Efficient And Versatile CRISPR/Cas9 Platform Facilitates Precise Genetic Modification In Mammalian Cell Lines

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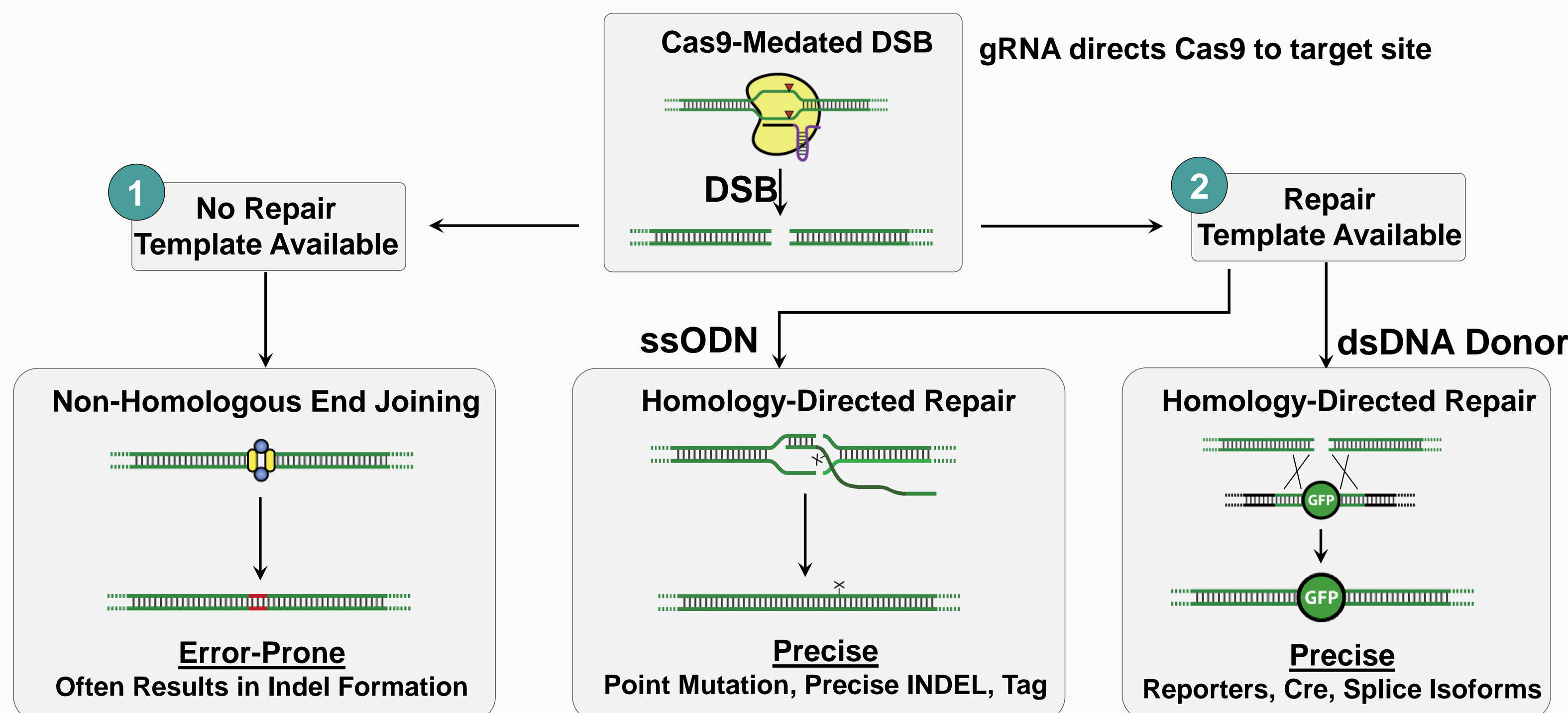
Introduction

- CRISPR/Cas9 technology has revolutionized genome engineering and has emerged as a reliable and versatile tool for genome editing in various organisms and cells.
- Precise mutations, gene disruption, mutation corrections and insertions has enabled better understanding of the genetics and mechanism of diseases.
- CRISPR gene editing in cell lines, the workhorse of preclinical and biomedical research, enables the generation of unlimited *in vitro* models with precise gene modifications and advanced gene expression design that are physiologically relevant.
- To accurately model diseases, as well as for precise single or bi-allelic manipulations, there is a strong dependency on homology directed repair (HDR) and gRNA selection strategies.
- Here, we present data demonstrating the versatility of the technology in editing cell lines:
  - Editing a variety of mammalian cell lines including hard-to-transfect blood lineage cells and stem cells
  - Case studies highlighting complex modifications such as double gene knockout, large knock-in of transgenes into specific endogenous locus and safe harbor locus; gRNA selection strategies to ensure specific mono- and bi-allelic modifications.
  - Efficiency of generating knock-in, knockout and point mutations in various cell lines, including pluripotent stem cells.

