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Statistical validation of the acceleration of the differentiation at the expense of the proliferation in human epidermal cells exposed to extremely low frequency electric fields

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Abstract

An acceleration of differentiation at the expense of proliferation is observed in our previous publications and in the literature after exposure of various biological models to low frequency and low-amplitude electric and electromagnetic fields. This observation is related with a significant modification of genes expression. We observed and compared over time this modification. This study use microarray data obtained on epidermis cultures harvested from human abdominoplasty exposed to ELF electric fields. This protocol is repeated with samples collected on three different healthy patients. The sampling over time allows comparison of the effect of the stimulus at a given time with the evolution of control group. After 4 days, we observed a significant difference of the genes expression between control (D4C) and stimulated (D4S) ($p < 0.05$). On the control between day 4 and 7, we observed another group of genes with significant difference ($p < 0.05$) in their expression. We identify the common genes between these two groups and we select from them those expressing no difference between stimulate at 4 days (D4S) and control after 7 days (D7C). The same analysis was performed with D4S–D4C–D12C and D7S–D7C–D12C. The lists of genes which follow this pattern show acceleration in their expressions under stimulation appearing on control at a later time. In this list, genes such as DKK1, SPRR3, NDRG4, and CHEK1 are involved in cell proliferation or differentiation. Numerous other genes are also playing a function in mitosis, cell cycle or in the DNA replication transcription and translation.

1. Introduction

The effect on cell proliferation and differentiation on different tissues after exposition to low frequency electric or electromagnetic fields, pulsed or sinusoidal stimulation is well described in the literature: Zhou et al. reported that while exposure to 50 Hz sinusoidal electromagnetic field inhibits the osteoblast proliferation, it significantly promotes differentiation (Zhou et al., 2011) and demonstrated that Alkaline phosphatase activity, marker for early phase osteoblast differentiation, was significantly increased when rat osteoblasts in vitro are exposed to 50 Hz 3.6 mT 0.5 h (Zhou et al., 2012); Hronik-Tupaj et al.

(2011) concluded that electrical stimulation is a useful tool to improve hMSC osteogenic differentiation; Manni et al. (2004) worked on primary human oral keratinocyte exposed to low electromagnetic field (50 Hz) and the results support the idea that exposure to electromagnetic field carries keratinocytes to higher differentiation level; data of Ciombor et al. (2002) suggested that chondrogenic differentiation occurs earlier and that cartilage extracellular matrix is synthesized to a greater degree and matures faster in response to low frequency pulsed EMF fields; Lohmann et al. (2000) wrote that his study on MG63 osteoblast-like cells showed enhanced differentiation as the net effect of pulsed electromagnetic fields as evidenced by decreased proliferation, increased alkaline phosphatase-specific activity, osteocalcin synthesis, and collagen production. The treatment with pulsed electromagnetic fields resulted in a more differentiated and mature osteoblastic phenotype.

Our laboratory observed on different in vitro and in vivo biological models exposed to a specific low frequencies and low-amplitude, asymmetric, charge-balanced, pulse-train modulated electromagnetic fields an acceleration of the cartilaginous matrix differentiation preceding the ossification (Hinsenkamp and Rooze, 1982; Hinsenkamp et al., 1985; Hinsenkamp, 1994; Rooze and Hinsenkamp, 1982, 1985).

This acceleration of the differentiation at the expense of proliferation has been shown on a human keratinocyte culture exposed to a similar pattern of electric field. On this model, observations made with planimetry, histologic examination, and [H3]-thymidine labeling techniques (Jercinovic et al., 1996; Hinsenkamp et al., 1997) were confirmed by microarray analysis (Collard et al., 2011).

In the present study, we used the gene expression data obtain with U133 Plus 2.0 microarray Affymetrix chips on a culture of epidermis on de-epidermized human skin exposed to a 40 Hz pulsed asymmetric charge-balanced carrier signal modulated by a fundamental frequency of 0.125 Hz and transmitted by two platinum electrodes. The complete protocol was performed on three healthy patients (Collard et al., 2011).

Considering the very coherent response of all our biological models, we want to compare statistically the modification in genes regulation induced by electrical stimulation to the natural genes expression over time. The sampling over time allows comparison of the effect of the stimulus at a given time with the evolution of the control group.

2. Materials and methods

2.1. Biological model

The in vitro model is prepared with skin harvested from human abdominoplasty after plastic surgery. Human epidermal explants were cultured at an air–liquid interface on decellularized human dermal supports. Two platinum electrodes are used to apply the electric signal (Fig. 1).

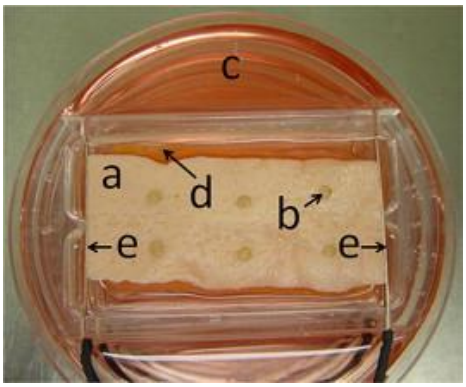

Fig. 1. In vitro model. Six epidermal explants placed on a dermal support. The culture medium, a foam support and two platinum electrodes complete the model. (a) Dermal support; (b) Epidermal explant; (c) Culture medium; (d) Foam; (e) 2 Platinum electrodes.

