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Electrotransfer of single-stranded or double-stranded DNA induces complete regression of palpable B16.F10 mouse melanomas

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Abstract

Enhanced tumor delivery of plasmid DNA with electric pulses *in vivo* has been confirmed in many preclinical models. Intratumor electrotransfer of plasmids encoding therapeutic molecules has reached Phase II clinical trials. In multiple preclinical studies, a reduction in tumor growth, increased survival, or complete tumor regression have been observed in control groups in which vector or backbone plasmid DNA electrotransfer was performed. This study explores factors that could produce this antitumor effect. The specific electrotransfer pulse protocol employed significantly potentiated the regression. Tumor regression was observed after delivery of single-stranded or double-stranded DNA with or without CpG motifs in both immunocompetent and immunodeficient mice, indicating the involvement of the innate immune system in response to DNA. In conclusion, this study demonstrated that the observed antitumor effects are not due to a single factor, but to a combination of factors.

Keywords

Electroporation; electrotransfer; melanoma; tumor regression

Introduction

Therapeutic gene delivery using electric pulses (electrotransfer) has been achieved in a variety of tissue types including skin, kidney, liver, testis, brain, cartilage, arteries, prostate, cornea, and skeletal muscle[1, 2]. Intratumor electrotransfer of plasmids encoding therapeutic genes has been developed in preclinical models[3, 4] and has been used in veterinary oncology[5] and clinical trials[6].

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Interestingly and apparently independent of specific genes, inhibition of tumor growth, increased survival time, and complete tumor regression have been observed after intratumor electrotransfer of control plasmids, either plasmid devoid of only therapeutic gene or plasmids encoding reporter genes to palpable tumors in preclinical models. These effects have been described in melanomas[7–11], lung carcinomas[12, 13], fibrosarcomas[14], pancreatic carcinomas[15], mammary tumors[16] and colorectal carcinomas[17, 18]. In two studies, antitumor effects were observed after intratumor electrotransfer of an oligonucleotide containing a CpG motif, although each oligonucleotide primarily functioned as an antisense molecule[19, 20]. The large number of variables between these studies limits the ability to explore the mechanism of this non-specific tumor regression.

This study explored potential factors that may play a role in this observed antitumor effect in the well-characterized mouse B16.F10 melanoma tumor model Intratumor plasmid electrotransfer by four electroporation pulse protocols that varied considerably in pulse number, length, and field strength was compared[12, 21–23]. Previous studies confirmed that application of pulses alone did not induce tumor regression[8, 12, 23]; combination with DNA is required. Therefore, the DNA composition was also explored.

Materials and Methods

Tumor and animal models

All procedures were approved by the Institutional Animal Use and Care Committee of the University of South Florida College of Medicine, Tampa, FL, USA (#R2736) and Veterinary Administration of The Ministry of Agriculture and the Environment of the Republic of Slovenia (#34401-12/2009/6). One million B16.F10 (ATCC CR6475) mouse melanoma cells in 50 μl PBS were injected subcutaneously in the left flank of female 7–8 week old C57Bl/6 (Jackson Laboratories, Bar Harbor, ME) or SCID (Harlan, Udine, Italy) mice. Tumors were allowed to grow approximately eight days to an approximate diameter of four mm before manipulation.

Plasmids and oligonucleotides

Plasmids gWizLuc and gWizBlank (Aldevron, Fargo, ND) were commercially prepared (Aldevron, Fargo, ND). Oligonucleotides were modified by the inclusion of phosphorothioate internucleotide bonds (IDT DNA, Coralville, IA or Invivogen, San Diego, CA) to produce nuclease resistance. Oligonucleotide sequences were ODN 1668, 5'-TCCATGACGTTCCTGATGCT-3' and ODN 1720, 5'-TCCATGAGCTTCCTGATGCT-3'[24, 25]. All molecules were suspended in sterile injectable saline at 2 mg/ml.

Electrotransfer in vitro

Confluent low passage B16.F10 cells were prepared in electroporation buffer at a concentration of 25×10^6 cells/mL[26]. Cells (80 μL of final cell suspension) were mixed with 20 μL of gWizBlank plasmid (1 mg/mL). One half of the mixture was placed between 2 mm gap steel electrodes and exposed to 8 square wave electric pulses (electric field intensity 600 V/cm, pulse duration 5 ms, frequency 1 Hz). The other half of the mixture served as control for plasmid exposure without the electric pulses. Electric pulses were generated by in-house build electroporator (University of Ljubljana, Faculty of Electrical Engineering, Slovenia). After the application of electric pulses, the cells were incubated at room temperature for 5 min and then plated for cell viability assay. The number of viable cells was determined by PrestoBlue Cell Viability Reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol every 24 hours for four consecutive days. Cell proliferation rate was quantified by the slope of the linear regression between $\log_{10}$ (cell proliferation) and time.

Electrotransfer in vivo

Deliveries were performed on day 0 (mean tumor diameter 4 mm), day 3, and day 7. Mice were anesthetized using a mixture of 2.5% isoflurane and 97.5% O_2. Tumors were injected with 50 μl of molecule solution and pulsed immediately with caliper electrodes moistened with electrode paste as described in each figure at a frequency of 1 Hz using a T820 Electrosquare porator (BTX Molecular Delivery Systems, Holliston, MA) or a Betatech Electro cell B10 porator (Betatech, Toulouse, France). Tumors were measured twice to three times weekly using a digital caliper. Tumor volume was calculated by the standard formula $v=ab^2\pi/6$, where a is the longest diameter, and b is the next longest diameter perpendicular to a. Any animal was considered incurable and humanely euthanized when the tumor volume reached 1000 mm^3 or when the animal's behavior indicated discomfort. Animals with tumors in regression were followed up to 100 days after the first electrotransfer. After that, if no tumor regrowth was observed, animals were considered to be in complete regression.