Cas9 Mediated Genome Engineering



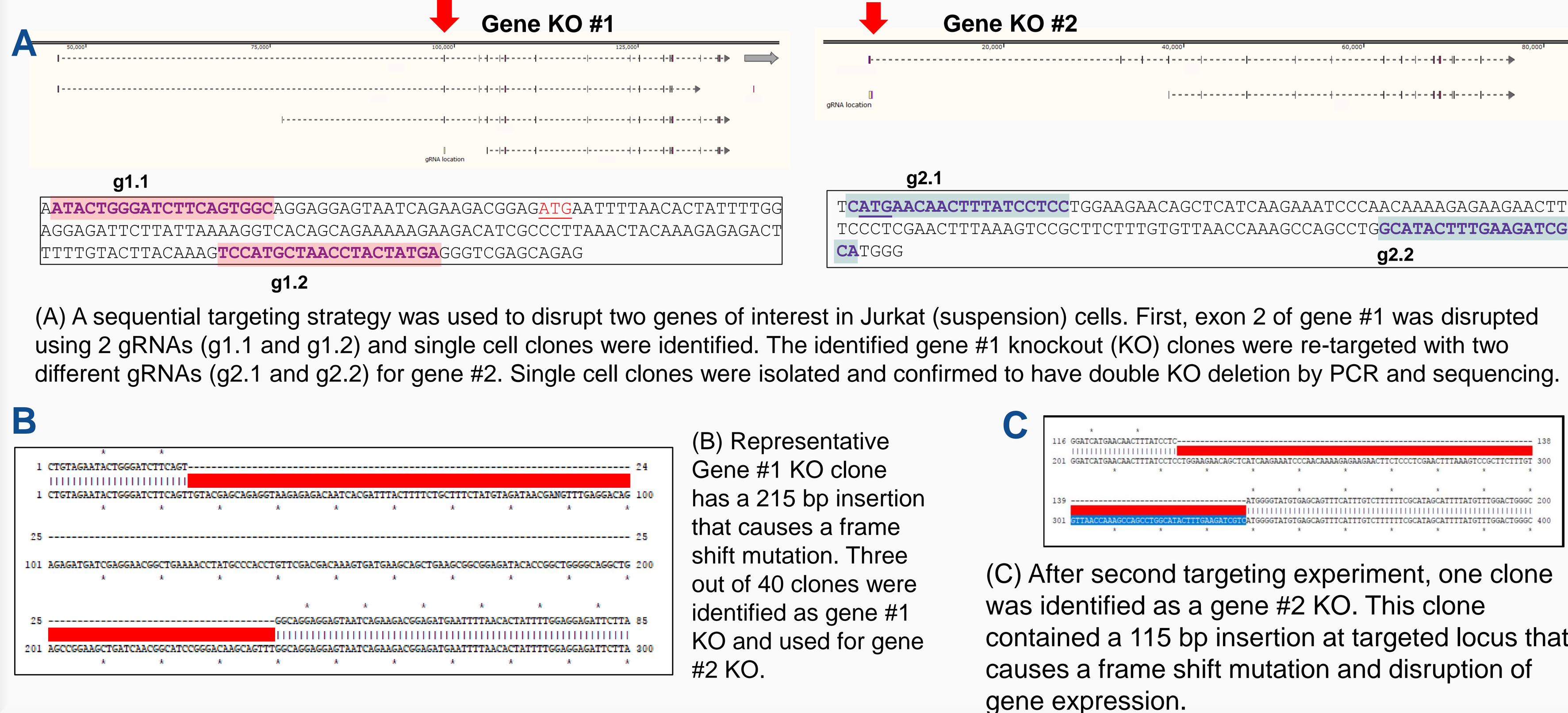
Cell Lines Amenable to CRISPR/Cas9 Gene Editing*

