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Improved Specificity of Gene Electrotransfer to Skin Using pDNA Under the Control of Collagen Tissue-Specific Promoter

Spela Kos[1] · Natasa Tesic[2] · Urska Kamensek[1] · Tanja Blagus[1] · Maja Cemazar[1,2] · Simona Kranjc[1] · Jaka Lavrencak[3] · Gregor Sersa[1]

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Abstract In order to ensure safe, efficient and controlled gene delivery to skin, the improvement of delivery methods together with proper design of DNA is required. Non-viral delivery methods, such as gene electrotransfer, and the design of tissue-specific promoters are promising tools to ensure the safety of gene delivery to the skin. In the scope of our study, we evaluated a novel skin-specific plasmid DNA with collagen (COL) promoter, delivered to skin cells and skin tissue by gene electrotransfer. In vitro, we determined the specificity of the COL promoter in fibroblast cells. The specific expression under the control of COL promoter was obtained for the reporter gene DsRed as well as for therapeutic gene encoding cytokine IL-12. In vivo, the plasmid with COL promoter encoding the reporter gene DsRed was efficiently transfected to mouse skin. It resulted in the notable and controlled manner, however, in lower and shorter expression, compared to that obtained with ubiquitous promoter. The concentration of the IL-12 in the skin after the in vivo transfection of plasmid with COL promoter was in the same range as after the treatment in control conditions (injection of distilled water followed by the application of electric pulses). Furthermore, this gene delivery was local, restricted to the skin, without any evident systemic shedding of IL-12. Such specific targeting of skin cells, observed with tissue-specific COL promoter, would improve the effectiveness and safety of cutaneous gene therapies and DNA vaccines.

Keywords Skin gene electrotransfer · Skin-specific promoter · Collagen promoter · Interleukin-12 · Electroporation · Gene delivery

✉ Gregor Sersa
gsersa@onko-i.si

[1] Department of Experimental Oncology, Institute of Oncology Ljubljana, Zaloska 2, 1000 Ljubljana, Slovenia

[2] Faculty of Health Sciences, University of Primorska, Polje 42, 6310 Izola, Slovenia

[3] Department of Cytopathology, Institute of Oncology Ljubljana, Zaloska 2, 1000 Ljubljana, Slovenia

Introduction

Skin is an attractive target for the gene therapy and vaccination, due to its accessibility, large surface area and numerous antigen presenting cells that are critical to elicit an effective immune response (Gothelf and Gehl 2010). To enhance the efficiency, specificity and temporal control of cutaneous gene therapy, and to ensure the safety of DNA vaccines, improvement of delivery methods together with proper design of the DNA plasmid itself is required (Glenting and Wessels 2005; Niidome and Huang 2002).

Non-viral gene delivery methods could circumvent some of the safety issues occurring with viral vectors such as endogenous viral recombination, systemic toxicity, oncogenic effects and unexpected immune response (Niidome and Huang 2002; Young et al. 2006). One of the most efficient and promising methods of non-viral gene delivery to target tissues is gene electrotransfer (Yarmush et al. 2014). It is based on electroporation, i.e. application of controlled electric pulses to cells or tissues, which increases the cell membrane permeability and allows normally non-permeant molecules to enter the cells (Orlowski et al. 1988). The method has been used to deliver genes to many tissues (Gothelf and Gehl 2010), including muscle (Aihara and Miyazaki 1998; Hojman et al. 2009; Muramatsu et al. 2001; Tevz et al. 2009; Todorovic et al. 2014), cornea (Blair-Parks et al. 2002), lung (Dean et al. 2003),

liver (Heller et al. 1996), kidney (Tsujie et al. 2001), testis (Widlak et al. 2003), bladder (Iwashita et al. 2004), tumour (Cemazar et al. 2002; Dolinsek et al. 2013; Kamensek et al. 2013; Lucas et al. 2002; Niu et al. 1999; Rols et al. 1998; Tevz et al. 2009) and skin (Drabick et al. 2001; Heller et al. 2001; Pavselj and Preat 2005). Electroporation into skin was tested both, for the topical delivery of small molecules (Denet et al. 2004) as well as for plasmid DNA (pDNA) (Gothelf et al. 2010; Heller et al. 2010; Pavselj and Preat 2005; Vandermeulen et al. 2007) or short interfering RNA molecules (siRNA) (Broderick et al. 2012) following intradermal injection, without any significant alteration of skin structure (Blagus et al. 2013; Guo et al. 2011).

