Short Communication

Highly efficient transfection of dendritic cells derived from esophageal squamous cell carcinoma patient: optimization with green fluorescent protein and validation with tumor RNA as a tool for immuno-genetherapy

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Abstract
This study was conducted to optimize a highly efficient mRNA transfection into dendritic cells (DC) derived from esophageal squamous cell carcinoma (ESCC) patients. Applying an electroporation technique, *in vitro* synthesized Green Fluorescent Protein (GFP) mRNA was transfected as an indicator into the DCs derived from a healthy donor. Flow cytometry revealed 84.9% transfection efficiency for DCs transfected with GFP mRNA. Optimized condition (500V/300 μs) yielded 79.8% efficiency in transfecting GFP mRNA into DCs from ESCC patient. Applying this efficient method, tumoral mRNA was transfected into DCs. T cells were then primed with tumor RNA/DCs and cytotoxicity assay revealed significantly higher lyses of tumor/DC vs. Mock DC; approving the optimized method for further establishment of the preclinical phase of DC-based immunotherapy for ESCC.
Keywords: Immuno-gene therapy; Esophageal squamous cell carcinoma (ESCC); Electroporation; mRNA transfection; Dendritic cell; GFP

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For vaccination, different methods have been used to load DCs with tumor-associated antigens (TAAs). It is accepted that, among non-viral gene transfer methods, electroporation is the most efficient for delivering mRNA into cells (Van Bockstaele *et al*., 2008). In this procedure, a mixture of cells and mRNA are pulsed at a specific voltage for a short period of time. Consequently, small pores are formed on the cell membrane which makes it possible for mRNA to pass into the cells (Mu *et al*., 2003). Gilboa *et al*. pulsed DCs with tumor antigen-encoding mRNA and reported an effective therapeutic tumor vaccination when it was administered in a murine model for the first time (Boczkowski *et al*., 1996).

Establishment and optimization of a highly efficient transfection method is the preliminary step in designing an immunotherapy modality for cancer in which transfection of tumoral mRNA into DCs will make them capable to enhance the immune response against tumor. In order to achieve the optimal conditions for a highly efficient transfection of mRNA, the K562 cell line was used for optimization of the transfection in the preliminary experiments. The optimized conditions were then applied to DCs generated from the monocytes of healthy controls. Finally, we approved the optimum parameters for the efficient

transfection of GFP mRNA into DC derived from peripheral blood monocytes of the ESCC patients.

Culture of DCs: Peripheral blood mononuclear cells were obtained from 30 ml of heparinized whole blood taken from a healthy donor or an ESCC patient. The generation of DC was performed on CD14 + cells isolated by the EasySep® Human Monocyte Enrichment Kit without CD16 Depletion (Stemcell Technologies). For 5 days, cells were seeded into six-well plates in supplemented RPMI1640. The production of immature DCs was detected by specific CD markers. The cells were electroporated with GFP mRNA on the sixth day, followed by the maturation of the culture with TNF-α 4 hours after electroporation.

Plasmid: pGEM4Z/GFP/A64 plasmid containing the poly (A) template (kindly provided by Dr. E. Gilboa, University of Miami, FA, USA) was utilized. This plasmid was transformed into *E. coli* Top10F and purified thereafter.

Generation of *in vitro*-transcribed EGFP mRNA: Vector linearization was performed by the restriction enzyme *SpeI*, followed by purification with phenol/chloroform extraction and ethanol precipitation. This purified DNA template was used for *in vitro* transcription under the control of a T7 promoter. *In vitro* capped mRNA was synthesized by the Mmessage mMACHINE kit (Ambion, Austin, TX, USA) according to manufacturer's instructions. To remove the linear plasmid template, DNase I was added. The amplified RNA was then purified through lithium chloride precipitation and resuspended in RNase-free water. EGFP mRNA was utilized as a reporter for subsequent quantitative analysis of transfection efficiency.

mRNA electroporation: Prior to electroporation, harvested K562 cells in a logarithmic phase and immature DCs (on day six), were washed twice with RPMI1640 and resuspended to a final concentration of 10×10^6 cells/ml in 100 µl Opti-MEM I (Gibco-BRL) medium. Subsequently, 100 µl of the cell suspension was mixed with 2-4 µg of *in vitro* transcribed mRNA and electroporated in a 2 mm cuvette (BioRad) at various voltages and pulse durations in order to obtain the optimized parameters for the most efficient transfection.

