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Efficient removal of LoxP-flanked genes by electroporation of Cre-recombinase mRNA

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Abstract

Introduction of Cre-recombinase in target cells is currently achieved by transfection of plasmid DNA or by viral-mediated transduction. However, efficiency of non-viral DNA transfection is often low in many cell types, and the use of viral vectors for transduction implies a more complex and laborious manipulation associated with safety issues. We have developed a non-viral non-DNA technique for rapid and highly efficient excision of LoxP-flanked DNA sequences based on electroporation of in vitro transcribed mRNA encoding Cre-recombinase. A K562-DSRed[EGFP] cell line was developed in order to measure Cre-mediated recombination by flow cytometric analysis. These cells have a stable integrated DSRed reporter gene flanked by two LoxP sites, and an EGFP reporter gene, which could only be transcribed when the coding sequence for DSRed was removed. The presented data show recombination efficiencies, as measured by appearance of EGFP-fluorescence, of up to 85% in Cre-recombinase mRNA-electroporated K562-DSRed[EGFP] cells. In conclusion, mRNA electroporation of Cre-recombinase is a powerful, safe, and clinically applicable alternative to current technologies used for excision of stably integrated LoxP-flanked DNA sequences.

Keywords: Electroporation; Cre-recombinase; mRNA electroporation

Cre-recombinase-mediated excision of LoxP-flanked DNA sequences is a powerful technology in transgenesis and in various gene therapy strategies [1]. Current technologies to establish stably transfected cells or cell lines involve co-selection for a drug selection marker [2] or a fluorescent protein [3]. However, if downstream applications for these transfected cells include animal experiments or human clinical trials, it might be advisable to remove the selection marker. For example, removal of a selection marker from modified embryonic stem cells avoids non-specific phenotypic changes in knockout mice [4,5]. Large numbers of human myogenic and endothelial cells for use in cell replacement or ex vivo gene therapy can be obtained by in vitro immortalization of primary cells with SV40 large-T antigen, giving them a significantly extended life span with retention of their cellular characteristics [6,7]. However, removal of the large-T antigen is mandatory before use in clinical settings in order to allow terminal differentiation in vivo and to prevent possible immune responses against large-T antigen. Also, commonly used selection markers, e.g., neomycin resistance and fluorescent proteins, are antigenic targets for cytotoxic T-cells upon re-introduction of gene-modified cells in immune competent animals or humans [8]. For these reasons, technologies were developed to remove undesired DNA sequences from stably transfected cells. Introduction of LoxP-sites in DNA constructs for generation of stable clones has become a widely used application. Cloned cells can be screened for integration in undesired DNA sequences, in order to avoid insertional mutagenesis, resulting in uncontrolled division of cells in vivo. To remove loxP-flanked DNA sequences, current technologies are based on the transfection of plasmid DNA encoding Cre-recombinase [9]

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or adenoviral-mediated transduction and expression of Cre-recombinase [10]. With these transfection techniques, additional integration of DNA sequences into the genome cannot be excluded, which might result in undesired side effects, although this is likely to occur with low frequency. Alternatively, mRNA encoding Cre-recombinase can be directly injected into cells in order to avoid the use of DNA [11]. However, single cell injection is a rather time-consuming process when large amounts of cells need to be processed. We have developed a non-viral non-DNA technique for rapid and efficient excision of LoxP-flanked DNA sequences in bulk cell populations based on electroporation of mRNA encoding Cre-recombinase. The use of mRNA for transfection of cell lines and primary cells has previously been demonstrated and is considered as a safe, highly efficient, and clinical applicable alternative to DNA-mediated gene transfer [12–14]. Moreover, electroporation of mRNA is not associated with the risk of additional DNA integration, and protein expression will rapidly disappear, thereby leaving cells in an untouched condition. We created an experimental model system, where human leukemic K562 cells were stably transfected with a DSRed reporter gene flanked by two LoxP sites. An EGFP coding sequence placed downstream of the DSRed reporter gene could only be transcribed when the coding sequence for DSRed was removed. These cells, described as K562-DSRed[EGFP] cells, were electroporated with in vitro transcribed mRNA encoding Cre-recombinase, in order to remove DSRed sequences. The presented data show recombination efficiencies, as measured by stable appearance of EGFP fluorescence and disappearance of DSRed fluorescence, reaching more than 80% in Cre-recombinase mRNA-electroporated K562-DSRed[EGFP] cells. These data demonstrate that there is a safe and clinically applicable alternative to the use of viral vectors for Cre-recombinase expression in order to efficiently remove loxP-flanked DNA sequences in a whole cell population.