Statistical analysis

Comparison of complete long-term regression levels between groups was performed using Holm Sidak test after one way ANOVA was performed and fulfilled or Dunnett's Method after Kruskal-Wallis One Way Analysis of Variance on Ranks (SigmaPlot, Systat, San Jose, CA).

Results

When plasmid DNA was electrotransferred into B16.F10 cells *in vitro*, a small but significant decrease in viability was observed (Figure 1a). This effect was not observed with plasmid addition or pulse application alone. Cell proliferation rate was calculated for each treatment group, and a significantly reduced cell proliferation rate was observed after plasmid DNA electrotransfer. Cell proliferation rate was not affected by plasmid exposure or pulse application alone (Figure 1b).

To determine the relative importance of the electrotransfer pulse protocol to the non-specific antitumor effect observed *in vivo*, tumor regression after vector plasmid delivery with four well-characterized electrotransfer protocols was assessed (Figure 2). Delivery of ten 5 ms pulses at a field strength of 800 V/cm, generated significant long-term tumor regression ($p<0.05$) in 25% of animals. Two other protocols, eight 20 ms pulses at a field strength of 500 V/cm and one 100 ms pulse at a field strength of 667 V/cm also induced a lesser but statistically significant regression levels ($p<0.05$). In contrast, application of six 50 ms 150 V/cm pulses did not induce tumor regression.

CpG motif DNA is a well-characterized immunostimulatory agent in the plasmid sequence that may be responsible for the observed antitumor effect[24]. Control and CpG oligonucleotides were delivered to determine if DNA containing this motif would induce regression of melanomas in immunocompetent C57Bl/6 (Figure 3a) and SCID mice (Figure 3b). In immunocompetent C57Bl/6 mice, electrotransfer of the Type B oligonucleotide (ODN) 1668[24, 25], containing a mouse specific motif (GA<u>CG</u>TT)[27], induced significant long-term and complete tumor regression in 36% of animals ($p<0.05$), while simple injection did not. Injection or electrotransfer of control ODN 1720, lacking this motif, did not induce regression. Intriguingly, electrotransfer induced long-term and complete tumor regression ($p<0.05$) in 20% of immunodeficient SCID animals receiving ODN 1668 (CpG motif) oligonucleotide and 40% of animals receiving control ODN 1720 (GpC motif) oligonucleotide. In addition, short term tumor regression was induced by electrotransfer of either oligonucleotide, apparently resulting in significant tumor growth delay ($p<0.05$),

being 15.7 ± 6.0 days after electrotransfer of Type B ODN 1668 and 8.5 ± 2.0 days after electrotransfer of control ODN 1720.

The typical appearances of tumors treated with electrotransfer of either of oligonucleotides in SCID mice are shown in Figure 4. When long-term tumor regression was achieved 100 days after electrotransfer of either of the oligonucleotides, only minimal scarring was observed at the site of electrode position, either completely without residual pigmentation or with small residual pigmentation at the site of tumor transplantation (Figs. 4 a, b). When only short-term tumor regression was achieved, the newly formed tumors regrew from the treated area (Figs. 4c, d). The short term regression was present for longer time in the group of mice that received Type B ODN 1668 (from 2–27 days) compared to mice that received control ODN 1720 (2–14 days).

Discussion

This study explored potential factors that may play a role in the antitumor effects observed in many preclinical studies after intratumor electrotransfer of control plasmids. *In vitro*, viability of B16.F10 cells and their proliferation rate were significantly reduced by plasmid DNA electrotransfer, which was not observed after exposure to plasmid DNA or electric pulses alone. Reduction of cell viability after application of electric pulses was expected as it was demonstrated previously that long electric pulses at low electric field strength that were used in the *in vitro* experiment affect cell viability[28]. Since cell proliferation and metabolism are interconnected via common regulatory pathways[29], it is possible that the decrease in cell proliferation rate is a reflection of changes in cellular metabolism in response to plasmid DNA. However, this decrease in cell proliferation and viability was not likely to explain the complete tumor regression observed *in vivo*.

Different electrotransfer pulse protocols delivering an identical lot and dosage of plasmid DNA produced different levels of tumor regression. This regression was not related to the quantity of plasmid successfully delivered as demonstrated by reporter expression[30] or to the pulse energy[31, 32], pulse number, pulse intensity or the pulse length. In groups where regression was observed, a relationship between regression and pulse field strength ($R^2 = 0.9976$) was observed. The rationale behind this strong association is not clear. However, the pulse regimen was a significant variable in the generation of tumor regression.

Another possible factor involved in the antitumor effect of control plasmids is the presence of CpG motifs[24] in the plasmid sequence. These motifs are bound by the endosomal toll-like receptor 9 (TLR9)[33], which is found primarily in dendritic and B cells, producing immune stimulation. The immune stimulation by CpG motif DNA has been utilized as a monotherapy or in combination therapies in clinical trials for cancer therapies and as vaccine adjuvants[34, 35]. In animals with palpable B16.F10 tumors, a survival increase and tumor growth inhibition was observed after multiple intratumoral[36] or peritumoral[37] injections of the Type B ODN 1826, which contains two mouse-specific CpG motifs. Significantly prolonged survival and tumor growth inhibition were observed in this model when intraperitoneal injection of Type A ODN 1585 was initiated simultaneously with intraperitoneal tumor cell injection[38]. Complete tumor regression was not described in these studies.