Studies imply that cells transfected by skin gene electrotransfer reside in different skin regions: in epidermis (Guo et al. 2013; Lin et al. 2001; Pavselj and Preat 2005), in dermal layer (fibroblasts and mononuclear cells) (Drabick et al. 2001; Lin et al. 2001; Pavselj and Preat 2005; Zhang et al. 2002) and even in the hypodermis and subcutaneous muscle layer (Roos et al. 2009). The transfection to the specific region of the skin depends on the electrode selection, electrical parameters, injection technique, animal species, dose of plasmid administered and the plasmid design (Guo et al. 2013). Furthermore, effective gene transfer to multiple cells and cell types within the electric field can pose a problem in terms of cell specificity of gene delivery. The ability to restrict gene delivery and its expression to particular cell type has great importance for various types of gene therapy. The ectopic expression of a transgene could lead to a deleterious host inflammatory responses or dysregulation of normal cell function (Dean 2013; Sardesai and Weiner 2011; Schalk et al. 2006). Therefore, multiple strategies for cell-specific targeting of genes are currently being developed (Dean 2013). An attractive strategy to improve the specificity and safety of gene therapy and vaccination is the design of plasmids containing tissue-specific promoters (Papadakis et al. 2004; Zheng and Baum 2008), which can lead to targeted transcription of cells. So far, different skin-specific promoters, which restrict transfection to epidermal keratinocytes or induce the gene expression of mature dendritic cells in epidermal and dermal layers, have been evaluated (Lin et al. 2001; Vandermeulen et al. 2009). To specifically target predominantly fibroblasts, an attractive skin-specific promoter would be the one that is under the control of the collagen type I transcription factors.

To restrict gene delivery to the skin, we constructed a plasmid DNA with collagen skin-specific promoter. With the aim to determine its cell specificity, we compared its transfection efficiency in different cell types in vitro. Furthermore, in vivo by gene electrotransfer to the mouse skin, we tested the specificity and the level and duration of its expression. To provide harmless skin gene delivery (Blagus et al. 2013; Guo et al. 2011; Heller et al. 2010), non-invasive multi-array electrodes were used. The results of the study show that gene electrotransfer of pDNA under the regulation of COL promoter is controlled, localized and effective approach for gene therapy to skin and DNA vaccination.

Materials and Methods

Cell Lines

Murine endothelial cell lines SVEC4-10 and bEnd-3 (American Type Culture Collection, Manassas, VA, USA) were cultured in advanced Dulbecco's modified Eagle medium (DMEM, Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 5 % FBS (Life Technologies), 10 mM/L L-glutamine (Life Technologies), 100 U/mL penicillin (Grünenthal, Aachen, DE) and 50 mg/mL gentamicin (Krka, Novo mesto, SI) in a 5 % CO_2 humidified incubator at 37 °C. Murine fibroblasts L929 and 3T6 (American Type Culture Collection) were cultured in advanced minimum essential medium (AMEM, Life Technologies) supplemented with 5 % FBS, 10 mM/L L-glutamine, 100 U/mL penicillin and 50 mg/mL gentamicin in a 5 % CO_2 humidified incubator at 37 °C. For experiments, cells were maintained in monolayers until they reached 80–90 % confluence.

Animals and Skin Preparations

All procedures were performed in compliance with the guidelines for animal experiments of the EU directive (2010/63/EU) and the permission from the Veterinary Administration of the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (permission no. 34401-4/2012/4). In the experiments, 8–12-week-old female Balb/c mice (Harlan Laboratories, Udine, IT), weighing between 20 and 25 g, were used. 3–5 mice were randomly assigned per each experimental group. Animals were housed in a specific pathogen-free condition with 12-h light cycles and provided food and water ad libitum. One day prior to the experiments, mice were shaved on the left and/or right flank and any remaining hair was removed by depilatory cream (Veet® Sensitive Skin, Reckitt Benckiser, UK). Before the plasmid administration, mice were anaesthetized in the induction chamber using 2 % isoflurane (Nicholas Piramal India, London, UK) in oxygen and remained under anaesthesia during the procedure.

Plasmids

Plasmids encoding the *Discosoma* red fluorescent protein DsRed (pCOL-DsRed) and interleukin-12 (pCOL-mIL-12) under the control of the COL promoter were constructed.

Their transfection efficiency and specificity were compared to two ubiquitous promoters: strong cytomegalovirus (CMV) promoter and the hybrid promoter for elongation factor-1α and human T cell leukaemia virus (EF-1α/HTLV).

The plasmids were prepared by standard molecular cloning methods of restriction and ligation. Source plasmid for COL promoter (plasmid pDD424) was a kind gift from Prof. David A. Dean (University of Rochester, Medical Center, NY, USA). The source of the DsRed reporter gene was pCMV-DsRed-Express2 (Clontech, Basingstoke, UK), and the source of the mouse IL-12 gene was pORF-mIL-12 (p40::p35) (Invivogen, Toulouse, FR), containing both subunits of IL-12 linked together with elastin linker. For construction of eukaryotic expression plasmids, a plasmid encoding enhanced green fluorescence protein (EGFP) with no CMV promoter (pEGFPnoCMV), the gift from Claudia Karl (Medical Center, University of Munich, Munich, DE), was used as a vector. Reporter plasmid with DsRed gene under the transcriptional control of a collagen promoter (pCOL-DsRed) was prepared by subcloning the sequence for COL promoter from pDD424 into the pEGFPnoCMV vector and then replacing the EGFP sequence with DsRed sequence. The therapeutic plasmid encoding mouse IL-12 under the transcriptional control of the COL promoter (pCOL-mIL-12) was prepared by replacing the EGFP sequence with an IL-12 sequence from the pORF-mIL-12 (p40::p35) plasmid. *Escherichia coli* strains JM107 and GM2163 (Thermo Scientific Molecular Biology, Vilnius, Lithuania) were transformed with prepared plasmids using the TransformAid Bacterial Transformation Kit (Thermo Scientific) according to the manufacturer's instructions. The identity of the newly constructed plasmids was confirmed by restriction analysis and sequencing.