The preparation of the dermal support consists, after removal of the epidermis, to decellularize the remaining dermis (20 successive freeze–thaw cycles of the dermis and gamma radiation at 7 kGy) and cutting 60 mm × 30 mm rectangle. Pairs of dermal support from the same skin donors were distributed equally to the stimulated and the control group.

For the explants, 3 mm diameter punch biopsies are removed from epidermis layer separated from the skin with a Wagner's dermatome.

Two dermal supports and 12 explants are planned for each sampling conditions thus 84 explants from the same healthy patient are distributed on 14 dermal supports (D −3 does not need dermal support: it is sampled directly after cutting with punch biopsies). The control and stimulated groups are placed inside the same incubator (37 ± 0.3 °C, 5% CO2). Stimulated group does not receive stimulation during the 3 first days of incubation (Fig. 2: gray time unit −3, −2, −1) to allow rest and explants attachment to dermal support. For the stimulated group, stimulation start at Day 1 (fourth day of incubation) and the control group does not receive stimulation.

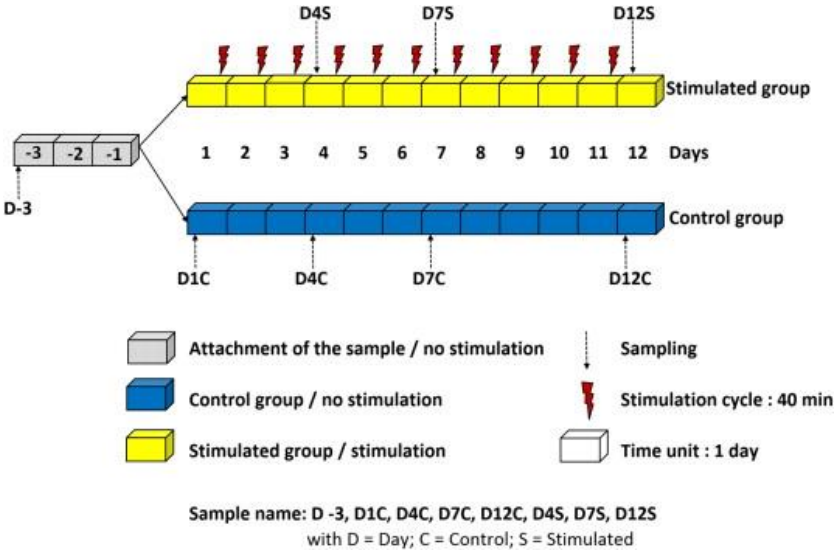

Fig. 2. Study design and sampling time: D −3: Control sample (just after the punch biopsies). D1C: Control sample (after the period of 3 days for explants attachment to the dermal support and before the first stimulation). D4C, D7C, D12C: Three samples in the control group at day 4, 7 and 12. D4S, D7S, D12S: Three samples in the stimulated group at day 4, 7 and 12 after 3, 6 and 11 period of stimulation.

We realized control samples: D −3 just after the punch biopsies, D1C (Day 1 Control group) after the period of 3 days for explants attachment to the dermal support and before the first stimulation. We collected three control samples at D4C, D7C, and D12C and three stimulated samples at D4S (Day 4 Stimulated group), D7S, and D12S, at days 4, 7, and 12, respectively (Fig. 2).

The details for biological model and culture protocol are explained in Collard et al. (2011).

2.2. Electrical stimulation pattern

The output of the generator consists of a biphasic, asymmetric, charge-balanced current stimuli, with a repetition frequency of 40 Hz modulated by a fundamental frequency of 0.125 Hz (Fig. 3a). The stimulus is repeated during 4 s followed by a 4 s break (Fig. 3b), for 40 min/day for 11 days (Fig. 2). Electrical stimulation is applied through two platinum (Pt) electrodes (50 mm × 2 mm × 0.5 mm) in contact with the dermal support (Fig. 1). The generator output current amplitude is 20 mA peaks.