In the study described here, electrotransfer of Type B ODN 1668 produced a significant antitumor effect in immunocompetent mice and a less pronounced effect in SCID mice. This difference is likely a consequence of activation of different signaling pathways involved in the stimulation of immune system[39]. Specifically, type B CpG oligonucleotides induce strong B cell activation and moderate NK cell activation[40]. SCID mice lack functional B-

cells[41], thus no response to B-cell mitogens is expected. However, to some extent, type B CpG oligonucleotides can activate NK cells[42, 43]. Hence, the observed antitumor effect in immunodeficient mice could be mediated through activation of NK cells. However, this should be confirmed by analysis of cytokine production in response to CpG ODNs in both types of mice.

In SCID mice, tumor regression was also observed after electrotransfer of the control oligonucleotide. Although both CpG and control oligonucleotides induced complete tumor regression in SCID mice, differences were observed between the two groups. Short-term tumor regression was more pronounced in mice receiving electrotransfer of CpG oligonucleotides, all mice in the group were tumor free for up to 12 days, while only 70% of mice were tumor free for up to 12 days after electrotransfer of control oligonucleotides. On the other hand, tumors regressed in only 20% of SCID mice after electrotransfer of CpG oligonucleotides as opposed to 40% after electrotransfer of control oligonucleotides. In previous studies, liposomal-mediated transfection of different oligonucleotides was used for determination of TLR9 activation. The authors demonstrated activation in response to oligonucleotides independently of the CpG motif and regardless of their methylation status[44, 45]. A switch from CpG to GpC motif in oligonucleotides creates a low affinity ligand for TLR9[46] and with natural DNA uptake pathway into the cells their concentration is too low to activate TLR9. These results support the hypothesis that upon enhanced endosomal translocation, in our case by electrotransfer, low affinity ligands in endosomes can reach the threshold concentrations required to drive TLR9 activation[44, 45]. Consequently, NK cells are activated and may exert antitumor effectiveness, which was observed in SCID mice. In any case, tumor regression was observed in SCID mice, so specific cellular and humoral immunity was not required. The lack of tumor response in immunocompetent mice after control ODN electrotransfer and its underlying mechanisms require further experimental studies.

In addition to TLR9 receptors, intracellular DNA can be recognized by several cytoplasmic double-stranded DNA sensors which, when bound to DNA, activate cascades producing inflammation and programmed cell death[47]. The specific ligands for these cytosolic sensors are not well characterized and may be redundant[48]. Similar to TLR9, these sensors regulate type I interferon production and mediate inflammatory responses[49]. DNA sensors are compartmentalized within the cell, and electrotransfer may be uniquely situated to activate both endosomal and cytosolic DNA sensors. Electrotransfer is primarily an endocytosis-driven process[50, 51] although DNA is observed in aggregates and in free form in the cytoplasm after electrotransfer[51, 52]. The half-life of cytosolic free DNA is short and probably is a dead end process as far as gene expression is involved[53]. However, its presence may be adequate to activate cytoplasmic DNA sensors. This DNA compartmentalization after electrotransfer may also explain the lack of correlation between tumor gene expression and tumor regression[30]. The production of pro-inflammatory and chemokines is observed in tumors[30] and in muscle[54] after DNA electrotransfer. This upregulation supports the concept that endosomal and/or cytosolic nucleic acid receptors, potentially including both TRL9 and cytosolic DNA sensors, are activated, generating an antitumor immune response.

In conclusion, our study confirmed sporadic observations in multiple preclinical tumor models of non-specific tumor regression after control vector electrotransfer in a single tumor type and single vector, including its concentration, batch, and diluent. The antitumor effects after electrotransfer of control vector plasmid DNA were due to multiple factors. Tumor regression was influenced by the pulse regimen, although the presence of DNA was required. Sequence motifs in DNA sequences contributed to tumor regression. Still, tumor regression was observed also in SCID mice after delivery of both CpG and GpC

oligonucleotides, indicating that innate immune system was likely activated through signaling pathways of multiple nucleic acid sensors. A basic understanding of the mechanism by which this regression occurs is important in any tumor electrotransfer therapy. The knowledge that additional signaling pathways are induced is important in therapeutic design as it may aid or hinder immune-based or other targeted cancer therapies. In delivery to other tissues, this understanding will also be important, both in the prediction of the therapeutic outcome after specific gene delivery, and, if necessary, in designing method to avoid pathway activation in situations where this induction is not desirable.

