

Research article

Open Access

Electric pulses used in electrochemotherapy and electrogene therapy do not significantly change the expression profile of genes involved in the development of cancer in malignant melanoma cells

Vid Mlakar¹, Vesna Todorovic², Maja Cemazar^{2,3}, Damjan Glavac¹ and Gregor Sersa^{*3}

Address: ¹Department of Molecular Genetics, Institute of Pathology, Faculty of Medicine, University of Ljubljana, Korytkova 2, SI-1000 Ljubljana, Slovenia, ²College of Health Care Izola, University of Primorska, Polje 42, SI-6310 Izola, Slovenia and ³Department of Experimental Oncology, Institute of Oncology Ljubljana, Zaloska cesta 2, SI-1000 Ljubljana, Slovenia

Email: Vid Mlakar - vid.mlakar@mf.uni-lj.si; Vesna Todorovic - vesna.todorovic@vszi.upr.si; Maja Cemazar - mcemazar@onko-i.si; Damjan Glavac - damjan.glavac@mf.uni-lj.si; Gregor Sersa* - gsersa@onko-i.si

* Corresponding author

Published: 26 August 2009

Received: 31 March 2009

BMC Cancer 2009, 9:299 doi:10.1186/1471-2407-9-299

Accepted: 26 August 2009

This article is available from: <http://www.biomedcentral.com/1471-2407/9/299>

© 2009 Mlakar et al; licensee BioMed Central Ltd.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/2.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Background: Electroporation is a versatile method for *in vitro* or *in vivo* delivery of different molecules into cells. However, no study so far has analysed the effects of electric pulses used in electrochemotherapy (ECT pulses) or electric pulses used in electrogene therapy (EGT pulses) on malignant cells. We studied the effect of ECT and EGT pulses on human malignant melanoma cells *in vitro* in order to understand and predict the possible effect of electric pulses on gene expression and their possible effect on cell behaviour.

Methods: We used microarrays with 2698 different oligonucleotides to obtain the expression profile of genes involved in apoptosis and cancer development in a malignant melanoma cell line (SK-MEL28) exposed to ECT pulses and EGT pulses.

Results: Cells exposed to ECT pulses showed a 68.8% average survival rate, while cells exposed to EGT pulses showed a 31.4% average survival rate. Only seven common genes were found differentially expressed in cells 16 h after exposure to ECT and EGT pulses. We found that ECT and EGT pulses induce an HSP70 stress response mechanism, repress histone protein H4, a major protein involved in chromatin assembly, and down-regulate components involved in protein synthesis.

Conclusion: Our results show that electroporation does not significantly change the expression profile of major tumour suppressor genes or oncogenes of the cell cycle. Moreover, electroporation also does not change the expression of genes involved in the stability of DNA, supporting current evidence that electroporation is a safe method that does not promote tumorigenesis. However, in spite of being considered an isothermal method, it does to some extent induce stress, which resulted in the expression of the environmental stress response mechanism, HSP70.

Background

Electroporation, as a physical method for the delivery of molecules into cells, was developed in 1982 [1]. However, since then it has been developed not only for *in vitro* use but also for *in vivo* use in a variety of applications [2]. Electroporation is of interest as a gene delivery method because, unlike transduction with viruses, it eliminates the risks and limitations linked to the use of viruses. In addition, in spite of extensive research, efficient and safe chemical vectors have not yet been developed for *in vivo* gene delivery [3]. Using appropriate electrical parameters, destabilization of the membrane is reversible, ensuring a high survival of permeabilized cells and the delivery of non-permeant molecules inside the cell, bypassing the normal internalisation route of these molecules [4].

The advantages of electroporation have recently been used by different groups for a novel approach to introducing chemotherapeutics in a variety of tumours, called electrochemotherapy [5-7]. Electrochemotherapy facilitates chemotherapeutic drug delivery into cells by increasing cell membrane permeability under specific electric pulses [4]. It is an effective local treatment for patients with cutaneous and subcutaneous tumour nodules, on the basis of the synergistic association of locally applied electric pulses and low permeant chemotherapeutics such as bleomycin and cisplatin. Moreover, several clinical trials with the same chemotherapeutics showed a good response of melanoma tumour nodules, as well as of other tumour types [5,6,8-10].

As mentioned earlier, electrochemotherapy is not the only application of electroporation. There are an increasing number of applications in which electroporation might be used. Electroporation is frequently used as a method of *in vitro* transfection of genetic materials into prokaryotic or eukaryotic cells. With the development of electric pulse generators, the method has also been used *in vivo* for naked DNA transfection in various rodent tissues, in order to treat various diseases and for vaccination [11-13]. The first clinical trial has also been reported for the treatment of melanoma nodules in patients with plasmid DNA encoding interleukin-12 [14].