Materials and methods

Cell lines. K562 cells were obtained from the American Type Culture Collection (ATCC no. CCL-243, Rockville, MD, USA). Cells were cultured in complete medium consisting of Iscove's modified Dulbecco's medium (IMDM) supplemented with L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), amphotericin B (1.25 μg/mL Fungizone), and 10% fetal calf serum (FCS; Sera Lab, Sussex, UK). Cells were maintained in logarithmic phase growth at 37 °C in a humidified atmosphere supplemented with 5% CO_2. All cell culture reagents were purchased from Invitrogen (Paisley, UK).

DNA constructs. For generating a stable transfected K562 cell line, the DSRed[EGFP] vector was constructed by subcloning the DSRed gene and SV40 polyA signal (pDSRedN-1, Clontech Laboratories, Palo Alto, CA) in the first multiple cloning site (MCS) of the pEGFP-N1 vector (Clontech). LoxP-sites were added on both sides of the DSRed gene by LoxP-sequence overhang. For DNA transfections, we used the pEGFP-N1 vector (Clontech) and the pTURBOCre vector (kindly provided by Dr. Timothey Ley, Washington University, St. Louis, MO, USA). For in vitro transcription of mRNA, we used the pGEM4Z-EGFP-A64 vector (kindly provided by Dr. E. Gilboa, Duke University Medical Center, Durham, NC, USA) and the pGEM4Z-CRE-A64 vector, which was constructed by *Hin*dIII-subcloning of the Cre-recombinase coding DNA sequence (pTURBOCre plasmid) into the pGEM4Z-A64 vector. All constructs were propagated in TOPO1 cells (Invitrogen, Paisley, UK) and purified by the Qiaprepspin miniprep kit (Qiagen, Chatsworth, CA).

Generation of stably-transfected K562 cells. K562 cells were transfected with the DSRed[EGFP] DNA construct using Fugene 6 (Roche Diagnostics, Brussels, Belgium), followed by 14 days selection in culture medium supplemented with G418 (Invitrogen). Next, viable DSRed positive cells were sorted on a FACS Vantage cell sorter (Becton–Dickinson, Erembodegem, Belgium) and further expanded in the presence of G418 (0.5 mg/mL).

Production of in vitro transcribed mRNA. The pGEM4Z-EGFP-A64 and pGEM4Z-CRE-A64 vectors were linearized with *Spe*I (MBI Fermentas GMBH, St. Leon-Rot, Germany), purified using a PCR purification Kit (Qiagen), and used as DNA templates for in vitro transcription reaction. Transcription was carried out in a final 20–100 μl reaction mix at 37 °C using the T7 MessageMachine Kit (Ambion, Austin, TX, USA) to generate 5′ capped in vitro transcribed (IVT) mRNA. Purification of mRNA was performed by DNase I digestion followed by LiCl precipitation, according to manufacturer's instructions. RNA was stored at −80 °C in small aliquots.

Cell transfections. Electroporation of DNA and mRNA was performed as described previously [14]. Briefly, prior to electroporation, K562-DSRed[EGFP] cells were washed twice with Optimix Washing Solution (EquiBio, Ashford, Middlesex, UK) and resuspended to a final concentration of 25×10^6 cells/mL in Optimix electroporation buffer (EquiBio). Subsequently 0.2 mL of the cell suspension was mixed with 20 μg plasmid DNA or 20 μg IVT mRNA and electroporated in a 0.4 cm cuvette at 300 V and 150 μF using an Easyject Plus device (EquiBio). After electroporation, fresh complete medium was added to the cell suspension and cells were further incubated at 37 °C in a humidified atmosphere supplemented with 5% CO_2.