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References

1. Heller LC, Heller R. In vivo electroporation for gene therapy. Hum. Gene Ther. 2006; 17(9):890–897. [PubMed: 16972757]
2. Cemazar M, Sersa G. Electrotransfer of therapeutic molecules into tissues. Curr Opin Mol Ther. 2007; 9(6):554–562. [PubMed: 18041666]
3. Heller, LC.; Heller, R. Translation of electroporation mediated DNA delivery to the clinic. In: Markov, M.; Miklavcic, D.; Pakhomov, A., editors. Advanced Electroporation Techniques in Biology and Medicine. CRC Press: 2010. p. 19-1-19-13.
4. Heller LC, Heller R. Electroporation gene therapy preclinical and clinical trials for melanoma. Current gene therapy. 2010; 10(4):312–317. [PubMed: 20557286]
5. Pavlin D, Cemazar M, Cör A, Sersa G, Pogacnik A, Tozon N. Electrogene therapy with interleukin-12 in canine mast cell tumors. Radiol Oncol. 2011; 45:30–39.
6. Daud AI, DeConti RC, Andrews S, Urbas P, Riker AI, Sondak VK, et al. Phase I Trial of Interleukin-12 Plasmid Electroporation in Patients With Metastatic Melanoma. J Clin Oncol. 2008; 26(36):5896–5903. [PubMed: 19029422]
7. Niu G, Heller R, Catlett-Falcone R, Coppola D, Jaroszeski M, Dalton W, et al. Gene therapy with dominant-negative Stat3 suppresses growth of the murine melanoma B16 tumor in vivo. Cancer Research. 1999; 59(20):5059–5063. [PubMed: 10537273]
8. Heller L, Coppola D. Electrically mediated delivery of vector plasmid DNA elicits an antitumor effect. Gene Therapy. 2002; 9(19):1321–1325. [PubMed: 12224015]
9. McCray AN, Ugen KE, Muthumani K, Kim JJ, Weiner DB, Heller R. Complete regression of established subcutaneous B16 murine melanoma tumors after delivery of an HIV-1 Vpr-expressing plasmid by in vivo electroporation. Molecular Therapy. 2006; 14(5):647–655. [PubMed: 16950655]
10. Ugen KE, Kutzler MA, Marrero B, Westover J, Coppola D, Weiner DB, et al. Regression of subcutaneous B16 melanoma tumors after intratumoral delivery of an IL-15-expressing plasmid followed by in vivo electroporation. Cancer Gene Therapy. 2006; 13(10):969–974. [PubMed: 16763607]
11. Marrero BSS, Heller R. Delivery of Interleukin-15 to B16 Melanoma by Electroporation Leads to Tumor Regression and Long-term Survival. Technol Cancer Res Treat. 2013 Aug 31. *e-pub ahead of print*:
12. Slack A, Bovenzi V, Bigey P, Ivanov MA, Ramchandani S, Bhattacharya S, et al. Antisense MBD2 gene therapy inhibits tumorigenesis. Journal of Gene Medicine. 2002; 4(4):381–389. [PubMed: 12124980]
13. Prud'homme GJ, Glinka Y, Khan AS, Draghia-Akli R. Electroporation-Enhl Gene Transfer for the Prevention or Treatment of Immunological, Endocrine aanced Nonviral Gene Transfer for the Prevention or Treatment of Immunological, Endocrine and Neoplastic Diseases In. 2006:243–273.

14. Grosel A, Sersa G, Kranjc S, Cemazar M. Electrogene therapy with p53 of murine sarcomas alone or combined with electrochemotherapy using cisplatin. DNA Cell Biol. 2006; 25(12):674–683. [PubMed: 17233116]
15. Deharvengt S, Rejiba S, Wack S, Aprahamian M, Hajri A. Efficient electrogene therapy for pancreatic adenocarcinoma treatment using the bacterial purine nucleoside phosphorylase suicide gene with fludarabine. Int. J. Oncol. 2007; 30(6):1397–1406. [PubMed: 17487360]
16. Radkevich-Brown O, Piechocki MP, Back JB, Weise AM, Pilon-Thomas S, Wei WZ. Intratumoral DNA electroporation induces anti-tumor immunity and tumor regression. Cancer Immunol. Immunother. 2010; 59(3):409–417. [PubMed: 19730859]
17. Wang YS, Tsang YW, Chi CH, Chang CC, Chu RM, Chi KH. Synergistic anti-tumor effect of combination radio- and immunotherapy by electro-gene therapy plus intra-tumor injection of dendritic cells. Cancer Lett. 2008; 266(2):275–285. [PubMed: 18397820]
18. Vidic S, Markelc B, Sersa G, Coer A, Kamensek U, Tevz G, et al. MicroRNAs targeting mutant K-ras by electrotransfer inhibit human colorectal adenocarcinoma cell growth in vitro and in vivo. Cancer Gene Therapy. 2010; 17:409–419. [PubMed: 20094071]
19. Elez R, Piiper A, Kronenberger B, Kock M, Brendel M, Hermann E, et al. Tumor regression by combination antisense therapy against Plk1 and Bcl-2. Oncogene. 2003; 22(1):69–80. [PubMed: 12527909]
20. Spugnini EP, Biroccio A, De Mori R, Scarsella M, D'Angelo C, Baldi A, et al. Electroporation increases antitumoral efficacy of the bcl-2 antisense G3139 and chemotherapy in a human melanoma xenograft. J Transl Med. 2011; 9:125. [PubMed: 21798045]
21. Rols MP, Delteil C, Golzio M, Dumond P, Cros S, Teissie J. In vivo electrically mediated protein and gene transfer in murine melanoma. Nat Biotechnol. 1998; 16(2):168–171. [PubMed: 9487524]
22. Lohr F, Lo DY, Zaharoff DA, Hu K, Zhang X, Li Y, et al. Effective tumor therapy with plasmid-encoded cytokines combined with *in vivo* electroporation. Cancer Research. 2001; 61(8):3281–3284. [PubMed: 11309280]
23. Cichon T, Jamrozy L, Glogowska J, Missol-Kolka E, Szala S. Electrotransfer of gene encoding endostatin into normal and neoplastic mouse tissues: Inhibition of primary tumor growth and metastatic spread. Cancer gene therapy. 2002; 9(9):771–777. [PubMed: 12189527]
24. Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature. 1995; 374(6522):546–549. [PubMed: 7700380]
25. Lipford GB, Sparwasser T, Bauer M, Zimmermann S, Koch ES, Heeg K, et al. Immunostimulatory DNA: sequence-dependent production of potentially harmful or useful cytokines. Eur. J Immunol. 1997; 27(12):3420–3426. [PubMed: 9464831]
26. Todorovic V, Sersa G, Mlakar V, Glavac D, Cemazar M. Assessment of the tumourigenic and metastatic properties of SK-MEL28 melanoma cells surviving electrochemotherapy with bleomycin. Radiol Oncol. 2012; 46(1):32–45. [PubMed: 22933978]
27. Bauer S, Kirschning CJ, Hacker H, Redecke V, Hausmann S, Akira S, et al. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc. Natl. Acad. Sci. U. S. A. 2001; 98(16):9237–9242. [PubMed: 11470918]
28. Rols MP, Teissie J. Electropermeabilization of mammalian cells to macromolecules: control by pulse duration. Biophysical journal. 1998; 75(3):1415–1423. [PubMed: 9726943]
29. Fritz V, Fajas L. Metabolism and proliferation share common regulatory pathways in cancer cells. Oncogene. 2010; 29(31):4369–4377. [PubMed: 20514019]
30. Heller LC, Cruz YL, Ferraro B, Yang H, Heller R. Plasmid injection and application of electric pulses alter endogenous mRNA and protein expression in B16.F10 mouse melanomas. Cancer Gene Therapy. 2010; 17(12):864–871. [PubMed: 20706286]
31. Henshaw J, Mossop B, Yuan F. Relaxin treatment of solid tumors: effects on electric field-mediated gene delivery. Mol. Cancer Ther. 2008; 7(8):2566–2573. [PubMed: 18723501]
32. Henshaw JW, Yuan F. Field distribution and DNA transport in solid tumors during electric fieldmediated gene delivery. J Pharm. Sci. 2008; 97(2):691–711. [PubMed: 17624918]
33. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. Nature. 2000; 408(6813):740–745. [PubMed: 11130078]