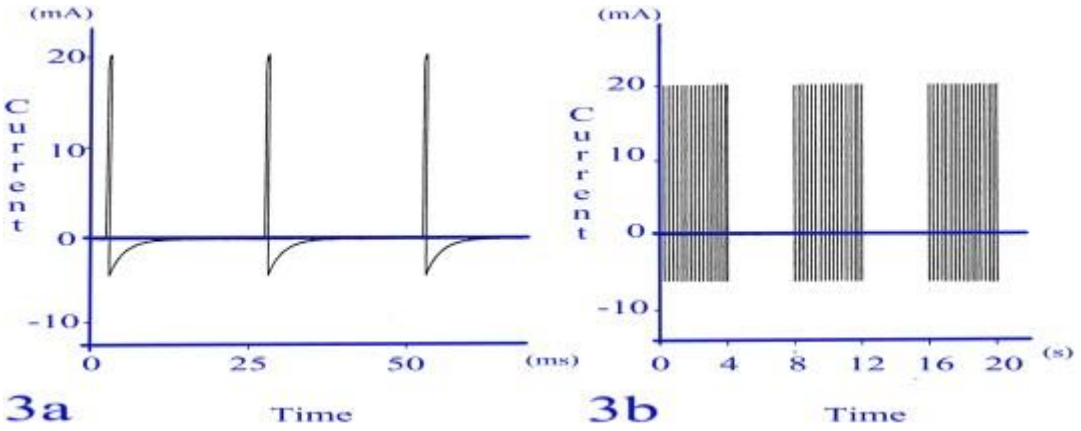

Fig. 3. Electrical pattern: (a) The output of the generator consists of a biphasic, asymmetric, charge-balanced current stimuli, with a repetition frequency of 40 Hz and a pulse width of 0.25 ms; (b) The stimulus is repeated during 4 s followed by a 4 s break, for 40 min/day during 11 days.

The electrical stimulation pattern details are explained in Collard et al. (2011).

2.3. RNA extraction, microarray and Real-time rtPCR

TotalRNA was extracted from a pool of 12 explants for each sample condition. Each pool is homogenized with a rotor-stator and total RNA was extracted using a Qiagen RNeasy Mini Kit (Dusseldorf, Germany) according to the recommended protocol. The RNA quality was measured with the capillary electrophoresis system from Agilent Technologies (Santa Clara, CA) and the RNA quantities were determined with NanoDrop Technologies spectrometer (Wilmington, DE).

Probe levels are measured using Affymetrix microarray U133 Plus 2.0 chips prepared and hybridized according to the Affymetrix (Santa Clara, CA) two-cycle technical protocol.

The protocol is repeated on three healthy patients. A total of 24 Affymetrix chips are processed.

Expression data and raw expression data (CEL files) were generated using GCOS Affymetrix software.

To validate the microarray data, we evaluated the expression of five transcripts up- or down-regulated in our results (TXNRD1, ATF3, MME, DKK1, and MACF1) with Real-Time rtPCR method. A normalization of the expression of all transcripts was made with two genes (TBP and B2M) proposed by Allen et al. (2008). All results obtained with this technique are consistent with results observed by microarray.

The details for RNA extraction, microarray protocol and Real-Time rtPCR procedure are explained in Collard et al. (2011).

2.4. Statistical analysis

Our statistical analysis identifies genes that are differentially expressed between two groups of samples (either stimulated and control at different sampling time or two different control groups corresponding to different sampling time). We performed differential expression analysis using LIMMA package (Smyth, 2004) to identify lists of differentially expressed genes (DEGs) between the following couples of groups of samples, as derived from Fig. 4:

1. D4S vs D4C (D7S vs D7C) – by performing differential analysis between the stimulated group and the control group from the same day we identify genes that could be indicators for the mechanisms that affects the cell by the stimulation with ELF. This analysis has been performed in our previous study (Collard et al., 2011) and revealed genes that are involved in the proliferation and the differentiation of the cell.

2. D7C vs D4C (D12C vs D4C, D12C vs D7C) – by performing differential analysis between the control groups at different sampling time we identify the genes that are naturally evolving (become up- or down-regulated) over time. We expect that these genes should be involved in the cells mechanisms that evolve with time such as cell differentiation.

3. D4S vs D7C (D4S vs D12C, D7S vs D12C) – by performing differential analysis between the stimulated group (early sampling time) and the control group (late sampling time), we identify the genes that have no difference in expression. If the p-value >0.05, a gene have no difference in his expression between the two groups.

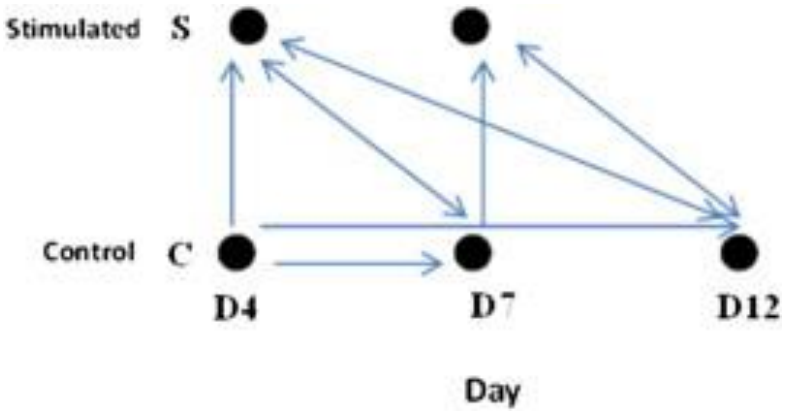

Fig. 4. All possible combinations for triangular analysis between the different groups of control samples and the different groups of stimulated samples.

The common genes found at point 1 and 2 could give clues about the cell mechanisms affected by the exposure to ELF.

