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Cellular processes involved in human epidermal cells exposed to extremely low frequency electric fields☆

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ABSTRACT

We observed on different tissues and organisms a biological response after exposure to pulsed low frequency and low amplitude electric or electromagnetic fields but the precise mechanism of cell response remains unknown. The aim of this publication is to understand, using bioinformatics, the biological relevance of processes involved in the modification of gene expression. The list of genes analyzed was obtained after microarray protocol realized on cultures of human epidermal explants growing on deepidermized human skin exposed to a pulsed low frequency electric field. The directed acyclic graph on a WebGestalt Gene Ontology module shows six categories under the biological process root: "biological regulation", "cellular process", "cell proliferation", "death", "metabolic process" and "response to stimulus". Enriched derived categories are coherent with the type of *in vitro* culture, the stimulation protocol or with the previous results showing a decrease of cell proliferation and an increase of differentiation. The Kegg module on WebGestalt has highlighted "cell cycle" and "p53 signaling pathway" as significantly involved. The Kegg website brings out interactions between FoxO, MAPK, JNK, p53, p38, PI3K/Akt, Wnt, mTor or NF-KappaB. Some genes expressed by the stimulation are known to have an exclusive function on these pathways. Analyses performed with Pathway Studio linked cell proliferation, cell differentiation, apoptosis, cell cycle, mitosis, cell death *etc.* with our microarrays results. Medline citation generated by the software and the fold change variation confirms a diminution of the proliferation, activation of the differentiation and a less well-defined role of apoptosis or wound healing. Wnt and DKK functional classes, DKK1, MACF1, ATF3, MME, TXNRD1, and BMP-2 genes proposed in previous publications after a manual analysis are also highlighted with other genes after Pathway Studio automatic procedure. Finally, an analysis conducted on a list of genes characterized by an accelerated regulation after extremely low frequency pulsed stimulation also confirms their role in the processes of cell proliferation and differentiation. Bioinformatics approach allows in-depth research, without the bias of pre-selection, on cellular processes involved in a huge gene list.

1. Introduction

We observed in our laboratory a biological response after exposure of the following different tissues to low frequency and low amplitude electric or electromagnetic fields:

- bones of mouse embryos *in vitro* [1],
- bones of chicken embryos *in vivo* [2],
- bones of quail embryo *in vivo* [3],
- human epidermal explant culture *in vitro* [4,5].

Considering the very coherent response of our biological models to specific low frequencies and low-amplitude asymmetric charge-balanced pulse-train modulated electric or electromagnetic fields, we tried to identify the mechanism involved at the cell level through microarray screening.

An analysis of potential metabolic networks and potential changes in cellular functions of all the up- and down-regulated data from microarray studies is a very useful method to maximize information. In 2006, Leszczynski [6] explained the interest on a screening approach using high-throughput transcriptomics techniques to determine the molecular targets of an electromagnetic field (EMF) on the sub-cellular level. He is convinced that a systematic screening will generate a large database that will allow scientists to formulate more accurate hypotheses concerning the mechanisms of biological effects of EMF.

To achieve our goal, we used a model of human epidermal explants cultured on deepidermized and devitalized human skin close to

Abbreviations: Akt, serine–threonine kinase; DAG, directed acyclic graph; DnC, sampling time at day n of a Control explant; DnS, sampling time at day n of a Stimulated explant; ELF, extremely low frequency; EMF, electromagnetic field; ERKs, extracellular signal-regulated kinases; FAK, focal adhesion kinase; FC, fold change; FoxO, Forkhead box O; GO, Gene Ontology; Jak/STAT, Janus kinase/signal transducers and activators of transcription; JNK, c-Jun N-terminal kinases; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinases; TGF-β1, transforming growth factor β1; WebGestalt, WEB-based GEne SeT AnaLysis Toolkit

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physiologic conditions [4,5]. Screening of gene expression variation on our biological model after electrical stimulation was realized with microarray chips U133 Plus 2.0 [5].

The amount of data obtained by microarray screening techniques is so huge that many analyses can be considered and published with the same data set [7]. We published in 2011 our first observations obtained after a manual analysis conducted on microarrays results [5,8] and in 2013, an original statistical approached allowed us to validate an hypothesis on the acceleration of the differentiation after extremely low frequency (ELF) stimulation [9]. It is still possible to find more information on these microarray screening data sets and bioinformatics can help generate a significant amount of results such as active links between genes or highlighted biological processes expressed by our stimulation.

The aim of this publication is to understand, with the help of bioinformatics, the biological relevance of the processes expressed by the modification of gene expression in our data set obtained in 2011.

WebGestalt (WEB-based GEne SeT AnaLysis Toolkit) [10] was used as an interface for a functional enrichment analysis using Gene Ontology (GO) [11] and Kyoto Encyclopedia of Genes and Genomes (KEGG) [12,13]. GO project provides a structured representation of a set of concepts and their relationships with defined terms representing gene properties. The analysis call "Gene-set enrichment" can be used to functionally profile a large set of genes and to determine which GO terms appear more frequently than would be expected by chance. This technique finds functionally coherent gene-sets, such as pathways, that are statistically over-represented in a given gene list. KEGG is a database resource for understanding high-level functions and utilities of the biological system from large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies. Pathway Studio software (Ariadne Genomics) [14] allowed the characterization of a biological association network supported by Medline publications related to the differentially expressed genes.

