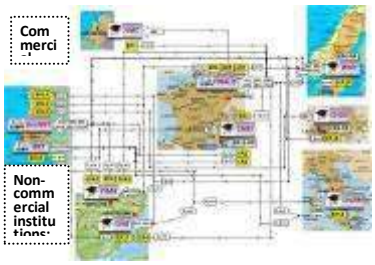
BrainVectors Newsletter n° 7

Marie Curie Industry-Academia Partnerships and Pathways - Contract n° 286071

BrainVectors secondments 2



BrainVectors project implementation: Secondments 2nd part

In this issue of the *BrainVectors* newsletters, we remind the introduction of the newsletter n° 4, in which the program of the cross-sectors secondments of researchers, was presented (see the figure here).

During the 2nd half of the project, the large majority of secondments were carried out, according to the agenda rescheduled with the Commission. In addition, the program of these visits was shortened of height months compared to the starting version of the agenda. This was due to the failure of availability of researchers, who were supposed to stay in the institutions of the partners for longer periods, as scheduled at the beginning of *BrainVectors*. However, despite the reduced time of the secondments, all results expected in the work program have been achieved.

We present here the reports of the most significant secondments; some reports derive directly from the ppt slides of the presentations that researchers made during the workshops presented in the last two newsletters (NL5 and NL6).

Table of content

1. AMC → FIRALIS (Atze DAS, July-August 2015)

Atze DAS, Associate professor (UHD) at AMC (Academisch Medisch Centrum – Amsterdam), Molecular biology of human immunodeficiency virus (HIV)

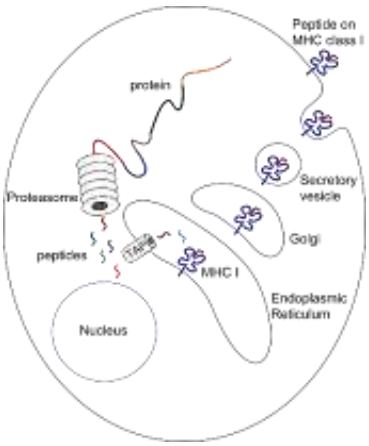
Title of work: ***In silico prediction of HLA A2 binding peptides from a new rtTA variant***

Introduction In order to access immunogenicity of the new rtTA variant generated at AMC, the analysis pipeline is the following:

- *In silico* determination of the candidate peptides eligible to bind to a given HLA (major histocompatibility complex class 1)
  For this approach, the following software has been used: http://www.cbs.dtu.dk/services/NetMHCpan/

- *In vitro* characterisation of the selected peptides candidate

For this approach, T2 cells have been used to study candidate peptide binding and complex stability.



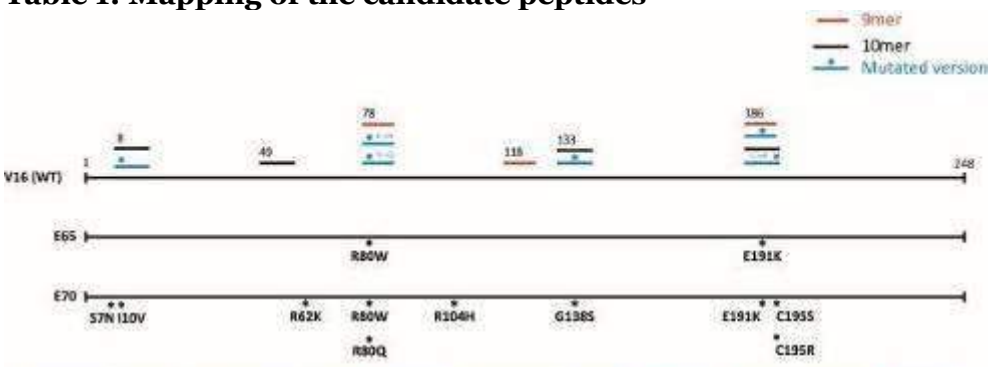
T2 cells are TAP deficient, therefore, they do not present endogenous peptides (Figure 1) and represent a suitable model for studying peptide binding.
T2 cells are expressing HLA*A2, therefore the in silico analysis is restricted to HLA*A2 for the prediction.
The read out is an indirect immune staining against HLA*A2 and a cytometer analysis.

