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ORIGINAL ARTICLE

Duration and level of transgene expression after gene electrotransfer to skin in mice

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In development of novel vaccines, attention is drawn to DNA vaccinations. They are heat stable and can be easily produced. Gene electrotransfer is a simple and nonviral means of transferring DNA to cells and tissues and is attracting increasing interest. One very interesting perspective with gene electrotransfer is that choice of tissue can determine the duration of transgene expression. With gene electrotransfer to muscle, long-term expression, that is beyond 1 year, can be obtained, whereas gene electrotransfer to skin gives short-term expression, which is desirable in, for example, DNA vaccinations. Level and duration of transgene expression after gene electrotransfer to skin is essential and here we present data from two independent quantitative studies. Using in vivo *bioimaging of a far-red fluorescent molecule, Katushka, allowing for continuous monitoring of local gene expression, compared with measurements of a systemic transgene, that is, serum erythropoietin (EPO) after gene electrotransfer with EPO to skin, we found a significant increase in transgene expression ($P < 0.01$) with a peak 9 days (Katushka) and 14 days (EPO) after transfection. Duration of expression could be 3–4 weeks, which is a suitable time frame for vaccinations and is applicable, for example, in gene therapy for wound healing or treatment of cancer.*

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Introduction

Gene electrotransfer is a nonviral means of transferring genes into cells and tissues and is regarded as an effective and safe procedure.[1–3] A vast number of studies have encompassed gene electrotransfer to muscles,[4–8] but the technique is also efficient in, for example, cornea,[9] lungs,[10] liver[11,12] kidney,[13] bladder,[14] testis,[15] skin[16,17] and tumor.[18–20]

One very interesting perspective with gene electrotransfer is that the choice of tissue can determine the duration of expression of the transgene. Gene transfection to muscle can give long-term expression lasting up to or beyond 1 year,[7,8,21] whereas gene transfer to skin has the advantage of easy accessibility but can in comparison with muscle only give short-term expression.[22,23] This is desirable in DNA vaccinations, where a long-term expression is not necessary.

In this context, increased attention is drawn at gene electrotransfer to skin. Skin contains antigen-presenting cells (Langerhans cells, dendritic cells), which are part of the immune surveillance system and is thus able to gain a response after vaccinations. Studies with DNA vaccinations in animal models have shown promising results in infectious diseases such as hepatitis B,[24–27] HIV,[28] malaria[29] and smallpox.[30] Vaccination studies using intradermal electroporation in mice with DNA coding for prostate-specific antigen (PSA) gave increased level of PSA-specific T cells [31,32] and a Phase I/II clinical trial (NCT00859729) is currently running.

Besides vaccinations, skin has the potential of creating a systemic response to gene electrotransfer. We have previously shown that gene electrotransfer with erythropoietin (EPO) to murine skin was able to achieve significant and relevant increase in hemoglobin and serum EPO compared to controls (Gothelf *et al*., accepted for publication[33]). We found a statistically significant increase in serum EPO 24 h after the gene electrotransfer procedure, and this increase remained significant until a peak was reached after 2 weeks.

In gene electrotransfer to skin, it is imperative to investigate the duration and the level of expression after the transfection, not only for vaccination purposes but also for transfection with other relevant compounds. Previous preclinical studies have primarily used luciferase[17,23,32,34–36] and have used either *in vitro* measurement of luciferase activity in tissue homogenates or *in vivo* bioluminescence scans. However some drawbacks exist regarding this construct; it is known for displaying large variations, often of several logs,[17] and thus the *in vitro* method warrants a large number of animals or samples to be processed.

Continuous monitoring of gene expression, which is possible with bioluminescence scans of luciferase or *in vivo* bioimaging of fluorescent molecules, not only reduces the number of animals used in experiments but also gets more reliable results as each animal is its own

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control. The advantage of using fluorescent molecules compared to luciferase is that no substrate is needed. In the case of luciferase, an injection of luciferin is needed, often injected intraperitoneally, to get the enzyme to react.

