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1 **Electroporation does not affect human dermal fibroblast proliferation and migration**  
2 **properties directly but indirectly via the secretome**

3

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6

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13

14 **Keywords**

15 Electrochemotherapy, gene electrotransfer, wound healing, scratch wound, conditioned  
16 medium, bystander effect

17

18

19

20

## 1 **Abstract**

2 Aesthetic wound healing is often experienced by patients after electrochemotherapy. We  
3 hypothesized that pulsed electric fields applied during electrochemotherapy (ECT) or gene  
4 electrotransfer (GET) protocols could stimulate proliferation and migration of human cutaneous  
5 cells, as described in protocols for electrostimulation of wound healing. We used  
6 videomicroscopy to monitor and quantify in real time primary human dermal fibroblast  
7 behavior when exposed *in vitro* to ECT and GET electric parameters, in terms of survival,  
8 proliferation and migration in a calibrated scratch wound assay. Distinct electric field intensities  
9 were applied to allow gradient in cell electropermeabilization while maintaining reversible  
10 permeabilization conditions, in order to mimic *in vivo* heterogeneous electric field distribution  
11 of complex tissues. Neither galvanotaxis nor statistical modification of fibroblast migration  
12 were observed in a calibrated scratch wound assay after application of ECT and GET  
13 parameters. The only effect on proliferation was observed under the strongest GET conditions,  
14 which drastically reduced the number of fibroblasts through induction of mitochondrial stress  
15 and apoptosis. Finally, we found that 24h-conditioned cell culture medium by electrically  
16 stressed fibroblasts tended to increase the migration properties of cells that were not exposed to  
17 electric field. RT-qPCR array indicated that several growth factor transcripts were strongly  
18 modified after electroporation.

19

## 1 **Introduction**

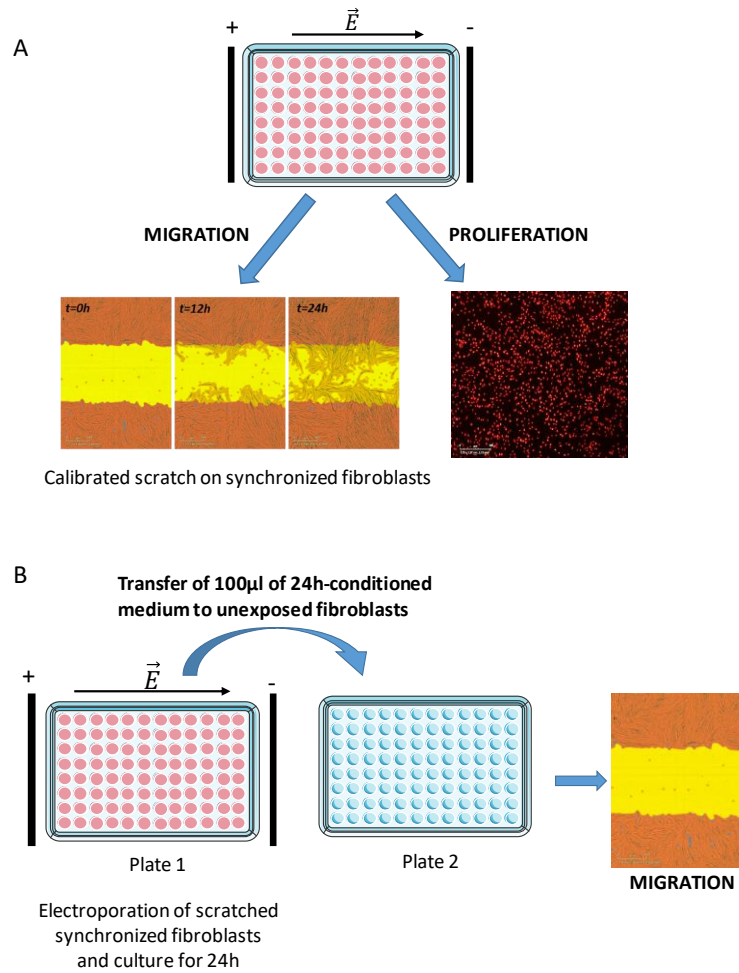
2       When a cell is exposed to an external pulsed electric field, the plasma membrane transiently  
3 becomes permeable for ions, hydrophilic drugs and molecules as large as plasmid DNA, which  
4 are otherwise unable to penetrate. This spatially and temporally localized physical phenomenon  
5 of cell electroporation is named “electroporation” [1,2]. Electroporation has many  
6 medical applications [3]. Among them, electrochemotherapy (ECT) [4,5] has reached an  
7 established position in local cancer treatment and is the main clinical use of electroporation in  
8 human and veterinary medicine. It consists in combining the injection of poorly permeant  
9 antitumor drugs, mainly bleomycin or cisplatin, with local application of calibrated electric  
10 field pulses to the tumor site. Thus, transient plasma membrane electroporation allows  
11 massive penetration of the cytotoxic drugs within the cell, potentiating its antitumor activities.  
12 Standardized protocols for ECT treatments have been published in the framework of the  
13 European Standard Operating Procedure on Electrochemotherapy (ESOPE) multicenter trial  
14 [6]. Interestingly, clinicians commonly observed aesthetic and functional wound healing of the  
15 tumor sites treated with electrochemotherapy [7–9]. This clinical outcome of ECT was  
16 confirmed by the patients themselves [10], as self-compiled questionnaires underlined an  
17 improvement in wound healing, bleeding, aesthetic impairment, and also in activities of daily  
18 life and social relations.

19       Another medical application of electroporation is gene electrotransfer (GET). Gene  
20 electrotransfer of plasmid encoding different growth factors is highly promising in cutaneous  
21 wound healing [11]. In an *in vivo* wound model, GET of plasmids encoding for example KGF-  
22 1 (keratinocyte growth factor) [12], VEGF (Vascular endothelial growth factor) [13], bFGF  
23 (fibroblast growth factor) [14] or antimicrobial peptide hCAP-18/LL-37 [15] led to an increased  
24 re-epithelialization rate of the wound, collagen synthesis, and angiogenesis. Other studies  
25 demonstrated a tendency [16] to a clear [17] beneficial effect of electroporation alone on wound

1 closure. While standard protocols for acute and chronic wound treatments are limited to  
2 antibiotic therapy and wound dressing, innovative therapies like electrostimulation have  
3 emerged as alternatives to conventional treatments [18]. Electrostimulation is based on a natural  
4 electric process that occurs after wounding. In an intact epidermis, there is an active segregation  
5 of Na<sup>+</sup> ions owing to the Na<sup>+</sup>/K<sup>+</sup>ATPase action and the presence of ENac channels, leading to  
6 a sodium gradient from the basal layer of the epidermis to the upper layer [19]. This process  
7 creates a transepithelial potential (TEP) within the epidermis. Depending on the body part, this  
8 TEP ranges from -10 to -50mV, while its average potential over all sites is -23 mV [20]. After  
9 skin damaging, the relocalization of these Na<sup>+</sup> ions leads to the creation of an endogenous local  
10 electric field of 100 to 200 mV/mm at the wound margins [21,22]. This field plays a major role  
11 in wound healing process, particularly through the activation of migration and proliferation of  
12 cutaneous cells, as well as the promotion of angiogenesis [23,24].

13 In this study, we hypothesized that ECT and GET electric parameters could stimulate  
14 cutaneous cell migration and proliferation and thus improve the quality of healing after ECT or  
15 GET treatment. To investigate this hypothesis, we worked with primary human dermal  
16 fibroblasts grown in monolayer. Firstly, classical hallmarks of cell electropermeabilization,  
17 viability and death were assessed after exposition to ECT and GET electric parameters.  
18 Secondly, we used videomicroscopy to monitor and quantify in real time cell proliferation and  
19 cell migration in a calibrated scratch wound assay, two properties that play a major role in  
20 cutaneous wound healing process. Because increases in electric conductivity due to  
21 electroporation and tissue heterogeneity (cell shape, extracellular matrix...) induce non-  
22 uniformity in the electric field distribution within the skin during electroporation, cells located  
23 in between the electrodes do not all experience the same field strength *in vivo* [25,26]. For that  
24 reason, in this study we decided to scan a wide range of electrical intensity, inducing various  
25 degrees of reversible cell electropermeabilization, and check dermal fibroblast proliferation and

1 migration properties. Finally, the indirect effect of electroporation on cell migration was  
 2 investigated by cultivating unexposed dermal fibroblasts in 24h-conditioned cell culture  
 3 medium. An overview of the experimental procedure is presented in schematic 1.



4

5 **Schematic 1: Overview of the experimental plan to test direct (A) or indirect (B) effects of**  
 6 **human dermal fibroblast electroporation on cell behaviour.** A. Migration and proliferation  
 7 properties of fibroblasts submitted to electroporation are followed by videomicroscopy. B. Assessment  
 8 of indirect effect of electroporation through the secretome. For these experiments, fibroblasts that were  
 9 not exposed to electric field are cultivated in cell culture medium conditioned for 24h by  
 10 electroporated fibroblasts. Migration capabilities are then followed by videomicroscopy

## 1 **Material and methods**

2 **Human cutaneous primary cell isolation and cell culture.** Primary dermal fibroblasts were  
3 isolated from 3-year old foreskin commercially bought (Icelltis) after posthectomy as  
4 previously described [27–29]. Cells were tested negative for mycoplasma using MycoAlert  
5 mycoplasma detection kit (Lonza) throughout the experiments. Dermal fibroblasts were grown  
6 in Dulbecco's Modified Eagles Medium (Gibco-Invitrogen) containing 4.5 g.L<sup>-1</sup> glucose,  
7 Glutamax, and 1mM pyruvate, supplemented with 10 % (v/v) of heat inactivated fetal calf  
8 serum, 100 U/ml penicillin, and 100 µg.mL<sup>-1</sup> streptomycin. Cells were maintained at 37°C in a  
9 humidified atmosphere containing 5 % CO<sub>2</sub> and cell culture media were changed three times a  
10 week.