For the experiments, all plasmids were isolated using the EndoFree Plasmid Mega Kit (Qiagen, Hilden, DE) according to manufacturer's instructions and diluted in endotoxin-free water to a concentration of 1 mg/mL, for in vitro analysis and to a concentration of 2 mg/mL, for in vivo experiments. Plasmid DNA concentration and pureness were determined spectrophotometrically (Epoch Microplate Spectrophotometer; Take3TM Micro-Volume Plate, BioTek, Bad Friedrichshall, DE) and by gel electrophoresis.

In Vitro Gene Electrotransfer

For in vitro gene electrotransfer, endothelial cells (SVEC4-10, b-End3) and fibroblasts (L929, 3T6) were trypsinized, washed with appropriate media, and centrifuged for 5 min at 4 °C at 1500 rpm. The cell suspension was prepared in ice-cold electroporation buffer (EP buffer: 125 mM sucrose, 10 mM K_2HPO_4, 2.5 mM KH_2PO_4, 2 mM $MgCl_2 \times 6H_2O$). The pH of EP buffer was 7.2, conductivity 2.1 mS/cm and osmolarity 160 mOsm/kg. Cell suspension (1×10^6 cells in 40 μL) was mixed with 10 μg/10 μL of plasmid DNA (pCMV-DsRed, pCOL-DsRed, pORF-mIL-12, pCOL-mIL-12) or endotoxin-free water, and the prepared mixture was pipetted between two stainless steel parallel plate electrodes with a 2-mm gap. Electroporation of cell suspension was performed with eight square-wave-shaped electric pulses with duration of 5 ms and frequency of 1 Hz at different amplitudes of electric pulses; 100 V (500 V/cm) was used for L929, SVEC4-10, b-End3 and 120 V (600 V/cm) for 3T6 based on our preliminary results. Pulses were generated by electric pulse generator GT-01 (Faculty of Electrical Engineering, University of Ljubljana, Ljubljana, SI). Immediately after electroporation, the cells were incubated for 5 min with 100 μL of 100 % FBS and plated in their corresponding medium for further assays.

In Vitro Transfection Efficiency

The transfection level and the fluorescence intensity of DsRed protein were quantified by flow cytometry. For the analysis, cells were trypsinized 48 h after transfection and centrifuged for 5 min at 25 °C at 1500 rpm. Cells were then resuspended in 200 μL of PBS, transferred to polystyrene round-bottom tubes (Becton–Dickinson, San Jose, CA, USA) and analysed by FASCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). To eliminate debris, 40,000 cells were first gated, and afterward histogram of gated cells against their fluorescence intensity was recorded. A number of fluorescent cells and their median fluorescence intensity were determined by BD FACSDiva V6.1.2 software. As a negative control, untreated, non-exposed cells to electroporation or plasmid DNA were used. Median fluorescence intensities of treatment groups were normalized to the median fluorescence of the control group. The experiments were repeated three times independently.

In Vitro Quantification of IL-12 Protein Concentration by Enzyme-Linked Immunosorbent Assay (ELISA)

To determine the IL-12 protein concentration in cell culture medium, ELISA Quantikine Mouse IL-12 p70 Immunoassay (R&D Systems, Minneapolis, MN, USA) was performed according to the manufacturer's instructions. Briefly, 48 h after gene electrotransfer of plasmids pCOL-mIL-12 and pORF-mIL-12, the whole media from cultured transfected SVEC4-10 and L929 cells was removed and centrifuged, and aliquots were stored at −80 °C for further processing. For the analysis, 50 μL of 100× diluted media, removed from cells transfected with pORF-IL-12, and 50 μL of undiluted media removed from the cells transfected

with pCOL-mIL-12, was assayed in duplicates. Absorbance was measured at 450 and 540 nm wavelengths using a microplate reader Tecan Infinite 200 (Tecan, Mannedorf, CH). Concentrations of IL-12 were determined from the slope of the standard curve and calculated as pg of IL-12 per mL of media.