The effect of electroporation on the level of cell genetic response has only been studied in muscle cells [15,16]. However, the effect of ECT and EGT pulses on malignant cells have not yet been analysed. In the present work, therefore, we studied the effect of ECT and EGT pulses on human malignant melanoma cells *in vitro*, in order to understand and predict the possible effect of electric pulses on gene expression and their possible effect on cell behaviour.

Methods

Cell line

Human malignant melanoma cells SK-MEL28 (HBT-72; American Type Culture Collection, USA) were grown as a monolayer in minimum essential medium (MEM) with Glutamax (Gibco, Paisley, UK), supplemented with 10% fetal bovine serum (FBS; Gibco) and gentamicin (30 µg/mL) (Gibco). Cells were routinely subcultured twice a week and incubated in an atmosphere with 5% CO₂ at 37°C.

Electroporation protocol

Confluent cell cultures were trypsinized, washed in MEM with FBS for trypsin inactivation and once in electroporation buffer (125 mM saccharose; 10 mM K₂HPO₄; 2.5 mM KH₂PO₄; 2 mM MgCl₂ · 6H₂O) at 4°C. The final cell suspension was prepared in electroporation buffer at 4°C, at a concentration of 22 × 10⁶ cells/mL. Aliquots of the final cell suspension (3 × 10⁶ cells) were placed between two parallel electrodes with a 2 mm gap and subjected to eight electric pulses for ECT pulses (electric field intensity 1300 V/cm, pulse duration 100 µs and frequency 1 Hz) or eight electric pulses for EGT pulses (electric field intensity 600 V/cm, pulse duration 5 ms and frequency 1 Hz). Electric pulses were generated by a GT-1 electroporator (Faculty of Electrical Engineering, Ljubljana, Slovenia). One aliquot of cell suspension was not subjected to any electric pulses and served as the control treatment. After electroporation, cells were incubated at room temperature for 5 minutes, diluted in MEM with FBS and then plated in culture flasks for 16 h for microarray assay.

Cell survival after electroporation

Clonogenic assay was used to determine cell survival after electroporation. After exposure to ECT and EGT pulses, SK-MEL28 were plated at a concentration of 500 cells/dish. After 16 days, colonies were fixed, stained with crystal violet and counted. The plating efficiency and the surviving fraction were calculated. The experiments were performed in triplicate and repeated three times.

RNA extraction

RNA from cells was isolated using TRI REAGENT™ (Sigma Aldrich, St. Louis, USA) and the PureLink™ Micro-to-Midi Total RNA Purification System (Invitrogen, Carlsbad, USA), according to the manufacturer's instructions. Briefly, 16 hours after electroporation, cells were trypsinized, washed in MEM with FBS for trypsin inactivation and resuspended in PBS. After centrifugation at 1500 × g for 5 min, all excess liquid was removed and 1 mL of TRI REAGENT™ was added to each sample. Samples were mixed by hand for 15 s and allowed to stand for 2 – 15 min at room temperature. The resulting mixture was cen-

trifuged at 12000 × g for 15 min at 4°C. The aqueous phase was transferred to a fresh microcentrifuge tube and an equal amount of 70% ethanol was added. Samples were transferred to a PureLink™ Micro-to-Midi Total RNA Purification System column (Invitrogen) and processed according to the manufacturer's protocol. All samples were washed from the column with 75 µl of RNase free water.

Analysis of RNA

The quality of RNA was checked on a Bioanalyzer 2100 (Agilent, Santa Clara, USA) using RNA 6000 Nano Labchip (Agilent, Santa Clara, USA) and 6000 RNA ladder as reference (Ambion, Austin, USA). The concentration and quantity of RNA were determined with ND-1000 (Nanodrop, Wilmington, USA).

Preparation of aaRNA

Preparation of aaRNA was performed with an Amino Allyl MessageAmp™ II aRNA Amplification Kit (Ambion) according to the manufacturer's recommendations. For each hybridization, we labelled 5 µg of non-exposed cells (Cy3) and 5 µg of cells exposed to either ECT or EGT pulses (Cy5) mRNA. After removing the excess dye, the RNAs were dissolved in Nexterion Hybridization solution (Schott Nexterion, Jena, Germany).