DEGs have been identified as genes having a low statistical significance ($p < 0.05$) for the corresponding t-test, while keeping high the difference in expression level (the difference in means) between the two groups of samples (Fold Change > 2).

The sampling over time allows comparison of the effect of the stimulus at a given time with the evolution of the control group. In order to find more evidence to confirm our previous findings that suggest an acceleration of cells differentiation at the expense of proliferation when exposed to ELF, we propose a triangular analysis of the gene expression at different sampling times (Fig. 4):

- Step 1: we compared gene expression profiles of stimulated samples with gene expression profiles of control samples for one sampling times (D4S vs D4C or D7S vs D7C); we select the genes list with a significant statistical difference between the two groups (p-value < 0.05).
- Step 2: we compared gene expression profiles in between the different groups of control samples at different sampling time. The early time is the sampling time selected in step 1 (D7C vs D4C or D12C vs D4C or D12C vs D7C); we select the genes list with a significant statistical difference between the two groups (p-value < 0.05).
- Step 3: we compared the later control samples selected in step2 with the stimulated samples selected at step 1 (D4S vs D7C or D4S vs D12C or D7S vs D12C); we keep the genes list with no significant statistical difference between the two groups (p-value > 0.05).

Then, we use the genes common to these three lists. By doing so, we aimed to investigate whether the stimulation with the ELF has the same effect on the cells as the normal evolution in time of a non-stimulated tissue over a time period.

3. Results

After 4 days, we observed a significant difference of the genes expression regulation between control (D4C) and stimulated (D4S) ($p < 0.05$). On the control between day 4 and 7, we observed another group of genes with significant difference ($p < 0.05$) in their genes expression. We select, from the common genes between these two groups, those with no more difference ($p > 0.05$) between stimulate at 4 days (D4S) and control after 7 days (D7C). The same analysis was performed with D4S–D4C–D12C and D7S–D7C–D12C.

3.1. Triangle D4S–D4C–D7C

There are 319 probes that meet the 3 conditions of the triangle D4S–D4C–D7C presented in materials and methods ($p < 0.05$ for D4S/D4C and D7C/D4C and $p > 0.05$ for D4S/D7C).

In this list, if we compare the fold change (FC) between D4S/D4C and D7C/D4C, 110 probes have exactly the same (if we consider two FC equal if the rounded value of the FC (log2) is not exceeding a difference of the tenth unit), 112 probes have an FC value greater for D4S/D4C than D7C/D4C and 97 probes have an FC value below for D4S/D4C than D7C/D4C. In this list, 271 probes are down-regulated by the stimulation and 48 up-regulated.

The list of 319 probes is reduced to 33 if we apply an FC cutoff equal 2 (log2FC = 1) (Table 1).

		D4S/D4C		D4S/D7C		D7C/D4C	
Probes	**Gene symbol**	**LogFC**	**p-value**	**LogFC**	**p-value**	**LogFC**	**p-value**
242074_at	---	-1.55	0.04033	-0.79	0.21379	-0.76	0.02683
239243_at	ZNF638	-1.63	0.00978	-0.76	0.14384	-0.87	0.02169
226877_at	RPL32P3	-1.48	0.00026	-0.31	0.49597	-1.17	0.03693
234032_at	---	-1.49	0.00780	-0.67	0.16530	-0.82	0.03070
240383_at	UBE2D3	-1.50	0.00537	-0.75	0.11777	-0.75	0.04697
239780_at	---	-1.41	0.00569	-0.77	0.07657	-0.65	0.02548
241837_at	---	-1.28	0.03474	-0.42	0.36349	-0.86	0.03323
244597_at	SPATS2L	-1.31	0.03003	-0.63	0.20934	-0.68	0.01144
238706_at	PAPD4	-1.34	0.00376	-0.56	0.16872	-0.78	0.02586
240458_at	---	-1.34	0.01775	-0.52	0.22680	-0.82	0.02682
229899_s_at	C20orf199	-1.15	0.01903	-0.48	0.21348	-0.68	0.00234
232500_at	RALGAPA2	-1.16	0.02718	-0.50	0.22275	-0.66	0.02871
228623_at	---	-1.18	0.02497	-0.65	0.13185	-0.52	0.03140
242693_at	---	-1.21	0.00532	-0.60	0.06221	-0.60	0.04118
1559232_a_at	SLC33A1	-1.18	0.00001	-0.03	0.62853	-1.15	0.00001
237107_at	PRKRA /// PRKRAP1	-1.20	0.01648	-0.03	0.94163	-1.17	0.00717
35493_at	---	-1.11	0.04241	-0.25	0.57701	-0.86	0.00231
235912_at	---	-1.12	0.02449	-0.54	0.17930	-0.59	0.01265
1559023_a_at	---	-1.13	0.00425	-0.37	0.84660	-0.76	0.04877
238736_at	REV3L	-1.14	0.03000	-0.16	0.69199	-0.98	0.00644
233595_at	USP34	-1.14	0.02496	-0.58	0.18200	-0.56	0.01136
239551_at	---	-1.14	0.01728	-0.23	0.52072	-0.91	0.02500
236951_at	NSFL1C	-1.10	0.04423	0.30	0.43224	-1.40	0.00136
239629_at	CFLAR	-1.03	0.01617	-0.49	0.19605	-0.54	0.03891
236492_at	PPP2R2A	-1.03	0.03335	-0.56	0.17362	-0.47	0.00698
202478_at	TRIB2	-1.00	0.01523	0.39	0.12578	-1.39	0.00136
218990_s_at	SPRR3	1.06	0.03100	-1.05	0.10149	2.10	0.01939
209159_s_at	NDRG4	1.21	0.04193	0.00	0.99838	1.21	0.00340
214455_at	HIST1H2BC	1.17	0.00453	-0.01	0.96893	1.18	0.00040
209277_at	TFPI2	1.26	0.04092	0.12	0.82942	1.14	0.04847
209114_at	TSPAN1	1.49	0.00901	0.73	0.08874	0.76	0.02764
205749_at	CYP1A1	1.45	0.04264	0.77	0.19367	0.69	0.00534
204602_at	DKK1	2.14	0.01417	-0.51	0.55390	2.65	0.01115

