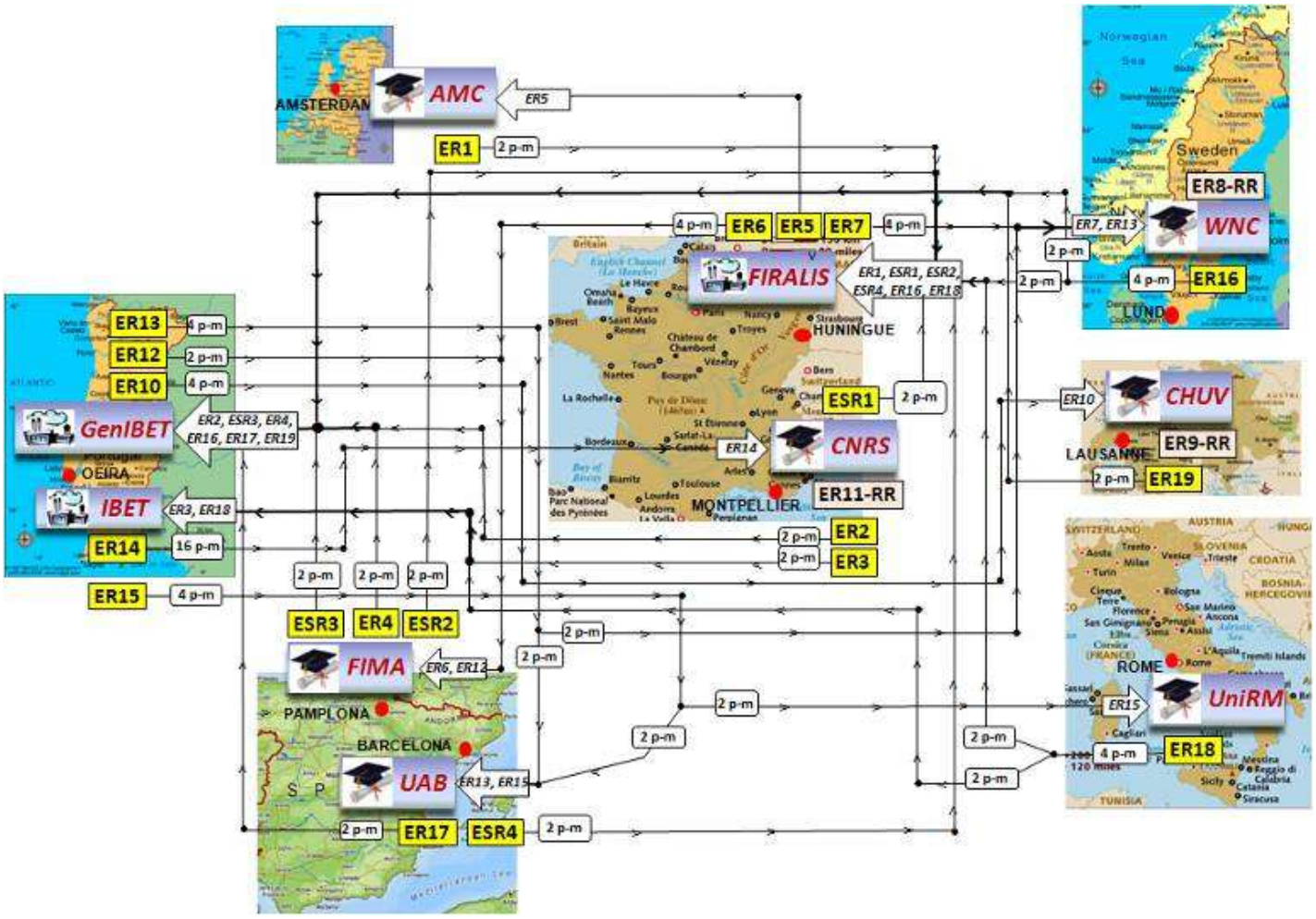
BrainVectors secondments

BrainVectors project implementation

To achieve the objectives of *BrainVectors* IAPP, the ten partners are committed to develop an integrated research program which will involve:

(i) the 3 researchers recruited for two years at the CHUV in Lausanne (CH), at the WNC in Lund (S) and at the CNRS in Montpellier (F)

(ii) a program of inter academia-industry secondments carried out by the staff of the partners institutions, who will visit the laboratories - as drawn in the graphic below - to develop the research activities scheduled in the workprogram.