In Vivo Gene Electrotransfer

Anaesthetized mice received a single intradermal injection of pCMV-DsRed (2 mg/mL) or pCOL-DsRed (2 mg/mL) in the volume of 50 μL in the right or/and left flank. Single injection was used over multiple, ensuring better control of the injected plasmid DNA (less leakage) and thus providing higher reproducibility of the results. For the plasmid administration, 29 G insulin grade syringe was used. Immediately after plasmid administration, high-voltage electric pulses with the amplitude of 570 V (1600 V/cm) and the duration of 100 μs were applied. The selected electrical parameters differ from that used for cells in vitro and were chosen according to the previous experience on skin delivery (Blagus et al. 2013). Gene electrotransfer was performed using the non-invasive multi-array electrodes (Iskra Medical, Podnart, SI) consisting of 7 spring loaded pins arranged in a hexagonal mesh and spaced 3.5 mm between each other. Electrodes were connected to the CLINIPORATOR^{TM} (IGEA s.r.l., Carpi, IT). A total of 24 electric pulses (2 electric pulses between each electrode pair) were delivered during the treatment. Expression of DsRed was followed under the Zeiss SteREO Lumar.V12 (Zeiss, Jena, DE) fluorescence stereomicroscope equipped with an MRc.5 digital camera (Zeiss). Mice were initially anaesthetized with inhalation anaesthesia in the induction chamber (2 % of isoflurane) and placed under the microscope, with their snout in the inhalation tube to remain anaesthetized during the measurement procedure. The first image was taken 24 h after gene electrotransfer, and the rest of them daily, until the fluorescence was undetectable. For the images, a suitable threshold was applied and the fluorescence intensity in the area of the electroporated skin was determined by means of AxioVision (Zeiss).

Quantification of IL-12 Protein Concentration

The concentration of cytokine IL-12 was measured in excised skin tissue and serum samples. Mice received multiple intradermal injections (4 × 20 μL) of pCOL-mIL-12 or pORF-mIL-12 in the left or right flank to ensure the detection of the proteins in the samples. Immediately after plasmid administration, electric pulses were applied, as described above. The control group received the intradermal injection of saline, followed by pulse application. Treated region of the skin was excised 48-h post-treatment, immediately weighted and snap frozen in liquid nitrogen. Frozen samples were mechanically macerated. Each sample was diluted in 500 μL of PBS containing protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche, Basel, CH), thoroughly mixed and centrifuged for 10 min at 3000 rpm. The supernatant was separated from the sediment and stored at −80 °C until analysis. Blood was collected from the intraorbital sinus into a blood collection tube (Vacuette serum tube with gel; Greiner Bio-One International AG, Kremsmünster, AU) and stored at 4 °C for 20 min until coagulated. Serum was extracted from blood samples by centrifugation at 2500 rpm for 5 min and immediately stored at −80 °C until further analysis. Both sets of samples were analysed using ELISA assay (R&D Systems). Due to highly comparable mass of samples, we presented the concentration of IL-12 as pg of IL-12 per mL of serum or mL of supernatant.

Statistical Analysis

For statistical analysis, Sigma Plot software (Systat software, London, UK) was used. Significance was determined by Student's *t* test or one-way analysis of variance (ANOVA) followed by Holm–Sidak test. Statistical significance was assumed at $P < 0.05$. The values were expressed as the arithmetic mean (AM) ± standard error of the mean (SEM).

Results and Discussion

Construction of the Plasmid with COL Promoter

Recombinant plasmids encoding DsRed and mIL-12 under the transcriptional control of COL promoter were successfully constructed (described in section of Materials and Methods), which was confirmed by restriction analysis (Fig. 1). The expression levels of plasmids with similar molecular weight were compared: for the expression efficiency and specificity of reporter gene, we compared pCMV-DsRed (4638 bp, 32.7 pmol) with pCOL-DsRed (4428 bp, 34.2 pmol), and for the therapeutic IL-12 gene, the comparison between pORF-mIL-12 (5840 bp, 25.9 pmol) and pCOL-mIL-12 was taken (5395 bp, 28.1 pmol). Due to similar molecular weight of both pairs of plasmids, all animals were treated with the same amount of plasmid DNA.

In Vitro Cell Specificity of Reporter Plasmid with COL Promoter

To evaluate the strength and specificity of COL promoter, the plasmid DNA, encoding DsRed protein, was electrotransfected into the two cell types: into fibroblasts that have a high level of collagen gene activators and into endothelial cells

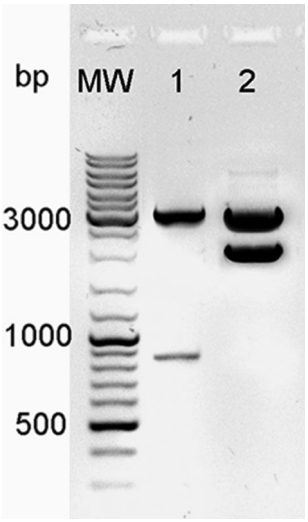

Fig. 1 Confirmation of successful construction of the plasmids using restriction analysis. Newly constructed plasmids pCOL-DsRed (*lane 1*) and pCOL-mIL-12 (*lane 2*) were cut with different combinations of restriction enzymes, with restriction sites in different parts of the construct (within the vector and the insert). The identity of the plasmid was confirmed by comparing the pattern of bands on the electrophoresis gel to the expected bands. *Lane MW* GeneRuler^{tm} DNA ladder mix (Fermentas); *lane 1* pCOL-DsRed cut with *SalI* and *MunI* restriction enzymes (Fermentas), expected bands: 3604 and 824 base bp (base pairs); *lane 2* pCOL-mIL-12 plasmid cut with *XhoI* and *MunI* restriction enzymes: expected bands, 3257 and 2164 bp