Table 1. List of the 33 probes for triangle D4S–D4C–D7C with an FC cutoff equal 2 (log2FC = 1).

3.2. Triangle D4S–D4C–D12C

There are 183 probes that meet the 3 conditions of the triangle D4S–D4C–D12C presented in materials and methods ($p < 0.05$ for D4S/D4C and D12C/D4C and $p > 0.05$ for D4S/D12C). In this list, if we compare the FC between D4S/D4C and D12C/D4C, 42 probes have exactly the same (if we consider two FC equal if the rounded value of the FC (log2) is not exceeding a difference of the tenth unit), 44 probes have an FC value greater for D4S/D4C than D12C/D4C and 97 probes have an FC value below for D4S/D4C than D12C/D4C. In this list, 159 probes are down-regulated by the stimulation and 24 up-regulated.

The list of 183 probes is reduced to 25 if we apply an FC cutoff equal 2 (log2FC = 1) (Table 2).

		D4S/D4C		D4S/D12C		D12C/D4C	
Probes	**Gene symbol**	**LogFC**	**p-value**	**LogFC**	**p-value**	**LogFC**	**p-value**
239243_at	ZNF638	-1.63	0.00978	-0.05	0.89923	-1.58	0.00021
242074_at	---	-1.55	0.04033	-0.74	0.22901	-0.81	0.00788
240383_at	UBE2D3	-1.5	0.00537	-0.23	0.63561	-1.26	0.01850
234032_at	---	-1.49	0.00780	-0.73	0.10014	-0.76	0.00829
226877_at	RPL32P3	-1.48	0.00026	0.19	0.34359	-1.67	0.00020
238706_at	PAPD4	-1.34	0.00376	-0.33	0.39005	-1.01	0.01157
241837_at	---	-1.28	0.03474	-0.32	0.49451	-0.96	0.03005
242859_at	---	-1.28	0.00308	-0.09	0.77245	-1.19	0.00681
232141_at	U2AF1	-1.27	0.00759	0.04	0.88993	-1.31	0.00299
242693_at	---	-1.21	0.00532	-0.28	0.36198	-0.92	0.01618
237107_at	PRKRA /// PRKRAP1	-1.2	0.01648	0.19	0.60863	-1.39	0.00074
1559232_a_at	---	-1.18	0.00001	0.05	0.29323	-1.23	0.00000
229899_s_at	C20orf199	-1.15	0.01903	-0.37	0.38673	-0.79	0.01814
238736_at	REV3L	-1.14	0.03000	-0.65	0.13102	-0.49	0.03792
239551_at	---	-1.14	0.01728	-0.29	0.37359	-0.85	0.01752
233595_at	USP34	-1.14	0.02496	-0.72	0.10799	-0.42	0.00889
235493_at	---	-1.11	0.04241	-0.43	0.38842	-0.69	0.01811
236951_at	NSFL1C	-1.1	0.04423	0.32	0.42073	-1.42	0.00181
239629_at	CFLAR	-1.03	0.01617	-0.41	0.28473	-0.62	0.03155
218990_s_at	SPRR3	1.06	0.03100	-1.36	0.13035	2.42	0.03237
214455_at	HIST1H2BC	1.17	0.00453	-0.95	0.10820	2.12	0.00544
209159_s_at	NDRG4	1.21	0.04193	0.24	0.63925	0.98	0.03913
205749_at	CYP1A1	1.45	0.04264	0.6	0.29570	0.85	0.00165
209114_at	TSPAN1	1.49	0.00901	0.8	0.06607	0.7	0.02930
204602_at	DKK1	2.14	0.01417	-1.51	0.10874	3.66	0.00236

Table 2. List of the 25 probes for triangle D4S–D4C–D12C with an FC cutoff equal 2 (log2FC = 1).