As for negative control of electroporation reaction, mock K562 and DCs were electroporated under the same conditions without the addition of mRNA. Square wave electroporation method was applied to enhance the efficiency of the mRNA transfer, in comparison with the exponential decay wave pulse electroporation method (Liu and Bergan, 2001).

FACS (Fluorescence-Activated Cell Sorting) analysis of immature DC: The following mouse monoclonal antibodies were used for the FACS analysis: anti-HLA DR, CD1a, CD80, CD86, CD83, FITC-conjugated (Fluorescein Isothiocyanate), and CD14 PE-conjugated (Phycoerytherin).

Analysis of EGFP expression in DCs: EGFP-transfected cells were checked for EGFP expression by flow-cytometry (FCM) analysis and fluorescent microscope 24-48 h after transfection. GFP was excited at 490 nm and emission was measured at 520 nm (Spiess *et al*., 2005). The efficiency of transfection is represented by the percentage of transfected cells in the live population. The introduction efficacy is calculated by multiplying the percentage of viable cells by the transfection efficiency (Ohshita *et al*., 2006).

DC transfection with tumor RNA: After optimizing an efficient transfection, DCs were loaded with the tumor RNA by the same method. To confirm the efficient transfection of DCs with tumor RNA and capability of enhancing an immune response, tumor-specific CTL was generated using tumor RNA-loaded DC. Briefly, the T-cell-enriched non-adherent fraction of PBMCs were obtained and suspended in supplemented RPMI. These cells were combined with transfected matured DCs and IL-7 and cultured at 37°C in 5% CO_2 for 8 days. IL-2 was added to the culture in the 3rd day. After 8 days, the effectors were harvested, washed, counted, and restimulated with newly transfected DCs. Adding IL-2 and IL-7 with the same concentrations to the culture, the cells were incubated for another 8 days to successfully induce tumor specific cytotoxic T lymphocyte (CTL).

Cytotoxicity test: To measure the enhanced cytotoxicity, the calcein-AM cytotoxicity assay was performed. Prior to Cytotoxicity assay, target cells were resuspended in RPMI-1640 complete medium at a final concentration of 10^6 cells/ml and incubated with 10 µM calcein-AM (Invitrogen inc., Grand Island, NY, USA) for 30 min at 37ºC with occasional shaking, treated with 50 µg/ml of mitomycin C for 30 min, and washed three times with RPMI-1640 medium. Effectors and calcein-labeled targets were co-cultured in U-bottom 96-well plates in triplicates for 4 h at 37 °

Table 1. Flow cytometry results for the expression of specific markers in immature and mature DCs derived from ESCC patient.

	Immature DC (%)	Mature DC (%)
CD14	0.07	0.10
CD1a	94.02	90.96
CD80	11.49	39.29
CD86	31.37	78.06
CD83	10.84	58.94
HLA-DR	95.10	64.50

C in a total volume of 200 µl with various effector/target (E/T) ratios i.e. 1:1, 3:1 and 9:1. Supernatant samples were measured using fluorescence spectrophotometer (FP-6200, Jasco, Japan; exciting filter: 485±9 nm; band-pass filter: 530±9 nm). Data were expressed as arbitrary fluorescent units (AFU). Percent cytotoxicity of the assay was calculated by the following formula: [(test release-spontaneous release)/(maximum release-spontaneous release)] × 100. The maximum release and the spontaneous release represent calcein release from the targets in the medium with and without 2% Triton X-100, respectively.

