemurafenib, also known as PLX4032, is a small molecule inhibitor that binds to the ATP-binding site of mutated BRAF kinase, inhibiting extracellular signal-regulated kinase (ERK) signaling only in tumor cells expressing BRAF^{V600E} (Joseph, et al. (2010) *supra*). Clinical use of PLX4032, to treat BRAF^{V600E} tumors, induces rapid

tumor regression and has revolutionized targeted therapy for melanoma (Boni, et al. (2010) *Cancer Res.* 70(13):5213-9; Khalili, et al. (2012) *Clin. Cancer Res.* 18(19):5329-40; Koya, et al. (2012) *supra*; Straussman, et al (2012) *Nature* 487(7408):500-4). However, resistance to PLX4032 typically develops within one year (Vultur, et al. (2011) *supra*; Joseph, et al. (2010) *supra*; Straussman, et al (2012) *supra*), underscoring the need for increased understanding of molecular mechanisms underlying BRAF^{V600E} and for developing additional therapeutic strategies.

[0004] The use of human melanoma cell lines is well established for *in vitro* and *in vivo* investigations of BRAF^{V600E} melanoma (Herlyn & Fukunaga-Kalabis (2010) *J. Invest. Dermatol.* 130(4):911-2; Søndergaard et al. (2010) *J. Translation. Med.* 8:39). However, human cell lines rely on xenografts in immune-compromised mice, which limit studies of host-tumor cell interactions in a genetically identical (syngeneic) background. Mouse models of BRAF^{V600E} malignant melanoma have provided researchers with the ability to investigate *in vivo* tumor responses to PLX4032 treatment (Dankort, et al (2009) *Nature Genetics* 41(5):544-52; Dhomen, et al (2009) *Cancer Cell* 15(4):294-303. Backcrossing these mice onto a pure background has allowed for *in vivo* testing of immunologic therapies in a syngeneic system (Hooijkaas, et al. (2012) *Oncoimmunology* 1(5):609-617; Hooijkaas, et al. (2012) *Am. J. Pathol.* 181(3):785-94). However, for *in vitro* analysis of the molecular mechanisms underlying BRAF^{V600E} melanoma, stable cell lines that recapitulate the human disease are needed. These lines could be used to identify critical mechanisms: signal transduction pathways, patterns of gene expression, and interactions with cells within the tumor microenvironment (Herlyn & Fukunaga-Kalabis (2010) *supra*).

[0005] The isolation of murine BRAF^{V600E} cell lines from mouse models of BRAF^{V600E} melanoma has been reported (Koya, et al. (2012) *supra*; Hooijkaas, et al. (2012) *supra*). However, given the heterogeneity of human BRAF^{V600E} cell lines, the establishment of only two murine cell lines may not adequately represent the true biology of BRAF^{V600E} cells *in vitro*. For example, both of the reported murine BRAF^{V600E} cell lines are only partially sensitive to PLX4032 and PLX4720 (Koya, et al. (2012) *supra*; Hooijkaas, et al. (2012) *supra*; Knight, et al. (2013) *J. Clin. Invest.* 123(3):1371-81), with respect to decreased pERK signaling and cellular proliferation. These *in vitro* results differ from *in vivo* results seen in a corresponding mouse model (Hooijkaas, et al. (2012) *supra*) and with human BRAF^{V600E} cell lines (Joseph, et al. (2010) *supra*; Yang, et al. (2010) *supra*; Straussman, et al (2012) *supra*).

[0006] Therefore, there is a need in the art for a transplantable tumor model, based on cells from a defined genetic background, which would enable more rapid and reproducible evaluations of tumor growth in multiple tissue compartments as well as a better understanding of the molecular mechanism underlying disease progression and resistance.