Human			
Cancer Cells	786-O (Renal carcinoma) A-375 (melanoma) Breast cancer cells CHLA-10 (Neuro-ectodermal) DLD-1 (Colon cancer) Gist-T1 (Gastrointestinal tumor) HCT 116 (Colon cancer) HEK293 (Kidney) HEK293T (Kidney) HEK293Braf HeLa (Cervical cancer) HepG2 (Liver sarcoma) HT1080 (Fibrosarcoma) HT29 (Colon cancer) KBM-7 (Myeloid leukemia) KM12-Luc (Colon carcinoma) LnCaP (Prostrate cancer) MDA-MB231 (Breast cancer) NCI-H2228 (Adenocarcinoma0 RKO (Colon cancer) TC32 (neuro-ectodermal cancer) SH-SY5Y (Glioblastoma)	Epithelial Cells	A-549 (lung) BEAS-2B (Bronchial) BT-474 (mammary gland) Follicular thyroid cell line HaCaT (Keratinocyte) HBE (Bronchial) Huh7 (Liver) MCF-10A (mammary gland) OCCM-30 (Cementoblats) RPE-1 (retinal epithelium) SK-MEL-31 (skin epithelium) U-2 OS (bone)
		Blood-derived Cells	BCWM-1 (Bone marrow) H929 (Bone marrow) Jurkat (T lymphocyte) K562 (Erythroleukemia) KHYG-1 (NK cell leukemia) MM.1s (B lymphocyte) MWCL-1 (lymphoma) NCI-H929 (Bone marrow) TF-1 (Leukemia) T2 cells (Lymphocyte)
Stem Cells	iPSC (Healthy and various disease models) Human multipotent adult progenitor stem cells Neural stem cells (Adult and fetal) Limbal stem cells	Fibroblasts & Adipocytes	Immortalized fibroblasts UCI 162 SGBS (preadipocyte/adipocyte)

Other Species	
Mouse	3617 (Adenocarcinoma) 4T1 (Mammary cancer) cTEC (Cortical thymic epithelial) C2C12 (Myoblast) GD25 (Fibroblasts) Embryonic stem cells (mESCs; C57BL/6) Induced pluripotent cells (miPSC) OCCM (Cementoblasts) Tonsil epithelial cells
Rat	CWSV-1 Cells (Hepatocytes) DAC8 embryonic stem cells (Dark agouti male) Immortalized Keratinocytes L77 embryonic stem cells (Fisher) PCC13 (Follicular thyroid cells) Chondrosarcoma cells
Primate	COS-7 (Kidney cells) GL37 (Kidney cells) Macaque ES cells
Hamster	CHO-K1 (Ovarian) CHO-S (Ovarian)
Insect & Others	Mosquito cells Wolf keratinocytes

** Selected list of cell lines successfully modified in Applied StemCell's Cell Biology Lab; CRISPR cell line editing is not limited to these cell lines.*