3.3. Triangle D7S–D7C–D12C

They are 329 probes that meet the 3 conditions of the triangle D7S–D7C–D12C presented in materials and methods ($p < 0.05$ for D7S/D7C and D12C/D7C and $p > 0.05$ for D7S/D12C). In this list, if we compare the FC between D7S/D7C and D12C/D7C, 85 probes have exactly the same (if we consider two FC equal if the rounded value of the FC (log2) is not exceeding a difference of the tenth unit), 131 probes have an FC value greater for D7S/D7C than D12C/D7C and 113 probes have an FC value below for D7S/D7C than D12C/D7C. In this list, 28 probes are down-regulated by the stimulation and 301 up-regulated.

The list of 329 probes is reduced to 65 if we apply an FC cutoff equal 2 (log2FC = 1) (Table 3).

		D7S/D7C		D7S/D12C		D12C/D7C	
Probes	**Gene symbol**	**LogFC**	**p-value**	**LogFC**	**p-value**	**LogFC**	**p-value**
235456_at	---	-1.65	0.02321	-0.56	0.40055	-1.09	0.02116
232035_at	HIST1H4H	-1.12	0.04822	0.27	0.69756	-1.39	0.03794
229351_at	---	-1.00	0.02464	-0.44	0.23328	-0.56	0.02100
227211_at	PHF19	1.00	0.04025	-0.20	0.57310	1.21	0.00963
232958_at	---	1.00	0.02618	-0.62	0.10256	1.62	0.00001
224771_at	NAV1	1.01	0.01307	0.21	0.40989	0.80	0.02327
222037_at	MCM4	1.01	0.00784	-0.44	0.37986	1.44	0.02251
213603_s_at	RAC2	1.02	0.00730	0.16	0.41808	0.87	0.01009
242560_at	FANCD2	1.02	0.00842	-0.07	0.81435	1.09	0.00384
206277_at	P2RY2	1.03	0.00202	0.21	0.24363	0.83	0.01489
222039_at	KIF18B	1.03	0.01195	0.18	0.50955	0.85	0.00531
222680_s_at	DTL	1.04	0.00990	0.14	0.55578	0.90	0.00664
214710_s_at	CCNB1	1.04	0.00751	0.11	0.66819	0.92	0.02961
203554_x_at	PTTG1	1.04	0.00122	0.38	0.10563	0.66	0.00590
216237_s_at	MCM5	1.05	0.00115	-0.09	0.75840	1.14	0.00491
209754_s_at	TMPO	1.05	0.01256	-0.15	0.20521	1.20	0.00838
219494_at	RAD54B	1.06	0.00639	0.34	0.17081	0.72	0.01864
218726_at	HJURP	1.07	0.00116	0.17	0.43677	0.91	0.00116
201506_at	TGFBI	1.08	0.02569	0.37	0.28894	0.72	0.01026
201896_s_at	PSRC1	1.08	0.00400	0.30	0.32473	0.79	0.04210
226936_at	CENPW	1.08	0.00036	0.28	0.38854	0.80	0.04884
202338_at	TK1	1.08	0.00131	0.16	0.50841	0.92	0.00401
205933_at	SETBP1	1.09	0.01325	0.40	0.12835	0.69	0.01774
242890_at	---	1.09	0.00164	0.30	0.36941	0.79	0.04970
204092_s_at	AURKA	1.10	0.00231	0.11	0.81412	1.00	0.04910
213008_at	FANCI	1.10	0.01423	-0.14	0.35692	1.24	0.00507
228729_at	CCNB1	1.10	0.00973	0.15	0.67555	0.95	0.03528
226875_at	DOCK11	1.10	0.02936	0.33	0.37737	0.77	0.02892
205394_at	CHEK1	1.10	0.02341	-0.33	0.56374	1.42	0.03497
218663_at	NCAPG	1.12	0.01042	0.38	0.30761	0.75	0.02087
224428_s_at	CDCA7	1.13	0.02846	-0.43	0.23863	1.56	0.00100
213007_at	FANCI	1.13	0.00022	0.38	0.24789	0.75	0.03423
204887_s_at	PLK4	1.13	0.00507	0.37	0.18491	0.76	0.03883
218662_s_at	NCAPG	1.13	0.00057	0.42	0.20417	0.71	0.03897
218349_s_at	ZWILCH	1.16	0.02263	0.03	0.90736	1.13	0.01839
205024_s_at	RAD51	1.18	0.00337	0.26	0.54978	0.92	0.04302
229538_s_at	IQGAP3	1.19	0.00664	-0.13	0.60192	1.31	0.01032
201930_at	MCM6	1.20	0.00107	0.20	0.45835	1.00	0.00525
203755_at	BUB1B	1.24	0.00351	0.07	0.82610	1.17	0.02405
209773_s_at	RRM2	1.26	0.00024	0.39	0.19351	0.87	0.01165