Microarrays

Microarrays were prepared with Human Apoptosis Subset v2.0 and Human Cancer Subset v3.0 (Operon, Ebersberg, Germany) 70 mer oligonucleotides and Nexterion 70 mer Oligo Microarraying Kit (Schott Nexterion) slides. A single array contained 2698 different genes, each gene being replicated at least 4 times on each array. Oligonucleotides were spotted using an MG1000 spotter (MicroGrid, Boston, USA), immobilised and stored according to the manufacturer's instructions (Schott Nexterion). All hybridisations were performed on HS400 in duplicate

(Tecan, Salzburg, Austria) according to the manufacturer's instructions (Schott Nexterion). We used an LS200 scanner (Tecan) at 6 µm resolution for scanning the microarrays.

Data analysis

We used Array-Pro Analyzer 4.5 (Media Cybernetics, Bethesda, USA) for feature extraction after imaging of microarrays. Acuity (Molecular devices, USA) was used for the filtration of bad signals, LOWESS fit and microarray data analysis. Features showing a signal intensity of more than 65000 were flagged as bad. Features with a signal less than 2 times the intensity of the background or coefficient of variation (CV, ratio between standard deviation of the background and the median feature intensity) greater than 0.3 were considered not significantly expressed and were filtered out. Log₂ ratios were normalized using LOWESS fit [17] and the median of four replicates was used to calculate the average gene expression for a single sample. We filtered out genes that were not expressed in all replicate samples at least 1.5 times.

The Gene Ontology Tree Machine [18] program was used for gene enrichment analysis. All other statistical analyses were done using SPSS 16 (SPSS inc., Chicago, USA).

Results

Cell survival after electroporation

After electroporation of cells, cell viability was assessed by clonogenic assay. Using this method, we determined a 68.8% average survival rate for cells exposed to ECT pulses and a 31.4% average survival rate for cells exposed to EGT pulses.

Microarrays

The difference in expression of genes involved in cancer development was obtained by comparison of malignant melanoma cells exposed to EGT or ECT pulses against the

Table 1: Genes differentially expressed in both ECT and EGT.

Name	RefSeq	Description	Median Log2 ratio
Down			
RPL31	NM_000993	RIBOSOMAL PROTEIN L31	-1.42
CD28	NM_006139	T CELL SPECIFIC SURFACE GLYCOPROTEIN	-0.78
H4FN	NM_175054	HISTONE H4	-0.72
Up			
HSPA1B	NM_014486	NEURONAL THREAD PROTEIN	0.58
CDC25C	NM_005346	HEAT SHOCK 70 KDA PROTEIN I	0.83
CDC25C	NM_001790	M PHASE INDUCER PHOSPHATASE 3	0.70
CCNF	NM_001761	G2/MITOTIC SPECIFIC CYCLIN F	0.79

same untreated malignant melanoma cells. In our experimental design, microarrays with 2698 different genes were used as a dual colour system in which exposed and non-exposed cells' mRNA were separately labelled, mixed and hybridised together on each array. Only microarrays expressing at least 50% of genes were used for further analysis. All oligonucleotides on the same array were spotted in quadruplicate and each microarray analysis was performed in duplicate, thus obtaining eight measurements of the same oligonucleotide. The acquired data were analysed with Acuity 4.0 to select reliable signals. Only genes, 1266 for ECT pulses and 1805 for EGT pulses, present in both duplicated microarrays were considered for further processing. We next checked the variability of replicate measurements on Operon's microarray platform. The average standard deviation of the Log2 ratio

(treated/untreated) of replicates exposed to ECT pulses was 0.21, and 0.17 for replicates exposed to EGT pulses. This gives a standard deviation of 1.16 fold and 1.12 fold from the median value of replicates for ECT pulses and EGT pulses, respectively. Out of 2698 different genes, 7 genes showed differential expression (Table 1), when the groups of ECT and EGT pulses were combined. This represents roughly 0.26% of all genes present on a microarray. However, looking at specifically different pulsation parameters, ECT pulses yielded 34 differentially expressed genes, which is roughly 1% of the interrogated genes, whereas EGT pulses yielded 26 differentially expressed genes, again accounting for roughly 1% of all interrogated genes. When using a cut of value of 2.0, we found only 3 deregulated genes in the treatment with ECT pulses and EGT pulses (Tables 2 and 3).

Table 2: Genes differentially expressed using ECT.