Until recently, *in vivo* bioimaging of skin after gene electrotransfer has been limited to green fluorescent protein and red fluorescent protein. Unfortunately green fluorescent protein may not be the optimal choice for evaluation of gene electrotransfer to skin due to autofluorescence from the skin itself.[17,37]

A new fluorescent marker molecule in the far-red area, Katushka, from the sea anemone *Entacmaea quadricolor*[38] has proven to be very useful for *in vivo* imaging after gene electrotransfer to muscle[39] and has a more intense signal than red fluorescent protein. With this new compound, it is now possible to investigate the duration and level of expression after gene electrotransfer to skin using continuous monitoring and *in vivo* bioimaging with time domain function. Time domain imaging allows quantitative analysis of the fluorescent marker due to precise determination of spatial and temporal distribution.[39]

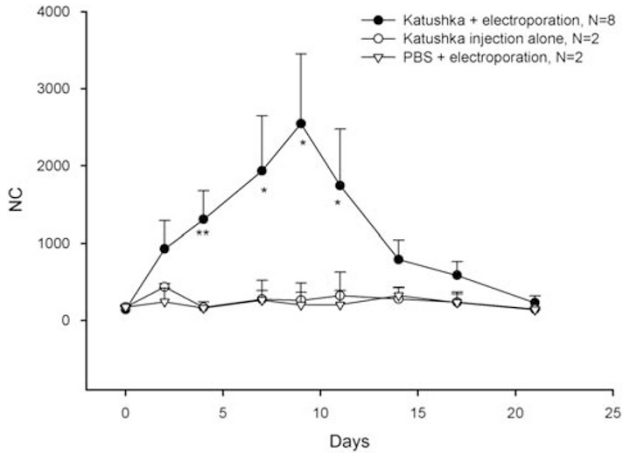

Figure 1 Level of intensity in normalized counts (NC) after gene electrotransfer with Katushka to mouse skin. The mean intensity increased after 2 days and was after 4 days statistically significant from controls ($P<0.01$). After 9 days a peak was reached, the curve declined and the level of intensity was similar to the controls after 3 weeks. The depicted *P*-values were based on Student's *t*-test comparing Katushka + electroporation with the PBS control. Similar *P*-values were obtained when Katushka + electroporation was compared with Katushka injection alone. The reported intensities are mean values; error bars represent standard deviations. $*P<0.05$, $**P<0.01$.

We therefore conducted a gene electrotransfer study to skin with a plasmid coding for Katushka and compared it to data from gene electrotransfer with EPO.

In addition, a study with consecutive *in vivo* bioluminescence scans to investigate the duration of expression after gene electrotransfer with luciferase to skin was performed.

Using these independent techniques, we have visualized local gene expression with continuous monitoring and measured serum levels of a systemic protein, and thus investigated the level and duration of expression of three different transgenes after gene electrotransfer to skin.

Results

In vivo *bioimaging of Katushka after gene electrotransfer to skin: duration of expression*

Two days after the gene electrotransfer procedure an increase in peak intensity was observed (mean 929 normalized counts (NC); Figures 1 and 2) and the difference between the transfected mice and the controls was statistically significant 4 days after the transfection, with mean 1303 NC opposed to Katushka injection alone (mean 171 NC, $P<0.01$) and phosphate-buffered saline (PBS)+electroporation (mean 161 NC, $P<0.01$). The intensity peaked 9 days after the transfection (mean 2545 NC) and was significantly increased compared to Katushka injection alone (mean 262 NC, $P<0.05$) and PBS injection with electroporation (mean 203 NC, $P<0.05$). The intensity was normalized after 3 weeks (mean 231 NC). Lifetime analysis of the area with high intensity showed a lifetime of 2.1 consistent with the fluorescent lifetime of Katushka (Figure 3).[39] The study was performed twice and reached the same conclusion.

Comparison of duration and level of expression of Katushka with EPO data

In Figure 4 the duration and level of expression are depicted for gene electrotransfer to skin with Katushka and EPO. The expression of Katushka peaks 9 days after the transfection, whereas EPO expression peaks after 14 days.

The level of intensity after gene electrotransfer with Katushka was normalized after 3 weeks, and serum EPO levels after gene electrotransfer with EPO to skin were declining after the peak to a near-baseline value after 4 weeks. Serum EPO was fully normalized after 8 weeks (data not shown).