Flow cytometric analysis. Transfected cells were analyzed by flow cytometry for DSRed (FL-2) and EGFP (FL-1) expression at different time points after transfection. Briefly, 1×10^6 cells were washed once in phosphate-buffered saline (PBS) supplemented with 1% FCS and resuspended in 0.5 mL PBS supplemented with 1% bovine serum albumin (BSA) and 0.1% sodium azide. Flow cytometric analysis was done on a FACScan analytical flow cytometer (Becton–Dickinson). Proper correction for spectral overlay of DSRed and EGFP was established by running single labelled controls in advance. FACS results were analyzed using WinMDI 2.8 software (designed by Joseph Trotter, URL: http://facs.scripps.edu/). For this, viable cells were gated based on bivariate forward scatter (FCS)/side scatter (SSC) profiles, followed by visualization of FL-1 versus FL-2 fluorescence in dot plot analysis.

Statistics. Results are expressed as means ± SD. Comparisons were validated using Student's t test. A p value $\leqslant 0.05$ was considered to be statistically significant.

Results and discussion

Characterization of K562-DSRed[EGFP] cells

In order to optimize Cre/LoxP-mediated recombination, we used leukemic K562 cells as a model cell line, because these cells are highly transfectable with DNA

and RNA as previously described [3,12,15]. K562 cells were stably transfected with a DSRed[EGFP] DNA construct in order to monitor Cre-mediated recombination of DSRed DNA sequences (Fig. 1). Upon excision of LoxP-flanked DSRed sequences, the EGFP reporter gene is under direct control of the CMV promoter and appearance of EGFP fluorescence can be used to monitor recombination efficiency [16]. Cultured K562-DSRed[EGFP] cells show a stable DSRed fluorescence, without marked interference of EGFP fluorescence, as compared to control K562 cells (Fig. 2A). To determine the transfectability of the newly generated K562-DSRed[EGFP] cell line with DNA and RNA, cells were electroporated with plasmid DNA and mRNA encoding EGFP. Dot plot analysis confirmed the transfectability of the K562-DSRed[EGFP] cell line (Fig. 2B). While EGFP DNA electroporation results in a transfection efficiency of $36 \pm 5\%$, EGFP mRNA electroporation results in a significantly higher efficiency of transfection, i.e., $87 \pm 8\%$, as measured 48 h after electroporation ($p = 0.009$).

Efficiency of Cre/LoxP-mediated recombination in K562-DSRed[EGFP] cells

K562-DSRed[EGFP] cells were electroporated with plasmid DNA or mRNA encoding Cre-recombinase. Using flow cytometric analysis, the appearance of green