11  
12 **Electroporation application to cell monolayers.** Cells were grown either on gelatin-coated  
13 round 1cm glass coverslip [30] or in 96-well plate 24h prior experiments. Cell culture medium  
14 was removed from the wells and replaced by 100 µL or 50 µL of pulsing buffer (10 mM  
15 K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, 250 mM sucrose, and 1 mM MgCl<sub>2</sub> in sterile water, pH 7.4) [31] respectively  
16 for glass coverslip or 96-well. Two stainless, flat, parallel electrodes, 1 cm interval (Megastil,  
17 Ljubljana, Slovenia) for round coverslip or 0.35 cm interval (Megastil, Ljubljana, Slovenia) for  
18 96-well plates were applied to the bottom of the well. Defined electric field for  
19 electrochemotherapy condition (ECT) (8 square-wave pulses of 100 µs, 1 Hz, ranging from 200  
20 to 800 V/cm) and gene electrotransfer (GET) (10 square-wave pulses of 5 ms, 1 Hz, ranging  
21 from 50 to 300 V/cm) was delivered at room temperature by Electro cell S20 generator  
22 (LeroyBiotech, Saint Orens de Gameville, France). An electric field intensity range was applied  
23 in experiments in order to better mimic the heterogeneous distribution of electric field that  
24 occurs within vascularized and rich in extracellular matrix tissue such as skin when submitted  
25 to electroporation. After electric field application, pulsing buffer was aspired and 200 µL of

1 fresh cell culture medium were added before cells were placed in a humidified atmosphere at  
2 37°C containing 5% CO<sub>2</sub> until analysis.

3

4 **Determination of cell electropermeabilization.** Plasma membrane electropermeabilization  
5 was first assessed through quantification by luminescence of ATP leakage within extracellular  
6 medium after cells were submitted to electroporation. For that purpose, cells were grown in 96-  
7 well plate and pulsed in 50 µL of pulsation buffer. 10 minutes after electric field application,  
8 40 µL of supernatant was place in white-96-well plate and topped up with 40µl of CellTiterGlo  
9 reagent (Promega), incubated for 5 minutes at room temperature and luminescence signal was  
10 read on plate reader (Clariostar, BMGLabtech). Secondly, plasma membrane  
11 electropermeabilization was assessed by flow cytometry using propidium iodide penetration.  
12 Propidium iodide is a non-permeant probe whose fluorescent intensity increases upon non-  
13 covalent binding to nucleic acids. 100 µM propidium iodide was added to pulsing buffer during  
14 application of electric fields on cells grown on round coverslip. Cells were grown on a 1cm  
15 coverslip in order to be submitted to electric field with 1cm plate electrodes. Immediately  
16 afterwards, the cells were washed twice with 300 µL of PBS before being trypsinized,  
17 centrifuged and suspended in PBS, placed on ice and analyzed by flow cytometry  
18 (FACSCalibur cytometer, BD Bioscience). Finally, to discriminate between reversible and  
19 irreversible electroporation, 30 minutes after electroporation cells were removed from the  
20 incubator and were incubated for 5 minutes with 100µM propidium iodide, washed, trypsinized  
21 and analyzed by flow cytometry.

22

23 **Cell viability and death measurement.** A clonogenic assay was used to finely determine cell  
24 viability after application of electric field. Briefly, cells grown on round coverslip were



1 submitted to electric field in 100 $\mu$ L of pulsing buffer. After replacing it by complete culture  
2 medium, cells were incubated for 1h at 37°C to recover. After this time, cells were trypsinized,  
3 counted and 250 cells were plated into 6-well plates and placed into the incubator. 7 days after  
4 seeding, cells were washed and colored with crystal violet in order to visualize and manually  
5 count cell colonies. The percentage of dead cells through apoptosis was quantified by  
6 videomicroscopy using the IncuCyte® Caspase-3/7 Green Apoptosis Assay Reagent  
7 (Sartorius), coupled with the Nuclight Red reagent (Sartorius) to determine the total number of  
8 cells according to the manufacturer protocol. Briefly, after application of electroporation, cells  
9 were grown in culture medium containing a permeable viable DNA stain named Nuclight Red  
10 Reagent (1/1000) and a caspase-3/7 recognition motif DEVD that labels cells undergoing  
11 apoptosis, i.e Caspase-3/7 green reagent (1/1000). Pictures were obtained at 10x with an  
12 IncuCyte ZOOM live cell analysis system (Sartorius), and analyzed by the MARS- associated  
13 software.

14

15 **Cell synchronization.** In order to assess only migration during the first 24h of the scratch  
16 wound assay, proliferation had to be inhibited. For this purpose, the overnight serum  
17 deprivation approach [32] was used as a biological way to synchronize primary dermal  
18 fibroblasts prior to scratch wound assay. Distribution of cells within cell cycle was checked by  
19 flow cytometry before the scratch experiments. Briefly, after being trypsinized and centrifuged,  
20 cells were suspended in PBS containing 5  $\mu\text{g.mL}^{-1}$  propidium iodide, 0.1% X100-Triton and  
21 2.5% fetal bovine serum. Cells were incubated for 20 min at room temperature in the dark  
22 before flow cytometry analysis on a FACSCalibur cytometer (BD Bioscience). Cell cycle  
23 distribution was analyzed using Modfit software.

24

1 **Calibrated scratch assay by videomicroscopy.** In order to perform the scratch wound  
2 experiment, cells were grown at post-confluency so that when the scratch was applied, a clear  
3 and calibrated wound remained (700-800  $\mu\text{m}$  wide). For this, 25 000 dermal fibroblasts were  
4 grown into ImageLock 96-well microplates (Sartorius). After 8 hours of culture, complete  
5 culture medium was removed and replaced by cell culture medium without fetal bovine serum  
6 in order to synchronize cells by serum deprivation overnight. The day of the experiment, a tool  
7 named WoundMaker (Sartorius) which is a 96-pin mechanical device designed to create  
8 homogeneous scratch wounds in cell monolayers was used. Floating scraped cells were  
9 removed and electroporation was performed in 50  $\mu\text{L}$  pulsing buffer. Attention had been paid  
10 to placing electrodes parallel to the scratch wound, the cathode and anode still at the same side  
11 of the wound. After replacing pulsing buffer by 200  $\mu\text{L}$  of complete cell culture medium, plates  
12 were then placed in IncuCyte ZOOM live cell analysis system (Sartorius) and pictures were  
13 taken at 10x every hour for 24h at least, and analyzed with the MARS- associated software,  
14 with the module dedicated to scratch wound assay. For galvanotaxis or electrotaxis  
15 measurements, the area colonized by fibroblasts in each wound side was quantified on Image J  
16 software (NIH) between the same picture at the moment of the scratch and 12h after. For  
17 experiments with conditioned media, 100  $\mu\text{L}$  of conditioned cell culture medium were  
18 harvested 24h after electric field application on synchronized dermal fibroblast wounded  
19 monolayer and immediately placed onto a freshly synchronized dermal fibroblast wounded  
20 monolayer. Cell migration within the wound that had not been submitted to an electric field  
21 was quantified by videomicroscopy for 24h.

22

23 **Cell proliferation quantification by videomicroscopy.** In order to allow cell proliferation to  
24 occur over several days, 5 000 dermal fibroblasts were plated in wells of 96-well plates, a  
25 density which approximately corresponds to a confluence of 30%. After being submitted to

1 electric field in pulsing buffer, cells were grown in 200  $\mu$ L of complete culture medium  
2 supplemented with 1/1000 Nuclight Red reagent (Sartorius) and placed within an IncuCyte  
3 ZOOM live cell analysis system (Sartorius). As a permeant DNA intercalant, it allows to clearly  
4 observe and quantify the number of nuclei over time. Pictures were taken at 10x every hour for  
5 at least 72h and analyzed with the MARS-associated software. Data obtained corresponded to  
6 the number of nuclei within the field of observation of the microscope.

