UNIVERZA V LJUBLJANI
MEDICINSKA FAKULTETA
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OPTIMIZACIJA GENSKEGA ELEKTROPRENOSA V KOŽO ZA NADZOROVAN VNOS PLAZMIDNE DNA

DOKTORSKA DISERTACIJA
Podiplomski študij

OPTIMIZATION OF SKIN GENE ELECTROTRANSFER FOR CONTROLLED DELIVERY OF PLASMID DNA

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Optimizacija genskega elektroprenosa v kožo za nadzorovan vnos plazmidne DNA

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Izjavljam, da sem avtorica doktorskega dela z naslovom **Optimizacija genskega elektroprenosa v kožo za nadzorovan vnos plazmidne DNA.**

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Špela Kos, mag. farm.

Ljubljana, marec 2017

»The important thing is not to stop questioning. Curiosity has its own reason for existing.«

Albert Einstein

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KAZALO VSEBINE

SEZNAM OKRAJŠAV

COL	kolagenski promotor
ConA	konkavalin A
DSRED (označen tudi DsRed)	*Discosoma* rdeč fluorescenten protein
ECT	elektrokemoterapija
EP	elektroporacija
FITC	dekstran označen z fluorescein-izotiocianatom
GET	genski elektroprenos
HV	visoko-napetostni električni pulzi (ang. *high voltage pulses*)
IL12 (označen tudi IL-12)	citokin interlevkin-12 (fuzijski protein α in β podenot IL12, ki se pri miših nahajata na lokusu p35 – *Il12a* in lokusu p40 – *Il12b*)
LV	nizko-napetostni električni pulzi (ang. *low voltage pulses*)
MEA	več-točkovna elektroda (ang. *multi-electrode array*)
OVA	ovalbumin

POVZETEK

Znanstveno izhodišče: Genska terapija s ciljanim vnosom plazmidne DNA v kožo postaja vse bolj zanimiv pristop zdravljenja tumorjev. Vnos plazmidne DNA v kožo predstavlja kompleksen proces, ki vključuje dostavo terapevtskih genov v kožne celice, specifično tkivno ciljanje, uravnavanje ravni in trajanja genskega izražanja, biološko aktivnost terapevtskih proteinov ter varnost vektorskih sistemov in genskih produktov. Varnost genskega vnosa v kožo lahko bistveno izboljšamo z uporabo ne-virusnih dostavnih metod, kot je genski elektroprenos. Genski elektroprenos je varna in učinkovita ne-virusna metoda za vnos genov v celice in tkiva. Učinek genskega elektroprenosa je odvisen od številnih parametrov, kot so izbor elektrode, napetosti električnih pulzov ter sestave plazmidne DNA. Optimizacija glavnih parametrov metode genskega elektroprenosa je tako ključna za zagotavljanje učinkovitosti in varnosti metode ter za doseganje optimalnega terapevtskega učinka.

Metode: V prvem sklopu smo optimizirali glavne parametre genskega elektroprenosa. Učinkovitost in varnost metode smo spremljali po uporabi različnih elektrod, pod različnimi električnimi parametri ali pri spremenjeni sestavi plazmidne DNA. Genski elektroprenos z vnosom referenčnih in terapevtskih genov v kožne celice smo izvedli v pogojih *in vitro* in *in vivo*. Genskemu izražanju in učinkovitosti transfekcije smo sledili na genskem in proteinskem nivoju ter s sledenjem terapevtskega učinka. V naslednjem koraku smo določali protitumorsko delovanje optimizirane metode na sarkomskem in melanomskem mišjem tumorskem modelu. Protitumorski učinek peritumoralne terapije smo spremljali lokalno na primarnih tumorjih in sistemsko na oddaljenih ne-tretiranih metastazah.

Rezultati: Z optimizacijo glavnih parametrov metode smo dokazali, da z izborom ustreznih parametrov genskega elektroprenosa vplivamo na transfekcijo različnih plasti kože, s čimer lahko prilagajamo trajanje izražanja genskega produkta ter uravnavamo lokalno ali sistemsko delovanje genskega vnosa. Dokazali smo, da lahko z ustreznim načrtovanjem protokolov in z uporabo optimiziranih parametrov zagotovimo učinkovit in varen način vnosa plazmidne DNA za peritumoralno zdravljenje tumorjev. S peritumoralnim vnosom plazmidne DNA z zapisom za citokin interlevkin-12 lahko dosežemo zaostanek v rasti tako primarnih tumorjev kot tudi sistemski učinek na oddaljene ne-tretirane tumorje.

Zaključek: Optimizirana metoda omogoča varen, učinkovit, nadzorovan in ciljan vnos plazmidne DNA v kožo. Metoda je učinkovit in varen način vnosa plazmidne DNA za peritumoralno zdravljenje tumorjev z lokalnim delovanjem na primarne tumorje ter sistemskim vplivom na lokoregionalne in oddaljene metastaze. Natančno optimizirana in nadzorovana metoda z dokazanim protitumorskim delovanjem predstavlja pomemben korak k nadaljnjemu razvoju metode in prenosu kožnega

genskega elektroprenosa v klinično prakso. Vnos terapevtskih genov v kožo dosega vse večje zanimanje predvsem na področju genske terapije raka, vakcinacije in celjenja ran.

ABSTRACT

Scientific background: Gene therapy with targeted delivery of plasmid DNA into skin is a promising approach for cancer treatment. The delivery of plasmid DNA into skin is a complex process, which includes the delivery of therapeutic genes to the skin cells, tissue-specific targeting, modulation of the level and duration of gene expression, the biological activity of therapeutic proteins and safety levels of delivery systems and transgenes. To enhance the efficiency and to ensure the safety of cutaneous gene therapy, the improvement of delivery methods is required. Gene electrotransfer is safe and efficient non-viral gene delivery method, widely used to deliver the plasmid DNA into different target tissues, including the skin. The effectiveness of gene electrotransfer relies on different parameters, such as the type of the electrodes, amplitude and duration of electric pulses and the design of the plasmid DNA. To provide optimal effectiveness of gene electrotransfer and to achieve the maximal therapeutic effect of gene therapy, the main parameters of gene electrotransfer need to be optimized.

Methods: In the scope of the study, the main parameters of gene electrotransfer were firstly optimized. The efficiency and safety of gene electrotransfer was evaluated after the application of different electrical parameters, after the utilization of different electrodes and after the delivery of the plasmid DNA with different construction. The delivery of reference and therapeutic genes into skin cells was performed *in vitro* as well as *in vivo* in mouse skin. Gene expression levels, protein production and therapeutic effect was followed to determine the transfection efficiency. The therapeutic anti-cancer effect of optimized method was later followed on sarcoma and melanoma tumor models. The local effect of peritumoral gene electrotransfer as well as systemic effect on distant metastasis was evaluated.

Results: With optimized parameters of gene electrotransfer, the depth of transfection into skin and the level and the duration of gene expression could be controlled, as well as the protein distribution (local or systemic). The feasibility of the optimized method for the purposes of cancer therapy was proven on different mouse tumor models. Peritumoral skin gene electrotransfer with plasmid DNA coding for the IL12 resulted in efficient growth delay of primary tumors and promoted the systemic effect on distant untreated metastases.

Conclusion: The optimization established safe, efficient and controlled method of skin gene electrotransfer. Well characterized and optimized method of skin gene electrotransfer with anticancer action could facilitate the translation of the method into the clinics. Specifically, gene electrotransfer with skin as a target tissue is a promising approach for many medical applications, such as cancer gene therapy, vaccination and wound healing.

UVOD IN PREDSTAVITEV HIPOTEZ

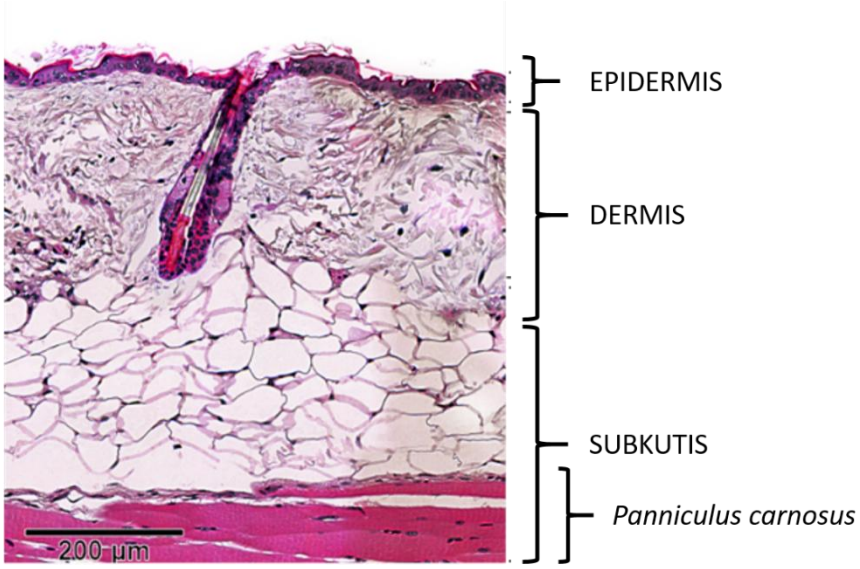
Genska terapija raka in genski elektroprenos

Slaba prognostična napoved preživetja bolnikov z nekaterimi oblikami raka, visoka stopnja umrljivosti in neželeni učinki klasične terapije so razlog za razvoj novih oblik zdravljenja raka. Genska terapija je eden izmed pomembnih novih pristopov zdravljenja (Becker, 2015), ki cilja na specifične molekule in signalne poti v tumorskih celicah ali aktivira imunski odziv za prepoznavo in uničenje tumorskih celic. Za vnos terapevtskih genov v tarčna tkiva za namen genske terapije so v uporabi različni pristopi, ki jih lahko razdelimo v virusne in ne-virusne metode vnosa. Kljub pogosto slabši učinkovitosti se lahko z uporabo ne-virusnih metod izognemo varnostnim zapletom virusnega vnosa, kot so sistemska toksičnost, endogena virusna rekombinacija, onkogeni učinki vključevanja v genom ter nepričakovane imunske reakcije (Niidome and Huang, 2002: Young et al., 2006). Izmed ne-virusnih metod je genski elektroprenos varna in učinkovita metoda za vnos genov v tarčne celice in tkiva (Mir, 2009).

Metoda genskega elektroprenosa temelji na elektroporaciji, t.j. aplikaciji električnih pulzov, ki povzročijo nastanek por v celični membrani in tako omogočijo vstop v celico manjšim molekulam, ki sicer ne prehajajo membrane (Orlowski et al., 1988; Yarmush et al., 2014). Kadar s pomočjo elektroporacije v tarčne celice vnašamo plazmidno DNA, metodo poimenujemo genski elektroprenos. Genski elektroprenos je široko uporabljena metoda za vnos genov v številna tarčna tkiva, kot so mišica (Aihara and Miyazaki, 1998; Tevz et al., 2009), jetra (Heller et al., 1996), ledvice (Tsujie et al., 2001), tumorji (Cemazar et al., 2002; Dolinsek et al., 2013; Lucas et al., 2002) pljuča (Dean et al., 2003) in koža (Drabick et al, 2001; Heller et al, 2001; Pavselj and Preat, 2005).

Koža kot tarčni organ za vnos genov

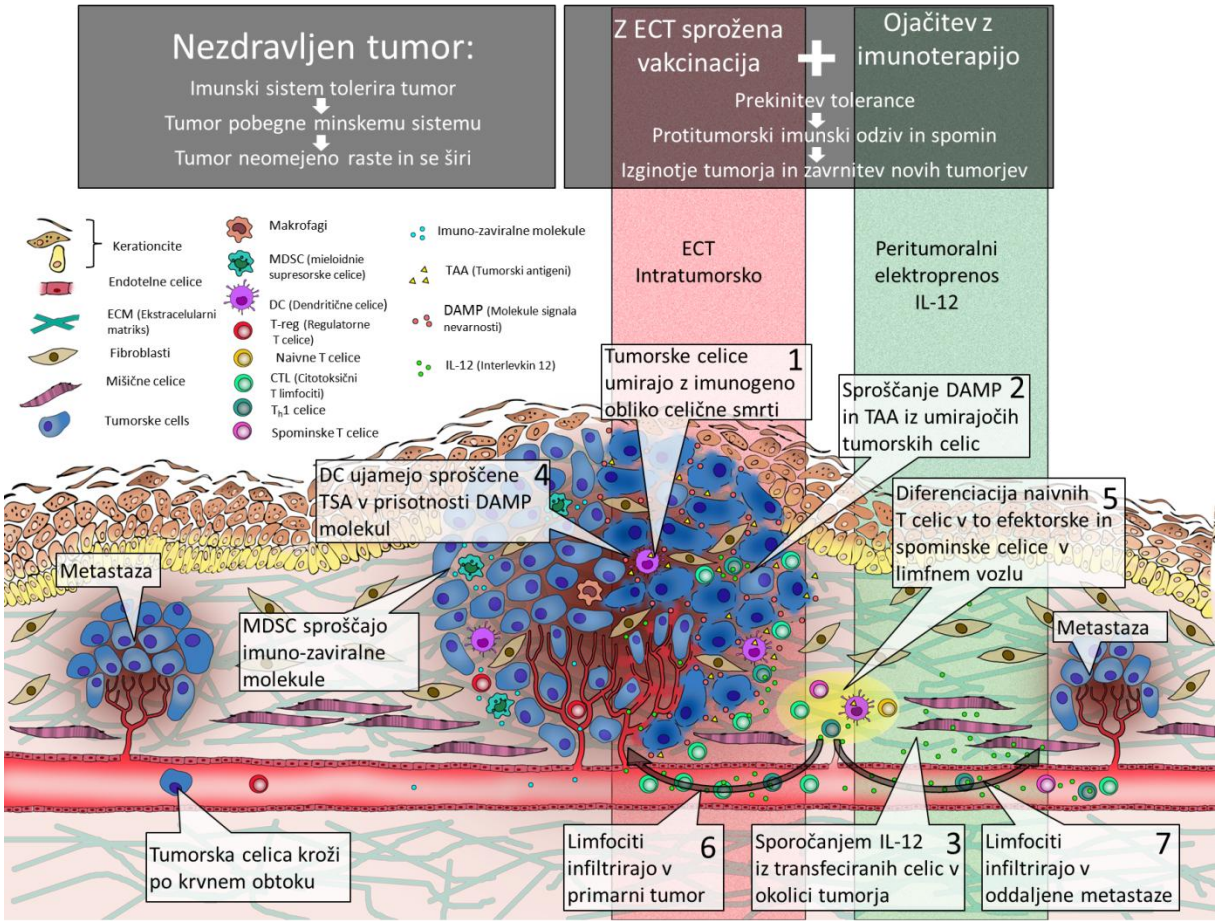
Koža je z vidika genske terapije zanimiv organ, predvsem zaradi enostavne dostopnosti tkiva, velike površine in številnih prisotnih kožnih antigen predstavitvenih celic, ki so ključne pri razvoju imunskega odgovora (Gothelf and Gehl, 2010). Koža je kompleksno tkivo, ki ga sestavlja več plasti celic z različno sestavo in različnimi lastnostmi *(Slika 1)*. Genski elektroprenos omogoča transfekcijo vseh plasti kože, od epidermisa na vrhu do najglobje plasti podkožja, *t. i.* mišična plast *panniculus carnosus*, ki je značilna za mišjo kožo. Zaradi svojih prednosti je koža zanimiv tarčni organ za vnos genov z vidika različnih kliničnih aplikacij, kot so zdravljenje kožnih bolezni (Gothelf and Gehl, 2010), vakcinacija (Drabick et al., 2001; Roos et al., 2009a; Roos et al., 2009b), celjenje ran (Chereddy et al., 2014; Ferraro et al., 2009; Steinstraesser et al., 2014) in genska terapija raka (Cemazar et al., 2016; Heller et al., 2001; Sersa et al., 2015).



Slika 1: Sestava mišje kože. Mišjo kožo sestavlja več plasti; epidermalna plast na vrhu, ki ji sledi dermalna plast. Pod njima leži podkožje z najgloblje ležečo mišično plastjo, imenovano *panniculus carnosus,* ki je značilna za mišjo kožo.

Koža kot tarčni organ v genski terapiji raka

Terapevtski geni, namenjeni genski terapiji raka s ciljanim vnosom v kožo, nosijo zapis predvsem za različne tumorske antigene (Brave et al., 2009; Roos et al., 2009b), antiangiogene molekule (Pedron-Mazoyer et al., 2007) in citokine (Heller et al., 2001; Sersa et al., 2015). Med citokini je zaradi svojega protitumorskega delovanja, dokazanega na več tumorskih modelih, zlasti zanimiv vnos gena, ki nosi zapis za IL12 (Cemazar et al., 2016; Heller et al., 2001; Hernandez-Alcoceba et al., 2016; Pavlin et al., 2009). Intradermalni vnos genov z zapisom za IL12 in ostalih citokinov v peritumoralno območje odkriva nove možnosti uporabe metode genskega elektroprenosa za namen ciljane genske terapije. Obetavne možnosti uporabe peritumoralnega genskega elektroprenosa se odkrivajo predvsem v kombinacijah z ablativnimi tehnikami (Calvet and Mir, 2016; Sersa et al 2015), kot so obsevanje in elektrokemoterapija. Kombinirana terapija lahko vodi do popolnih ozdravitev primarnih tumorjev z dodatnim sistemskim učinkom na oddaljene ne-tretirane tumorje *(Slika 2)*. Kljub vzpodbudnim rezultatom na področju genskega zdravljenja raka s ciljanim genskim vnosom v kožo, pa širšo uporabo in hitrejši prenos metode v kliniko omejujejo pomanjkljivosti in nepopolno poznavanje glavnih parametrov genskega elektroprenosa.

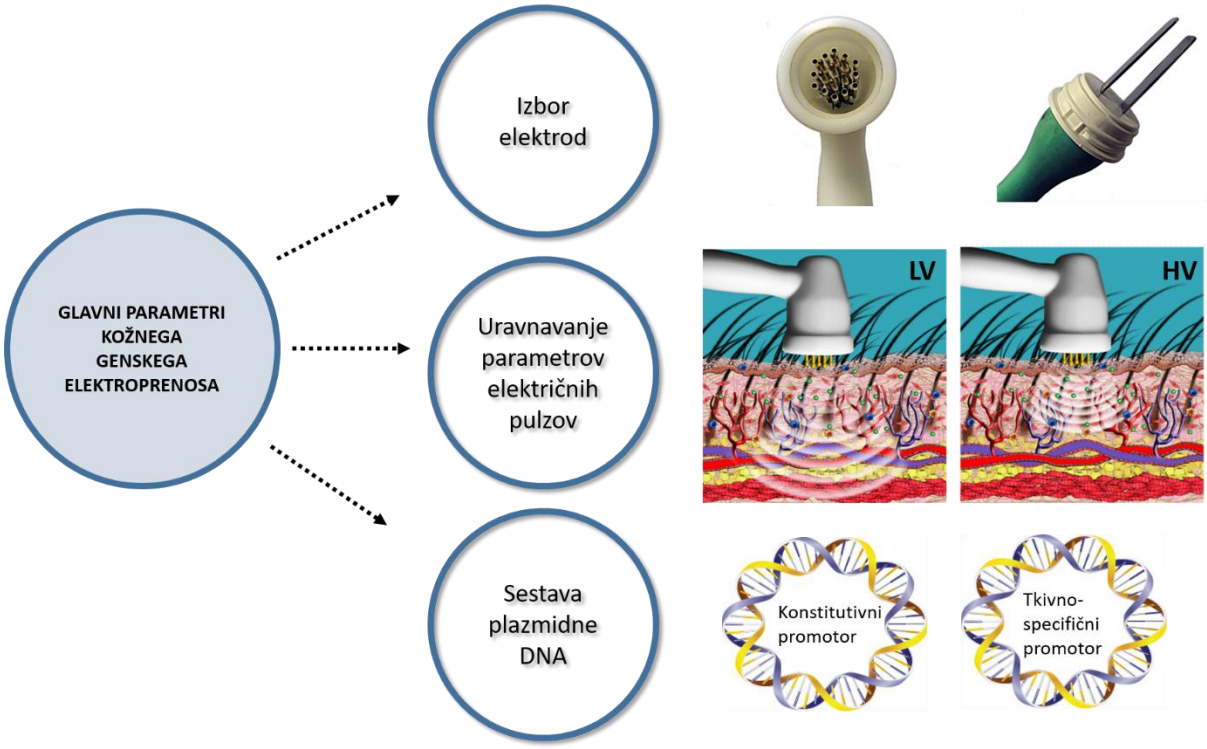


Slika 2: Predlagan model elektrokemoterapije (ECT) v kombinaciji s peritumoralnim genskim elektroprenosom z vnosom gena z zapisom za IL12. ECT povzroči imunogeno smrt tretiranih tumorskih celic (1), kar vodi do sproščanja antigenov iz umirajočih tumorskih celic (2). Elektroprenos omogoči izražanje in sproščanje IL12 iz transfeciranih celic v okolici tumorja (3), kar olajša imunsko prepoznavo sproščenih antigenov (4, 5) in spodbudi imunsko uničenje tumorja. Kombinacija terapij vodi do aktivacije imunskega sistema, usmerjenega lokalno proti celicam primarnega tumorja kot tudi sistemsko proti oddaljenim metastazam (6,7). Povzeto po: Sersa et al., 2015.

Pomanjkljivosti genskega elektroprenosa s ciljanim vnosom plazmidne DNA v kožo in možne izboljšave

Genski elektroprenos s ciljanim vnosom v kožo je odvisen od številnih parametrov (Shirley et al., 2014), med najpomembnejšimi so izbor napetosti in trajanja električnih pulzov, sestava in količina plazmidne DNA ter izbor primerne elektrode *(Slika 3)*. Pomanjkljivost obstoječe metode genskega elektroprenosa se kaže v variabilnosti parametrov električnih pulzov med različnimi študijami, pomanjkanju tkivno-specifičnih promotorjev, ki bi omogočili ciljano transfekcijo kože, ter neprimernosti obstoječih elektrod, ki zaradi slabe učinkovitosti vnosa v kožo ali invazivnosti niso primerne za uporabo v kliniki. Pomanjkljivosti glavnih parametrov tako omejujejo varno, učinkovito, ciljano in nadzorovano uporabo kožnega genskega prenosa. Razvoj poteka predvsem v iskanju kožno specifičnih promotorjev (Dean, 2013; Vandermeulen et al., 2009), preučevanju učinka različnih

napetosti in trajanja električnih pulzov (Dean, 2013; Gothelf and Gehl, 2010; Pavselj and Preat, 2005) ter oblikovanju novih elektrod za ciljano transfekcijo v kožo (Daugimont et al., 2010; Gothelf et al., 2011).



Slika 3: Glavni parametri kožnega genskega elektroprenosa, ki vplivajo na varnost in učinkovitost metode in omogočajo nadzorovan in ciljan vnos plazmidne DNA v kožo.

Varnost in učinkovitost metode genskega elektroprenosa lahko izboljšamo z ustreznim izborom elektrode. Razvoj elektrod za kožni vnos je potekal od začetnih invazivnih elektrod, kot so igelne elektrode (Gothelf et al., 2011; Maruyama et al., 2001), do vse manj invazivnih elektrod, ki vključujejo mikro-igle (Daugimont et al., 2010) in ne-invazivne ploščate elektrode (Gothelf and Gehl, 2010; Gothelf and Gehl, 2012). Učinkovitost ter varnost vnosa se je bistveno izboljšala z nadaljnjim razvojem ne-invazivnih več-točkovnih elektrod (MEA, *multi-electrode array*) (Guo et al., 2011; Heller et al., 2010). Več-točkovne elektrode praviloma za enako učinkovitost omogočajo dovajanje električnih pulzov nižjih napetosti in vodijo do zmanjšanih mišičnih krčev ob apliciranju (Heller et al., 2010). Ena takšnih elektrod MEA je bila razvita tudi v sodelovanju z industrijskim partnerjem in z našo raziskovalno skupino (Blagus et al., 2013) ter uporabljena pri raziskovalnem delu, zajetem v doktorski nalogi.

Za doseganje nadzorovane metode je ključno zagotavljanje ciljane transfekcije v posamezne plasti kože. S transfekcijo različnih tipov kožnih celic namreč vplivamo na odgovor po genski terapij. Glede

na globino transfeciranih celic ter hitrost delitve transfeciranih celic lahko uravnavamo trajanje genskega izražanja ter omogočimo tako lokalno kot tudi sistemsko porazdeljevanje transgena. Tako lahko po transfekciji dolgo-živečih mišičnih celic pričakujemo daljše gensko izražanje, medtem ko bo genski produkt po vnosu v hitro-deleče keratinocite v koži prisoten krajši čas (Gothelf and Gehl, 2010; Vandermeulen et al., 2007). Po transfekciji globlje ležečih plasti lahko pričakujemo vstopanje genskega produkta v krvni obtok in posledično sistemsko delovanje terapije, medtem ko lahko z vnosom plazmidne DNA v višje ležeče plasti pričakujemo zadrževanje produkta v koži ter lokalno delovanje terapije. Poznanih je več pristopov, s katerimi lahko dosežemo ciljano transfekcijo posameznih kožnih celic ter posledično omogočimo uravnavanje trajanja, jakosti genskega izražanja in porazdeljevanja genskega produkta (Dean, 2013). Med možnimi načini zagotavljanja ciljanega in nadzorovanega vnosa genov v kožo sta tudi prilagajanje parametrov električnih pulzov in uporaba plazmidne DNA s kožno-specifičnimi promotorji (Lin et al., 2001, Vandermeulen et al., 2009). Med obetavnimi promotorji za ciljano transfekcijo fibroblastov v koži je tudi kolagenski promotor COL, ki smo ga uporabili pri raziskovalnem delu v sklopu doktorske naloge.

Optimizacija glavnih parametrov metode genskega elektroprenosa je tako ključna za zagotavljanje učinkovitosti in varnosti metode. Z izborom optimalnih parametrov genskega elektroprenosa ter ustreznim načrtovanjem protokolov lahko bistveno vplivamo na učinkovitost in izid protitumorske genske terapije z vnosom različnih plazmidnih molekul DNA na različnih tumorskih modelih (Shirley et al., 2014). Le natančno optimizirana in nadzorovana metoda predstavlja odskočno desko za prenos metode v klinično prakso.

Namen dela, hipoteze in zastavljeni potek študije

Namen doktorske naloge je bil z optimizacijo glavnih parametrov metode genskega elektroprenosa razviti varno, učinkovito in ciljano metodo genskega elektroprenosa za vnos terapevtskih plazmidov v kožo. Razvito metodo smo uporabili za ugotavljanje protitumorskega delovanja peritumoralne genske terapije za različne tumorske modele ter za preučevanje učinka terapije na lokoregionalne in oddaljene metastaze.

Pričakovani rezultati so združeni v treh hipotezah:

Hipoteze

1 Z uporabo kolagenskega tkivno-specifičnega promotorja dosežemo ciljano in nadzorovano gensko terapijo v kožo.

2 S spreminjanjem električnih parametrov vplivamo na transfekcijo različnih plasti kože, s čimer prilagajamo trajanje izražanje genskega produkta ter lokalno ali sistemsko delovanje genskega vnosa.

3 Optimizirana metoda intradermalnega vnosa plazmidne DNA v peritumorskem področju je učinkovit in varen način zdravljenja kožnih tumorjev in zasevkov.

Delo zajeto v doktorsko nalogo je potekalo *in vitro* na izbranih celičnih linijah, predvsem pa *in vivo* na različnih mišjih modelih. Za doseganje namena doktorske naloge in potrjevanje hipotez smo delo razdelili v dva sklopa. Prvi sklop predstavlja optimizacijo glavnih parametrov metode genskega elektroprenosa, kot so izbor elektrode, optimizacija električnih parametrov ter sestave plazmidne DNA. V drugem sklopu smo tako optimizirano metodo uporabili za določanje protitumorskega delovanja peritumoralnega genskega elektroprenosa s ciljano transfekcijo v kožo. Protitumorsko delovanje smo spremljali po vnosu gena, ki nosi zapis za IL12, na različnih mišjih tumorskih modelih. Terapevtski učinek peritumoralne genske terapije smo spremljali tako lokalno na primarnih tretiranih tumorjih kot tudi sistemsko na oddaljenih metastazah. Tako optimizirana metoda predstavlja odskočno desko za nadaljnjo uporabo metode v veterinarski in humani onkologiji. Genska terapija raka pa je le ena izmed možnih aplikacij genskega elektroprenosa s ciljano dostavo v kožo. V sklopu doktorske naloge predstavljamo tudi ostale možne klinične aplikacije optimizirane metode, kot so vakcinacija in celjenje ran.

UPORABLJENE METODE

V oklepaju je označeno poglavje, v katerem je metoda navedena:

In vitro metode:

- Standardne molekularne metode za pripravo plazmidne DNA (3)
- Izolacija in ovrednotenje plazmidne DNA (3-5)
- Gojenje celic *in vitro* (3)
- Genski elektroprenos *in vitro* na izbranih celičnih linijah (3)

Delo z živalmi:

- Nastanitev in oskrba živali (1-5)
- Anestezija miši (1-5)
- Intradermalni vnos modelnih učinkovin (fluorescentno označen dekstran – FITC in doksorubicin) v kožo (1)
- Intradermalno injiciranje plazmidne DNA v bok miši (1-5)
- Aplikacija električnih pulzov *in vivo* z uporabo več-točkovne elektrode (1-5)
- Genski elektroprenos *in vivo* (1-5)
- Priprava celične suspenzije za nasaditev podkožnih tumorjev (2, 4, 5)
- Nasaditev podkožnih tumorjev (2, 4, 5)
- Nasajanje kontralateralnih tumorjev (5)
- Peritumoralni genski elektroprenos *in vivo* (4, 5)
- Merjenje velikosti tumorjev (2, 4, 5)
- Izračunavanje zaostanka v rasti in izris rastnih krivulj (2, 4, 5)
- *In vivo* test celjenja ran (2, 4)
- Vakcinacija (2)

Odvzem vzorcev in metode spremljanja učinka genskega elektroprenosa:

- Priprava vzorcev kože za histološko analizo (fiksiranje v formalinu, vklapljanje v parafin, rezanje rezin, barvanje s hematoksilininom in eozinom) (1, 2, 4)
- Histološka priprava zmrzlih rezov kože (4)
- Odvzem krvi mišim (2-4)
- Odvzem mišje vranice in izolacija splenocitov (2)
- Odvzem vzorcev kože za nadaljnjo analizo (3, 4)
- Izolacija mRNA in kvantitativna verižna reakcija s polimerazo v realnem času (4)

- Spremljanje luminiscence z uporabo sistema IVIS (2)
- Spremljanje fluorescentnega signala z uporabo fluorescenčnega mikroskopa (1, 3, 4)
- Pretočna citometrija (3)
- Imunokemijski test ELISA (2-4)
- Statistične metode (1-5)

POGLAVJE 1

Poglavje 1: Uporaba več-točkovnih elektrod za vnos učinkovin in genov v mišjo kožo

Utilization of Multi-array Electrodes for Delivery of Drugs and Genes in the Mouse Skin

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Povzetek

Ena od pomanjkljivost kožnega genskega elektroprenosa je uporaba elektrod, ki so pogosto invazivne ali premalo učinkovite. Tako je potreben razvoj novih ne-invazivnih elektrod, ki bi zagotavljale učinkovito permeabilizacijo kožnih celic in vnos terapevtskih molekul brez spremljajočih poškodb kože. Razvoj gre predvsem v smeri novih ne-invazivnih več-točkovnih elektrod. Ena takšnih je bila razvita v sklopu naše raziskovalne skupine in ovrednotena glede na doseganje učinkovitosti in varnosti vnosa molekul v kožo. Rezultati potrjujejo, da je elektroporacija z uporabo več-točkovne elektrode učinkovit dostavni sistem za vnos tako modelnih učinkovin (dekstran in doksorubicin) kot tudi plazmidne DNA v mišjo kožo. Tako aplicirani električni pulzi povzročajo le minimalne poškodbe na koži. Poleg dobre učinkovitosti in varnosti je prednost tovrstne elektrode tudi možnost prilagajanja števila in razporeditve elektrodnih konic znotraj aplikatorja. S prilagajanjem krožne razporeditve konic lahko razširimo možnost uporabe elektrode za različne klinične aplikacije, kot so celjenje ran ali peritumoralno zdravljenje tumorjev. Enostavna uporaba, prilagodljivost, varnost in učinkovitost elektrode so razlogi za uporabo elektrode v nadaljnjih študijah vnosa plazmidne DNA v kožo.