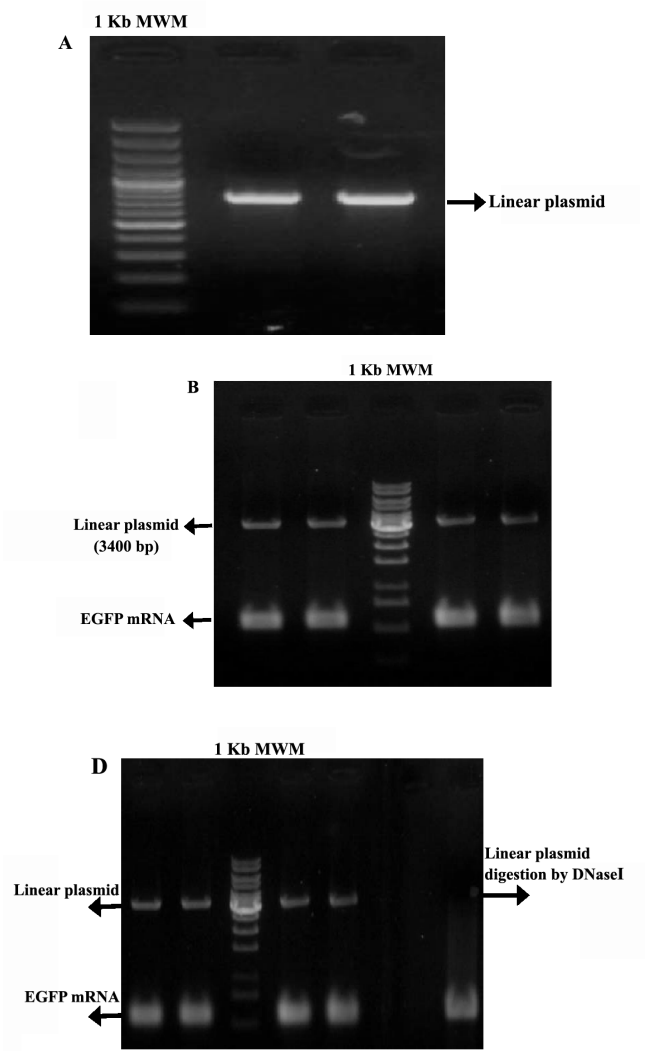

Figure 1. A: EGFP plasmid is digested by SpeI enzyme. The produced linear plasmid is detected as a 3400 bp band after electrophoresis on 1.5% Agaros gel. B: *In vitro* transcription of GFP mRNA is approved with 800 bp band. C: Linear plasmid DNA (3400 bp band) is removed after treatment with DNase I.

Production of monocyte-derived DCs from an ESCC patient was confirmed by flow cytometry for CD1a, CD14, CD80, CD86, CD83, and HLA-DR, acting as specific DC markers. CD14 was decreased to less than 1% and CD1a was increased to the average of approximately 95% representing transformation of monocytes into DCs. After 2 days, FACS analysis was performed to detect specific CD markers of matured DCs. CD83 was increased to average of 58.9% confirming the maturation process. The results of flow cytometry for expression of these markers are shown in Table 1.

***In vitro* transcription of GFP mRNA:** Figure 1 outlines the sequential steps of *in vitro* transcription of GFP mRNA. EGFP plasmid was cloned in *E. coli* and extracted, then followed by a SpeI digestion. Linear plasmid was produced and represented as a 3400 bp band after electrophoresis on 1.5% Agaros gel (Figure 1A). Linear plasmid was applied as a template for the *in vitro* transcription reaction. Transcribed GFP mRNA was visualized as a 800 bp band on electrophoresis gel (Figure 1B) and linear plasmid DNA was removed after digestion with DNase I (Figure 1C). *In vitro* transcription of 1 µg digested plasmid yielded approximately 25 µg mRNA in each reaction.

Optimization of *in vitro* transcribed mRNA transfection into K562 and DC: In order to establish the optimized condition for transferring genes into monocyte-derived DC, the transfection efficacy of *in vitro* transcribed GFP mRNA into K562 cell line was initially investigated. Successful GFP expression, as a reporter gene, was assessed by fluorescent microscopy and FACS analyses. Flow cytometry analyses are shown in Figure 2.