34. Krieg AM. Development of TLR9 agonists for cancer therapy. The Journal of clinical investigation. 2007; 117(5):1184–1194. [PubMed: 17476348]
35. Vollmer J, Krieg AM. Immunotherapeutic applications of CpG oligodeoxynucleotide TLR9 agonists. Advanced Drug Delivery Reviews. 2009; 61(3):195–204. [PubMed: 19211030]
36. Sharma S, Karakousis CP, Takita H, Shin K, Brooks SP. Intra-tumoral injection of CpG results in the inhibition of tumor growth in murine Colon-26 and B-16 tumors. Biotechnol. Lett. 2003; 25(2):149–153. [PubMed: 12882291]
37. Kunikata N, Sano K, Honda M, Ishii K, Matsunaga J, Okuyama R, et al. Peritumoral CpG oligodeoxynucleotide treatment inhibits tumor growth and metastasis of B16F10 melanoma cells. J Invest Dermatol. 2004; 123(2):395–402. [PubMed: 15245441]
38. Ballas ZK, Krieg AM, Warren T, Rasmussen W, Davis HL, Waldschmidt M, et al. Divergent therapeutic and immunologic effects of oligodeoxynucleotides with distinct CpG motifs. J Immunol. 2001; 167(9):4878–4886. [PubMed: 11673492]
39. Krieg AM. Immune effects and mechanisms of action of CpG motifs. Vaccine. 2000; 19(6):618–622. [PubMed: 11090712]
40. Yu YZ, Li N, Ma Y, Wang S, Yu WY, Sun ZW. Three types of human CpG motifs differentially modulate and augment immunogenicity of nonviral and viral replicon DNA vaccines as built-in adjuvants. European journal of immunology. 2013; 43(1):228–239. [PubMed: 23037552]
41. Belizário JE. Immunodeficient Mouse Models: An Overview. The Open Immunology Journal. 2009; 2:79–85.
42. Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdorfer B, Giese T, et al. Quantitative expression of toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol. 2002; 168(9):4531–4537. [PubMed: 11970999]
43. Ballas ZK, Rasmussen WL, Krieg AM. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. J Immunol. 1996; 157(5):1840–1845. [PubMed: 8757300]
44. Yasuda K, Rutz M, Schlatter B, Metzger J, Luppa PB, Schmitz F, et al. CpG motif-independent activation of TLR9 upon endosomal translocation of "natural" phosphodiester DNA. European journal of immunology. 2006; 36(2):431–436. [PubMed: 16421948]
45. Yasuda K, Yu P, Kirschning CJ, Schlatter B, Schmitz F, Heit A, et al. Endosomal translocation of vertebrate DNA activates dendritic cells via TLR9-dependent and -independent pathways. J Immunol. 2005; 174(10):6129–6136. [PubMed: 15879108]
46. Lenert PS. Classification, mechanisms of action, and therapeutic applications of inhibitory oligonucleotides for Toll-like receptors (TLR) 7 and 9. Mediators of inflammation. 2010; 2010:986596. [PubMed: 20490286]
47. Desmet CJ, Ishii KJ. Nucleic acid sensing at the interface between innate and adaptive immunity in vaccination. Nat Rev Immunol. 2012; 12(7):479–491. [PubMed: 22728526]
48. Hornung V, Latz E. Intracellular DNA recognition. Nat Rev Immunol. 2010; 10(2):123–130. [PubMed: 20098460]
49. Keating SE, Baran M, Bowie AG. Cytosolic DNA sensors regulating type I interferon induction. Trends in immunology. 2011; 32(12):574–581. [PubMed: 21940216]
50. Wu M, Yuan F. Membrane binding of plasmid DNA and endocytic pathways are involved in electrotransfection of mammalian cells. PLoS One. 2011; 6(6):e20923. [PubMed: 21695134]
51. Rosazza C, Phez E, Escoffre JM, Cezanne L, Zumbusch A, Rols MP. Cholesterol implications in plasmid DNA electrotransfer: Evidence for the involvement of endocytotic pathways. Int J Pharm. 2012; 423(1):134–143. [PubMed: 21601622]
52. Golzio M, Teissie J, Rols MP. Direct visualization at the single-cell level of electrically mediated gene delivery. Proc Natl Acad Sci U S A. 2002; 99(3):1292–1297. [PubMed: 11818537]
53. Vaughan EE, Dean DA. Intracellular trafficking of plasmids during transfection is mediated by microtubules. Mol Ther. 2006; 13(2):422–428. [PubMed: 16301002]
54. Mann CJ, Anguela XM, Montane J, Obach M, Roca C, Ruzo A, et al. Molecular signature of the immune and tissue response to non-coding plasmid DNA in skeletal muscle after electrotransfer. Gene Ther. 2012; 19(12):1177–1186. [PubMed: 22170344]