Utilization of Multi-array Electrodes for Delivery of Drugs and Genes in the Mouse Skin

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Abstract— **To take full advantage of electroporation mediated delivery of therapeutic molecules to skin, it is important to establish an efficient and safe delivery system. Current electrode systems typically induce muscle twitching and pain, which is related to the distance between the electrodes and the applied voltage. Hence, the use of non-invasive electrodes, by which the delivery of electric pulses does not damage the skin, but does successfully permeabilize the skin cells and deliver the desired molecules into them is necessary. For these purposes, a system consisting of an array of electrodes was developed and evaluated in our study. In the scope of our study, the multi-array electrodes combined with electroporation proved to be effective for the delivery of model drugs (dextran and doxorubicin) as well as plasmid DNA to the mouse skin, with minimal skin damage caused by electric pulses. Furthermore, the advantage of the multi-array electrodes is to adjust the number of addressable electrode pairs that are contained within the electrode applicator, which was also demonstrated in our study. The use of multi-arrays with adjustable pins would be a valuable approach for the delivery of genes around the skin area for wound healing as well as for peritumoral delivery for cancer gene therapy. These results suggest that electroporation with multi-array electrodes can be efficient and non-invasive skin delivery method for therapeutic drugs and genes, with less adverse side effects than other electroporation delivery systems, and with promising clinical applications.**

Keywords— **Multi-array electrodes, drug delivery, gene electrotransfer, skin.**

I. INTRODUCTION

Skin is an attractive target for the drug delivery and gene therapy, due to its accessibility, large surface area and numerous antigen presenting cells that are critical to elicit an effective immune response [1]. To enhance the efficiency, specificity and temporal control of cutaneous therapy it is of utmost importance to establish an efficient and reproducible delivery system [2]. Electroporation (EP) is a physical method for the delivery of molecules into the cells and tissues [3]. Currently its biomedical applications are the delivery of chemotherapeutics [4, 5], naked plasmid DNA or RNA for tumor treatment and DNA vaccine [6, 7] or drugs/DNA delivery in or across the skin [8]. Delivery of molecules to skin and skin damage varies substantially according to the electrodes used. Several different invasive and non-invasive electrode types, from plate, needle, needle free microelectrode array to multi-electrodes array were used [1]. Such electrodes, the multi-electrode array (MEA), were designed by Heller et al. and have proved to be effective for the delivery of plasmid DNA to the skin when an intradermal injection of plasmid DNA was combined with EP [2, 9]. With the MEA, the applied voltage was minimized by maintaining a short electrode distance [2]. This diminished or eliminated the muscle twitching and pain associated with the application of the pulsed electric field.

In the scope of our study, we evaluated our novel multi-array electrodes with the similar design, for the purposes of delivery the molecules in the mouse skin. First, the histological analysis of the skin section after EP treatment was performed to determine the extent of potential tissue damage, caused by electric pulses applied by multi-array electrodes. Since the application of electric pulses to the skin by the novel electrode caused minimal skin damage, its efficiency was tested for the topical delivery of small molecules and electrotransfer of the plasmid DNA to the mouse skin.

II. MATERIALS AND METHODS

A. Reagents

The 4 kDa fluorescein-isothyocianate (FITC) labeled dextran (FD) (Sigma-Aldrich, St. Louis, MO, USA) and Doxorubicin hydrochloride (DOX) obtained from Teva (Teva Pharmaceutical Industries Ltd, Pharmachemie B.W., Haarlem, NL) were used as a model drugs to study the extent of the transport into the mouse skin.

B. Plasmids

In the experiment, the reporter plasmid encoding the red fluorescent protein (pCMV-DsRed) was used to observe the level and duration of its expression in the mouse skin. 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 2 mg/mL.

C. Animals and Skin Preparations

All procedures were performed in compliance with the guidelines for animal experiments of the EU directive

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(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 weeks old female Balb/c mice (Harlan Laboratories, Udine, IT) were used. Three to six mice were randomly assigned per each experimental group. 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, Slough, UK). Before the plasmid administration, mice were anesthetized in the induction chamber using 2% isofluorane (Nicholas Piramal India, London, UK) in oxygen and remained under an aesthesia during the procedure.

D. Electrodes

The non-invasive multi-array electrodes, consisting of 7 spring loaded pins arranged on hexagonal mesh and spaced 3.5 mm between each other, were provided by Iskra Medical (Ljubljana, SI). Electrodes were connected to the CLINIPORATOR™ (IGEA s.r.l.,Carpi, IT). A total of 24 electric pulses (2 electric pulses between each electrode pairin frequency of 5 kHz) were delivered during the treatment. For the optimization study, the central pin was removed. The electrode consisted of 6 remaining pins arranged in the circle.

E. Histology

Possible skin damages due to application of EP were observed in the exposed region of the skin. Potential skin burns, erythema, edema, hair follicle damage or the damage of the vasculature were evaluated. The region of the skin exposed to the treatment with different amplitudes of EP pulses was excised at 1, 12, 24 and 48 h post-treatment. The excised skin was fixed in formalin for 24 h and then stored in 70% ethanol until embedding in paraffin. Subsequently, 5 µm thick sections were cut in the direction perpendicular to the skin layers and stained with hematoxyline and eosine. The prepared slides were observed with BX-51 microscope (Olympus, Hamburg, GE).

F. Drug Delivery

In all experiments a circular patch served as a reservoir for molecules or drug application. In the case of EP the patch soaked with FD or DOX was applied on the depilated mouse flank region. It was removed prior to administering EP. Different short high voltage square wave pulses (amplitude between 70 and 570 V) with duration of 100 µs were applied. The increase in fluorescence intensity in the area of the electroporated skin was determined by Zeiss SteREO Lumar.V12 (Zeiss, Jena, DE) fluorescence stereomicroscope and analyzed by means of AxioVision (Zeiss).

G. Gene Electrotransfer

Anaesthetized mice received a single intradermal injection of pCMV-DsRed (2 mg/mL) in the volume of 50 µL in the right or/and left flank or four injections in the volume of 20 µL (4 × 20 µL). Immediately after plasmid administration, high voltage electric pulses with the amplitude over a distance ratio of 570 V (1600 V/cm) and the duration of 100 µs were applied. Expression of DsRed was followed under the Zeiss SteREO Lumar.V12. 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). For statistical analysis Sigma Plot software (Systat software, London, UK) was used.

III. Results

A. Macroscopical and Histological Evaluation of the Skin After EP

In order to evaluate the possible skin damage due to application of EP using the multi-array electrodes the histology analysis was performed. Macroscopically, no skin damage was observed; neither skin burns, erythema or edema. Microscopically, small presence of edema at 1 h and 12 h, mild parakeratosis and hyperplasia of epidermis were observed after applications of EP of higher amplitudes. However, hair follicles damage or severe damage of vasculature was not observed.

B. Drug Delivery

EP with novel multi-array electrodes was performed to deliver the molecules (FD and DOX as a model drugs) to the mouse skin. To determine the efficiency of topical delivery, the skin area where the delivery was performed was monitored by fluorescence stereo-microscope. Application of electric pulses by multi-array electrodes proved to be effective approach for delivery of both molecules to skin tissue. Efficiency of topical delivery of FD was observed even at the lowest amplitude of electric pulses, and it steadily increased with increasing the amplitude up to 570 V. This data was supported with the fluorescence measurement of DOX in the skin tissue. Topical delivery was again depended on the amplitude of electric pulses.

C. Gene Electrotransfer

In order to determine the efficiency and duration of plasmid DNA expression in skin tissue, plasmid encoding the *Disscosoma* red fluorescent protein DsRed with constitutive promoter (pCMV-DsRed) was delivered to skin. DsRed expression after single intradermal injection of

plasmid DNA followed by electrotransfer was monitored by non-invasive fluorescence imaging. Using the ubiquitous CMV promoter, the fluorescent signal of DsRed was first detected at 24 h post-treatment. The expression peaked at 48 h after EP and then started to decline in the next two weeks (Fig. 1). Namely, under our EP parameters, multi-array electrodes proved to be effective for delivery of plasmid DNA in mouse skin.

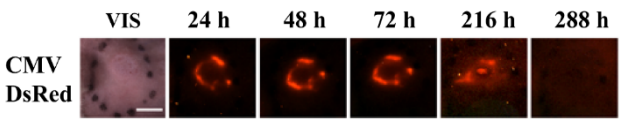

Fig. 1 Expression of red fluorescence protein in the skin. After single intradermal injection of pCMV-DsRed, followed by electric pulse application, fluorescence signal was observed under fluorescence stereomicroscope at various time points. Scale bar: 5mm. VIS - images were taken under visible light

D. Variability in Number of Addressable Electrode Pairs within the Applicator

The central pin of the electrodes was removed and the remaining 6 pins arranged in the circle were used to deliver the pCMV-DsRed in the skin. Plasmid DNA was administered by the single injection (1 x 50 µL) in the middle of treated area or by the multiple injections (4 x 20 µL) around the circle with the same diameter as electrode applicator (Fig 2-D). After administration of a single plasmid DNA injection and the use of electrode with removed central pin around the injection side, expression of DsRed was not observed. On contrary, after the administration of multiple injections, the statistical difference in expression level between the applicator with or without central pin was not identified (Fig. 2-A,B). This indicates that the removal of the central pin does not affect the transfection efficiency, when the plasmid DNA is administered at multiple sites around the treated area.

IV. Discussion

As it was presented in previous studies, after gene electrotransfer with the use of multi-array electrodes minimal skin damage were induced, that were completely recovered [10]. Similar observation was also demonstrated with our novel-multi-array electrodes, consisting of 7 spring loaded pins arranged in hexagonal mesh. Under our EP parameters, the use of novel electrodes caused minimal damage to the skin, without burns, severe edema or erythema, with only microscopically visible parakeratosis and hyperplasia. These results suggest that EP with multi-array electrodes is a non-invasive procedure for skin treatment, which can greatly reduce the adverse effects of needle or plate electrodes [10]. The use of such electrodes is thus warranted in further studies of delivery therapeutic molecules or genes to the skin.

In the scope of our study, a successfully delivery of molecules as well as plasmid DNA was achieved after the application of electric pulses provided by novel multi-array electrodes. For both model drugs, FD and DOX, the increase of fluorescence signal was detected after administration of EP with multi-array electrodes. Topical delivery was depended on the amplitude of electric pulse; higher amplitude provided larger permeabilized area and also higher quantity of molecules in the skin [8]. Under selected EP parameters, successful topical delivery as well as efficient gene electrotransfer to skin was observed [11]. The expres-

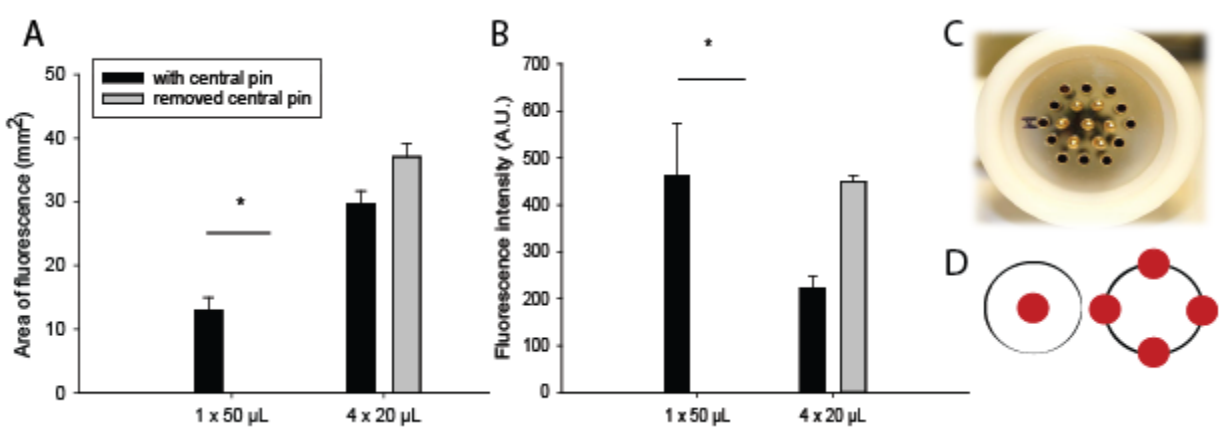

Fig. 2 The expression of DsRed protein in mouse skin using the multi-array electrodes with or without central pin. A) Area of fluorescence and B) Fluorescence intensity of DsRed protein in a mouse skin. Statistical significance was assumed at $P < 0.05$. The values were expressed as the arithmetic mean ± standard error of the mean. n= 6samples per experimental group. C) Multi-array electrode, consisting of 7 spring loaded pins arranged on hexagonal mesh and spaced 3.5 mm between each other. D) Injection protocol for pCMV-DsRed delivery to skin: 1 × 50 µL (left) and 4 × 20 µL (right).

sion profile of reporter gene coding for the DsRed was comparable to duration and level of expression to other reporter genes delivered to skin, as reported in the literature [1].

The advantage of the multi-array electrodes is a capability to adjust the number of addressable electrode pairs that are contained within the electrode applicator. Our results indicate that the removal of the central pin does not affect the transfection efficiency, when the plasmid DNA is administered at multiple sites around the treated area. These results are of utmost importance for clinical applications, when the area of transfection should be precisely regulated and when the transfection of therapeutic genes around the treated area is required. The use of multi-arrays with adjustable pins would be a valuable approach for the delivery of genes around the skin area for the wound healing assay as well as for peritumoral delivery for cancer gene therapy. The establishment of the efficient and reproducible delivery system with the capability to adjust the transfected area by varying the number of electrode pairs would promote the electroporation-mediated drug and gene delivery in the clinical practice.

V. CONCLUSIONS

To conclude, electrotransfer with multi-array electrodes promoted the effective and non-invasive delivery of drugs as well as plasmid DNA in skin tissue. Furthermore, the improvements to the electrode applicator described in our study could facilitate the translation of electrically mediated gene delivery to the clinic for the use with DNA vaccines, cancer gene therapy and wound healing assay.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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POGLAVJE 2

Poglavje 2: Genski elektroprenos v kožo z uporabo ne-invazivnih več-točkovnih elektrod za namen vakcinacije in celjenja ran

Gene electrotransfer into skin using noninvasive multi-electrode array for vaccination and wound healing

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Povzetek

Zaradi lahke dostopnosti kože in njenih imunoloških prednosti je koža zanimiv tarčni organ za vnos genov in uporabo genskega elektroprenosa. Uporaba obstoječih elektrod, namenjenih za vnos genov v kožo, je pogosto invazivna in boleča. Tako je na področju kožnega genskega elektroprenosa nujen razvoj novih ne-invazivnih elektrod, kakršna je nova več-točkovna (MEA) elektroda s krožno razporeditvijo elektrodnih konic. Z vnosom reporterskega gena za luciferazo in sledenjem luminiscentnega genskega produkta smo dokazali, da je uporaba elektrode MEA učinkovitejša za vnos plazmidne DNA v kožo v primerjavi s »klasično« ploščato elektrodo. V naslednjem koraku smo določali učinkovitost metode na primeru vakcinacije in celjenja ran. Po vnosu DNA vakcine, ki nosi zapis za modelno molekulo ovalbumin, in uporabo elektrode MEA smo izmerili porast protiteles v serumu, usmerjenih proti ovalbuminu, porast IFN-γ v splenocitih ter zaznali zaostanek v rasti nasajenih tumorjev, označenih z ovalbuminom. Vakcinacija z uporabo elektrode MEA tako vodi do močne aktivacije celičnega in humoralnega imunskega odziva in vpliva na vzpostavitev imunskega spomina. Poleg vakcinacije je elektroda MEA zaradi krožne razporeditve konic zanimiva tudi v terapiji celjenja ran, kar smo dokazali s hitrejšim celjenjem ran po vnosu terapevtske plazmidne DNA hCAP-18/LL-37 in uporabi elektrode MEA za genski elektroprenos v kožo. Enostavna uporaba in visoka učinkovitost elektrode MEA tako omogoča lažji prenos metode genskega elektroprenosa v kliniko in je kot dokazano obetavna z vidika vakcinacije ali celjenja ran.

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Gene electrotransfer into skin using noninvasive multi-electrode array for vaccination and wound healing

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ABSTRACT

Skin is an attractive target for gene electrotransfer due to its easy accessibility and its interesting immune properties. Since electrodes are often invasive and frequently induce discomfort during pulse application, there is a fundamental need for non-invasive electrodes for skin delivery. We developed circular pin non-invasive multi-electrode array (MEA), suitable for different clinical applications. MEA was first employed to deliver a luciferase reporter gene. Then, it was used to deliver a DNA vaccine coding for ovalbumin or a plasmid encoding hCAP-18/LL-37 for promoting wound healing. The results demonstrated a strong gene expression and an efficient delivery of both, DNA vaccine and wound healing agent, dependent on the pulses applied. The use of MEA to deliver the ovalbumin plasmid demonstrated a strong immune response, as evidenced by the presence of antibodies in sera, the IFN-gamma response and the delayed tumor growth when the mice were subsequently challenged with B16-OVA cells. The delivery of a plasmid encoding hCAP-18/LL-37 significantly accelerated wound closure. The easy applicability and non-invasiveness of MEA make it suitable for various clinical applications that require gene electrotransfer to skin. Specifically, by adapting electric pulses to the expected action of a transgene, non-invasive MEA can be employed either for vaccination or for wound healing.

1. Introduction

Gene electrotransfer (GET) is a safe and efficient non-viral gene delivery method that has been used to deliver naked DNA or RNA to various tissues. Among these, skin is a promising target for gene delivery [1–4], due to its easy accessibility, large treatment area and the presence of many antigen-presenting cells, which are critical for eliciting an effective immune response [5]. Therefore, skin GET has been used in many medical applications, including vaccination [2,6,7], wound healing [8–10] and cancer treatment [11,12].

In order to bring GET into broader clinical applications, delivery needs to be patient-friendly, i.e. non-invasive with minimal discomfort induction [5]. The proper design of clinically applicable electrodes is very important as it conditions their safe use and their efficiency for gene delivery. Intradermal GET can be easily performed by simple electrode devices such as plate or caliper electrodes. However, with such devices the depth of penetration of electric field is hard to control [13,14], resulting in low efficiency of intradermal gene expression. Furthermore, these electrodes require high voltages for efficient enhancement of nucleic acid delivery and could thus cause tissue damage [15,16]. To increase efficiency and to minimize skin damage, other types of non-invasive electrodes are being explored [1] [15,17]. One of the minimally invasive approaches, allowing less painful delivery of DNA into the skin while guarantying an adequate distribution of the electric field, is the utilization of microneedles [5,18]. Further advancement has been made by using non-penetrating electrodes. One example of such non-invasive electrodes is multi-electrode arrays (MEA), which were designed by two groups and have proved to be effective for the delivery of the plasmid DNA into the skin [15,19–22]. MEA minimized the necessary voltage by maintaining a short electrode distance. Consequently, the muscle twitching and pain due to the application of the electric field are decreased or eliminated [15].

Here, the hypothesis was that the easy applicability and versatility of MEA could make it suitable for various clinical applications of skin GET. In particular, by adapting the electric pulses to the specific action of the

Abbreviations: MEA, multi-electrode array; GET, gene electrotransfer; OVA, ovalbumin; ConA, concanavalin; IFN-γ, interferon gamma; HV, high voltage electric pulses; LV, low voltage electric pulses.

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transgene expressed from the plasmid, the circular pin non-invasive MEA (Fig. 1) could be employed either for vaccination or wound healing.

2. Materials and methods

2.1. Plasmids

The plasmids pVAX2, pVAX2-LUC, pVAX2-OVA and pQE-hCAP-18/LL-37 were previously constructed [10,23]. All plasmids were isolated using the EndoFree Plasmid Mega or Giga Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions and diluted in PBS. Plasmid DNA concentration was determined spectrophotometrically (Epoch Microplate Spectrophotometer; Take3TM Micro-Volume Plate, BioTek, Bad Friedrichshall, Germany) at 260 nm. Plasmid quality was assessed by the 260/280 nm ratio and by agarose gel electrophoresis.

2.2. Cell culture

B16F10-OVA, a melanoma cell line from C57BL/6 mice that stably expresses OVA, was cultured in minimum essential medium (MEM, Life Technologies, Carlsbad, CA, USA) supplemented with GlutaMAX with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 U/mL penicillin (Life Technologies) in a 5% CO_2 humidified incubator at 37 °C.

2.3. Animals

C57BL/6 mice were obtained from Janvier (Le Genest-Saint-Isle, France) or Envigo (Udine, Italy). Mice were between 6 and 8 weeks-old at the beginning of the experiments, weighing between 18 and 20 g. They were anesthetized by intraperitoneal injection of ketamine (10 mg/ml; Anesketin, euroVet, Heusden-Zolder, Belgium) and xylazine (1.3 mg/ml; Sigma, Diegem, Belgium). Before electroporation, mice were shaved using a rodent shaver (Aesculap Exacta shaver, AgnTho's, Lidingö, Sweden). All experimental protocols in mice were approved by the Ethical Committee for Animal Care and Use of medical Sector of the Université Catholique de Louvain (permission no. 2011/UCL/MD/007) and had permission from the Veterinary Administration of the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (permission no. 34401-4/2012/4). The experimental procedures were performed in compliance with the guidelines for animal experiments of the EU directive (2010/63/EU).

2.4. Plasmid injection and electroporation

For all the experiments, 50 μg of the plasmids were first injected intradermally using an insulin syringe. They were injected either as a single injection in a volume of 30 μl (for vaccination) or as a multiple injection (4 × 20 μl, for wound healing) around the wound created by using a 5 mm diameter biopsy punch (Kai Medical, Solingen, Germany). 6 mice were randomly assigned for each experimental group. Two different types of electrode were applied: (1) 2 mm spaced plate electrodes for delivery of short high voltage pulse (700 V/cm 100 μs) immediately followed by a low voltage pulse (200 V/cm 400 ms) and (2) MEA used to deliver short high voltage pulses (HV, 1600 V/cm 100 μs) or long low voltage pulses (LV, 170 V/cm 150 ms). MEA (Iskra Medical, Podnart, Slovenia) consists of 7 spring loaded pins arranged on hexagonal mesh and spaced 3.5 mm from each other (Fig. 1). A total of 24 electric pulses (2 electric pulses between each electrode pair in frequency of 5 kHz in opposite polarity) were delivered during the treatment. The electric pulses were generated by a Cliniporator system (Cliniporator, IGEA, Carpi, Italy). For all the experiments, a conductive gel was used to ensure electrical contact with the skin (Aquasonic 100, Parker, Fairfield, USA).

2.5. Luciferase expression

C57BL/6 mice were injected with 50 μg of pVAX2-LUC (single or multiple injections) and electroporated as described above. To follow the luciferase expression over time, mice were injected intraperitoneally with luciferin (150 mg/kg) in 100 μl PBS and optical images were acquired using an IVIS 50 system (Xenogen Corporation, Alameda, CA, USA). The luminescence was quantified by ImageJ software (National Institute of Mental Health, Bethesda, MD, USA) and expressed as integrated density, i.e. the sum of intensities of all pixels.

2.6. Immunization

C57BL/6 mice were immunized by intradermal injections of 50 μg of pVAX-OVA or pVAX2 as a control plasmid. The mice which received pVAX2 were considered as a naïve group. Six mice were included in each experimental group. Immediately after the plasmid injection, electroporation was performed using the plate or MEA electrodes with HV or LV pulses. Mice received one priming and two booster doses with a two weeks interval between each administration. Two weeks after the last immunization procedure, the spleen and blood were either collected or the mice were challenged with tumor cells (Scheme 1).

An ELISA assay was performed to quantify the total immunoglobulin titers and the isotypes of anti-OVA antibodies in the serum samples. 96-

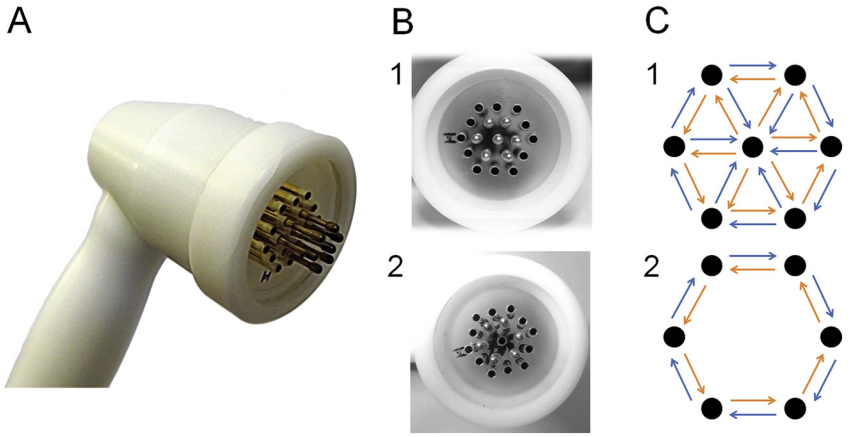
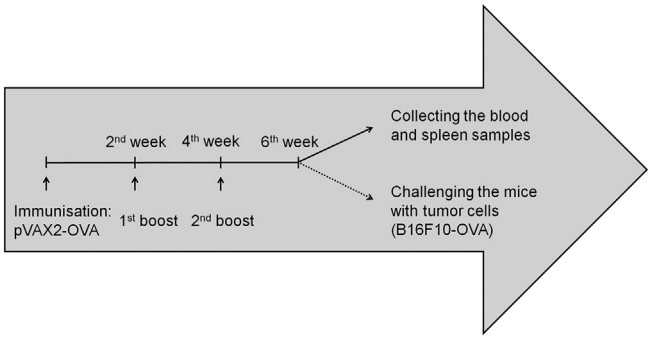


Fig. 1. Non-invasive multi-electrode array (MEA) developed for different therapeutic approaches. The figure was modified from *Mol Ther Nucleic Acids* [27]. (A) Applicator of MEA. (B) MEA consists of 7 spring loaded pins arranged on hexagonal mesh (1). The central pin can be removed and the rest 6 pins remain arranged in a circle (2). (C) The distribution of electric pulses between the pins arranged in hexagonal mesh (1) or in a circle (2).



Scheme 1. Immunization protocol. Immunization protocol included one priming and two boosts with plasmid pVAX-OVA.

well plates were coated overnight with 10 μg/ml of OVA protein (Sigma) diluted in sodium bicarbonate solution. After washing with PBS/Tween 20 0.1%, plates were blocked with 5% dry milk in PBS for 0.5 h in a humid chamber at room temperature. Subsequently, plates were washed and incubated for 2 h at room temperature with serial dilutions of serum samples diluted in 1% solution of bovine serum albumin (BSA) in PBS. After washing, peroxidase-labeled LO-MGCOC-2 (IMEX, UCL, Brussels, Belgium) and TMB substrate revelation (Calbiochem, San Diego, CA, USA) were used to determine total immunoglobulin titers, defined as the dilution factor giving an optical density at 450 nm equal to the limit of quantification (LOQ, mean blank value plus 10 SDs). Isotypes of anti-OVA antibodies were determined using appropriate secondary antibodies (LO-MG1-13 for IgG1 and LO-MG2A-9 for IgG2a quantification).

The spleens of the treated mice were collected aseptically. Red blood cells were lysed with ACK lysis buffer (Lonza, Walkersville, USA) and splenocytes were washed with PBS, counted and adjusted to a concentration of 10^6 cells/well. They were cultured in 96-well tissue culture plates (Becton Dickinson, Erembodegem, Belgium) in RPMI medium supplemented with 10% FBS, 1% penicillin/streptomycin, 1% sodium pyruvate and 5×10^{-5} M 2-mercapto-ethanol. Cells were restimulated by the addition of 100 μL of OVA protein solution (0.1 mg/mL), 100 μL concanavalin A (5 μg/mL) as a positive control or 100 μL of the culture medium as a negative control. After 72 h of incubation (37 °C, 5% CO_2), supernatants were collected and assayed for IFN-γ (DuoSet ELISA development kits, R&D systems Europe Ltd., Abingdon, United Kingdom).

Two weeks after the delivery of the last boost, mice were challenged with the tumor cells. As a challenge, 10^5 OVA expressing B16F10 cells in a volume of 100 μL of PBS were injected subcutaneously into the mouse flank and the tumor size (length × width × height, in mm^3) was measured with an electronic digital caliper. Mice were sacrificed when the tumor volume became larger than 1500 mm^3 or when they were in poor condition. The results are expressed using Kaplan-Meier curves.

2.7. Wound healing assay

C57BL/6 mice were anesthetized using an intraperitoneal injection of ketamine (1 mg/ml, Narektan®, Vetoquinol, Ittigen, Switzerland), xylazine (5 mg/ml, Chanazine; Chanelle Pharmaceuticals, Loughrea, Ireland) and acepromazine (0.4 mg/ml, Promace; Fort Dodge Animal Health, Iowa, USA). The dorsal surface was shaved and treated with depilatory cream (Veet® Sensitive Skin, Reckitt Benckiser, United Kingdom) in order to remove any remaining hair. The dorsum of each anesthetized animal was disinfected with polyvidone iode (Braunoderm, Braun, Melsungen, Germany). Two full-thickness wounds, one on the each mouse flank, were created using 5 mm round skin biopsy punch (kai Europe GmbH, Solinger, Germany). To avoid cross-contamination both wounds were administered with the same treatment. Plasmids in the total volume of 80 μl (50 μg per wound) were intradermally injected in four sites around the wound (4 × 20 μl). The wounds were electroporated either using plate or MEA with removed central pin (Fig. 1) [20]. Between the 6 pins arranged in a circular mesh, a total of 12 electric pulses were applied (2 electric pulses between each electrode pair in opposite polarity).