Legend: ER: experienced researcher; ESR: early stage researcher. These researchers are staff members of the institutions, who visit the partners' institutions

72 person-months are scheduled during the overall duration of the IAPP for these visits

Secondment UniRM-IBET

Staff member:

Stefania PIERSANTI

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Dates: **01 August – 30 September 2013**

Title of the secondment: **3D cultures to characterize the biological interaction of HD-CAV-2 with 3D cultured human neuroprogenitors**

The secondment herein reported was intended to integrate Uniroma1 and IBET expertise to investigate the response of neuronal human cultures to viral vectors as a preliminary step to the analysis of the response of this system to HD-CAV-2 transduction. During Uniroma1/IBET secondment, the whole transcriptome of dopaminergic differentiated human midbrain neural progenitor (hmNPCs) 3D cultures (neurospheres) was analyzed to fully characterize the 3D system.

14 days (14Diff) differentiated neurospheres (Fig.1) where tested for RNA modulations as compared to undifferentiated 2D hmNPCs. Biological replicates of both undifferentiated and differentiated samples were analyzed using the Affymetrix HG U133 plus2 gene chip. A paired t-test was applied between differentiated and undifferentiated groups selecting genes with a False Discovery Rate (FDR) value ≤ 0.01 and with a fold change ≥ 2 for upregulated genes and ≤ -2 for downregulated genes. The gene expression profile demonstrated that 807 probes, which correspond to 664 unique genes, were differently modulated following neurospheres differentiation (Fig. 1).

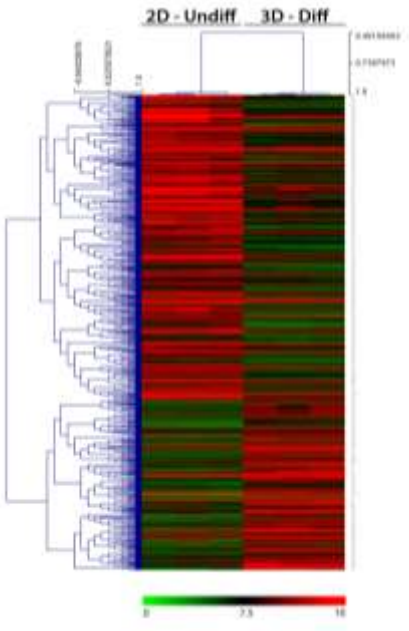

Figure 1. Hierarchical custering of modulated 807 probes, corresponding to 664 unique genes, significantly altered in differentiated neurospheres as compared to the undifferentiated 2D hmNPCs (FDR ≤0.01, 2-fold change in upregulated and downregulated genes). Data of 3 independent cultures; each column represents an independent sample. Color bar represents the scale of RMA-normalized log2-transformed expression value of each transcript.

Gene Ontology analysis performed on modulated genes revealed that the main categories significantly enriched in 3D differentiation are “cell cycle” (corrected p-value (FDR) 4.21E-18),

"cell differentiation" (FDR 3.22E-04), "cytoskeleton organization" (FDR 1.03E-04) and "proteinaceous extracellular matrix" (FDR 2.5E-03) (Fig. 2).