7

8 **Mitochondrial characterization.** Mitochondrial membrane potential was assessed using  
9 MitoView 633 (Biotium) ( $\lambda_{ex} = 622\text{nm}$ ;  $\lambda_{em} = 648\text{nm}$ ) which is a mitochondrial membrane  
10 potential-sensitive, fluorogenic dye that rapidly accumulates in mitochondria. Staining is  
11 dependent on mitochondrial membrane potential and is lost when mitochondria become  
12 depolarized [33]. Cells were labeled according to the manufacturer protocol. Briefly, cells  
13 grown in monolayer in 96-well plate were washed with PBS and then incubated for 20 minutes  
14 in the dark in a humidified atmosphere at 37°C containing 5% CO<sub>2</sub> with 50nM MitoView 633  
15 in cell culture medium. Once charged with MitoView 633, cells were submitted to GET  
16 electroporation and 1h after treatment fluorescence was measured within Incucyte  
17 videomicroscope and analyzed with the MARS-associated software. Mitochondrial oxidative  
18 stress was assessed through MitoSOX Red Mitochondrial SuperOxide indicator (ThermoFisher  
19 scientific) which is a fluorogenic dye for a selective detection of superoxide in the mitochondria  
20 of living cells [34]. Once in the mitochondria, this non-fluorescent reagent is oxidized by  
21 mitochondrial superoxide when mitochondrial oxidative stress occurs to exhibit red  
22 fluorescence [35]. Cells were incubated in a 5 $\mu$ M of MitoSOX reagent solution and incubated  
23 10 minutes at 37°C protected from light. MitoSOX fluorescence ( $\lambda_{ex} = 510\text{nm}$ ;  $\lambda_{em} = 580\text{nm}$ )  
24 was observed by videomicroscopy on Incucyte for 12h after electroporation treatment. Pictures  
25 were analyzed with the MARS-associated software.

1

2 **Genes expression analysis by PCR array.** Three conditions were analyzed 4h after electric  
3 field application: control condition (n=3), ECT condition at 600V/cm (n=3) and GET condition  
4 at 200V/cm (n=2). Total RNAs of adherent dermal fibroblasts grown on 6 glass coverslips for  
5 each condition were isolated using RNeasy Micro Kit (Qiagen) strictly according to the  
6 manufacturer's instruction. The quality and quantity of isolated RNA were checked on a  
7 Nanodrop 2000 (ThermoFisher) and RNAs were then stocked at -80°C until rapid use. Reverse  
8 transcription was performed on 500ng of RNAs with the RT<sup>2</sup> HT First Strand Kit (Qiagen)  
9 according to the manufacturer's protocol. It allows an efficient first-strand cDNA synthesis and  
10 genomic DNA elimination in RNA samples. The Human Wound Healing RT<sup>2</sup> Profiler PCR  
11 Array profiles the expression of 84 key genes central to the wound healing response. For this  
12 study, we focused on growth factors sub-panel. Obtained cDNAs were directly subjected to  
13 qPCR using RT<sup>2</sup> SYBR Green qPCR Mastermix (Qiagen) and RT<sup>2</sup> Profiler PCR Array Human  
14 Wound Healing kit (Qiagen #PAHS-121Z) strictly according to the manufacturer's protocol.  
15 96-well plate was processed into a CFX96 CFX Real-Time PCR Detection System (BioRad).  
16 Data were statistically analyzed with the associated-excel datasheet proposed by Qiagen for this  
17 gene panel. Actin, Beta-2-microglobulin, GAPDH, Hypoxanthine phosphoribosyltransferase 1  
18 (HPRT1), ribosomal large protein P0 (RPLP0) transcripts were used for normalization.  
19 HeatMap was carried out on GraphPad Prism 8 to represent significantly up and down regulated  
20 transcripts.

21

22 **Growth factor quantification by ELISA.** Supernatants conditioned for 24h by dermal  
23 fibroblasts submitted to electroporation with ECT (600V/cm) and GET (200V/cm) electric  
24 parameters were stocked at -80°C until use, within a month. Vascular Endothelial Growth  
25 Factor A (VEGFA) was quantified in these supernatants using Human VEGF-A ELISA Kit

1 (Sigma # RAB0507, Merck KGaA, Darmstadt, Germany) according to manufacturer's  
2 protocol. Absorbance was read using a Synergy H1 (Biotek, Winooski, VT, USA) hybrid Multi-  
3 Mode Reader. Non-parametric Mann Whitney test was used to determine statistical differences  
4 between each Control, ECT, GET condition (n=5).

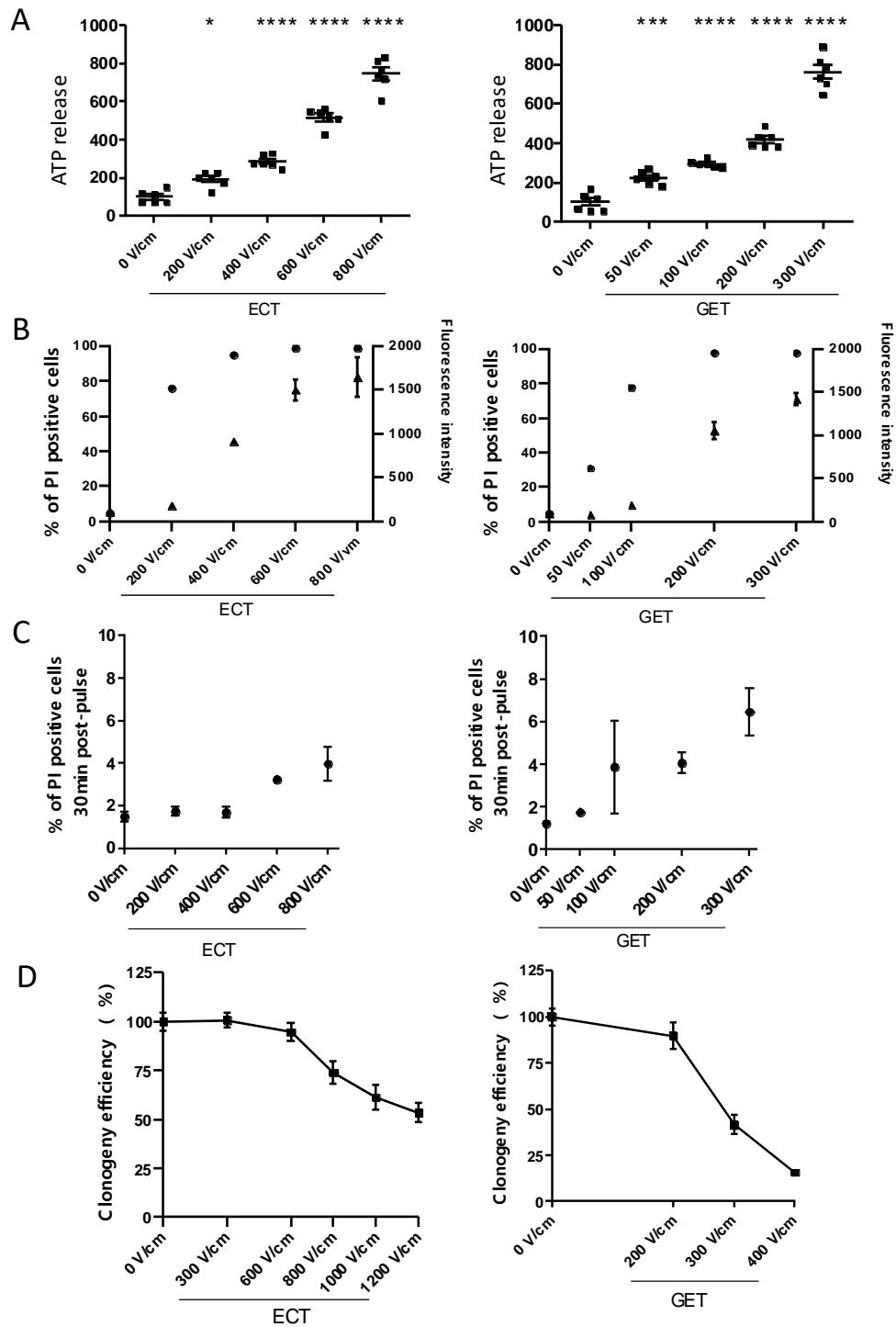
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6 **Statistical analyses.** Data analysis was performed using GraphPad Prism 8 program (GraphPad  
7 Software, Inc., La Jolla, CA, USA) and data were expressed as mean  $\pm$  SEM (standard error to  
8 the mean). Number of experiments are indicated in figure legends, where N indicates the  
9 number of independent experiments and n the total number of biological replicates. Multiple  
10 comparisons were performed using one-way analysis of variance (ANOVA) followed by  
11 Dunnett's post-test to compare every mean to the control (0V/cm) mean or two-way ANOVA  
12 followed by Bonferroni post-test to compare means within each time point. Statistics are  
13 expressed relative to the control condition (0V/cm). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 and  
14 \*\*\*\*p < 0.0001.

