Optimization of the double fluorescence reporter assay and establishment of a CRISPR/Cas9-mediated protein knock-out for characterization of syncytin-1 in cell fusion context

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ABSTRACT

Cell fusions are important physiological processes and take place in fertilization, placentation, the development of bones and muscles or in wound healing[1]. In contrast, it is known that cell fusion also occurs in pathophysiological processes, such as viral infections or cancer development and progression. Fusion of a cancer cell and another cell leads to the formation of hybrid cells, which often show new malignant properties, such as a high metastatic potential[2]. A variety of different proteins and signaling molecules regulate the cell fusion process, including the retroviral fusion protein syncytin-1. Different studies show that syncytin-1 is often highly expressed in different kinds of tumors[3].

In this study a CRISPR/Cas9 vector was established, which was designed to knock out the retroviral fusion protein syncytin-1. Different guide RNAs (gRNA) were tested by transfecting M13SV1 breast epithelial cells with the CRISPR/Cas9 vector, extracting the genomic DNA and performing a T7 Endonuclease 1 (T7E1) assay. For optimization and production of a homogenous knock-out cell line, the most effective gRNA was ligated into a new vector expressing a bicistronic sequence (Cas9-p2A-PuroR), which enables the selection of transfected cells by antibiotic pressure.

Furthermore, fluorescence double reporter assay, which was used in our working group before, was improved by creating a tricistronic vector containing the sequence mCherry-p2A-iCre-p2A-PuroR (miCP) or iCre-T2A-mCherry-p2A-PuroR (iCmP). On the one hand the use of codon improved Cre recombinase leads to the expression of a more efficient protein which will translocate into the nucleus with the help of a nuclear localization sequence (NLS). On the other hand, the use of a tricistronic sequence with the puromycin resistance to be expressed last guarantees the expression of all three proteins simultaneously.

METHODS

Cre-loxP fluorescence double reporter assay

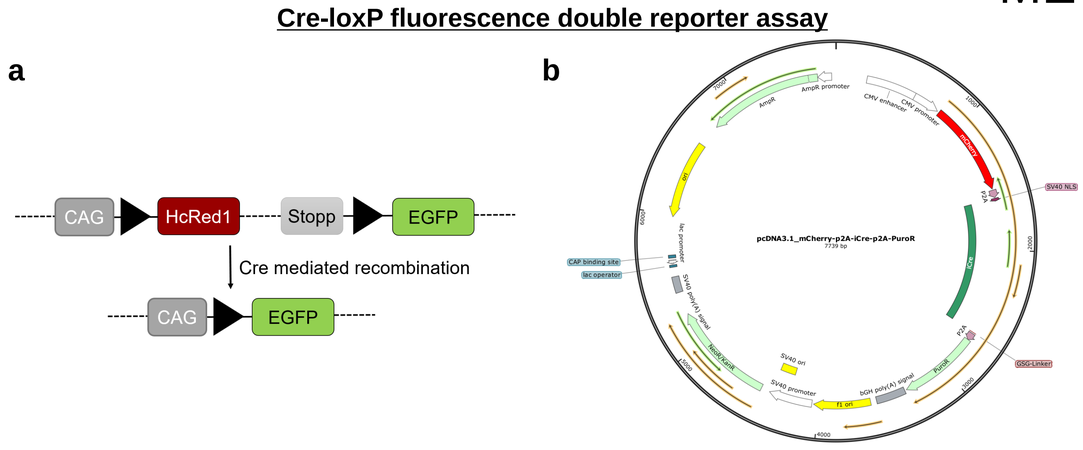


Fig. 1: Cre-mediated recombination of the fluorescence double reporter vector (pFDR.2) (a) and new Cre expressing vector to be evaluated (b).
a Simultaneous expression of Cre recombinase and pFDR.2 vector in a cell leads to the excision of the HcRed1 gene at the loxP sites and expression of the EGFP gene instead. **b** pcDNA3.1_mCherry-p2A-iCre-p2A-PuroR (miCP) vector. Optimized tricistronic vector with codon-improved Cre recombinase (iCre) and additional puromycine resistance. A second vector with the sequence iCre-T2A-mCherry-p2A-PuroR (iCmP) was also established.