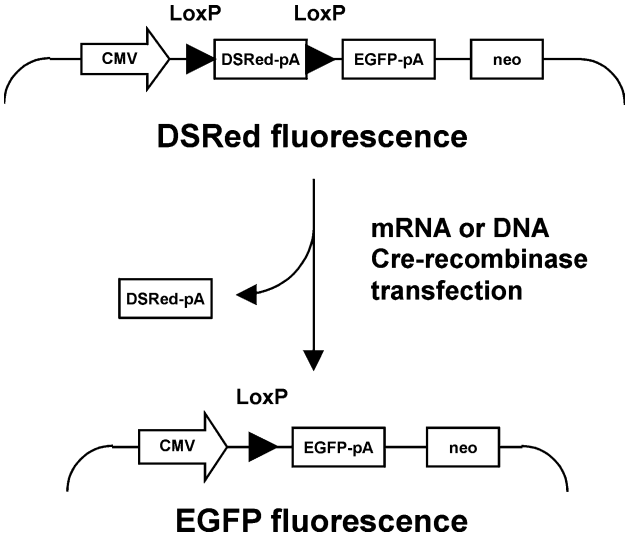

Fig. 1. Principle of Cre-mediated recombination of a LoxP-flanked reporter gene. A DSRed[EGFP] vector was constructed for stable transfection of K562 cells. The DSRed[EGFP] vector contains a CMV promoter followed by the DSRed coding sequence (CDS) and SV40 polyA (pA) signal between two LoxP-sites. Downstream in this vector, a promoter-less EGFP gene followed by a SV40 polyA signal serves as a reporter gene for detection of Cre-recombinase mediated removal of the DSRed CDS. Upon transfection of Cre-recombinase DNA or mRNA into K562 cells stably transfected with the DSRed[EGFP] vector, the loxP-flanked DSRed sequence can be excised, followed by transcription and expression of EGFP.

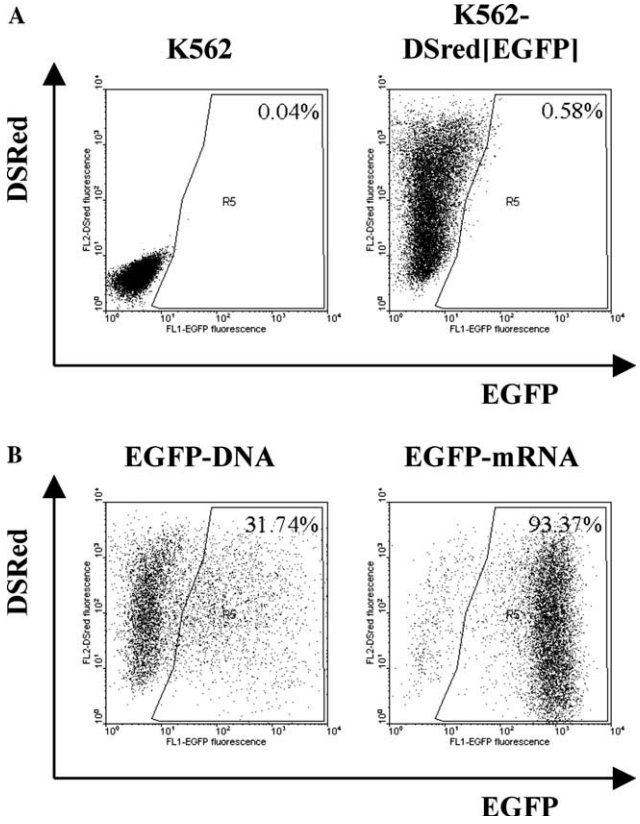

Fig. 2. Generation and transfectability of the K562-DSRed[EGFP] cell line. (A) K562 cells were stably transfected with the DSRed[EGFP] plasmid. Dot plots show flow cytometric analysis of EGFP-fluorescence (*x*-axis) versus DSRed-fluorescence (*y*-axis) of parental K562 cells (dot plot on the left) and K562-DSRed[EGFP] cells (dot plot on the right). The percentage indicated is the number of cells positive for EGFP-fluorescence. (B) K562-DSRed[EGFP] cells were electroporated with EGFP DNA (dot plot on the left) and EGFP mRNA (dot plot on the right), and were analyzed by flow cytometry for EGFP fluorescence (*x*-axis) versus DSRed fluorescence (*y*-axis) after 48 h of culture. The percentage indicated is the number of cells positive for EGFP fluorescence. Data are representative for two electroporations with EGFP DNA and two electroporations with EGFP mRNA.

fluorescence, indicating efficient removal of the DSRed DNA sequence, was followed with time (Fig. 3). After 7 days of culture, K562-DSRed[EGFP] cells electroporated with Cre-recombinase mRNA showed a significantly higher number of EGFP-positive cells as compared to K562-DSRed[EGFP] cells electroporated with Cre-recombinase plasmid DNA. The recombination efficiency after Cre-recombinase mRNA electroporation was $78 \pm 2\%$ as compared to $34 \pm 10\%$ for electroporation with Cre-recombinase plasmid DNA ($p < 0.001$). Remarkably, appearance of EGFP fluorescence is rather slow after electroporation with Cre-recombinase plasmid DNA, as compared to the appearance of EGFP positive cells following electroporation with Cre-recombinase mRNA. This can be accounted to the direct translation of electroporated mRNA into protein upon arrival in the cytoplasm, while

