Assessing the modulation of cells by modified mRNAs *in vitro* and *in vivo*

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Introduction

The transfer of genetic information to cells allows deciphering the function of genes and proteins as well as programming of cells. Current methods for transient gain- or loss-of-function experiments and modification of genes by recombinases are mainly based on transfer of DNA. However, delivery of DNA is limited by the defense mechanisms of cells and potentially genotoxic. In our study we aimed at exploring the versatility of RNA to i) extend DNA-free gain-of-function experiments to cells that have been difficult to address *in vitro* and *in vivo* and ii) to increase the efficacy of recombination events.

Results

1 Generation of animal component–free, sterile, modified mRNAs

We have set up a standardized workflow to generate mRNAs. The cDNA of a gene of interest, optimized with regard to human codon usage, is cloned into a basic vector system containing the T7 promotor and a 5′ and 3′ UTR. RNA is generated in an animal component–free production process by T7-based transcription and subsequent 5′ capping and 3′ polyadenylation. Optionally, Ψ-UTP- and 5-mCTP-modified nucleotides are introduced to reduce innate immune responses against RNA in downstream applications. RNA is DNase treated, sterile filtered, and lyophilized.

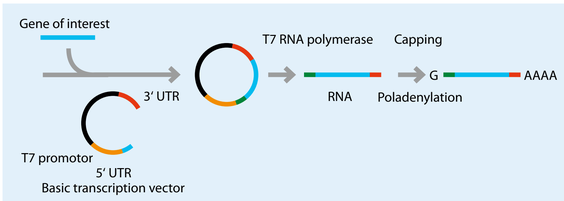

Figure 1

2 StemMACS™ mRNAs show a very fast, efficient, and non-toxic expression *in vitro*

We have validated the performance of mRNA generated with the new process on different cell lines, primary cells, and pluripotent stem cells. We first aimed at optimizing the transfection of primary human fibroblasts. To this end, we screened for reagents showing the highest transfection efficiency and lowest toxicity. One particular reagent (now called StemMACS™ mRNA Transfection Reagent) showed a transfection efficiency for fibroblasts of up to 90% (n=12) with an average survival of 90–99%.

(A) RNA transfection of fibroblasts. 2.5×10⁴ human fibroblasts of different origins were plated (24-well plate, 500 µL/well) overnight and transfected the next day with 0.6 µL of StemMACS mRNA Transfection Reagent and with indicated amounts of StemMACS eGFP mRNA. GFP expression was determined 24 h later on the MACSQuant® Analyzer using the MFI of viable cells and the percentage of GFP-positive cells among viable cells. Transfection efficiency and GFP expression depended on the ratio of StemMACS Transfection Reagent and RNA. Overall cell viability was above 95% for all cells and ratios tested.

(B) RNA electroporation of hematopoietic stem cells. CD133⁺ cells were isolated from cord blood by magnetic cell separation (MACS® Technology), cultured overnight, and subsequently electroporated with GFP mRNA (2.4–8 µg per sample). 24 h after electroporation 97+/–2.9% of the cells were GFP⁺ (n=10), and the overall viability was 45+/–17.8%. Viability of cells electroporated with mRNA relative to mock-electroporated cells was about 100%, showing that the mRNA is not toxic (data not shown). After electroporation of cells with 8 µg GFP mRNA, GFP expression persisted for at least seven days, indicating a high stability of GFP expression (mean+/–SD; n=3).

(C) RNA transfection of cultured myoblasts. Satellite cells were isolated from mouse skeletal muscle using MACS Technology, based on the depletion of cells positive for lineage markers (CD31, CD45, CD11b, Sca-1). As a proof of concept, nuclear GFP mRNA (100 ng per well) was transfected into myoblasts cultured for five days in 24-well plates, resulting in a transfection efficiency higher than 75%. We tested up to four consecutive transfections and did not observe cell death or other negative effects.