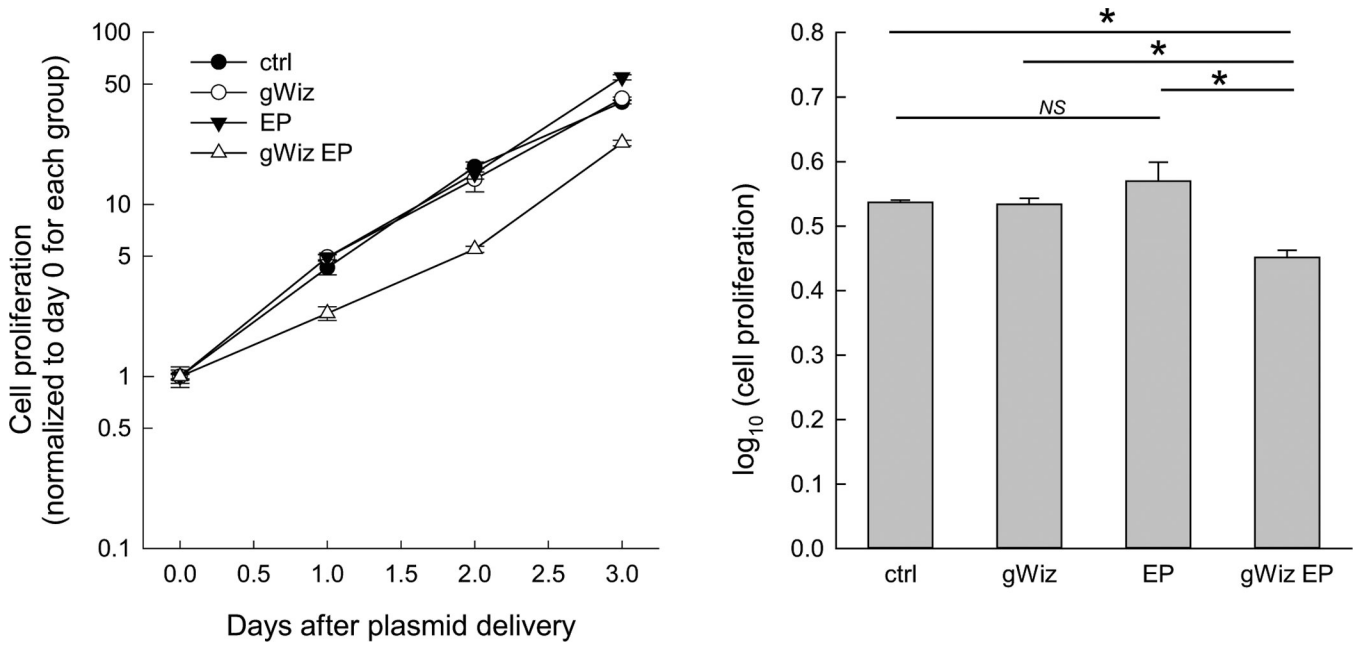

Figure 1. Effect of DNA electrotransfer on cell proliferation *in vitro*

(a) Electrotransfer of gWizBlank plasmid induced a significant decrease in viability, that was not observed by plasmid alone or application of electric pulses alone. 20 μg of plasmid (1 mg/mL) was mixed with 2×10^6 B16.F10 cells and half of this mixture was exposed to eight 5 ms 600 V/cm electric pulses with a plate electrode, whereas the other half served as control for plasmid alone. Open circles, untreated; filled circles, electroporation; open triangles, gWizBlank without pulses; filled triangles, gWizBlank with pulses. ANOVA two sided, $P<0.05$ * statistical significance. (b) Electrotransfer of gWizBlank plasmid induced a significant decrease in cell proliferation rate in comparison to control cells, that was not observed by plasmid alone or application of electric pulses alone. Bars: mean cell proliferation rate ± st. error of the mean. One way ANOVA, $P<0.05$ * statistical significance, NS not significant.

