CRISPR-Cas9 Engineered Isogenic Luciferase-expressing Cell Lines as *In Vitro* and *In Vivo* Models for Cancer Research

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Abstract

The CRISPR-Cas9 system provides a robust gene-editing tool for basic research in biology and for the development of disease models for translational research. The objective of this study is to use advanced technologies including CRISPR-Cas9 and bioluminescence to generate novel human cell lines for use as both *in vitro* and *in vivo* models in cancer research. Approximately 50% of melanoma patients have the $BRAF^{V600E}$ mutation and often become resistant to current BRAF inhibitors after several months of treatment. $KRAS^{G13D}$ is an acquired mutation associated with resistance to these inhibitors. In this study, CRISPR-Cas9 was used to knock-in the $KRAS^{G13D}$ point mutation into the A375 malignant melanoma cell line, which also contains the targetable $BRAF^{V600E}$ mutation. The resulting $KRAS^{G13D}$ mutant isogenic line A375, which has been validated at the genomic, transcript, and protein bio-functional levels, exhibits significant resistance to the BRAF inhibitors dabrafenib and vemurafenib when studied both in traditional 2D and 3D cell culture. Based on the *in vitro* model described above, we developed additional models for use in live-animal bioluminescence imaging by introducing a stable luciferase reporter into the isogenic A375 and $KRAS^{G13D}$ A375 cell lines. Both the relative and absolute bioluminescence signals within the cells were quantified and found to emit 4.9 x 10^5 photons/cell/sec (A375) and 3.5 x 10^5 photons/cell/sec ($KRAS^{G13D}$ A375). A subcutaneous xenograft model was utilized in this study and the *in vivo* live bioluminescence signal was quantified using the Xenogen IVIS™ imaging system to correlate tumor growth with luciferase expression. Both A375-Luc2 and $KRAS^{G13D}$ A375-Luc2 grew as subcutaneous tumors with increasing levels of bioluminescence when injected into nude mice. In addition, a portfolio of 5 human isogenic luciferase reporter cell line pairs and 18 human and mouse luciferase reporter cell lines were developed for the study of various cancer types. In conclusion, the combination of two technologies CRISPR-Cas9 technology and stable luciferase expression allows for the generation of isogenic luciferase-expressing cell lines, which are valuable tools for elucidating mechanisms involved in tumorigenesis and for studying drug responses *in vitro* and *in vivo*.

Results

CRISPR Gene Editing $KRAS^{G13D}$ Knock-in Design

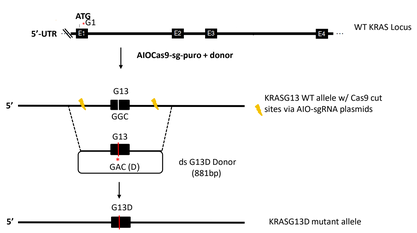


Figure 1. Gene-editing knock-in strategy for the generation of the $KRAS^{G13D}$ allele in A375 cells. A375 parental cells were transfected with the respective gRNA and knock-in donor, along with the Cas9-expressing plasmid. The transfected cells were then subjected to transient antibiotic selection and the surviving pooled cells were used for single cell sorting. The expanded single cell clones were then subjected to genotyping to identify clones harboring the desired knock-in mutations.

Verification of $KRAS^{G13D}$ Alleles at the Genomic Level

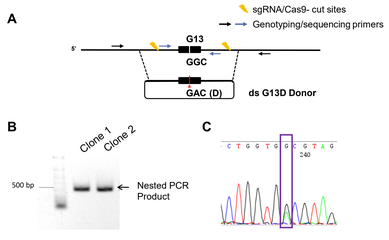


Figure 2. Confirmation of the $KRAS^{G13D}$ knock-in mutation. (A) Screening for the $KRAS^{G13D}$ point mutation integration in recovered clones was carried out using genotyping PCR primers (black) and sequencing primers (blue). (B) Gel image of the PCR products. (C) Introduction of the $KRAS^{G13D}$ point mutation into the cell line was confirmed via Sanger sequencing. Boxed in purple is the expected G>A mutation.

Verification of KRASG13D Alleles at the Transcript Level

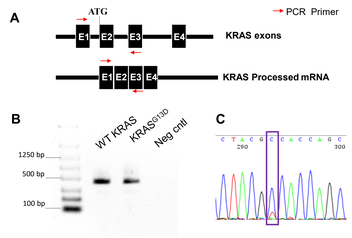


Figure 3. Confirmation of the $KRAS^{G13D}$ mutation at the transcript level. (A) Screening for the $KRAS^{G13D}$ point mutation in recovered clones was carried out via generating cDNA from cells and performing PCR (red arrows). (B) Gel image of the PCR products. (C) Introduction of the $KRAS^{G13D}$ point mutation into the cell line was confirmed via Sanger sequencing on the right. The expected point mutation is boxed in purple.