## 1 **Results**

2 **Distinct electric field intensities allow gradient of electroporation of dermal**  
3 **fibroblasts.** When electroporation is applied to skin tissue, the electric field distribution is  
4 heterogeneous, meaning that all the cells within the tissue do not experience the same electric  
5 field [26]. Therefore, one of the aims of this study was to submit dermal cells to distinct electric  
6 field intensities. For ECT condition, we applied a classical calibrated sequence of electric pulses  
7 (i.e 8 pulses lasting 100 $\mu$ s at a frequency of 1Hz) with increasing intensity, from 200V/cm to  
8 800V/cm. For GET condition, 10 pulses lasting 5ms were applied at 1Hz frequency, with  
9 intensities ranging from 50V/cm to 300V/cm. By increasing the electric field intensity both for  
10 ECT and GET conditions, a gradual but significant increase in leakage of ATP was measured  
11 in the extracellular medium as soon as 10 minutes after electric field application (Figure 1A).  
12 To confirm that the observed ATP leakage was due to plasma membrane defaults caused by  
13 electroporation, a complementary experiment was performed by flow cytometry to quantify  
14 propidium iodide penetration within cells (Figure 1B). Both the percentage of  
15 electroporated cells as well as the fluorescence intensity of these cells increased when  
16 increasing the electric field intensity in ECT and GET conditions. 100% of  
17 electroporated cells were reached at 600V/cm in ECT condition and 200V/cm in GET  
18 condition. In order to be sure that the electric parameters applied induced solely a reversible  
19 and not an irreversible permeabilization, a plasma membrane resealing experiment was carried  
20 out. 30 minutes after electroporation, cells were incubated with 100 $\mu$ M propidium iodide and  
21 fluorescence signal was quantified by flow cytometry (Figure 1C). Less than 5% of cells were  
22 labelled with propidium iodide, regardless of the electric field intensity applied in ECT and  
23 GET condition. This means that the applied electric parameters did not induce irreversible  
24 permeabilization. The long-term cell viability after exposition to electroporation was assessed  
25 by clonogenicity (Figure 1D). In the ECT condition, cell viability was not affected up to 600V/cm

1 but an approximately 25% loss of viability was observed at 800V/cm. The GET condition  
 2 appeared to be more severe. Below 200V/cm cell viability was not affected but it fell to 50% at  
 3 300V/cm. It seems that even if irreversible electroporation was not induced by the chosen  
 4 electric parameters, they still affected the long-term cell viability.



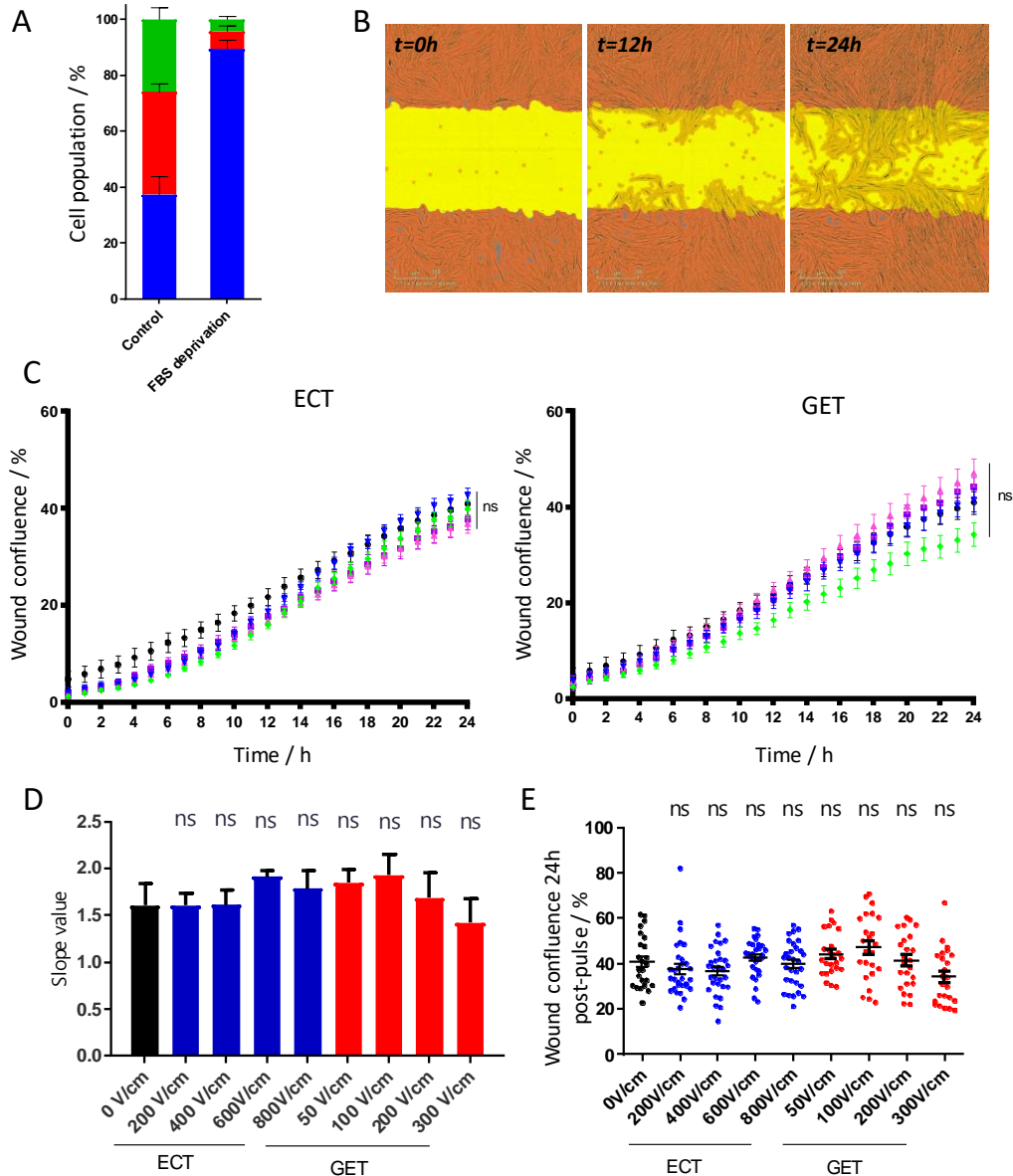
5

1 **Figure 1: Determination of electric field intensity (V/cm) ensuring efficient transient cell**  
2 **electropermeabilization without affecting cell viability.** A. Quantification of ATP release 10 min after  
3 the application of ECT and GET electrical parameters, expressed as % of control. N=2; n=6. Statistical  
4 analysis by One-way ANOVA \*=p<.05; \*\*\*=p<.001; \*\*\*\*=p<.0001. B. Monitoring of cell  
5 electropermeabilization immediately after the application of ECT and GET electrical parameters. Left  
6 axis: % of propidium iodide (PI) positive cells (dot ●); Right axis: fluorescence intensity (triangle ▲).  
7 n=3. C. Monitoring of plasma membrane resealing 30min after the application of ECT and GET  
8 electrical parameters. n=3. D. Long term cell viability after the application of ECT and GET electrical  
9 parameters, N=3; n=9. Data are represented as the mean value ± SEM. ECT: electrochemotherapy; GET:  
10 gene electrotransfer.  
11

12 **Migratory properties of human primary dermal fibroblasts are not statistically affected**  
13 **by the application of ECT and GET electric parameters.** In scratch wound assay, cells  
14 migrate and proliferate to fill the gap, thus, it is classically desired to suppress proliferation  
15 component in order to measure only cell migration during the first 24h [36]. The *in vitro*  
16 synchronization of dermal fibroblasts was achieved using overnight serum deprivation, a non-  
17 pharmaceutical method. Cellular DNA content was determined by staining cells with propidium  
18 iodide and measuring fluorescence by flow cytometry. The proportion of cells in the different  
19 phases of cell cycle were calculated depending on the cell's DNA content (n): G1 (2n), S  
20 (between 2n and 4n), and G2 (4n). As shown in Fig 2A, primary dermal fibroblasts were highly  
21 responsive to this method since approximately 90% of the cells were synchronized in the  
22 growing phase G1 using this method. With its scratch wound module, the IncuCyte ZOOM live  
23 cell analysis system allows to properly measure cell migration in calibrated scratch wound  
24 assay. Wound confluence was determined each hour after electric field application for 24h using  
25 image processing with masks (Figure 2B and Movie 1),. Movie 2 showed that wound closure  
26 by dermal fibroblasts was a highly dynamic process. As previously explained, wound closure  
27 was analyzed only for the first 24h after application of electric field on synchronized cells in  
28 order to take into account solely the migration component. The absence of cell division events  
29 (rounding cell before splitting in two round cells) on the movie during the first 24h confirmed  
30 this assumption. After 24h, cell confluence within the wound was approximately 40%, whatever  
31 the ECT or GET condition (Figure 2C). It is noteworthy that cell migration occurred



1 immediately after wounding, and continued regularly over hours, with or without application  
2 of ECT or GET electric parameters. No statistical difference was observed on the time course  
3 of the migration depending on the electric parameters applied, but a linear regression was  
4 performed to obtain the speed of migration of each condition during the first 12h (Figure 2D).  
5 Even if no statistical difference was observed whatever the conditions were, taking account  
6 standard error to the mean, ECT 600V/cm and 800V/cm as well as GET 50V/cm and 100V/cm  
7 conditions presented a slight increase in speed of migration. Finally, the wound confluence 24h  
8 post-electric field application was finely analyzed (Figure 2E). GET conditions at 50V/cm and  
9 100V/cm seemed to improve dermal fibroblast migration and thus wound healing, but despite  
10 numerous repetitions of the experiments and biological replicates (until 30) no statistical  
11 difference was observed, so it remained a trend.