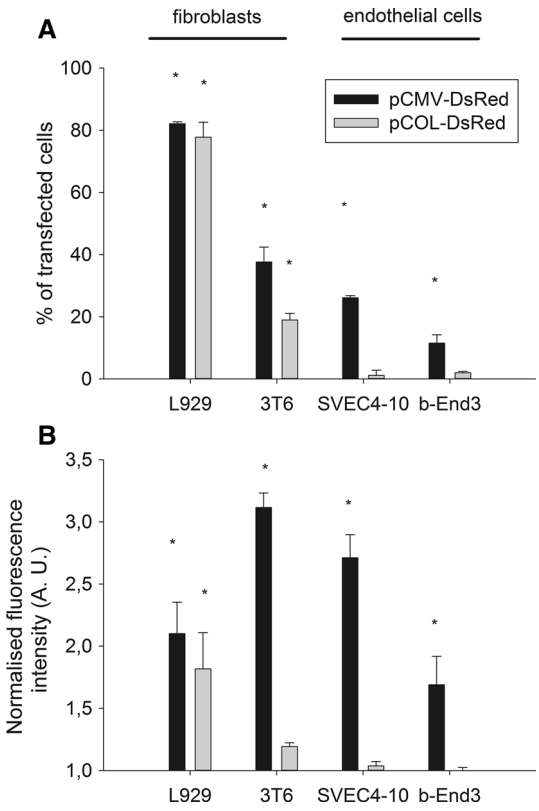

Fig. 2 Transfection level and the fluorescence intensity in fibroblasts and endothelial cells. **a** Percentage of the cells expressing DsRed protein. The results represent the transfection efficiency under the optimal electric conditions. $*P < 0.05$ compared to the control group of untreated cells with 0 value of transfection efficiency. **b** The fluorescence intensity of fibroblasts and endothelial cells. Fluorescence intensity is expressed as the median fluorescence intensity in treated cells normalized to the median fluorescence in untreated cells. $*P < 0.05$ compared to the control group of untreated cells. *Error bars* indicate SEM. Results represent three independent experiments, each containing three samples

with lower levels of activators of COL promoter. Differential transfection efficiency of plasmid DNA encoding DsRed under the control of COL and CMV promoters in fibroblasts and endothelial cells was determined by flow cytometry 48 h after the gene electrotransfer, by comparing the percentage of the cells expressing DsRed (Fig. 2a). Transfection efficiency with pCOL-DsRed was most pronounced in L929 cells (77.8 %); furthermore, approximately 4-times lower expression of the DsRed protein under the control of tissue-specific promoter COL in 3T6 (19.0 %) cells was observed, while in both endothelial cell lines, only up to 2 % cells expressed DsRed protein.

Cytomegalovirus (CMV) promoter is a strong viral promoter that induces expression in a wide range of cell types (Kamensek et al. 2011). Due to its widespread transfection efficiency, the gene electrotransfer with pCMV-DsRed resulted in a high percentage of transfected fibroblasts (up to 85 % in L929 cells) as well as the endothelial cells (up to 30 % in SVEC4-10 cells). On the contrary, skin-specific promoter COL was activated in fibroblasts, without any significant expression in endothelial cells. Furthermore, these results were supported by the fluorescence intensity measurements (Fig. 2b), which represent the amount of protein present in cells (Bosnjak et al. 2014). We obtained 1.8 ± 0.30-fold increase of fluorescence intensity in L929 fibroblasts compared to control group, while the fluorescence intensity of endothelial cells did not differ between electrotransfected group and the control group. A general increase of fluorescence intensity of endothelial cells and fibroblasts after the transfection of plasmid with the ubiquitous promoter and the specific transfection of fibroblasts after the gene electrotransfer of plasmid DNA under the control of COL promoter indicate that this plasmid promoted the expression in skin cells, while it did not affect the protein expression in the non-specific endothelial cell. Therefore, the constructed plasmid showed highly fibroblast-specific activity in vitro, indicating the potential cell specificity also in vivo in mouse skin.

Cell Specificity of IL-12 Therapeutic Plasmid with COL Promoter

As demonstrated, the COL promoter specifically induced the expression of the DsRed reporter gene in fibroblast cells. To confirm these results, a plasmid DNA with the

therapeutic gene for IL-12 was constructed, and again, tested for the cell specificity. It was tested in the two cell lines that provided the highest transfection efficiency with the reporter gene (fibroblasts L929 and endothelial cells SVEC4-10). Reporter genes, such as green fluorescent protein (GFP), red fluorescent protein (DsRed), luciferase or beta-galactosidase, are widely used in skin gene transfer (Gothelf and Gehl 2010). With these plasmids, it is possible to quantify and visualize the extent and the amount of protein expression and to a certain degree predict the expression of the therapeutic gene. Since the reporter genes encode the exogenous products, the level of expression could vary, due to different biological effects, such as methylation of the viral promoter (Kamensek et al. 2011) and also in vivo immune response to reporter protein (Guo et al. 2011; Stripecke et al. 1999; Vandermeulen et al. 2009). These are most likely the cause of the change in expression patterns and could lead to unpredicted expression profile. Therefore, the important step in plasmid DNA optimization and evaluation is the translation from the use of reporter genes to the therapeutic system.