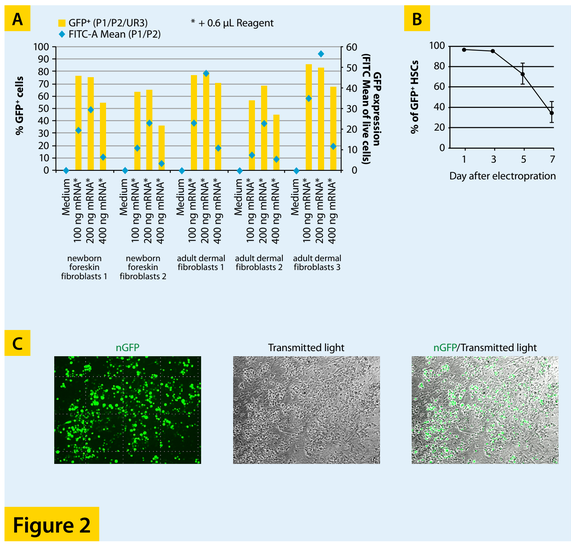

Figure 2

3 *In vivo* electroporation with mRNA is more efficient than with DNA

(A) Efficiency of *in vivo* electroporation of the mouse postnatal forebrain with mRNA or DNA was analyzed by *in vivo* analysis of GFP expression. DNA- or mRNA-containing solutions were directly injected into the lateral ventricles. After application of electrical pulses, neural stem cells (NSC) in the subventricular zone (SVZ) were transfected. **(B)** Seven days after electroporation with a DNA construct, GFP was expressed in all derivatives of NSC – from the SVZ to the olfactory bulb (OB). **(C)** Compared to electroporation with 5 µg cDNA (Pcx-EGFP-N1), electroporation with the same amount of GFP-RNA led to a considerably stronger GFP expression in more NSC lining the ventricular wall. **(D)** Left: *In vivo* electroporation with RNA of the ventricular wall at p5 allowed efficient expression of GFP in ependymocytes, a cell population that is refractory to DNA transfection. Right: transfection of GFP in ependymocytes validated by S100-beta staining.

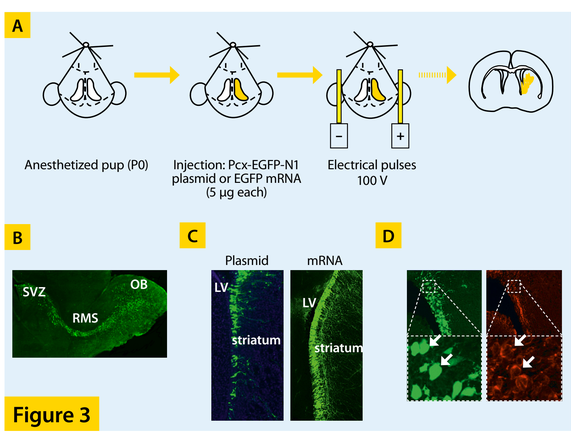

Figure 3

4 Efficient reprogramming of fibroblasts into iPS cells

(A) Workflow for efficient reprogramming of human fibroblasts into iPS cells. After adaption of the cells to the reprogramming medium, cells are transfected daily for 12 days with modified mRNAs coding for reprogramming transcription factors. Medium is replaced 4 h after transfection and immune suppressor B18R is added to inhibit innate immune responses. iPS cell colonies can be detected usually at day 14. **(B)** Oct-4 staining of reprogrammed human newborn fibroblasts at day 14 (12 consecutive transfections). Starting cell numbers were 7000, 4000, and 2000 in 6-well plates. At day 14, cells were fixed and stained with an anti-human Oct-4 antibody. **(C)** Alkaline phosphatase staining of iPS colonies of reprogrammed human newborn fibroblasts at day 14 in 24-well plates (12 consecutive transfections). **(D)** Example of iPS colonies.

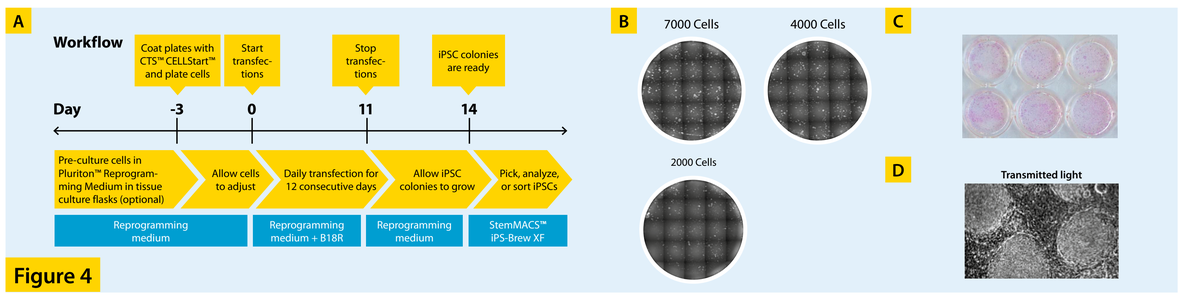

Figure 4

5 Transfection of iCre and FlpO mRNA yields very high recombination rates

(A) We explored the recombination efficiency of iCre and FlpO mRNA when transfecting (by lipofection) or electroporating mES cells carrying a GFP ON-OFF cassette (GAGG-Stop (flox)-eGFP(FRT)). **(B)** We observed a very high recombination efficiency in electroporated mES cells. The rate of GFP⁺ cells correlated with the amount of iCre mRNA, indicating the possibility to control the degree of recombination. **(C)** The recombination efficiency of up to 90% after electroporation or lipofection with mRNA was about 10 times higher than after electroporation with DNA. **(D)** Electroporation with FlpO mRNA also resulted in an about 3-fold higher recombination efficiency than electroporation with FlpO DNA.

