COST EMF - MED (Action BM1309):
European network for innovative uses of EMFs in biomedical applications

STSM Report:

Electromagnetic fields (EMFs) delivery of an active recombinant human glycogen debranching enzyme (GDE) into Glycogen Storage Disease type III (GSDIII) fibroblasts: novel insights for GSDIII management

Researcher: Dr. ROSELLA FRANCONI. email: rosella.franconi@enea.it

Home Institution: ENEA, Italian National Agency for New Technologies, Energy and Sustainable Economic Development, Casaccia Research Center, Rome, Italy. Contact: Dr. Carmela Marino, Division of Health Protection Technologies (Director). email: carmela.marino@enea.it

Host Institution: Laboratory of Vectorology and Anticancer Therapeutics (UMR 8203), Joint research Unit of the Centre National de la Recherche Scientifique (CNRS), the University Paris-Saclay and the Gustave-Roussy, Villejuif Cedéx, France. Contact: Dr. Lluis M. Mir (Director). email: luismir@igr.fr

STSM Reference: ECOST-STSM-BM1309- 39785

STSM dates: FROM: 25th March 2018 TO: 08th April 2018

Abstract:

Glycogen storage disease type III (GSDIII) is a rare disease due to glycogen-debranching enzyme (GDE) deficiency, a cytosolic protein with two distinct enzymatic activities. GSDIII patients present abnormal glycogen accumulation in liver, skeletal and cardiac muscle. The unique available treatment is based on a strict diet.

We recently obtained recombinant GDE as a soluble, stable and active protein. Further, we over-expressed the relevant synthetic gene in HEK 293 cells by lipofectamine-mediated transfection. However, this was not possible with fibroblasts derived from GSDIII patients.

During the STSM we worked to develop protocols for the electro-transfection (gene or protein) of GDE-defective human fibroblasts, with the long-term goal of contributing to a regenerative medicine for GSDIII patients.

A. Purpose of the STSM

Glycogen storage disease type III (GSDIII or Cori/Forbes disease; OMIM #232400) is a rare disorder of glycogenolysis, due mutations of the *Agl* gene, that cause glycogen-debranching enzyme (GDE) deficiency. GDE is a freely diffusible, large monomeric protein (about 170 kDa) with prevalent cytoplasmic localization and two distinct enzymatic activities, 4-α-glucantransferase and amylo-α-1,6-glucosidase plus a glycogen binding domain.

GSDIII patients present abnormal glycogen ('phosphorylase limit dextrin', PLD) accumulation in liver and/or both skeletal and cardiac muscle, with a large heterogeneity among individuals: from an acute disease in childhood (with hepatomegaly as the major symptom), GSDIII develops into a chronic, progressive disease in adulthood, affecting liver, heart, skeletal muscle and bones, and a high risk of developing Type 2 diabetes mellitus.

The unique treatment for GSDIII, at the moment, is based on diet. Gene therapy approaches and enzyme replacement therapy (ERT) are being considered. In the latter case, the main conceptual obstacle is represented by the fact that, contrary to most ERTs developed so far for lysosomal diseases, an exogenous GDE protein should be delivered into the cytosol.

With the aim to develop novel strategies for GSDIII treatment, we recently obtained a synthetic codon-optimized human *Agl* gene. Surprisingly, only this gene (and not the one with the original human nucleotide sequence) could be expressed as a soluble, stable and active protein in *E. coli.* (Demurtas *et al.,* manuscript in preparation). The same gene was introduced by lipofectamine-mediated transfection in Human Embryonic Kidney cells 293 (HEK 293) where it was overexpressed.

We applied the same strategy to human cell lines defective for the *Agl* gene (*Agl*$^{-}$ GM02523 and GM00111 cells, fibroblasts derived from GSDIII patients) that we had previously characterized by immunoblotting, immunofluorescence and electron microscopy demonstrating that GDE is absent as well as the relevant debranching activity (Triggiani *et al.,* manuscript in preparation). However, we were not able to deliver the mammalian expression vector, containing the gene coding for GDE, into these cells by lipofectamine-mediated transfection.

The aim of the STSM performed at UMR 8203, Villejuif, was to explore the potential of using Electro Magnetic Fields (EMFs), and in particular electroporation (EP), on such GDE-defective human fibroblasts to introduce the missing function (as a gene or as a protein). In particular, we worked on optimizing protocols for electro-transfection with the long-term goal of effective introduction of biomolecules (DNA or recombinant enzyme) to be applied for regenerative medicine of GSDIII patients.