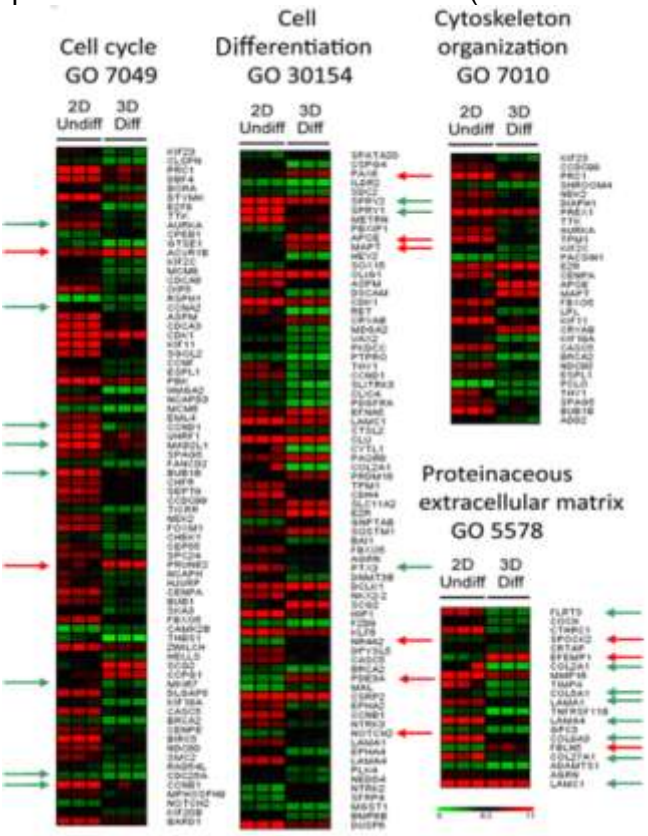

Figure 2. Heat maps of the expression patterns (RMA normalized values) of genes altered in each replicate of four significantly enriched GO categories (corrected p-value≤ 0.01), according to a color scale ranging from green to red. Enriched biological clusters consist in "Cell cycle" (FDR $4.2x10^{-18}$), "Cell differentiation" ($3.2x10^{-4}$), "Cytoskeleton organization" ($2.58x10^{-3}$), "Proteinaceous extracellular matrix" ($6.9x10^{-3}$).

To further characterize the system, targeted transcripts analysis performed at 14 (14Diff) and 32 days (additional 18 days of maturation, 18Mat) of neurosphere differentiation (Diff) with respect to 3D undifferentiated hmNPCs (7Agg) was performed. This revealed a positive modulation of specific dopamine related markers. Extending the differentiation process by additional 18 days in presence of cAMP led to a significant upregulation of these markers (400- and 16-fold increase for TH and DRD2 respectively). Expression of the genes coding for other enzymes involved in dopamine biosynthesis pathway, such as aromatic L-amino acid decarboxylase (AADC), dihydropteridine reductase (DHPR) and GTP cyclohydrolase (GTPCH) was already detected in undifferentiated cells, with no significant upregulation upon differentiation. Neurotrophic receptors such as TRK1 and especially TRK2, receptors of NGF and BDNF respectively, which play a critical role in neuronal differentiation and survival (Jiang et al, 2005), were positively modulated upon differentiation with a 2- and 28-fold increase at 18Mat, respectively . To evaluate genes associated to neuronal activity, we analyzed the expression of transcripts involved in synaptic formation and neuron homeostasis. Expression of different presynaptic markers, such as synaptotagmin I (SYT1) and synaptophysin (SYP), gradually increased during differentiation, reaching up to 6- and 2-fold greater levels by the end of the differentiation process.

Summarizing the data, the characterization of 3D dopaminergic differentiated neurospheres based on a global transcriptional analysis revealed the downregulation of cell cycle genes and of several markers of neural stem cells along with the upregulation of transcripts involved in neurogenesis and maintenance of neuronal differentiation. Noteworthy is the modulation of specific extracellular matrix components, consisting in repression of collagen, laminin and fibronectin interactors and activation of the more physiological glycosaminoglycans binders. A detailed investigation displayed the induction of dopaminergic genes along with neurotrophic receptors and synaptic components, suggestive of mature and functional neurospheres and indicative of a cellular model pertinent for disease modeling and for further viral vectors applications. These results contribute to **Brainvector deliverables 5.1 and 5.2** and will be

integrated with results on the effect of viral vectors on this system in 2D and 3D cultures to fulfill 5.1 and 5.2 deliverables.