Figure 1: Normal processing in MCH class 1

- *In vivo* validation of the immunogenic peptides, which has not been done during this secondment.

Results The different peptides predicted by the netMHC software have been mapped on the rtTA sequence (Table1). The V16 is considered as the WT sequence and the E65 and E70 are 2 variants encompassing several mutations.

Table 1: Mapping of the candidate peptides



Start Position	9/10mer	WT seq	Affinity HLA-A*02 (nM)	Affinity Mamu B:00101 (nM)	Mutated seq	Affinity HLA-A*02 (nM)	Affinity Mamu B:00101 (nM)	Mutation	ΔAffinity HLA-A*02 (WT/Mut)	ΔAffinity Mamu B:00101 (WT/Mut)
133	10mer	ALSAVGHFTL	27,8	25378	ALSAVSHFTL	17,6	23699	G138S	0,63	1,07
186	9mer	FLFGLELI	30,6	15494	FLFGLKLI	47,3	22792	E191K	1,54	0,68
8	10mer	KIINSALELL	238,4	24570	KIVNSALELL	696,8	30864	I10V	2,92	0,80
186	10mer	FLFGLELIIC	461,7	41311	FLFGLELIIR	4656,5	41852	C195R	10,09	0,99
78	9mer	FLRNNAKSY	23711	40788	FLWNNAKSY	6929,4	25908	R80W	0,29	1,57
					FLQNNAKSY	17745,7	34327	R80Q	0,75	1,19
49	10mer	ALLDALPIEM	14,2		N/A					
118	9mer	FLCQQGFSL	17,7		N/A					

Conclusion

This secondment allow us to identify the different peptide candidates for rtTA V16 and its mutant counterpart. Preliminary Flow cytometry experiments have been performed to fine tune the setting parameters.

2. FIRALIS → CHUV (François LEGUEUX, July-August 2015)

Seconded researcher &
project manager
François LEGUEUX

Title of work: ***Biomarkers for the monitoring of gene therapy in clinical trials***

Variety of gene therapy approaches have emerged for the treatment of diverse diseases. However, there are significant challenges to transfer preclinical results into clinically acceptable treatment. Among these challenges, immune response against transgene and vector constituents is a key parameter that needs to be addressed for an efficient gene therapy allowing a long term in vivo transgene expression. In particular, immune responses to Tet-on proteins such as the rtTA transcription factor have been reported, raising concerns about their occurrence in humans.

Within the BrainVectors consortium project, Firalis aims to compare immune safety of the dox-sensitive rtTA (rtTA-V16) and its corresponding mutant displaying an immune escape phenotype (rtTA-V16-imm) obtained from the group of Atze Das (AMC partner). Based on a multistep approach, we investigated the immune response against transactivator cassette. To do so, we predicted in silico the class I major histocompatibility complex (MHC) HLA-A*0201 restricted epitopes within rtTA Transactivator. Then HLA-A*0201 binding assays are performed to address in vitro binding on T2 cells. Results showed that the strongest signal is obtained when cells are incubated with the highest concentration of peptide (100mM) but technical limitations occurred du to presence of background signal.