Summary of the Invention

[0007] This invention is a method for producing a BRAF^{V600E} mouse melanoma cell line. The method involves the steps of (a) excising a first melanoma tumor from a mouse, wherein cells of the tumor carry a Cre-activated allele of BRAF^{V600E}; (b) dissociating cells of the excised tumor, (c) injecting the dissociated tumor cells into an immune-compromised mouse, without culturing the dissociated tumor cells, (d) allowing the injected tumor cells to develop into a second

tumor, (e) excising the second tumor from the mouse, and (f) culturing the excised tumor cells to produce a BRAF^{V600E} melanoma cell line. A BRAF^{V600E} mouse melanoma cell line produced by the method is also provided.

Detailed Description of the Invention

[0008] Mouse models of BRAF^{V600E} provide a system for studying melanoma *in vivo*. Accordingly, melanoma cell lines have now been established from the conditional mouse model of metastatic melanoma, *Tyr::CreER; Braf^{CA}; Pten^{lox4-5/lox4-5}*. The melanoma cell lines grow readily in culture, are sensitive to PLX4032, and are transplantable in syngeneic hosts. Given that the cells of the invention recapitulate human disease, they provide a valuable model for studying the molecular mechanisms of malignant melanoma *in vitro*, as well as a transplantable model of melanoma progression *in vivo*.

[0009] Accordingly, the present invention is a method for producing a BRAF^{V600E} mouse melanoma cell line. The method of the invention comprises or consists of the steps of excising a first melanoma tumor from a mouse, wherein cells of the tumor carry a Cre-activated allele of BRAF^{V600E}; dissociating cells of the excised tumor; injecting the dissociated tumor cells into an immune-compromised mouse, without culturing the dissociated tumor cells; allowing the injected tumor cells to develop into a second tumor; excising the second tumor from the mouse; and culturing the excised tumor cells to produce a BRAF^{V600E} melanoma cell line.

[0010] As is known in the art, melanoma is a malignant tumor arising from melanocytes in the skin or other sites, and which may contain dark pigment. In certain embodiments, the cells of the melanoma cell line of this invention have

a mutation in the v-raf murine sarcoma viral oncogenes homolog B1 (BRAF) isoform of the RAF kinase. Ninety percent of *braf* mutations are accounted for by a thymine to adenine single-base change at position 1,799. This missense mutation, located in exon 15, results in a change at residue 600 of the BRAF protein that substitutes glutamine for valine (V600E; Davies, et al. (2002) *Nature* 417:9490-54). BRAF^{V600E} can gain 500-fold increased activation, stimulating the constitutive activation of MEK/ERK signaling in tumor cells. Furthermore, it allows activation of this signaling cascade in the absence of any extracellular stimuli, allowing the cell to become self-sufficient in growth signals within this pathway. Accordingly, in particular embodiments, the cells of the melanoma cell line carry the BRAF^{V600E} mutation.

[0011] Mutation of BRAF can be carried out by conventional methods. In particular embodiments, the mutation is carried out using the Cre-Lox system to create a conditionally active (CA) mutant. By way of illustration, DNA encompassing mouse BRaf exons 14-16 can be modified such that exon 16 is replaced with a HSV-thymidine kinase cassette. A LoxP-flanked cassette containing the 3' 382 base pairs (bp) of intron 14, the human BRAF cDNA containing exons 15-18, the mouse BRaf polyadenylation sequences, and a PGK-neo cassette can be inserted into intron 14 upstream of a modified exon 15 that encodes BRafV600E. The LoxP-flanked cassette can be introduced into mouse embryonic stem cells by conventional methods. Subsequently, embryonic stem cell clones can be screened to identify targeting of BRAF. Clones with a single copy integration of the mutant BRAF can be injected into C57Bl/6 blastocysts and germline transmission assessed by, e.g., PCR. Chimeric mice can be crossed to yield homozygous

($BRaf^{CA/CA}$) or heterozygous ($BRaf^{CA/+}$) mice. See, e.g., Dankort, et al. (2007) *Genes Dev.* 21:379-84. Furthermore, $BRaf^{CA}$ mice can be backcrossed, e.g., to C57Bl/6 mice, to achieve a pure background (e.g., less than 5%, 4%, 3%, 2% or 1% of the genome is from another mouse line).