To reduce wound contraction, wounds were then fixed with a silicone splint, cut from a 0.5 mm thick silicone sheet (Grace Bio-Labs, Sigma-Aldrich, Oregon, USA). Donut-shape splints with an outer diameter of 15 mm and an inner diameter of 6 mm were placed around the wound so that the wound was centered within the splint. An immediate-bonding adhesive was applied to fix the splint to the skin, followed by 5-0 Mersilk non-absorbable sutures (Ethilon, San Lorenzo, USA). Wounds were washed with saline and covered by non-adhesive patches (Tosama, Domzale, Slovenia) and Tegaderm on top of it as a transparent occlusive wound dressing (Tegederm, Neuss, Germany). After the surgery mice received intramuscular analgesia (butorphanol, 0.3 mg/kg, Torbugesic; Fort Dodge Animal Health), repeated on the next day. Every second day wounds were digitally captured by Zeiss SteREO Lumar. V12 (Zeiss, Jena, Germany) fluorescence stereomicroscope equipped with an MRc.5 digital camera (Zeiss). The optical zoom was maintained constant throughout the experiment. Wound areas were quantified using AxioVision software (Zeiss). Results were expressed as the time needed for complete wound closure [9,10].

Five days after wound formation and treatment, mice were sacrificed and the wounds with the surrounding skin tissue were excised for the histological analysis. Excised samples were fixed in zinc fixative (BD PharMingen™, BD Bioscience, San Diego, CA, USA) for 24 h and then stored in 70% ethanol until being embedded in paraffin for assessing the wound healing histologically. Five micrometer thick sections were cut in the direction perpendicular to the skin layers and stained with hematoxylin and eosin (HE) to assess the predominant stages of healing, or with Masson's trichrome (MT) to study the extent of collagen deposition in healed tissue. MT staining enabled the visualization of collagen fibers labeled in blue. The samples were observed using a BX-51 fluorescence microscope (Olympus, Hamburg, Germany) equipped with a digital camera DP72 (Olympus). All histological analyses were performed on at least 4 wounds per group and the images presented are representatives of all replicates.

2.8. Statistical analysis

For statistical analysis Sigma Plot software (Systat software, London, United Kingdom) or GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) was used. Significance was determined by the Student *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 arithmetic mean (AM) ± standard error of the mean (SEM).

3. Results

3.1. MEA promoted a high efficiency and a long duration of luciferase gene expression in mouse skin

Electrotransfer of the luciferase reporter gene to mouse skin was used to evaluate the impact of MEA on both the level and the duration of gene expression in the mouse skin. After a single injection of the plasmid DNA, a high level of expression was achieved in the groups treated by MEA, either under HV or LV conditions (Fig. 2A). At day 3 post-treatment, the expression level of the luciferase gene was ~7-fold higher using HV pulses applied by MEA, compared to the untreated control group, and 6-fold higher using LV pulses applied by MEA (Fig. 2C). The luminescent signal was detected up to 30 days post-treatment. The expression level in the group treated with the plate electrodes did not statistically differ from the untreated group.

To simulate the experimental conditions of wound healing experiments, multiple injections of the plasmid DNA around the wounded

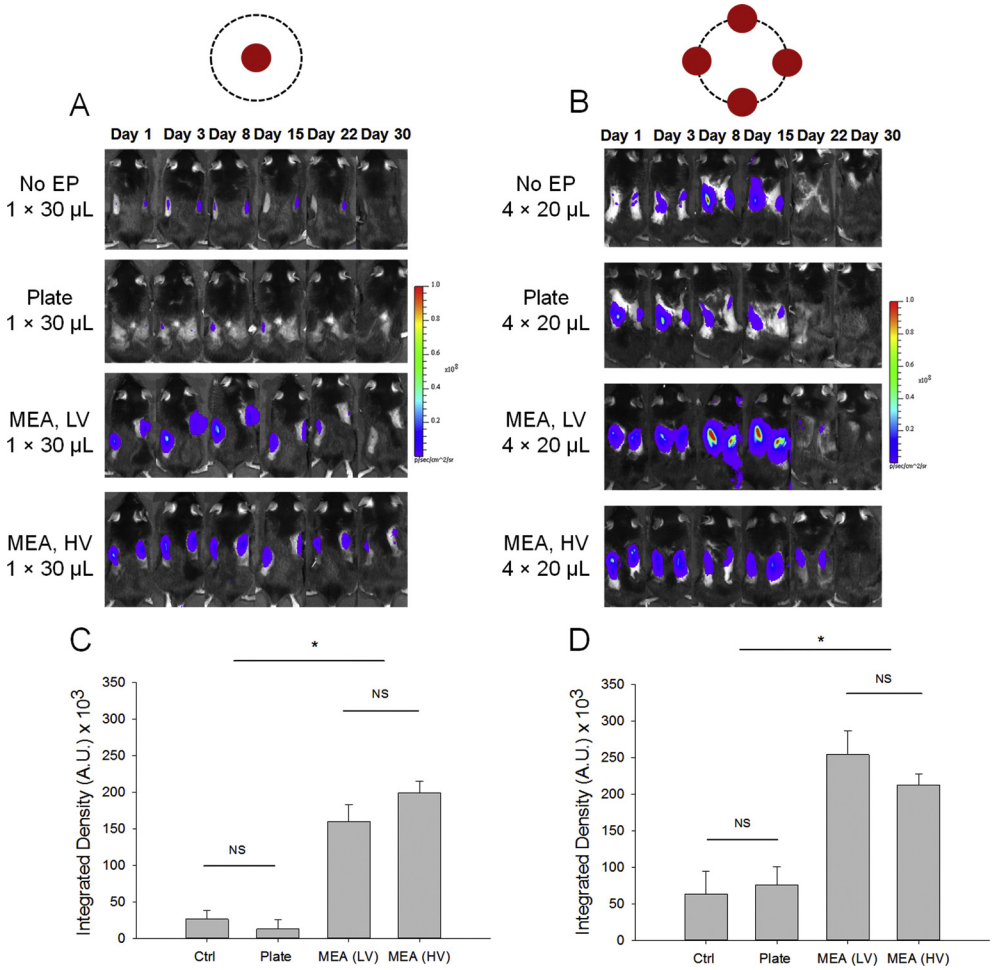


Fig. 2. The expression of the luciferase gene in mouse skin. (A) The expression of the luciferase gene after a single injection of plasmid pVAX2-LUC. (B) The expression of the luciferase gene after multiple injections of plasmid pVAX2-LUC around the treated area (wounded skin). (C) The quantification of the intensity of luciferase gene expression after a single injection of plasmid pVAX2-LUC. The quantification is presented for day 3 post-treatment. No EP = the group was not treated by electroporation. (D) The quantification of the intensity of luciferase gene expression after multiple injections of plasmid pVAX2-LUC around the treated area. The quantification is presented for day 3 post-treatment. Error bars indicate SEM. *P value < 0.05 between the selected groups. NS = no statistically significant difference. N = 6 mice per group.

area were performed (Fig. 2B). Similar results than those obtained with a single plasmid injection were observed; 3 days post-treatment the luciferase gene expression was 3-fold higher under HV conditions and 4-fold higher under LV pulses applied by MEA, compared to the control groups (without electroporation treatment or with the plate electrodes) (Fig. 2D). As observed with the single injection treatment, the use of MEA induced a prolonged gene expression which was maintained for several weeks.

3.2. Utilization of MEA resulted in high immunoglobulin antibodies production

To assess the efficiency of MEA for the purpose of vaccination, mice were immunized with pVAX2-OVA plasmid. A strong immune response against ovalbumin was obtained in mice when the plasmid was electrotransfered to the skin with MEA. Namely, after one priming and two boosts delivered every two weeks, mice that were vaccinated via MEA showed ~100-fold higher total anti-OVA IgG levels compared to the treatment with the plate electrodes (Fig. 3A1). Significantly higher total anti-OVA IgG titers were obtained either after HV or LV pulses application. To investigate whether the application of HV or LV pulses influences the bias of the immune response, the IgG1 (Fig. 3A2) and IgG2a (Fig. 3A3) specific titers were measured after the immunization via MEA. IgG1 and IgG2a levels did not statistically differ between the application of HV and LV pulses. Although, IgG1/IgG2a ratio under LV pulses tended to be higher (22.2 ± 10.7) compared to HV conditions (9.2 ± 3.1), indicating a Th1-shifted response when HV pulses were applied.

3.3. Utilization of MEA resulted in high cytokine production by stimulated splenocytes

To further evaluate the effect of the electrodes on the generation of the immune response, the secretion of IFN-γ cytokine was measured after restimulation of the splenocytes collected from animals immunized by pVAX-OVA plasmid injection and following electroporation. A significant increase of IFN-γ was observed for all treated groups, using either plate electrodes or MEA (Fig. 3B). Although, higher levels of IFN-γ were obtained after the immunization performed with MEA compared to the plate electrodes application. Particularly under LV conditions (22,312 ± 3142 pg/mL), significantly higher cytokine levels were measured compared to immunization with plate electrodes (11,803 ± 1805 pg/mL).

3.4. Plate electrodes and MEA were equally efficient in delaying B16F10 tumor growth and improving mouse survival

After one priming and two booster doses delivered every two weeks, the vaccinated mice were challenged with OVA-expressing B16F10 tumor cells, to test long term specific memory of the immune system

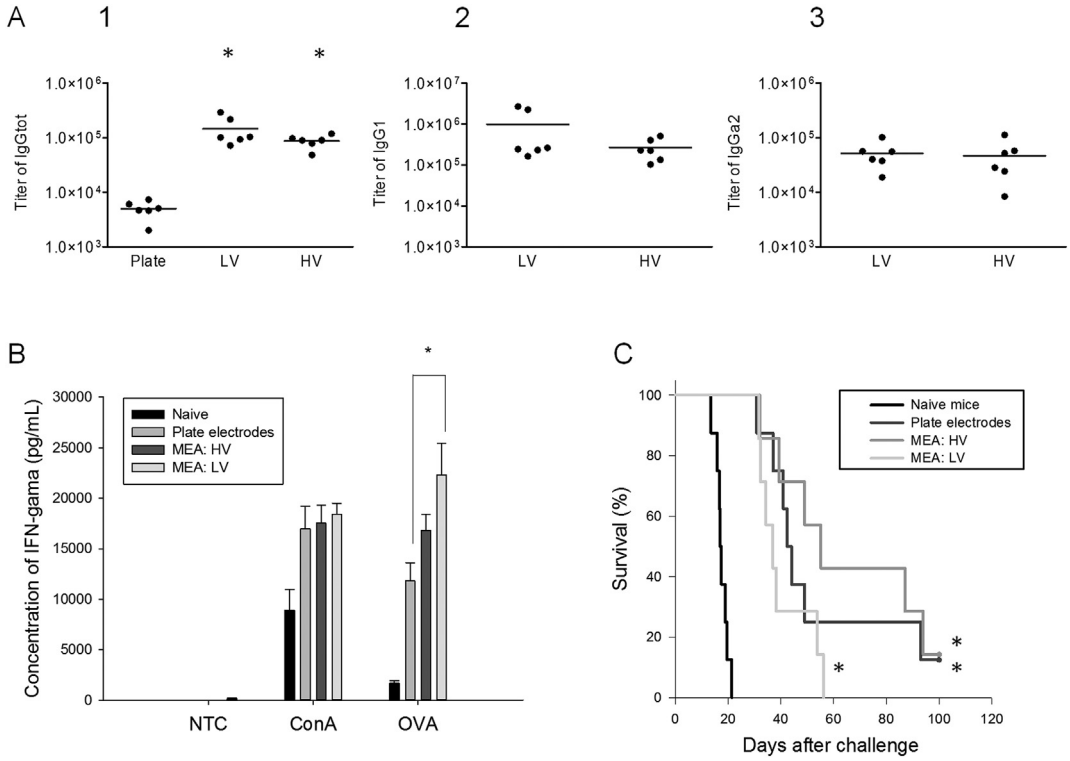


Fig. 3. A) Immunoglobulin titers measured in the serum of the immunized mice. (1) Total anti-OVA IgG levels measured after GET via the plate electrodes or MEA under LV conditions or MEA under HV conditions. *P value < 0.05 compared to the group treated by plate electrodes. (2) IgG1 isotypes or (3) IgGa2 isotypes of anti-OVA antibodies measured after GET via MEA under LV conditions or MEA under HV conditions. B) Concentration of IFN-γ measured in the spleen samples of the immunized mice. Error bars indicate SEM. *P value < 0.05 between the groups. N = 6 mice per group. OVA = ovalbumin; ConA = concanavalin A; NTC = culture medium as a negative control. C) Survival curves of immunized mice after the tumor challenge. The results are expressed using Kaplan-Meier curves. *P value < 0.05 between the naïve group and the treated groups. N = 8 mice per group.

against OVA antigen. Naïve mice failed to induce rejection of B16F10 cells, whereas the vaccinated mice significantly impaired B16F10 tumor growth and prolonged the survival of mice (Fig. 3C). The immunization was effective either with MEA or plate electrodes, but no statistically relevant difference was noted between the HV or LV pulses delivered with MEA.

3.5. Easy applicability and high efficiency of MEA make it promising for wound healing applications

To compare the effect of different electrodes on wound healing, the control plasmid encoding LL-37 was electrotransferred around the wounded area and the pulses were applied via plate electrodes or by

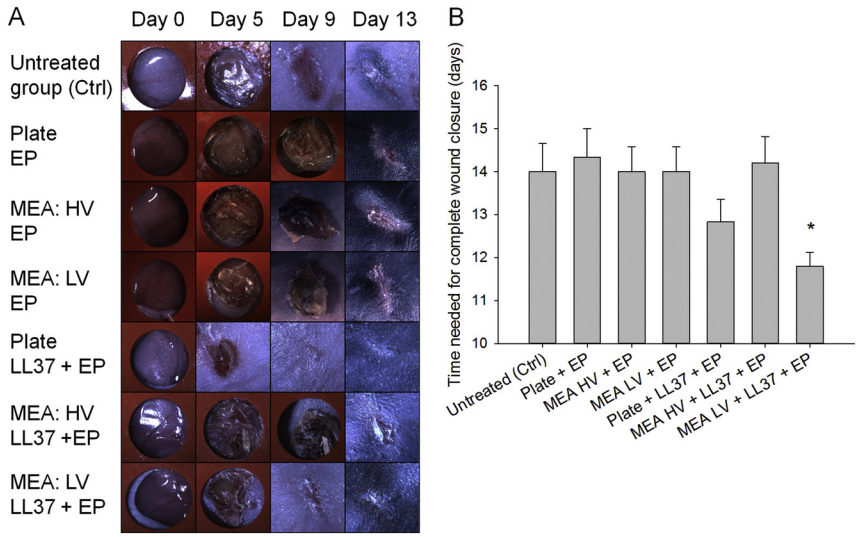


Fig. 4. Time needed for the complete wound closure after the intradermal delivery of the plasmid encoding LL37 around the wounded area. (A) Images of the wounds taken under the stereomicroscope on the designated times. EP = the group was treated by application of electric pulses; LL37 = hCAP-18/LL-37 encoding plasmid. B) The number of days needed for the complete wound repair. Error bars indicate SEM. *P value < 0.05 compared to the control group. N = 6 mice per group.

MEA. To determine the effectiveness of the electrodes, the time needed for complete wound repair was followed and histological analysis of the treated wounds was performed. As demonstrated in previous studies, plasmid hCAP-18/LL-37 promoted the wound healing process [9,10]. Hence, shorter healing time in experimental groups indicated better treatment efficiency. The highest effectiveness of the therapy was achieved in the group treated with LV pulses applied by MEA (Fig. 4A). The electrotransfer of the plasmid DNA by LV pulses significantly shortened the time needed for the complete wound repair (11.8 ± 0.3 days) compared to the untreated group (14.0 ± 0.7 days). Moreover, shorter healing time was observed in the group treated by plate electrodes (12.8 ± 0.5 days). When the pulses were applied by MEA under HV conditions, the treatment was not effective with a comparable healing time (14.2 ± 0.6) to the untreated group (Fig. 4B). Healing times of control groups, treated with plate electrodes or MEA in the absence of plasmid DNA did not statistically differ from the untreated control group. Therefore, electric pulses alone did not damage the skin around the wounded area, and did not impact on wound healing time.

Histological evaluation of the skin with HE and MT staining presented insights into the morphology of skin layers and collagen production during the healing process. At day 5, the skin sections of the untreated control group revealed pronounced inflammation with strong infiltration of immune cells (Fig. 5A). In the untreated group, skin was poorly vascularized without evident collagen production. Similar results were observed after HV pulse application. In the group treated with MEA under LV conditions as well as in the group treated with the plate electrodes, less inflammation, high vascularization and intensive collagen production were observed (Fig. 5B). Interestingly, the histological evaluation appeared predictive of wound healing efficiency with noticeable difference between control or HV (with no influence on wound healing) and plate electrodes or LV (that induced an accelerated healing of wounds).

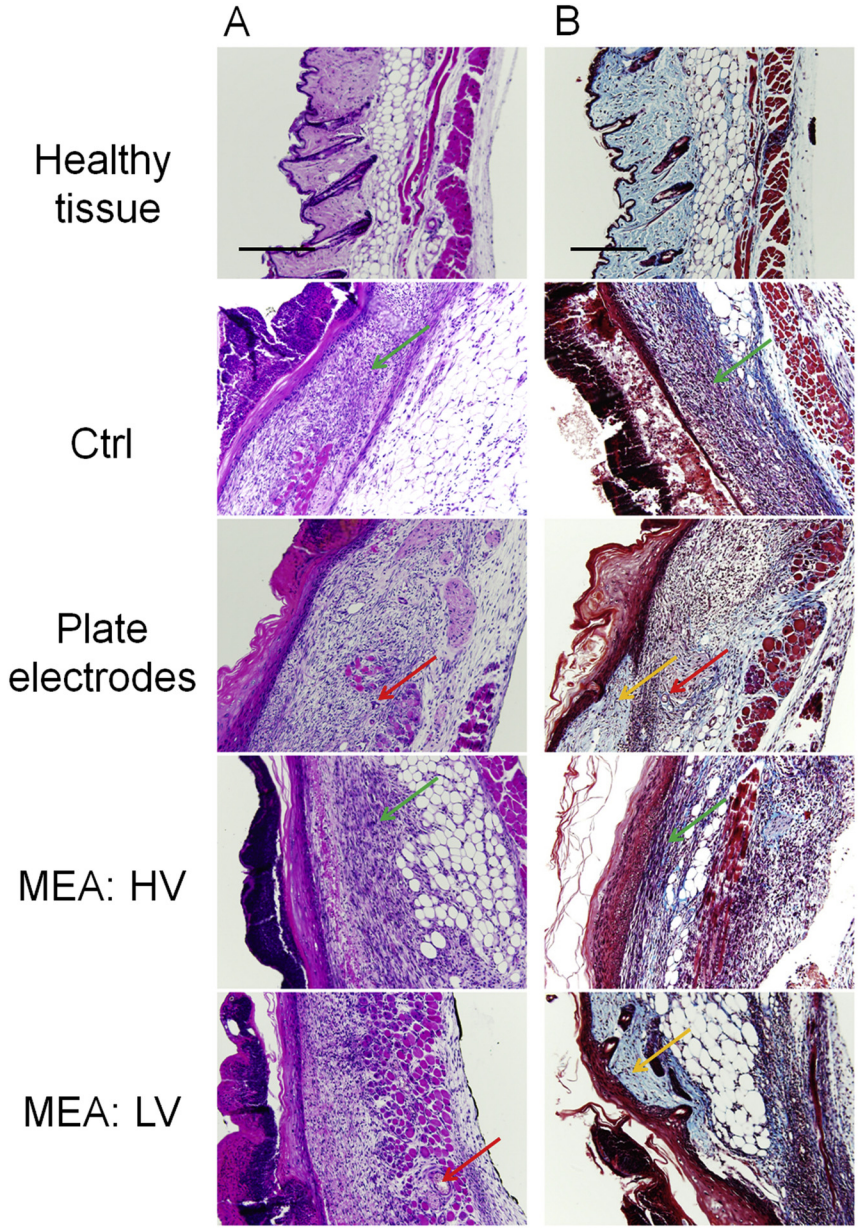


Fig. 5. Histological analysis of the treated wounds. GET was performed either using plate electrodes, MEA under high voltage conditions (HV) or MEA under low voltage conditions (LV). 5 μm thick sections were cut in the direction perpendicular to the skin layers and stained with (A) hematoxylin and eosin, or stained with (B) Masson's trichrome. Vascularization is presented by red arrows, pointed to representative blood vessels in dermis. Area of immune infiltration in dermal layer is presented by green arrows and collagen production with yellow arrows. The area of collagen production is stained blue, due to the Masson's trichrome staining. *Scale bar* 200 μm.

4. Discussion

In this study, we demonstrate high effectiveness of the non-invasive MEA to deliver ovalbumin or hCAP-18/LL-37 encoding plasmid for the purposes of vaccination and wound healing, respectively. We focused not only on the type of electrode used but also on the magnitude of the electric pulses.

First, the efficiency of skin GET using the different electrodes was determined from the intensity and duration of luciferase gene expression. GET parameters for each electrode were selected based on the data published in previous studies [20,22–24]. The plasmid injection protocols were optimized either for vaccination by a single injection of the plasmid DNA or for wound healing treatment by multiple injections around the wounded area. In both ways, the GET via MEA resulted in higher levels and longer duration of luciferase gene expression, compared to the plate electrodes application. Furthermore, gene expression could be enhanced by increasing the site of the treated skin area [19]. These results could facilitate the utilization of MEA for vaccination and wound healing applications.

Second, the induction of an immune response was studied to compare the efficiency of MEA and the plate electrodes for the purposes of vaccination. After the priming and two boosts with pVAX-OVA plasmid, humoral as well as the cellular immune response were determined. The vaccination via MEA resulted in 100-fold higher total anti-OVA IgG levels compared to the treatment with the plate electrodes, indicating a stronger activation of humoral immune response when the vaccination was performed with MEA. The activation of cellular immune response resulted in significantly higher IFN-γ levels in the isolated splenocytes, when GET was performed with MEA. The tumor growth was similarly delayed for all the treated mice, suggesting that the cellular response was sufficient even after delivery of the DNA vaccine through plate electrodes. We presume that more evident differences between the electrodes would be highlighted, if mice received a lower dosage (<50 μg/mouse) of the DNA vaccine.

Third, the efficiency of gene delivery with MEA was followed on wound healing model. The control plasmid encoding peptide LL37, which promotes wound healing and angiogenesis [9], was electroporated around the wounded area. The best efficiency, i.e. the shortest healing time, high vascularization of the treated skin region and intensive collagen production, was obtained when MEA under LV conditions was applied. Here, the important advantage of MEA was taken to deliver the pulses around the wounded area. From the hexagonal pattern of the MEA, the central pin was removed and each wound was caught in between the rest of the 6 pins arranged in a circle [20]. The adjustment of the pin number inside the applicator provides an easy applicability of MEA for different treatment purposes [15] (Fig. 1). Apart from wound healing, the electrodes are also promising in cancer gene therapy to administer plasmid DNA coding for cytokines [25] peritumorally to the skin, in order to boost the immune reaction of the local ablative techniques. Such an approach was already proposed for the electrochemotherapy in the combination of peritumorally delivered IL-12 [12,26].

As was evident from the wound healing results, the HV and LV pulses did not show the same efficiency for plasmid delivery. For instance, the wound healing and therapeutic effect of LL37 was promoted under LV pulses, but not under HV pulses. As was explained in our previous study, varying the pulses applied by MEA allowed a modulation of the depth of the transfection impacting the local or systemic influence of the treatment [27]. It was demonstrated that HV pulses promote the transfection efficiency in the superficial skin layers and result in local distribution of the transgene, while LV pulses promote the transfection also in the deeper skin layers and are optimal for the GET of the molecules with systemic mode of action [27]. For the purposes of vaccination, which targets the local immune response of the skin cells, the HV pulses would optimally improve local gene expression in the skin. Nevertheless, this study did not reveal any major difference between HV and LV pulses in terms of vaccine efficiency. Under both electrotransfer parameters the IgG titers as well as cytokine levels were very high. This could be possibly suggested by the high immunogenicity of ovalbumin, resulting in immune response too high to observe any significant difference between the pulses. Even though, a lower IgG1/IgG2a ratio and slightly improved antitumor effectiveness potentially suggested a better efficiency of HV pulses for the purposes of vaccination. For future studies, we hypothesize that significant differences would be observed if mice were vaccinated with less immunogenic molecules, or with a lower dosage of the plasmid DNA, as discussed above.

Although adequate therapeutic results could be achieved by plate electrodes administration, the development of novel non-invasive electrodes is required to fulfil the safety issues of GET. As was confirmed in the previous studies, the use of novel MEA electrodes causes minimal damage to the skin without burns, severe edema or erythema, with only microscopically visible parakeratosis and hyperplasia observed after application of pulses of higher amplitudes [20,22]. These results suggest that GET with MEA is a non-invasive procedure for skin treatment, which can greatly reduce the adverse effects of needle or plate electrodes [19] and contribute to better compliance with the patients.

5. Conclusion

To conclude, this study demonstrates high efficiency of MEA for the purposes of GET into the mouse skin. In particular, electroporation through MEA resulted in high gene expression levels in the skin and elicited a humoral and cellular immune response against ovalbumin in vaccinated mice. In addition, shorter healing time of the wounds was observed when MEA was used to deliver LV pulses as compared to the "classical" plate electrodes. Hence, to provide the optimal effectiveness of MEA, it is of the utmost importance to carefully select the electrical parameters according to the mode of action of the delivered molecule and adjust them to different therapeutic approaches. The advantages of MEA, such as non-invasiveness, high efficiency for gene delivery and easy applicability of the electrode, make the electrode attractive for clinical applications, such as wound healing, cancer therapy and vaccination.

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POGLAVJE 3

Poglavje 3: Izboljšana učinkovitost genskega elektroprenosa v kožo z uporabo plazmidne DNA s kolagenskim tkivno-specifičnim promotorjem

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

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Povzetek

Da bi zagotovili varen, učinkovit in nadzorovan vnos plazmidne DNA v kožo, je potrebno izboljšati tako dostavne sisteme kot sestavo plazmidne DNA. Varnost genskega vnosa v kožo lahko bistveno izboljšamo z uporabo ne-virusnih dostavnih metod, kot je genski elektroprenos, kot tudi z razvojem tkivno-specifičnih promotorjev. V okviru opisane raziskave smo ocenili plazmidno DNA z novim kolagenskim kožno-specifičnim promotorjem (COL), ki smo ga vnesli v kožne celice in kožno tkivo s pomočjo metode genskega elektroprenosa. Specifičnost promotorja COL za transfekcijo kožnih celic smo določali tako *in vitro* kot *in vivo*. *In vitro* smo specifično izražanje plazmidne DNA s promotorjem COL v fibroblastih zaznali tako v primeru vnosa reporterskega gena DsRed kot terapevtskega gena, ki nosi zapis za IL12. V nadaljevanju smo *in vivo* na mišjem modelu dosegli učinkovit vnos reporterskega in terapevtskega gena v kožo. Plazmid s kolagenskim promotorjem se je v koži izražal šibkeje in krajši čas v primerjavi s konstitutivnim promotorjem. Kljub šibkejšemu izražanju plazmida s promotorjem COL, pa le ta, v nasprotju s konstitutivnim promotorjem, omogoči lokalno in specifično izražanje genskega produkta v koži, brez sistemskega porazdeljevanja transgena. Tako doseženo specifično ciljanje kožnih celic s tkivno-specifičnim promotorjem je pomemben korak k izboljšanju učinkovitosti in varnosti kožne genske terapije in DNA vakcin.

