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Defining the role of NRAS in melanoma maintenance

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Defining the Role of NRAS in Melanoma Maintenance

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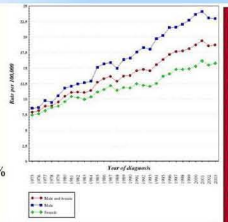
Melanoma is the most rapidly increasing malignancy among young people in the U.S.

- The incidence of melanoma has increased by more than 600 percent over the last forty years (ACS statistics www.cancer.org)
- Melanoma is the leading cause of cancer death in women aged 25-29
- 5-yr survival for advanced stages of the disease is <20%



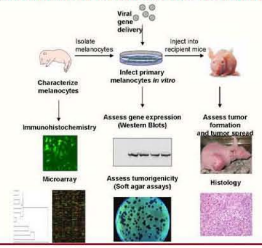
- **Goal**: to generate an in vivo model of melanoma that is resistant to inhibition of MAPK signaling in the context of mutant NRAS.
- **Hypothesis**: resistant tumors will develop that are no longer dependent on NRAS for continued growth.
- **Method**: to use a novel mouse model of melanoma, generated through the somatic introduction of NRAS-encoding avian retroviruses into transgenic mice expressing the avian retroviral receptor, TVA, specifically in melanocytes to induce melanoma in vivo. The MAPK pathway will be inhibited genetically by doxycycline mediated suppression of NRAS expression to select for resistant tumors.
- **Long-term goal**: to identify additional targets for rational combination therapy for advanced melanoma.

Molecular Analysis of Human Melanoma*

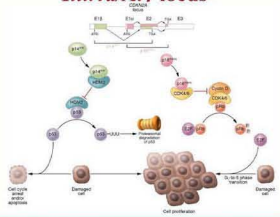
Gene/Locus	Familial(F)/Sporadic(S)	Alterations
9p21: $p16^{INK4a}p19^{ARF}$	F, S	Point mutation,deletion, promoter^{M}
p53	S	Point mutation
p27	S	Decreased expression
10q23: PTEN	S	LOH and Point mutation
11q22-23	S	LOH
NRAS	S	Point mutation
C-myc	S	Overexpression
B-RAF	S	Point Mutation
MC1R (melanocortin receptor)	S	Point mutation

*Adapted from Castellano and Parmiani Melanoma Research 1999:9;421-432

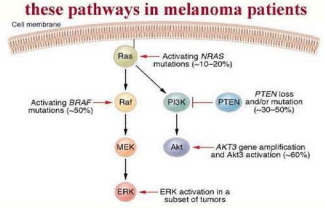
Initial Validation of Melanoma Associated Genes



Ink4a/Arf locus



Schematic of the canonical RAS effector pathways RAF-MEK-ERK and PI3K-Akt and the mutations that most often activate these pathways in melanoma patients



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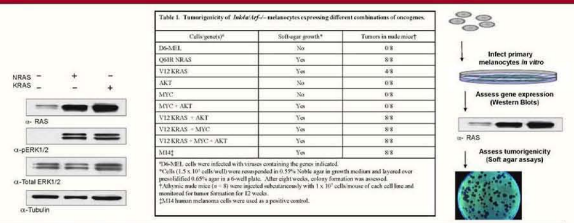


Table 1. Tumorigenicity of *Ink4a/Arf*−/− melanocytes expressing different combinations of oncogenes.

Cells (gene(s))*	Soft-agar growth*	Tumors in nude mice†
D6/MEL	No	0/8
Q61R NRAS	Yes	8/8
V12 KRAS	Yes	4/8
AKT	No	0/8
MYC	No	0/8
MYC + AKT	Yes	0/8
V12 KRAS + AKT	Yes	8/8
V12 KRAS + MYC	Yes	8/8
V12 KRAS + MYC + AKT	Yes	8/8
MH‡	Yes	8/8

*D6/MEL cells were infected with viruses containing the genes indicated.
†Cells (1.5 x 10^5 cells/well) were resuspended in 0.35% Noble agar in growth medium and layered over preestablished 0.68% agar in a 6-well plate. After eight weeks, colony formation was assessed.
‡Athymic nude mice (n = 8) were injected subcutaneously with 1 x 10^7 cells/mouse of each cell line and monitored for tumor formation for 12 weeks.
‡MH human melanoma cells were used as a positive control.