In order to test the specificity and efficiency of COL promoter to drive the therapeutic gene expression, the concentration of IL-12 protein in the supernatant was measured 48 h after the gene electrotransfer of pDNA. As expected, IL-12 levels in supernatants of the both cell lines were higher after gene electrotransfer of pDNA under the control of a ubiquitous promoter, compared to the cells transfected with plasmid with tissue-specific promoter. Several lines of evidence demonstrate that ubiquitous promoters are stronger promoters than tissue-specific promoter (Nakamura et al. 2008; Vandermeulen et al. 2009). Nevertheless, the specificity of the COL promoter was demonstrated by the measurement of IL-12 in supernatant of 2 different cell lines, fibroblast (L929 cells) and endothelial cells (SVEC4-10). The concentration of IL-12 protein was statistically higher in supernatant of L929 cells transfected with pCOL-mIL-12 compared to the concentration of IL-12 protein in SVEC4-10 cells (Fig. 3), whereas no difference in the concentration of IL-12 protein in the supernatant of the two cell lines transfected with plasmid DNA containing a ubiquitous promoter was observed. Our results demonstrate the specific transfection of fibroblasts with the pDNA with COL promoter. Thus, the evidence provided with plasmid coding for the reporter protein was confirmed by the plasmid encoding the therapeutic gene IL-12. The importance of the IL-12 in cancer therapy (Pavlin et al. 2011), predominantly in skin cancer, like melanoma, is promising (Cemazar et al. 2010; Cha and Daud 2012; Daud et al. 2008). Therefore, the translation from the use of the reporter genes to a therapeutic system with verification of the specificity is of utmost importance and sets the stage for the in vivo evaluation.

Expression of Plasmids in the Mouse Skin

In order to translate in vitro data, an in vivo experiment was performed to demonstrate the expression level of plasmid DNA and its duration in mouse skin. DsRed expression after single injection of plasmid DNA, to ensure high reproducibility, followed by electrotransfer was monitored by non-invasive fluorescence imaging. Using the ubiquitous CMV promoter, the expression peaked on the second day after the treatment, and then started to decline in the next 2 weeks (Fig. 4). The steady regression of fluorescence intensity after second day post-treatment could be caused by methylation of the CMV promoter causing the transcriptional inactivation (Kamensek et al. 2011). Beside the unspecific expression in the wide range of cell types, the graduate inactivation of CMV promoter could be the limitation in terms of controlled gene delivery. Using the tissue-specific promoter COL, the onset of gene expression was detected at the third day post-treatment with approximately fourfold lower fluorescence intensity, compared to the ubiquitous promoter-regulated expression. The signal was detected up to a week post-treatment with the steady fluorescence intensity. This might indicate on lower susceptibility of the COL promoter to inactivation by methylation. Despite the presumed absence of methylation effect, the use of plasmid with tissue-specific promoter resulted in lower and shorter expression (Fig. 5), but still notable, localized and in more controlled manner, as compared to that obtained with ubiquitous promoter. In addition, lower rate of transfection under the tissue-specific promoter could be due to the fact that different or more specific cells were targeted (Gothelf and Gehl 2010). Namely, if COL promoter targeted just the specific skin cells, intensity of measured fluorescence is consequently lower due to the smaller percentage of cells expressing the DsRed protein.

Knowledge of the tissue localization, the magnitude and persistence of gene expression will allow better application of gene therapy protocols. The use of reporter genes, such as DsRed, is an essential tool for the in vivo evaluation of therapeutic gene delivery. However, the expression efficiency of reporter gene is not necessarily consistent with therapeutic gene transfection and its effectiveness. As was described above, the translation of the use of reporter genes to therapeutic system is of utmost importance for successful evaluation of plasmid effectiveness. Therefore, the aim of the further experiments was to compare the specificity and efficiency of tissue-specific and ubiquitous plasmids encoding therapeutic gene for interleukin 12 (IL-12).

Localized and Systemic Distribution of IL-12

Since the plasmid with COL promoter transfects fibroblasts with reasonably high specificity, we presumed that the

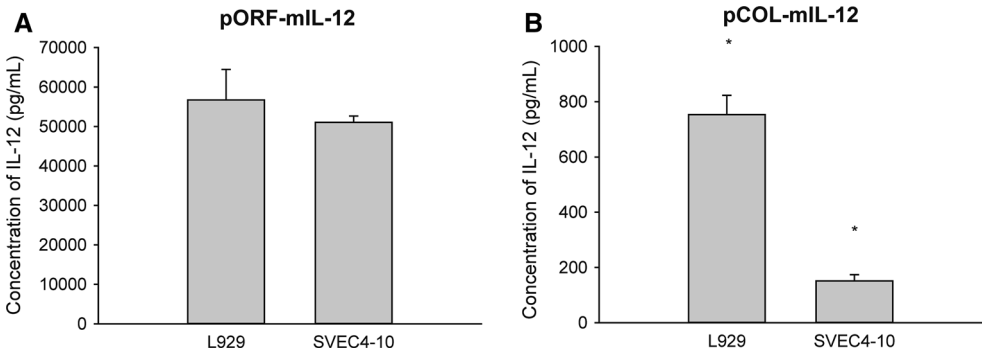

Fig. 3 Concentration of protein IL-12 (pg/mL) in L929 and SVEC4-10 cells. Concentration was measured in extracted cell medium 48 h after gene electrotransfer of (**a**) pORF-mIL-12 and (**b**) pCOL-mIL-12. $^*P < 0.05$ between L929 and SVEC4-10 cell lines. The results represent three independent experiments, each containing four samples. *Error bars* indicate SEM