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

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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

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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),

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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™ (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

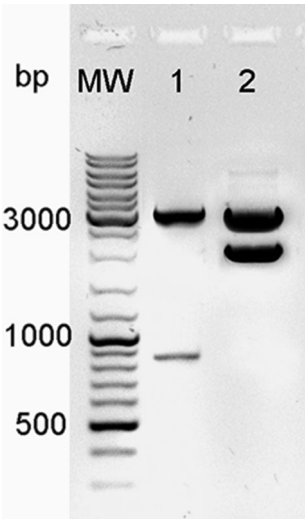

Fig. 1 Confirmation of successful construction of the plasmids using restriction analysis. Newly constructed plasmids pCOL-DsRed (*lane 1*) and pCOL-mIL-12 (*lane 2*) were cut with different combinations of restriction enzymes, with restriction sites in different parts of the construct (within the vector 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

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

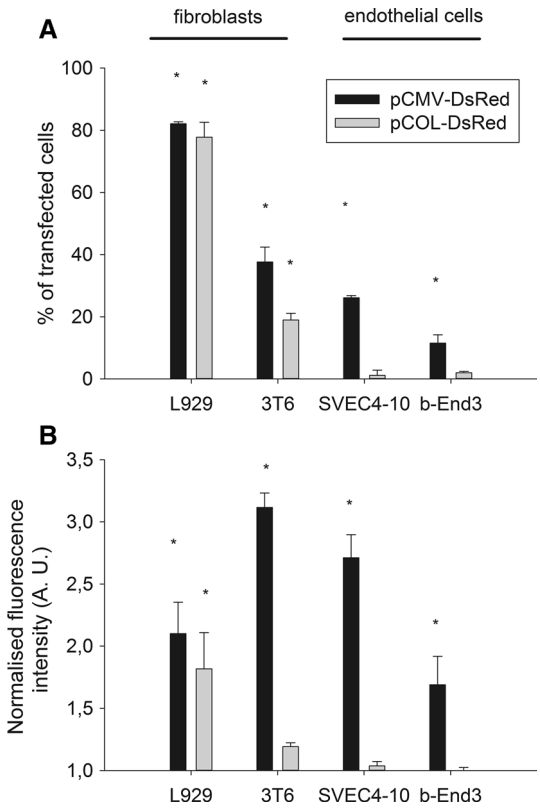

Fig. 2 Transfection level and the fluorescence intensity in fibroblasts and endothelial cells. **a** Percentage of the cells expressing DsRed protein. The results represent the transfection efficiency under the optimal electric conditions. $*P < 0.05$ compared to the control group of untreated cells 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

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

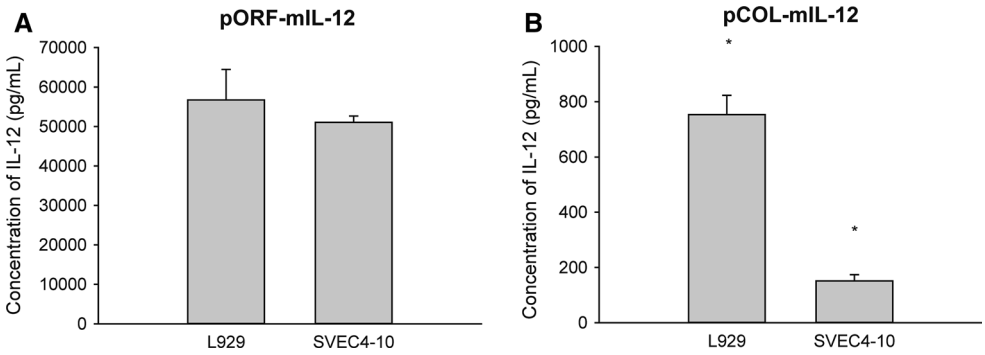

Fig. 3 Concentration of protein IL-12 (pg/mL) in L929 and SVEC4-10 cells. Concentration was measured in extracted cell medium 48 h after gene electrotransfer of (**a**) pORF-mIL-12 and (**b**) pCOL-mIL-12. $*P < 0.05$ between L929 and SVEC4-10 cell lines. The results represent three independent experiments,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

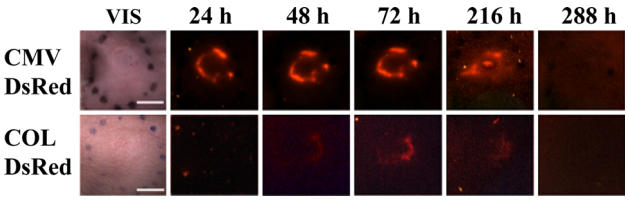

Fig. 4 Expression of red fluorescence protein in the skin under the control of ubiquitous or tissue-specific promoter. After single intradermal injection of plasmid DNA, followed by electric pulse application, fluorescence signal was observed under fluorescence stereomicroscope at various time points. 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

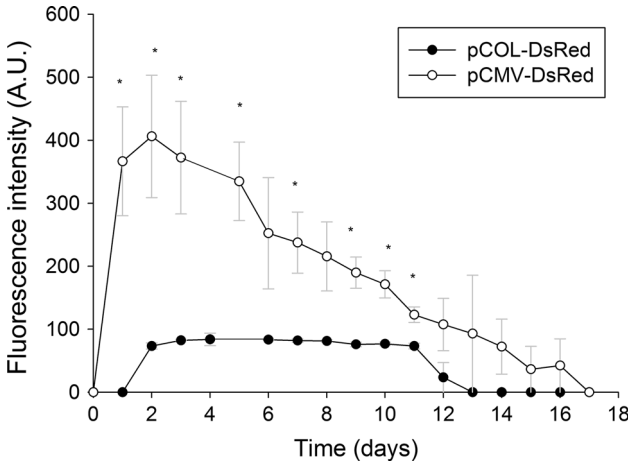

Fig. 5 Fluorescence intensity of DsRed protein expressed in the skin at different time points. Fluorescence was measured after single intradermal injection of pCOL-dsRed or pCMV-DsRed in the volume of 50 µL, followed by application of electric pulses (570 V/100 µs). To measure fluorescence intensity, 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

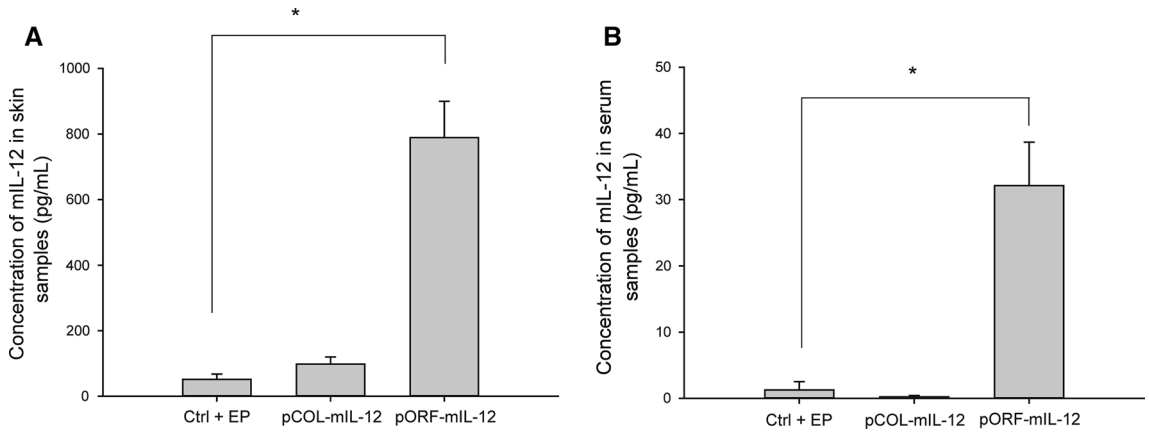

Fig. 6 Concentration of IL-12 (pg/mL). **a** Concentration of IL-12 (pg/mL) measured in skin samples. **b** Concentration of IL-12 (pg/mL) measured mouse serum. For the detection, ELISA analysis was performed. $*P < 0.05$ compared to the control group of samples without plasmid DNA delivery, only with 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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POGLAVJE 4

Poglavje 4: Parametri elektroprenosa kot orodje za doseganje kontroliranega in ciljanega genskega izražanja v koži

Electrotransfer parameters as a tool for controlled and targeted gene expression in skin

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Povzetek

Genski elektroprenos je varna in učinkovita ne-virusna metoda, ki se pogosto uporablja za dostavljanje gole DNA ali RNA v različna tarčna tkiva. Eno izmed bolj zanimivih tkiv z vidika genskega elektroprenosa je koža, ki kot tarčni organ za dostavo DNA predstavlja izziv predvsem za vakcinacijo, zdravljenje ran ter v terapiji raka. Na varnost in učinkovitost metode lahko vplivajo številni dejavniki, med drugim tudi izbor električnih parametrov. Z vnosom reporterskih in terapevtskih genov smo dokazali, da lahko s prilagajanjem trajanja in napetosti električnih pulzov vplivamo na globino transfekcije v mišji koži, trajanje in stopnjo genskega izražanja ter lokalno ali sistemsko porazdeljevanje transgenih molekul. Pokazali smo, da kratki visoko-napetostni pulzi povzročijo transfekcijo vrhnjih plasti kože in vodijo k lokalnemu izražanju transgena, medtem ko dolgi nizko-napetostni pulzi povzročijo transfekcijo tudi globljih plasti kože in vodijo do dolgotrajnejšega genskega izražanja s sistemskim porazdeljevanja transgena. Z izborom optimalnih parametrov genskega elektroprenosa lahko tako zagotovimo kontroliran vnos molekul DNA v kožo ter varno in učinkovito delovanje terapije.

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Electrotransfer parameters as a tool for controlled and targeted gene expression in skin

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Skin is an attractive target for gene electrotransfer. It consists of different cell types that can be transfected, leading to various responses to gene electrotransfer. We demonstrate that these responses could be controlled by selecting the appropriate electrotransfer parameters. Specifically, the application of low or high electric pulses, applied by multi-electrode array, provided the possibility to control the depth of the transfection in the skin, the duration and the level of gene expression, as well as the local or systemic distribution of the transgene. The influence of electric pulse type was first studied using a plasmid encoding a reporter gene (DsRed). Then, plasmids encoding therapeutic genes (IL-12, shRNA against endoglin, shRNA against melanoma cell adhesion molecule) were used, and their effects on wound healing and cutaneous B16F10 melanoma tumors were investigated. The high-voltage pulses resulted in gene expression that was restricted to superficial skin layers and induced a local response. In contrast, the low-voltage electric pulses promoted transfection into the deeper skin layers, resulting in prolonged gene expression and higher transgene production, possibly with systemic distribution. Therefore, in the translation into the clinics, it will be of the utmost importance to adjust the electrotransfer parameters for different therapeutic approaches and specific mode of action of the therapeutic gene.

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Introduction

Gene electrotransfer (GET) is a safe and efficient nonviral gene delivery method based on electroporation, *i.e.*, the application of controlled electric pulses to cells or tissues.[1] The method has been used in gene therapy to deliver naked DNA or RNA to different tissues, including the skin.[2–4] Specifically, skin is an attractive target for gene therapy and vaccination due to its easy accessibility, large treatment area and the presence of many antigen-presenting cells, which are critical for eliciting an effective immune response.[5]

Skin GET has been used to effectively elicit both local and systemic expression of transgenes and has been used in many medical applications, including vaccination,[2,6–9] wound healing,[10–12] and cancer treatment,[3,13] as well as local and systemic skin disorders.[14] Due to the increasing use of skin GET for medical purposes, the gene expression induced in the skin should be well characterized and controlled. An important aspect of regulating the duration and the effects of gene delivery is the targeted transfection of specific skin layers.

Skin is a complex tissue that is divided into different layers consisting of different cell types.[14,15] The diversity of cell types in skin could be of the utmost importance in gene therapy and DNA vaccination because different cell types can theoretically elicit different responses to gene transfection. Genes transfected into muscle cells can have long-term expression because muscle fibers rarely divide, while genes transfected into keratinocytes show short-term expression due to the faster cell turnover.[14,16] In addition to the duration and level of gene expression, the specific targeting of different types of skin cells or layers could influence the local, paracrine or systemic effectiveness of the gene therapy. If keratinocytes in superficial skin layers are transfected, local or paracrine action of the transgene is expected. However, when cells in deeper skin layers are transfected, a systemic effect can also be expected due to shedding of the protein into the bloodstream. This presumption was confirmed in our previous study, where with noninvasive electrodes and varied electrotransfer parameters, local or systemic distribution of the therapeutic molecule doxorubicin was observed.[17] In this study, we hypothesized that varied parameters of GET applied with the same electrodes could affect the localization and expression of the delivered transgene.

The transfection of a specific region in the skin depends on different parameters, such as electrode selection, electrical parameters,[14,18–20] injection technique, animal species, dose of plasmid administered, and plasmid design.[21] The aim of our study was to evaluate the different parameters of electrotransfer as a tool for targeted gene delivery to different layers of the skin and to distinct between the local or systemic effects of such gene therapy. As in our previous study for the delivery of small molecules into skin,[17] pulses were applied using a multi-electrode array (MEA). Two different types of

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electric pulses were compared: (i) short, high-voltage (HV) pulses and (ii) long, low-voltage (LV) pulses. Mouse skin was selected as the model to evaluate the effects of varied GET parameters. Namely, mouse skin has an additional subcutaneous muscle layer called the *panniculus carnosus*. This layer enables us to distinguish between superficial and deeper transgene delivery as well as to study the therapeutic outcome of GET. We anticipated that with different electrotransfer parameters, we could control the depth of transfection into the skin and the level and the duration of gene expression, as well as whether the transcribed protein distribution was systemic or local. We found that the GET parameters could be adjusted for the desired transgene mode of action (*i.e.*, local or systemic) and optimized for maximum therapeutic effectiveness, when MEA is used. Similar effects could be anticipated in human skin, where the transfection of deep, well-vascularized skin layers could lead to systemic transcribed protein distribution.

Results

Electrical pulses influenced both the intensity and the duration of gene expression

The intensity and duration of DsRed reporter gene expression after intradermal plasmid injection and the application of LV and HV electric pulses to the skin were monitored using noninvasive fluorescence imaging of the transfected mouse skin. When the plasmid was injected without the electroporation as a delivery method, the fluorescence signal was not detected. When HV electric pulses were applied, the expression peaked at day 2 post-treatment and then declined for the next 2 weeks. The onset of gene expression was similar with LV electric pulses, but after 2 days, the fluorescence signal started to blur and spread in the shape of muscle cells, indicating the transfection of deeper skin layers (**Figure 1a**). Fluorescence was detected for up to 5 days longer with LV pulses than with HV pulses (**Figure 1b**).

The transfection of deeper skin layers with LV pulses was further supported by the observation that no expression was observed after the subcutaneous injection of plasmid DNA followed by HV pulses. In contrast, significant fluorescence signals were detected after the administration of LV pulses (**Figure 1c**). To further validate these results, histological analysis of the excised skin was performed.

The depth of transfection of pCMV-DsRed was evaluated by imaging the fluorescence of frozen skin sections. The first samples were excised at day 2 post-treatment. After intradermal injection of pCMV-DsRed followed by HV pulses, DsRed expression was observed in upper layers of the skin (*i.e.*, epidermis and dermis), while LV pulses promoted transfection in the epidermis, dermis and subcutis, including the deepest muscle layer (*panniculus carnosus*). In the skin sections excised at day 6 after the HV pulse treatment, pCMV-DsRed gene expression was not detected. On the contrary, the fluorescence signal was still observed in the upper layers, as well as in the muscles, after the application of LV pulses (**Figure 2**). Therefore, the histological analysis proved the effect of different electrical parameters on the duration of transgene production and the selective transfection of different skin layers, which was observed by noninvasive fluorescence imaging of the transfected skin.

Local and systemic expression of the IL-12 therapeutic gene was influenced by the applied pulses

The gene expression of IL-12 in the skin was evaluated by qRT-PCR analysis after the intradermal injection of pORF-mIL-12, which was followed by the application of LV and HV pulses. Under both electrical conditions, *i.e.*, LV and HV pulses, GET resulted in significantly increased expression levels of IL-12 mRNA at day 2 post-treatment, compared with the untreated control group (**Figure 3a**). Four days after GET, the IL-12 mRNA levels rapidly decreased. These results illustrate notable gene expression in the skin with brief mRNA persistence.

Subsequently, the protein levels were measured after intradermal GET of the therapeutic plasmid coding for IL-12. The local protein expression was measured in excised skin samples, while the systemic distribution of IL-12 was determined in mouse serum. Locally in the skin (**Figure 3b**), the application of LV pulses resulted in significantly higher IL-12 production compared with the application of HV pulses. In both cases, the expression peaked at day 2 after the treatment (1,213.3 ± 71.8 pg/ml for LV; 553.5 ± 99.0 pg/ml for HV), and it steadily decreased for the next few days. At the last detection time point, 6 days post-treatment, IL-12 was still detectable in low concentrations. In serum samples (**Figure 3c**), high concentrations of IL-12 were measured 2 days after the delivery of LV pulses (379.0 ± 80.3 pg/ml). The systemic distribution rapidly decreased over the next 2 days. In contrast, after the delivery of HV pulses, the systemic concentration of IL-12 was minimal, significantly lower compared with the application of LV pulses and completely reduced at day 6 post-treatment. Therefore, these results suggest that transgene is produced locally in the skin after intradermal injection of pORF-mIL-12 followed by the application of LV or HV pulses, while only LV pulses promote systemic protein distribution, whereas HV pulses restrict the expression locally in the skin.

The antitumor effect is stronger after IL-12 GET using LV pulses

The antitumor effectiveness of GET with plasmid DNA encoding IL-12 was evaluated on the B16F10 melanoma tumors as proof of principle. IL-12 is responsible for the antiangiogenic effect of the therapy as well as for local and systemic immunomodulation.[13,22] The antitumor efficiency of systemically distributed IL-12 was presented in previous studies with GET into the mouse muscle.[23,24] This time, tumor growth was monitored after the peritumoral injection of plasmid DNA followed by the application of LV or HV pulses. A significant increase ($P < 0.05$) in mouse survival was observed in both HV and LV pulse groups compared with that in the untreated control group and the group treated with the control plasmid pControl. The LV pulses prolonged mouse survival up to 8 weeks post-treatment. The HV pulses prolonged survival up to 3 weeks after the therapy (**Figure 4**). Therefore, the LV pulses, which induce the transfection of the deeper *panniculus carnosus* layer and also lead to the systemic distribution of IL-12, exhibited significantly ($P < 0.05$) better antitumor effectiveness compared with the HV pulses, which only exerted local effectiveness.

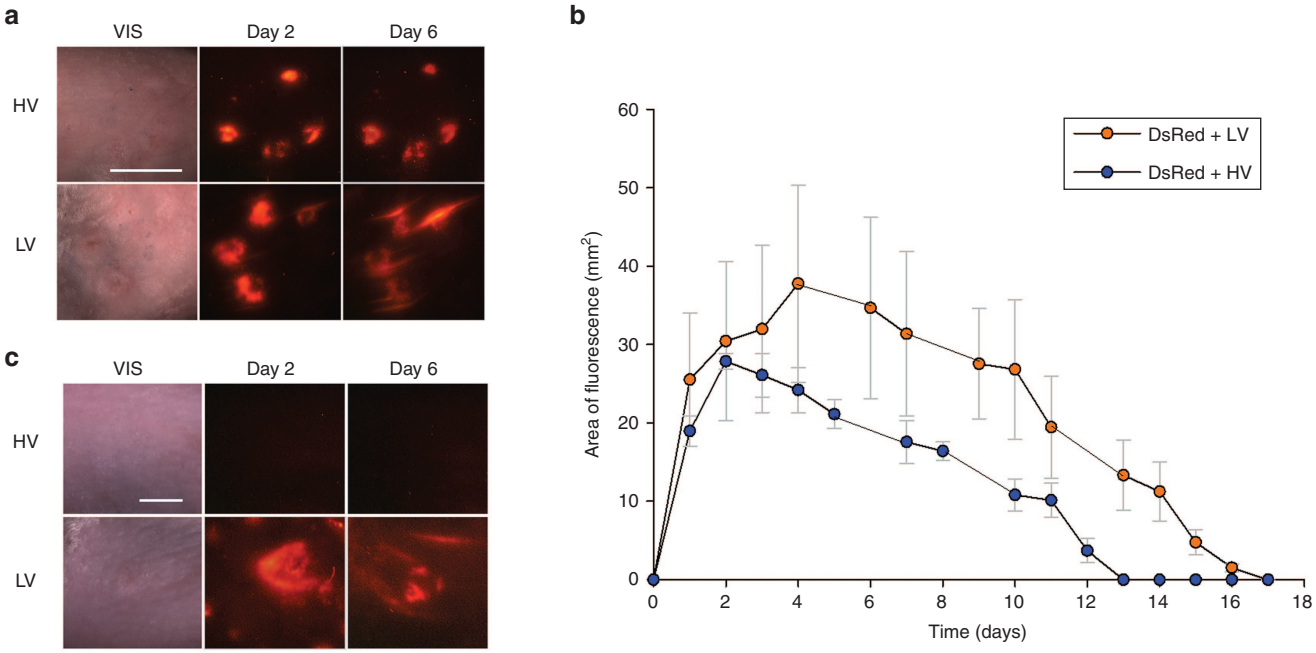

Figure 1 Fluorescence of DsRed protein following electrotransfer of the reporter plasmid coding for DsRed in mouse skin. (**a**) Representative images of the treated region of mouse skin showing the increased fluorescence signal due to *intradermal injection* of plasmid DNA followed by the application of high-voltage (HV) or low-voltage (LV) pulses. Images were acquired using the fluorescence stereomicroscope at the designated times. Scale bar: 5 mm. (**b**) Fluorescence intensity of DsRed protein obtained in mouse skin at different time points. Fluorescence was measured after multiple *intradermal injections* of pCMV-DsRed, followed by the application of HV or LV pulses. To measure the fluorescence intensity, images were analyzed using the same exposure criteria; $n = 6$ mice per group. Error bars indicate stanadard error of the mean. (**c**) Representative images of a treated mouse skin region showing increased fluorescence signal due to *subcutaneous injection* of plasmid DNA followed by the application of HV or LV pulses. Images were captured using the fluorescence stereomicroscope at the designated times.

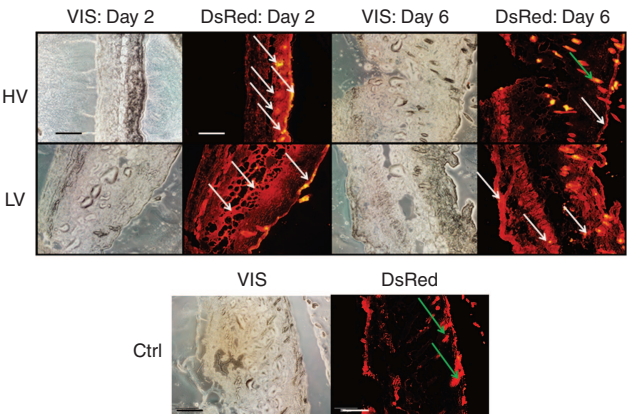

Figure 2 Depth of transfection of pCMV-DsRed into the skin after the application of high-voltage (HV) and low-voltage (LV) pulses. The fluorescence of DsRed in different skin layers was observed at day 2 and day 6 after the therapy in 20 µm thick frozen skin sections using the fluorescence stereomicroscope. Areas with gene expression are marked with white arrows, and the green arrow represents the auto fluorescence of hair follicles. VIS, images were taken under visible light; Ctrl, control group with pCMV-DsRed injection, but without the electroporation. Scale bar: 200 µm.

Pulse parameter choice is dependent on the transgene mode of action demonstrated in the wound-healing model

The wound-healing assay was used as a model for evaluating the effect of HV and LV pulses on the therapeutic outcome of GET. The efficiency and the mechanisms of action of all the plasmids used in the experiment were evaluated and described elsewhere.[12,13,22,25–27] For this study, they were selected based on already established mechanisms of action and were used as a model molecules. Three different plasmids with antiangiogenic action were selected, encoding IL-12, shRNA against melanoma cell adhesion molecule (MCAM) and shRNA against endoglin. As described above, IL-12 is responsible for local and systemic immunomodulation and the antiangiogenic effect of the therapy. The delivery of shRNA against endoglin, as well as the expression of shRNA against MCAM, typically has local, targeted vascular effects, as has been shown in previous studies of tumor models.[25–27] The plasmid encoding the peptide LL-37 was used as a positive control because this peptide promotes the wound-healing process.[12] The model is based on the theory that antiangiogenic molecules interfere with the revascularization and reepithelialization of the skin and would slow down the healing process. A longer healing time after therapeutic plasmid delivery would indicate a higher gene expression and GET efficiency.

According to our results, the average time for complete wound repair is 14.2 ± 0.4 days, as observed after treatment with miliQ water or pControl delivery. Electroporation with LV pulses significantly prolonged wound-healing time in the group treated with IL-12 (**Figure 5a**). After the IL-12 delivery and the application of LV pulses, complete wound repair was achieved after 17.1 ± 0.9 days, *i.e.*, 3 days longer than in the

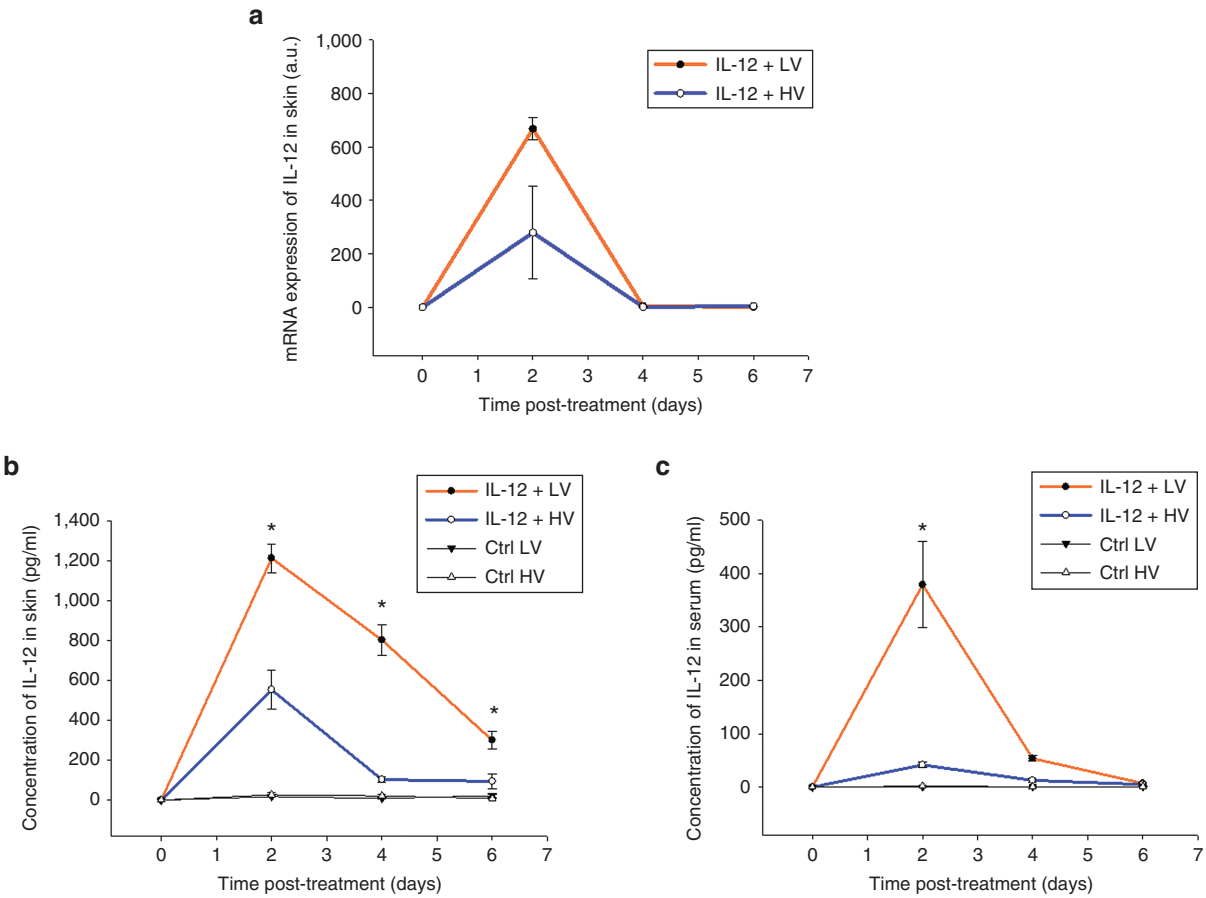

Figure 3 IL-12 mRNA and protein expression levels measured in skin and serum samples. (**a**) mRNA expression of IL-12 in skin samples. Real-time PCR analysis was performed at days 2, 4, and 6 post-treatment. (**b**) The concentration of IL-12 (pg/ml) measured in skin samples. The protein production was measured by ELISA at days 2, 4, and 6 after the treatment. (**c**) The concentration of IL-12 (pg/ml) measured in serum samples. The IL-12 protein was detected in the serum only after the administration of LV pulses and not after the application of HV pulses. The values are expressed as the arithmetic mean ± standard error of the mean; *P value indicates a significant difference ($P < 0.05$) between the groups treated either with LV or HV pulses. IL-12 + LV, intradermal injection of pORF-mIL-12 followed by the application of LV pulses; IL-12 + HV, intradermal injection of pORF-mIL-12 followed by the application of HV pulses; Ctrl LV or HV, intradermal injection of miliQ water followed by the application of LV or HV pulses.

control group (**Figure 5b**). Healing time after the treatment with IL-12 and the application of HV pulses did not differ from that of the control group. Electroporation with HV pulses prolonged wound-healing time in the group treated with shRNA against MCAM and in the group treated with shRNA against endoglin. Under the HV conditions, the healing time lasted 16.2 ± 0.9 days in the group treated with anti-endoglin shRNA and 19.0 ± 2.9 days in the group treated with anti MCAM shRNA. Wound healing was not significantly improved in those two groups after the delivery of LV pulses. Electroporation of phCAP-18/LL-37 under the LV conditions led to earlier wound closure and a healing process that was up to 3 days shorter than that of the control groups (**Figure 5b**).

The results indicate that all the therapeutic approaches had antiangiogenic effectiveness; however, due to different modes of action, their therapeutic effectiveness was varied with different electrotransfer parameters. The effectiveness of IL-12 is mediated systemically and was greater with LV pulses that transfected deeper skin layers, whereas the effectiveness of shRNA against endoglin and MCAM is mediated locally and was greater with HV pulses. GET with phCAP-18/LL-37 as a positive control efficiently decreased healing time. In addition, better responses were observed under LV conditions, indicating that the optimal effectiveness of the peptide occurs when deeper skin layers are transfected, similarly as with IL-12.

Evaluation of skin damage

Electroporation side effects were evaluated by macroscopic observation of skin damage caused by the application of HV or LV pulses at days 2, 7, and 14 after the treatment. Examination of the treated skin areas revealed no skin damage after the application of HV pulses (**Figure 6a**). There was no topical skin damage at days 2 and 7 post-treatment. Fourteen days after the application of HV pulses, hair had completely regrown in the treated area. On the contrary, the application of LV electric pulses (**Figure 6b**) caused noticeable skin damage (hyperkeratosis) observed at day 2 post-treatment. The damage was observed in the hexagonal pattern, indicating that mild skin burns had occurred under each electrode pin (**Figure 6c**). The damage was not severe and had completely healed in 14 days. However, no hair regrew in the treated regions where the pins had contacted the skin.

Under the same electrical conditions, the experiment was repeated with the application of the conductive gel on the mouse skin before the pulses were applied; this was done both to avoid thermal skin damage and to improve the contact between the electrodes and the tissue.[11,12,28,29] The use of conductive gel did not affect the degree of the skin damage, as observed in a series of treated animals (**Figure 6b**), indicating that the addition of conductive gel placed on the treated skin region did not ameliorate the skin damage caused by the applied electric pulses. Exposure of the skin to the LV pulses caused the same skin damage that resulted without the application of conductive gel. Once again, the HV pulses did not cause any noticeable skin damage.

The histological analysis of the treated skin (**Figure 6d**) revealed mild skin damage, which was mainly present in the epidermis. The treatment with HV pulses resulted in minor keratinocyte damage observed 2 days after the treatment. The thicker epithelium that was observed 6 days after pulse application indicates active recovery of the epidermis[30,31] after the damage caused by the electric pulses. The keratinocyte damage was more evident after the application of LV pulses; as the LV pulses were more harmful to the skin cells, the thickness of the epithelium indicates extensive recovery of the epidermal cells. Histological analysis confirmed the macroscopically observed hair regrowth. The HV application led to normal hair regrowth, while the application of LV pulses destroyed the hair follicles underneath where the electrode pins were in contact with the skin.

Discussion

Our study shows that with different electrical pulses applied by noninvasive pin electrodes, the depth of transfection and the level of gene expression can be controlled. Therefore, we can optimize the electrotransfer parameters for different medical applications and adjust the pulses for local or systemic action of a transgene.

Electrotransfer parameters regulate the depth of transfection in the skin, duration of the gene expression, and local or systemic protein distribution

A large variety of pulses have been used for GET. The optimal pulse conditions reported are not the same for all applications, but they could depend on the type of tissue, type of electrode and injected DNA.[5] The electric pulses chosen for gene transfection into skin have been either short duration (~100 μs) HV pulses alone,[2,32] long duration (>100 ms) LV pulses alone,[33] or a combination of one or several HV and LV pulses.[4,9,34,35] The amplitude and the duration of electric pulses used for GET are two critical parameters, which can affect the efficiency transgene expression as well as tissue damage.[36] Based on these published studies, we decided to test two different types of electric pulses, short HV pulses with an amplitude-over-distance ratio of 1600 V/cm and duration 100 μs, and long LV pulses with an amplitude-over-distance ratio of 170 V/cm and duration 150 ms. They were selected based on their efficiency to promote the local or systemic delivery of small molecules into the skin, as was demonstrated in our previous study.[17]

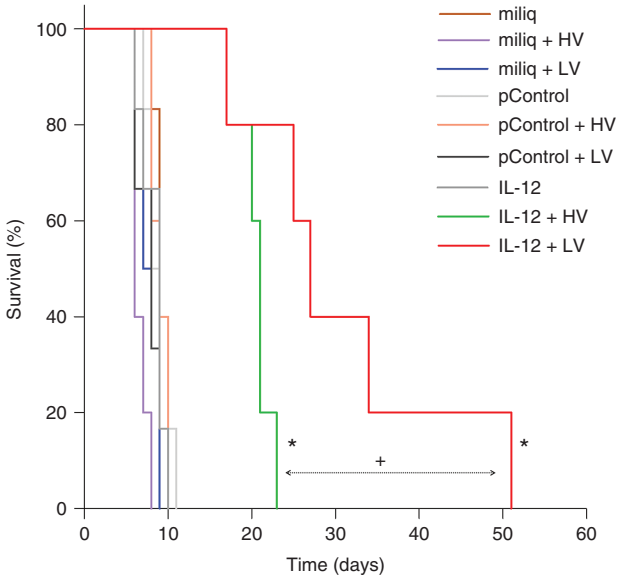

Figure 4 Mouse survival curves after treatment of the B16F10 melanoma tumors. *P value indicates a significant increase ($P < 0.05$) in mouse survival observed either with IL-12 + HV or IL-12 + LV treatment compared with the untreated control group and the group treated with pControl. +P value indicates statistically a significant difference ($P < 0.05$) between the IL-12 + HV and IL-12 + LV groups. pControl, intradermal injection of control plasmid; IL-12, intradermal injection of pORF-mIL-12; LV, application of low-voltage pulses; HV, application of high-voltage pulses.