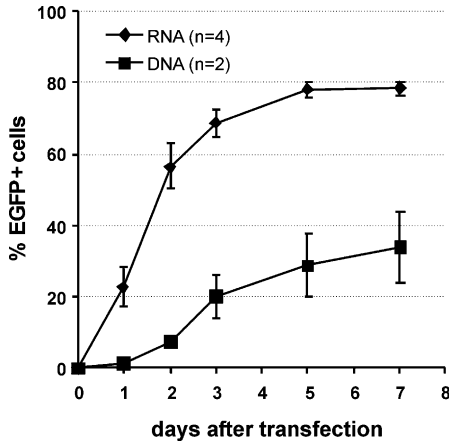

Fig. 3. Efficiency of Cre-mediated excision of LoxP-flanked DSRed CDS. K562-DSRed[EGFP] cells were electroporated with Cre-recombinase DNA ($n = 2$) and Cre-recombinase mRNA ($n = 4$). At different intervals after electroporation, the number of EGFP positive cells (i.e., cells with excised DSRed CDS) was determined by flow cytometric analysis for EGFP fluorescence. Data are shown as means ± SD.

plasmid DNA first has to reach the nucleus for initiation of transcription. Interestingly, although electroporation with EGFP mRNA showed a high level of protein expression as fast as 3 h after electroporation, the almost maximal efficiency of Cre-recombinase-mediated excision of DSRed sequences was only achieved 5 days after electroporation. This demonstrates that Cre-mediated recombination of LoxP-flanked DNA sequences is a rather slow process, but that a single electroporation of Cre-recombinase mRNA is sufficient to achieve high-level recombination efficiency. Moreover, the fact that the number of EGFP-positive cells is increasing over time (until up to three weeks) might be explained by the stability of Cre-recombinase mRNA and/or protein. However, for the latter no information is yet available.

Kinetics of DSRed disappearance in Cre-recombinase DNA- and mRNA-electroporated K562-DSRed[EGFP] cells

Because we have used a combined DSRed/EGFP reporter system, the appearance of EGFP fluorescence and the disappearance of DSRed fluorescence can both be observed by flow cytometric analysis (Fig. 4). While the number of EGFP-positive cells is increasing with time, DSRed-fluorescence in EGFP-positive cells decreases. After 21 days of culture, the majority of EGFP positive cells have become negative for DSRed fluorescence. However, a small population remains positive for both EGFP and DSRed fluorescence. This is in accordance with previous reports showing that the function of Cre-recombinase has an efficiency of around 95% [17]. On day 21, plasmid DNA electroporation of Cre-recombinase resulted in DSRed excision in 39 ± 17% of