2. Materials and methods

2.1. Biological model

Human epidermal explants were cultured on deepidermized and devitalized human skin close to physiologic conditions. Strips of skin were obtained from abdominoplasty after plastic surgery. Epidermal and hypodermal layers from the harvested skin were removed and cells contained in the dermal layer were destroyed by freeze–thaw cycles and gamma radiation. Finally, this dermis, which will served as culture support, is cut into a 60 mm × 30 mm rectangle. For epidermis explants, a thin layer of epidermis is removed from the skin with a Wagner's dermatome and a punch biopsy is used to cut a 3 mm circle from this harvested epidermis thin layer. Six epidermal explants are placed on the dermal support itself lying on foam. The model is completed by the culture medium. The explant culture was repeated on three different subjects. Eighty-four explants per subjects were divided into control and exposed groups and distributed in 14 Petri dishes. The details for the biological model and culture protocol are explained in Collard et al. [5].

2.2. Electrical stimulation

After 3 days, when the explants are attached to the dermal support, two platinum electrodes are placed on each side of the dermal support and are used to apply the electric signal. Stimulation is realized with a generator producing a pulsed biphasic, asymmetric, charge-balanced current signal with a carrier frequency of 40 Hz and peak current amplitude of 20 mA. The stimulus is repeated during 4 s followed by a 4 s break, for 40 min/day for 11 days. The control group will not be connected to the generator and did not receive stimulation. The electrical stimulation pattern details are explained in Collard et al. [5].

2.3. RNA extraction, microarray and real-time rtPCR

Samplings for microarray analysis were done at days 1, 4, 7 and 12 (Fig. 1). After sampling, the total RNA was extracted from a pool of 12 explants in each sampling condition.

Total RNA of each pool was extracted using a Qiagen RNeasy Mini Kit (Dusseldorf, Germany) after homogenization by a rotor-stator. RNA quality and quantities were measured respectively with the capillary electrophoresis system of Agilent Technologies (Santa Clara, CA) and the spectrometry system of NanoDrop Technologies (Wilmington, DE).

The gene expressions are analyzed following the procedure recommended by Affymetrix (Santa Clara, CA) using Affymetrix microarray U133 Plus 2.0 chips. Quality control was assessed based on 3′/5′ ratios of glyceraldehyde 3-phosphate dehydrogenase and b-actin control probe sets. Normalization and statistical analysis of microarray data were performed using ArrayAssist1 Expression Software (Agilent Technologies-Stratagene Products, La Jolla, CA) for the analysis of variance (ANOVA) and k-means analysis. For Student's t-tests, p-values were performed individually for each sampling time comparison.

To validate the microarray results, real-time rtPCR was performed using an Applied Biosystems 7500 Fast Real-Time PCR System and Power SYBR Green PCR Master Mix (Foster City, CA) to evaluate the expression of five transcripts in all of the RNA samples used for microarray analyses. The results showed similar expression in control and stimulated samples as microarray data.

The details for RNA extraction, microarray and real-time rtPCR are explained in Collard et al. [5].

2.4. Bioinformatics

For this analysis, we used the entire list of probes differentially expressed found in D4S *vs.* D4C and D7S *vs.* D7C and D12S *vs.* D12C comparisons in order to have all genes having their expression modified by the stimulation in early and later times to highlight a maximum metabolic interaction. The complete list was presented in "Supplementary Table 1" with the denomination "List A".

Probe sets were defined as differentially expressed if the fold change (FC) was ≥ 2 or ≤ -2 and the p-value was ≤ 0.05 after unpaired Student's *t*-test for one of the following ratio: $D(4, 7, \text{or } 12)_{\text{stimulated}}$ *versus* $D(4, 7, \text{or } 12)_{\text{control}}$ time points.

Different tools were used to analyze "List A": GO module on WebGestalt, KEGG module on WebGestalt, KEGG website and Pathway Studio.

To facilitate reading, the various analyses performed on "List A" with bioinformatics tools (GO module on WebGestalt, KEGG module on WebGestalt, KEGG website and Pathway Studio) will be presented just before each result.

3. Results

3.1. WebGestalt

WebGestalt website is designed for large-scale genetic studies from which high-throughput datasets are generated. A gene can be visualized and organized by a user-selected method (GO, KEGG *etc.*). He can also perform a statistical analysis to suggest areas of interest with respect to the set of genes selected.

Parameters introduced in the WebGestalt stating page analysis are "List A" for the Data, "hsapiens" for Organism, the Id Type was "affy_hg_u133_plus_2", the Ref Set was "affy_hg_u133a_plus_2", "Hypergeometric" for Statistic, the level of significance was fixed to 0.01, the multiple test adjustment was set to "Benjamini & Hochberg (1995)" and the minimum number of genes for a category was fixed at "2".

The analysis, using these parameters, generated the following output: the total number of IDs in "List A" was 836; 622 IDs can