Name	RefSeq	Description	Median Log2 ratio
Down			
RPL31	NM_000993	RIBOSOMAL PROTEIN L31	-1.52
RPS17	NM_001021	40S RIBOSOMAL PROTEIN S17	-1.09
TBCA	NM_004607	TUBULIN SPECIFIC CHAPERONE A	-1.08
PPIA	NM_021130	PEPTIDYLPROLYL CISTRANS ISOMERASE A	-0.97
S100B	NM_006272	S100 PROTEIN, BETA CHAIN	-0.94
	NM_006471	MYOSIN REGULATORY LIGHT CHAIN 2	-0.93
RPA3	NM_002947	REPLICATION PROTEIN A 14 KDA SUBUNIT	-0.89
NQO1	NM_000903	NAD(P)H DEHYDROGENASE [QUINONE] I	-0.88
RPS6	NM_001010	40S RIBOSOMAL PROTEIN S6	-0.81
H4FN	NM_175054	HISTONE H4	-0.80
EEF1A1	NM_001402	ELONGATION FACTOR I ALPHA I	-0.77
ITGB4	NM_000213	INTEGRIN BETA4 PRECURSOR	-0.76
CD28	NM_006139	T CELL SPECIFIC SURFACE GLYCOPROTEIN CD28	-0.75
H3F3A	NM_002107	HISTONE H3.3	-0.74
CASP9	NM_001229	CASPASE 9 PRECURSOR	-0.73
TNFRSF14	NM_003820	TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY MEMBER 14	-0.65
CGB5	NM_033142	CHORIOGONADOTROPIN BETA CHAIN PRECURSOR	-0.64
RPH3AL	NM_006987	RABPHILIN 3 ALIKE	-0.61
TFDP1	NM_007111	TRANSCRIPTION FACTOR DP-1	-0.60
CST3	NM_000099	CYSTATIN C PRECURSOR	-0.59
Up			
RB1	NM_000321	RETINOBLASTOMA I	0.55
RIN2	NM_018993	RAS ASSOCIATION (RALGDS/AF6) DOMAIN CONTAINING PROTEIN	0.56
DNAJB1	NM_006145	DNAJ HOMOLOG SUBFAMILY B MEMBER I	0.56
MATR3	NM_018834	MATRIN 3	0.57
HOXA4	NM_002141	HOMEBOX PROTEIN HOXA4	0.65
RBBP4	NM_005610	CHROMATIN ASSEMBLY FACTOR I SUBUNIT C	0.70
CDC25C	NM_001790	M PHASE INDUCER PHOSPHATASE 3	0.70
	NM_014486	NEURONAL THREAD PROTEIN	0.73
GLIPR1	NM_006851	GLIOMA PATHOGENESIS RELATED PROTEIN	0.75
CRABP2	NM_001878	RETINOIC ACID BINDING PROTEIN II	0.77
RBL2	NM_005611	RETINOBLASTOMA LIKE PROTEIN 2	0.78
CCNF	NM_001761	G2/MITOTIC SPECIFIC CYCLIN F	0.79
HSPA1B	NM_005346	HEAT SHOCK 70 KDA PROTEIN I	0.89
IL6	NM_000600	INTERLEUKIN 6 PRECURSOR (IL6)	0.98

To find the possible biological processes involved in the response to the electroporation procedure, we used the Gene Ontology Tree Machine [18] program for gene enrichment analysis. Our original dataset of genes was compared against differentially expressed genes given in Tables 2 and 3 to check whether there was any significant gene enrichment in comparison to the original gene set. Interestingly, we found significant enrichment of down-regulated genes involved in biosynthesis, regulation of viral genome replication and viral genome replication and significant enrichment of deregulated genes involved in cytokine production in melanoma cells exposed to ECT pulses (Figure 1). Deregulated genes involved in cell division, response to unfolded proteins and response to protein stimulus, were enriched in melanoma cells exposed to EGT pulses (Figure 2).

In order to enable other users comprehensively to interpret and evaluate our results, the original tables of complete microarray results are available in the supplementary data (see the GEO website at [http://](http://www.ncbi.nlm.nih.gov/projects/geo)

www.ncbi.nlm.nih.gov/projects/geo Series entry: GSE15420).

Discussion

In this study, we analysed the expression profile of malignant melanoma cells for genes known to be involved in the development of cancer. This was done in order to assess whether electroporation could lead to an altered expression profile of cells, possibly making them more detrimental to patients. Our results show only minor differences in the expression of genes involved in cancer development. Overall, microarrays showed differential expression of only 7 genes, when using a threshold value of 1.5 fold and only 1 gene when using a threshold value of 2.0 fold (Table 1). When calculating the standard deviation of measurements across the microarray, we found it to be very low (1.16 and 1.12 fold for ECT and EGT pulses replicates) showing that the 1.5 fold threshold change is a reasonable and reliable cut-off value. The results obtained are also in agreement with studies performed so far [15,16]. However, these studies used mouse muscle cells

Table 3: Genes differentially expressed using EGT.