CRISPR/Cas9-mediated protein knock-out

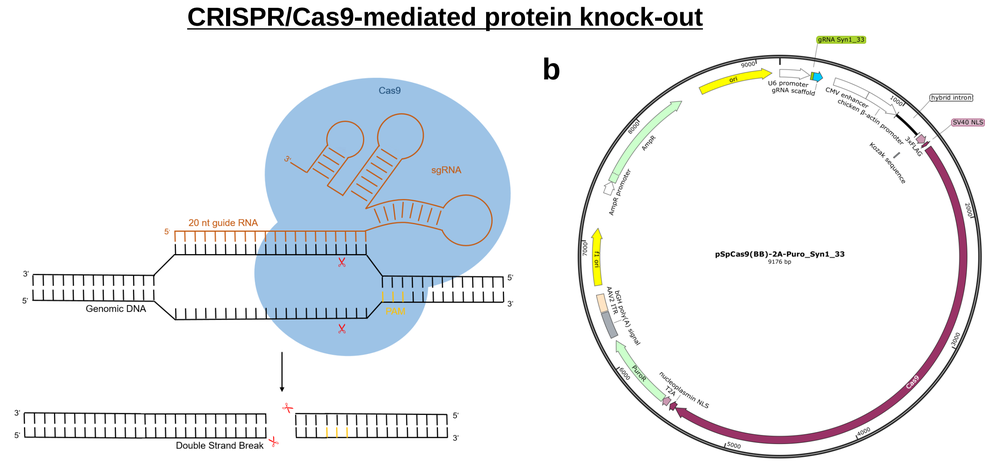
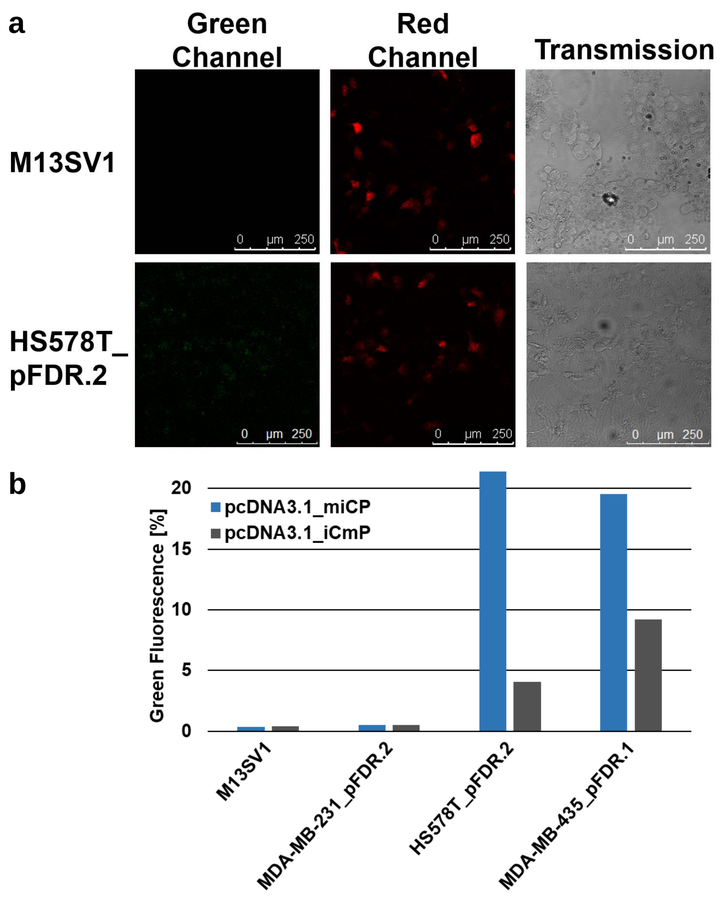