K562: An optimal electroporation condition was achieved as a single pulse 300V, 500 µs for K562. The viability percentage was 95.9% (as compared with 96.8% in the negative control) with approximately 94.4% of viable cells successfully transfected. The introduction efficacy was 90.5%.

DC: Three different conditions were tested in order to

achieve the highest efficacy for gene transfer. The results for a healthy control and ESCC patient are tabulated in Table 2 and flow cytometry results are presented in Figure 3. Approximately similar results were obtained in three conditions with a slightly better efficacy (79.2%) for 500 V/300 μs (Viability percentage and transfection efficacy 95.5% and 82.9% respectively).

DCs derived from an ESCC patient were then subjected to GFP mRNA transfection under the optimized condition. The viability percentage of the population in the procedure was 85.7%, compared with 93.9% for Mock cells. Transfection efficiency was 79.8%. Figure 4 provides the flow cytometry results of the ESCC patient.

Cytotoxicity results: Cytotoxicity of primed lymphocytes (CTL) with tumoral mRNA-loaded DCs derived from the ESCC patient was assessed by measuring the cytotoxicity of CTLs against DC targets loaded with tumoral RNA and mock DC as negative control. Cytotoxicity percentage against DCs loaded with tumoral RNA was 35% while 1% cytotoxicity was yielded with Mock DC ($p<0.001$) (Fig. 5).