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Secondment UAB → IBET

Staff member:
Maria ONTIVEROS
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Dates: 12 may - 12 July 2014

Title of the secondment: **Large scale AAV production and purification in GMP conditions**

A. Project objectives for the period

The objectives of my secondment at Genibet-IBET were:

1. **To learn how to work with viral vectors in GMP conditions**. To this aim, I visited Genibet to review and discuss different topics related to Good Manufacturing Practices conditions, and we discussed how to apply them to our specific viral vector production procedure.
2. **To learn procedures to produce and purify AAV vectors at large-scale**. To this aim, I collaborated in the production and purification of a new AAV1-tetON* vector harbouring a novel candidate immune-escape mutant tet transactivator. This vector will be compared in the future with the AAV1-tetON non-mutant vector.

B. Work progress and achievements during the period.

During the secondment at Genibet-IBET the following goals were achieved:

1. **Viral vectors in GMP conditions.** This first section included:
   - Visiting the Genibet facilities: Bacterial Unit, Cell Culture Unit, Virus Unit, Fill and Finish Unit, Water for Injection (WFI) System.
   - Genibet presentation.
   - GMP presentation: introduction to GMP.
   - GMP Documentation: Good Documentation Practices, Non conformances, Change Controls, Deviations, Out of Specification Results, Concept of Traceability, Qualifications, Calibrations and Maintenace, Standard Operating Procedures.
   - GMP Regulations: PICs, ISO Standards, Eudralex (EU), Europeian Pharmacopoeia (EU), FDA Regulations (USA), US Pharmacopoeia (USA).
   - GMP Quality Control: Product Reception (approval, rejection, quarentine), Warehouse storage, Weighing Room, Sampling of RM, QC Analysis, QC Registers, Water Analysis.

- GMP aseptic handling.
- Units Cleaning and Environmental Monitoring.
- Technology Transfer and Confidentiality.
- Practical: try to design a GMP Batch Production Record for our specific process.

2. Large-scale production and purification of AAV vectors. The following procedures were performed at IBET:

- Development and optimization of a large-scale transfection protocol. This procedure was tested during the production of the AAV1-V16immesc-GFP vector.
- Production of two different AAV1-V16immesc-GFP batches using the double transfection system with PEI in cell factories (CF10), one batch before optimization and a second batch after optimization of the transfection procedure.
- Purification of the former AAV1-V16immesc-GFP batch. A purification protocol combining PEG precipitation, iodixanol gradient purification and Amicon concentration was performed. The resulting purified viral vector preparation was used for further characterization.
- Development and optimization of a Real Time Q-PCR protocol specific for our viral vector titration. Determination of the vector titers using this procedure.
- Quality Control analysis of the former AAV1-V16immesc-GFP batch: determination of contaminant proteins, total DNA determination by Picogreen assay, Nanosight analysis, SDS-PAGE analysis...

Once defined and established, these procedures will be used for the production and characterization of the AAV1-tetON-V16mutant-hGDNF vector and its non-mutant homologue.

C. Conclusions and perspectives:

A large-scale production, purification and characterization procedure for the future production of AAV1-tetON-V16mutant-hGDNF has been developed and optimized. Although further improvement is needed in order to adapt this protocol to viral vector production in GMP conditions, the resulting quality level of the vector preparations is expected to be appropriate for its use in preclinical studies.

Secondment FIMA → IBET

PIGNATARO Diego

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Date of the secondment: May –June 2014

My resarch in BrainVectors

Our research group is mainly engaged in the study of the neurobiology of Parkinson disease, with a multiple focus on a number of different topics related to the pathophysiology of this disease, as follows:

1. Analysis of brain circuits underlying the pathophysiology of Parkinson disease. By using relevant animal models of the disease, we study the ongoing changes in basal ganglia circuits following dopaminergic neurodegeneration.

3. Controlled release of neurotrophic factors (GDNF and relatives) in animal models of parkinsonism. In this regard we have a dual strategy: on the one hand, we are using GDNF-loaded microspheres; on the other hand, we are currently preparing adeno-associated viral vectors (AAVs) for the controlled release of GDNF into the brain areas of interest.

4. Cell therapy in Parkinson disease. Together with a number of European collaborators, we have recently started a research project focused on the use of direct reprogramming techniques converting skin fibroblast onto dopaminergic neurons that will further be used for autologous transplantation in relevant animal models of parkinsonism.