1

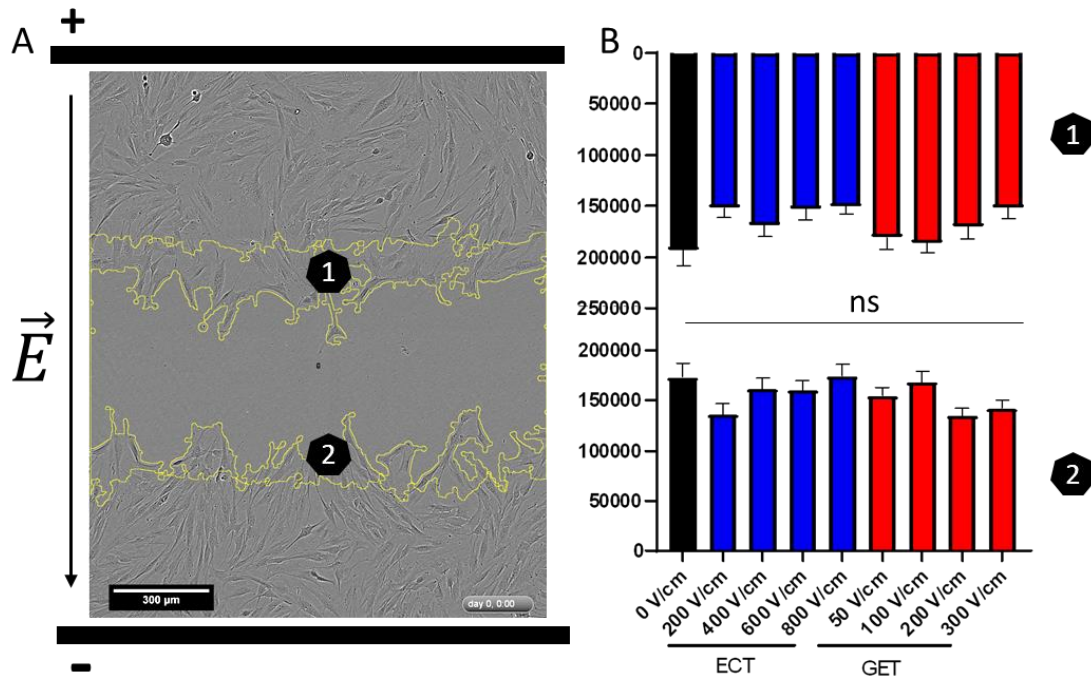
2 **Figure 2: ECT and GET electrical parameters did not affect dermal fibroblast migration**  
 3 **properties in scratch wound assay.** A. Cell cycle synchronization after overnight fetal bovine serum  
 4 (FBS) deprivation. Phases G1 in blue, S in red and G2 in green. N=3; n=8. B. Image processing with  
 5 masks to quantify dermal fibroblasts migration in scratch wound assay. C. Time course of cell migration  
 6 after the application of ECT (x8 pulses lasting 100 $\mu$ s, 1Hz frequency, at 0V/cm (●), 200V/cm (■), 400  
 7 V/cm (▲), 600V/cm (▼), 800V/cm (◆)) and GET (x10 pulses lasting 5ms, 1Hz frequency, at 0V/cm (●),  
 8 50V/cm (■), 100 V/cm (▲), 200V/cm (▼), 300V/cm (◆)) electrical parameters on synchronized  
 9 fibroblasts wounded monolayer. N=5; n= 30 Statistical analyses by Two-Way ANOVA. D. Slope values  
 10 calculated by linear regression during the first 24h. N=3, n=9. E. Wound confluence (%) 24h after the  
 11 application of ECT and GET electrical parameters. N=5; n= 30. Statistical analysis by One-Way  
 12 ANOVA. Data are represented as the mean value  $\pm$  SEM. ECT: electrochemotherapy; GET: gene  
 13 electrotransfer.

14

15 **No galvanotaxis of dermal fibroblasts is induced by ECT and GET electric parameters.**

16 The external electric field is a major physical cue of the microenvironment that guides

1 directional migration. This phenomenon is called galvanotaxis or electrotaxis. Since the plate  
 2 electrodes were systemically placed parallel to the wound, anode on the top and cathode on the  
 3 bottom, we were able to determine if fibroblasts at the margin of the wound displayed  
 4 preferential directed migration (Figure 3). No statistical differences were observed, meaning  
 5 that dermal fibroblasts migrated equally toward the cathode or anode side.

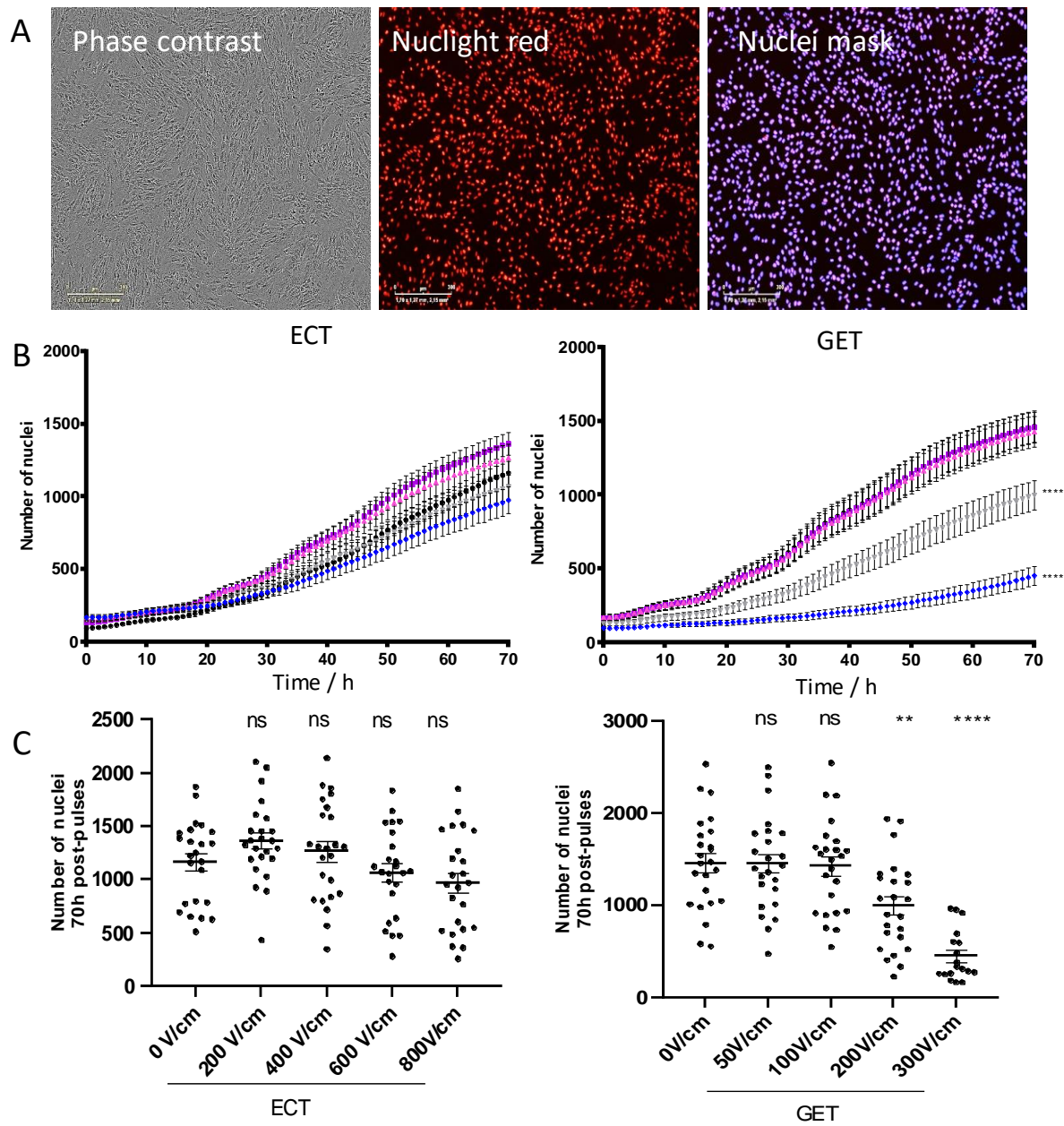


6  
 7 **Figure 3: No electrotaxis was observed in dermal fibroblasts when submitted to ECT and GET**  
 8 **electrical parameters.** A. Schematic representation of electrodes position at the wound site. Yellow  
 9 line delimits cell-occupied area in the anode panel (1) and cathode panel (2). B. Cell-occupied area  
 10 ( $\mu\text{m}^2$ ) either upper panel of the wound (1) or lower panel of the wound (2) 12h after electric field  
 11 application. Data are represented as the mean  $\pm$  SEM. Statistical analysis by One-Way Anova. N=3;  
 12 n=18.

13  
 14 **High GET electric parameters drastically reduce the number of dermal fibroblast .**  
 15 Evolution of cell number over 72h after ECT and GET application was followed by  
 16 videomicroscopy (Movie 3) through automatically counting fluorescently labelled nuclei within  
 17 the microscope field (Figure 4A). ECT electric parameters did not lead to statistical difference  
 18 between the control condition and the distinct electric field intensity applied, even if 200V/cm

1 and 400V/cm growth curves tended to be higher than the control one (0V/cm) (Figure 4B). For  
2 GET conditions, three distinct behaviors had to be differentiated. Cells submitted to electric  
3 fields of 50 and 100V/cm behaved similarly to the control condition. On the other hand, from  
4 200V/cm, the number of nuclei decreased with a significant delay, and this phenomenon was  
5 increased for higher electric field intensity (300V/cm). This observation was confirmed by  
6 analyzing the number of nuclei within the microscope field 70h after electric field application  
7 (Figure 4C). The delay in cell growth can be explained either by a stop in cell proliferation or  
8 by a dynamic equilibrium between dying cells and proliferative cells, as we observed by  
9 clonogenic assay that GET at 300V/cm altered long-term cell viability by 60% (Fig 1D).