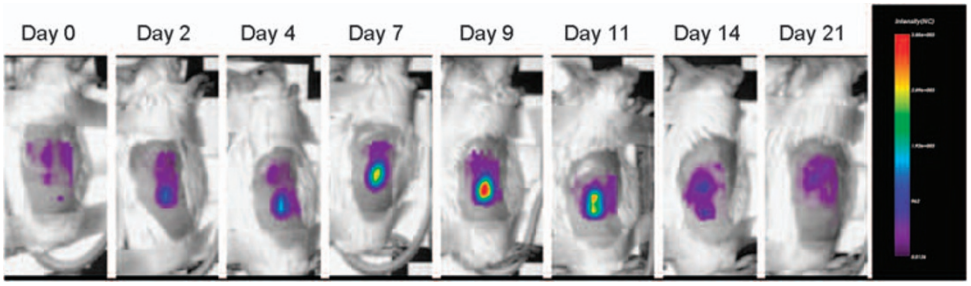

Figure 2 *In vivo* bioimaging of skin after gene electrotransfer with Katushka to skin. With this modality, it is possible to perform consecutive scans and thus continuous monitoring of the same animal. In this study, we have scanned the animals at different time points and in this figure it is possible to visualize the peak in expression observed at day 9.

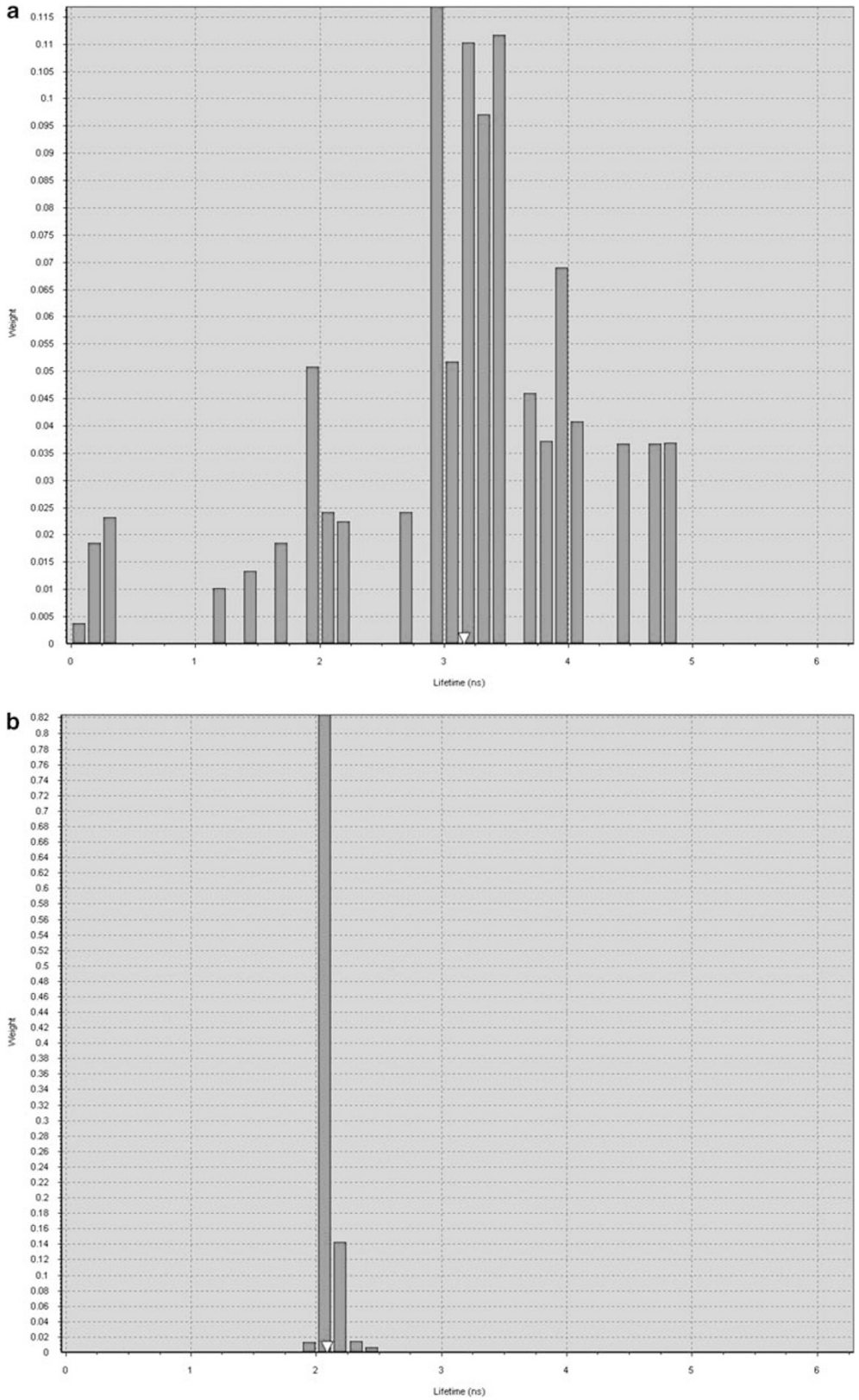

Figure 3 Comparison of lifetime histograms between day 0 and 9. In the software Optiview, it is possible to detect the fluorescent lifetime of each scanned point. The lifetime is characteristic for each fluorochrome and indicates the time in nanoseconds (ns) the fluorescent molecule stays in its excited state before emitting a photon. Before gene transfection with Katushka to skin, the scanned area has no particular lifetime (**a**). After gene electrotransfer with Katushka to skin, the lifetime is narrowed around 2.1 ns if the area expresses Katushka (**b**).

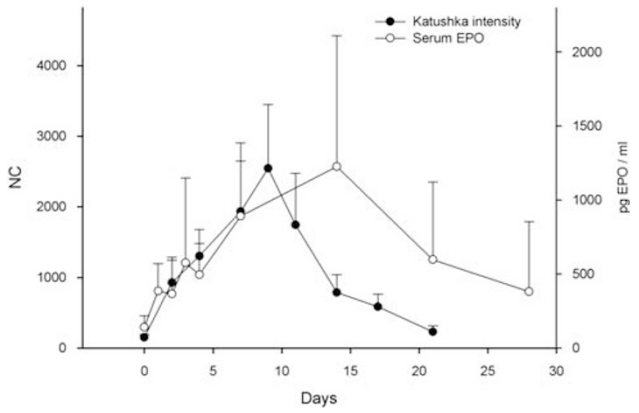

Figure 4 Comparison of gene electrotransfer to skin with Katushka with EPO in terms of duration and level of expression. With Katushka a peak in expression (in NC) is reached after 9 days, whereas serum EPO (in pg EPO per ml) seems to peak after 14 days.

Investigation of the optimal time point for bioluminescence scanning after i.p. injection of luciferin

