Design of CRISPR/Cas9 system components for visualization of specific genomic editing sites in tumor cell lines

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Various strategies for imaging living cells altered by genome editing with CRISPR/Cas9 system have been implemented since discovery. Catalytically inactive Cas9 (dCas9) has multiple applications with the most useful being the activation/repression of transcription. The use of dCas9 may assist in mapping of genes within chromatin structure at the level of individual cells and intact tissue. The goal of our study was in developing and testing of dCas9 orthologs expressed under an inducible promoter to ensure regulated expression of chimeras in cells and in animal models of cancer.

All chimeric constructs were obtained using the third-generation lentiviral vector FU-tet-o-hOct4 (Addgene Plasmid # 19778, USA) as a precursor, in which gene expression is regulated by doxycycline-inducible Tet-On system. Two nuclear localization sequences (NLS) - fused catalytically inactive mutant dCas9 orthologs from Streptococcus thermophilus (St) and Neisseria meningitides (Nm) as well as fluorescent protein (FP) marker were chosen at the ratio of 1:1. The pair of FPs: EGFP and mCherry were selected due to their ability to engage in Forster resonance energy transfer (FRET)as a donor and acceptor, respectively. FU-tet-o-linker construct was designed to carry a polylinker with unique restriction sites for dCas9 and FP orthologs in various combinations. FU-Tet-o-StdCas9-EGFP and FU-Tet-o-NmdCas9-mCherry were engineered by following standard protocols to enable lentiviral particle production (Fig.1-2).

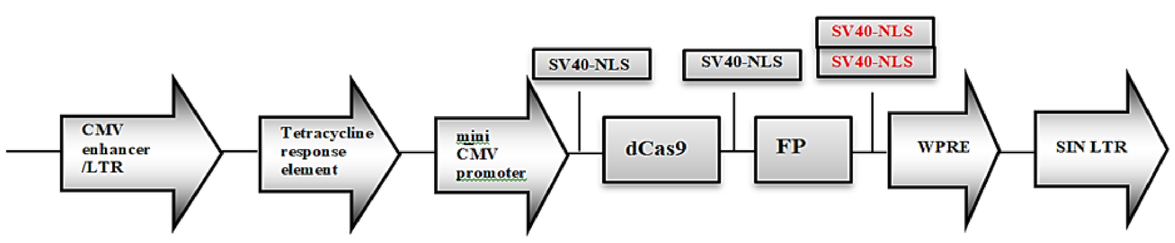

Figure 1 - Scheme of a genetically engineered construct St dCas9-EGFP and NmdCas9-mCherry

Figure 2 - Scheme of a genetically engineered construct pLego-St sgRNA-T and pLego-St sgRNA-T

Results: 1. Cells expressing St dCas9-EGFP and Nm dCas9-mCherry showed normal morphology with predominantly nuclear dual fluorescence. Before FP chimera induction with Dox there was no fluorescent signal detectible in cells proving tight regulation of chimera expression. FP expression was observed in vivo 1 day post induction and fluorescent signal was progressively decreasing during the course of 4-5 days following the withdrawal of Dox (Fig.3).