1



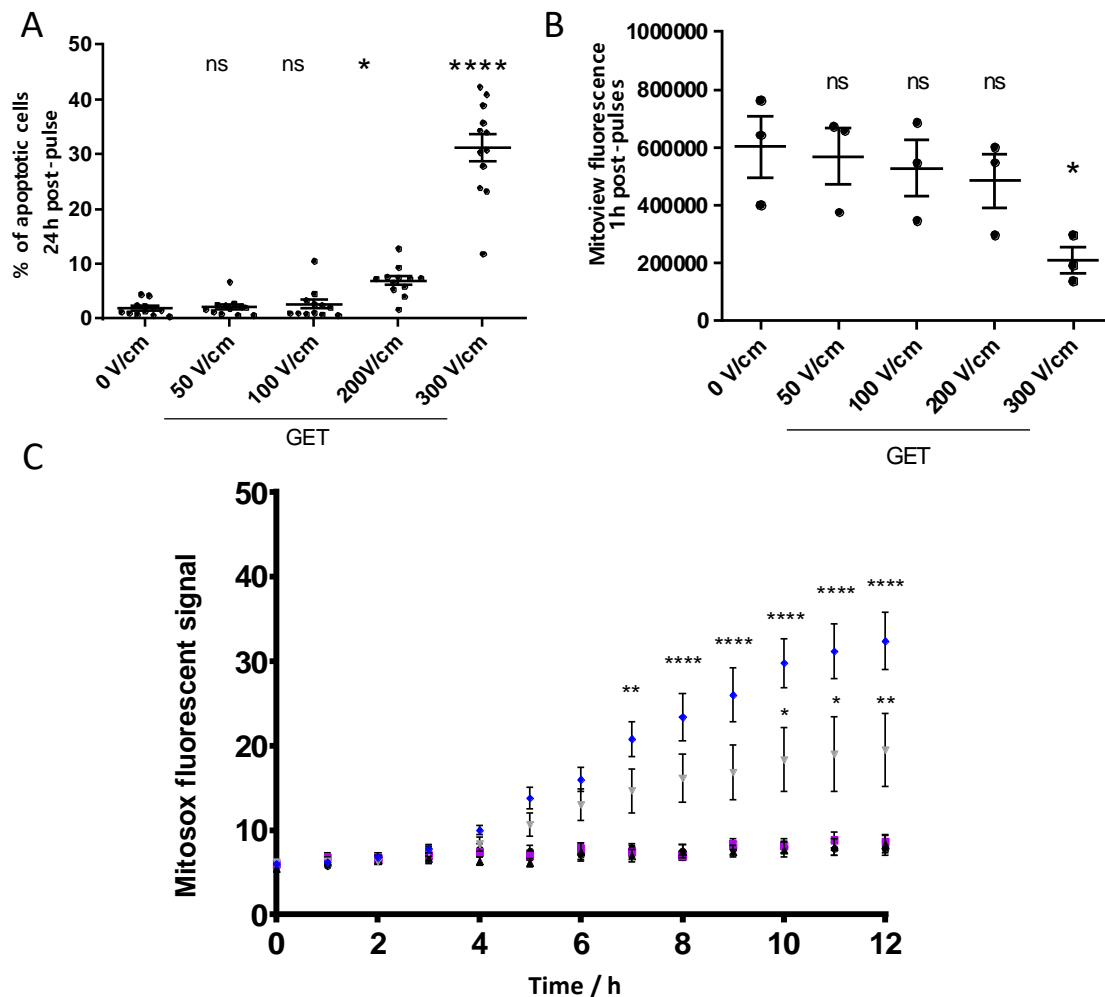
2

3 **Figure 4: ECT electrical parameters did not affect dermal fibroblast proliferation while some**  
 4 **GET parameters reduced cell number.** A. Image processing with masks to quantify dermal fibroblasts  
 5 proliferation. B. Dermal fibroblasts proliferation after application of ECT (x8 pulses lasting 100 $\mu$ s, 1Hz  
 6 frequency, at 0V/cm (●), 200V/cm (■), 400 V/cm (▲), 600V/cm (▼), 800V/cm (◆)) and GET (x10  
 7 pulses lasting 5ms, 1Hz frequency, at 0V/cm (●), 50V/cm (■), 100 V/cm (▲), 200V/cm (▼), 300V/cm  
 8 (◆)) electrical parameters. Statistical analysis by Two-Way ANOVA \*\*\*\*= $p < .0001$  C. Number of  
 9 nuclei 70h after the application of ECT and GET electrical parameters. N=6; n=24. Statistical analysis  
 10 by One-Way ANOVA \*= $p < .05$ ; \*\*= $p < .01$ ; \*\*\*\*= $p < .0001$ . Data are represented as the mean value  $\pm$   
 11 SEM.

12

1 **High GET electric parameters induce dermal fibroblast apoptosis through mitochondrial**  
2 **stress.** Only certain electric field intensities in GET conditions have shown anti-proliferative  
3 results on cell monolayers, thus we decided to focus on GET electric parameters for apoptosis  
4 and mitochondrial stress analysis. In order to explain the delay observed in cell growth after  
5 GET electric parameter application, apoptosis was investigated 24h after electroporation (figure  
6 5A). The percentage of cells dying by apoptosis increased when the electric field intensity  
7 increased. At 200V/cm approximately 10% of the cells displayed activated caspases3/7 and this  
8 percentage reached 30% at 300V/cm 24h after electric field application. This 30% of apoptotic  
9 cells in 300V/cm condition differed from the one observed by clonogenic assay (loss of 60%  
10 of long-term cell viability, Fig 1D), meaning that other cell death mechanisms could be  
11 involved, such as necrosis. We previously showed that mitochondria ultrastructure was rapidly  
12 altered after cells were submitted to electroporation [37]. Consequently, we focused subsequent  
13 experiments on mitochondrial characterization. A MitoView fluorescent probe was used to  
14 follow the mitochondrial membrane potential after application of GET electric parameters  
15 (Figure 5B). The fluorescent staining depends on the mitochondrial membrane potential and is  
16 lost when mitochondria become depolarized [33]. A significant loss of staining was observed  
17 on live cells 1h after GET electric field application at 300V/cm, meaning that mitochondria  
18 underwent depolarization, which could lead to initiation of cell death processes, especially  
19 apoptosis [38]. We used videomicroscopy to follow mitochondrial oxidative stress in live cells  
20 after GET application using the MitoSOX reagent (Figure 5C). This probe selectively targets  
21 mitochondria and becomes highly fluorescent after oxidation specifically by mitochondrial  
22 superoxide [34]. No immediate mitochondrial oxidative stress was observed after GET electric  
23 parameters application, but a delayed effect was observed for 200V/cm and 300V/cm intensity  
24 from 4h after pulses, and became statistically significant 7h after pulses. This means that at

1 200V/cm and even more at 300V/cm, oxidant mitochondrial stress was generated as soon as 4h  
 2 after electroporation, through generation of mitochondrial superoxide.