The results of this study showed the differences in the depth of transfection of plasmids into mouse skin resulting from using LV or HV electric pulses for GET. Specifically, the use of HV pulses promoted the transfection in upper skin layers (*i.e.*, epidermis and dermis), while the use of LV pulses promoted the transfection of deeper layers of the skin with muscle cells (*i.e.*, *panniculus carnosus*). The targeted transfection of different skin layers by the application of short HV or long LV pulses was demonstrated for the intradermal delivery of a reporter plasmid encoding DsRed, as well as for a therapeutic plasmid encoding IL-12. The transfection of cells in deeper layers of the skin achieved by LV pulses resulted in longer expression of the plasmid DNA with systemic distribution of the transcribed protein, as was demonstrated by the elevated serum levels of IL-12 at day 2 post-treatment. On the contrary, the transfection of cells in the epidermis and dermis that was achieved after the application of HV pulses resulted in local expression of the transgene without any systemic distribution. Therefore, electrotransfer parameters could regulate the depth of transfection in the skin and could essentially contribute to the duration of gene expression as well as regulate the local or systemic distribution of the transcribed protein (**Figure 7**). The therapeutic relevance of different electrotransfer parameters was presented with the B16F10 tumor treatment and the wound healing was performed as a proof of principle of variable effects of electrical conditions.

The mode of action of the delivered molecule requires the proper selection of HV or LV pulses

Different therapeutic plasmids have been transfected into skin via electrotransfer.[5,14] Transgene molecules differ in mode of action as well in the dosage needed for optimal

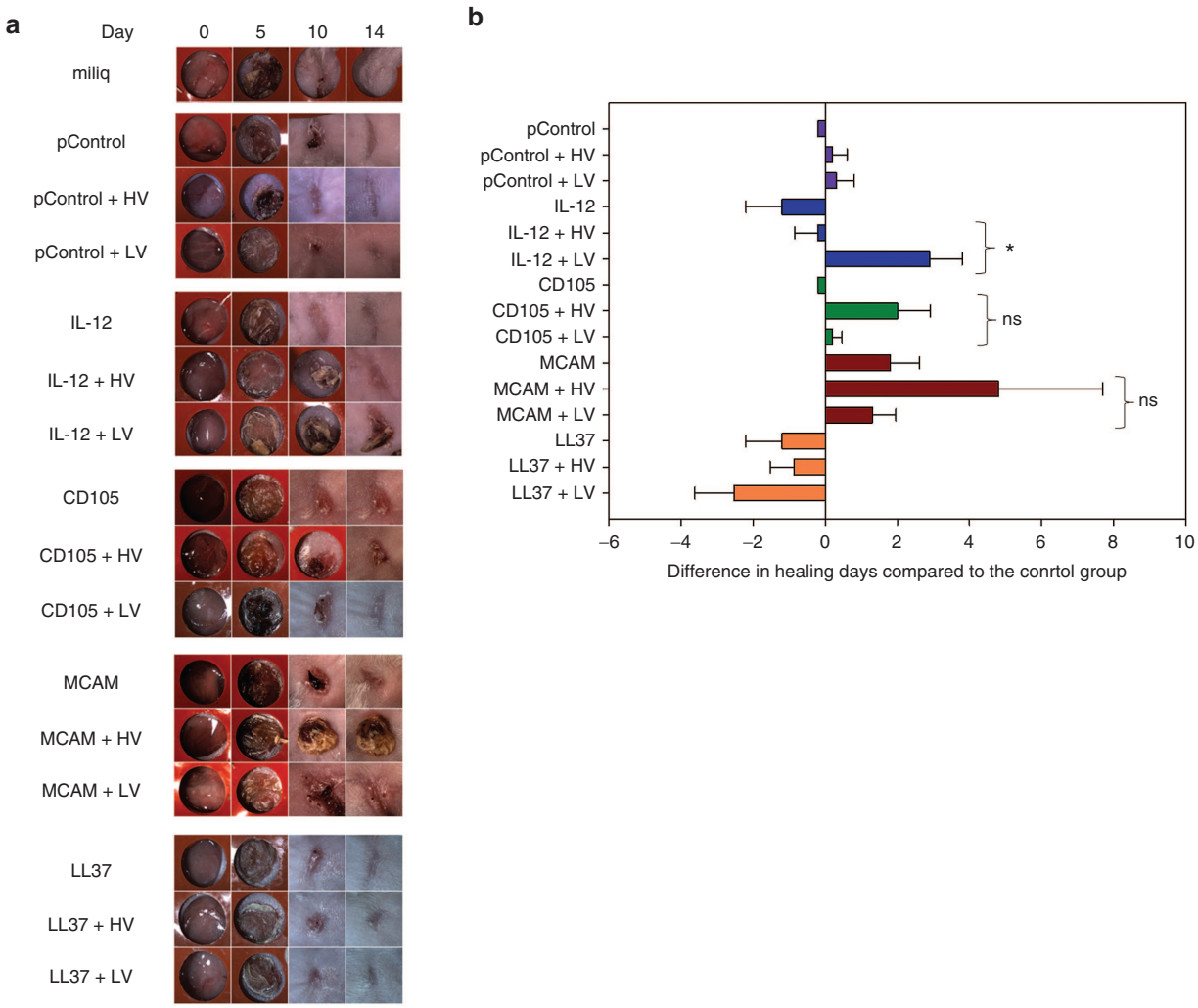

Figure 5 Therapeutic effect of plasmid DNA delivery after the application of HV or LV pulses. (**a**) Images of the wounds were captured with the digital camera connected to the stereomicroscope at the designated times. Wounds were created using a 5 mm round skin biopsy punch. The inner diameter of the red silicone ring is 6 mm. (**b**) To quantify the results of the wound-healing model, the wound images were analyzed using AxioVision software. The results are expressed as the number of days for complete wound repair, normalized to the control group. Error bars indicate standard error of the mean. *P value < 0.05 between the selected groups; ns, no statistically significant difference between the groups; pControl, intradermal injection of control plasmid pControl; IL-12, intradermal injection of pORF-mIL-12; CD105, intradermal injection of pU6-antiCD105, coding for shRNA against endoglin (CD105); MCAM, intradermal injection of pU6-antiMCAM, coding for shRNA against melanoma cell adhesion molecule (MCAM); LV, application of low-voltage pulses; HV, application of high-voltage pulses.

therapeutic effectiveness. First, molecules delivered intradermally can act locally in the skin or elicit systemic effects when the products reach the bloodstream. As presented in this study, it is of the utmost importance to select the optimal electrical parameters according to the mode of action of the transcribed molecule. We demonstrated that for plasmids encoding molecules with a local mode of action, such as shRNA against endoglin[26,27,37–39] or shRNA against MCAM,[25] the best effectiveness was obtained with HV pulses, which transfect the cells in the superficial mouse skin layers. In that way, the HV pulses would restrict the distribution of molecules with a local mode of action locally in the skin, thus avoiding possible systemic side effects due to their distribution in the bloodstream. In contrast, with molecules that have paracrine and systemic modes of action, such as IL-12, we obtained better effectiveness with LV pulses that promote the transfection of deeper skin layers. Compared with HV pulses in the wound-healing model, better IL-12 effectiveness was obtained with LV pulses, which were required to achieve optimal systemic effects. Furthermore, the prolonged survival of B16F10 tumor-bearing mice clearly indicates an antitumor effectiveness of IL-12 with LV pulses applied in the peritumoral area than that resulting from the HV conditions.

Aside from the types of transfected cells, the optimal therapeutic effectiveness of gene therapy also depends on the amount of produced therapeutic transgene and the duration of expression. Therefore, the dosing needs to be optimized for each specific therapeutic plasmid. Although there have been several studies that have attempted to improve the dosage of plasmid DNA available for gene expression in the skin, the highest possible dosage does not always lead to the best therapeutic results.[40] Therefore, to reach optimal therapeutic

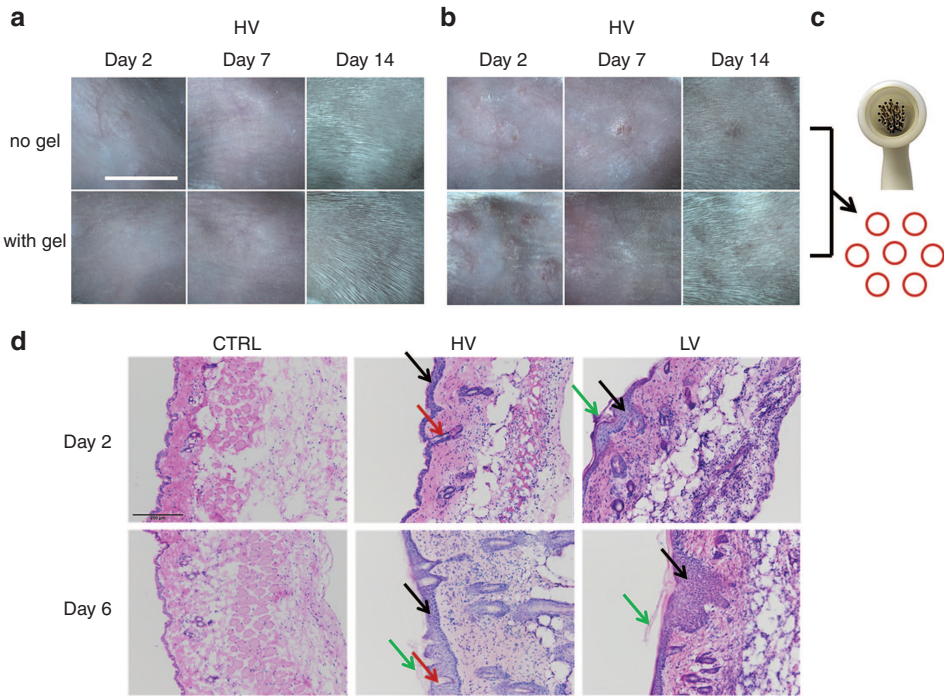

Figure 6 Macroscopic and microscopic evaluation of skin damage. Damage was observed after multiple miliQ injections followed by the application of (**a**) high-voltage (HV) pulses or (**b**) low-voltage (LV) pulses. Pulses were applied immediately after the intradermal injection of miliQ water (no gel) or after the preliminary administration of conductive gel (with gel). Mouse skin was observed under the stereomicroscope 2, 7, and 14 days after the application of HV and LV pulses. For each experimental condition, four treated skin areas were evaluated. Representative images of the skin damage in the treated region. *Scale bar* 5 mm. (**c**) Skin damage caused by LV pulses was observed in the same hexagonal pattern as that of the pins inside the MEA. *Scale bar* 5 mm. VIS images were taken under visible light. (**d**) Histological evaluation of skin damage. Skin sections were excised at day 2 or day 6 post-treatment and stained with hematoxylin and eosin. The keratinocyte damage is marked with green arrows, the extended thickness of epithelium is marked with black arrows, and the hair follicles are marked with the red arrows. *Scale bar* 200 µm. LV, low-voltage pulses; HV, high-voltage pulses; CTRL, untreated group.

effectiveness of GET, the level and duration of expression of the transgene transfected into the skin should be precisely regulated. The results of our study provide the evidence that with noninvasive electrodes and different electrotransfer parameters, the depth of skin transfection can be controlled, resulting in different levels and durations of IL-12 expression.

From mouse skin to clinical practice

Due to the specific structure of mouse skin with the subcutaneous *panniculus carnosus* muscle layer, mouse skin was selected as a model to assess the effect of different electrical parameters. The differential characteristics of mouse skin layers, including the muscle layer, provide an easy means of detecting of the depth at which a transgene is expressed. Although only rudimentary forms of this subcutaneous muscle layer can be found in humans, such as in the small muscles of the face and neck or the platysma,[15] the results of this study provide clear evidence that the parameters of electrotransfer influence the depth of transfection into the skin. Targeting deeper skin layers with LV pulses would lead to the transfection of cells with slower turnover, such as muscle cells, and thus, longer duration of gene expression. Because deeper, well-vascularized skin layers are transfected with LV pulses, the resulting systemic effect could be expected, even in the absence of the *panniculus carnosus* in human skin. The use of different electrical parameters, which regulate the depth of transfection and distinguish between local or systemic response, would contribute to the translation of therapeutic GET into the clinic. These parameters present a promising tool for improving the safety and effectiveness of GET for various medical applications, such as wound healing, vaccination and cancer therapy. As demonstrated in this study, the electrical conditions should be carefully selected for each application. For instance, for the purposes of vaccination, which targets the local immune response of the skin cells, the HV pulses would optimally improve local gene expression in the skin. A similar example is the application of HV pulses in the wound-healing process, where a transgene should act locally, or in the paracrine mode. In that case, it is sufficient for the transgene to affect the local environment, as there is no need for a systemic response.[14]

On the other hand, LV pulses would be recommended for the delivery of molecules with systemic action and expected abscopal effects. One example is the delivery of plasmid DNA encoding IL-12 in cancer therapy,[3,23,41–43] as was also demonstrated in our study. One specific promising approach is the administration of plasmid DNA encoding IL-12 peritumorally into skin to boost the immune reaction of the local ablative techniques.[13,44] Such an approach was already proposed for electrochemotherapy in combination with IL-12 delivered by electrotransfer peritumorally and demonstrated effectiveness in client-owned dogs with spontaneous mast cell tumors.[44]

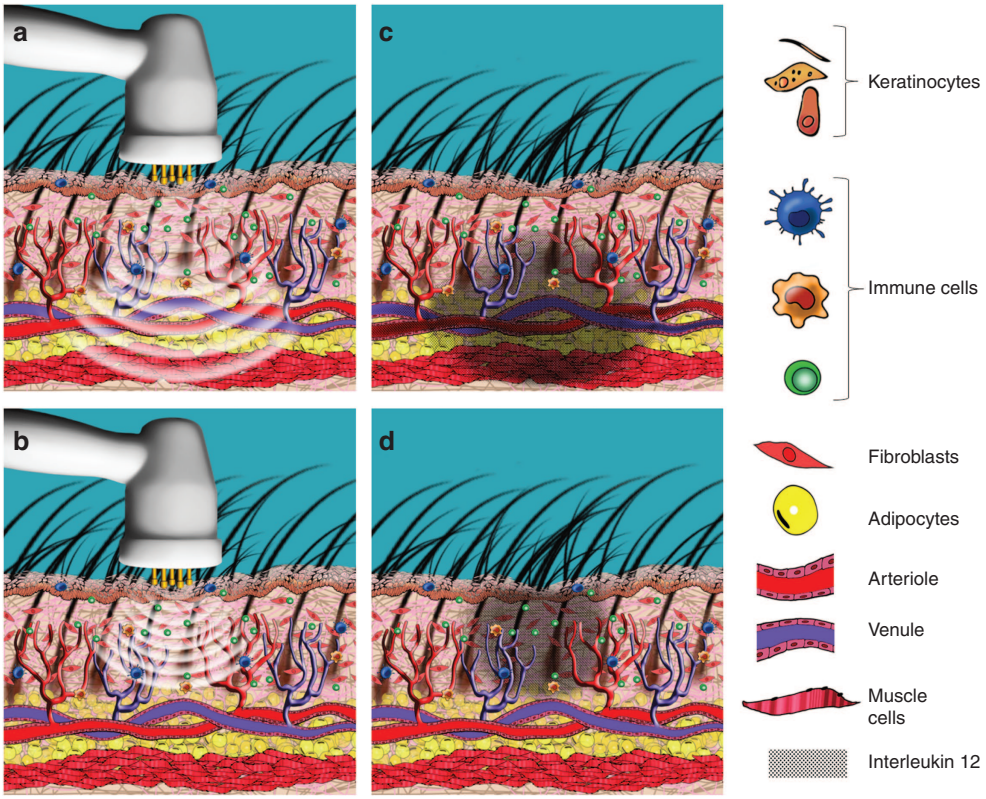

Figure 7 The depth of transfection in mouse skin and transgene distribution after high-voltage (HV) or low-voltage (LV) pulses, applied by MEA. LV pulses promote the transfection of deeper skin layers (**a**), while HV pulses promote the transfection of cells in the superficial layers of mouse skin (**b**). The deeper transfection achieved by LV pulses resulted in longer plasmid DNA expression with systemic protein distribution, as was demonstrated by the increased IL-12 serum levels (**c**). The transfection of the superficial skin layers by the HV pulses resulted in local plasmid DNA expression without any systemic transgene distribution (**d**).

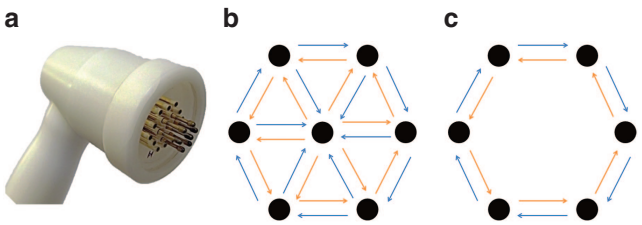

Figure 8 The distribution of electric pulses within the multi-electrode array (MEA). (**a**) Applicator of noninvasive MEA. (**b**) The electrode consists of seven spring-loaded pins arranged in a hexagonal mesh and spaced 3.5 mm apart. When the central pin was inserted, a total of 24 electric pulses (2 electric pulses between each electrode pair) were delivered during the treatment. (**c**) For wound healing and tumor treatment, the central pin was removed from the applicator; 6 pins remained on the electrode, delivering 12 electric pulses (2 electric pulses between each electrode pair).

MEA for the delivery of genes into the skin

Different types of electrodes have been used to deliver genes into the skin, from needle electrodes[45,46] microneedles,[47] and plate electrodes[36,48] to the more recent noninvasive MEA.[49,50] One of the novelties of our study is the utilization of the circular MEA for wound healing and tumor treatment. In previous studies, the use of MEA was effective for the delivery of model drugs (*e.g.*, dextran, fentanyl and doxorubicin),[17] as well as plasmid DNA, into mouse skin[32] with minimal skin damage caused by electric pulses.[17] Particularly the HV pulses provided harmless gene delivery, without noticeable skin damage. Although the LV pulses could result in more evident skin damage, potentially caused by longer pulse duration, the damage was not severe and had completely healed in 14 days. Therefore, the skin damage is not critical component to pulse selection and the use of the LV pulses should not cause the concerns for further application. The advantage of the MEA is that the number of addressable electrode pairs that are contained within the electrode applicator can be adjusted,[51] which was also demonstrated in the current study with the removal of the central pin. As demonstrated by our results, the use of MEA with adjustable pins represents a valuable approach for the effective delivery of genes into the skin for wound healing, as well as for peritumoral delivery for cancer gene therapy. Hence, MEA opens the new possibilities to control the gene expression in the skin, which were not described for any other type of electrode so far.

Conclusion

Different skin layers and controlled gene expression in the skin can be achieved by selecting the appropriate electrotransfer parameters. Specifically, the application of LV or HV pulses provides the possibility to control depth and, consequently, the types of cells transfected; in this way, the systemic or local action of the transgene can also be controlled. Therefore, in the translation into the clinics, it will be of the

utmost importance to adjust the parameters of GET for different therapeutic approaches, desired therapeutic genes and their modes of action.

Materials and methods

Plasmids. The plasmids used for the experiments include the reporter plasmid encoding the *Discosoma* red fluorescent protein (DsRed), the control plasmid (pControl), and the therapeutic plasmids encoding hCAP-18/LL-37, interleukin 12 (IL-12), shRNA against MCAM, and shRNA against endoglin (CD105). The DsRed reporter gene was encoded on the pCMV-DsRed-Express2 plasmid (Clontech, Basingstoke, UK), and the mouse IL-12 gene was encoded on the pORF-mIL-12 plasmid (InvivoGen, Toulouse, FR). The precise construction of the control plasmid without the therapeutic gene (pControl)[52] and the plasmids against MCAM (pU6-antiMCAM)[25] and against endoglin (pU6-antiCD105)[27] is described in our previous studies. The construction of the plasmid encoding hCAP-18/LL-37 is described elsewhere.[12] All plasmids were isolated using the EndoFree Plasmid Mega Kit (Qiagen, Hilden, DE) according to the manufacturer's instructions and diluted in endotoxin-free water (miliQ) to a concentration of 2 mg/ml. Plasmid DNA concentration and purity were determined both spectrophotometrically (Epoch Microplate Spectrophotometer; Take3 Micro-Volume Plate, BioTek, Bad Friedrichshall, DE) and by gel electrophoresis.

Animal ethics. All procedures were performed in compliance with the guidelines for animal experiments of the EU directive (2010/63/EU) and with permission from the Veterinary Administration of the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (permission no. 34401–4/2012/4, 34401-1/2015/7). Animals were housed in pathogen-free conditions with 12-hour light cycles and were provided food and water *ad libitum*.

In vivo GET and transfection efficiency. In the experiments, 8–12-week-old female BALB/c mice (Envigo, Udine, IT) weighing between 20 and 25 g were used; three to five mice were randomly assigned into each experimental group. One day prior to the experiments, the mice were shaved on the left and/or right flank, and any remaining hair was removed with depilatory cream (Veet Sensitive Skin, Reckitt Benckiser, UK). Before the plasmid injections, the mice were anesthetized in the induction chamber using 2% isoflurane (Nicholas Piramal India, London, UK) in oxygen, and they remained under isoflurane anesthesia during the procedure.

To observe the intensity and duration of gene expression in the skin, the anesthetized mice received multiple intradermal injections (4 × 20 µl) of pCMV-DsRed (2 mg/ml) in the right and/or left flank. A 29-G insulin grade syringe (Terumo, Terumo Europe N.V., Leuven, BE) was used for the plasmid injection. To evaluate transfection depth, single intradermal and subcutaneous injections of 50 µl of pCMV-DsRed (2 mg/ml) were administered during the experiments, immediately after the plasmid injection electric pulses were applied. Two different electrotransfer parameters were used for plasmid delivery: either HV electric pulses with an amplitude-to-distance ratio of 1,600 V/cm (amplitude 570 V, duration of pulse 100 µs) or LV electric pulses with an amplitude-to-distance ratio 170 V/cm (amplitude 60 V, duration 150 ms). GET was performed using a noninvasive MEA (Iskra Medical, Podnart, SI) consisting of seven spring-loaded pins arranged in a hexagonal mesh (**Figure 8a,b**) and spaced 3.5 mm apart. The MEA was gently pressed directly onto the depilated skin without the application of conductive gel. Due to the fact that the array was designed based on the commercially available hexagonal electrode from IGEA, the MEA was connected to the Cliniporator (IGEA s.r.l., Carpi, IT). A total of 24 electric pulses (2 electric pulses between each electrode pair in opposite direction) were delivered during the treatment. Expression of DsRed was monitored using a Zeiss SteREO Lumar.V12 (Zeiss, Jena, DE) fluorescence stereomicroscope equipped with an MRc5 digital camera (Zeiss). The mice were initially anesthetized with inhalation anesthesia in the induction chamber (2% of isoflurane in oxygen) and were then placed under the microscope with their snout in the inhalation tube to remain anesthetized during the measurement procedure. The mice were imaged daily from day 1 after the GET until the fluorescence was undetectable. A threshold suitable for the images was applied, and the fluorescence intensity in the area of the electroporated skin was determined using AxioVision software (Zeiss).

Histological determination of DsRed distribution and skin damage. For the histological determination of DsRed distribution in the skin, frozen sections were prepared. The mice received a single intradermal injection of 50 µl of pCMV-DsRed (2 mg/ml) in the left or right flank, and the electrotransfer was performed as described above. At day 2 and day 6 post-treatment, the mice were humanely sacrificed, and the region of the skin exposed to the treatment was excised. The excised skin was immediately frozen in liquid nitrogen. Subsequently, skin samples were embedded in medium designed for frozen tissue specimens (Tissue-Tek 4583, Sakura Finetek Europe B.V., Alphen aan den Rijn, NL), and 20 µm thick sections were cut in the direction perpendicular to skin layers. Additionally, some samples were fixed in zinc fixative (BD PharMingen, BD Bioscience, San Diego, CA) for 1 day and then stored in 70% ethanol until being embedded in paraffin for assessing skin damage histologically; 5 µm thick sections were cut in the direction perpendicular to the skin layers and stained with hematoxylin and eosin. The samples were observed using a BX-51 fluorescence microscope (Olympus, Hamburg, GE) equipped with a digital camera DP72 (Olympus).

Total mRNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. The BALB/c mice received multiple intradermal injections (4 × 20 µl) of pORF-mIL-12 in the left or right flank. Electric pulses were applied immediately after plasmid administration, as described above. The control group received intradermal injections of miliQ water, followed by electric pulses. To determine IL-12 expression in the mouse skin, RNA extraction and qRT-PCR analysis were performed at days 2, 4, and 6 after GET of pORF-mIL-12. The mice were sacrificed, and the treated skin regions were excised and snap-frozen in liquid nitrogen. Skin samples were later homogenized, and total

RNA was extracted with the TRIzol Plus RNA Purification system (Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. RNA concentration and purity were determined spectrophotometrically (Epoch Microplate Spectrophotometer; Take3TM Micro-Volume Plate, BioTek, Bad Friedrichshall, DE). cDNA transcription was performed using 1,000 ng of total RNA extract using a SuperScript VILO cDNA Synthesis Kit (Life Technologies), according to the manufacturer's instructions. For the qRT-PCR analysis, mixtures of transcribed cDNA diluted 10-fold were used as templates using SYBR Select master Mix (Life Technologies). Custom primers (forward primer sequence: 5′-CGGCAG-CAGAATAAATATGAG-3′, reverse primer sequence: 5′-GAG TTCTTCAAAGGCTTCATC-3′) were designed to amplify the fragments of mouse IL-12 cDNA. The reaction was performed using a QuantStudio 3 analyzer (Applied Biosystems, Life Technologies), and the products were analyzed with the associated QuantStudio Design & Analysis Software (Life Technologies). The optimized thermal cycling conditions were as follows: activation of uracil-DNA glycosylase (2 minutes at 50 °C), hot start activation of AmpliTaq Gold Enzyme (10 minutes at 50 °C), 45 cycles of denaturation (15 seconds at 95 °C), annealing and extension (1 minute at 60 °C). The IL-12 mRNA expression levels in mouse skin are presented as the threshold cycle value (C_t). The relative quantification of cDNA expression was normalized to β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by the $2^{-\Delta\Delta Ct}$ method, calculated via the QuantStudio 3 software (Applied Biosystems, Life Technologies).

Quantification of IL-12 protein concentration. The concentration of the cytokine IL-12 was measured in excised skin and serum samples. BALB/c mice were treated as they were for the qRT-PCR analysis. At days 2, 4, and 6 post-treatment, the mice were sacrificed, and the treated region of the skin was excised, immediately weighed and snap-frozen in liquid nitrogen. The frozen samples were mechanically macerated. Each sample received 500 µl of PBS containing protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche, Basel, CH); the samples were then thoroughly mixed and centrifuged for 10 minutes at 3,000 rpm. The supernatant was separated from the sediment and stored at –80 °C until further 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 minutes until it had coagulated. Serum was extracted from the blood samples via centrifugation at 2,500 rpm for 5 minutes, and the serum was immediately stored at –80 °C until further analysis. Both sets of samples were analyzed using enzyme-linked immunosorbent assay (ELISA) (ELISA Quantikine Mouse IL-12 p70 Immunoassay, R&D Systems, Minneapolis, MN). Due to the highly comparable mass of the samples, the IL-12 concentrations are presented as pg of IL-12 per ml of serum or ml of supernatant.

Evaluation of antitumor effectiveness. Tumors were induced by a subcutaneous injection of 1×10^6/0.1 ml of B16F10 melanoma cells into the right mouse flank; these cells are syngeneic to C57Bl/C mice (Envigo). The mice in this experiment were 8–10 weeks old, weighing between 18 and 20 g. When the tumors reached an approximate volume of 40 mm^3, the mice were randomly divided into experimental groups, and the treatments were administered; this point constituted day 0 of our study. The tumors were treated with multiple intradermal peritumoral injections (4 × 20 µl) of pORF-mIL-12 in the concentration of 2 mg/ml; mice in the control group received miliQ water. Immediately after the plasmid injections, the peritumoral region was covered with conductive gel, and the LV or HV pulses were applied via an MEA with the central pin removed (**Figure 8c**). Thereafter, the tumors were measured in three perpendicular directions (a, b, c) every 2–3 days using a digital Vernier caliper. Tumor volume was calculated using the following formula: $V = a \times b \times c \times \Pi/6$. The results are expressed using Kaplan-Meier curves; overall survival is presented as a percentage of mice present in the experiment at different time points post-treatment. The mice were sacrificed and disregarded from the experiment when the tumor reached a volume of 350 mm^3.

Wound model and electroporation of wounded skin. BALB/c female mice (Envigo) weighing between 18 and 20 g were used in this experiment. At the time of treatment, the mice were 6–8 weeks old. Using the younger mice, it ensured that the skin structure was intact. The mice were anesthetized using an intraperitoneal injection of ketamine (1 mg/ml, Narektan, Vetoquinol, Ittigen, CH), xylazine (5 mg/ml, Chanazine; Chanelle Pharmaceuticals, Loughrea, IRL) and acepromazine (0.4 mg/ml, Promace; Fort Dodge Animal Health, Fort Dodge, IA). The injection volume was adjusted to the weight of the mouse and ranged from 70–90 µl.