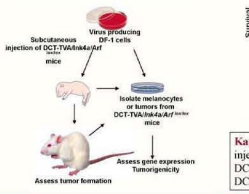
Expression of RAS in D6-MEL cells and growth in soft agar. Cell lysates from uninfected D6-MEL melanocytes (–) or cells infected with retroviruses containing either Q61R NRAS or V12 KRAS (+) were collected in SDS lysis buffer, separated on a 4-20% gradient gel and immunoblotted for total RAS expression (α-RAS), activated phosphorylated ERK p44/42 (α-pERK1/2), total ERK p44/42 expression (α-Total ERK1/2), and α-tubulin.

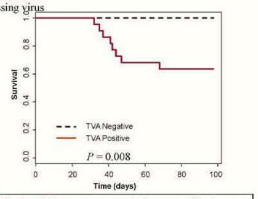
RCAS/TVA Melanoma Mouse Model System

TVA: receptor for subgroup A Avian Leukosis Virus (ALV-A)
- Confers susceptibility to ALV-A infection when introduced into cells normally resistant to infection, can be expressed in a tissue-specific manner allowing efficient gene targeting

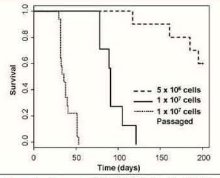
- RCAS: replication competent retroviral vector (in avian cells), derived from ALV
- In replicating mammalian cells expressing TVA, RCAS can stably integrate into the DNA and express the inserted gene at high levels
  - Replication defective in mammalian cells; does not spread in target animals. Multiple oncogenic alterations can be introduced into the same cell or animal without the cost of generating multiple strains of transgenic mice
- No endogenous viral sequences with which these vectors could recombine
- Gene expression can be reduced by RNAi using a miRNA expressing virus

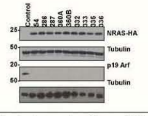
Delivery of RCASBP(A) viruses to murine Cells *in vitro* and *in vivo*



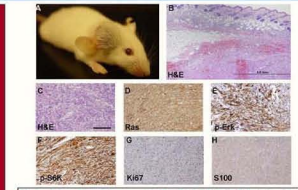


Kaplan-Meier percent survival curve. All mice were injected with *NRASQ61R* and *Cre* viruses at birth. For DCT-TVA negative mice $n = 16$ (dashed line) and for DCT-TVA-positive mice $n = 22$ (solid line).

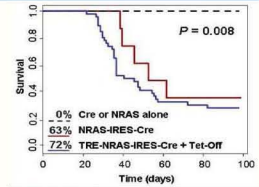




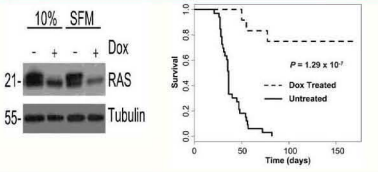
Kaplan-Meier percent survival curve. DCT-TVA/*Ink4a/Arf* $^{lox/lox}$ mice were injected subcutaneously with a cell line derived from an explanted melanoma: 5 × 106 cells $n = 10$ (large dashed line) and 1× 10^7 cells $n = 7$ (solid line). 1× 10^7 cells that had been passaged *in vivo* were re-injected $n = 17$ (small dashed line). **Protein expression in explanted melanoma cells.** Expression of $NRAS^{Q61R}$-HA from eight different mice (54, 286, 287, 360A, 360B, 332, 333, 335 and 336). The A and B designation indicates two separate tumors from the same mouse. Expression was detected with an antibody to the HA epitope tag on NRAS. D6-MEL immortal melanocytes were used as a negative control for HA expression and normal mouse astrocytes were used as a positive control for p19 Arf expression. The blots were re-probed with a-tubulin as a loading control.

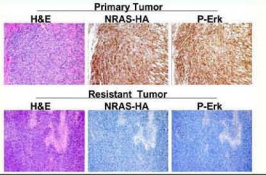


Induction of melanoma in DCT-TVA/*Ink4a/Arf lox/lox* mice. Mice were injected with *NRASQ61R* and *Cre* viruses at birth. (A) A representative mouse (age 42 days) with a tumor at the site of injection. (B-C) H&E stained tumor sections. (D) IHC for RAS. (E) IHC for phosphorylated Erk (p-Erk) (F) IHC for phosphorylated p70 S6 kinase (p-S6K). (G) IHC for Ki67. (H) IHC for S100. All sections were counterstained with hematoxylin. Scale bars represent 1.0 mm for (B) and 200 μm for (C-H).