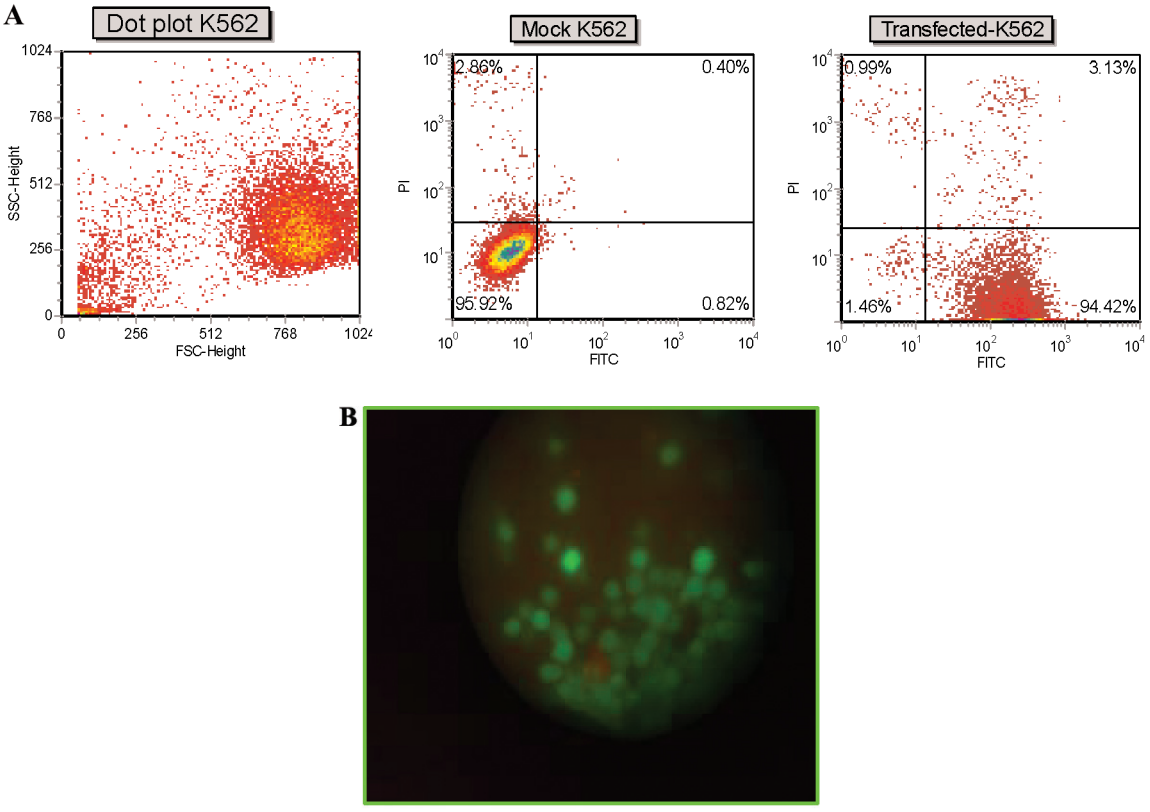

Figure 2. GFP mRNA delivery into K562 cell line using electroporation. A: Viability percentage was 95.9% in transfected cells as compared with 96.8% in Mock control. Transfection efficiency was 94.4%. B: Expression of GFP was also detected with fluorescent microscope.

Table 2. Transfection efficacy and viability percentage of electroporation of DCs in different conditions.

Source of DC	Voltage- Duration	Viability%	Efficiency%	Introduction Efficacy%
Healthy donor	300 V – 500 μs	95.8	81.3	77.9
	500 V – 300 μs	95.5	82.9	79.2
	800 V – 200 μs	92.3	84.9	78.3
ESCC patient	500 V – 300 μs	85.7	79.8	68.4

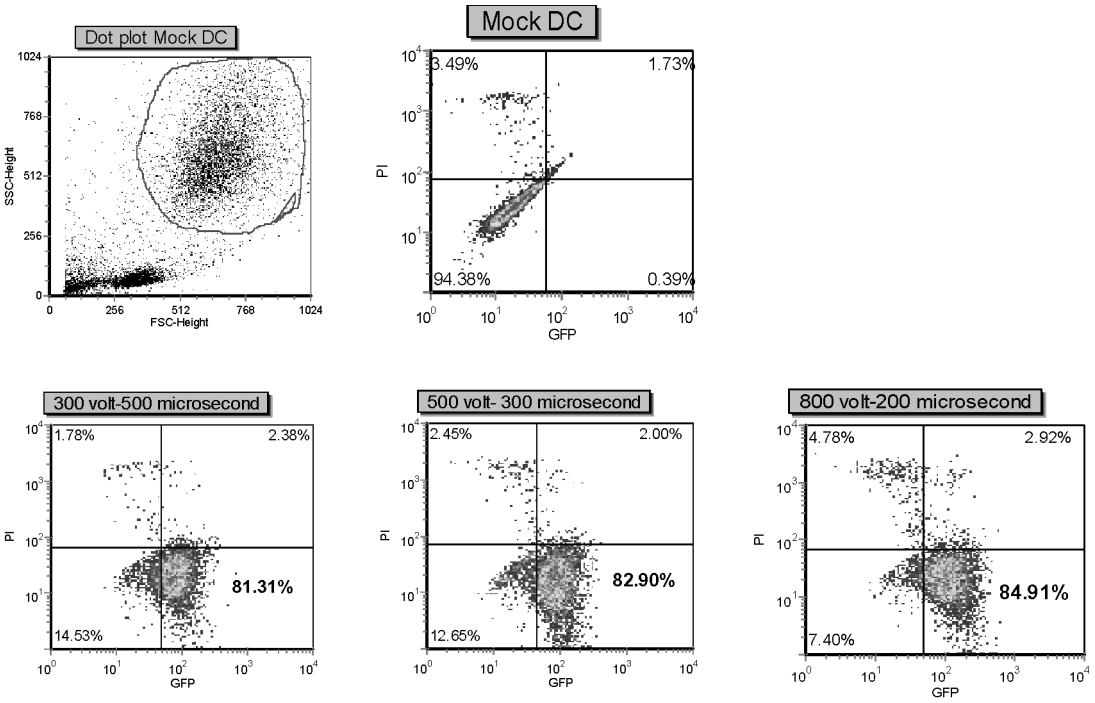

Figure 3. GFP mRNA delivery into DCs derived from a healthy donor with electroporation. FACS analysis of three different parameters is shown with Mock as a control. Viability percentages are 95.8%, 95.5% and 92.3% with 300V, 500V and 800V respectively, in transfected cells as compared with 94.8% in Mock control. Transfection efficiency in transfected cells was 81.3%, 82.9%, 84.9% with 300V, 500V and 800V respectively.