Seven mice received gene electrotransfer with luciferase to skin and were scanned for bioluminescence 48 h after the transfection procedure (Figure 5). Three minutes after the injection of luciferin the intensity in NC has increased significantly from mean 10 to mean 27 NC ($P<0.01$). After 12 min the intensity has reached a plateau on a logarithmic scale. We thus concluded that scanning for bioluminescence 20 min after intraperitoneal (i.p.) injection of luciferin would be representative of the degree of transfection. The controls remained consistently at baseline intensity.

Expression of luciferase over time after gene electrotransfer to skin

Twenty-four hours after the transfection, the mean intensity, in the luciferase transfected group, was increased from 15.6 to 174.4 NC ($P<0.01$, Figure 6). After 48 h a peak of intensity was reached (mean 434.1 NC, $P<0.01$). The intensity declined hereafter and was near the baseline level 17 days after the transfection. The controls had consistently intensity levels from 15 to 20 NC.

Discussion

Skin is a good target for gene therapy due to its accessibility and the easiness in which it can be evaluated. It is capable of expressing genes transfected by electroporation, locally as is the case with Katushka and luciferase, but also systemically, as we have shown with EPO.[33] Compared to gene electrotransfer to muscle, short-term expression must be expected. Muscle cells do not (or seldom) divide, whereas cells in the skin (epidermal and dermal cells) are subject to constant mitotic activity, cell division, cell death and in the case of keratinocytes, desquamation from the surface.