All necessary materials and reagents related to STSM were prepared, or purchased, and delivered to UMR 8203 before the STSM period. In particular:

- GM00111 and GM02523 fibroblasts, derived from GSDIII patients.
- pVAX-sGDE plasmid (0.45 □g/□l in H_2O), Endotoxin-Free.
- Purified recombinant GDE protein (1 mg/ml in 100 mM Histidine, 5 mM EDTA, pH 6.0, 'Histidine buffer').

Antibodies: a) immunofluorescence: primary Ab, PA5-12142 (Thermo Fisher), rabbit anti-Human GDE; secondary Ab, A-11034 (Thermo Fisher) goat anti-rabbit IgG (H+L), Alexa Fluor® 488 conjugate; b) immunoblotting: primary Ab, AS09454 (Agrisera) rabbit anti-GDE; c) secondary 31460 (Thermo Fisher) goat anti-rabbit IgG (H+L).

B. Work Description

Electroporation (EP) experiments with GM00111 and GM02523 fibroblasts (or GSDIII fibroblasts) were performed using, basically, the protocol described in Liew A. et al (2013), and human Mesenchymal Stem Cells (hMSCs) and pCMV-GFP, available at UMR8203, as controls.

In general, cells (250.000/cuvette in 25 µl), detached by using TrypLE™ Express (Gibco, Life Technologies Corporation), centrifuged at 400 x g for 10 min, and resuspended in MEM modified for suspension cultures (without calcium and without glutamine, S-MEM) were mixed with the control pCMV-GFP plasmid, carrying the GFP reporter gene under the control of the cytomegalovirus promoter (PlasmidFactory, Bielefeld, Germany, 1 µg/µl, 12.5 µg /cuvette) or the pVAX-sGDE plasmid (25 µl or 12.5 µl, corresponding to 11.25 µg or 5.625 µg /cuvette, respectively)**.** They were transferred to a 1 mm electroporation cuvette (Cell Projects), and electrotransfection was performed using the Cliniporator device (IGEA, Italy) by applying a train of eight square

electric pulses (100 µsec) at amplitude ranges of 1500–2000 V/cm at a 1Hz repetition frequency (8 p.x 1500 V/cm, 100 µs, 1Hz). All steps were performed at room temperature. 15 minutes after electrotransfection, cells were collected and transferred to a 15 ml tube containing 6 ml of complete media. For each EP sample, 1 ml was transferred into each of 3 wells of a 12 well-plate (containing coverslips pre-treated with H_2O, acetone, propanol, ethanol, PBS- for IF) while the remaining 3 ml were put in a 6 well-plate (for Immunoblotting).

Electrotransfection experiments with the recombinant GDE protein diluted in Histidine buffer were performed using three different electric field conditions: 10 p.x 600V/cm, 5ms, 1Hz; 10 p.x 500V/cm, 5ms, 1Hz; 10 p.x 700V/cm, 5ms, 1Hz.

After electrotransfection, cells were analyzed at different time points (in a range included between 3 - 120 hours) by phase contrast, direct fluorescence and/or immunofluorescence microscope analysis. Where relevant, cells were collected and stored at – 80°C for subsequent immunoblotting analysis. Electrotransfected cells were compared with sham electrotransfected controls (no plasmid and no pulse).

For immunofluorescence analysis, cells grown on sterilized coverslips, in a 12 multi-well plate, were washed in PBS. Fixation and permeabilization were performed with 100% methanol and 100% acetone, respectively. After washing, cells were blocked with 1% BSA/PBS for 30'. Primary antibody (PA5-12142, Thermo Fisher), diluted 1:100 in 1% BSA/PBS, was added for 1.5 h. After washing, cells were incubated for 1 h at room temperature in the dark with the secondary antibody (A-11034 Thermo Fisher), diluted 1:1000 in 1% BSA/PBS. Coverslips were mounted with a drop of Fluoromont-G, with DAPI (eBioscience 00-4959-52, Affimetrix Inc. San Diego, CA, USA) and stored in the dark at room temperature until microscope analysis.

C. Results

The main problem encountered, during the all STSM period, was that the GSDIII fibroblasts (delivered in cryotubes in dry ice) once in culture in the host laboratory, had a very slow growth rate, and the number of cells/electroporation experiments was always sub-optimal (≤250.000/cuvette). This represented a main limiting factor for electroporation success.