The dorsal surface was shaved and treated with depilatory cream (Veet Sensitive Skin) to remove any remaining hair. The dorsum of each anesthetized animal was disinfected with polyvidone iodine (Braunoderm, Braun, Melsungen, GE). Two full-thickness wounds, one on the each mouse flank, were created using a 5 mm, round skin biopsy punch (Kai Europe GmbH, Solinger, GE). To avoid cross-contamination, both wounds received the same treatment. Plasmids in a total volume of 80 µl (160 µg per mouse) were intradermally injected in four sites around each wound (4 × 20 µl). Mice in the control group received injections of miliQ water. Each wound was caught in between MEA, consisting of six spring-loaded pins (**Figure 8c**) spaced 3.5 mm apart, and the Cliniporator (IGEA s.r.l., Carpi, IT) was used. The same electrodes were used as described in the previous experiment, this time with the central pin removed.[51] Both HV and LV pulses were tested. To reduce wound contraction, the wounds were fixed with silicone splints that had been cut from a 0.5 mm thick silicone sheet (Grace Bio-Labs, Sigma-Aldrich). Donut-shaped splints with an outer diameter of 15 mm and an inner diameter of 6 mm were placed around each wound so that the wound was centered within the splint. An immediate-bonding adhesive was applied to fix the splint to the skin, followed by 5-0 Mersilk, nonabsorbable sutures (Ethicon, San Lorenzo, Puerto Rico). The wounds were washed with saline and covered by nonadhesive patches (Tosama, Domzale, SI) and transparent, occlusive wound dressings (Tegaderm, Neuss, GE). After the surgery, the mice received intramuscular analgesia (butorphanol, 50 µl of 3 mg/kg, Torbugesic; Fort Dodge Animal Health), which was repeated the next day. At days 0,

3, 5, 7, 10, and 14, the wounds were digitally imaged using a Zeiss SteREO Lumar.V12 (Zeiss, Jena, GE) fluorescence stereomicroscope equipped with an MRc5 digital camera (Zeiss). The same optical zoom was maintained throughout the experiment. If the treatment prolonged the healing process, the wounds were observed on additional days until the healing was completed. Wound area was quantified using the AxioVision software (Zeiss). The results are expressed as the percentage of wound coverage compared with day 0 postsurgery and are normalized to the control group treated with miliQ water or pControl.

Evaluation of skin damage caused by electric pulses. BALB/c mice received multiple intradermal injections (4×20 µl) of miliQ water in the left or right flank. Immediately after the administration of miliQ water, HV or LV electric pulses were applied, as described in the section above. The application was performed with or without the conductive gel (AquaUltra Basic, Ultragel, Budapest, HU). Potential skin damage was assessed using the stereo microscope (Zeiss). The treated skin regions were digitally imaged at days 2, 7, and 14 post-treatment. Subsequently, the effect of different electrotransfer parameters on skin damage was compared; in addition, the effect of conductive gel for the prevention of potential skin damage was evaluated.

Statistical analysis. Sigma Plot software (Systat software, London, UK) was used for statistical analysis. Significance was determined by Student's *t*-test or one-way analysis of variance followed by the Holm-Sidak test. The analysis of survival after the tumor treatment was performed using the log-rank test. A $P < 0.05$ was considered significant. Values are expressed as the arithmetic mean ± standard error of the mean.

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POGLAVJE 5

Poglavje 5: Peritumoralna genska terapija s ciljanim vnosom plazmidne DNA v kožo

Targeted delivery of plasmid DNA into the skin for the purpose of peritumoral gene therapy

1 UVOD

Genska terapija predstavlja način zdravljenja bolezni, pri katerem v somatske celice vnašamo gene z namenom odpravljanja genetske motnje ali zagotavljanja izražanja terapevtsko učinkovitih proteinov (Cross and Burmester, 2006). Vneseni geni v primeru genske terapije raka lahko povzročijo smrt transfeciranih celic, zaustavijo delitev celic ali zavrejo rast tumorskega žilja. V razvoju je več metod vnosa izbranih genov v tarčne celice, med katerimi je genski elektroprenos ena bolj varnih in učinkovitih ne-virusnih metod vnosa (Mir, 2009).

Genski elektroprenos omogoča učinkovit vnos plazmidne DNA v celice in tkiva, med drugim tudi v kožo. Zaradi številnih prednosti kože kot tarčnega organa so v dosedanjih študijah preučevali protitumorski učinek intradermalno vnesenih tumorskih antigenov, vnos antiangiogenih molekul ter citokinov, ki aktivirajo imunski sistem za uničenje tumorskih celic (Gothelf and Gehl, 2010). Med citokini je za vnos v kožo zlasti zanimiv interlevkin-12 (IL12), ki kaže velik potencial za zdravljenje malignega melanoma (Daud et al., 2008), poleg tega izkazuje protitumorsko delovanje na sarkomskem tumorskem modelu po intratumoralnem vnosu ali intradermalnem injiciranju v peritumoralno področje (Pavlin et al., 2009). IL12 po genskem elektroprenosu uspešno zavre rast različnih vrst tumorjev in zmanjša možnost ponovnega nastanka raka (Pavlin et al., 2009).

Učinkovitost genskega elektroprenosa v kožo je odvisna od številnih dejavnikov (Shirley et al., 2014), kot so količina in sestava plazmidne DNA, vrsta elektrod ter izbor parametrov električnih pulzov. Dokazali smo, da lahko z optimiziranimi parametri genskega elektroprenosa dosežemo ciljani vnos plazmidne DNA v posamezne plasti kože. Tako omogočimo možnost prilagajanja časa izražanja genskega produkta ter vplivamo na lokalno ali sistemsko delovanje terapije (Kos et al., 2016). Tako razvita in optimizirana metoda genskega elektroprenosa v kožo predstavlja učinkovito orodje za vnos terapevtskih genov v kožo, kar lahko v naslednjem koraku uporabimo v klinične namene, kot je genska terapija raka. Protitumorski učinek optimizirane metode, s ciljanim vnosom plazmidne DNA v peritumoralni predel kože, smo določali na melanomskem in sarkomskem tumorskem modelu. Kot terapevtsko molekulo smo uporabili plazmidno DNA z zapisom za mišji IL12. Poleg delovanja na primarne tumorje smo spremljali tudi sistemski učinek terapije na lokoregionalne in oddaljene metastaze.

2 MATERIALI IN METODE

2.1 Plazmidi

Kot terapevtski plazmid smo uporabili pORF-mIL-12-ORT. Plazmid s konstitutivnim promotorjem ORF nosi zapis za mišji IL12 (fuzijski protein α in β podenot IL12, ki se pri miših nahajata na lokusu p35 –

Il12a in lokusu p40 – *Il12b*) brez zapisa za odpornost na antibiotike. Kot kontrolni plazmid smo uporabili pControl-ORT z enako sestavo kot terapevtski plazmid, a brez zapisa za IL12. Plazmide smo pripravili s pomočjo standardnih molekularnih metod restrikcije in ligacije (Kamensek et al., 2017). Izolacija plazmidov je potekala s pomočjo reakcijske mešanice EndoFree Plasmid Mega Kit (Qiagen, Hilden, Nemčija) po navodilih proizvajalca. Izolirano plazmidno DNA smo do želene koncentracije redčili v prečiščeni vodi brez endotoksina (miliQ). Ustreznost in čistost izoliranih plazmidov smo določali spektrofotometrično (Epoch Microplate Spectrophotometer; Take3 Micro-Volume Plate, BioTek, Bad Friedrichshall, Nemčija) ter z uporabo agarozne elektroforeze.

2.2 Poskusne živali in tumorski modeli

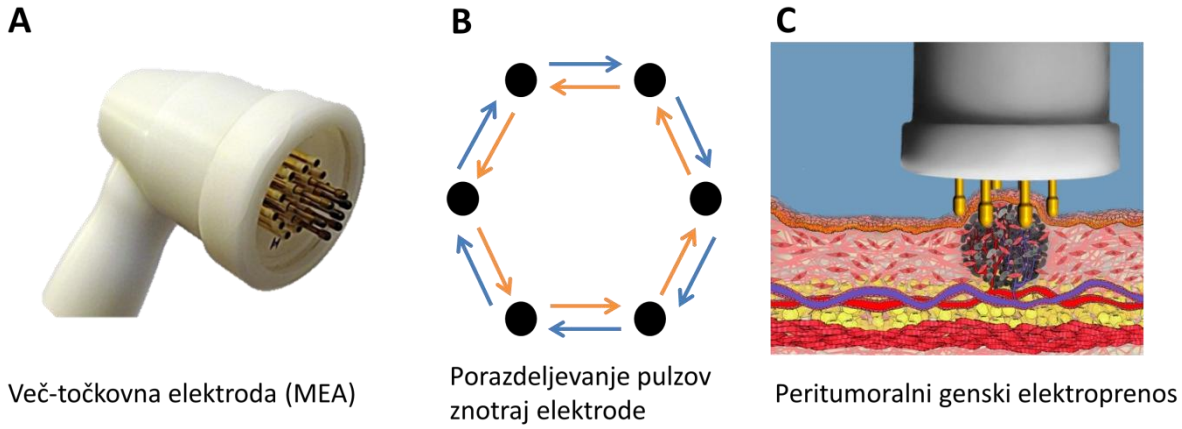
Raziskavo smo izvedli na dveh tumorskih modelih, mišjem melanomskem modelu B16F10 ter mišjem sarkomskem modelu LPB. Oba tumorska modela sta singena mišji liniji C57BL/6. Miši so izvirale iz registrirane vzrejne organizacije Envigo (Videm, Italija). Živali smo vzdrževali v živalski koloniji, prosti specifičnih patogenih organizmov. Zagotavljali smo kontroliran dnevno-nočni cikel (12/12 ur) ter okolje s stalno temperaturo 21 °C in 55% vlažnostjo. Hrano in vodo smo priskrbeli *ad libitum*. Do vključitve v poskus so bile miši najmanj 14 dni v karanteni. V poskuse smo vključili zdrave, 8-12 tednov stare živali ženskega spola, ki so ob pričetku poskusov tehtale med 18 in 20 g. Raziskave so potekale v skladu z dovoljenjem Ministrstva za kmetijstvo, gozdarstvo in prehrano Republike Slovenije (dovoljenje za izvajanje poskusov na živalih št. 34401-4/2012/4) ter v skladu evropske direktive za poskuse na živalih (2010/63/EU). V izogib povzročanju nepotrebne bolečine in nelagodja živalim, smo med terapijo miši uspavali z inhalacijsko anestezijo z izofluranom (Nicholas Piramal India LTD, London, Anglija). Miši smo evtanazirali z metodo cervikalne dislokacije z izkrvavitvijo ali z metodo zadušitve z inhalacijo CO_2. Poskuse so izvajale samo osebe z ustreznim dovoljenjem za izvajanje poskusov na živalih.

2.3 Tumorski model

Eksperimentalne tumorje smo inducirali z injiciranjem suspenzije živih melanomskih ali sarkomskih celic (1 x 10^6 celic B16F10 ali LPB v 100 µL fiziološke raztopine) v podkožje na predhodno obritem desnem boku miši. Ko so tumorji dosegli volumen 30 – 40 mm^3, smo živali naključno razporedili v poskusne skupine ter pričeli z izvajanjem peritumoralne genske terapije.

2.4 Peritumoralna genska terapija in vivo

Ob pričetku poskusa smo miši uspavali z inhalacijsko anestezijo. Nato smo v peritumoralno območje intradermalno injicirali 80 µL plazmidne DNA (4 x 20 µl krožno ob tumorju) s koncentracijo 1,25 µg/µL (100 µg DNA) ali 0,63 µg/µL (50 µg DNA). Kontrolnim skupinam smo injicirali vodo miliQ ali kontrolni plazmid pControl-ORT. Takoj po injiciranju smo na mesto injiciranja ob tumorju nanesli prevodni gel (AquaUltra Basic, Ultragel, Budimpešta, Madžarska) in dovedli električne pulze. Pri tem smo uporabili več-točkovno elektrodo (MEA) s krožno razporeditvijo šestih elektrodnih konic (Iskra Medical, Podnart, Slovenija) na razdalji 3,5 mm med vsako konico *(Slika 4A, 4B)*. Elektrodo smo namestili na mesto injiciranja ob tumorju, tako da smo tumor zaobjeli (*Slika 4C*), in dovedli električne pulze s pomočjo generatorja Cliniporator (Cliniporator, IGEA, Carpi, Italy). Pri tem smo uporabili dolge nizko-napetostne pulze z napetostjo 60 V in trajanjem 150 ms (LV, 170 V/cm), ki so se v predhodnih poskusih izkazali kot optimalni pulzi za dostavo plazmida, ki nosi zapis za IL12. Glede na terapevtsko skupino smo terapijo izvedli enkrat ali jo v posameznih skupinah še dvakrat ponovili na dan 2 in dan 4 po prvi terapiji.



Slika 4: Uporaba več-točkovne (MEA) elektrode. (A) Aplikator elektrode MEA s krožno razporeditvijo šestih elektrodnih konic. (B) Krožno porazdeljevanje električnih pulzov znotraj elektrode. (C) Uporaba elektrode MEA za peritumoralni genski elektroprenos.

2.5 Spremljanje zaostanka v rasti tumorjev

Vpliv genske terapije na rast melanomskih in sarkomskih tumorjev smo spremljali z merjenjem premerov tumorjev vsak drugi dan. Z digitalnim kljunastim merilom smo merili tri razdalje: a-najdaljši premer, b-premer pravokoten na a ter c-debelina tumorja. S pomočjo enačbe za prostornino elipsoida smo izračunali prostornino tumorjev (V = a x b x c x Π/6). Velikost tumorjev smo spremljali do doseženega volumna 350 mm^3. Miši z večjimi tumorji od 350 mm^3 smo humano usmrtili. Rezultate

smo prikazali v obliki rastnih ali preživetvenih krivulj (krivulje Kaplan-Meier). Rastne krivulje prikazujejo povprečne vrednosti volumna tumorjev pri posameznih dnevih po terapiji. Preživetvene krivulje prikazujejo število miši v poskusu, s tumorji manjšimi od 350 mm^3, ob posameznih dnevih po terapiji.

2.6 *Spremljanje sistemskega učinka terapije na lokoregionalne in oddaljene metastaze*

Za spremljanje sistemskega učinka terapije na oddaljene metastaze, smo mišim nasadili po en tumor na vsakem boku; prvi – primarni tumor smo tretirali, med tem ko smo sistemski učinek protitumorske genske terapije spremljali na nasprotnem netretiranem – kontralateralnem tumorju. Za spremljanje zaostanka v rasti kontralateralnih tumorjev smo primarni tumor (1 x 10^6 celic B16F10 v 100 µL fiziološke raztopine) in tumor na nasprotnem boku (0,8 x 10^6 celic B16F10 v 100 µL fiziološke raztopine) injicirali istočasno. Ko je primarni tumor dosegel velikost 30 – 40 mm^3 smo izvedli peritumoralni genski elektroprenos ter merili volumen kontralateralnih tumorjev. Postopek terapije primarnih tumorjev je opisan v odstavku *2.4 Peritumoralna genska terapija in vivo*.

2.7 Statistična obdelava

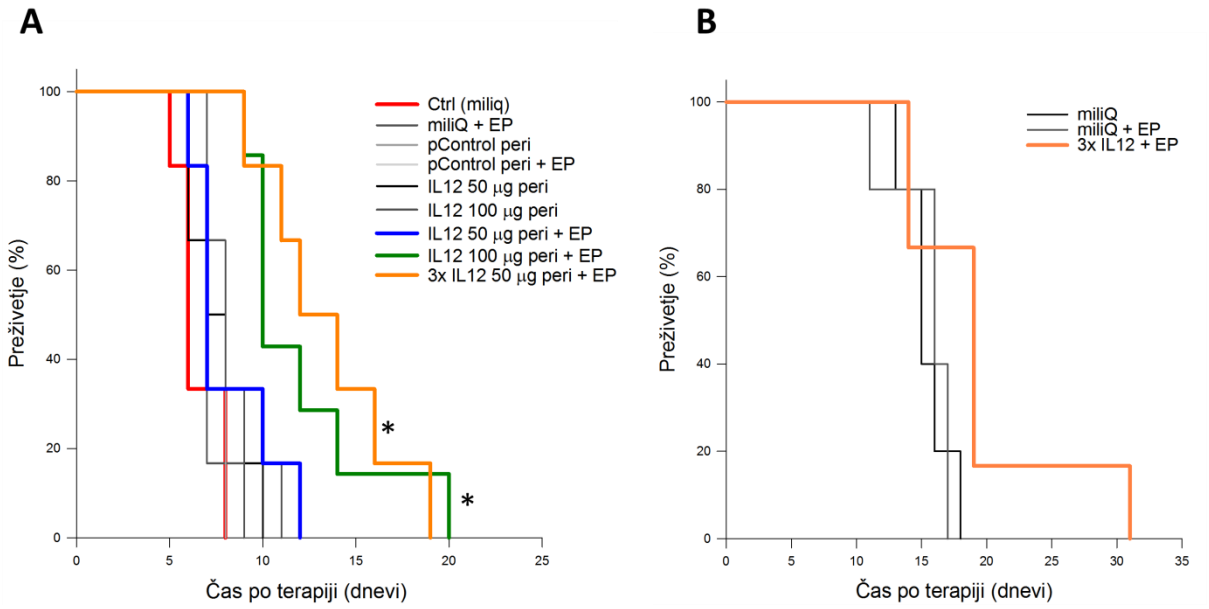
Za vse podatke smo obravnavali test normalne porazdelitve podatkov (test Shapiro-Wilk). Za vse skupine v poskusih smo določili srednjo vrednost in standardno napako ter ugotavljali statistično značilno razliko med skupinami s testom enosmerne analize variance (One-Way ANOVA) oz. t-testom. Če so bile razlike med skupinami statistično značilno različne, smo razlike med skupinami primerjali s Holm-Sidakovim testom. Statistično značilne razlike med rastnimi krivuljami med posameznimi skupinami smo določali s testom Survival log-rank. Vrednosti $p<0,05$ smo obravnavali kot statistično značilne. Statistično obdelavo podatkov smo izvedli s programskim orodjem SigmaPlot 12 (Systat Software Inc., London, Anglija).

3 REZULTATI

3.1 *Vpliv peritumoralne genske terapije z vnosom gena za IL12 na preživetje miši s sarkomskim ali melanomskim tumorskim modelom*

Na sarkomskem (LPB) in melanomskem (B16F10) modelu smo po intradermalni genski terapiji z vnosom gena za IL12 v peritumoralno področje sledili deležu preživelih miši ob posameznih dnevih po terapiji. Rezultati so predstavljeni v obliki preživetvenih krivulj (*Slika 5*). Na melanomskem modelu (*Slika 5A*) se je najboljši učinek terapije pokazal pri miših, tretiranih z višjo dozo plazmidne DNA (100 µg/tumor) ali pri miših, pri katerih smo terapijo večkrat ponovili (3x IL12 + EP). Pri obeh skupinah smo dosegli statistično značilno podaljšano preživetje miši v primerjavi z ne-tretirano kontrolno skupino.

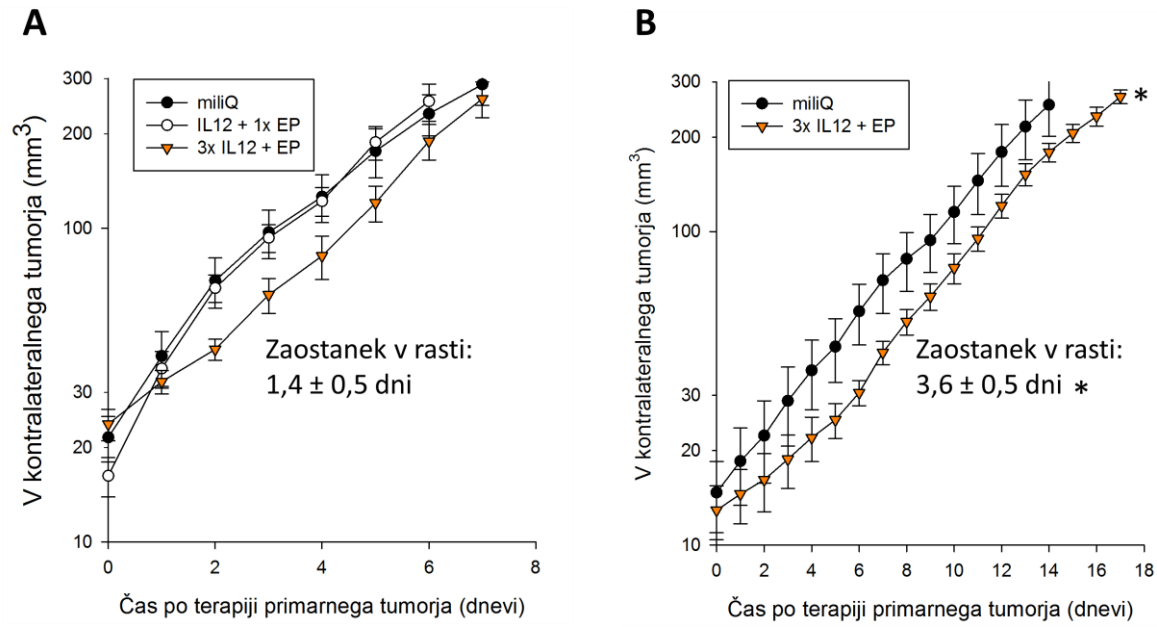
Terapevtsko skupino, ki je na melanomskem modelu najbolje odgovorila na terapijo, smo vključili v testiranje delovanja terapije na sarkomskem modelu. Tako kot na melanomskem tudi na sarkomskem modelu (*Slika 5B*) trikratna terapija z vnosom gena za IL12 izkazuje zaostanek v rasti primarnih tumorjev ter podaljšano preživetje miši v primerjavi s kontrolno skupino.



Slika 5: Preživetvene krivulje po terapiji primarnih mišjih tumorjev. A) Melanomski tumorski model B16F10. B) Sarkomski tumorski model LPB. *P < 0,05 med izbranimi skupinami in kontrolno ne-tretirano skupino.

3.2 *Sledenje rasti kontralateralnih tumorjev in sistemski vpliv peritumoralne genske terapije*

Za spremljanje sistemskega učinka terapije, smo mišim istočasno nasadili po dva tumorja; primarnega smo tretirali, na nasprotnem netretiranem – kontralateralnem tumorju pa smo spremljali učinek peritumoralne genske terapije. V primeru tumorskega modela B16F10 (*Slika 3A*) smo opazili zaostanek v rasti kontralateralnih tumorjev v skupini s trikratnim genskim elektroprenosom z vnosom gena za IL12 (zaostanek v rasti = 1,4 ± 0,5 dni) v primerjavi s kontrolno skupino, tretirano z miliQ. V primeru enkratne peritumoralne terapije zaostanek v rasti kontralateralnih tumorjev ni bil opazen. Na sarkomskem tumorskem modelu LPB (*Slika 3B*) smo po trikratni terapiji primarnega tumorja pokazali statistično značilen zaostanek v rasti (zaostanek v rasti = 3,6 ± 0,5 dni) kontralateralnih tumorjev v primerjavi s kontrolno skupino. S sledenjem zaostanka v rasti kontralateralnih tumorjev, ki posnemajo lokoregionalne ali oddaljene metastaze, smo tako na melanomskem kot na sarkomskem modelu potrdili sistemsko delovanje peritumoralne terapije z vnosom gena za IL12.



Slika 6: Rastne krivulje kontralateralnih tumorjev. A) Melanomski tumorski model B16F10. B) Sarkomski tumorski model LPB. *P < 0,05 med izbranimi skupinami in kontrolno ne-tretirano skupino.

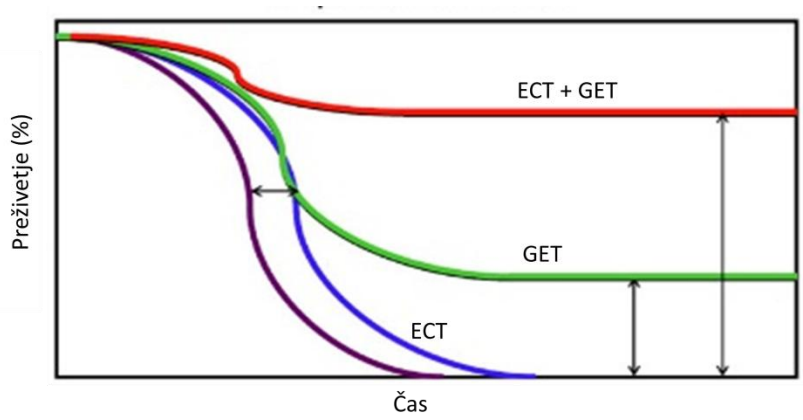
4 RAZPRAVA

V predhodnih raziskavah smo dokazali, da optimizacija metode genskega elektroprenosa vodi do varnega, učinkovitega in ciljanega vnosa plazmidne DNA v kožo (Kos et al., 2015a, 2016). V naslednjem koraku smo na melanomskem in sarkomskem modelu določali, ali je tako optimizirana metoda učinkovita v genski terapiji raka za doseganje protitumorskega delovanja. Protitumorski učinek optimizirane metode genskega elektroprenosa s ciljano dostavo peritumoralno v kožo smo spremljali po vnosu plazmidne DNA, ki nosi zapis za citokin IL12.

Optimizacija glavnih parametrov genskega elektroprenosa, kot so izbor elektrode, optimizacija električnih parametrov ter sestave plazmidne DNA, ključno pripomore k varni in učinkoviti uporabi metode za namen genske terapije (Kos et al., 2016; Shirley et al., 2014). Tako smo za določanje protitumorskega delovanja uporabili nizko-napetostne pulze (LV), ki so se v postopku optimizacije izkazali za najbolj optimalne v primeru izražanja transgena s sistemskim ali parakrinim delovanjem, kakršen je IL12 (Kos et al., 2016). Za namen dovajanja električnih pulzov smo uporabili novo ne-invazivno več-točkovno elektrodo, ki s svojo krožno razporeditvijo točkovnih elektrod omogoča učinkovit vnos plazmidov v peritumoralno področje, kot smo predhodno pokazali v postopku optimizacije elektrode (Kos et al., 2015b). Novost na področju intradermalnega vnosa genov je tudi vnos plazmidne DNA z uporabo plazmida brez zapisa za antibiotično rezistenco, kar omogoča lažji prenos metode v klinično prakso (Kamensek et al., 2017).

Uporaba optimiziranih parametrov in prilagajanje protokolov z izborom količine plazmidne DNA in večkratnega ponavljanja terapije tako ključno prispeva k doseganju optimalnega terapevtskega učinka peritumoralne genske terapije. Optimizirana metoda izkazuje protitumorsko delovanje tako na primarnih tumorjih kot tudi sistemsko na oddaljenih metastazah. Najboljši učinek peritumoralne genske terapije smo na primarnih tumorjih dosegli v primeru vnosa plazmidne DNA z višjo dozo (100 µg/tumor) ali v primeru trikratne peritumoralne terapije. Sistemsko delovanje peritumoralne terapije z IL12 se kaže z zaostankom v rasti kontralateralnih tumorjev tako na melanomskem kot tudi na sarkomskem tumorskem modelu. Opazen učinek remisije oddaljenih ne-tretiranih metastaz imenujemo tudi »abscopal« učinek, k čemur stremi razvoj novejših oblik zdravljenja (Sersa et al., 2015).

Tako optimizirana metoda z dokazanim protitumorskim delovanjem na primarnih in oddaljenih tumorjih predstavlja odskočno desko za nadaljnjo uporabo ter prenos metode v klinično prakso. Zanimanje za uporabo peritumoralnega genskega elektroprenosa se kaže predvsem v imunoterapiji raka z uporabo peritumoralne genske terapije v kombinaciji z lokalnimi ablativnimi tehnikami. Tako predlagana kombinirana terapija predstavlja peritumoralni genski elektroprenos z vnosom gena za IL12 v kombinaciji z intratumoralno elektokemoterapijo (ECT) z vnosom cisplatina ali bleomicina (Calvet and Mir, 2016; Sersa et al., 2015). Prednost predlagane kombinirane terapije je stopnjevanje učinka vsake posamezne terapije, kar bi vodilo ne le do zaostanka v rasti tretiranih tumorjev, ampak tudi do popolnih ozdravitev z dodatnim sistemskim delovanjem na oddaljene ne-tretirane metastaze (*Slika 4*). Prve raziskave v veterini že potrjujejo učinkovito delovanje tovrstne kombinirane terapije (Cemazar et al., 2016).



Slika 7: Pričakovani učinek kombinirane terapije peritumoralnega genskega elektroprenosa (GET) z elektrokemoterapijo (ECT).

5 ZAKLJUČKI

Optimizirana metoda genskega elektroprenosa je učinkovit in varen način vnosa plazmidne DNA za peritumoralno zdravljenje tumorjev z lokalnim delovanjem na primarne tumorje ter sistemskim vplivom na lokoregionalne in oddaljene metastaze.

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POGLAVJE 6

Poglavje 6: Klinični pomen elektroporacije na področju genske terapije in vnosa DNA vakcin

Clinical potential of electroporation for gene therapy and DNA vaccine delivery

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Povzetek

Elektroporacija omogoča učinkovit vnos DNA v tarčne celice in s tem bistveno izboljša izražanje terapevtsko učinkovitih ali imunogenih proteinov, za katere zapis nosi vnešena plazmidna DNA. Enostavna in vsestransko uporabna metoda elektroporacije nosi velik potencial za zdravljenje in preprečevanje različnih bolezni. Pregledni članek ponuja pregled nad dosedanjimi in tekočimi kliničnimi študijami s področja genskega elektroprenosa. Osredotoča se na uporabljene protokole in rezultate zdravljenja. Na podlagi rezultatov kliničnih študij in novih dognanj predlaga izboljšave obstoječe metode genskega elektroprenosa za doseganje boljše učinkovitosti in boljše sprejemljivosti s strani bolnikov. Izboljšave metode so mogoče z dodajanjem adjuvantov k DNA vakcinam, ustreznim izborom tarčnega mesta vnosa ter izborom primernih elektrod in parametrov električnih pulzov. Vse večje število kliničnih študij in obetavni rezultati zdravljenja nakazujejo vse večjo uspešnost in zanimanje za metodo genskega elektroprenosa v klinični praksi. Kljub vsemu pa ostajajo odprta vprašanja za prihodnje študije. V prihodnje je bistveno nadgraditi znanje o osnovnih mehanizmih metode, vzpostaviti in poenotiti elektroporacijske protokole ter nadaljevati z iskanjem novih terapevtskih aplikacij genskega elektroprenosa.

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REVIEW

Clinical potential of electroporation for gene therapy and DNA vaccine delivery

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ABSTRACT

Introduction: Electroporation allows efficient delivery of DNA into cells and tissues, thereby improving the expression of therapeutic or immunogenic proteins that are encoded by plasmid DNA. This simple and versatile method holds a great potential and could address unmet medical needs such as the prevention or treatment of many cancers or infectious diseases.

Areas covered: This review explores the electroporation mechanism and the parameters affecting its efficacy. An analysis of past and current clinical trials focused on DNA electroporation is presented. The pathologies addressed, the protocol used, the treatment outcome and the tolerability are highlighted. In addition, several of the possible optimization strategies for improving patient compliance and therapeutic efficacy are discussed such as plasmid design, use of genetic adjuvants for DNA vaccines, choice of appropriate delivery site and electrodes as well as pulse parameters.

Expert opinion: The growing number of clinical trials and the results already available underline the strong potential of DNA electroporation which combines both safety and efficiency. Nevertheless, it remains critical to further increase fundamental knowledge to refine future strategies, to develop concerted and common DNA electroporation protocols and to continue exploring new electroporation-based therapeutic options.

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1. Introduction

In vivo transfection of a DNA plasmid (pDNA) makes targeted tissues capable of producing a specific protein. pDNA can encode either a therapeutic protein or an antigen and is thus a promising tool to treat a wide range of diseases. To make this gene transfer efficient, the delivery method is critical. Indeed, since the phospholipid bilayer of the plasma membrane has a hydrophilic exterior and a hydrophobic interior, any polar molecules, such as DNA, are unable to freely pass through the membrane. Many methods have been developed to increase transfection efficiency.[1] Among them, electroporation (EP) takes advantage of the relatively weak nature of the phospholipid bilayer's hydrophobic/hydrophilic interactions. EP is based on application of controlled electric pulses to cells or tissues, which increase the cell membrane permeability and allow polar molecules to pass through. EP is a highly attractive method and has been applied in several medical applications, namely for electrochemotherapy (ECT), gene electrotransfer (GET), nonthermal irreversible EP and transdermal drug delivery.[2–4] This review will focus on GET and will depict its potential for DNA vaccines and gene therapy.