[0012] Resulting $BRaf^{CA}$ mice harbor the germ-line conditional $BRaf^{V600E}$ allele, the expression of which can be initiated at physiological levels under the control of the gene's chromosomal promoter by the action of Cre recombinase. When the $BRaf^{CA}$ allele is combined with a conditionally active Cre recombinase, e.g., CreER^{T2} that is specifically expressed in melanocytes (Bosenberg, et al. (2006) *Genesis* 44:262-7) and exposed to 4-hydroxytamoxifen (4-HT), highly pigmented lesions appear by 21-28 days after 4-HT administration. As is seen in human compound melanocytic nevi, $BRaf^{V600E}$ -induced pigmented lesions of the transgenic mouse are frequently located at the dermal/epidermal junction with extension into the dermis. See Dankort, et al. (2009) *Nat. Genet.* 41:544-552.

[0013] As is known in the art $BRAF^{V600E}$ elicits sustained activation of the $BRAF \rightarrow MEK1/2 \rightarrow ERK1/2$ MAP kinase pathway. While contributing to the aberrant pathophysiological characteristics of the melanoma cell (Wellbrock, et al. (2004) *Nat. Rev. Mol. Cell Biol.* 5:875-85; Mercer & Pritchard (2003) *Biochim. Biophys. Acta* 1653:25-40), progression to malignant melanoma is often accompanied by silencing of one or more tumor suppressor genes, most commonly *PTEN* (phosphatase and tensin homolog), *INK4A* (cyclin-dependent kinase 4 inhibitor A) and/or *ARF* (alternate reading frame) (Garraway, et al. (2005) *Nature* 436:117-22; Wellbrock, et al. (2004) *Cancer Res.* 64:2338-4; Chin, et al. (2006) *Genes Dev.* 20:2149-82; Lin, et al. (2008) *Cancer Res.* 68:664-73). Thus, in particular

embodiments of this invention, the tumors of the instant method are malignant and, in addition to carrying a Cre-activated allele of BRAF^{V600E}, said tumors are deficient in one or more tumor suppressor genes. In particular embodiments, the tumors are deficient in one or more of *PTEN*, *INK4A* or *ARF*. A tumor deficient in a tumor suppressor gene is intended to mean that the cells does not express the tumor suppressor gene, e.g., as the result of a gene knockout, gene disruption, or siRNA-mediated gene silencing.

[0014] As a first step in the method of the invention, a melanoma tumor of a mouse carrying a Cre-activated allele of BRAF^{V600E} is excised, e.g., by excisional biopsy, incisional biopsy, needle biopsy, surgical biopsy. An "excisional biopsy" refers to the removal of an entire tumor mass with a small margin of normal tissue surrounding it. An "incisional biopsy" refers to the removal of a wedge of tissue that includes a cross-sectional diameter of the tumor. A "needle biopsy" generally contains a suspension of cells from within the tumor mass. Biopsy techniques are discussed, for example, in *Harrison's Principles of Internal Medicine*, Kasper, et al., eds., 16th ed., 2005, Chapter 70, and throughout Part V.

[0015] Once excised, the BRAF^{V600E} cells of the tumor are dissociated, e.g., by enzymatic dissociation with collagenase and DNase, by mechanical dissociation in a blender, by teasing with tweezers, using mortar and pestle, cutting into small pieces using a scalpel blade, and the like, to yield single, isolated cells. In some embodiments, the isolated primary tumor cells are briefly cultured (e.g., 24 to 72 hours) in culture medium (e.g., Dulbecco's Modified Eagle Medium (DMEM), DMEM/F-12 advanced medium, Roswell Park Memorial Institute (RPMI) medium, and the

like) with animal serum (e.g., fetal bovine serum, human serum or an appropriate substitute thereof) to ensure and/or maintain viability. In other embodiments, the isolated primary tumor cells are directly injected into an immune-compromised mouse without culturing the dissociated tumor cells. Examples of immune-compromised mice of use in the invention include, but are not limited to, nonobese diabetic (NOD)/severe combined immunodeficient (SCID) mice, NOD/SCID/ γ chain^{null} (NOG, NSG or DKO) mice and Nu/Nu (Nude) mice.