Name	RefSeq	Description	Median Log2 ratio
Down			
RET	NM_020975	PROTOONCOGENE TYROSINEPROTEIN KINASE RECEPTOR	-1.364
RPL31	NM_000993	RIBOSOMAL PROTEIN L31	-1.218
PC	NM_022172	PYRUVATE CARBOXYLASE	-1.009
	NM_006590	SNRNP ASSEMBLY DEFECTIVE 1 HOMOLOG	-0.931
IGFALS	NM_004970	INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN	-0.895
CD28	NM_006139	T CELL SPECIFIC SURFACE GLYCOPROTEIN	-0.845
CYP2A7	NM_000764	CYTOCHROME P450 2A7	-0.844
POLR2F	NM_021974	DNA DIRECTED RNA POLYMERASE II	-0.840
	NM_007013	WW DOMAIN CONTAINING PROTEIN I	-0.806
	NM_004881	QUINONE OXIDOREDUCTASE HOMOLOG	-0.795
TIMP2	NM_003255	METALLOPROTEINASE INHIBITOR 2 PRECURSOR	-0.795
	NM_005851	DOC1 RELATED PROTEIN (DOC1R)	-0.753
H4FN	NM_175054	HISTONE H4	-0.648
FGFR1	NM_023111	BASIC FIBROBLAST GROWTH FACTOR RECEPTOR 1 PRECURSOR	-0.599
LIG3	NM_013975	DNA LIGASE III	-0.582
SKP2	NM_032637	S PHASE KINASE ASSOCIATED PROTEIN 2	-0.550
MUC4	NM_004532	MUCIN 4, ISOFORM D	-0.549
PSMB7	NM_002799	PROTEASOME SUBUNIT BETA TYPE 7 PRECURSOR	-0.544
PTPN21	NM_007039	PROTEIN TYROSINE PHOSPHATASE	-0.539
SULT1C1	NM_001056	SULFOTRANSFERASE	-0.520
HSPB2	NM_001541	HEATSHOCK PROTEIN, BETA2	-0.519
Up			
	NM_014486	NEURONAL THREAD PROTEIN	0.575
HSPA1B	NM_005346	HEAT SHOCK 70 KDA PROTEIN I	0.747
CDC25C	NM_001790	MPHASE INDUCER PHOSPHATASE 3	0.759
TTYH1	NM_020659	TWEETY HOMOLOG I	0.775
CCNF	NM_001761	G2/MITOTICSPECIFIC CYCLIN F	0.777