Then why bother to transfect genes into skin? Would it not be better to use muscles instead? In many cases this would be right; the muscle cell can easily function as a 'protein-factory' and compared to skin much less DNA is

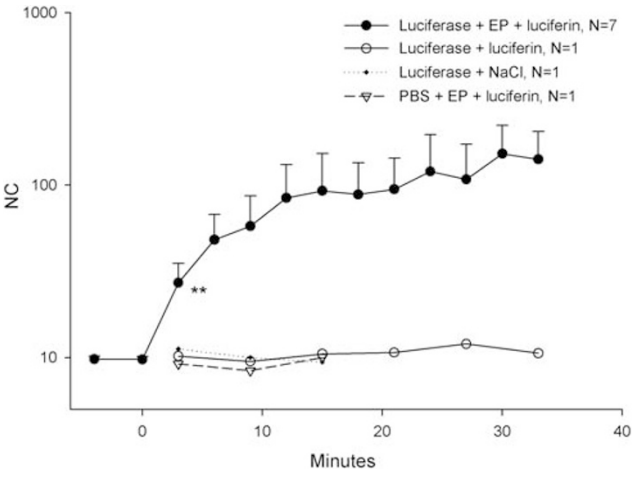

Figure 5 Investigation of optimal time for bioluminescence scanning after i.p. injection of luciferin. Mice were transfected with luciferase to skin and 48 h later they were scanned for bioluminescence. They received 3 mg luciferin (10 mg ml^{-1}) i.p. and were scanned consecutively. Three minutes after the luciferin injection, a statistical significant increase ($P<0.01$) in intensity was observed and after 12 min, a plateau was reached on a logarithmic scale. Previous studies have shown that merely the combination of luciferase plasmid injection, electroporation and luciferin injection before scans yielded response, why we chose to use as few controls as possible. EP electroporation, $^{**}P<0.01$.

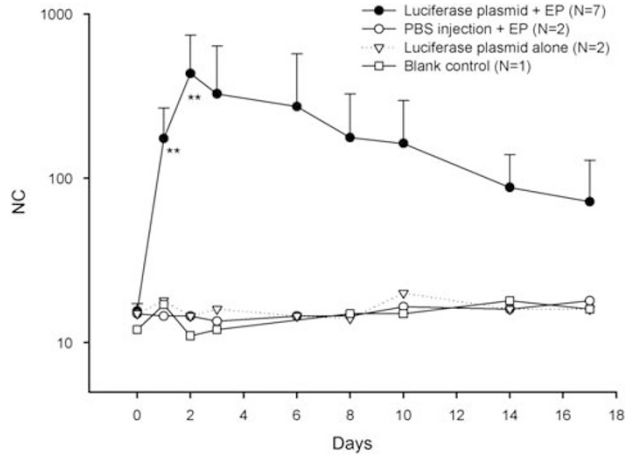

Figure 6 Duration of expression of luciferase after gene electrotransfer to skin. Mice were transfected with luciferase and scanned on different time points after i.p. administration of 3 mg luciferin. Bioluminescence was measured in normalized counts (NC). After 24 h there was an increase in intensity from mean 15.6 to mean 174.4 NC. The intensity peaked at 48 h and declined hereafter slowly toward the baseline intensity level. The controls had no increase in intensity but remained consistently at the baseline level. EP electroporation, $^{**}P<0.01$.

needed to give the same response.[7,39] However, in other cases a long-term expression is not warranted. In DNA vaccinations, the transfection only needs to be efficient long enough for the immune system to respond and in, for example, wound healing there is no need for extra production of growth factors once the wound has closed.[40] Furthermore, skin contains antigen-presenting cells and would with optimal conditions probably be more efficient in creating an antigen response after gene electrotransfer with DNA vaccines than muscle.[29]

If we want gene electrotransfer to progress as a treatment modality and enter clinical trials, it is of utmost importance to know the duration and level of the transgene expression.

Few studies have been conducted with the focus on duration and level of transgene expression and mostly with luciferase.[17,23,41] Heller *et al*.[23] found a peak in expression 2 days after gene electrotransfer with 100 μg luciferase, but the level of expression remained substantially elevated compared to controls 2 weeks after the transfection, and data from Roos *et al*.[36] confirmed these results. We found a peak in expression 2 days after gene electrotransfer with luciferase, which is comparable to the results reported in the literature. Surprisingly, the peak in expression of luciferase is different than the results obtained after gene electrotransfer with Katushka and EPO, which showed a peak after 1–2 weeks, and it remains to be investigated what causes this difference.

In vivo bioimaging has the possibility of continuous monitoring, which gives a good resolution of the curve and allows for more data points without using too many animals in the process. Each animal serves as its own control, which reduces variation.