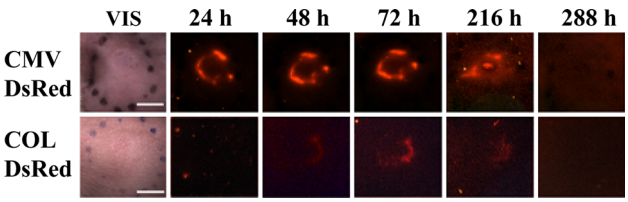

Fig. 4 Expression of red fluorescence protein in the skin under the control of ubiquitous or tissue-specific promoter. After single intradermal injection of plasmid DNA, followed by electric pulse application, fluorescence signal was observed under fluorescence stereomicroscope at various time points. For the best transparency, the images are presented under different exposure criteria, best fitted for each image. *Scale bar* 5 mm. VIS images were taken under visible light

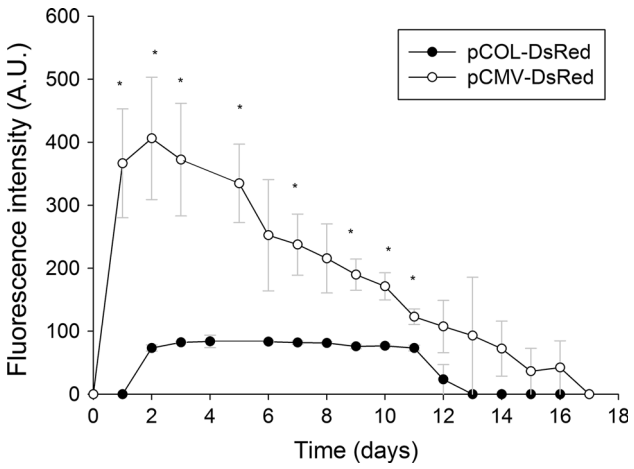

Fig. 5 Fluorescence intensity of DsRed protein expressed in the skin at different time points. Fluorescence was measured after single intradermal injection of pCOL-dsRed or pCMV-DsRed in the volume of 50 µL, followed by application of electric pulses (570 V/100 µs). To measure fluorescence intensity, images were analysed under the same exposure criteria. $n = 3$–5 mice per group. $^*P < 0.05$ between the groups treated with ubiquitous or COL promoter. *Error bars* indicate SEM

transgene expression would be predominantly localized, restricted to the skin and controllable, meaning that its action would be paracrine. To explore this, an experiment was performed where we measured the level of expressed cytokine IL-12 locally in the skin, and followed the systemic release of the protein in the mouse serum. The levels of the IL-12 in the skin and serum samples after the delivery of plasmid DNA encoding IL-12 under the control of COL and ORF promoters into the mouse skin were measured using the ELISA assay. In the excised skin tissues, IL-12 was detected at the concentrations of 788.7 ± 111.0 pg/mL, when the expression was controlled under the ubiquitous promoter. The expression of IL-12 under the COL promoter was measured in the concentration of 97.8 ± 21.6 pg/mL (Fig. 6a). The results indicate significant, up to 8 times lower production of IL-12 after the delivery of the plasmid DNA with COL promoter compared to a strong ubiquitous promoter, similar to the in vitro results. However, in these experiments, we could not demonstrate significantly higher production of IL-12 in the skin with pCOL-mIL-12 compared to the control. This might be due to the fact that electroporation can cause an inflammation reaction, attracting immune cells, which consequently leads also to the production of IL-12. This was already demonstrated in muscle and skin tissues, however, using different types of electrodes (Chiarella et al. 2008; Markelc et al. 2012). On the other hand, these results also indicate that there is some local production of IL-12 in the skin that could be beneficial for boosting the immune response, when applied in combination with local ablative techniques, such as electrochemotherapy.

Cytokine IL-12 was measured also in the serum samples (Fig. 6b), as an indicator of the systemic shedding of the protein encoded by transgene from the skin. The difference in the concentrations of IL-12 in the systemic circulation between the ubiquitous and tissue-specific pDNA was observed. Namely, the cytokine IL-12 was not detected in the serum samples collected 48-h post-treatment with

tissue-specific plasmid, while it was measured in low, but significant amount after delivery of plasmid with the ubiquitous promoter in the skin. The very low level of the IL-12 in the serum after pCOL-mIL-12 plasmid could be due to the low expression of the IL-12 in the different types of skin cells, or to the fact that the IL-12 was expressed only in the target cells, i.e. fibroblasts, as can be speculated based on our in vitro results. Indeed, the in vitro data support the notion that pCOL-mIL-12 is expressed in fibroblasts and though its expression is in more controllable fashion compared to the pORF-mIL-12. However, to demonstrate this at the skin level, a detailed histological analysis should be performed. Thus, we speculate that more controllable and localized expression of the transgene could be obtained using COL promoter for the plasmid construction, which may be in favour in certain situations.