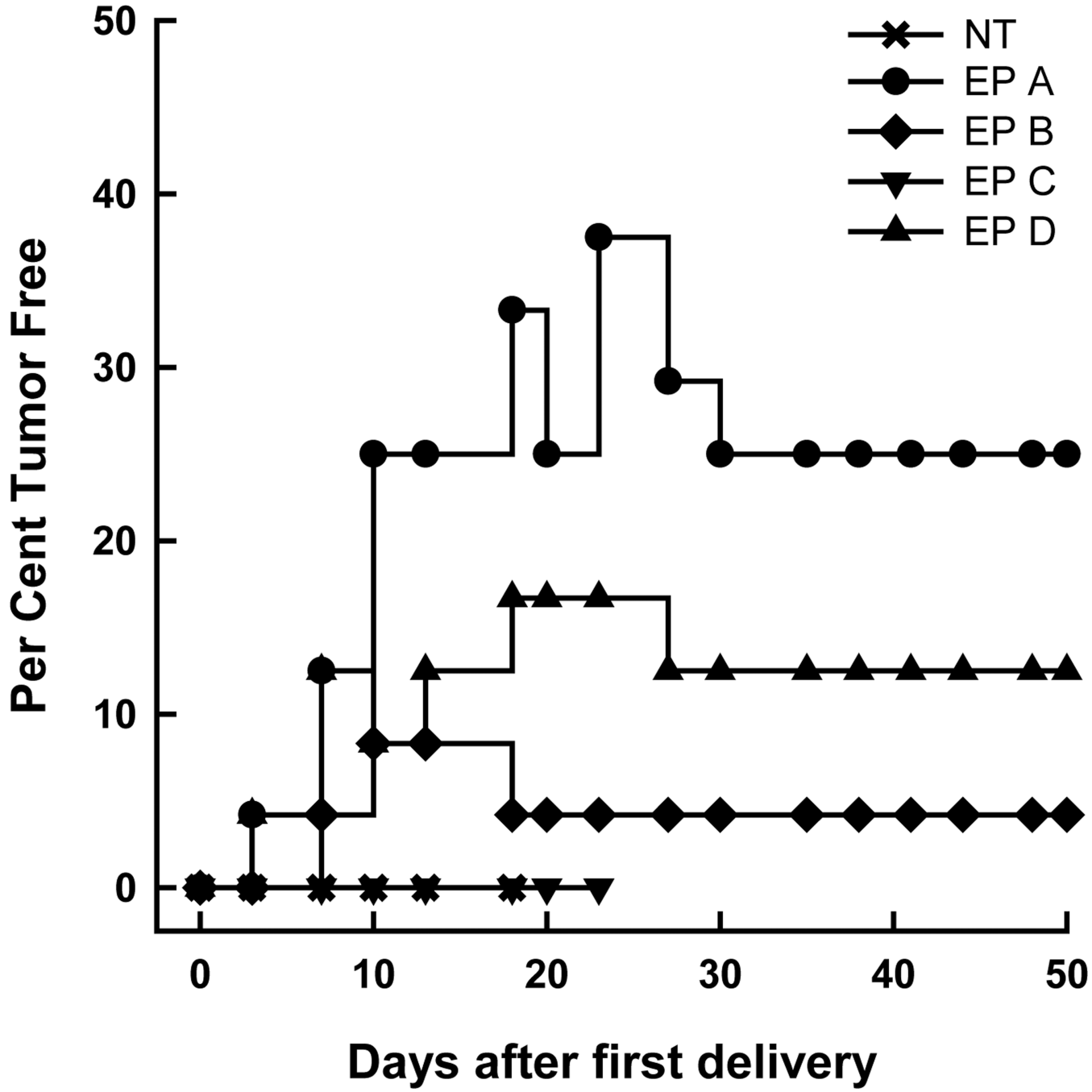

Figure 2. Long-term tumor regression after electrotransfer using several pulse protocols

100 μg commercially prepared gWizBlank was delivered by electrotransfer into palpable melanoma tumors in the flanks of C57Bl/6 mice using various pulse protocols: X, untreated; filled circles, ten 5 ms 800 V/cm pulses with a caliper electrode (EP A)[21]; filled diamonds, eight 20 ms 500 V/cm pulses with a caliper electrode (EP B)[12]; filled inverted triangles, six 50 ms 150 V/cm pulses with a caliper electrode (EP C)[22]; filled triangles, one 100 ms 667 V/cm pulse with a caliper electrode (EP D)[23]. Tumor growth or regression was monitored as described in methods. n=24 for all groups.

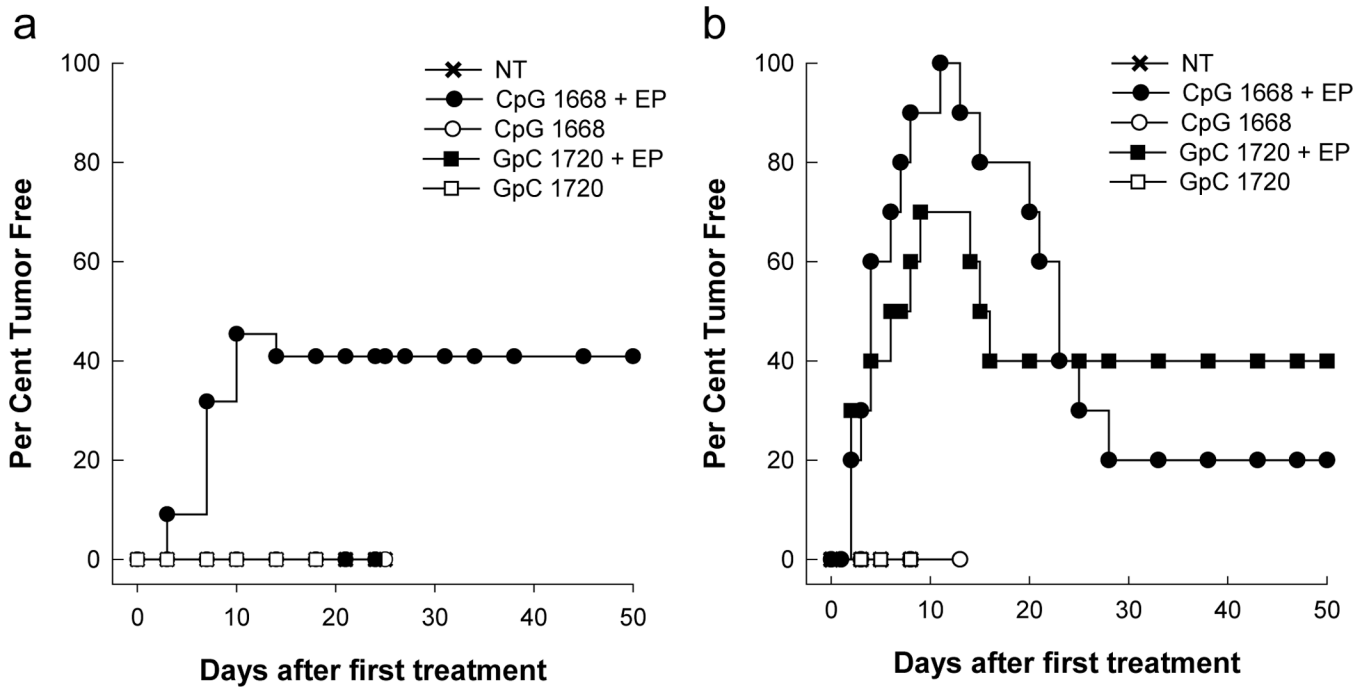

Figure 3. Long-term tumor regression after electrotransfer of CpG motif oligonucleotides

