EDITING RUNX2 KO IN B16 MELANOMA CELLS WITH CRISPR-CAS9 AS A POTENTIAL STRATEGY TO ENHANCE IN VIVO TUMOR RESPONSE TO THERAPEUTIC APPROACHES

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BACKGROUND

Cutaneous melanoma (CM) is a highly heterogeneous and aggressive skin cancer with unpredictable clinical behavior. Despite recent progress, the search for effective therapeutic strategies is still ongoing. RUNX2 is a transcription factor implicated in key oncogenic processes, including apoptosis, epithelial–mesenchymal transition (EMT), and cancer stemness. It modulates major signaling pathways such as WNT, NOTCH, BMP, and RAS[1, 2], contributing to tumor progression and resistance. This project presents a workflow for the CRISPR/Cas9-mediated generation of RUNX2 knockout (KO) B16 mouse melanoma cells, with the goal of developing a model to investigate RUNX2's role in therapy resistance and explore novel treatment approaches in CM.

WORKFLOW

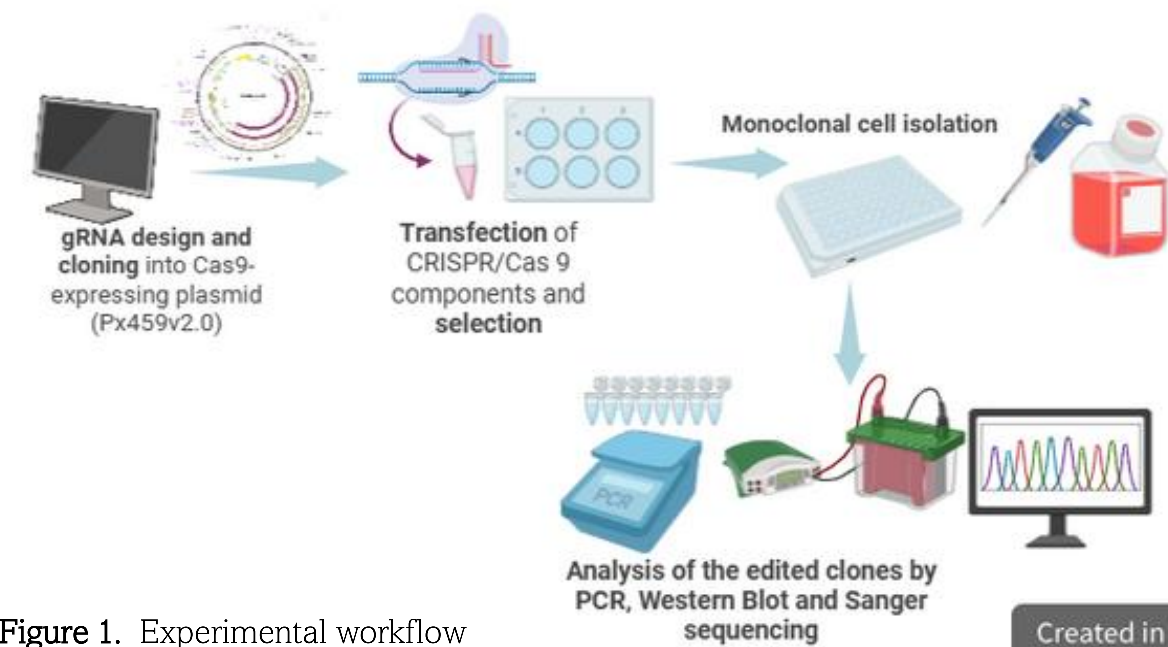

Figure 1. Experimental workflow

MATERIALS AND METHODS

Two gRNAs targeting exon 4 (gRNA1: CCCATCTGGTACCTCTCCGA; gRNA2: AAATCTCAGATCGTTGAACC) of RUNX2 were designed and cloned into px459v2.0 plasmid. The B16 mouse melanoma cells were transfected with 2.5 μg of plasmid DNA by Lipofectamine 3000 in DMEM (w/o antibiotics). After one day, cells were selected with complete DMEM containing 1μg/ml puromycin for six days. Cells were further subjected to monoclonal cell isolation by seeding 0.3 cells/well into 96-well plates. Genomic DNA was extracted from isolated clones, PCR amplified with primers spanning the gRNA-targeting region (Primer FW: AGGGAAGGAGATGCTACTTCG; Primer REV: ATTCCCGCAGACAGCCTAAC). Amplicons were Sanger sequenced with forward primer and editing results were analysed by DECODR v3.0 (https://decodr.org/). For Western blot detection of RUNX2, a rabbit monoclonal anti-RUNX2 antibody (D1H7, Cell Signaling #8486) at 1:1000 dilution was used. β-actin was used as housekeeping control.

RESULTS

Clones with 108 bp deletion, as shown by PCR (Fig.2) were selected for Sanger sequencing and Western blot analysis.