Fig. 2: Induction of a DNA double strand break with Cas9 (a) and Cas9-2A-PuroR expressing vector with ligated gRNA for syncytin-1 (b).
a Cas9 recognizes its target DNA sequence with the help of a gRNA. A double strand break is induced by nuclease domains at a specific site. Cells will repair the double strand break and thereby integrate insertions or deletions (Indels) in the DNA sequence causing a non-functional protein. **b** pSpCas9(BB)-2A-PuroR vector ligated with gRNA for syncytin-1. After transfection cells can be selected by antibiotic pressure to ensure Cas9 expression.

RESULTS



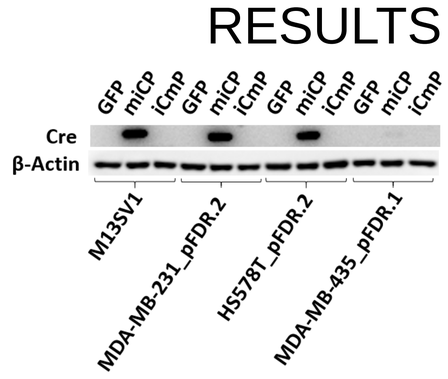


Fig. 3: Evaluation of the functionality of miCP and iCmP vectors.
a Confocal microscopy shows the expression of mCherry in M13SV1 cells transfected with miCP vector and a fluorescence switch in HS578T_pFDR.2 cells transfected with miCP indicating a functional iCre recombinase. **b** Flow cytometry for green fluorescent cells in different cell lines with or without pFDR.1/2 vectors transfected with miCP or iCmP shows highest values for HS578T_pFDR.2 and MDA-MB-435_pFDR.1 cells transfected with miCP vector, indicating best Cre expression and functionality in these cell lines. **c** Western Blot with cell lysates from cell lines transfected with miCP or iCmP vector shows protein bands of 42 kDa for Cre in cell lines transfected with miCP vector.

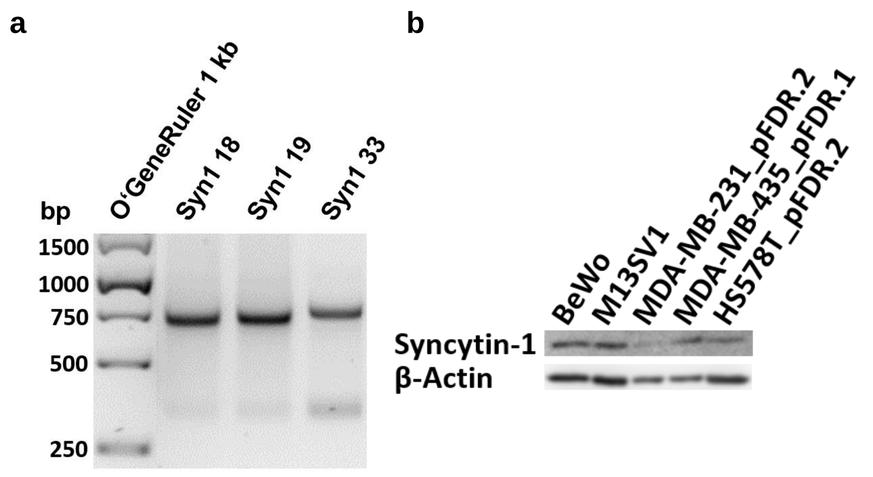


Fig. 4: Evaluation of CRISPR/Cas9 functionality for syncytin-1 knock-out with T7 Endonuclease-1 (T7E1) assay (a) and Western Blot (b).
a M13SV1 cells were transfected with vectors coding for Cas9 and different gRNAs for syncytin-1. Genomic DNA was extracted and T7E1 assay was done. T7E1 detects Indels caused by Cas9 in genomic DNA and cuts it which results in two bands in an agarose gelelectrophoresis. gRNA for syncytin-1_33 showed best results. **b** Western Blot with cell lysates of different cell lines with a syncytin-1 polyclonal antibody. This antibody can be used to detect protein levels of syncytin-1 in cells after transfection with pSpCas9(BB)-2A-Puro vector.