Various cells of the immune system have been targeted for Immuno-genetherapy, and dendritic cells as a powerful arm, are one of the best candidates subjected in many studies. Transfer of genetic material into DCs is a crucial step in the development of DC vaccines.

Electroporation has proved to be a more efficient method of gene transfer than many other techniques such as simple incubation in which prolonged incubation of labile RNA could cause its degradation, or the lipofection method which is also toxic to cells and thereby limits the amount of RNA that can be applied (Mu *et al*., 2003).

Thus, electroporation method was utilized in order to achieve efficient mRNA transfer.

For optimal mRNA transfection by electroporation electric field strength and pulse duration are key parameters in maximizing transfection efficiency and maintaining cell viability. Square wave and exponential decay wave are two different forms through which the electroporation pulse is generated. While exponential decay waves were more frequently used in previous studies, square wave has been shown to result in higher efficiency (Heiser, 2000; Liu and Bergan, 2001). Under the conditions examined, the highest efficiency and viability rates were 84.9% and 95.8% respectively. Different ranges of transfection efficiency were reported in previous studies, varying from 11% to 95% (Mu *et al*., 2003; Van Tendeloo *et al*., 2001; Strobel *et al*., 2000). Van Tendeloo *et al*. first reported highly efficient mRNA transfection of k562 and DCs from normal volunteers and hemochromatosis patients. Applying exponential decay wave in electroporation, they reached to 89% efficiency (85% viability) for k562 and 63±9% efficiency for DCs (Van Tendeloo *et al*., 2001). In the current study, we applied the more efficient method of electroporation i.e. square wave, and achieved higher viability (95% in k562 and healthy donor) and more efficient transfection of DCs (approximately 84% in healthy donor). The introduction efficacy of this study, which includes viability and transfection efficiency, indicated that these results based on the optimal parameters, are among the most efficient conditions reported to date. Moreover, DCs derived from ESCC patients were subjected as the main target for further establishment of DC-based vaccine in future studies on designing immunotherapy of ESCC. Similar promising results were achieved as well in cancer-derived DCs.

The discovery of vaccination with DCs loaded with tumor antigens so as to create a potent immune

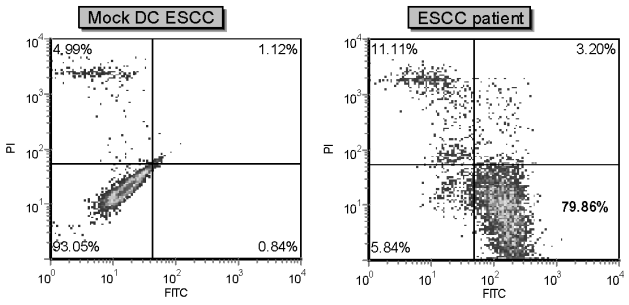

Figure 4. GFP mRNA delivery into DCs derived from an ESCC patient with electroporation (500V, 300 μs). FACS analysis of transfected DC and Mock as a control is shown. Viability percentage was 85.7% in transfected cells as compared with 93.9% in Mock control. Transfection efficiency was 79.8%.

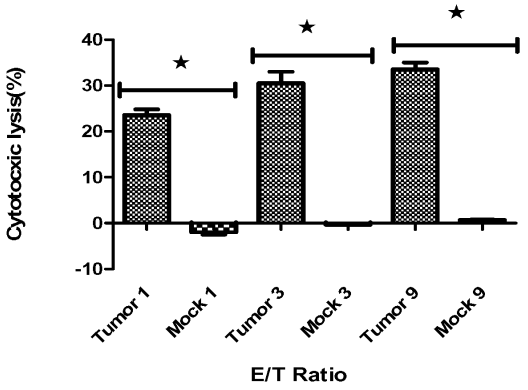

Figure 5. Cytotoxicity results representing cytotoxic activity of CTLs induced by DC/Tumor-mRNA. Cytotoxicity assay was performed against DC/Tumor mRNA, DC/normal mRNA and DC/Mock as targets at various Effector/Target (E/T) ratios assessed by Calcein-AM release assay. Experiments were repeated three times, and representative data of similar results are shown. Cytotoxicity against DC/tumor-mRNA, and DC/Mock was 35% and 1% respectively (* $p < 0.05$).