Generation of Double Gene Knockout in Hard-to-Engineer Jurkat Cell Lines

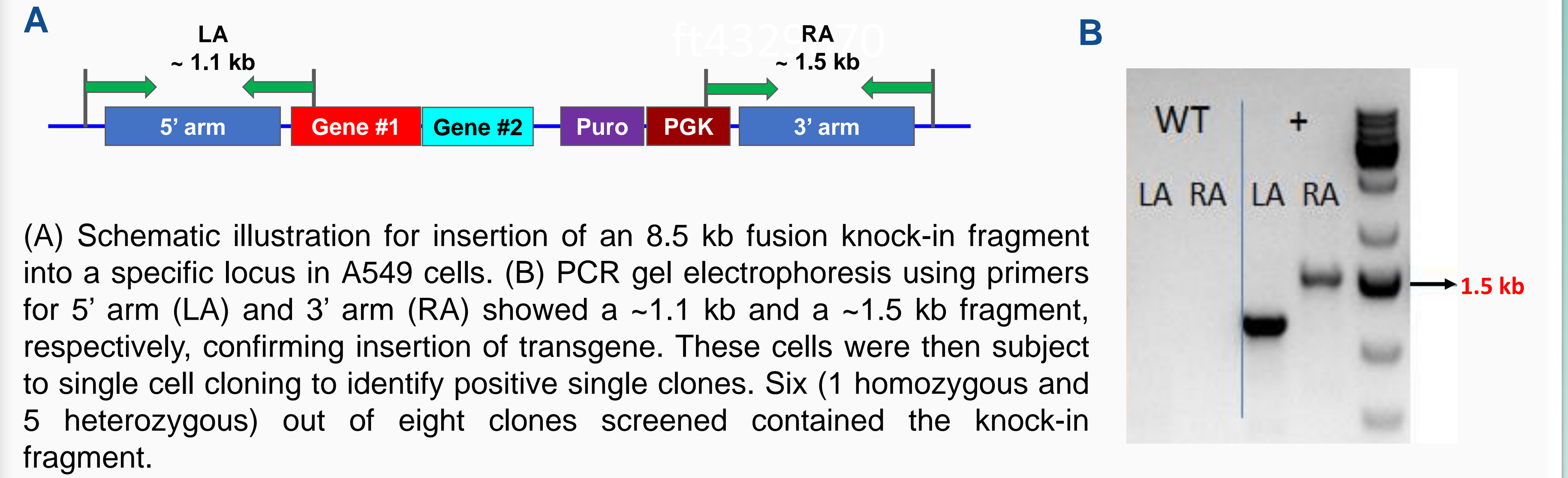


(A) A sequential targeting strategy was used to disrupt two genes of interest in Jurkat (suspension) cells. First, exon 2 of gene #1 was disrupted using 2 gRNAs (g1.1 and g1.2) and single cell clones were identified. The identified gene #1 knockout (KO) clones were re-targeted with two different gRNAs (g2.1 and g2.2) for gene #2. Single cell clones were isolated and confirmed to have double KO deletion by PCR and sequencing.

(B) Representative Gene #1 KO clone has a 215 bp insertion that causes a frame shift mutation. Three out of 40 clones were identified as gene #1 KO and used for gene #2 KO.

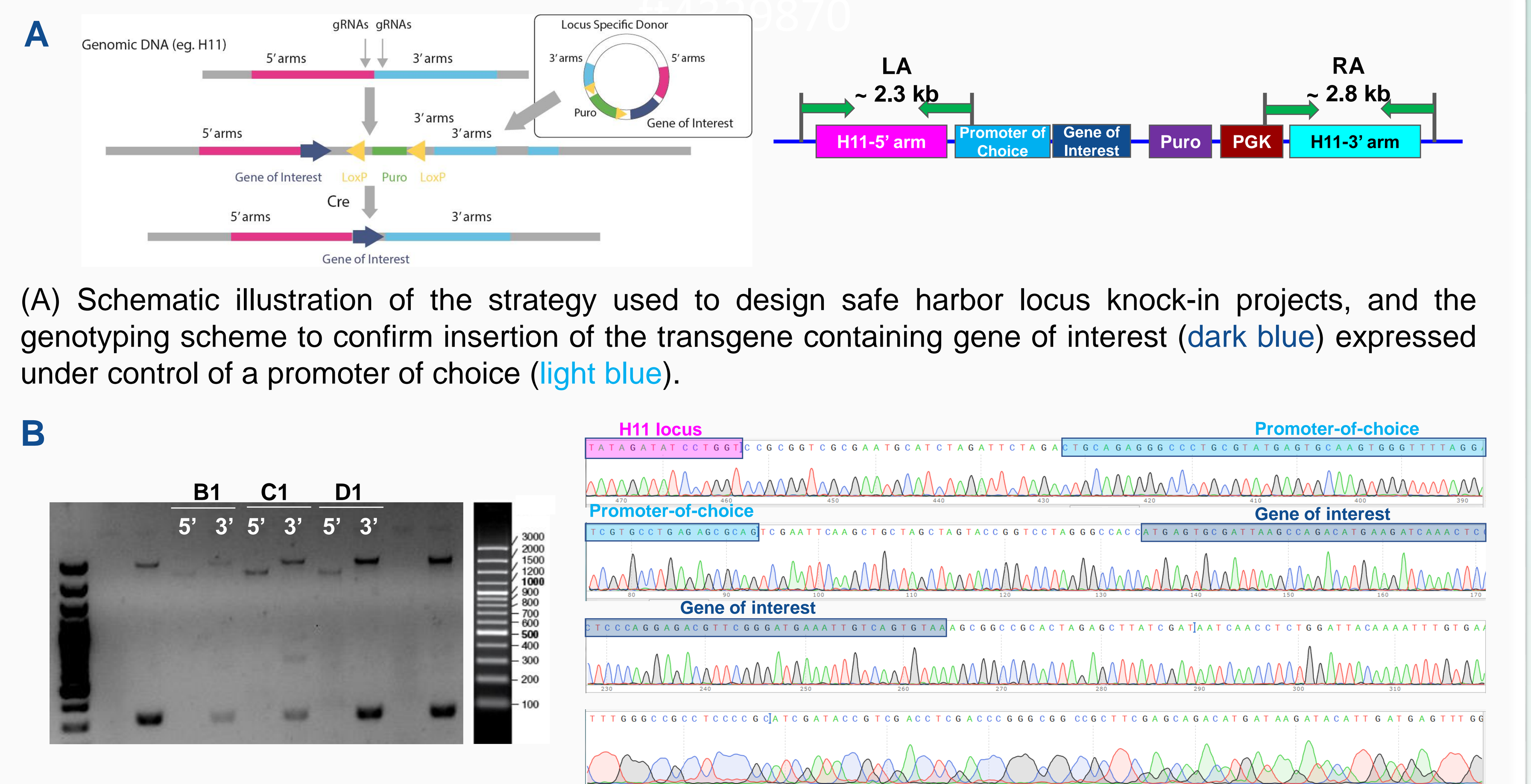
(C) After second targeting experiment, one clone was identified as a gene #2 KO. This clone contained a 115 bp insertion at targeted locus that causes a frame shift mutation and disruption of gene expression.