218039_at	NUSAP1	1.27	0.00757	0.31	0.40395	0.96	0.03510
203968_s_at	CDC6	1.28	0.02246	0.29	0.32191	0.99	0.01957
201890_at	RRM2	1.29	0.00016	0.50	0.12085	0.79	0.03793
214079_at	DHRS2	1.29	0.03254	0.11	0.56399	1.18	0.04970
229674_at	SERTAD4	1.29	0.02296	-0.44	0.26281	1.72	0.00080
202870_s_at	CDC20	1.30	0.00089	0.02	0.95673	1.28	0.00860
202094_at	BIRC5	1.31	0.00174	0.38	0.26229	0.94	0.02502
232238_at	ASPM	1.31	0.00799	-0.08	0.71797	1.39	0.00562
225834_at	FAM72A /// FAM72B /// FAM72C /// FAM72D	1.34	0.02458	0.05	0.91000	1.29	0.02075
225655_at	UHRF1	1.35	0.00046	0.33	0.23152	1.02	0.01107
204162_at	NDC80	1.35	0.01855	0.47	0.34435	0.87	0.04341
204962_s_at	CENPA	1.36	0.00366	0.29	0.34593	1.07	0.02966
220651_s_at	MCM10	1.39	0.00985	0.15	0.55465	1.24	0.01717
218585_s_at	DTL	1.39	0.00054	0.30	0.24195	1.09	0.01046
221521_s_at	GINS2	1.43	0.00238	0.38	0.24930	1.05	0.00177
201291_s_at	TOP2A	1.43	0.00006	0.20	0.44896	1.23	0.00406
223307_at	CDCA3	1.44	0.00038	0.40	0.29179	1.04	0.03791
218355_at	KIF4A	1.48	0.00161	0.47	0.10761	1.02	0.01805
220085_at	HELLS	1.48	0.00294	-0.52	0.11590	2.00	0.00293
203418_at	CCNA2	1.49	0.01691	0.13	0.69952	1.36	0.00939
202095_s_at	BIRC5	1.50	0.01903	0.03	0.93683	1.48	0.00495
204822_at	TTK	1.60	0.00492	0.37	0.42681	1.22	0.03391
209642_at	BUB1	1.69	0.00057	0.29	0.21780	1.41	0.00431
219978_s_at	NUSAP1	1.79	0.00229	0.41	0.19096	1.38	0.00200
209714_s_at	CDKN3	2.06	0.01192	1.07	0.11025	0.99	0.02394

Table 3. List of the 65 probes for triangle D7S–D7C–D12C with an FC cutoff equal 2 (log2FC = 1).

4. Discussion

The triangular analysis performed based on Fig. 4 gives a list of genes where the electromagnetic stimulation causes the same up- or down-regulation of gene expression that we observe in a control sample collected at a later time. So we observe a phenomenon that occurs naturally in the control group but happens faster in the stimulated group.

When analyzing the FC values, we observe that the modification of gene expression induced by stimulation may be exactly the same, slightly lower or slightly higher than the change, in the expression of control at different sampling moments ($p < 0.05$). It shows that genes whose regulation is accelerated by the electrical stimulation can put a longer or shorter time in the control group to reach the same level of regulation.

It is interesting to note that stimulation accelerates a majority of down-regulation in gene expression at time D4 (triangle D4S–D4C–D7C or D4S–D4C–D12C) while for the time D7 (triangle D7S–D7C–D12C) stimulation accelerates mainly the up regulation of some gene.

The Reference Sequence (RefSeq) Project (The NCBI handbook [Internet]) can give us some information on the functions of genes listed in Table 1, Table 2 and Table 3. For example,

- MCM4, MCM5, MCM6, MCM10 encoded for proteins that are essential for the initiation of eukaryotic genome replication

- CDC20 appears to act as a regulatory protein interacting with several other proteins at multiple points in the cell cycle
- CDC6 as a regulator at the early steps of DNA replication
- UHRF1 and CDCA3 play a role in the cell division cycle
- RRM2 encodes subunits for ribonucleotide reductase. This reductase catalyzes the formation of deoxyribonucleotides from ribonucleotides
- ZNF638 is associated with packaging, transferring, or processing of the transcripts
- TSPAN1 mediate signal transduction events that play a role in the regulation of cell development, activation, growth and motility
- ZWILCH plays a role in the M phase of mitotic cell cycle
- U2AF1 plays a role in RNA splicing and mRNA processing
- TOP2A: this nuclear enzyme is involved in processes such as chromosome condensation, chromatid separation, and the relief of torsional stress that occurs during DNA transcription and replication.
- TMPO: the protein encoded by this gene resides in the nucleus and may play a role in the assembly of the nuclear lamina, and thus help maintain the structural organization of the nuclear envelope

In this list of genes, with an accelerated regulation in the stimulated compared with the control, we identify also genes that play a role in cell proliferation and differentiation:

• DKK1 (Table 4)

	Probes	**Gene symbol**	**LogFC**	**p-value**	**LogFC**	**p-value**	**LogFC**	**p-value**
			D4S/D4C		**D4S/D7C**		**D7C/D4C**	
Triangle D4S-D4C-D7C	204602_at	DKK1	2.14	0.01417	-0.51	0.55390	2.65	0.01115
			D4S/D4C		**D4S/D12C**		**D12C/D4C**	
Triangle D4S-D4C-D12C	204602_at	DKK1	2.14	0.01417	-1.51	0.10874	3.66	0.00236

Table 4. D4S–D4C–D7C and D4S–D4C–D12C triangles data for DKK1 gene.