response, has been reinforced in the field of cancer immunotherapy (Kyte *et al*., 2006). The optimization of parameters for RNA transfection into DCs by electroporation is a major step. Therefore, in this study GFP mRNA was employed as an optimization tool for transfecting DCs. After achieving optimized parameters for the DCs from a healthy donor, the DCs derived from an ESCC patient were transfected. The results were approximately in the same range. In an ESCC patient, autocrine and paracrine cytokines and molecules might affect the function of DCs and make them more vulnerable to physical and chemical stress. The slight difference between a healthy donor and an ESCC patient is probably due to this fact. The optimized transfection of tumoral DC with GFP was then applied to transfect the ESCC-derived DCs with tumor RNA. Enhanced cytotoxicity of CTL against tumor RNA-loaded DCs, while Mock DC did not show any cytotoxicity, approved the optimized method for further establishment of the preclinical phase of DC-based immunotherapy for ESCC.

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References

Boczkowski D, Nair SK, Snyder D, Gilboa E (1996). Dendritic cells pulsed with RNA are potent antigen-presenting cells *in vitro* and *in vivo*. *J Exp Med.* 184: 465-472.

Heiser WC (2000). Optimizing electroporation conditions for the transformation of mammalian cells. *Methods Mol Biol.* 130: 117-134.

Kyte JA, Mu L, Aamdal S, Kvalheim G, Dueland S, Hauser M (2006). Phase I/II trial of melanoma therapy with dendritic cells transfected with autologous tumor-mRNA. *Cancer Gene Ther.* 13: 905-918.

Liu Y, Bergan R (2001). Improved intracellular delivery of oligonucleotides by square wave electroporation. *Antisense Nucleic Acid Drug Dev.* 11: 7-14.

Mu LJ, Gaudernack G, Saeboe-Larssen S, Hammerstad H, Tierens A, Kvalheim G (2003). A protocol for generation of clinical grade mRNA-transfected monocyte-derived dendritic cells for cancer vaccines. *Scand J Immunol.* 58: 578-586.

Ohshita A, Yamaguchi Y, Minami K, Okita R, Toge T (2006). Generation of tumor-reactive effector lymphocytes using tumor RNA-introduced dendritic cells in gastric cancer patients. *Int J Oncol.* 28: 1163-117.

Spiess E, Bestvater F, Heckel-Pompey A, Toth K, Hacker M, Stobrawa G, Feurer T, Watzlaw C, Berchner-Pfannschmidt U, Porwol T, Acker H (2005). Two-photon excitation and emission spectra of the green fluorescent protein variants ECFP, EGFP and EYFP. *J Microsc.* 217: 200-204.

Strobel I, Berchtold S, Gotze A, Schulze U, Schuler G, Steinkasserer A (2000). Human dendritic cells transfected with either RNA or DNA encoding influenza matrix protein M1 differ in their ability to stimulate cytotoxic T lymphocytes. *Gene Ther.* 7: 2028-2035.

Van Bockstaele F, Pede V, Naessens E, Van Coppernolle S, Van Tendeloo V, Verhasselt B, Philippe J (2008). Efficient gene transfer in CLL by mRNA electroporation. *Leukemia* 22: 323-329.

Van Tendeloo VF, Ponsaerts P, Lardon F, Nijs G, Lenjou M, Van Broeckhoven C, Van Bockstaele DR, Berneman ZN (2001). Highly efficient gene delivery by mRNA electroporation in human hematopoietic cells: superiority to lipofection and passive pulsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells. *Blood* 98: 49-56.