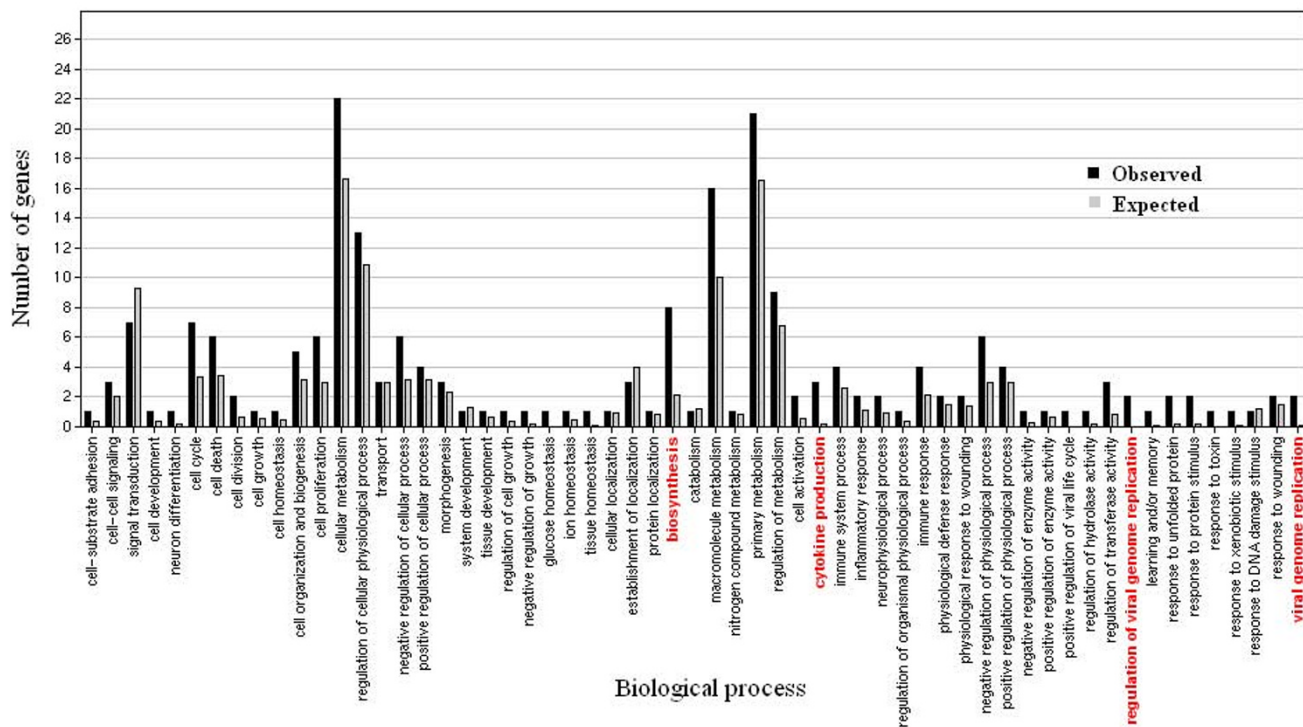


Figure 1
Gene enrichment analysis for cell line exposed to ECT pulses. Expected – number of genes expected to be differentially expressed. Observed – number of genes differentially expressed. In red are GO biological functions significantly enriched in the cell line exposed to ECT pulses.

to account for any damage to tissue or difference in expression profile made by electroporation for immunization purposes. Hojman *et al.* observed only minor histological changes and no changes in muscle performance or the gene expression profile of genes involved in cell death, inflammation or muscle regeneration [15]. Similar results were also obtained by Rubenstrunk *et al.* when they used Stress/Toxicology Atlas cDNA expression arrays. The group found only 2 genes out of 140 to be differentially expressed and concluded that electroporation does not induce expression of genes involved in stress and toxic response [16]. Therefore, despite the fact that only single cell line was used in our study, it is reasonable to expect that other cell lines would behave in similar way.

Interestingly, one of the seven differentially expressed genes is the stress response gene HSPA1B. HSPA1B is a member of the HSPA family of HSP70 proteins and is the strongest stress inducible member of the HSPA family [19]. It has been proposed that HSPA1B is a part of the molecular chaperon network that protects the proteome against environmental stress [20]. This shows that cells exposed to either of the electroporation protocols are

exposed to the stress arising to some extent from a protein denaturation, and therefore over-express HSP70. This observation is also supported by the overexpression of DNAJB1 in cells treated with ECT pulses. DNAJB1, containing a conserved sequence motif (HPD) in the J domain is known to be critical for the acceleration of the ATPase activity of HSP70 [19].

Another interesting observation was downregulation of the histone protein H4 in both treatment protocols and a significant enrichment of downregulated genes involved in protein synthesis. Both results indicate a stall in DNA assembly to chromosomes and biosynthesis of proteins, which could arise from stress.

Conclusion

Overall, our results clearly show that electroporation does not significantly changes the expression profile of major tumour suppressor genes or oncogenes of the cell cycle. Electroporation also does not change the expression of genes involved in the stability of DNA, therefore supporting the notion that electroporation is a safe method that does not promote tumorigenesis. However, in the present