My secondment to IBET

1. Development of AAV vectors expressing GFP constitutively or under the control of a doxycycline inducible promoter

FIMA has developed two AAV vector expressing GFP under the control of the Chicken beta actin (CBA) promoter and the glial fibrillary acidic protein (GFAP) promoter. FIMA has also constructed an AAV vector that express the transactivator V16 (developed by AMC) and GFP under the control of Doxycycline inducible promoter. These vectors have been tested in the neuroblastoma cell line Neuro2-a (CCL-131™ - N2a).

2. Analysis of the transduction efficiency of AAV vectors in 3D cultured human neuroprogenitors and characterization of the biological interaction

The vectors developed in the activity 1 will be tested in in 3D cultured human neuroprogenitors. First we will determine the transduction efficiency using different Multiplicities of Infection of the vector by determining the cells expressing GFP using the vectors constitutively expressing GFP. Next we will test Doxycycline inducible promoter, we will determine the inducibility of the system and the optimal doxycycline concentration.

The effect of AAV transduction and doxycycline administration over human neuroprogenitors transcritome will be evaluated and compare with the data obtained after Adenovirus and lentivirus infection.

Secondment CHUV → GenIBET

Staff member:
Liliane TENENBAUM
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Dates: 12 May - 11 Juillet 2014

Title of the secondment:

Production and Quality Control of mid-scale AAV vectors batches incorporating doxycycline-sensitive and potentially-immune escape mutants of the reverse tetracycline transactivator (rtTA).

A. Project objectives for the period

-Optimize the conditions for the production, titration and analysis of tetracycline-inducible AAV vectors batches of serotype 1. For the second part of the project, 2 batches will be produced at a sufficient scale and purity to allow immune response analysis in a transgenic immunologically-humanized mice model: AAV1-tetON-V16-hGDNF incorporating a mutant rtTA responding to low doses of doxycycline (AMC) and AAV1-tetON-V16im-hGDNF, a potentially immune-escape mutant, (AMC).
-Discuss with GenIBET's personal the GMP requirements for clinical applicability of the AAV-tetON-GDNF vector.

B. Work progress and achievements during the period.

Coordination of the work of BrainVectors team (Maria Ontiveros (UAB), Diego Pignataro (FIMA), Vanessa Bandeira (IBET), Manuel Garrido (GenIBET);
-Definition of goals.
-Experiments planning.
-Data analysis and discussions.
-Decision making.

Work progress

(see also reports from Vanessa Bandeira, Maria Ontiveros and Diego Pignataro for more details)
In order to optimize the conditions for mid-scale production and purification of the AAV1-tetON-GDNF vectors, we have first produced batches of AAV1-V16imm-GFP, in order to use the GFP reporter gene to easily evaluate the transfection efficiency as well as the biological activity of the recombinant virus.
We have used a double transfection method in cell factories followed by 3 steps of purification/concentration: i) benzonase treatment to remove nucleic acids, ii) PEG 8000-assisted protein precipitation, ii) iodixanol gradient and iii) buffer exchange/concentration in Amicon concentrators.
A titration method of viral genomes was optimized and used to evaluate the recovery of viral particles at each purification/concentration step.
Finally, the quality control consisted in the determination of contaminant proteins by SDS-PAGE analysis, of total DNA by Picogreen Assay and of viral particles by Nanosight analysis. In the future, in addition to these tests, an ELISA kit (Progen) will be used to quantify AAV1 viral capsids and detection of endotoxin will be performed using a kit.

However, the first batch which has been purified until the end of the above described protocol, appeared to contain a high level of contaminant proteins. After discussions between researchers used to purify AAV (Maria Ontiveros, Diego Pignataro, Liliane Tenenbaum, Manuel Garrido), our hypothesis is that the manual recovery of the iodixanol fractions resulted in unprecise harvesting of fractions containing the virus and perturbation of the gradient layers. Therefore it was decided that IBET will order a fraction collector to reproducibly isolate fractions containing the virus and separate them from fractions containing contaminants.
A second batch was produced using the optimized transfection conditions and titrated using the optimized q-PCR conditions. This stock has been transferred to CHUV where it will be further purified and titrated before being sent back to IBET for the quality control.