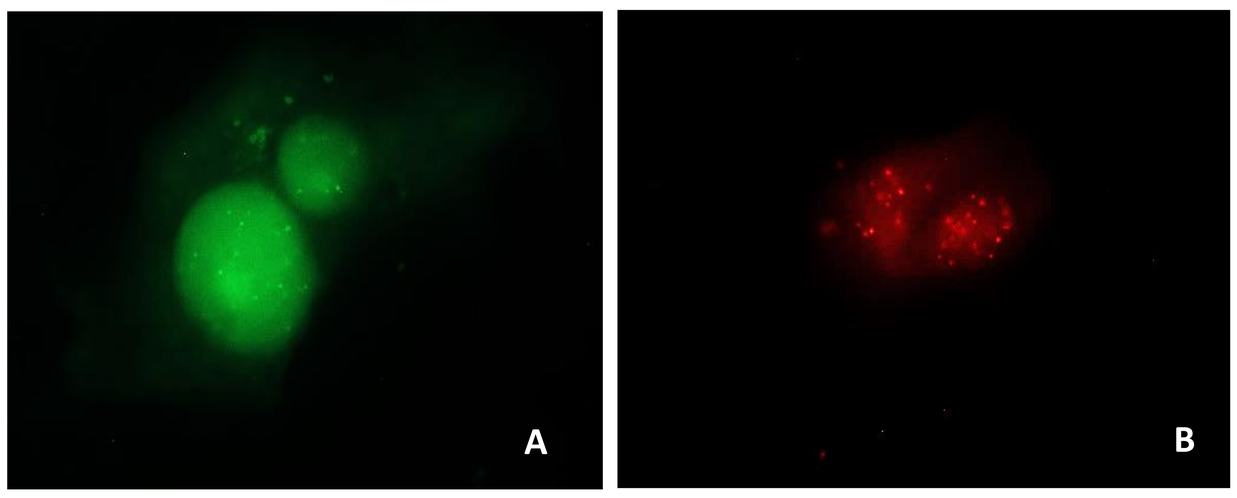

Figure 4 - Fluorescence microscopy of A549 StdCas9-EGFP with expression of St guide RNA (A) and A549 NmdCas9-mCherry with expression of Nm guide RNA (B) 48 h after Tet- induced expression of chimeras by doxycycline (1 μg/ml). Zeiss Axio Observer Z1, DI-AxioCam-HRm, x100 (Scale (μm/pixel): 100x/1.4 px = 0.063 micron), A - green cube Ex. BP 470/40, BS FT 495/ Em. BP 525/50; B - red cube: Ex. BP 550/25 BS FT 570/Em. BP 605/70.

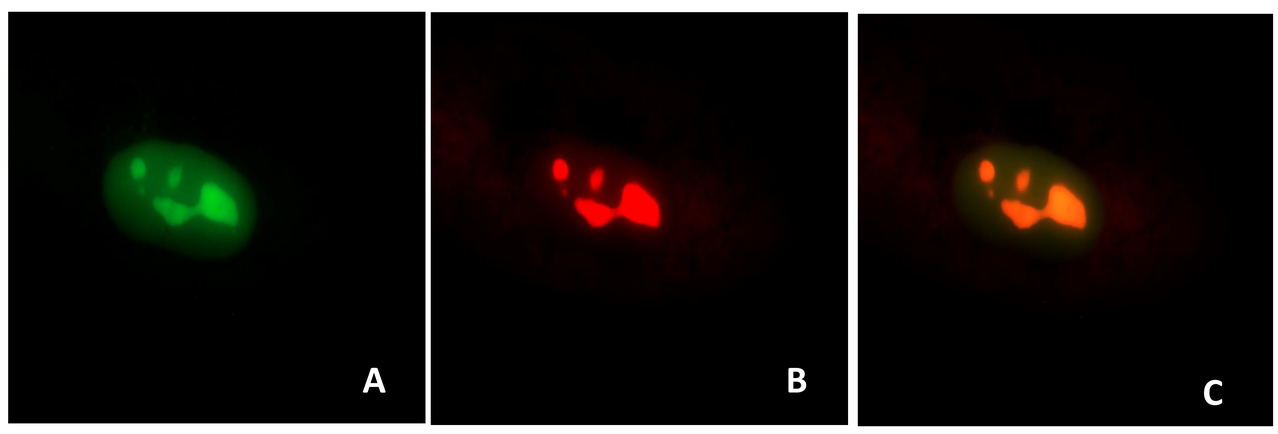

Figure 3 - Fluorescence microscopy of A549 StdCas9-EGFP-Mn mCherry 48 h after Tet- induced expression of chimeras by doxycycline (1 μg/ml). Zeiss Axio Observer Z1, DI-AxioCam-HRm, x100 (Scale (μm/pixel): 100x/1.4 px = 0.063 micron), A - green cube Ex. BP 470/40, BS FT 495/ Em. BP 525/50; B - red cube: Ex. BP 550/25 BS FT 570/Em. BP 605/70; C- the ooverlay of images (A) and (B).