Knock-in of a Large "Fusion" Transgene into a Specific Locus in a Human Cancer Cell Line



(A) Schematic illustration for insertion of an 8.5 kb fusion knock-in fragment into a specific locus in A549 cells. (B) PCR gel electrophoresis using primers for 5' arm (LA) and 3' arm (RA) showed a ~1.1 kb and a ~1.5 kb fragment, respectively, confirming insertion of transgene. These cells were then subject to single cell cloning to identify positive single clones. Six (1 homozygous and 5 heterozygous) out of eight clones screened contained the knock-in fragment.

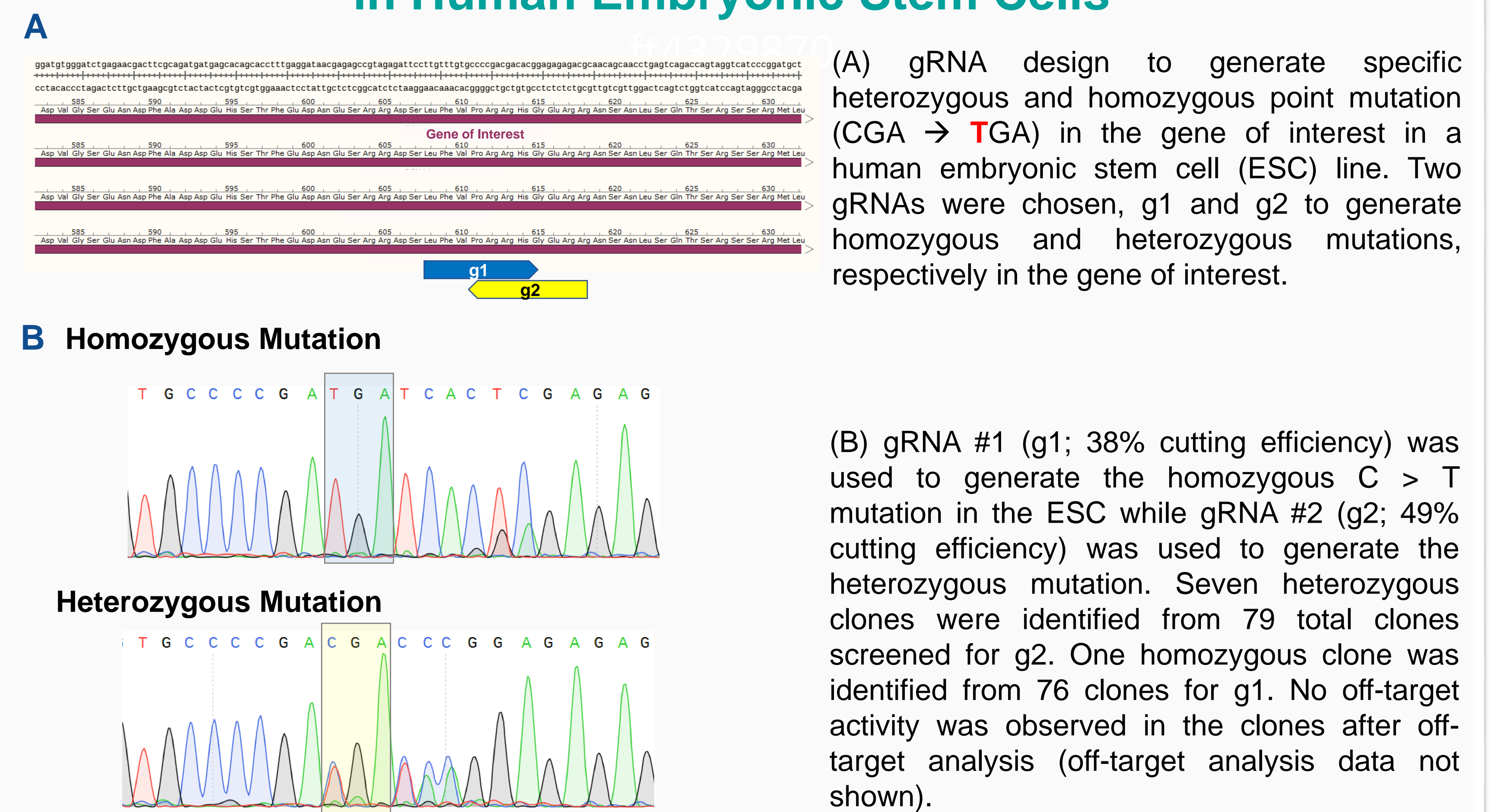
Large Transgene into hH11 Safe Harbor Locus in Induced Pluripotent Stem Cells