(E) Efficient transfection and iCre recombination in primary mesenchymal stem/stromal cells (MSC) from murine adipose tissue using nuclear GFP and iCre mRNA. Primary MSCs were isolated from collagenase-digested murine subcutaneous fat, using a two-step procedure involving MACS Technology, resulting in TER119⁻CD31⁻CD45⁻ Sca-1⁺ cells. The cultured cells were transfected with 250 ng of nuclear GFP or iCre mRNA. Three days following transfection, iCre-mediated recombination was assessed by measuring expression of the floxed gene. Relative mRNA expression was determined by qRT-PCR.

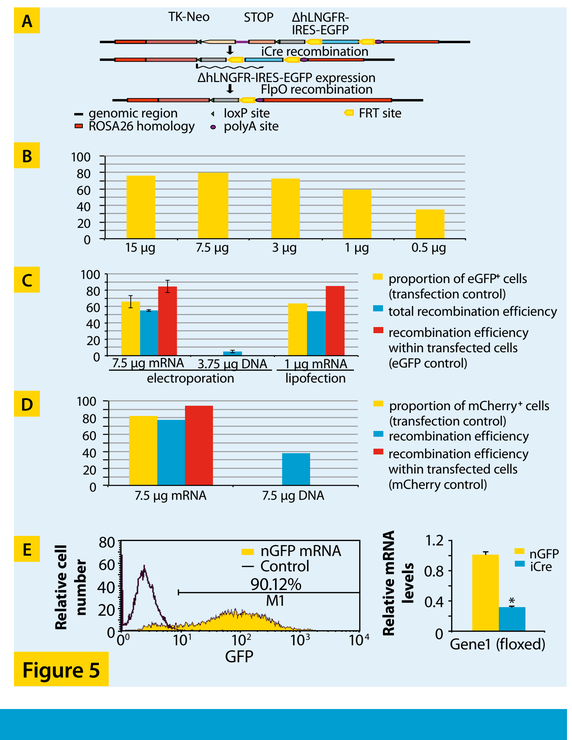

Figure 5

6 mRNA-based expression of transcription factors

We have set up and validated by immunocytochemistry a number of mRNAs coding for transcription factors known to modulate cell fate towards neural (Ascl1 (Mash1), Lmx1a, Nurr1 (Nr4a2), Myt1l, NeuroG2, Pou3F2, NeuroD1), cardiac (Gata4, Mef2c, Mesp1) and brown adipocyte (Cebpb, Pparg) lineages.

Primary fibroblasts were seeded in 24-well or 96-well plates and transfected with 50 to 83 ng mRNA per 10,000 cells. These RNAs will be used to explore the RNA technology for directed conversion of fibroblasts.

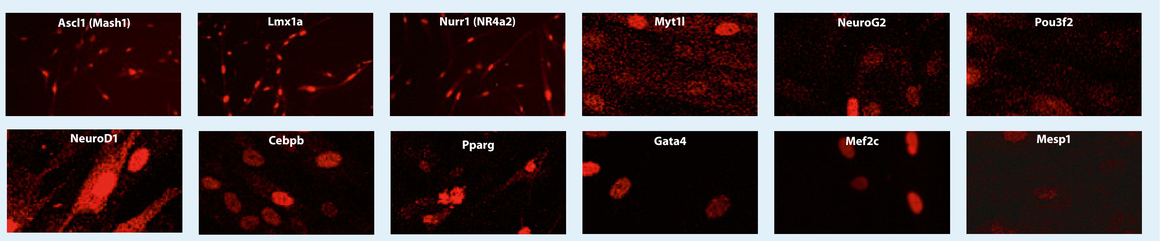

Figure 6

Conclusion

- We developed a workflow for the animal component–free generation of sterile, modified mRNAs.
- Modified mRNAs showed a very fast, efficient, and harmless expression *in vitro* and *in vivo* in a variety of primary cell types, exceeding expression rates observed with DNA transfection.
- Consecutive transfections with reprogramming mRNAs efficiently converted newborn and adult patient fibroblasts into iPS cells.
- Transfection of a variety of transgenic cells with iCre or FlpO showed a much higher recombination rate than observed with iCre or FlpO DNA.
- A number of mRNAs coding for transcription factors known to modulate cell fate towards different lineages could be generated and expressed in primary fibroblasts.

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