Development of Cellular Model with *CALR* Gene Mutations Using Genome Editing Tool CRISPR/Cas9 System

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Objective

BCR/ABL negative classic myeloproliferative neoplasms (MPN) include primary myelofibrosis, polycythemia vera and essential thrombocythemia. Mutated calreticulin (*CALR*) was discovered to be involved in MPN pathogenesis. Although *CALR* involvement in MPN pathogenesis is an area of active research, there is no commercially available cell line with specific *CALR* mutations, characteristic to MPN. CRISPR/Cas9 system is one of the newest and the most advanced methods for cell genome editing. We hypothesized that CRISPR/Cas9 system is appropriate tool for *CALR 52bp del* and *5bp ins* mutations initiation in cell culture.

The aim of this study was to initiate *CALR* gene mutations that are specific to MPN pathogenesis, and provide electroporation settings to deliver designed constructs into cell culture model.

Methods

After selection of potential DNA targets in genomic DNA, corresponding tabs were cloned into vector (pSpCas9(BB)-2A-Puro (PX459) V2.0, Addgene plasmid #62988) that is optimized for Cas9 and RNA-guided expression in eukaryotic cells. The effectivity of CRISPR/Cas9 system in *CALR* gene mutations (*52bp del* and *5bp ins*) induction was assessed in HEK293T cells. Designed plasmids were chemically transfected ("*TurboFect*" Transfection Reagent, Thermo Scientific) into HEK293T and after 48 hours the analysis was done (Figure 1). To assess the effectiveness of double-stranded break induction by Cas9, *T7E1* nuclease method was used (T7 Endonuclease I, New England Biolabs). This method enables finding the deletions and insertions that occur after NHEJ (non-homologous end joining). The *T7EI* results were quantified using the *ImageJ* software. The editing efficiency was calculated using the following formula: Indel (%)=(1-(1-B+C/A+B+C))$^{1/2}$)x100, where, A = uncut DNA, B,C = digestion products.

Furthermore, we provided electroporation settings to balance highest possible transfection efficiency with hematological UT-7 cell line viability and growth post-electroporation. UT-7 cells were transfected with green fluorescent protein plasmid (pMaxGFP) by electroporation at various conditions using the electroporation system BTX T820 (BTX, San Diego, Calif). The transfection efficiency was determined by the counting pGFP positive cells 48 hours after post-transfection.

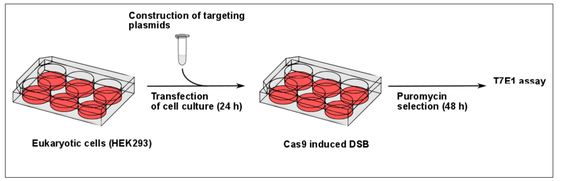

Figure 1. *Experimental design. The transfection protocol used in this study is shown.*

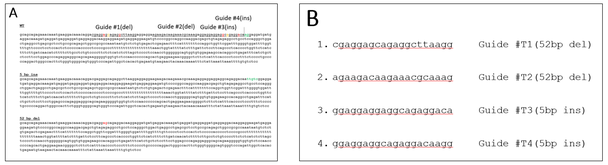

Figure 2. (A, B) *The target sites used in this study targeting human CALR gene (exon 9) are shown with corresponding PAM sequences.*

Results

Widely used type II CRISPR-Cas9 system derives from *Streptococcus pyogenes*. It is known that for DNA cleavage, the corresponding Cas9 protein depends on binding to its cognate 5'-NGG protospacer adjacent motif (PAM). We screened human *CALR* gene to identify matching target sites that respect the PAM requirements. Four sites (two for each mutation type) with lowest off-target rate effect were found using online tool available at ZHANG LAB website (Figure 2).

After selection of potential DNA targets in genomic DNA, corresponding tabs were cloned into vector pSpCas9(BB)-2A-Puro (PX459) V2.0. To generate the plasmids containing *CALR 52bp del* and *5bp ins* were used standard procedures and some specific modifications were applied.