2. EP for gene delivery: GET

2.1. Basic mechanism of EP

Among the various theories that have been proposed to explain the effect of EP at molecular level, the consensual explanation consists in the formation of aqueous pores in cell membrane. Basically, some water molecules can enter into the lipid bilayer, leading to membrane rearrangement to orient the polar heads of phospholipids toward water. Consequently, nanopores are formed in cell membrane.[3] This event occurs spontaneously, but it is neither frequent nor thermodynamically stable.[5] During the EP process, exposure of cell membrane to an electric field polarizes membrane and thus reduces the energy required for water penetration in the bilayer. Therefore, the probability and stability of pore formation is improved.[3] However, a direct visualization of pores after EP is still challenging and the term electropermeabilization is sometime preferred to prevent any misuse by describing not fully elucidated molecular events.[6,7]

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Article highlights.

- Electroporation is a promising method for gene delivery [gene electrotransfer (GET)] by increasing plasmid uptake and protein expression.
- The good safety profile allows repeated GET, making it very attractive for vaccine delivery.
- An increasing number of clinical trials are using GET for cancer treatment and vaccination against cancer or infectious diseases.
- In clinical trial, electroporation has only mild and transient side effects.
- Many strategies can be considered for further improvement of clinical outcomes and tolerability of GET.

This box summarizes key points contained in the article.

2.2. GET procedure and parameters affecting transfection efficiency

GET requires pDNA injection into the targeted tissue before pulse application. The application of electric pulses has two complementary effects promoting both permeabilization and electrophoresis of the negatively charged pDNA, necessary for its transfer across the cell membrane.[1,8] EP devices are pulse generators that deliver current through electrodes. Potential difference (V) between electrodes generates an electric field (V/cm) around the cells that causes an induced transmembrane voltage ($\Delta\varphi$). For isolated and regularly shaped cells, a first-order approximation of key parameters is given by the Schwan equation [9]:

$$\Delta\varphi = \frac{3}{2} ER \cos\sigma \left(1 - e^{-\frac{t}{\tau}}\right)$$

where E is the strength of the electric field, R is the radius of the cell, σ is the polar angle measured from the center of the cell with respect to the direction of the field, t is the time elapsed since the onset of the field and τ is the time constant of the membrane charging, which varies with medium conductivity. For irregularly shaped cells or cells that are not in dilute suspension, $\Delta\varphi$ has to be determined experimentally or by numerical analysis.[10,11]

The electric field strength, the number and duration of the applied electric pulses are key factors to control membrane permeabilization. At low range or low amplitude of electric pulses, no molecular transport by EP occurs. More intense or longer electric pulses lead to reversible EP that creates the temporary and limited molecular pathways required for GET. Irreversible EP is observed at higher electric field leading to permanent permeabilization of the membrane and cell death initiation. Finally, thermal damage and molecule release occurs in sufficiently strong fields.[3,12] Besides the amplitude of electric pulses, the efficiency and safety of GET depends on different parameters, such as cell radius, medium conductivity or osmotic pressure. Some of the parameters are difficult to control while some others are easy to adjust: first, the electric field strength needs to be adapted to observe an appropriate $\Delta\varphi$. Another key parameter is the electrode design as it influences the strength, homogeneity, shape and total energy distribution from the electric field to the targeted cells.[13]

2.3. Advantages and drawbacks

EP not only increases the number of transfected cells and enhances the magnitude of gene expression compared to the injection of pDNA alone, but also allows the co-transfection of several plasmids.[14,15] Moreover, the versatility of GET merits to be highlighted: it can be used with nearly all cell types and at all stages of the cell cycle.[16] It is effective for pDNA delivery to several tissues, explaining its attractiveness for *in vivo* applications.[15,17] Finally, unlike viral vectors for gene delivery, GET does not induce unwanted specific immunity against viral proteins and lowers the risk of integration into the host genome or environmental spread.[18,19] GET is of particular interest for the development of vaccines. First, pDNA can lead to stimulation of both the humoral and the cellular arms of the adaptive immune system. Interestingly, pDNA acts as an adjuvant, triggering inflammatory signal upon cytosolic recognition and transfer to innate immune system, thanks to the stimulator of interferon gene molecule .[20] GET increases both the intensity and the duration of the immune responses induced by DNA vaccines. This is not only due to a higher antigen expression but also to the infiltration of inflammatory cells with cytokine release at the EP site owing to a moderate tissue injury.[21,22]

Nevertheless, GET has also some drawbacks. First, it can cause strong cell damages or even cell death if the electric pulses are not appropriate. Second, cell specificities that influence the $\Delta\varphi$ must be taken into account and care must be paid to the surrounding tissue to avoid unwanted damages. In addition, the mass transport of material inside and outside the cell through the existing pores caused by EP is nonspecific. This can potentially lead to ionic imbalance.[23] EP requires the empirical optimization of many parameters in order to be able to target the right cells. Moreover, specialized equipment is necessary, with the choice of an appropriate generator and electrodes.[13,24,25] Last but not least, it is crucial to adapt the procedure for reaching effective gene expression level and duration in order to trigger adequate clinical effect.[8]

3. Past and current clinical trials

3.1. Overview

There is a long clinical experience about clinical use of EP in a context of ECT. Combining injected chemotherapy and EP of tumors to render them more susceptible to the treatment is a method used now in many hospitals to treat cancer patients. More recently, several clinical trials have been using GET against several infectious diseases and cancer. Up to now, 61 gene therapies have been recorded (www.clinicaltrials.gov), and this number is increasing continuously.[26] Most of them are currently in phase 1 and, only few have been completed (Figure 1A). DNA vaccination is encountering the largest success for GET as it accounts for 84% of the trials. The remaining 16% consists in gene therapy trials (Figure 1B). The pathologies addressed are many forms of cancer and infectious diseases (Figure 1C).

3.2. Tolerability and safety of GET

During GET, the direct nerve stimulation and the intense muscle contraction can potentially induce a local acute pain. Noticeably, pain level depends on the injection site, type of electrodes and pulse parameters. The most important parameters to reduce patient discomfort are the amplitude and duration of the electric field. Similarly, waveform, rate and number of pulses globally influence patient compliance.[27] In clinical studies, the adverse events associated to GET are mild and transient. EP can be easily performed after local anesthesia of the delivery area.[28] However, in several clinical trials, the EP procedure did not request the use of a local anesthetic.[29–31] Several studies have been evaluating the safety and tolerability of different devices and their use as a gene delivery technique. In all these studies, the common patient reaction is mild pain lasting for a few minutes. GET is well tolerated whatever the delivery site [intramuscular (IM), intradermal (ID) or intratumoral (IT)] with no systemic toxicity or clinically relevant side effects (Table 1).

3.3. DNA vaccines against infectious diseases

Infectious diseases have been the main targets of the vaccination strategies developed in the last decades, and the vaccination approaches currently available have allowed an effective control of several fatal diseases. To further broaden the number of preventable or curable infectious diseases, several DNA vaccines have been developed and delivered by GET. They mainly target HIV, influenza, hepatitis B or C but also hemorrhagic fever, malaria, Ebola, etc. (Table 2). Many clinical trials are currently ongoing, and there are only a few studies with results already published. ADVAX vaccine, a HIV-1 vaccine encoding the gag, env, pol, nef and tat antigens was delivered by IM EP to healthy volunteers. The EP procedure appeared acceptable for a prophylactic vaccine. Moreover, an increased magnitude of HIV-1-specific cell mediated immunity over IM injection was observed and EP improved also the number of antigens to which the response was detected.[32] A good

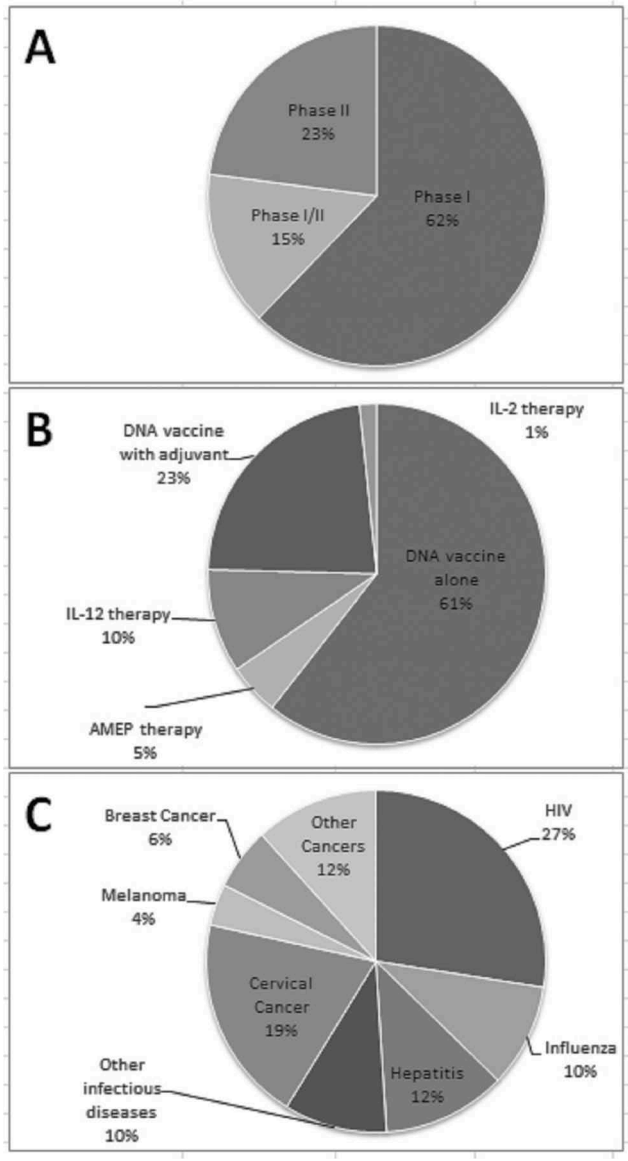

Figure 1. **Clinical trials using GET.** (**A**) Distribution of the clinical phases reached by the ongoing trials. (**B**) Distribution of the type of trials using EP for gene delivery: DNA vaccine alone, combined with adjuvants or gene therapy. (**C**) Distribution of the pathologies addressed in the GET trials. AMEP: antiangiogenic metargidin peptide; EP: electroporation; GET: gene electrotransfer.

Table 1. EP devices used in clinical trials.

Name of the device	Route of delivery	Common side effects	Common duration	Reference
CELLECTRA®	IM : 5 needles, 18 mm depth ID : 3 needles, 3 mm depth	Injection pain (IM > ID) Involuntary muscle contraction after ID EP Local grade 1 erythema	Few minutes (< 10) One day in one patient One day	[31]
Easy Vax™	ID : 80 needles, 600 µm depth	Burning sensation Tingling	Few seconds Few minutes (<5)	[27]
MedPulser	IM : 4 needles, 1.5 cm depth IT (no more information available)	IM injection pain Transient local reaction	One minute Few minutes	[30]
Trigrid	IM : 4 needles ID (no more information available)	Mild–moderate local pain Local reaction	Few minutes One day	[32,38,39]
Dermavax™	ID : 4-4-2 or 4-6-2 parallel row needle electrodes, 2 mm depth	Momentary muscle fasciculation Minor grade 1 skin reaction	Brief but not further specified	[29]
OncoSec	IT IM (no more information available)	Transient grade 1 pain Grade 1 injection site reaction	Brief but not further specified	
Cliniporator	IT : 2 rows of 4 linear needles, 4 mm between needle arrays	Treatment was performed under local anesthesia Good tolerability Mild local toxicity: erythema around the treated lesion Contraction of the underlying muscle	Transient Brief	[28]

The name, the delivery route, the common side effects and their duration are described.
EP: electroporation; ID: intradermal; IM: intramuscular; IT: intratumoral.

tolerability and a superior immunogenicity after EP were also observed for the PENNVAX HIV DNA vaccine combined with an adjuvant plasmid encoding human interleukin-12 (hIL-12) and more than 71% and 88% of volunteers developed either a CD4(+) or CD8(+) T-cell response after the second or third vaccination, respectively.[35] Very recently, results from the HIV-MAG phase I trial have been released. This DNA vaccine was delivered by IM EP in combination with an IL-12 encoding plasmid to healthy volunteers and was well tolerated. A moderate immunogenicity was observed, but, in that study, no adjuvant effect of IL-12 was observed.[36] In another study, a DNA vaccine against the hepatitis B virus (HBV), enhanced by IL-2/interferon (IFN)-γ fusion protein expression was electrotransfered and evaluated in patients suffering from chronic hepatitis B and co-treated with lamivudine chemotherapy. GET was safe and immunologically effective. Interestingly, the HBV-specific T-cell responses showed a correlation with the suppression of viral replication in patients. A DNA vaccine against the hepatitis C virus (HCV) was also found to be safe and immunogenic, in combination with standard-of-care therapy in patients with chronic HCV infection. Transient effects on the viral replication were shown by the decrease in the serum levels of HCV RNA.[37] DNA vaccines against hemorrhagic fever with renal syndrome expressing genes from Hantaan and Puumala viruses have been tested alone or in combination in a phase I clinical trial. No serious adverse effects were reported after delivery by IM EP and both vaccines were immunogenic when delivered alone or mixed together.[38] Immunization against multiple antigens through the unique injection of several plasmids followed by EP appears, therefore, as an attractive strategy.

3.4. DNA vaccine against cancer

In clinical trials using plasmid EP, the most common strategy against cancer is the use of pDNA that encodes tumor-associated antigens (TAA) overexpressed in a particular cancer type. In some cases, the plasmid coding for the TAA is used in combination with an adjuvant, typically encoding human IL-12. pDNA vaccines can also be combined with other therapies (radiotherapy, surgical removal, chemotherapy or endocrine therapy) to mutually enhance their potency. Most of the current GET clinical trials against cancer use an IM injection of pDNA followed by EP (Table 3). The cost-effectiveness and the safety profile of the pDNA vaccines allow repeated low-dose administrations (in the order of milligram) for a long-term protection.[39] Most of the studies are still in progress, and only few results are available. In a phase I clinical trial, pDNA coding for the melanoma tyrosinase, a melanocytic differentiation antigen expressed by several melanoma specimens, able to induce CD8+ T-cell response, was tested. At the maximal dose (1.5 mg), 40% of patients developed Tyr-reactive CD8 + T-cell responses and 14% of those that received all the five doses had an increase in Tyr-reactive CD8+ IFN-γ^+ T cells.[33] It was also shown that a plasmid encoding human papilloma virus (HPV) E6 and E7 viral oncoproteins that promote p53 and retinoblastoma degradation decreased the possibility of cervical cancer

Table 2. Clinical trials using DNA vaccines against infectious diseases.

Biological	Phase	Year	Adjuvant	Delivery site	Dose	Electroporation device	Additional information
DNA vaccines against HIV (14 clinical trials)							
ADVAX vaccine = HIV-1 vaccine encoding the gag, env, pol, nef, and tat antigens	I	2007	/	IM	Three dosing levels Prime and boost	TriGrid	- NCT00545987, Ho et al. - Healthy volunteers - Results published in [32]
PENNVAX-G = Mixture of pDNA encoding HIV-1 env A, C, and D, and consensus gag	I	2009	plasmid encoding hIL-12	Not mentioned	3mg with or without 1mg adjuvant 3 doses over 3 months	Not mentioned	- NCT00991354 - Healthy volunteers - Results published in [33]
	I	2010	/	IM	3mg 4 doses over 4 months	CELLECTRA	- NCT01082692, Tebas et al. - Concurrent antiretroviral therapy (ART)
	I	2010	/	IM	4mg 2 doses over 1 month	CELLECTRA	- NCT01260727, Ake et al. - Healthy volunteers - Comparison with Biojector 2000 needleless device - Boost with attenuated modified vaccinia ankara vaccine at month 3 or 6
	I	2015	plasmid encoding hIL-12	ID or IM	0.6 to 8mg with or without 0.2 to 1mg adjuvant 4 doses over 6 months	CELLECTRA	- NCT02431767, Edupugant et al. - Healthy volunteers
HIV-MAG = Mixture of pDNA encoding HIV-1 gag/pol and HIV-1 nef/tat/vif, env	I	2010	plasmid encoding hIL-12	IM	3mg with 0 to 1mg adjuvant 3 doses over 3 months	Not mentioned	- NCT01266616, Jacobson et al. - Concurrent antiretroviral therapy (ART)
	I	2011	plasmid encoding hIL-12	IM	3mg with 0 to 1mg adjuvant 1 to 3 doses over 2 months	TriGrid	- NCT01496989 - Healthy volunteers - Boost with adenovirus vaccine at month 4 or 6 - Results published in [34]
	I	2012	plasmid encoding hIL-12	IM (2 sites)	2x1.5mg with 0 to 2x0.75mg adjuvant 3 doses over 3 months	TriGrid	- NCT01578889, Hay et al. - Healthy volunteers - With or without boost with VSV vaccine at month 6
	I	2013	plasmid encoding hIL-12	IM (2 sites)	2x1.5mg with 0 to 2x0.5mg adjuvant 4 doses over 9 months	TriGrid	- NCT01859325, Sneller et al. - Concurrent antiretroviral therapy (ART) - Boost with VSV vaccine at months 6 and 12 months
DNA vaccine encoding a pool of HIV peptides	II	2012	/	ID Zetajet	2x0.3mg or 1x0.6mg	Dermavax	- NCT01697007 - Healthy volunteers - With modified vaccinia Ankara boost delivered IM
HIV clade B'/C DNA vaccine	II	2012	/	Not mentioned	4mg 3 doses over 2 months	Not mentioned	- NCT01705223, Wu, Zhang, Shao et al. - Healthy volunteers - Boost with recombinant vaccinia virus at month 6

(*Continued*)

Table 2. (Continued).

Biological	Phase	Year	Adjuvant	Delivery site	Dose	Electroporation device	Additional information
HIV-1 vaccine GTU-MultiHIV B clade = MultiHIV antigen (synthetic fusion protein) built up by full-length polypeptides of Rev, Nef, Tat, p17 and p24 with more than 20 Th and CTL epitopes of protease, reverse transcriptase (RT) and gp160 regions of an HAN2 HIV-1 B clade isolate	I	2014	/	IM (2 sites)	2x2mg 3 doses over 3 months	TriGrid	- NCT02075983, McCormack et al. - Healthy volunteers - Comparison with ID and TC route without EP
	I/II	2015	/	IM (2 sites)	2x2mg 3 doses over 3 months	TriGrid	- NCT02457689, McCormack et al. - Concurrent antiretroviral therapy (ART)
pSG2.HIVconsv DNA	I/II	2014	/	IM	4mg 3 doses over 2 months	Not mentioned	- NCT02099994, Jaoko et al. - Healthy volunteers - With or without boost with adenovirus vaccine at month 3 and poxvirus vaccine at month 5
DNA VACCINES AGAINST INFLUENZA (5 clinical trials)							
VGX-3400 = Mixture of pDNA encoding the hemagglutinin (HA), neuraminidase (NA), and M2e-NP antigen of the H5N1 avian influenza virus	I	2010	/	IM	0.6 to 6mg 2 doses over 1 month	Not mentioned	- NCT01184976, Park et al. - Healthy volunteers
VGX-3400X = Mixture of pDNA targeting the proteins of the H5N1 avian influenza virus	I	2010	/	IM	0.6 to 6mg 2 doses over 1 month	CELLECTRA	- NCT01142362, Hull, Ghosh et al. - Healthy volunteers
INO-3401 = Mixture of pDNA targeting the proteins of the H5N1 avian influenza virus	I	2011	/	ID	0.9mg/ml 2 doses over 3 months	CELLECTRA	- NCT01403155, Bagarazzi et al. - Healthy volunteers previously vaccinated with VGX-3400X
Multiple Combinations of H1 and H5 Influenza Hemagglutinin Plasmids (variant strains of H5N1 and H1N1 with antigenically modified HA)	I	2011	/	ID	0.9mg total dose 3 doses over 6 months	CELLECTRA	- NCT01405885, Bagarazzi et al. - Healthy volunteers
FVH1 = Mixture of two different H1 HA pDNA	I	2012	/	ID	0.9mg 3 doses over 7 months or 1 dose	CELLECTRA	- NCT01587131, Kobinger, Racine et al. - Elderly (≥ 65 years) - If 1 dose, boost with trivalent seasonal influenza vaccine at week 15
DNA VACCINES AGAINST HEPATITIS (6 clinical trials)							
CHRONVAC-C encoding full-length codon optimized NS3/4A gene of HCV genotype 1a	I/II	2007	/	IM	Several doses (low, medium, high)	Not mentioned	- NCT00563173, Vahlne, Sällberg et al. - Chronic HCV patients
	II	2011	/	IM	0.5mg 2 doses over 1 month	MedPulser	- NCT01335711, Vahlne, Sällberg et al. - Chronic HCV patients - With Standard of Care (SOC): Peg-IFN-α-2a and Ribavirin
HBV envelope- and nucleocapsid-based DNA vaccines	II	2011	dual-plasmids expressing Th1 type cytokines (IL-2 and IFN-γ) as fusion proteins	Not mentioned	4mg 4 doses over 3 months	Not mentioned	- NCT01487876, Yang et al. - Chronic HBV patients - Concurrent lamivudine chemotherapy - Results published in [82]

(*Continued*)

Table 2. (Continued).

Biological	Phase	Year	Adjuvant	Delivery site	Dose	Electroporation device	Additional information
HB-110	I	2012	/	Not mentioned	1 to 4mg	TriGrid	- NCT01641536, Yoon et al. - Chronic HBV patients - Concurrent entecavir chemotherapy - Preclinical study results in [83]
VGX-6150 = pDNA covering hepatitis C virus (HCV) genotypes 1a and 1b and targeting the antigens NS3/4A	I	2014	/	IM	1 to 6mg 4 doses over 3 months	CELLECTRA	- NCT02027116, Hoon, Heo et al. - Chronic HCV patients - Results published in [35]
INO-1800 = Mixture of pDNA encoding HBsAg and HBcAg	I	2015	plasmid encoding hIL-12	IM	0.3 to 9mg with 0 or 0.25mg adjuvant From 3 to 6 doses	Not mentioned	- NCT02431312, White et al. - Chronic HBV patients - Concurrent entecavir/tenofovir chemotherapy
DNA VACCINES AGAINST HEMORRHAGIC FEVER (2 clinical trials)							
HTNV and PUUV DNA vaccines (Hantaan virus and Puumala virus) = plasmid expressing two of the three gene segments of HTNV (the M and S segments)	I	2011	/	IM	2mg/ml HTNV or PUUV DNA vaccine or 1mg/ml of each DNA vaccine 3 doses over 2 months	TriGrid	- NCT01502345, Moon et al. - Healthy volunteers - Results published in [36]
	II	2014	/	IM	1 or 2mg of the DNA vaccine mixture 3 or 4 doses over 6 months	TriGrid	- NCT02116205, Paolino et al. - Healthy volunteers
DNA VACCINES AGAINST OTHER INFECTIOUS DISEASES							
Venezuelan Equine Encephalitis Virus (VEEV) DNA Vaccine	I	2013	/	ID or IM	0.5 or 2mg/ml	TriGrid	- NCT01984983 - Healthy volunteers
EP-1300 = DNA-based polyepitope vaccine against malaria	I	2010	/	IM	0.25 to 4mg 3 doses over 2 months	TriGrid	- NCT01169077 - Healthy volunteers
INO-4212 = INO-4201 encoding past Ebola Zaire virus outbreak and INO-4202 encoding the current Ebola virus outbreak strain. When given together, the DNA vaccine is called INO-4212	I	2015	plasmid encoding hIL-12	ID or IM	INO-4201 or INO-4202 or combination of both, with or without adjuvant	CELLECTRA	- NCT02464670, White et al. - Healthy volunteers

A description of the vaccine, the year and the current trial phase are provided. Information regarding the use of adjuvant, the delivery site, the dose, the EP device and additional key parameters are presented. The clinicaltrials.gov identifier of the study and the principal investigator's name (when available) are provided.

ART: antiretroviral therapy; EP: electroporation; HBV: hepatitis B virus; HCV: hepatitis C virus; hIL-12: human interleukin-12; HTNV: Hantaan virus; ID: intradermal; IFN: interferon; IL: interleukin; IM: intramuscular; PUUV: Puumala virus; SOC: standard of care; TC: Transcutaneous; VEEV: Venezuelan Equine Encephalitis Virus; VSV: Vesicular stomatitis virus.

Table 3. Clinical trials using DNA vaccines against cancer.

Biological	Phase	Year	Adjuvant	Additional treatments	Delivery site	Dose	Electroporation	Additional information
————DNA vaccines against HPV-induced cancers———— **(10 clinical trials)**								
VGX-3100 = pDNA encoding E6 and E7 oncogenes (responsible of cellular transformation) of HPV types 16 and 18	I	2008	/	/	IM	0.6, 2 or 6mg 3 doses over 3 months	CELLECTRA	- NCT00685412, Chu, Parker, Sunyecz, Morales et al.
	I	2010	/	/	IM	6mg 1 dose	CELLECTRA	- NCT01188850, Parker, Sunyecz, Morales et al. - Adult females who have been previously immunized with three doses of VGX-3100
	II	2011	/	/	IM	1ml 3 doses over 3 months	CELLECTRA	- NCT01304524, Trimble, Parker, Valenzuela et al.
	I/II	2014	plasmid encoding hIL-12	R, CR	IM	6mg of VGX-3100 and 1mg adjuvant 1 dose	CELLECTRA	- NCT02172911, Gelder et al.
	I/IIa	2014	plasmid encoding hIL-12	S, CR	IM	6mg of VGX-3100 and 1mg adjuvant 4 doses	CELLECTRA	- NCT02163057, Yang et al.
GX-188E = DNA plasmid encoding E6 and E7 proteins of HPV16 and HPV18 fused to extracellular domain of Flt3L and the signal sequence of tissue plasminogen activator (tpa)	I	2012	/	/	IM	1, 2 and 4 mg; The highest dose, 4 mg of GX-188E was split into 2+2 mg 1 dose	Trigrid	- NCT01634503, Kim et al. - Results published in [39]
	I	2014						- NCT02100085, Kim et al. - Follow-up of patients who completed the phase I GX-188E trial
	II	2014	/	/	IM	1 or 4 mg 3 doses over 3 months	Trigrid	- NCT02139267, Park, Kim, Lee, Cho et al.
	II	2015	/	/	IM	1 or 4mg 3 doses	Not mentioned	- NCT02411019, Park, Kim, Lee, Cho et al.
pNGVL4a-CRT/E7 (detox) = pNGVL4a vector coding for CTR (calreticulin) and E7 (detox), a mutated form of E7 genes	I	2011	/	C	IM	0.5, 1, 2 or 4mg/dose 3 doses over 43 days	Trigrid	- NCT01493154, Califano et al.
————DNA VACCINES AGAINST MELANOMA———— *(2 clinical trials)*								
pINGmuTyr = DNA vaccine encoding the melanosomal antigen tyrosinase (Xenogeneic Tyrosinase)	I	2007	/	R, CR, C	IM	0.2, 0.5 or 1.5 mg 5 doses every 3 weeks	Trigrid	- NCT00471133, Wolchok et al. - Results published in [38]
SCIB1 = Plasmid DNA encoding a human antibody molecule engineered to express T cell epitopes derived from the TRP2 and gp100 and two helper T cell epitopes	I/II	2010	/	/	IM	0.4, 2.0, 4.0 and 8.0 mg 5 doses over 6 months	Trigrid	- NCT01138410, Patel, Lorigan, Mulatero, Ottensmeier, Pandha et al. - Part 1 of the study will escalate through 0.4, 2.0, 4.0 and 8.0 mg dose level cohorts - In part 2, the 4.0 and 8.0 mg doses will be administered

(*Continued*)

Table 3. (Continued).