100 μg oligonucleotide was injected with or without pulses into palpable melanoma tumors using ten 5 ms 800 V/cm pulses with caliper or plate electrode three times in one week. (a) C57Bl/6 mice. X, untreated (n=16); filled squares, CpG ODN 1668 with pulses (n=15); open squares, CpG ODN 1668 without pulses (n=6); filled triangles, GpC ODN 1720 with pulses (n=15); open triangles, GpC ODN 1720 without pulses (n=6). (b) SCID mice, identical symbols, n=10 per group. Tumor growth or regression was monitored as described in methods.

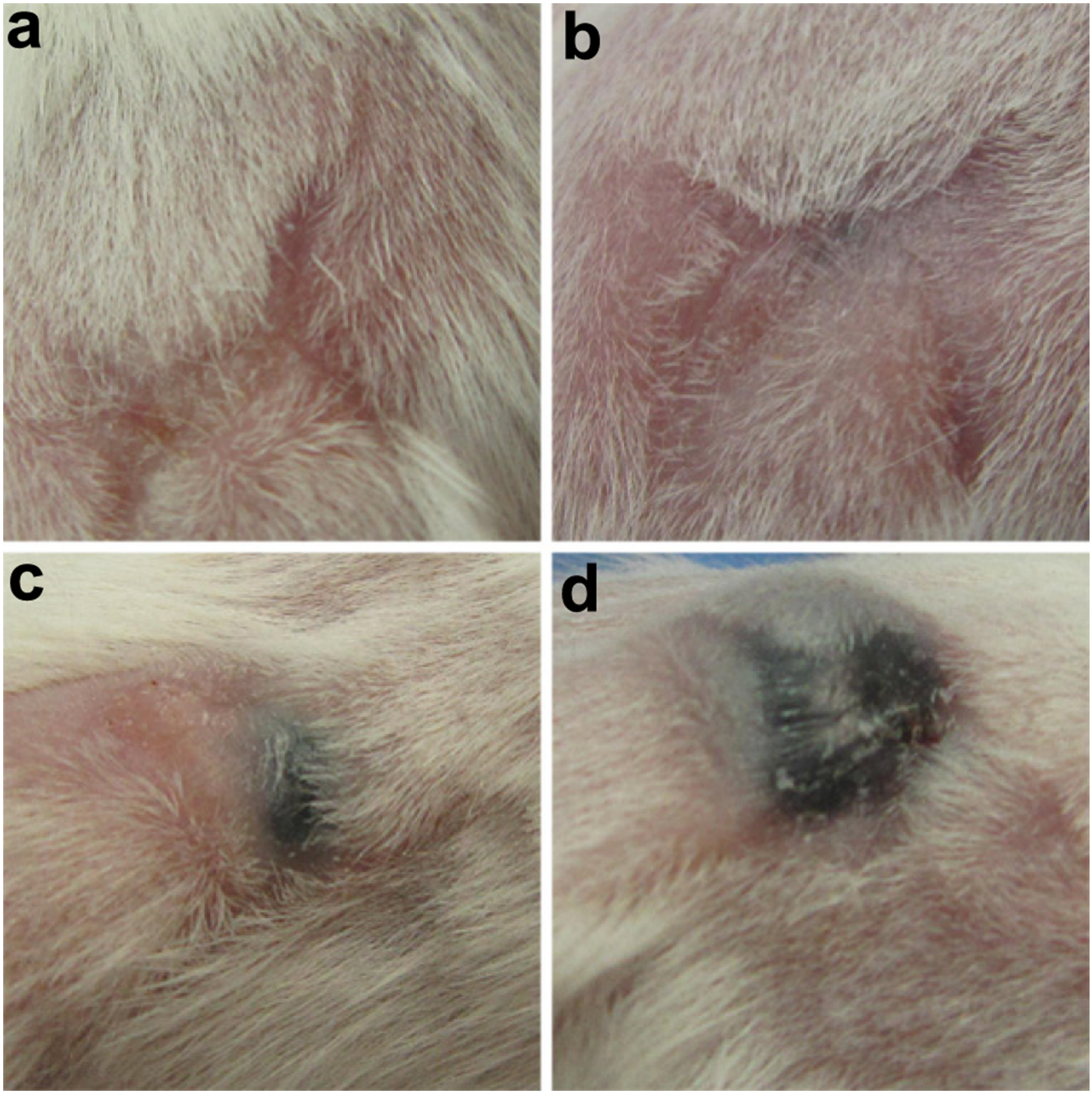

Figure 4. Tumor regression after electrotransfer of CpG motif oligonucleotides in SCID mice

On day 23 after first electrotransfer of oligonucleotides using ten 5 ms 800 V/cm pulses with a plate electrode, different outcomes were observed: (a) minimal scarring at the tumor site after electrotransfer of Type B ODN 1668 and (b) electrotransfer of control ODN1720, where pigmentation at the site of tumor regression is still present; (c) tumor regrowth at the tumor site after electrotransfer of Type B ODN 1668; and (d) tumor regrowth was quicker after electrotransfer of control ODN 1720.