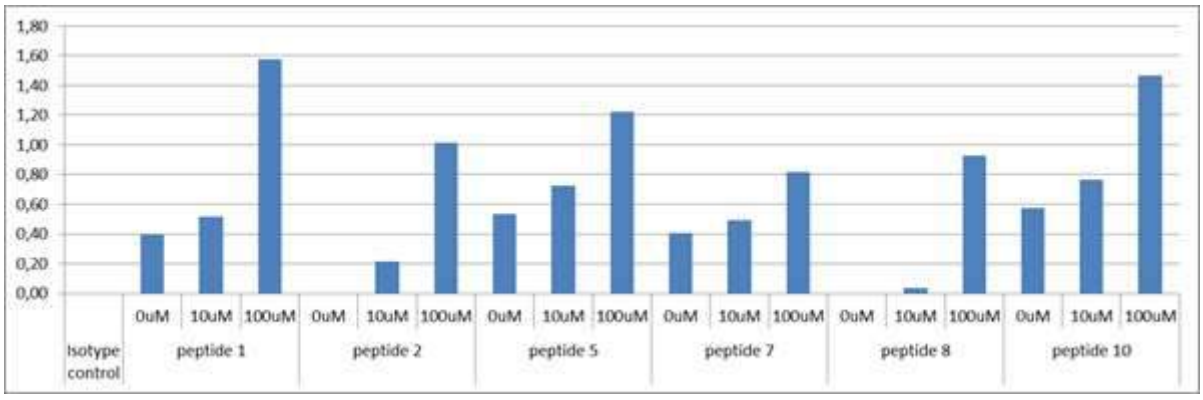
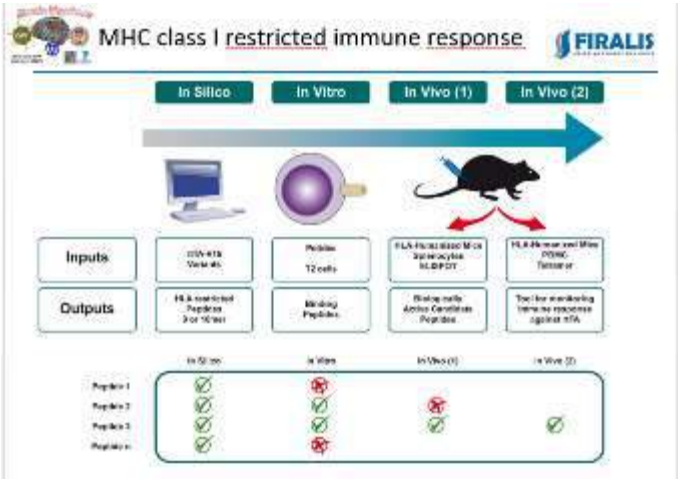


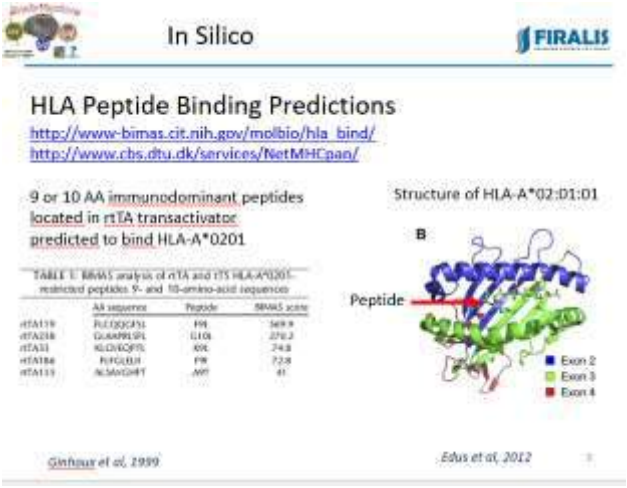
Figure legend: Flow cytometry results of 6/10 peptides. Mean fluorescence intensity signals for the different conditions subtracted by the background signal obtained by control secondary antibody alone.

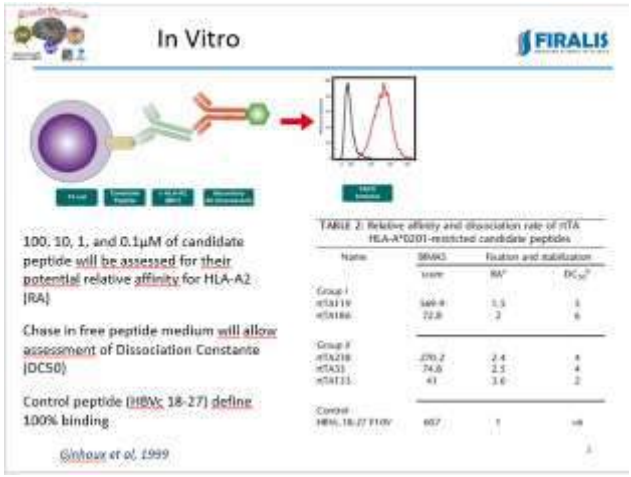
In conclusion, we developed an analytical pipeline and tools to follow immune responses against the transgene GDNF expression cassette in preclinical settings (using HLA-A*0201 humanized mice) and later in clinical studies. Further characterization will include measure of epitope specific T cell count using HLA-A2.1 restricted peptide loaded tetramers and functional T cell counting using epitopic peptide-loaded APCs and ELISPOT essays.