Relative Drug Response of KRAS Isogenic Clones in 2D and 3D Cell Culture

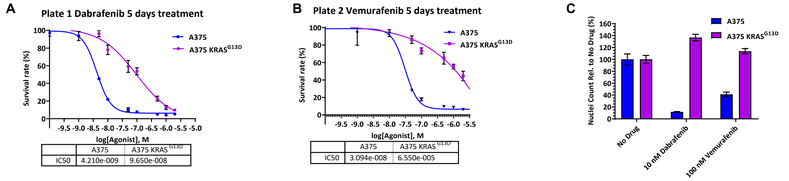
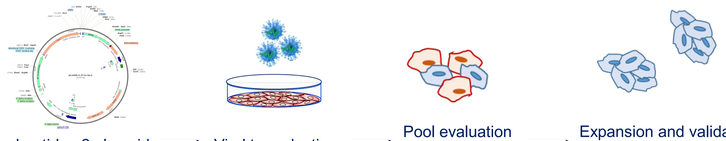


Figure 4. Dose response curves for cells treated with BRAF inhibitors dabrafenib and vemurafenib. Isogenic $KRAS^{G13D}$ cells has selective drug resistance to BRAF inhibitors dabrafenib (A) and vemurafenib (B) relative to the parental A375 cell line. Data represent mean ± SD; n=4. (C) BRAF inhibitor resistance of the $KRAS^{G13D}$ cell line in 3D cell culture was analyzed after treating 3D spheroids with either 25 nM dabrafenib or 100 nM vemurafenib and counting the nuclei in a confocal z-stack projection. Data represent mean ± SD; n=3

Workflow of Luciferase-expressing Cell Line Generation



In vitro Confirmation of Luciferase Expression

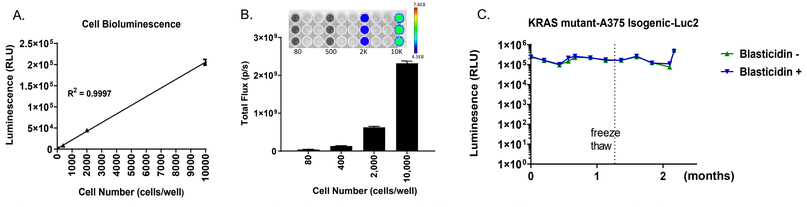


Figure 5: Linearity, activity and stability of luciferase in KRAS mutant-A375 Isogenic-Luc2. Cells were seeded in 96-well plates at the indicated number of cells per well. Bright-Glo™ (Promega) was used for the luciferase assay. (A) The luminescence was read using a luminescence plate reader, and determined to have a linear correlation of bioluminescence intensity with cell numbers. (B) The plate was imaged using a Xenogen IVIS Spectrum to quantify the photons emitted per cell. (C) To verify the luciferase expression stability, the cells were maintain in culture media with or without blasticidin, and luciferase expression was monitored twice a week for over 2 months using the luciferase assay.

Confirmation of Cell Morphology

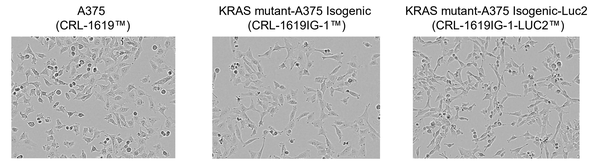


Figure 6: Morphology of A375, $KRAS^{G13D}$ A375, and $KRAS^{G13D}$ A375-Luc2. Cell were maintained according to ATCC recommended culture conditions. Cell morphology was observed under microscopy and images were captured by digital camera.

Tumor Model and *In Vivo* Bioluminescence Imaging

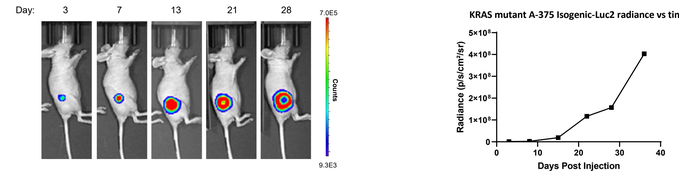


Figure 7: *In vivo* detection of luciferase activity of $KRAS^{G13D}$-A375 Isogenic-Luc2. $KRAS^{G13D}$-A375 Isogenic-Luc2 cells (3 x 10^6) were injected subcutaneously into the dorsal region near the thigh of female nude mice. Tumor growth was monitored weekly using a Xenogen IVIS Spectrum. *In vivo* bioluminescence imaging demonstrated the progression of tumors.