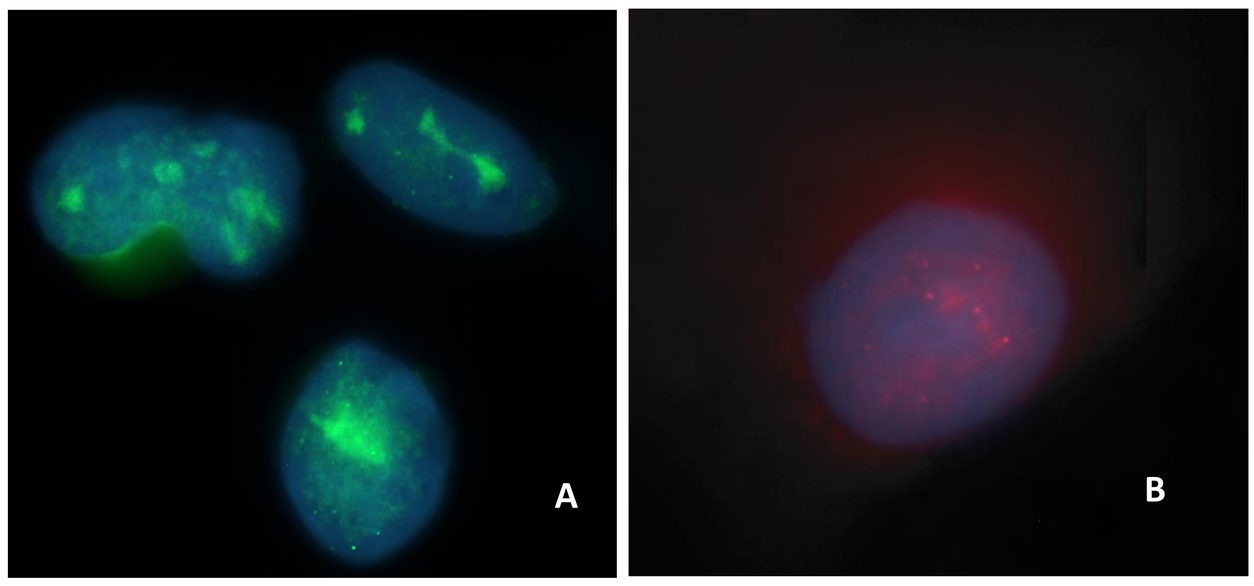

Figure 5 - In situ fluorescent hybridization in A549 cells using a green fluorescent-labeled probe, complementary telomeric sequence recognition for StsgRNA (A) and NmsgRNA (B)

Results: 2. Cells expressing St dCas9-EGFP and Nm dCas9-cherry simultaneously with the corresponding guide RNA showed the presence of a large number of bright small dots in the cell nucleus region, which is a visualization of the marking of telomeric sequences. (Fig.4). The obtained results of labeling telomeric sequences were confirmed using the FISH method using fluorescently labeled probes complementary to telomeric sequences and repeating the sequence of guide RNAs. (Fig. 5).

Conclusions: The possibility of obtaining cells expressing double (red and green fluorescent) chimeric proteins based on dCas9 in transduced human cells has been demonstrated. Tet-dependent induction of expression of these chimeric fluorescent orthologs over time in vivo has been shown. Effective use of constructs expressing guide RNAs for telomere labeling has been shown. It is shown that as a result of the expression of dCas9-FP-sgRNA complexes, there is a change in the localization of the fluorescent signal in telomeric regions, which is confirmed by FISH analysis. The use of the optical purification method made it possible to obtain high-contrast visualization of FP expression in tumor xenografts.

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