[0016] Upon introduction into the immune-compromised mouse, the tumor cells are allowed to develop into a second tumor, e.g., a tumor in the range of 5 to 20 mm in diameter, or more preferably 10 to 15 mm in diameter. The secondary tumor is subsequently excised, dissociated, and cultured in a suitable culture medium as described herein. Cells obtained by this method of the invention express high constitutive phosphorylation of ERK, are inhibited by Vemurafenib (PLX4032), are readily transplantable into mice, and show no difference in growth rates in either NSG or syngeneic C57Bl/6 mice.

[0017] BRAF-mutated tumors have been correlated with poor response to traditional chemotherapy and poor prognosis in melanoma, thyroid, and colon cancers (Tang & Lee (2010) *J. Chin. Med. Assoc.* 73:113-28; Houben, et al. (2004) *J. Carcinog.* 3:6). Targeted therapies are of great interest for these cancer types and the elucidation of the structure and functions of the BRAF kinase is the subject of ongoing research. The approach of targeting oncogenic kinases has been successful in the treatment of cancers, with activating mutations in the kinase gene that drives their progression (Sawyers (2004) *Nature* 432:294-7). For example, Imatinib has been used in the treatment of chronic myeloid

leukemia, which is driven by a characteristic mutant fusion protein Bcr-Abl, resulting in constitutive activation of the Abl kinase. Imatinib effectively blocks the Abl kinase producing an effective clinical response that can be correlated to the presence of the activating kinase mutation.

[0018] Similarly, BRAF-specific inhibitors such as GDC-0879 and PLX4720 effectively block BRAF^{V600E} and thus block BRAF^{V600E}-induced ERK activation. PLX4720 was designed according to the atomic structure of BRAF^{V600E} and as a result has a 10-fold increased potency for BRAF^{V600E} over the wild-type kinase (Tsai, et al. (2008) *Proc. Natl. Acad. Sci. USA* 105:3041-6). These first-generation BRAF inhibitors were found to be effective in preclinical trials but underwhelmed in clinical trials. The emergence of second-generation inhibitors, such as the PLX4720 analogue PLX4032, is proving much better in clinical trials. The details of a phase I trial for PLX4032 announced an 80% response rate (as determined by complete or partial tumor regression) among patients with BRAF^{V600E} mutations whereas patients without the mutation did not respond (Flaherty, et al. (2010) *N. Engl. J. Med.* 363:809-19). This analysis provides proof of concept for the identification of other small BRAF^{V600E} inhibitors.

[0019] In this respect, the BRAF^{V600E} melanoma cell line of this invention is of use in correlating *in vitro* studies on molecular mechanisms of melanoma with *in vivo* investigations on pathology, immunogenicity, and signaling in response to Vemurafenib or other targeted signal transduction inhibitors. In addition, the cell line of the invention will allow the *in vivo* induction of tumors in syngeneic or immune-compromised mice for the evaluation of targeted or combinatorial therapy in a well-controlled

tumor model. Thus, agents that inhibit melanoma formation and may therefore be used in prophylactic treatment regimens or in the therapy of existing melanoma can be identified.

[0020] The following are provided as specific embodiments of the present invention. Other modifications of this invention will be readily apparent to those skilled in the art, without departing from the scope of this invention.

Example 1: Murine BRAF^{V600E} Cell Lines

[0021] Mouse BRAF^{V600E} cell lines were produced by backcrossing the transgenic mouse model, *Tyr::CreER;Braf^{CA};Pten^{lox/lox}* (Dankort, et al. (2009) *supra*) to a C57BL/6 (B6) background to achieve <98% non-B6 DNA; these mice are hereafter referred to as Braf/Pten mice. Initially, the establishment of stable cell lines from this model was difficult likely due to the heterogeneous population of cells in the primary tumor and the slow growth of the tumor cells in culture. Accordingly, a technique was developed, which was used to establish several stable cell lines that represent the *in vivo* biology of tumors in Braf/Pten mice.