DKK1 function is clearly identified as an inhibitor of WNT signaling pathway and evidence is provided that down-regulation of Wnt signaling is required for the induction of cells differentiation (Boyden et al., 2002; van der Horst et al., 2005) and the reduction of cells proliferation (Pasca di Magliano et al., 2007).

• SPRR3 (Table 5)

	Probes	Gene symbol	LogFC	p-value	LogFC	p-value	LogFC	p-value
			D4S/D4C		D4S/D7C		D7C/D4C	
Triangle D4S-D4C-D7C	218990_s_at	SPRR3	1.06	0.03100	1.05	0.10150	2.10	0.01939
			D4S/D4C		D4S/D12C		D12C/D4C	
Triangle D4S-D4C-D12C	218990_s_at	SPRR3	1.06	0.03100	-1.36	0.13035	2.42	0.03237

Table 5. D4S–D4C–D7C and D4S–D4C–D12C triangles data for SPRR3 gene.

SPRR3 is strongly induced during differentiation of human epidermal keratinocytes in vitro and in vivo (Gibbs et al., 1993) and SPRR is expressed in close association with epidermal differentiation in normal skin (Koizumi et al., 1996).

• NDRG4 (Table 6)

	Probes	Gene symbol	LogFC	p-value	LogFC	p-value	LogFC	p-value
			D4S/D4C		D4S/D7C		D7C/D4C	
Triangle D4S-D4C-D7C	209159_s_at	NDRG4	1.21	0.04193	0.0008	0.99838	1,216	0.00340
			D4S/D4C		D4S/D12C		D12C/D4C	
Triangle D4S-D4C-D12C	209159_s_at	NDRG4	1.21	0.04193	0.24	0.63925	0.98	0.03913

Table 6. D4S–D4C–D7C and D4S–D4C–D12C triangles data for NDRG4 gene.

The NDRG (N-Myc downstream-regulated gene) family, consisting of NDRG1, NDRG2, NDRG3, and NDRG4, are a group of structurally related proteins with roles in cell proliferation, differentiation, apoptosis, stress responses, and cell migration/metastasis. The expression of these proteins is up-regulated during cell differentiation and suppressed in several tumor cells (Qu et al., 2002; Shimono et al., 1999; Zhou et al., 2001).

NDRG4 protein may participate in regulating processes that lead to cellular differentiation and neurite formation (Ohki et al., 2002).

• CHEK1 (Table 7)

			D7S/D7C		D7S/D12C		D12C/D7C	
	Probes	Gene symbol	LogFC	p-value	LogFC	p-value	LogFC	p-value
Triangle D7S-D7C-D12C	205394_at	CHEK1	1.10	0.02341	-0.33	0.56374	1.42	0.03497

Table 7. D7S–D7C–D12C triangle data for CHEK1 gene.

The overexpression of CHEK1 caused a mitosis G2 cell cycle arrest (Tapia-Alveal et al., 2009). CHEK1 plays an essential but poorly defined role in the proliferation of unperturbed cells (Wilsker et al., 2008).

We can also observe that some genes are linked to known biological pathway:

• UBE2D3 (Table 8)

	Probes	Gene symbol	LogFC	p-value	LogFC	p-value	LogFC	p-value
			D4S/D4C		D4S/D7C		D7C/D4C	
Triangle D4S-D4C-D7C	240383_at	UBE2D3	-1.50	0.00537	-0.75	0.11777	-0.75	0.04697
			D4S/D4C		D4S/D12C		D12C/D4C	
Triangle D4S-D4C-D12C	240383_at	UBE2D3	-1.5	0.00537	-0.23	0.63561	-1.26	0.01850

Table 8. D4S–D4C–D7C and D4S–D4C–D12C triangles data for UBE2D3 gene.

UBE2D3 plays a role in BMP signaling pathway (Chen et al., 2004). The role of BMPs in osteogenesis and fracture healing has already been recognized (Hinsenkamp and Collard, 2011).

5. Conclusions

The triangular analysis performed here helps us to investigate whether the ELF stimulation accelerates some natural cellular processes. Our results show that ELF stimulation accelerates the up or down-regulation of some genes which in normal circumstances will follow that particular trend (up or down-regulation) but in a slower manner.

Many of genes listed in Table 1, Table 2 and Table 3 play a role in mitosis, cell cycle, cell development, DNA replication transcription or translation. The other genes not described have either the same type of function or functions not yet well known, unclear, unprecise or some probes have not yet received a gene symbol and does not provide specific function information.

Some genes identifies within this study are involved in cell proliferation and differentiation. These new results provide further justification for the previous observations: the acceleration of the differentiation at the expense of proliferation is observed on different in vitro and in vivo biological model exposed to a specific electric fields.

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