Data from two different quantitative methods are presented here: continuous *in vivo* bioimaging of Katushka and bioluminescence scans after gene electrotransfer with luciferase, which both define the local expression, and measurements of serum EPO, which is a proof of systemic distribution of the transgene.

From the transfection procedure to the initiation of production of protein encoded by the transgene, studies have shown less than 30 min elapse,[36] which make our findings of an increase in expression already after 24 h valid.

We found a peak of expression after 9 days for Katushka and 14 days for EPO, and that the intensity in NC from the Katushka scanning was normalized after 3 weeks, whereas the serum EPO level after gene electrotransfer was nearly normalized after 4 weeks. We thus conclude from the experiments that the duration of the expression could be 3–4 weeks.

This assumption is based on the data obtained in the studies presented and hence the kinetics of the produced transgene is not taken into account. To define the exact duration of expression directly in the skin, techniques such as PCR could be of value. This would enlighten whether a measured value is in fact due to continued expression or due to remnants of the transgene. However, because the $T_{\frac{1}{2}}$ for EPO is in the range of 3–7 h,[42,43] serum EPO measurements can be a valid alternative for determination of the duration of expression.

From the curves, we can speculate that the $T_{\frac{1}{2}}$ of Katushka is not longer than the $T_{\frac{1}{2}}$ for EPO but further data on Katushka expression will be needed to investigate this point.

The fact that a peak in expression of transgene is achieved after 1–2 weeks and that the expression probably continues for 3–4 weeks can have an impact on the choice of issues, where gene electrotransfer to skin can be an advantage. In wound healing, small wounds could obtain a considerable improvement in 2–3 weeks and if necessary, it would be possible to re-transfect the new margin of the wound. Another issue is cancer therapy. Gene electrotransfer to skin could be an option because the 3–4 weeks duration of the expression of the transgene correlates to courses of chemotherapy; often administrated every 3 weeks.

Finally, in vaccinations against various infectious diseases, the duration of the transgene expression is prolonged compared to the standard vaccines, which are degraded after few days, and thus would work as a vaccine with sustained release.

In conclusion, we present data from continuous monitoring of a local transgenic fluorescent marker molecule, Katushka, and from a systemically secreted protein (EPO) and find these two compounds relevant supplements in estimating efficiency of gene electrotransfer to skin.

Gene electrotransfer to skin has proven to be an efficient and safe means of transfecting genes into skin, and if we want this technique to become an option in future clinical trials with DNA vaccinations and gene therapy, duration and level of expression is of utmost importance and must be defined. On the basis of the results from two different transgenes and two different means of evaluation, we conclude that a peak in expression will occur 1–2 weeks after the transfection and probably last for 3–4 weeks.

Materials and methods

Animals

Female NMRI mice 13- to 15-week-old own breed (Copenhagen University Hospital Herlev) were used in the studies presented here. Mice were kept in a pathogen-free environment in a 12 h light/darkness cycle with food and water *ad libitum*. During the experiments mice were anesthetized with Hypnorm (0.4 ml/kg; Janssen Saunderton, Buckinghamshire, UK) and Dormicum (2 mg/kg; Roche, Basel, Switzerland) and had the hair on the right side of the back removed by depilatory cream. At termination of the studies, the animals were killed with quick cervical dislocation. All studies were performed with approval from the Danish Animal Experiments Inspectorate and in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimentations.

Plasmid constructs

Luciferase, CMV-luc (Promega Corporation, Madison, WI, USA), was used in the bioluminescence studies and a new construct with emission in the far-red area, Katushka, pTurboFP635-c (Evrogen, Moscow, Russia) both controlled by a CMV promoter, was used for *in vivo* bioimaging. The plasmids were purified using Nucleobond AX Maxiprep kits (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. In all transfections, the injected volume of plasmid was 100 μg plasmid dissolved in 100 μl PBS (final concentration 1 μg μl^{-1}).

Gene electrotransfer procedure and electrical parameters

For each transfection, 100 μg plasmid (1 μg μl^{-1}) was carefully injected intradermally with a 29G insulin syringe and within 2 min the injected area was electroporated. We used custom-made plate electrodes with a distance of 3 mm between the plates connected to a Cliniporator (IGEA, Carpi, Italy) for delivery of the

electric pulses. The electrodes were coated with electrode gel, EKO-GEL (Ekkomarine Medico, Holstebro, Denmark), to secure proper contact with the skin. The pulses consisted of one high-voltage pulse, 1000 V cm^{-1}, 100 μs of length and one low-voltage pulse, 100 V cm^{-1} and duration of 400 ms. There was a lag of 1 s between the pulses.