The selected constructs were used to introduce *CALR 52bp del* and *5bp ins* in relatively easy to transfect HEK293T cell line and after 48 hours the *T7EI* analysis was done. This method enabled finding *CALR 52bp del* and *5bp ins* mutations that occurred after NHEJ in HEK293T cells.*T7EI* activity was detected only in samples, in which catalytically active Cas9 proteins were expressed, confirming that Cas9 proteins have their DNA-cleavage properties. Based on this, it was possible to choose optimal tabs which effectively guide Cas9 complex to cut genomic DNA at the selected location. According to our results, only in samples with *CALR 52bp del* significant *T7EI* activity was detected and reached 28-30% (Figure 3). In samples with induced *5bp ins* the amount of cleavage was below the 5% detection limit of the assay and was omitted (Figure 4).

Because hematological UT-7 cells are recalcitrant to transfection with standard liposomal or calcium phosphate methods, we have used electroporation as a technique for delivery of plasmid into UT-7 cell line. Pilot experiments was carried out with GFP constructs in order for electroporation settings optimization. The best results, represented by 76.05±1.20% GFP-positive cells, were obtained in the combination of 1.4kV/cm (electric field strength), 100μs (duration) and 3HV (high voltage pulses) regimen. 100 μg/ml of the pMaxGFP was appropriate concentration for carried experiments. Cell viability under these conditions was 22.60 ±0.05% (Figure 4).

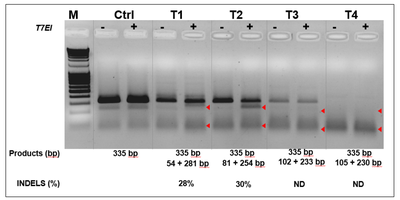

Figure 3. *Cas9 cleavage activity. HEK293T cells were transfected with plasmid expressing Cas9 and its corresponding gRNA. The cleavage activity was assessed via T7 endonuclease I. The example gel pictures show a T7EI assay performed at CALR. The arrow indicates the expected position of the cleaved product. Numbers at the bottom show the average percentage of modified alleles (n=3). Abbreviations: M: DNA ladder (100 bp); Ctrl: untreated genomic DNA from the same HEK293T cells used for transfection was used as a negative control, where only 335 bp band can be appreciated; T1-T4 – guide sequences; T7EI + or -, treated or not with the T7EI endonuclease; ND: not detected.*

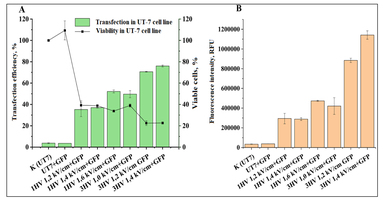

Figure 4. *(A) In vitro transfection efficiency and cell viability in UT-7 cells at 48 h post-transfection. Fluorescence based evaluation of the percentage of GFP-positive cells (bars) and percentage of viable cells (lines) at different electroporation regimen. Values represent mean ±SD (n=3). (B) Fluorescence intensity on UT-7 cell line transfected by pGFP. Graph represent mean±SD (n=3). Abbreviations: K: control UT-7 cell line (no electroporation, no added GFP); GFP: green fluorescent protein plasmid; HV: high voltage; kV/cm: kilovolt/centimeter.*

Conclusions

CALR gene mutations initiation with CRISPR/Cas9 system was appropriate. Construct with *CALR 52bp del* was designed properly and can be transfected in cell culture relatively easily. Despite successful construction of plasmid containing *CALR 5bp ins*, further experiments with corresponding plasmid delivery into cell culture are warranted. Additionally, there is a need of improving UT-7 cell line viability during electrotransfection.

Key words

Calreticulin, myeloproliferative neoplasms, CRISPR/Cas9, UT-7 cell line, electroporation