Kaplan-Meier percent survival curves. DCT-TVA/*Ink4a/Arf* $^{lox/lox}$ mice were injected at birth with the $NRAS^{Q61R}$ and Cre viruses indicated. Dashed line: Cre alone $n = 20$ and NRAS alone $n = 15$. Black line: $NRAS^{Q61R}$ and Cre viruses in combination $n = 22$. Blue line: TRE-NRAS-IRES-Cre and Tet-off $n = 46$. Red line: NRAS–IRES-Cre ($n = 8$). The comparison between the solid lines and the dashed line (controls) was highly significant ($P = 0.008$) demonstrating that both Cre and NRAS are required for tumor formation in this context.

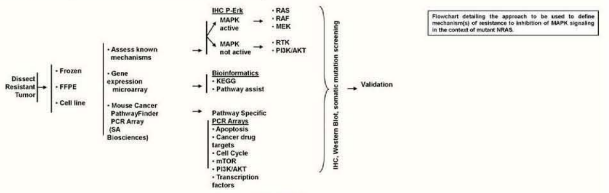




Protein expression in explanted melanoma cells. Expression of $NRAS^{Q61R}$-HA from an explanted tumor sample derived from a DCT-TVA/*Ink4a/Arf* $^{lox/lox}$ mouse injected with TRE-NRAS-IRES-Cre and Tet-off viruses. The cells were grown in the absence (-) or presence (+) of Dox for 1 week. NRAS expression was detected with a pan-RAS antibody. The virally delivered NRAS is larger than endogenous RAS due to the HA epitope tag on NRAS. The blots were re-probed with an antibody that recognizes a-tubulin as a loading control. **Kaplan-Meier percent survival curves.** DCT-TVA/*Ink4a/Arf lox/lox* mice were injected at birth with TRE-NRAS-IRES-Cre and Tet-off. Dashed line: Tumor-bearing mice were treated with Dox when tumors reached 1,000 mm3 $n = 12$ Black line: untreated $n = 33$. The comparison between the two groups was highly significant ($P = 1.29$ x 10-7) demonstrating that inhibition of NRAS expression in this context significantly increases survival. **Comparison of primary and resistant tumors.** Tumors were induced in DCT-TVA/*Ink4a/Arf lox/lox* mice by delivery of viruses containing TRE-NRAS-IRES-Cre and Tet-off. Top Panel: Representative H&E, NRAS-HA IHC, and P-Erk IHC of a primary tumor. Bottom Panel: Representative H&E, NRAS-HA IHC, and P-Erk IHC of a tumor that became resistant while on Dox treatment. As expected, virally delivered NRAS-HA expression was detected in the primary tumor but not in the resistant Dox treated tumor.

Results and Conclusions

- Both *INK4a* and *ARF*, DCT-TVA mice were crossed to *Ink4a/Arf lox/lox* mice to generate DCT-TVA;*Ink4a/Arf lox/lox* mice.
- No tumors were detected in TVA-negative mice but in 12 weeks, more than one-third of DCT-TVA;*Ink4a/Arf lox/lox* mice developed melanoma that was histologically similar to the human disease.
- Delivery of a virus in which *NRASQ61R* and *Cre* expression was linked by an internal ribosomal entry site (IRES) resulted in tumor formation in nearly two-thirds of TVA positive mice. Short term cultures from the primary tumors were established and were syngeneic with the DCT-TVA/*Ink4a/Arf lox/lox* strain, forming tumors in all recipient mice.
- 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. Using this approach in the context of Tet-off and the TRE-NRAS-IRES-Cre vector we observed tumor formation in 72% of DCT-TVA/*Ink4a/Arf lox/lox* mice.
- In contrast to tumors from mice not exposed to Dox, resistant tumors from Dox treated mice lacked exogenous NRAS expression indicating that tight Dox-mediated NRAS regulation remained intact. Because reactivation of the MAPK pathway is one possible mechanism of resistance, we used IHC to detect active phosphorylated Erk (P-Erk) in the resistant tumors. In contrast to the primary tumors, very little P-Erk expression was detected in the resistant tumors.
- These results suggest that reactivation of the MAPK pathway upstream of Erk is not the mechanism of resistance. Several other mechanisms of resistance are possible, including activation of alternative signaling pathways.



Future Directions

- Preservation and evaluation of samples to identify the mechanism(s) of resistance to either genetic or pharmacological inhibition of the MAPK pathway.
- Evaluation of resistant tumor samples to ensure suppression of virally delivered NRAS expression by both IHC of FFPE tissue and by Western blot analysis from established cell lines.
- Gene expression analysis to identify novel mechanisms that may be responsible for resistance in this context using GeneChip 12X135k microarrays.

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