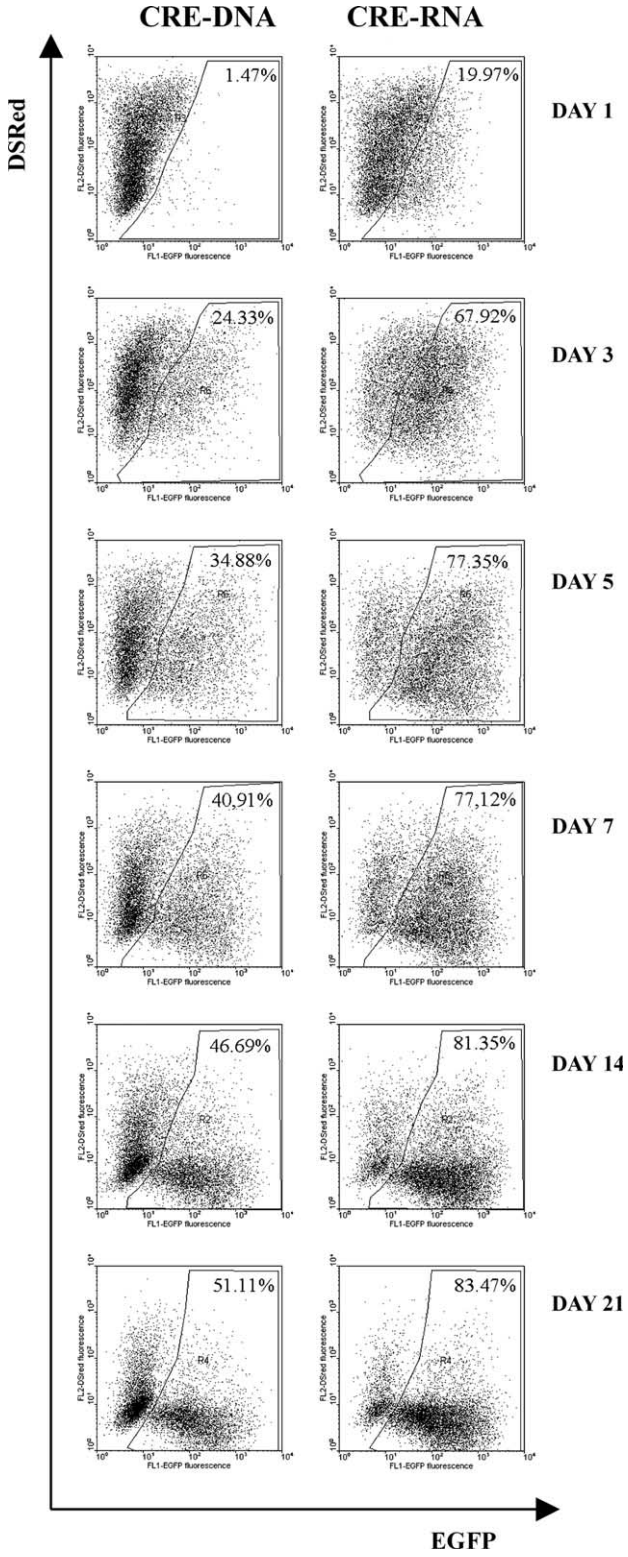

Fig. 4. EGFP expression after Cre-mediated recombination of DSRed CDS. K562-DSRed[EGFP] cells were electroporated with Cre-recombinase DNA and Cre-recombinase mRNA. At different intervals after electroporation, cells were analyzed by flow cytometry for EGFP fluorescence (*x*-axis) versus DSRed fluorescence (*y*-axis). The percentage indicated is the number of cells positive for EGFP-fluorescence. Data are representative for two electroporations with Cre-DNA and four electroporations with Cre-mRNA.

cells, of which $4.3 \pm 2.7\%$ were still double positive for EGFP and DSRed, presumably because of incomplete excision of all integrated DSRed sequences. On day 21 after transfection, electroporation of Cre-recombinase mRNA resulted in DSRed excision in $85 \pm 2\%$ of cells, of which $6.7 \pm 1.9\%$ were still positive for EGFP- and DSRed-fluorescence. These data show a significantly higher recombination efficiency using Cre-recombinase mRNA electroporation as compared to Cre-recombinase plasmid DNA electroporation ($p = 0.002$). Moreover, there is no significant difference in the number of cells remaining positive for both EGFP and DSRed when comparing mRNA and DNA electroporation of Cre-recombinase ($p = 0.136$).

Conclusions

In the present study, we describe an improved, safe, and clinically applicable technology for highly efficient removal of undesired LoxP-flanked DNA sequences based on electroporation of Cre-recombinase mRNA. Although this study has been performed using the highly transfectable K562 cell line, many other cell lines and primary cells are refractory to plasmid DNA transfection. For these non-transfectable cells, the only option is to use viral vectors, which carries the risk of additional non-specific integration of DNA fragments. Previous reports from our laboratories have shown efficient gene-transfer by mRNA electroporation in various human hematopoietic cell lines, in primary human cells including dendritic cells [12,14], monocytes [18] and hematopoietic progenitor cells, and in mouse embryonic stem cells (unpublished data). Therefore, the presented technology opens new perspectives in cell therapy and in embryonic stem cell research.

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