In spite of this, several experiments could be performed. A preliminary experiment was performed using hMSCs, and for which electrogene transfer (EGT) parameters are well established. Subsequently, other EP experiments using GM00111 and GM02523 fibroblasts were performed. In particular, we demonstrated that:

1) The EGT parameters already set up for the hMSCs and the control pCMV-GFP plasmid can be applied also to the pVAX-sGDE plasmid. In fact, after immunofluorescence on fixed transfected cells, a signal, corresponding to GDE expression, could be detected. Thus, the transfection protocol is reproducible, irrespective of DNA source/dimension (pVAX-sGDE size is about the double of pCMV-GFP, 7605 bp versus 3487 bp, respectively).

2) The same electrogene transfer parameters can be applied to GM00111 and GM02523 fibroblasts that are able to survive after pulsing. Further, when using pCMV-GFP, a direct fluorescence could be detected, demonstrating that these cells can be electrotransfected when a suitable DNA plasmid is used (Fig 1a, b).

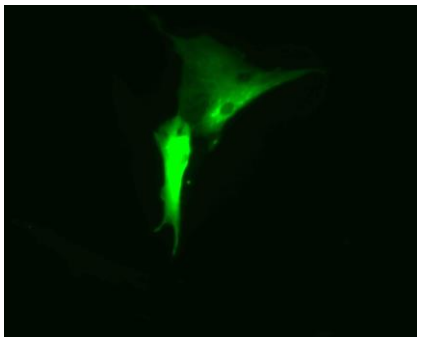
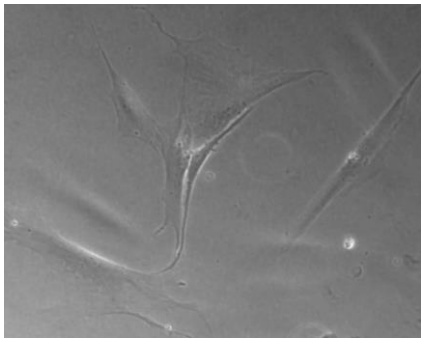
a)  b) 

Fig 1) GM02523 fibroblasts after transfection with pCMV-GFP (same field). a) Direct Fluorescence (GFP) b) phase contrast.

3) GM00111 and GM02523 cells could not be clearly electrotransfected when using the pVAX-sGDE plasmid. Here, a toxic effect of the plasmid was observed, with significant cell death. In some cases, immunofluorescence with the anti-GDE antibody revealed a signal, although this could not always be clearly distinguishable as positive with respect to the background. Experiments with pVAX-sGDE and GM00111/GM02523 cells need to be repeated and optimized (see below).

4) In an attempt to improve viability of GSDIII fibroblasts after electrotransfection with pVAX-GDE, we decreased plasmid concentration of two fold (from 11.5 µg in 25 µl to 5.625 µg in 12.5 µl /cuvette) by addition of S-MEM. In this case, although the toxic effect slightly decreased, again no clear data of occurred transfection could be recorded. Anyway, GSDIII fibroblasts look more sensible to foreign DNA introduction (Fig. 2a, b)

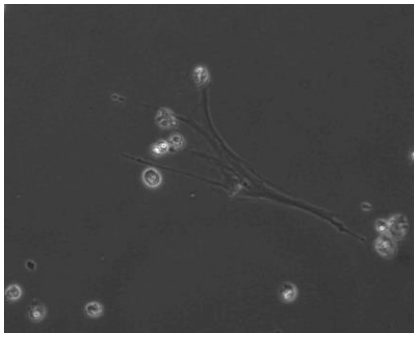
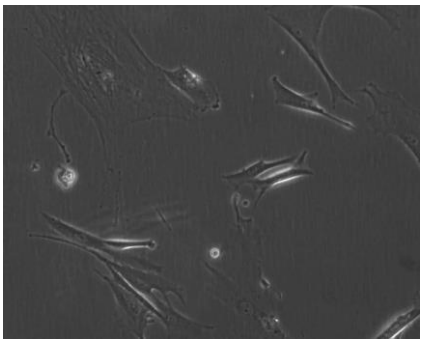
a)  b) 

Figure 2: GM00111 fibroblasts after transfection with pVAX-sGDE (72 h, phase contrast). a) 25 µl of plasmid solution; b) 12.5 µl of plasmid solution

5) GM00111 cells were permeabilized when pulsed at 600 V/cm in the presence of Yo-Pro, 3 µM.

6) GSDIII fibroblasts retain viability after pulsing at 500 V/cm with the purified recombinant GDE protein. However, protein internalization could not be clearly demonstrated. A high mortality occurred after pulsing at 600 and 700 V/cm.