In therapeutic constructs, it is extremely important to precisely regulate the transcriptional activity of gene expression system in order to ensure the safety of gene-therapeutic drug for normal tissue (Kuzmin et al. 2010) or restrict the local effect of gene delivery in the skin. Specific targeting of skin cells is essential for the delivery of DNA vaccines to cutaneous antigen presenting cells to elicit the local immune response without the systemic toxicity, such as development of systemic auto-immune reactions or modulation of immune response (Glansbeek et al. 2002; Schalk et al. 2006). So far, different therapeutically relevant plasmids have been transfected to the skin. They were delivered to skin cells in order to treat malignant melanoma (Daud et al. 2008), to improve wound repair (Ferraro et al. 2009; Marti et al. 2004; Steinstraesser et al. 2014) or as DNA vaccines to elicit immune response to an antigen (Donate et al. 2011; Drabick et al. 2001; Vandermeulen et al. 2007).

One of the advantages of ubiquitous promoters is their high transfection efficiency compared to tissue-specific promoters (Papadakis et al. 2004), as was obtained also in our in vitro and in vivo experiments. However, high level of transgene production is not always beneficial for successful gene therapy. Specifically, for IL-12, it was demonstrated that high local gene expression did not contribute to the better therapeutic outcome of melanoma treatment (Shirley et al. 2014). This suggests that high amount of expressed protein is not always crucial for effective therapeutic result. In the scope of our study, we can conclude that the low expression level of tissue-specific plasmid in skin is not a drawback, since the tissue-specific promoter for the plasmid enables the cell-specific transgene expression, with controlled and localized effectiveness.

Furthermore, the use of non-invasive multi-array electrodes is also of advantage for the cutaneous gene therapy, due to non-invasiveness and almost without damage to the skin. Minimal skin damage was testified as well as complete recovery after gene electrotransfer was observed (Guo et al. 2011). Gene electrotransfer with the multi-array electrodes greatly reduced the muscle contraction with comparable or even higher gene expression levels compared to needle or plate electrodes (Donate et al. 2011; Guo et al. 2011). They can be used for drug delivery after topical administration, as we have already demonstrated (Blagus et al. 2013), as well as for gene delivery, presented in this study. The use of such electrodes is thus warranted in further studies of gene electrotransfer to the skin.

In conclusion, this study indicates on more controlled and localized expression of the plasmid with tissue-specific promoter, such as collagen promoter, predominantly expressed in fibroblasts. Skin gene electrotransfer proved to be effective for both the plasmid DNA with ubiquitous and

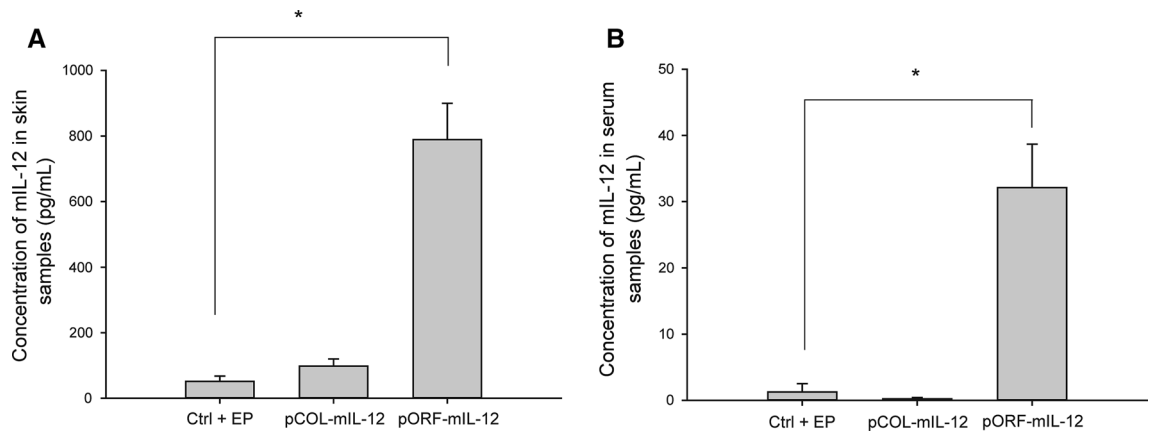

Fig. 6 Concentration of IL-12 (pg/mL). **a** Concentration of IL-12 (pg/mL) measured in skin samples. **b** Concentration of IL-12 (pg/mL) measured mouse serum. For the detection, ELISA analysis was performed. $*P < 0.05$ compared to the control group of samples without plasmid DNA delivery, only with EP application. $n = 4$–6 samples per each experimental group. *Error bars* indicate SEM

tissue-specific promoters, for the reporter gene and also therapeutic IL-12 gene. The lower expression of the plasmid DNA with the tissue-specific promoter was localized to the skin with paracrine action and no systemic shedding of IL-12. Such controlled transfection is important for safe and effective translation of gene therapy or vaccination into the clinic.

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