[0022] It was found that the establishment of stable cell lines from Braf/Pten mice required a protocol of sequential *in vivo* and *in vitro* growth. Induced tumors (10 mm in diameter) were resected from Braf/Pten mice and dissociated into single cells by collagenase digestion according to established methods. It was observed that DMEM advanced/5% FBS media was optimal for survival of cells isolated from the primary tumor. However, these cells often grew slowing in culture, making them unsuitable for *in vitro* studies. To verify the tumorigenicity of dissociated primary tumor cells and cultured primary tumor cells, the cells were

directly injected into NOD/SCID/ γ chain^{null} (NSG) mice, an immune-compromised, permissive host. When secondary tumors from the host mice reached 10-12 mm in diameter, they were surgically excised and cultured using the same dissociation protocol and culture media. Of the seven tumors that were processed with this sequence, five stable D4M (Dartmouth Murine Mutant Malignant Melanoma) cell lines were successfully developed from both male and female Braf/Pten mice, demonstrating that cell lines with this protocol can routinely be established.

Example 2: Characterization of D4M Cell Lines

[0023] Since both human BRAF^{V600E} and mouse Braf^{V600E} tumors have constitutive activation of MAPK signaling cascades, levels of downstream pERK was evaluated in three D4M cell lines, 3A, 5A, and 7A. In keeping with a mutational activation of Braf (Dankort, et al. (2009) *supra*), D4M cell lines had high constitutive levels of pERK compared to B16F1 cells, which are *Braf*^{wt}. Two D4M cell lines, D4M.3A (from a male donor) and D4M.7A (from a female donor), were used for subsequent studies. Although these cell lines were generated using different modifications of the protocol described herein (3A, with culture before re-injection, 7A, without culture before re-injection), they were morphologically similar in culture. All D4M cell lines generated were unpigmented, which is also observed in human melanoma cell lines such as VMM5 and VMM12 cells (Blackburn, et al. (2007) *Cancer Res.* 67:10849-58; Blackburn, et al. (2009) *Oncogene* 28:4237-4248; Croteau, et al. (2012) *J. Cell. Physiol.* 228:773-780; Huntington, et al. (2004) *J. Biol. Chem.* 279:33168-176; Yamshchikov, et al. (2005) *J. Immunol.* 174:6863-71). Further, both D4M.3A and D4M.7A grew in advanced DMEM/F-12 media with or without

serum. Survival and growth in serum-free conditions, though significantly less than in serum-containing conditions ($P > 0.05$), allows for analysis of secreted factors released from these cell lines *in vitro*.

[0024] PLX4032 binds V600E mutant BRAF with high affinity and inhibits ERK signaling output. Thus, the sensitivity of cultured D4M cells to this inhibitor was tested by first evaluating cell signaling after treatment with PLX4032, using DMSO-treated cells as negative controls and U0126-treated cells as positive controls. U0126 is a selective inhibitor of MEK1 and MEK2, blocking downstream signals including phosphorylation of ERK. Treatment of D4M.3A, D4M.5A and D4M.7A cells with 3 μ M PLX4032 for 45 or 90 minutes, led to decreased pERK, with no change in levels of pAKT. Treatment with U0126 decreased pERK levels after 10 minutes. VMM5 and VMM12 cells, human BRAF^{V600E} cell lines of malignant melanoma (Huntington, et al. (2004) *J. Biol. Chem.* 279(32):33168-76), showed similar responses to PLX4032 and U0126, with decreased pERK levels at 45 and 10 minutes respectively, and no change in pAKT. D4M cell lines were, thus, comparable to human BRAF^{V600E} cell lines in their cell signaling response to PLX4032 treatment (Joseph, et al. (2010) *supra*; Yang, et al. (2010) *supra*; Straussman, et al (2012) *supra*). This analysis indicated that D4M cell lines have characteristics similar to human BRAF^{V600E} melanoma and BRaf^{V600E} mouse models.