7) Immunoblotting experiments are currently being performed on cell pellets of relevant samples collected after most electrotransfection experiments.

We reasoned about the 'toxic effect' of pVAX-sGDE, that could be attributable to several factors, such as:

a) pVAX-sGDE is more diluted than pCMV-GFP. Thus, plasmid DNA concentration might determine the success of EGT with different cell types. Further, we cannot exclude that the size of pVAX-sGDE, while not significantly affecting hMSCs, is toxic for GSDII fibroblasts.

b) the original human nucleotide sequence of the gene encoding GDE has a GC content of 40.17%, while the sequence optimized for insect expression (sGDE) has a GC content of 52.45%. We cannot exclude that pVAX-sGDE contains more (unmethylated) CpG DNA sequences, that might affect cell viability.

D. Future collaboration with host institution

Adult primary cells, like GSDIII fibroblasts, are difficult to transfect with traditional techniques, as we already demonstrated with lipofectamine-mediated transfection. This limits their use as model host cells for studies of efficacy of new biomolecules *in vitro*. DNA electrotransfer represents a safe method of gene transfer since it does not involve the introduction of potentially harmful foreign particles such as viruses and square-wave electric pulses have already been proven safe and effective in their use in clinical settings. Further, for the recombinant GDE protein, it guarantees proper compartmentalization in the cytosol.

We used defined squarewave electric pulses (8 p. x 1500 V/cm, 100 μs, 1Hz) and buffer (S-MEM with 40% H_2O) that do not rely on a specific electroporator or undisclosed commercial buffers, allowing for simple, cheap, and easy optimization.

The experiments with GSDIII fibroblasts need to be repeated but, nevertheless we showed that they can be transfected with the control plasmid pCMV-GFP by electroporation.

This suggests that it would be worthwhile continuing the research on this topic and that improved protocols (that could not be developed during the STSM for reason of time limitations) could be developed in the future.

As an example, since it is known that increase in transfection efficiency correlates with an increase in plasmid concentration, pVAX-sGDE could be concentrated by evaporation and resuspended in a more concentrated formulation, in water (or another appropriate buffer).

There is also room for developing improved/effective electrotransfection protocols for the GDE protein. Further work has to be performed in order to set up the best transfection conditions, taking into consideration that changes in transfection medium osmolarity modulate the transfection efficiency *in vitro*. Indeed, the buffer in which the protein is diluted ('Histidine buffer', necessary for proper folding and enzymatic activity retention) has an osmolarity of about 200 mosm. Improvement in cell viability and transfection efficiency could be obtained by increasing the amount of water in the protein buffer.

This work, currently supported by AIG (Associazione Italiana Glicogenosi, ONLUS, Via Roma, 2/G 20090 Assago, Milan, Italy) could also be of interest for AGF, L'Association Francophone des Glycogenoses, France (http://www.glycogenoses.org/) for the preparation of joint proposals.

Further, since the aim of this STSM was to further contribute to the cooperation between the two laboratories (CNRS UMR 8203 and ENEA), possible future collaborations can be envisaged also in the field of genetic vaccines (prophylactic and/or therapeutic) against infectious diseases and/or cancer. The ENEA group is working on this topic since several years and the superior effects of the association of genetic vaccines and electroporation has been previously described for Human Papillomavirus (HPV) associated cancers (Massa S, et al. 2017; Paolini F, et al. 2017; Massa S, et al. 2011), in particular when using a novel animal model for HPV-associated oral cancers (Paolini F, et al 2013; Cordeiro MN, et al. 2015). On these subjects, new IP has also been developed (Franconi R, et al. 2016, PCT/IT2017/050008; Franconi, R et al., European patent EP2456785, notified in Italy, France and Germany). Thus, it would be of interest to explore the possibility to enhance the efficacy *in vivo* of the novel DNA vaccines in combination to novel, optimized EP protocols.

E. Expected Publications

As already anticipated, results need to be confirmed and further experiments are needed. However, as a first step, we aim to submit an abstract (deadline for submission: 4 June 2018) to present our preliminary data at **EMF-Med 2018**, 1st EMF-Med World Conference on Biomedical Applications of Electromagnetic Fields that will be held in Split (Croatia), 10-13 September 2018 by

Confirmation by the host institution of the successful execution of the STSM:

We confirm that (Researcher's Name) has performed the research work as described above.

Contact Person of Host Institution

Signature

Name of researcher