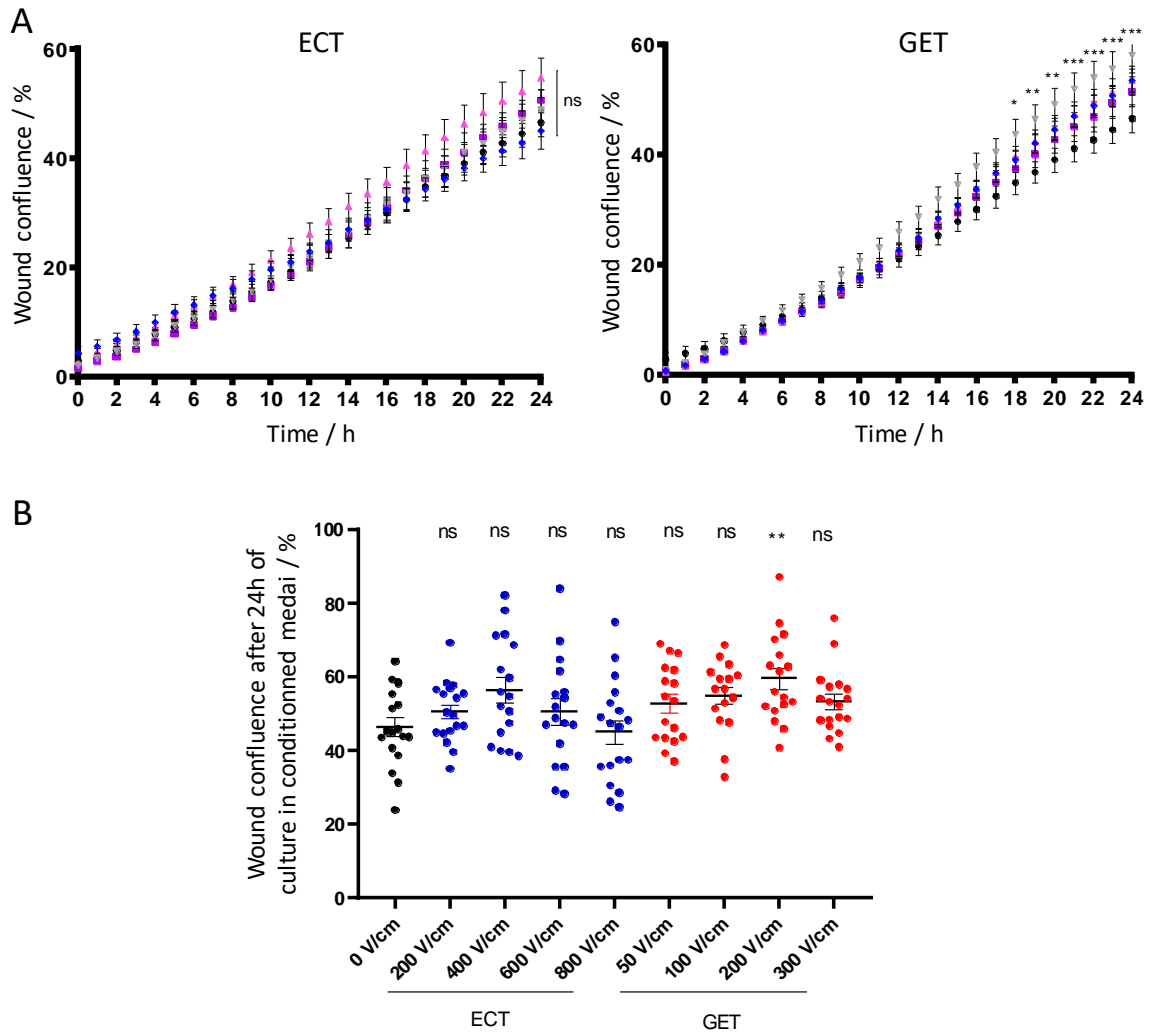
3  
 4 **Figure 5: Some GET electrical parameters induced apoptosis through mitochondrial stress.** A.  
 5 Percentage of apoptotic cells. N=2; n=12. Statistical analysis by One-way and Two-way ANOVA  
 6 \*=p<.05; \*\*\*\*=p<.0001 B. Quantification of mitochondrial membrane potential loss 1h after electric  
 7 field application. N=1; n=3. Statistical analysis by One-way ANOVA \*=p<.05 C. Fluorescent detection  
 8 of generation of mitochondrial superoxide anion O<sub>2</sub><sup>-</sup> after application of GET (x10 pulses lasting 5ms,  
 9 1Hz frequency, at 0V/cm (●), 50V/cm (■), 100 V/cm (▲), 200V/cm (▼), 300V/cm (◆)) electrical  
 10 parameters. N=2; n=12. Data are represented as the mean value ± SEM. Statistical analysis by Two-way  
 11 ANOVA \*=p<.05; \*\*=p<.01; \*\*\*\*=p<.0001.  
 12

13 **Unexposed fibroblast migration properties were stimulated by 24h-conditioned media.** In  
 14 order to assess the indirect effects of electric field application on the migration properties of  
 15 dermal fibroblasts, scratch wound assays with cells cultured in conditioned media were  
 16 performed (Figure 6). In this condition, analyzed dermal fibroblasts were never submitted to  
 17 electric field, but only exposed to cell culture medium conditioned for 24h by dermal fibroblasts

1 that had themselves been exposed to different conditions of ECT and GET. Statistical analyses  
2 on a large number of replicates indicated that over the first 24h the conditioned media of GET  
3 200V/cm condition improved wound closure compared to control condition. A trend emerged  
4 from 10h, which became statistically significant at 18h and then increased over the last 6 hours  
5 of the experiment. However, these analyses did not reveal statistical differences for the other  
6 GET conditions nor for the ECT conditions (figure 6A). Cell confluence within wound area  
7 after 24h of culture in conditioned media indicated a global trend for all conditioned-media  
8 conditions to stimulate cell migration (Figure 6B). However, only the conditioned media of  
9 GET 200V/cm condition statistically improved the wound healing in the scratch wound assay.  
10 It has to be underlined that the electric parameters applied in this condition were the ones  
11 leading to moderate apoptosis. We thus hypothesized that dermal fibroblasts exposed to pulsed  
12 electric fields and undergoing light apoptosis secreted several factors stimulating unexposed  
13 fibroblasts to migrate in the wound area.



1

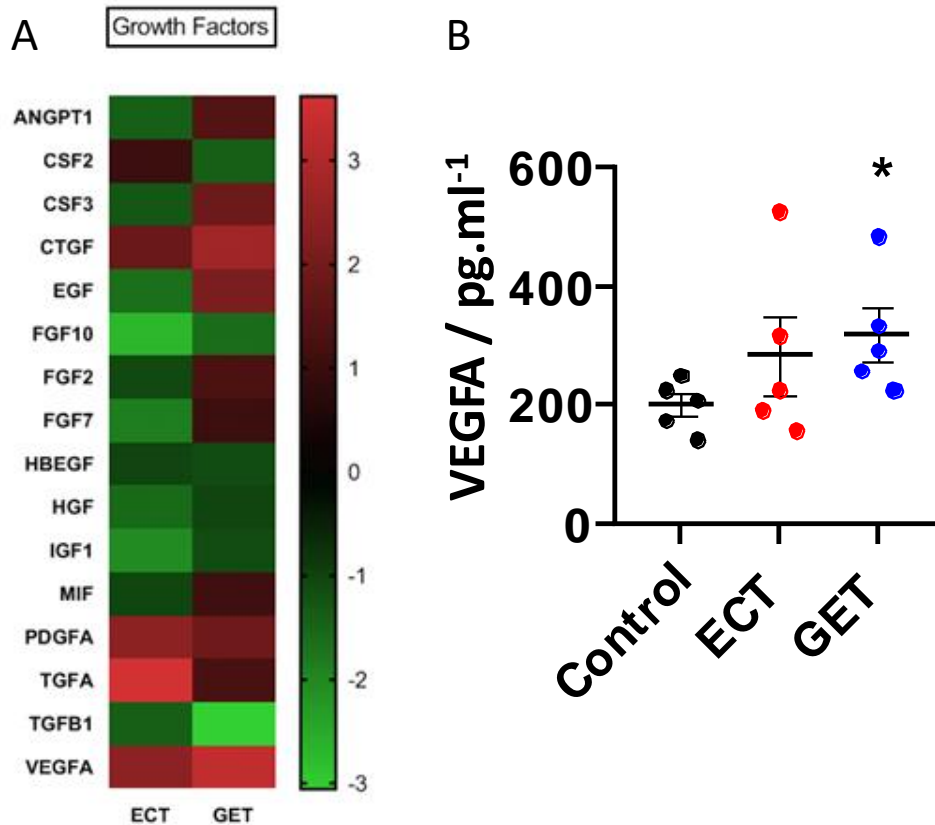


2

3 **Figure 6: Conditioned media from electroporated-fibroblasts stimulated the migration of**  
 4 **unexposed fibroblasts.** A. Migration of dermal fibroblasts cultured in 24h ECT-(x8 pulses lasting  
 5 100 $\mu$ s, 1Hz frequency, at 0V/cm (●), 200V/cm (■), 400 V/cm (▲), 600V/cm (▼), 800V/cm (◆)) and  
 6 and GET-(x10 pulses lasting 5ms, 1Hz frequency, at 0V/cm (●), 50V/cm (■), 100 V/cm (▲), 200V/cm  
 7 (▼), 300V/cm (◆)) conditioned cell culture media. N=3; n= 18. Only the 200V/cm condition showed  
 8 statistical difference with the control condition (0V/cm). Statistical analysis by Two Way ANOVA  
 9 \*= $p < .05$ ; \*\*= $p < .01$ ; \*\*\*= $p < .001$  B. Wound confluence after 24h of culture in 24h ECT- or GET-  
 10 conditioned media. N=3; n=18. Data are represented as the mean value  $\pm$  SEM. Statistical analysis by  
 11 One-way ANOVA \*\*= $p < .01$ .  
 12

13 **ECT and GET electric parameters strongly modulate growth factor transcripts.** In order  
 14 to understand the above results, a commercially available qPCR array dedicated to wound  
 15 healing was performed 4h after exposure to ECT and GET electric parameters. This analysis  
 16 revealed significant variations in the number of growth factor transcripts (Figure 7A).

1 Overexpressed genes (in red in the figure) in both conditions include Connective Tissue Growth  
2 Factor (CTGF), Vascular Endothelial Growth Factor A (VEGFA), Platelet Derived Growth  
3 Factor Subunit A (PDGFA), and Transforming Growth Factor Alpha (TGF $\alpha$ ) with 1.2- to 3.6-  
4 fold increases compared to non-electroporated controls. Several growth factors were also under-  
5 expressed (in green in the figure) 4h after exposure to ECT and GET electric parameters  
6 including TGF- $\beta$ 1 a well-known protagonist in dermal fibrosis. For GET parameters we also  
7 noted specific over-expressions of immunity-related factors like Colony stimulating factor 3  
8 (CSF3), as well as angiogenic factors like Epidermal growth factor (EGF). This experiment  
9 confirmed that the expression of several growth factors transcripts was rapidly and strongly  
10 modulated after cell electroporation. To confirm that this modulation of growth factors  
11 transcripts was also observed at protein level, we quantified one using ELISA in cell culture  
12 medium 24h after electroporation of dermal fibroblasts (Figure 7B). We focused on Vascular  
13 Endothelial Growth Factor A (VEGFA) because it was the more over-expressed at the RNA  
14 level (2.4 times in ECT and 3.3 times in GET conditions). More VEGFA protein was indeed  
15 found in cell culture supernatant 24h after electroporation both in ECT condition (even if not  
16 statistically significant) and in GET condition (statistically significant). We thus confirmed at  
17 protein level that electroporation, especially GET condition, increases the expression of  
18 VEGFA growth factor, as observed at transcriptional level.