[0025] Cellular proliferation of D4M cell lines in response to PLX4032 was subsequently evaluated. Treatment with 3 μ M PLX4032 significantly ($P < 0.05$) arrested *in vitro* growth of D4M.3A and D4M.7A cells over 2-3 days. Human tumors with BRAF^{V600E} are dependent on constitutive activation of ERK signaling for cellular proliferation; thus, addition of PLX4032 causes growth arrest of BRAF^{V600E} tumor cells in

vitro (Joseph, et al. (2010) *supra*). In keeping with reports of human BRAF^{V600E} cell lines (Joseph, et al. (2010) *supra*; Yang, et al. (2010) *supra*), the response of the D4M cells to PLX4032 appeared to be primarily cytostatic. Previously published murine BRaf^{V600E} cell lines are relatively resistant to PLX4032 and PLX4720 treatment compared to human BRAF^{V600E} cell lines, with respect to decreased pERK signaling and cellular proliferation (Koya, et al. (2012) *supra*; Hooijkaas, et al. (2012) *supra*; Knight, et al. (2013) *supra*).

[0026] To assess the tumorigenicity of D4M melanoma cells, a range (300-300,000) of cells were intradermally injected into B6 and NSG host mice. Each concentration of cells was injected into four B6 and four NSG mice. Of the eight mice injected with 300 cells, no tumors developed in any of the mice after 27 weeks. In contrast, it took only two weeks for tumors to be observed in mice that received $\geq 3,000$ cells, and only one week for tumors to be observed in mice that received 300,000 cells (Table 1). Tumors arose with similar kinetics in B6 and NSG host mice; there was no significant difference in the average tumor size between NSG and B6 mice injected with 3,000 D4M.3A cells, at any time point. Histologic examination revealed no gross morphologic differences between tumors in B6 or NSG hosts. It was concluded that the D4M cells were equally tumorigenic in both immune-compromised and syngeneic hosts.

TABLE 1

Host	Number of cells injected	Tumor incidence ratio (mice with tumors/total injected mice)				
		1 week	2 weeks	3 weeks	4 weeks	27 weeks
NSG	300,000	3/4	4/4			
	30,000	2/4	4/4			
	3,000	0/4	2/4	4/4		
	300	0/4	0/4	0/4	0/4	0/4
B6	300,000	4/4				
	30,000	1/4	4/4			
	3,000	0/4	1/4	3/4	4/4	
	300	0/4	0/4	0/4	0/4	0/4

Bold values indicate when 100% of mice developed tumors.

[0027] PLX4720 (a compound similar to PLX4032; Tsai, et al. (2008) *Proc. Natl. Acad. Sci USA* 105:3041-46) was also tested for efficacy in inhibiting tumor progression and phosphorylated ERK signaling *in vivo*. D4M.3A cells were injected intradermally into B6 mice (300,000 cells/mouse). When tumors reached 5 mm in diameter (8 days post-injection), mice were either given chow containing PLX4720 (Plexxikon; Berkeley, CA) or control chow. Tumors were measured 4 and 7 days after the start of treatment and tumor volumes were calculated. After 7 days of treatment, mice that were fed the PLX4720 chow had significantly smaller tumors than mice that were fed the control chow ($P < 0.05$). Mice injected with D4M.7A cells showed comparable results, with smaller tumors after 7 days in mice fed the PLX4720 chow compared with those fed the control chow. In addition, tumors in mice fed the PLX4720 chow for 7 days had decreased pERK levels compared to tumors in mice fed the control chow.

[0028] To further verify that D4M cell lines have melanoma characteristics, expression of the murine melanoma marker, pmel, was examined. Several melanocyte differentiation antigens (MDAs), such as gp100 (also referred to as Pmel)