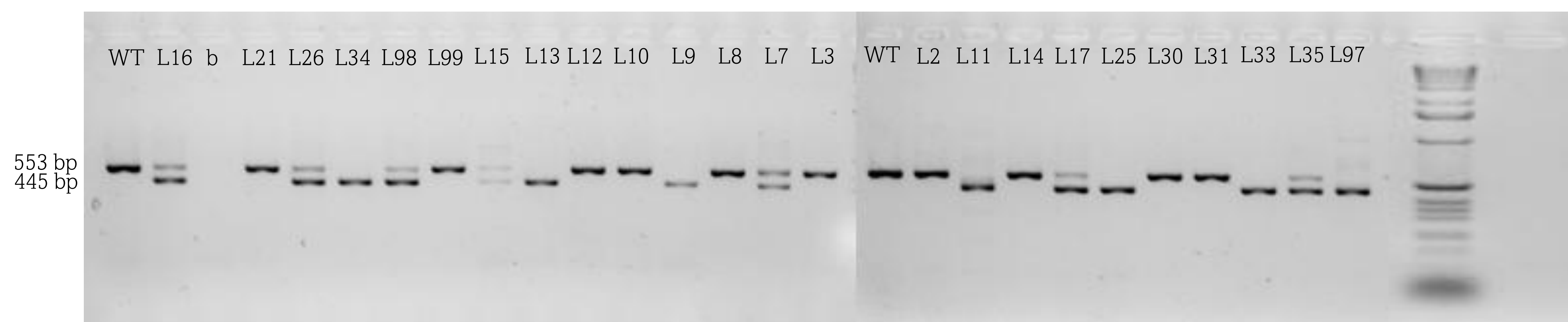

Figure 2. PCR amplification products resolved on a 2% agarose gel. DNA fragments were separated to assess the editing events in B16 clones. 1Kb Plus DNA ladder was used as a molecular weight marker.

Three clones (L13, L25, L34) were homozygous, each carrying a -108 bp in-frame deletion within the *RUNX2* Runt domain. Clone L17 was heterozygous, showing a combination of a -108 bp deletion and a small insertion (+2+1), both in-frame. Clones L9, L16, and L26 were heterozygous, harboring alleles with both in-frame and out-of-frame deletions in varying proportions (Tab.1).

Figure 3. The image below reports an example of sequence alignment between WT and edited clones presenting the 108 bp deletion

clone name	editing event	in/out-of-frame	homozyg. /heterozyg.
L13	-108	in-frame	homozygous
L25	-108	in-frame	homozygous
L34	-108	in-frame	homozygous
L17	-108; +2+1	in-frame	heterozygous
L9	-108; -107	In-frame (65.5%); out-of-frame (34.5%)	heterozygous
L16	-108; +1	In-frame (85.4%); out-of-frame (14.6%)	heterozygous
L26	-108; -2	In-frame (84.1%); out-of-frame (15.9%)	heterozygous

Table 1. Sequencing analysis of seven B16 clones revealed distinct editing profiles

These seven clones showed no RUNX2 protein expression by Western Blot. (Fig. 4, Fig.5).

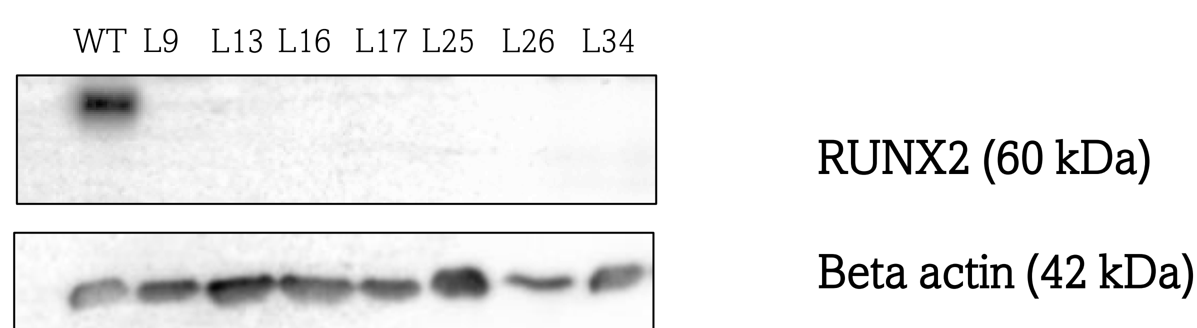

Figure 4. Western blot of seven B16 clones

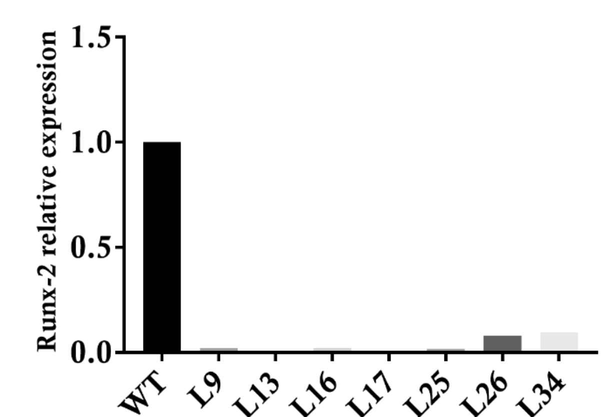

Figure 5. Runx2 relative expression in Western blot

CONCLUSIONS

Our study outlines a robust workflow for generating genetically engineered B16 melanoma clones, a valuable tool for exploring the biological functions of RUNX2 in syngeneic mouse models. Interestingly, several clones harboring a 108 bp in-frame deletion within the Runt domain exhibited no detectable RUNX2 protein expression. This suggests that deletion of the Runt domain may induce protein instability and degradation, or alternatively, may interfere with antibody recognition, warranting further investigation with different detection strategies. In mouse cells, the generated clones carry targeted deletions of the Runt domain, a critical functional region of the protein. These models provide a promising starting point for dissecting RUNX2-dependent mechanisms in melanoma biology and therapy resistance in *in vivo* mice models.

REFERENCES

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[2]Dalle Carbonare L, Minoia A, Vareschi A, et al. Exploring the Interplay of RUNX2 and CXCR4 in Melanoma Progression. Cells. 2024;13(5):408. Published 2024 Feb 27. doi:10.3390/cells13050408

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