1

2 **Figure 7: Gene expression analysis of growth factor related genes in dermal fibroblasts 4h after**  
3 **exposure to ECT and GET electric parameters.** A. Gene expression was quantified by RT-qPCR  
4 array. Fold changes are expressed compared to the control condition. Control and ECT condition  
5 (600V/cm) n=3, GET condition (200V/cm) n=2. Angiopoietin 1 (ANGPT1); Colony stimulating factor  
6 2,3 (CSF2,3); Connective Tissue Growth Factor (CTGF); Epidermal growth factor (EGF); Fibroblast  
7 growth factor 2,7,10 (FGF2,7,10); Heparin binding EGF like growth factor (HBEGF); Hepatocyte  
8 growth factor (HGF); Insulin like growth factor 1 (IGF1); Macrophage migration inhibitory factor  
9 (MIF); Platelet Derived Growth Factor Subunit A (PDGFA); Transforming Growth Factor Alpha  
10 (TGF $\alpha$ ); Transforming Growth Factor Beta 1 (TGF- $\beta$ 1); Vascular Endothelial Growth Factor A  
11 (VEGFA). B. VEGFA protein expression quantified by ELISA in cell culture medium 24h after dermal  
12 fibroblasts electroporation with ECT (600V/cm) and GET (200V/cm) electric parameters N=1, n=5.  
13 Statistical analysis by Mann Whitney test \*=p<.05.

## 1 **Discussion**

2 The existence of a physiological transepithelial electric potential within the skin makes  
3 skin cells a good target for testing modulation of behavior through electroporation application.  
4 Endogenous electric fields are important to guide cell migration and often predominate over  
5 chemical or topographic signals [39]. Directional migration along an electric field guiding cue  
6 has already been shown in fibroblast cells [24,40,41]. Human dermal fibroblasts submitted to  
7 an electric field of 50–200 mV mm<sup>-1</sup> under direct current took more than 1 hour to exhibit  
8 measureable directional migration toward the anode [24,41]. Zhao *et al.* have identified  
9 Phosphatidylinositol 3 Kinase (PI(3)K) and Phosphatase and tensin homolog (PTEN) as  
10 essential genes controlling the electrotaxis [24]. Indeed, the electric field directs cell migration  
11 by acting as the primary directional signal, triggering Tyrosine-protein Kinase Src and Inositol-  
12 phospholipid signalling pathways. The role of Phosphatidylinositol 3 Kinase in electro-  
13 induced migration was confirmed by Guo and colleagues in 2010 [41]. Another suggested  
14 mechanism is the asymmetric redistribution of EGFR membrane receptors on the cell surface  
15 [42–44]. Nevertheless, these studies mainly applied long exposure direct currents, whereas in  
16 this study we used a pulsed electric field. The difference in the nature and intensity of the  
17 applied electric fields could explain the absence of significant results in migration and  
18 galvanotaxis experiments with electroporation.

19 High-voltage pulsed galvanic stimulation has been shown to have a proliferative effect on  
20 human foetal lung fibroblasts IMR-90 and to stimulate protein synthesis [45]. This study  
21 indicated an increase in protein synthesis rates 2h post stimulation and in DNA synthesis  
22 between 2 and 24h post stimulation. The maximum increases in protein and DNA synthesis  
23 occurred with electric parameters of 100 pulses/seconds and 50 and 75 V/cm respectively. In  
24 this study, electrical parameters were applied for at least 20 min, far from the pulsed electric

1 field applied in electroporation. Thus, the involved molecular mechanisms may differ and  
2 explain the absence of proliferation after ECT and GET application.

3 Reactive oxygen species (ROS) play a major role in wound healing response [46,47].  
4 Interestingly, it has previously been shown that electroporation generates ROS such as H<sub>2</sub>O<sub>2</sub>  
5 [48,49]. Studies on lipid peroxidation in cell membranes after electroporation have been  
6 reported [48–50]. Lipids peroxidation and ROS concentration increase with electric field  
7 intensity, pulse duration and number of pulses and is correlated to membrane  
8 electropermeabilization and to cell death when the amount of cellular damage is too high [51].  
9 ROS can be involved in the recruitment of immune cells [52,53], in the regulation of  
10 angiogenesis processes [46,54] and they present bacteriostatic effects at wound sites [55].  
11 However, high production of ROS may lead to impaired wound repair [56]. In these conditions  
12 ROS will give rise to the secretion of pro-inflammatory cytokines, of metalloproteases, and can  
13 impair the function of dermal fibroblasts and keratinocytes [57]. Moreover, ROS are generally  
14 associated with skin ageing, fibroblast senescence and autophagy [58]. In our experimental  
15 conditions, electroporation of dermal fibroblasts with the highest electric field intensities in  
16 GET condition (200V/cm and 300V/cm) blocked migration process and induced cell death.  
17 These results are in agreement with those reported in the literature showing the deleterious  
18 effects of H<sub>2</sub>O<sub>2</sub> on dermal fibroblasts [59,60].

19 We demonstrated that modulating the intensity of the electric fields led to a gradient of cell  
20 electropermeabilization. We can hypothesize that cell responses to these different plasma  
21 membrane defaults will be different. Indeed, apoptotic cell death induction was observed for  
22 the most stressful GET electrical parameters (i.e 200V/cm and 300V/cm). The secretome in a  
23 culture medium that was conditioned in these electric conditions improved the ability of  
24 unexposed dermal fibroblasts to migrate. We can theorize that this bystander effect can occur  
25 *in vivo*, where dying cell would secrete specific factors modifying surrounding cells behavior.

1 It was previously demonstrated in electroporation that electric field alone induced the  
2 externalization of calreticulin [61] while calreticulin is usually an endoplasmic reticulum-  
3 resident protein. When externalized, it becomes a danger-associated molecular pattern  
4 molecule (DAMP) associated with the induction of immunogenic cell death [62]. Interestingly,  
5 studies showed that calreticulin externalization played a significant role in cutaneous wound  
6 healing by stimulating dermal fibroblast and keratinocyte proliferation, migration, and  
7 differentiation [63]. Several studies have already shown that nucleotides released from damaged  
8 tissues, such as extracellular ATP or purines and pyrimidines released following cell death and  
9 DNA fragmentation, regulate many important functions of skin cells (proliferation, migration,  
10 contraction) and can promote wound healing [64].

11 Further experiments would be needed to finely describe what kind of factors are secreted  
12 or released by the dying cells. We obtained encouraging results on the modulation of a number  
13 of growth factors transcripts by electroporation. One of them, VEGFA, was quantified at protein  
14 level by ELISA. In our experimental condition, we demonstrated that electroporated dermal  
15 fibroblasts released in cell culture a significant amount of VEGFA. However, comprehensive  
16 analyses of both growth factors and cytokines secreted as a result of electroporation should be  
17 exhaustively conducted at protein level (ELISA, proteomic, secretome profiling) and functional  
18 tests are essential to conclude in our experimental conditions. Interestingly, cues can be found  
19 in the literature to enrich the reflection on this bystander effect. Some authors used pulsed  
20 electric field to activate platelets. They showed that electroporation stimulates the release of  
21 growth factors such as PDGF-AA, TGF- $\beta$ 1 or EGF, contained in granules within the cells [65].  
22 A modulation of pulsed electric field parameters led to a differential growth factors release [66].

23 Bystander effect induced by electroporation was previously investigated in the context of  
24 melanoma tumor cells [67]. In this context, a negative bystander effect leading to death of cells  
25 cultivated in conditioned medium was observed and could be due to release of microvesicles

1 (<500nm). Interestingly, the bystander effect and microvesicles amounts depended on electric  
2 parameters (pulse amplitude and repetition frequency), confirming that in tissue cell response  
3 will be different depending on electric field distribution. Since electroporation affects plasma  
4 membrane integrity, it would be of utmost importance to characterize well and screen the  
5 induced extracellular vesicles, which can contain proteins, RNA molecules and other agents,  
6 able to influence signaling pathways in distant cells.

7 In this line of thought on communication between cells, co-culture of cells would be a  
8 complementary approach to the one adopted in this study. In our case, a co-culture of dermal  
9 cells (primary fibroblasts) with epidermal cells (primary keratinocytes) would be relevant. To  
10 go forward, *in vitro* cell culture models should switch from 2D to 3D tissue models. Even if 2D  
11 cell cultures are easier to manage, it is now widely accepted that 3D models better mimic *in*  
12 *vivo* situations [68]. In skin context, some *in vitro* 3D tissue models such as spheroids [69],  
13 dermal cell sheets [29,70] or recellularized human skin biopsy [71] have been developed and  
14 used in electroporation study. The next step would be to use them for fundamental studies to  
15 better understand links between electroporation and wound healing.

16

17 **Author Contributions.** conceptualization, L.G.; investigation and formal analyses, S.G., A.D.,  
18 L.G.; writing-original draft preparation, S.G. and L.G.; writing-review and editing, S.G.,  
19 AF.M., P.V., MP.R., L.G.; scientific discussion, S.G., A.D., AF.M., P.V., MP.R., L.G.;  
20 supervision, L.G.; funding acquisition, MP.R., L.G.

21

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5  
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