are widely expressed in human melanoma and recognized by tumor-infiltrating lymphocytes (Kawakami (1994) *Proc. Natl. Acad. Sci. USA* 91(14):6458-6462), making it a potentially useful biomarker. Relatively low levels of pmel expression were observed in D4M.3A cultured cells, however, expression increased in tumors that arose when D4M.3A cells were injected into either NSG or B6 mice. Further, when these tumors were excised and re-cultured once or twice, pmel levels fell in culture, but increased again upon implantation into mice ($P < 0.0005$). This trend was also observed with Mart-1, tyrosinase and Tryp-1 expression. This indicates that D4M cells maintain the ability to express pmel, *in vivo* and *in vitro*. Further, the *in vivo* upregulation of pmel indicates that D4M cells are responding to host factors within the tumor microenvironment. The fact that equal levels of increase were seen upon transplantation of the D4M cells into either NSG or B6 mice indicates that non-immune cells may be mediating most of this increase. Indeed, the stromal compartment of adjacent fibroblasts has been implicated as one prominent modulator of tumor cell behavior *in vivo* (Bhowmick, et al. (2004) *Nature* 432(7015):332-7; Smalley, et al. (2005) *Exp. Rev. Anticanc. Ther.* 5(6):1069-78; Eck, et al. (2009) *J. Autoimmun.* 33(3-4):214-21; Marsh, et al. (2013) *Biochim. Biophys. Acta* 1832(7):1070-8). In addition, discrepancies in the levels of gene expression between cultured human melanoma cells and those excised from nude mice (Croteau, et al. (2012) *J. Cell. Physiol.* 228:773-780) have been found, underscoring the importance of utilizing both *in vitro* and *in vivo* model systems.

[0029] Investigations with human melanoma cell lines and clinical tissue samples demonstrate that PLX4720 (a precursor of PLX4032) treatment leads to increased

expression of MDAs such as Pmel (Boni, et al. (2010) *Cancer Res.* 70:5213-9; Frederick, et al. (2013) *Clin. Cancer Res.* 19:1225-31). Since D4M cells were sensitive to PLX4032, cells were treated with PLX4032 and mRNA levels of pmel were analyzed. In D4M.3A and D4M.7A cell lines, basal levels of pmel were relatively low and comparable to levels in 3T3 cells. However, an approximate 4-fold increase ($P>0.05$) in pmel expression was observed 48 hours after the addition of PLX4032. Similar increases were observed in two D4M cell lines but not in *Braf*^{wt} 3T3 cells. Similarly, when the two human melanoma cell lines (VMM5 and VMM12) were treated with PLx4032 for 48 hours, pmel levels significantly increased, while no effect was observed in human fibroblasts.

[0030] For functional analysis of Pmel antigen recognition, a cytotoxicity assay was used to assess melanoma cell sensitivity to lysis by Pmel TCR transgenic T cells (Overwijk, et al. (2003) *J. Exp. Med.* 198:569-80). Activated Pmel T cells were added to DMSO- or PLX4032-treated D4M.3A cells at different E:T ratios. PLX4032 treatment increased lysis of D4M.3A cells by Pmel T cells compared to DMSO treatment. EL-4 (antigen-negative) cells either pulsed or unpulsed with Pmel peptide were used as control targets, and Pmel T cells specifically recognized and lysed peptide-pulsed, but not unpulsed, EL-4 cells. Taken together, these data are consistent with increased MDA expression in human melanoma cell lines in response to BRAF^{V600E} inhibition (Boni, et al. (2010) *supra*), and further validate the D4M cell lines as *bone fide* melanoma cells and an appropriate murine model system. Although D4M cell lines do not have high endogenous levels of MDAs *in vitro*, the data herein illustrate that D4M.3A cells express Pmel in a functionally-relevant manner.

[0031] D4M cell lines recapitulated human BRAF^{V600E} melanoma *in vitro*. However, unlike human cell line xenografts, D4M cell lines were readily transplantable into syngeneic host mice, which allows for immunological studies. The fact that there was no significant difference between tumorigenicity and tumor volume in NSG and B6 hosts indicates that D4M cell lines are relatively non-immunogenic, like murine B16 cells (Leveson, et al. (1979) *Cancer Res.* 39:582-6; Ashley & Kotlarski (1986) *Cell. Immunol.* 101:156-67). This is consistent with the expression of low levels of MDAs in culture and the relative paucity of immune cells in the tumor tissue (as seen by histology).