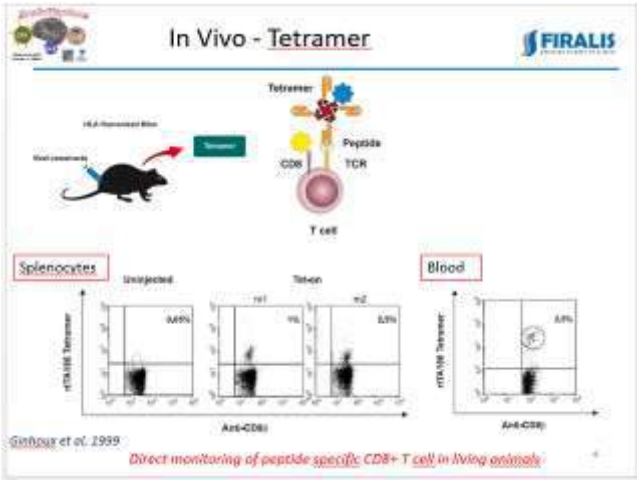
Ultimately, these findings will generate Biomarker based tools to track immune responses against the transactivator, to allow personalized therapeutic approaches using various immune-modulators and to improve safety monitoring of the gene therapy clinical protocols.

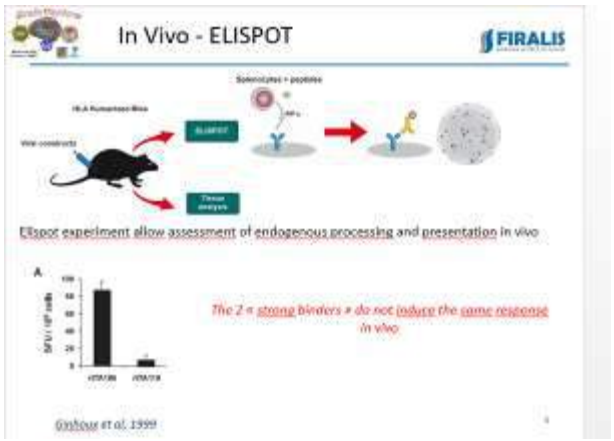
Relevant slides concerning the project

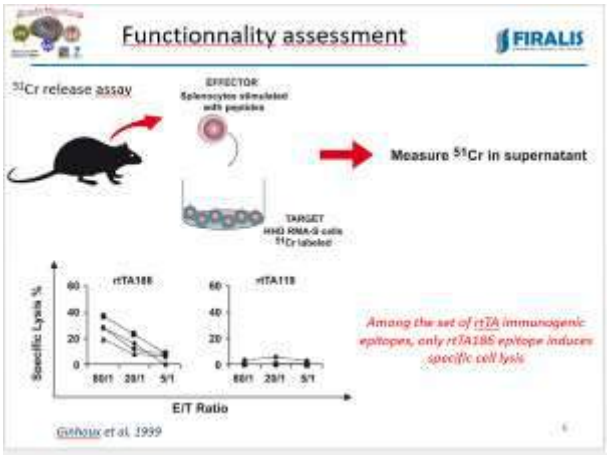












3. CHUV – FIRALIS (Liliane TENENBAUM, January - July 2016)

Seconded researcher:
Liliane TENENBAUM

Project manager:
François LEGUEUX

Summary

During my secondment, I discussed and did troubleshooting of several optimized conditions of the methodologies and technologies transferred from CHUV and ULB to the infrastructure existing at FIRALIS.
I also trained scientists from FIRALIS to the field of gene therapy by giving seminars and discussions.
I prepared the last workshop of the BrainVectors consortium and applied to different grant providers to generate additional funding (FNS, FBM) see Annex. I was helped in this matter by Dr Humbert-Claude (CHUV) who applied for additional funding from Suisse Parkinson and Prof. Marc Levivier from Medtronic.This organization was finalized in Aug, until 12 Sept. Thanks also to the help of Dr Otto Merten (EASCO) for the scientific program and round tables organisation. Dr Humbert-Claude (CHUV) managed the local organization committee and together with EASCO (Mauro Mezzina and Otto Merten) helped me for the follow-up of the workshop: report (see attached), reimbursements, invoices management, etc…
We also used the BRAINVECTORS secondments to discuss and plan new collaborative project proposals that we intend to submit to future calls (e.g. SME-driven projects). A draft project has been written and preliminary experiments have been started to obtain data for a patent filing.

Research Projects:

Glial cell line-derived neurotrophic factor (GDNF) and Neurturin (NRTN) bind to a receptor complex consisting of a GFR-□ family member and the Ret tyrosine kinase. Both factors were shown to protect nigro-striatal dopaminergic neurons and reduce motor symptoms when applied terminally in toxin-induced Parkinson's disease models. However clinical trials based on intraputaminal GDNF protein administration or adeno-associated virus (AAV)-mediated NRTN gene delivery have been disappointing. In addition to its anti-apoptotic and neurotrophic properties, GDNF also interferes with DA homeostasis via time and dose-dependent effects such as: stimulation of DA neuron excitability, inhibition of DAT activity, tyrosine hydroxylase (TH) phosphorylation and inhibition of TH transcription. Depending on the delivery parameters, the net result of this intricate network of