A List of ATCC Luciferase-expressing Cell Lines

Luciferase cell line	ATCC© No.	Species	Tissue/disease	Photons/ cell/sec*	*In vivo* model
B16-F1-Luc2	CRL-6323-LUC2™	Mouse	Melanoma	336,669	Syngeneic
B16-F10-Luc2	CRL-6475-LUC2™	Mouse	Melanoma	207,727	Syngeneic
4T1-Luc2	CRL-2539-LUC2™	Mouse	Breast cancer	2,564,375	Syngeneic
EL4-Luc2	TIB-39-LUC2™	Mouse	Lymphoma	277,331	Syngeneic
LL/2-Luc2	CRL-1642-LUC2™	Mouse	Lung cancer	29,104	Syngeneic
A549-Luc2	CCL-185-LUC2™	Human	Lung cancer	22,325,000	Xenograft
EML4-ALK A549-Luc2	CCL-185IG-LUC2™	Human	Lung cancer	19,356,250	Xenograft
A375-Luc2	CRL-1619-LUC2™	Human	Melanoma	491,308	Xenograft
KRAS G13D A375-Luc2	CRL-1619IG-1-LUC2™	Human	Melanoma	347,619	Xenograft
NRAS Q61K A375-Luc2	CRL-1619IG-2-LUC2™	Human	Melanoma	1,192,822	Xenograft
A-431-Luc2	CRL-1555-LUC2™	Human	Epidermoid carcinoma	603,085	Xenograft
TF-1-Luc2	CRL-2003-LUC2™	Human	Leukemia	112,759	Xenograft
IDH2 mutant TF-1-Luc2	CRL-2003IG-LUC2™	Human	Leukemia	64,768	Xenograft
HL-60-Luc2	CCL-240-LUC2™	Human	Leukemia	123,330	Xenograft
U-87 MG-Luc2	HTB-14-LUC2™	Human	Glioblastoma	8,601,250	Xenograft
IDH1 mutant U-87MG-Luc2	HTB-14IG-LUC2™	Human	Glioblastoma	8,165,625	Xenograft
HT-1080-Luc2	CCL-121-LUC2™	Human	Fibrosarcoma	2,125,000	Xenograft
HCT 116-Luc2	CCL-247-LUC2™	Human	Colon cancer	2,770,000	Xenograft
PANC-1-Luc2	CRL-1469-LUC2™	Human	Pancreatic cancer	2,175,533	Xenograft
PC-3-Luc2	CRL-1435-LUC2™	Human	Prostate cancer	397,640	Xenograft
LNCaP clone FGC-Luc2	CRL-1740-LUC2™	Human	Prostate cancer	1,187,513	Xenograft
U-2 OS-Luc2	HTB-96-LUC2™	Human	Osteosarcoma	990,500	Xenograft
AGS-Luc2	CRL-1739-LUC2™	Human	Gastric adenocarcinoma	1,847,925	Xenograft
HCT-15-Luc2	CCL-225-LUC2™	Human	Colon cancer	1,894,946	Xenograft
FaDu-Luc2	HTB-43-LUC2™	Human	Pharyngeal carcinoma	2,297,406	Xenograft
MCF7-Luc2	HTB-22-LUC2™	Human	Breast cancer	2,180,843	Xenograft
SW480-Luc2	CCL-228-LUC2™	Human	Colon cancer	7,161,294	Xenograft

*The *in vitro* bioluminescence was quantified by Xenogen IVIS Spectrum (subject to imaging and cell culture condition).

Conclusion

We have successfully created a $KRAS^{G13D}$ A375 melanoma isogenic cell line via CRISPR/Cas9 gene editing, and further stably expressed the luciferase gene within the edited cells. Used together with the A375 parental cell line, the $KRAS^{G13D}$ A375 isogenic line and $KRAS^{G13D}$ A375-Luc2 luciferase-expressing cell line are valuable tools for elucidating mechanisms involved in KRAS-associated melanoma drug resistance, studying melanoma tumors *in vivo*, and screening anti-cancer compounds for drug discovery and development. A portfolio of luciferase reporter cell lines have been developed for the study of various cancer types.

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