[0032] Given the heterogeneity of human BRAF^{V600E} melanoma cell lines, having multiple murine BRAF^{V600E} cell lines is an important resource for the scientific community. The established BRAF^{V600E} SM1 cell line has been a useful model in supporting the therapeutic potential of combining BRAF inhibitors with immunotherapy (Knight, et al. (2013) *supra*). D4M cells are expected to be similarly useful in developing models of new therapeutic modalities. In addition, the fact that, at 3 μ M PLX4032, the D4M cells show substantial decreases in pERK and decrease in cell proliferation, indicates that these cells are distinct from those described in the art.

What is claimed is:

1. A method for producing a BRAF^{V600E} mouse melanoma cell line comprising:

(a) excising a first melanoma tumor from a mouse, wherein cells of the tumor carry a Cre-activated allele of BRAF^{V600E};

(b) dissociating cells of the excised tumor,

(c) injecting the dissociated tumor cells into an immune-compromised mouse, without culturing the dissociated tumor cells,

(d) allowing the injected tumor cells to develop into a second tumor,

(e) excising the second tumor from the mouse, and

(f) culturing the excised tumor cells to produce a BRAF^{V600E} melanoma cell line.

2. A BRAF^{V600E} mouse melanoma cell line produced by the method of claim 1.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 14/62852

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 5/00; C12N 5/07; C12N 15/00; C12N 15/87 (2014.01)

CPC - A61K 35/12; A01K 2227/105; C12N 15/87; C12N 2800/30

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - C12N 5/00; C12N 5/07; C12N 15/00; C12N 15/87 (2014.01) - see keyword below

CPC - A61K 35/12; A01K 2227/105; C12N 15/87; C12N 2800/30; USPC - 435/325; 435/354; 435/455; 435/462

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

IPC(8) - C12N 5/00; C12N 5/07; C12N 15/00; C12N 15/87 (2014.01) - see keyword below

CPC - A61K 35/12; A01K 2227/105; C12N 15/87; C12N 2800/30; USPC - 435/325; 435/354; 435/455; 435/462 - see keyword below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PubWEST(USPT,PGPB,EPAB,JPAB); PatBase; Medline, Google: BRAF, V600E, Val600Glu, V599E, tumor, melanoma, cell line, mouse, cre-activated, loxP, tamoxifen, induction, dissociated, excising, remove, producing, establish, generate, culture, SCID, nude, immune-compromised, immunodeficient, B-Raf proto-oncogene, serine/threonine kinase, NS7, BRAF1, RAF

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HOOIJKAAS et al. Targeting BRAFV600E in an Inducible Murine Model of Melanoma. Am J Pathol. 2012, Vol. 181(3), p. 785-94. Epub 2012 Jul 11. Abstract; pg 786, col 2, para 1, para 3, and last para; pg 787, col 1, top para and last para, and col 2, para 2 and 3; pg 790, col 2, para 1; and pg 792, col 1, para 1 and para 2	1-2
Y	US 2008/0263685 A1 (Chen) 23 October 2008 (23.10.2008), para [0008], [0011], [0024], [0048], [0049], [0100], [0113], and [0120]	1-2
A	FEIL et al. Inducible Cre mice. Methods Mol Biol. 2009, Vol. 530, p. 343-63. Entire documentation, especially Abstract; pg 344, last para; and pg 345, Fig 18.1	1-2
A	KIM et al. Tumor Self-Seeding by Circulating Cancer Cells. Cell. 2009, Vol. 139(7), p. 1315-26. Entire documentation, especially Abstract; pg 1317, col 1, last para and Table 1; pg 1319, col 1, para 1, and Fig 3; and pg 1324, col 2, para 1;	1-2
P, A	JENKINS et al. Multiple murine BRaf(V600E) melanoma cell lines with sensitivity to PLX4032. Pigment Cell Melanoma Res. 2014 May, Vol. 27(3), p. 495-501. Epub 2014 Mar 6. Entire documentation	1-2

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

16 December 2014 (16.12.2014)

Date of mailing of the international search report

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Name and mailing address of the ISA/US

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