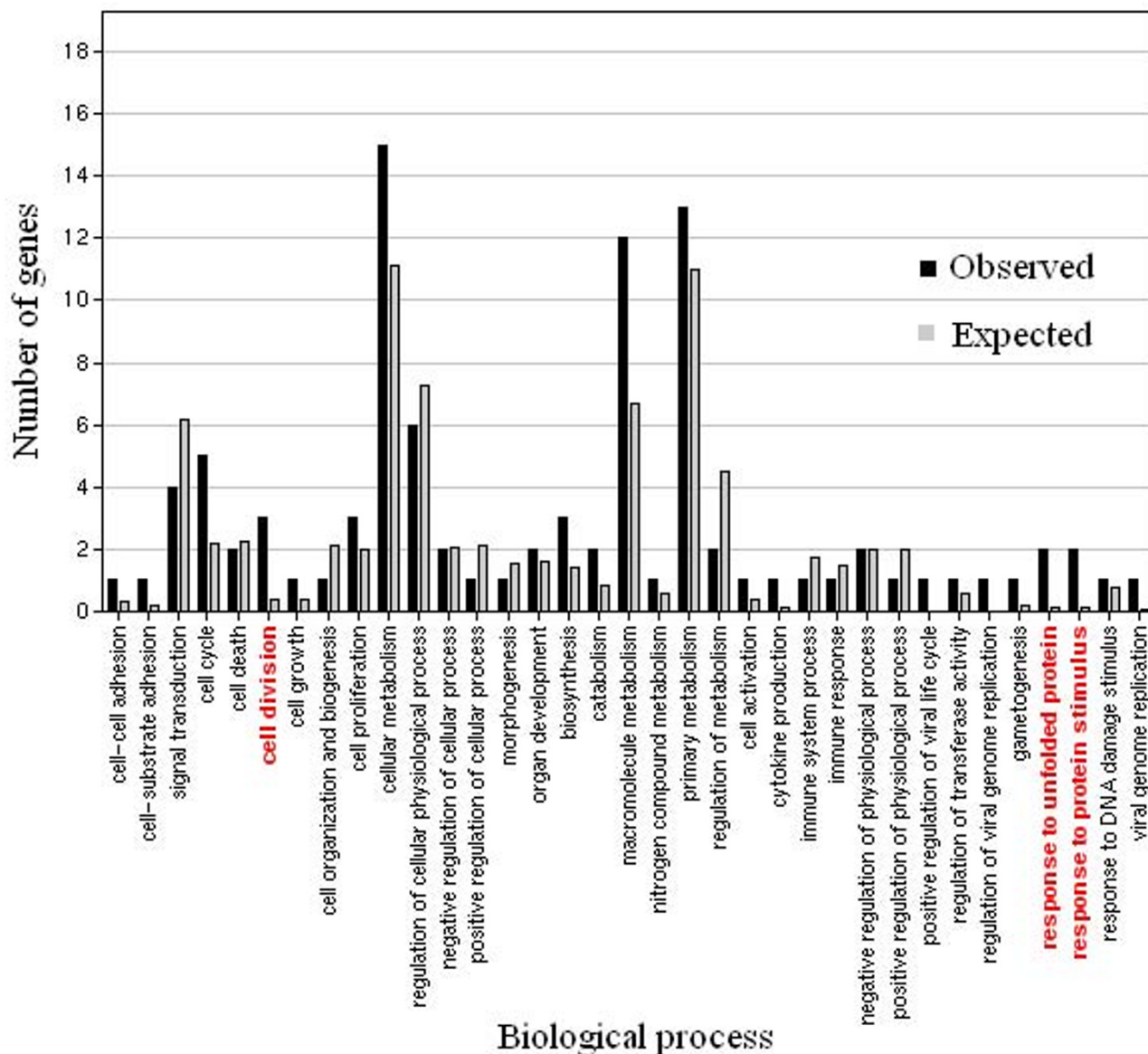


Figure 2
Gene enrichment analysis for cell line exposed to EGT pulses. Expected – number of genes expected to be differentially expressed. Observed – number of genes differentially expressed. In red are GO biological functions significantly enriched in the cell line exposed to EGT pulses.

study, we showed that to some extent electroporation induces HSP70, resulting in the activation of the environmental stress response mechanism.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

VM carried out the isolation of RNA, microarray experiments, data analysis and drafted the manuscript. VT and

MC performed cultivation of cells, electroporation and cell survival. VT also helped to draft the manuscript. MC, DG and GS conceived the study and participated in its design and coordination and critically revised the draft. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by Slovenian Research Agency in program P3-0003 and P3-054.