(A) Schematic illustration of the strategy used to design safe harbor locus knock-in projects, and the genotyping scheme to confirm insertion of the transgene containing gene of interest (dark blue) expressed under control of a promoter of choice (light blue).

(B) PCR gel image of clones containing the transgene inserted into the hH11 locus in human induced pluripotent stem cells (iPSCs). The 5' and 3' junction PCRs at hH11 locus showed a 2.3 kb and 2.8 kb fragment, respectively. Sequence chromatogram of a representative clone confirmed the presence of both promoter-of-choice and gene of interest at the right locus. Our gene insertion strategy generated 6 heterozygous clones among a total of 30 screened clones containing the knock-in fragment at the H11 locus.

Targeted Heterozygous and Homozygous Point Mutations in Human Embryonic Stem Cells



(A) gRNA design to generate specific heterozygous and homozygous point mutation (CGA → TGA) in the gene of interest in a human embryonic stem cell (ESC) line. Two gRNAs were chosen, g1 and g2 to generate homozygous and heterozygous mutations, respectively in the gene of interest.

(B) gRNA #1 (g1; 38% cutting efficiency) was used to generate the homozygous C > T mutation in the ESC while gRNA #2 (g2; 49% cutting efficiency) was used to generate the heterozygous mutation. Seven heterozygous clones were identified from 79 total clones screened for g2. One homozygous clone was identified from 76 clones for g1. No off-target activity was observed in the clones after off-target analysis (off-target analysis data not shown).

Efficiency of CRISPR-Mediated Genome Editing in Cell Lines

Types of Projects	% Efficiency Homozygous	% Efficiency Heterozygous	% Total Efficiency	# of Projects
Knock-in	13.51	40.91	40.35	17-28
Knockout	14.38	Not Applicable	14.38	23
Point Mutation	8.73	6.69	10.99	19-37

The efficiency of CRISPR-mediated gene modifications was calculated as the number of heterozygous or homozygous clones normalized to the total number of clones screened. The total efficiency denotes the total number of clones (heterozygous and homozygous clones) identified versus the total number of clones screened.

Conclusions

- CRISPR/Cas9 technology is a versatile gene editing technology and can be used for modifying a variety of cell lines including hard-to-transfect blood lineage cells such as Jurkat, bone marrow cell lines, and pluripotent stem cells.
- CRISPR can also be used to efficiently and precisely modify genes: knockout/ disruption, point mutations and transgene knock-in. As well, with proper gRNA and targeting design, off-target activity can be avoided or minimized.
- CRISPR technology also enables the generation of complex genetically engineered cell line models, double knockout, and inducible gene expression models, in addition to being easily manipulated to generate specific bi-allelic and mono-allelic clones.
- In summary, CRSIPR gene editing in cell lines provides an unlimited source of physiologically relevant *in vitro* models for basic research, drug target discovery and initial stage drug screening, and has tremendous potential for cell replacement therapeutic applications.

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