Training
GenIBET 19th -23rd May. "Initiation on Good Manufacturing Practices" including:

- -GeniBET presentation and visit to the facilities
- -GMP presentation
- -GMP quality system: Good Documentation Practices, Non-Conformances, Change Controls, Deviations, Out of Specification Results, Concept of Traceability, Qualifications/Validations, Calibrations and Maintenance, SOPs.
- -Guidelines: PICs, ISO Standard, ICH, Eudralex (EU), European Pharmacopeia (EU), FDA Regulations (USA), US Phamacopeia (USA).
- -Quality control: Reception Materials, Approved/Rejected/Quarantine, Warehouse storage Weighing Room, Sampling of Raw Materials, QC analysis and Registers, Water Analysis (WFI), Cleaning and Envornmental monitoring, Presentation on aseptic handling.
- -Production / operations: Tech Transfer, Requesr for Proposal, Confidentiality, How to create a batch production record.

Oral presentations

- 6th June:.*Seminar at Genibet*: "Towards a clinically-acceptable tetracycline-regulatable vector for neurotrophic factors delivery in Parkinson's disease."
- 26th June: *Lab Meeting at IBET*: "The BrainVectors project : advancement and challenges."

Other
Enrolment as external expert in the thesis committee of a new PhD student, Catarina Pinto.

C. Conclusions and perspectives:

- The requirements for clinical applicability of the AAV1-tetON-GDNF vector have been thoroughly discussed at GenIBET with researchers experienced in the release of biomedical products. A particular emphasis has been put on the risks of the use of antibiotics in the context of increasing incidence of antibiotic resistance in the world. It was decided to contact regulatory authorities in order to ask an advice.
- A common protocol for mid-scale production of rAAV1 has been established.
- The purification steps still require improvements that will hopefully be completed durng the stay of Vanessa Bandeira from IBET at CHUV (Oct.-Nov 2014). During this secondment, batches of AAV1-tetON-V16-GDNF and AAV1-tetON-V16imm-GDNF already produced at CHUV (by Marie Humbert-Claude, RR) will be purified and their quality assessed by the personal of IBET.
- These batches will be used to assess the immune response to both vectors in collaboration with FIRALIS.

Secondment WNC → IBET

Staff member:
Luis QUINTINO
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Dates:
Step 1- 08-09-2014 to 26-09-2014
Step 2- Last 24 months of the BRAINVECTOR project

Title of the secondment:
Methodologies and applications of LV production in CNS

A. Project objectives for the period

The BRAINVECTORS project is designed to generate productive scientific exchange between different countries and research backgrounds. Two of its members Instituto de Biologia Experimental e Tecnológica (IBET) and Lund University are particularly suited to reap the benefits of such environments. IBET in Oeiras is a European expert of biopharmaceutical product development, manufacturing processes and method optimization. At Lund University, the CNS Gene Therapy group has been at the interface of neurodegenerative disease research and cutting-edge lentiviral vector development.

The secondment will be divided into 2 steps:

- Step 1 (September 2014)
  - Learning vector production methodologies under Good Manufacturing Practices (GMP)
  - Define step 2 of the secondment, where both institutions will collaborate in short-term projects to exchange scientific expertise and generate synergies for future collaborations.
- Step 2 (last 24 months of the BRAINVECTOR project)
  - Learning vector production strategies under Good Manufacturing Practices (GMP)
  - Evaluate cell specificity of vectors produced in Lund on human 3D culture systems developed at IBET.
  - Determine the necessary downstream scalable methods optimal for CNS gene delivery using Lentiviral Vectors.

B. Work progress and achievements during the period.

Step1-

During the first part of the secondment the researcher from Lund University was able to assess the workflow, methodologies and expertise present at IBET. Due to unforeseen circumstances it was not possible to learn GMP vector production methodologies and GENIBET during this period. This step will also be performed on step 2 of the secondment. Nevertheless, the researcher was introduced to the upstream and downstream virus/vector production methodologies, namely production in bioreactors and chromatography purification techniques that are applicable to LV production under GLP/GMP conditions.