Bioimaging procedure

For *in vivo* bioimaging, we used an Optix MX-2 Time Domain Optical Imaging (ART Advanced Research Technologies, Montreal, Canada). The time domain function allows for accurate determination of the spatial and temporal distribution of the emitted light and thus to measure the fluorescent molecule quantitatively. Furthermore, the fluorescent lifetime, which is distinctive for each fluorochrome, can be evaluated.

Before the procedure, animals were anesthetized, placed on a platform and carefully fixated. For *in vivo* imaging of Katushka, a pulsing laser with a 635 nm wavelength was used for excitation of the tissue and emission was detected with a 650 nm long pass filter. The intensity was reported as peaks in normalized counts (NC) after background subtraction and fluorescent lifetime of transfected area was estimated.

In studies with mice transfected with luciferase, *in vivo* bioluminescence was detected using the Optix MX-2, and the intensity of luciferase expression was reported as peaks in NC as well.

For data analysis, background subtraction, and lifetime analysis Optiview 2.2 software (ART Advanced Research Technologies) was used.

Duration of expression after gene electrotransfer with Katushka to skin

Eight mice were injected intradermally with 100 μg Katushka plasmid (1 μg μl^{-1}) and were electroporated as described above. Two mice were injected with Katushka plasmid but received no electroporation and two mice had intradermal injection of PBS with subsequent electroporation. Mice were scanned 2, 4, 7, 11, 14, 17 and 21 days after transfection.

Kinetic study of i.p. injection of luciferin

A study was conducted with the purpose of finding the optimal time for bioluminescence scans after i.p. injection of luciferin. Six mice were injected intradermally with 100 μg luciferase plasmid (1 μg μl^{-1}) and subsequently electroporated. After 48 h the animals were anesthetized and had an i.p. injection of 3 mg luciferin (Promega Corporation) in NaCl (10 mg ml^{-1}) and each mouse was scanned for bioluminescence at different time points up to 35 min. Controls were injection of luciferase without electroporation and intradermal injection of PBS with electroporation. An additional control was gene electrotransfer with luciferase but i.p. injection of NaCl before scanning instead of luciferin.

Expression of luciferase over time after gene electrotransfer to skin

To investigate the duration of luciferase gene expression, we conducted a kinetic study using *in vivo* detection of bioluminescence. Seven mice were injected intradermally with 100 μg luciferase plasmid (1 μg μl^{-1}) and electroporated as described above. As controls served two mice receiving luciferase plasmid injection without electric pulses, two mice receiving intradermal PBS injection with electroporation, and one mouse was a blank control. At different time points the animals were anesthetized, had the hair on the treated area removed if necessary, injected with luciferin intraperitoneally and scanned for bioluminescence. At day 17, the study was terminated and the animals were killed.

Evaluation of serum EPO levels after gene electrotransfer to skin with plasmid coding for EPO

These studies are described in detail by Gothelf *et al*.[33] The plasmid used was pUHD-EPO controlled by the doxycycline-dependent promoter system Tet-On and Tet-S, and for each EPO injection a solution of 50 μg pUHD-EPO, 50 μg Tet-On and 50 μg Tet-S diluted in 100 μl PBS (final concentration 1.5 μg μl^{-1}) was administered.

Mice were anesthetized and had the hair on the right side of the back removed. Groups of mice were treated with (1) two i.d. injections of EPO plasmid solution and subsequent electrotroporation, (2) two i.d. injections of EPO plasmid solution alone or (3) two i.d. injections of 100 μl PBS and subsequent electroporation. At different time points, mice were anesthetized and exsanguinated to measure serum EPO levels with enzyme-linked immunosorbent assay (Quantikine ELISA kit; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

Statistics

In the study encompassing duration of expression after gene electrotransfer with Katushka, data were analyzed with Student's *t*-test, Katushka transfection versus Katushka injection alone and Katushka transfection versus the PBS control for each condition. In both studies involving gene transfection with luciferase, Student's *t*-test was used as well. *P*-values <0.05 were reported as statistically significant.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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