Biological	Phase	Year	Adjuvant	Additional treatments	Delivery site	Dose	Electroporation	Additional information
————DNA VACCINES AGAINST BREAST CANCER———— *(3 clinical trials)*								
Personalized polyepitope DNA vaccine	I	2015	/	/	IM	4 mg 3 doses over 57 days	Trigrid	- NCT02348320, Gillanders et al.
Mammaglobin-A DNA Vaccine	I	2014	/	E	IM (2 sites)	2X3 doses over 84 days	Not mentioned	- NCT02204098, Gillanders et al.
INO-1400 = Immunotherapy designed to target a gene known as the human telomerase reverse transcriptase (hTERT)	I	2014	plasmid encoding hIL-12	/	IM	2 or 8mg of INO-1400 with or without 0.5mg or 2mg adjuvant 4 doses over 3 months	Not mentioned	- NCT02327468, Vonderheide et al. - Also tested for lung and pancreatic cancers
————DNA VACCINES AGAINST OTHER TYPES OF CANCER———— *(6 clinical trials)*								
CEA DNA = Carcinoembryonic antigen (overexpressed in a variety of cancer cell types) fused to a tetanus toxoid T helper epitope	I/II	2010	GM-CSF	C	ID	400µg 2 doses over 3 months	DermaVax	- NCT01064375, Liljefors et al. - Pathology: colorectal cancer
pVAXrcPSAv53I = DNA encoding rhPSA (Rhesus Prostate Specific Antigen), 89% homologous to human PSA	I/II	2009	/	/	ID	50 or 150 or 400 or 1000 or 1600µg 5 doses, 4 weeks apart	DermaVax	- NCT00859729, Yachnin et al. - Pathology: prostate cancer - Results published in [29]
pDOM-WT1-37 = First domain of fragment C (FrC) of tetanus toxin (pDOM) fused to the human Wilms' Tumor gene-1-derived MHC class I-binding epitope	II	2011	/	/	IM	1mg 6 times at 4 weekly intervals	Not mentioned	- NCT01334060 - Pathology: leukemia - Responders may continue vaccination 3 monthly to maximum of 24 months
INVAC-1 = DNA vaccine encoding human telomerase reverse transcriptase (hTERT)	I	2014	/	/	ID	100, 400 or 800 µg 4 weeks X 3 cycles	Not mentioned	- NCT02301754, Culine et al. - Pathology: solid tumors
INO-3106 = Vaccine against HPV6	I	2014	plasmid encoding hIL-12	/	Not mentioned	3 mg or 6 mg INO-3106 alone or in combination with 1 mg adjuvant 4 doses over 9 weeks	Not mentioned	- NCT02241369, Yang et al. - Pathology: aerodigestive malignancies (e.g. squamous cells carcinoma)
V934/V935 hTERT = Cancer vaccine directed against human telomerase reverse transcriptase (hTERT)	I	2008	/	/	IM	2X10^9 vector genomes/mL (low dose) or 2X10^11 vector genomes/mL (high dose) every 2 weeks	Not mentioned	- NCT00753415 - Pathology: solid tumors

A description of the vaccine, the year and the current trial phase are provided. Information regarding the use of adjuvant, the delivery site, the dose, the EP device and additional key parameters are presented. Combinations with other treatments are mentioned: chemotherapy (C), radiotherapy (R), chemoradiotherapy (CR), surgery (S) and endocrine therapy (E). The clinicaltrials.gov identifier of the study and the principal investigator's name (when available) are provided.

hTERT: human telomerase reverse transcriptase; ID: intradermal; IM: intramuscular; TPA: tissue plasminogen activator.

progression. pDNA was electrotransfered IM, using TriGrid Delivery System in both deltoid muscles. The level of antibodies against DNA in the blood of patients was below the detection limit. Furthermore, 22% of patients developed an IFN-γ response after a single immunization, and it reaches 55% after the second vaccination. High levels of IFN-γ were observed even at week 24, suggesting the vaccine induction of E6/E7-specific memory T cells. After the second immunization, half of the patients showed HPV and lesions clearance at week 12 and other 22% reached the same result after week 36.[34] Encouraging phase 1 safety, tolerability and immunogenicity results were reported for a therapeutic HPV16/18 candidate vaccine, VGX-3100, delivered by *in vivo* EP in women previously treated for high-grade cervical dysplasia. The vaccine was well tolerated and induced antigen-specific humoral and Th1-biased cellular immune responses. Strong CTL response, an important determinant for successful immunotherapy, was observed upon DNA vaccination.[40] More recently, VGX-3100 vaccine was involved in a randomized, double-blind, placebo-controlled phase 2b trial. The results indicate clinical efficacy against cervical intraepithelial neoplasia 2/3 following vaccination by EP and the trafficking and tissue infiltration of antigen-specific CD8+ T cells in the targeted lesions. VGX-3100 is the first therapeutic vaccine to show efficacy against cervical intraepithelial neoplasia 2/3 associated with HPV-16 and HPV-18 and could present a new nonsurgical therapeutic treatment.[41] In a clinical study against prostate cancer, pDNA coding for rhesus macaque prostate-specific antigen (PSA) was delivered ID using a DermaVax EP system. Twenty percent of the patients showed an increased response to self PSA. After 1 year of follow-up, only 13% of the subjects developed metastatic disease. This was the first clinical trial in which EP was combined with ID injection for tumor treatment and the anti-PSA antibodies were less efficiently produced than after IM DNA vaccination.[29]

3.5. Other cancer gene therapies

GET has been used not only for vaccination purpose but also for delivering plasmid encoding proteins that act as cancer therapies. Plasmids coding for cytokines (IL-2 or IL-12) have been delivered to tumor by EP in order to treat melanoma (NCT00223899, Kharkevitch et al.; NCT00323206, Daud et al.; NCT01502293, Le et al.), Merkel cell carcinoma (NCT01440816, Bhatia et al.), cutaneous lymphoma (NCT01579318, Le et al.) and head and neck squamous cell carcinoma (NCT02345330, Le et al.). In a phase I clinical trial with patients suffering from melanoma, IL-12 plasmid EP-induced minimal systemic toxicity and local transient pain was the main adverse effect. As previously demonstrated in mice, untreated distal lesions were affected by the therapy, suggesting the involvement of the immune system. Indeed, 10% of patients showed complete regression of all metastasis and 42% showed disease stabilization or partial response.[42,43] Based on these promising results for the treatment of both local and metastatic cancers, four clinical studies in phase II focusing on IL-12 plasmid EP have been launched. GET of antiangiogenic metargidin peptide (AMEP), a plasmid devoid of antibiotic resistance gene and encoding the recombinant disintegrin domain of human metargidin, has also been explored for the treatment of metastatic tumors (NCT01045915, Attali et al.; NCT01664273, Gehl et al.). In a first phase I study, AMEP was delivered by 2 weekly EP of a cutaneous lesion. AMEP and its delivery procedure by EP appeared safe. Three weeks after the last administration, treated lesions were stable in diameter for all patients but response was not observed in distant lesions.[28] The ongoing phase I/II clinical trial aims at evaluating the safety and efficacy of AMEP delivered by muscle EP in the same indication in order to generate a systemic response (NCT01764009, Vasseur et al.).

4. Possible strategies to optimize GET therapeutic outcome

Several parameters need to be considered to set up an efficient GET. Figure 2 summarizes the different actors that are involved in GET process and states how they can be handled to impact the treatment outcome.

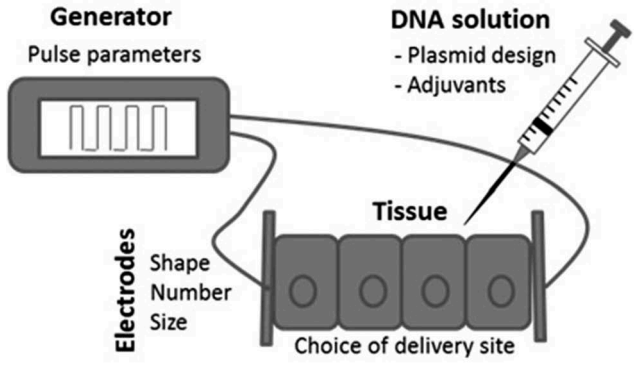

Figure 2. Schematic representation of EP procedure for GET and description of the protocol parameters that can be handled to optimize treatment outcome. pDNA is injected prior electric field application via electrodes. The plasmid design, the use of adjuvants, the choice of delivery site, appropriate electrodes and pulse parameters have to be accurately adjusted to obtain optimal patient compliance and therapeutic efficacy. EP: electroporation; GET: gene electrotransfer.

4.1. Plasmid optimization

One key requirement to optimize protein expression and to obtain a great therapeutic efficacy of pDNA treatments is the plasmid design. To achieve these objectives, it is possible to act on synonymous codon replacement, use of various promoters and introduction of immunostimulatory motifs in the plasmid sequence. Furthermore, the use of antibiotic resistance-free plasmids is in compliance with Regulatory Agencies, concerning the safety in clinical applications.

Codon optimization (CO) is a gene engineering technique that uses synonymous codon replacement to increase protein production, taking advantage of the degenerate nature of the genetic code.[44,45] The same polypeptide can be produced in different quantities, depending on its mRNA structure. CO acts mainly at two different levels, increasing mRNA stability and its translation kinetic.[44,46] It has been suggested that CO can also increase the mRNA quantity.[47] In the last decade, several codon adaptation algorithms have been developed to manipulate gene sequences in order to increase protein production while maintaining the activity and folding properties.[44]

Another strategy to increase protein production is the use of nonmammalian promoter to control plasmid expression, like cytomegalovirus (CMV) or simian virus 40 promoters. They are ubiquitous nonspecific promoters, active in a wide range of cells and tissues. Most of the time the intron A of the CMV immediate-early gene is included in the complete CMV promoter as it can increase mRNA stability and gene expression.[48,49] Although the constitutive promoters, such as CMV, can induce high levels of gene expression, they can pose a problem in terms of cell specificity of gene delivery. Tissue-specific promoters are attractive to improve the specificity and safety of gene therapy and vaccination. Therefore, different promoters have been designed to specifically activate the transcription in certain cell types or tissues, such as liver, muscle, skin, tumors, endothelium tissue, erythroid tissue and neurons.[50–53]

For DNA vaccine design, immunomodulators can be added to strengthen the immune response. This is the case of genetic adjuvants, which can be co-encoded or co-administered. Additional strategies to stimulate the immune system have been described such as the use of conventional adjuvants including alum particles and MF59 emulsion.[48] A more recent strategy takes advantage of unmethylated CpG motifs abundantly present in prokaryotic DNA but rare (~1%) in the eukaryotic DNA.[49] During infections, the released bacterial DNA exposes CpG motifs to cells that express the toll-like receptor 9 (TLR9), commonly antigen-presenting cells (APCs). This may subsequently increase both innate and adaptive immune response against the pathogen, supporting maturation, differentiation and proliferation of NK, T cells and B cells. A number of CpG motifs can be inserted in pDNA, and their ability to induce Th1 immune response make them particularly promising for cancer vaccines.[54] However, it has been suggested that DNA vaccines elicit immune response by multiple mechanisms and that TLR9 and CpG motifs are not essential for DNA vaccine action. Indeed, TLR9-deficient mice responded to DNA and CpG motifs appeared to play mainly an adjuvant role.[48,55,56]

To avoid a loss of plasmid during cell division and to favor the survival of the transformed bacteria, it is necessary to insert a selection marker into the plasmid. A common strategy consists in the use of a gene conferring resistance to an antibiotic. Nevertheless, possible side effects such as horizontal gene transfer or allergy development limit its applicability in clinical trials.[57] Because of that, the use of antibiotic resistance genes as selection markers is becoming more restricted or even forbidden by the authorities. Alternatives have been developed to take over. One of them is based on the inactivation or modification of a gene responsible for bacterial growth in the bacteria strain. The active gene is then inserted in the plasmid construct. Other strategies include toxin–antitoxin system, operator–repressor system, antisense RNA regulators and have been reviewed elsewhere.[57] Antibiotic resistance-free plasmids confer a great safety profile and avoid a negative impact on mammalian gene expression.[48]

4.2. Genetic adjuvants

GET is promising to set up vaccine strategies as both pDNA and EP possess several intrinsic adjuvant properties. In addition, genes can be designed to encode immunomodulatory proteins that act as genetic adjuvants. Advantages of using genetic adjuvants are their low cost and simplicity of administration. They avoid the need of unstable recombinant cytokines and potentially toxic conventional adjuvants.[58] Currently, the most common genetic adjuvants are plasmid coding for cytokines and only these molecules have been involved in clinical trials.[59] In particular, plasmids encoding hIL-12 are often chosen as adjuvant. This cytokine is known to enhance Th-1-biased cellular immunity.[60] Other Th-1-inducing cytokines such as IL-2 and IFN-γ are also under clinical investigation. Another strategy consists in the use of GM-CSF encoding plasmid. This pro-inflammatory cytokine helps in the recruitment, activation and

enhancement of activity of APCs.[61] The genetic adjuvant field is under intense wave of research. Several promising strategies have been considered in preclinical studies and hold a huge potential for future clinical investigations.[62,63] For instance, an innovative strategy is the use of local ablative techniques, such as ECT, as *in situ* vaccination that can be boosted by peritumoral GET using immunomodulatory gene such as IL-12.[64] Such strategy has been applied for the canine tumor therapy with success (submitted publication).

4.3. Choice of the delivery site

The most usual delivery sites are muscle and skin. Delivery to muscle requires a lower electric field and allows a long duration in gene expression and a delivery of a large amount of liquid; however, muscle contraction can be painful. Skin is more accessible and contains many dendritic cells. These characteristics consent a less invasiveness, thanks to a reduced needle depth, a better tolerable delivery and a great immune response.[31] In preclinical model, the choice of the delivery site for GET influenced not only the magnitude but also the type of immune response.[65]

4.4. Improvement of electrodes

In order to bring gene delivery into broader clinical applications, the delivery needs to be compliant with the patients, that is, noninvasive and not inducing discomfort. To this end, both the design of electrodes and the choice of electrical parameters are very important. The electrodes used for GET can be divided into two groups: noninvasive and invasive electrodes.[8,66] The invasive electrodes consist of different needle configurations that are penetrated to the skin, muscle or tumor [8,15,66–68] (Figure 3). Well-covered electric field achieved by pulse delivery in between the pairs of electrodes and large electrotransfered tissue area results in high transfection efficiency. The commercially available electrodes used in EP clinical trials belong to this category and are additionally modified for more feasible clinical use.

However, the invasiveness and discomfort of GET with such electrodes initiated the studies with less invasive electrodes. One of the minimally invasive approaches is the utilization of microneedles, which provide less painful delivery of DNA and achieve sufficient electric field distribution for effective GET.[69] Further advancements have been made using nonpenetrating electrodes such as calliper and plate electrodes. The depth of penetration of electric field is rather small,[70] basically in the skinfold, which is in certain

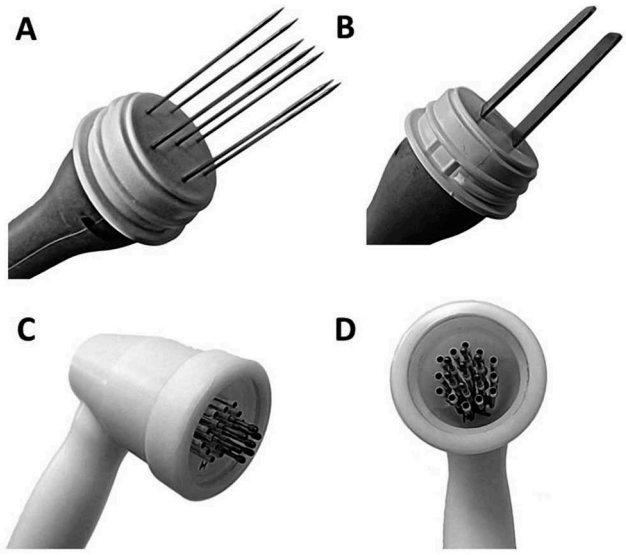

Figure 3. **Different types of electrodes:** (**A**) needle electrode; (**B**) plate electrode; (**C** and **D**) multielectrode array.

situations not convenient,[70] and would not be optimal for use in humans. Furthermore, these electrodes require high voltages (HVs) to enhance delivery and therefore can cause tissue damage.[71] To this end, other types of noninvasive electrodes are being explored. Such noninvasive electrodes, the multielectrode array (MEA), were designed by several groups and have proved to be effective for the delivery of pDNA to the skin [53,71,72] and have promising expectations in cancer gene therapy.[71] With the MEA, the applied voltage was minimized by maintaining a short electrode distance. This diminished or eliminated the muscle twitching and pain associated with the application of the pulsed electric field.[71] The design of novel electrodes, therefore, leads to less adverse side effects than other EP delivery systems with efficient gene delivery and high immune response and proposes promising clinical applications.

4.5. Optimization of pulse parameters

A large variety of pulses has been used for GET. The electric pulses chosen for gene transfection have been either short HV pulses alone,[53,73] low voltage (LV) pulses alone [74] or a combination of one or several HV and LV pulses.[51,75–77] The border between HV and LV is not strictly defined, but, in general, the HV pulses (> 400 V/cm) are typically short (50 – 100 μs), whereas the LV pulses (<400 V/cm) are longer, typically in milliseconds (10 – 400 ms).[66] The optimal pulse conditions reported are not the same but could depend on the type of transfected tissue, the type of electrode and also the type of injected DNA. To ensure efficient

GET electrical parameters need to be adjusted for transfection of different tissues, due to differences in tissue structure, cell size, and DNA and electric field distribution. Typically, muscles are easier to transfect compared to skin or tumors, what requires lower field strengths to reach the efficient gene delivery into muscle cells.[77] Besides the duration and amplitude of electric pulses, also the number of pulses and pulse polarity varies between the studies. To minimize tissue damages, the tendency is to decrease the voltage and pulse length and to reduce the number of pulses and change the pulse polarity.[78,79] Therefore, to find the best compromise between the potential damages caused by electric pulses and the efficiency and specificity of gene delivery, the electrical parameters need to be carefully selected for each targeted tissue in order to reach the expression for optimal therapeutic effectiveness.

5. Conclusion

Although the use of genes is attractive and opens a promising method of combatting many diseases, their delivery in patients' cells remains challenging. EP appears as a safe and effective transfection technique, presenting many advantages over other strategies. An increasing number of clinical trials using EP have been recorded over the past years. They consist mainly in DNA vaccines against infectious diseases and cancers. Nevertheless, EP requires precise and specific protocol optimization in order to obtain the best treatment outcome while avoiding side effects.

6. Expert opinion

The last 10 years have shown a growing number of clinical trials based on DNA EP. Compared to the other methods of gene delivery, EP benefits from excellent transfection efficiency. After EP, the transgene is locally and transiently expressed. However, systemic effects can be observed such as immune response against an encoded antigen or secretion of a therapeutic protein that can be distributed systemically. Consequently, EP has been used for treating a wide range of diseases and many dynamic research groups focus on the improvement of the current approaches, from the optimization of plasmids to the improvement of electrodes and devices. If patients were initially worried about the use of electrical pulses for GET, it appears from all human studies that EP is well tolerated and only mild and transient side effects have been observed.

EP holds the potential of treating or preventing many diseases such as cancer. Nevertheless, EP is a complex process and the ultimate goal would be development of an approach for effective, controlled and painless gene delivery to various tissues. Providing a way to facilitate the setting up of optimal protocols is a global challenge. First, further refinement of the electrodes and electrical parameters and adjustment to the clinical situation are required. To help physicians in their clinical practice, it could be of interest to develop monitoring tools. For instance, imaging methods can be useful to establish a treatment planning that models electrode position and determine the number, amplitude, duration and frequency of the electric pulses to obtain the desired effect on the targeted tissue.[3,80] In addition, it is important to increase the current knowledge on how GET parameters (such as electric field, electrode design and delivery site) influence clinical outcome. For instance, studies must be carried out to better understand the basic mechanism of membrane modification at molecular, cellular and tissue level. In particular, the effect of EP procedure itself and the effect of GET on the early inflammatory events and innate immune activation would merit a deep investigation. Nevertheless, a better understanding is not the only key to success. As illustrated in this review, all the parameters are interconnected and the results of the clinical studies are somewhat difficult to compare. Therefore, a way to organize the data is needed; to facilitate adoption of the common protocols would lead to integrative approach as a platform technology for gene delivery.

Apart from its use as a delivery method for DNA vaccines or therapies, it has been recently demonstrated that EP holds also a potential for inducing immunogenic cell death (ICD). Indeed, in certain circumstances depending on the initiating stimulus, dying tumor cells behave as a cancer vaccine and elicit a cytotoxic immune response. It involves the exposure of damage-associated molecular patterns that are recognized by innate immune cells, which subsequently prime effector T-cell response against dead-cell-associated antigens. It has been shown that ECT was able to induce ICD.[81] We believe that GET of certain pDNA could similarly trigger ICD and thus further extend the range of GET clinical applications in the future.

In conclusion, the multiplication of clinical trials and the results already available demonstrate the strong potential of GET. Nevertheless, it remains critical to further increase fundamental knowledge about EP to refine future strategies and to continue exploring new EP-based therapeutic options. Finally, long-term observation is needed to confirm the safety of GET.

Declaration of interest

L Lambricht is a FNRS research fellow (Fonds de la Recherche Scientifique, Belgium); A Lopes is a research assistant at UCL; V Préat is a full Professor at UCL, Belgium; S Kos is a young researcher and G Sersa is a full Professor both at Institute of Oncology Ljubljana, Slovenia; G Vandermeulen is a FNRS post-doctoral researcher at UCL. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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•• of considerable interest

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Optimizacija metode genskega elektroprenosa za vnos plazmidne DNA v kožo

Genska terapija z uporabo metode genskega elektroprenosa s ciljano transfekcijo v kožo predstavlja kompleksen proces, učinek terapije pa je odvisen predvsem od glavnih parametrov metode (Shirley et al., 2014). Uporabljeni parametri genskega elektroprenosa med dosedanjimi študijami močno variirajo, hkrati pa uporaba neprimernih parametrov, kot so neustrezne elektrode (Vandermeulen et al., 2015), vodijo do neučinkovitega terapevtskega učinka. Pred vpeljavo metode v klinično prakso so tako za doseganje optimalnega učinka in varnosti genske terapije nujne izboljšave glavnih parametrov genskega elektroprenosa ter optimizacija protokolov.

V prvem sklopu doktorske naloge smo se osredotočili na optimizacijo treh glavnih parametrov genskega elektroprenosa: elektrod za dostavo električnih pulzov, sestave plazmidne DNA ter parametrov električnih pulzov. Z vidika prenosa metode v klinično prakso je pomembno, da je metoda ne-invazivna, ne povzroča poškodb in je sprejeta s strani bolnikov. K temu lahko bistveno pripomoremo z ustrezno izbiro tako elektrode kot parametrov električnih pulzov (Shirley et al., 2014). Za vnos molekul v kožo so se izkazale predvsem ne-invazivne več-točkovne elektrode (MEA: multi electrode-array), ki zaradi svoje tesne razporeditve elektrodnih konic zagotavljajo učinkovit vnos učinkovin in plazmidne DNA v kožo, hkrati pa pripomorejo k manj invazivni in manj boleči aplikaciji električnih pulzov v primerjavi z obstoječimi elektrodami (Guo et al., 2011; Guo et al., 2013; Heller et al., 2007; Heller et al., 2010). Ena takšnih elektrod je bila razvita tudi v sodelovanju z našo raziskovalno skupino (Blagus et al., 2013). V sklopu doktorske naloge smo preskusili učinkovitost in varnost tovrstne elektrode za vnos plazmidne DNA. Rezultati kažejo na minimalne poškodbe kožnega tkiva po uporabi elektrode MEA, hkrati pa na močno povečano učinkovitost vnosa plazmidne DNA v primerjavi s klasično ploščato elektrodo. S tem smo dokazali, da visoka stopnja genskega izražanja v koži po uporabi elektrod MEA, ne-invazivnost in enostavno rokovanje omogočajo uporabo novih elektrod MEA za namen genskega elektroprenosa v kožo.

Naslednji parameter metode, ki močno vpliva na bolnikovo sprejemljivost, varnost in učinkovitost metode, je tudi izbor parametrov električnih pulzov. Na podlagi dosedanjih študij je razvidno, da širok nabor pulzov različnih napetosti in dolžine trajanja vodi do učinkovite transfekcije v kožo (Labricht et al., 2015). Kljub na videz nepomembni izbiri pulzov za namene genskega elektroprenosa v kožo, smo v sklopu doktorske naloge pokazali, da izbor parametrov električnih pulzov ne vpliva le na jakost izražanja transgena v koži, temveč vpliva tudi na globino transfekcije, sloj transfecirane kože ter posledično na čas izražanja in porazdeljevanja transgena (lokalno ali sistemsko). Pokazali smo, da

kratki visoko-napetostni pulzi (HV) povzročijo transfekcijo vrhnjih plasti kože in vodijo k lokalnemu izražanju transgena, medtem ko dolgi nizko-napetostni pulzi (LV) povzročijo transfekcijo tudi globljih plasti kože in vodijo do dolgotrajnejšega genskega izražanja s sistemskim porazdeljevanja transgena. Z izborom ustreznih parametrov električnih pulzov tako zagotovimo kontroliran vnos plazmidne DNA v kožo, ki ga lahko prilagajamo glede na namen terapije in terapevtsko delovanje transgena (Kos et al., 2016).

Naslednji parameter genskega elektroprenosa, ki prav tako vpliva na nadzorovan in ciljan vnos genov v kožo, je uporaba plazmidne DNA pod kontrolo kožno-specifičnega promotorja. V sklopu doktorske naloge smo uporabili kolagenski promotor COL, ki je pod nadzorom transkripcijskih faktorjev kolagena tipa I. Kožno specifičnost plazmidne DNA s promotorjem COL smo dokazali tako *in vitro* s transfekcijo fibroblastov kot *in vivo* na mišjem modelu (Kos et al., 2015a). Uporaba promotorja COL zmanjša učinkovitost transfekcije v primerjavi z konstitutivnim promotorjem, vendar vodi do specifične transfekcije kožnih celic z lokalnim zadrževanjem transgena v koži. Vnos plazmidne DNA pod kontrolo promotorja COL je tako zaželen v primerih, ko želimo zamejiti izražanje transgena v koži in s tem preprečiti sistemsko toksičnost ali zmanjšati toksični vpliv transgena na sosednja tkiva.

Prvi sklop doktorske naloge smo tako zaključili z optimizirano metode genskega elektroprenosa in s tremi novostmi na področju vnosa genov s ciljano transfekcijo v kožo: (1) vpeljavo nove elektrode MEA, (2) dokazano specifičnostjo promotorja COL za kožne celice ter (3) možnostjo kontroliranega vnosa s prilagajanjem električnih pulzov. Optimizirani protokoli tako zagotovijo varno, učinkovito, ciljano in kontrolirano uporabo metode za terapevtske namene. V drugem sklopu doktorske naloge smo uporabili tako optimizirano metodo za ugotavljanje protitumorskega delovanja peritumoralne genske terapije za različne tumorske modele.

Protitumorski učinek peritumoralne genske terapije

Za namen protitumorske genske terapije s ciljano dostavo plazmidne DNA v kožo je med citokini zlasti zanimiv IL12, ki kaže velik potencial za zdravljenje malignega melanoma (Daud et al., 2008), poleg tega pa izkazuje protitumorsko delovanje na sarkomskem tumorskem modelu po intradermalnem injiciranju v peritumoralno področje (Pavlin et al., 2009). Zlasti peritumoralno območje je zanimivo z vidika imunoterapije, saj lahko s ciljanjem in transfekcijo zdravih kožnih celic ob tumorju omogočimo prepoznavo ter uničenje tumorskih celic (Sersa et al., 2015).

V sklopu doktorske naloge smo protitumorski učinek peritumoralne genske terapije s ciljano dostavo v kožo sledili na dveh mišjih tumorskih modelih; melanomskem (B16F10) in sarkomskem (LPB). V obeh primerih smo kot terapevtski gen vnesli plazmidno DNA z zapisom za mišji IL12, pri čemer smo

uporabili optimizirano metodo genskega elektroprenosa. Tako na melanomskem kot na sarkomskem modelu se učinek terapije kaže z zaostankom v rasti tretiranih tumorjev ter statistično značilnim podaljšanim preživetjem miši v terapevtskih skupinah. Največji učinek peritumoralne terapije je bil dosežen pri vnosu plazmidne DNA z višjo koncentracijo ali pri skupinah s ponovljeno - večkratno terapijo. Poleg učinka na primarne tumorje, je peritumoralna genska terapija z vnosom gena za IL12 izkazala tudi sistemski učinek na oddaljnene ne-tretirane tumorje. Tako smo z zdravljenjem primarnih tumorjev dosegli zaostanek v rasti tudi na kontralateralnih ne-tretiranih tumorjih. Sistemsko delovanje terapije se kaže tako na melanomskem kot tudi na sarkomskem modelu, pri katerem smo dosegli statistično značilen zaostanek v rasti kontralateralnih tumorjev.

Kljub zaostanku v rasti in dodatnemu sistemskemu učinku peritumoralne terapije z IL12, v nobenem primeru nismo dosegli popolne ozdravitve tumorjev. S tem potrjujemo hipotezo že obstoječe literature (Calvet and Mir, 2016; Sersa et al., 2015), ki predpostavlja, da peritumoralna genska terapija z vnosom citokinov ni optimalna kot samostojna terapija, ampak je smiselna v kombinaciji z ablativnimi tumorskimi tehnikami. Predlagana kombinacija je intratumoralna elektrokemoterapija (ECT) v kombinaciji s peritumoralnim genskim elektroprenosom z vnosom IL12. V predpostavljeni kombinaciji terapij bi z ECT uničili tumorske celice, izražanje IL12 v okoliških nepoškodovanih celicah pa bi omogočilo prepoznavo sproščenih antigenov ter posledično uničenje tumorja (Sersa et al., 2015). Peritumoralno izražanje IL12 bi prispevalo k dodatnemu sistemskemu učinku na oddaljene metastaze.

Uporaba optimizirane metode genskega elektroprenosa za vnos DNA vakcin in celjenje ran

V sklopu doktorske naloge smo se osredotočili na uporabo optimizirane metode za namen genske terapije raka. Poleg onkologije uporaba optimizirane metode veliko obeta tudi na področju vakcinacije in genske terapije celjenja ran. Pokazali smo, da lahko z uporabo več-točkovnih elektrod in optimiziranih parametrov v kožo uspešno vnesemo plazmidno DNA z zapisom za modelno vakcino ovalbumin. Uporaba optimizirane metode zagotovi močno aktivacijo humoralnega in celičnega imunskega odziva, usmerjenega proti genskemu produktu DNA vakcine, hkrati pa vliva na vzpostavitev dolgotrajnejšega imunskega spomina. Možnost nadzorovanega lokalnega izražanja plazmidne DNA v koži ima poleg vakcinacije velik pomen tudi v terapiji celjenja ran. Genski elektroprenos omogoča vnos plazmidne DNA z zapisom za molekule, ki s svojim delovanjem pospešijo celjenje ran. Eden takšnih plazmidov je tudi hCAP-18/LL-37, ki smo ga uporabili v sklopu doktorskega dela. Dokazali smo, da z uporabo optimiziranih parametrov in ne-invazivne elektrode lahko zagotovimo lokalno izražanje transgena in pospešeno celjenje ran v mišji koži. Uporaba

optimizirane elektrode je tako zanimiva z vidika celjenja ran, predvsem kroničnih ran, pri katerih obstoječe zdravljenje pogosto ni učinkovito.

Doprinos k znanosti in vpogled v prihodnje študije

Z razvojem varne, učinkovite in ciljane metode genskega elektroprenosa in potrditvijo protitumorskega delovanja tako optimizirane metode na primarnih in oddaljenih tumorjih lahko potrdimo vse tri hipoteze, predlagane v uvodnem delu. Novost doktorske naloge je obravnava kože kot možnega tarčnega tkiva za vnos genov, uporaba novih ne-invazivnih elektrod in ovrednotenje glavnih električnih parametrov genskega elektroprenosa, s katerimi lahko dosežemo ciljano in nadzorovano transfekcijo v kožo. Z naštetimi novostmi doktorska naloga prispeva k znanju na področju genske terapije, olajšajo pripravo protokolov genskega elektroprenosa ter pripomore k lažjemu in hitrejšemu prenosu metode v klinično prakso. Optimizirana metoda genskega elektroprenosa s protitumorskim delovanjem na različnih mišjih tumorskih modelih odpira vrata za nadaljnje študije, predvsem pri iskanju kombiniranih terapij. Rezultat doktorske naloge predstavljajo izziv za nadaljnje študije v veterinarski in humani onkologiji in podajajo alternativno možnost klasičnim pristopom zdravljenja raka, kot so kirurgija, radioterapija in sistemsko zdravljenje (citostatiki, hormonsko zdravljenje, biološka zdravila).

ZAKLJUČKI

Najpomembnejše sklepe doktorskega dela lahko strnemo v naslednje zaključke:

- Nova več-točkovna (MEA) elektroda je varnejša in bolj učinkovita za vnos plazmidne DNA v kožo v primerjavi s »klasičnimi« ploščatimi elektrodami.
- Z uporabo kolagenskega tkivno-specifičnega promotorja dosežemo ciljano in nadzorovano gensko terapijo v kožo.
- S spreminjanjem parametrov električnih pulzov vplivamo na transfekcijo različnih plasti kože, s čimer prilagajamo trajanje izražanja genskega produkta ter lokalno ali sistemsko delovanje genskega vnosa.
- Optimizirana metoda intradermalnega vnosa plazmidne DNA v peritumorskem področju je učinkovit in varen način zdravljenja kožnih tumorjev in zasevkov.
- Peritumoralna terapija s ciljanim vnosom plazmidne DNA v kožo predstavlja pomemben korak k nadaljnji uporabi metode na področju onkologije, predvsem v kombinacijah z lokalnimi ablativnimi tehnikami.
- Optimizirana metoda odpira nove možnosti uporabe genskega elektroprenosa s ciljanim vnosom plazmidne DNA v kožo tudi na področju vakcinacije in celjenja ran.

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