Moreover it was possible to draw a workplan for the upcoming part of the secondment. For the second part of the secondment the Lund University and IBET will exchange expertise in the following short-term projects:

1. Evaluate cell specificity of vectors produced in Lund on human 3D culture systems developed at IBET.
2. Determine the necessary downstream scalable methods optimal for CNS gene delivery using Lentiviral Vectors.

Secondment IBET → CHUV

Staff member:
Vanessa BANDEIRAS
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Dates: October 1st – November 30, 2014

Title of the secondment: Large scale production and purification of GDNF-AAV vectors and assessment of their efficiency with in vivo gene transfer

Project objectives for the period

A. Purification of the 2nd production of AAVs produced in IBET in a cell factory platform (CF10): For the 2nd batch production of AAVs encoding GFP (positive control) produced at IBET we did all the purification of the vectors up to 'PEG precipitation' step. With the absence of a more precise system for the recovery of the AAVs from the 'iodixanol gradient' step it was between the counterparts that the remaining purification process would be finished at the CHUV, as well as some of the quality controls needed to analyze the purity of the final viral preparation. Below are the steps to be performed in CHUV:

A.1. Iodixanol gradient (using the Fraction Collector System) to the sample sent from IBET (already precipitated with PEG);

A.2. Concentration of the purified sample with Amicon Centriplus 100;

A.3. Quality control analysis of the final purified and concentrated AAVs sample (SDS-Page Silver Staining, qRT-PCR, etc);

A.4. Aliquots of the final sample will be sent to Cristina Peixoto (IBET) for analysis of capsid proteins, endotoxins, DNA contaminants, etc.

If the final sample is pure enough we will proceed with the purification of both GDNF lysates at CHUV using the same methodologies used before. If not, a discussion between IBET and CHUV will be done, in order to find a better purification method (e.g. chromatographic column).

B. Purification of the GDNF lysates produced in the CHUV: GDNF lysates produced in the CHUV will be purified with the methodology used before, namely, treatment with endonuclease (+EDTA for inactivation), precipitation with PEG, iodixanol gradient (using Fraction Collector) and quality control assays. Again aliquots of the final samples will be sent to IBET for analysis. If the final AAV GDNF samples are pure enough they will be used for *in vivo* experiments with mice. If not, a new discussion with Cristina Peixoto will be performed.

C. *In vivo* experiments with mice If possible and if it is within the period of staying it is also planned to learn and perform *in vivo* experiments with rat perfusions and rat injections, as well as histology tests and analysis of the results obtained.

Previous work. Since 2009 I have been involved in research in cell biology and genetics. This scientific activity began with the development of my master's thesis, which aimed to analyze the virological factors involved in mother-to-child transmission of HIV-1 and that allowed me to learn some new methodologies (DNA extraction and analysis and sequencing of viral genes).

With the entrance in the Animal Cell Technology Unit in 2010 this interest could be deepened, more specifically in the development of viral vectors and cell lines, allowing the acquisition of various techniques of molecular and cellular biology. During these last four years I have been involved in several research projects funded either by national (FCT) or international (European Network of Excellence Clinigene and BrainVectors) networks, as well as in international services for biopharmaceutical and/or biotechnology companies.
These projects allowed me to be in contact with a large numbers of techniques, namely:
- Molecular biology (cloning, production of bacterial plasmid, RNA extraction, cDNA Synthesis, and Real-Time PCR);
- Protein analysis (Western blot, SDS-PAGE, Mass Spectrometry, ELISA);
- Development of cell lines (culture of animal and insect cells in static and suspension system, and in both small and large scale; cryopreservation of cell stocks, monitoring of cellular metabolic profile, methods of transfection and infection);
- Production and manipulation of viral vectors (retroVLPs, adeno-associated virus, baculovirus, retrovirus and lentivirus);
- Purification of viral vectors using PEG precipitation, iodixanol gradient, ultracentrifugation or monolithic column techniques;
- Expertise in phase contrast microscopy and flow cytometry.
All this experience has allowed me to acquire various professional skills, improving my spirit of teamwork, teaching me to ask the right questions and to develop the appropriate strategies in order to respond to certain questions, forcing me to think "outside the box" and cope with unexpected results. In short, be persistent and able to work under tight deadlines and targets