CONCLUSION & OUTLOOK

The results obtained show the functionality of the new vectors created for optimization of the fluorescence double reporter assay and syncytin-1 protein knock-out with CRISPR/Cas9.
The CRISPR/Cas9 vector used to test the gRNAs for syncytin-1 shows the best functionality for gRNA syncytin-1_33 in T7E1 assay. The new vector with Cas-P2A-PuroR sequence will also be tested for its functionality on DNA and protein level using T7E1 assay and Western Blot. The tricistronic vector miCP shows red fluorescence in M13SV1 cells, which shows correct expression of mCherry fluorescent protein. Furthermore green fluorescence, which is caused by a fluorescence switch in a Cre-loxP system, implicated the functionality of codon-improved Cre recombinase in HS578T_pFDR.2 and MDA-MB-435_pFDR.1 cells. The puromycin resistance needs to be evaluated by putting transfected cells under antibiotic pressure, which will be done to obtain a homogenous cell population expressing the three desired proteins. The planned experiment on cell fusion with the two vectors is shown in figure 5.

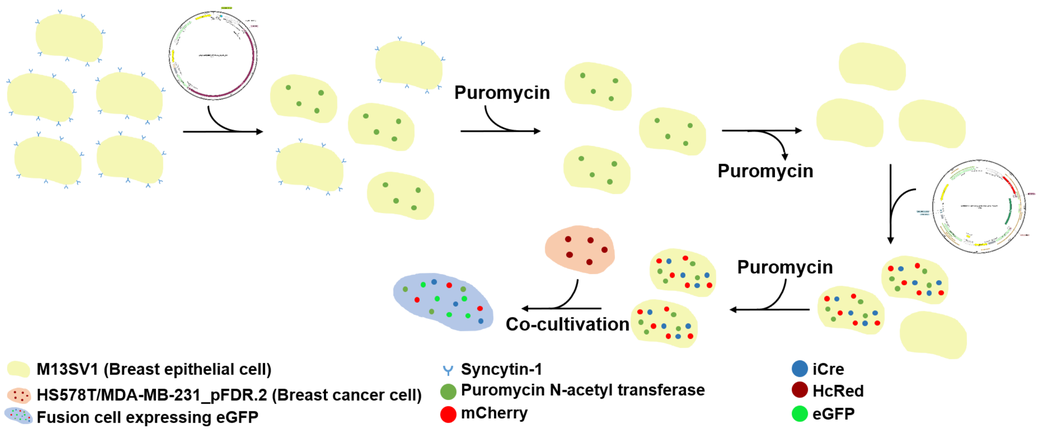


Fig. 5: Planned experiment to determine the role of syncytin-1 in cell fusion between breast epithelial and breast cancer cells.
M13SV1 breast epithelial cells will be transfected with pSpCas9-(BB)-2A-PuroR_Syn1_33 vector to achieve syncytin-1 knock-out. Afterwards the cells will be transfected with miCP vector and cell fusion experiments will be done with breast cancer cell lines containing pFDR.1/2 vector. The results of the cell fusion studies will give insights whether syncytin-1 play a role for cell fusion between breast epithelial and breast cancer cells.

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1 Larsson, L. I. *et al.* Cell fusions in mammals. *Histochem Cell Biol* **129**, 551–561 (2008).
2 Weiler, J., Dittmar, T. Cell Fusion in Human Cancer: The Dark Matter Hypothesis. *Cells* **8** (2019).
3 Huang, Q. *et al.* Epigenetic and non-epigenetic regulation of syncytin-1 expression in human placenta and cancer tissues. *Cell Signal* **26**, 648–656 (2014).