References

1. Neuman E, Schaefer-Ridder M, Wang Y, Hofschneider PH: **Gene transfer in mouse lymphoma cells by electroporation in high electric fields.** *EMBO J* 1982, **1**:841-845.
2. Gehl J: **Electroporation: theory and methods, perspectives for drug delivery, gene therapy and research.** *Acta Physiol Scand* 2003, **177**:437-47.
3. Barteau B, Chèvre R, Letrou-Bonneval E, Labas R, Lambert O, Pitard B: **Physicochemical parameters of non-viral vectors that govern transfection efficiency.** *Curr Gene Ther* 2008, **8**:313-323.
4. Mir LM, Orłowski S, Belehradek J Jr: **Biomedical applications of electric pulses with special emphasis on antitumor electrochemotherapy.** *Bioelectrochem Bioenerg* 1995, **38**:203-207.
5. Heller R, Jaroszeski M, Glass F, Messina JL, Rapaport DP, DeConti RC, Fenske NA, Gilbert RA, Mir LM, Reintgen DS: **Phase I/II trial for the treatment of cutaneous and subcutaneous tumors using electrochemotherapy.** *Cancer* 1996, **77**:964-971.
6. Marty M, Serša G, Garbay JR, Gehl J, Collins CG, Snoj M, Billard V, Geertsen PF, Larkin JO, Miklavčič D, Pavlović I, Paulin-Kosir SM, Žemazar M, Morsli N, Soden DM, Rudolf Z, Robert C, O'Sullivan GC, Mir LM: **Electrochemotherapy – An easy, highly effective and safe treatment of cutaneous and subcutaneous metastases: Results of ESOP (European Standard Operating Procedures of Electrochemotherapy) study.** *EJC Suppl* 2006, **4**:3-13.
7. Sersa G, Miklavcic D, Cemazar M, Rudolf Z, Pucihar G, Snoj M: **Electrochemotherapy in treatment of tumours.** *Eur J Surg Oncol* 2008, **34**:232-240.
8. Quaglino P, Mortera C, Osella-Abate S, Barberis M, Illengo M, Rissone M, Savoia P, Bernengo MG: **Electrochemotherapy with intravenous bleomycin in the local treatment of skin melanoma metastases.** *Ann Surg Oncol* 2008, **15**:2215-2222.
9. Campana LG, Mocellin S, Basso M, Puccetti O, De Salvo GL, Chiarion-Sileni V, Vecchiato A, Corti L, Rossi CR, Nitti D: **Bleomycin-based electrochemotherapy: clinical outcome from a single institution's experience with 52 patients.** *Ann Surg Oncol* 2009, **16**:191-199.
10. Sersa G, Cemazar M, Miklavcic D, Rudolf Z: **Electrochemotherapy of tumours.** *Radial Oncol* 2006, **40**:163-174.
11. Heller LC, Heller R: **In vivo electroporation for gene therapy.** *Hum Gene Ther* 2006, **17**:890-897.
12. Cemazar M, Sersa G: **Electrotransfer of therapeutic molecules into tissues.** *Curr Opin Mol Ther* 2007, **9**:554-562.
13. Bodles-Brakhop AM, Heller R, Draghia-Akli R: **Electroporation for the Delivery of DNA-based Vaccines and Immunotherapeutics: Current Clinical Developments.** *Mol Ther* 2009, **17**:585-592.
14. Daud AI, DeConti RC, Andrews S, Urbas P, Riker AI, Sondak VK, Munster PN, Sullivan DM, Ugen KE, Messina JL, Heller R: **Phase I trial of interleukin-12 plasmid electroporation in patients with metastatic melanoma.** *J Clin Oncol* 2008, **26**:5896-5903.
15. Hojman P, Zibert JR, Gissel H, Eriksen J, Gehl J: **Gene expression profiles in skeletal muscle after gene electrotransfer.** *BMC Mol Biol* 2007, **8**:56.
16. Rubenstrunk A, Mahfouli A, Scherman D: **Delivery of electric pulses for DNA electrotransfer to mouse muscle does not induce the expression of stress related genes.** *Cell Biol Toxicol* 2004, **20**:25-31.
17. Cleveland WS, Devlin SJ: **Locally weighted regression: an approach to regression analysis by local fitting.** *J Am Stat Assoc* 1988, **83**:596-610.
18. Zhang B, Schmoyer D, Kirov S, Snoddy J: **GOTree Machine (GOTM): a web-based platform for interpreting sets of interesting genes using Gene Ontology hierarchies.** *BMC Bioinformatics* 2004, **5**:16.
19. Vos MJ, Hageman J, Carra S, Kampinga HH: **Structural and functional diversities between members of the human HSPB, HSPA, and DNAJ chaperone families.** *Biochemistry* 2008, **47**:7001-7011.
20. Albanese V, Yam AY-W, Baughman J, Parnot C, Frydman J: **System analyses reveal two chaperone networks with distinct functions in eukaryotic cells.** *Cell* 2006, **124**:75-88.

Pre-publication history

The pre-publication history for this paper can be accessed here:

<http://www.biomedcentral.com/1471-2407/9/299/prepub>

Publish with **BioMed Central** and every scientist can read your work free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime."

Sir Paul Nurse, Cancer Research UK

Your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours — you keep the copyright

Submit your manuscript here:
http://www.biomedcentral.com/info/publishing_adv.asp