regulations could be either beneficial or deleterious. Further unraveling of the mechanism of action of GDNF gene delivery in relevant animal models is still needed to optimize the clinical benefits of this new therapeutic approach. This is the subject of a collaborative project between several partners of the BrainVectors consortium (ULB, CHUV, Lund, AMC, FIMA and the group of Tomas Gonzalez-Hernandez, Spain). This work has allowed to publish 2 articles in 2016 (Chtarto et al., Mol.Ther.Meth.& Clin. Dev. 2016; Barrosso-Chinea et al., Neurobiol. Dis. 2016) and to submit a review to Frontiers in Neuroanatomy (Humbert-Claude and Tenenbaum) for the topic edited by Dr Jose-Luis Lanciego (FIMA).
With the goal to obtain a clinically-useful regulatable viral vector for neurotrophic gene delivery, we have designed a doxycycline-inducible AAV vector which allows to finely adjust the GDNF dose and period of administration in response to doxycycline administered orally at clinically-acceptable doses (Chtarto et al., Mol.Ther.Meth.& Clin. Dev. 2016). However, there is still a major limitation for the safe use of this vector in clinical trials: the immune response to the tetracycline transactivator (rtTA).
The secondments from and to FIRALIS aimed at dissecting the humoral and cellular immune response to the dox-sensitive rtTA mutant (rtTA-V16 previously obtained by the AMC partner)

used in our studies and compare it to a potentially immune-escape variant obtained after *in vivo* screening by the group of Atze Das (AMC). Thanks to FIRALIS expertise, cutting-edge tools are (or will be) made available to the consortium: software and know-how for *in silico* epitope analysis; GDNF monoclonal antibodies (ELISA kit development); human lymphocytic cell line (obtained from the group of François Lemonnier, Paris) and rtTA peptides for FACS and Elispot analysis; mice expressing human HLA (obtained from the group of François Lemonnier).

(i) Evaluating the comparative immune response to the tetracycline transactivator mutant rtTAV16 and a potentially immune-escape mutant thereof (rtTAV16-immEsc) after intramuscular and intra-cerebral injections.

In order to more easily analyze the immune response to rtTA, an immunologically-inert reporter gene was introduced in tetracycline-inducible and constitutive (CMV promoter) vectors: the murine secreted alkaline phosphatase (MuSeAP; work of Dr A.Chtarto, ULB seconded at FIRALIS). The first assays to set-up techniques were performed using a plasmid constitutively expressing MuSeAP. Although measurements of the MuSeAP gene product in supernatants of transfected cells were successful (using a commercially available kit; work of Dr François Legeux during his secondement at CHUV), no transgene expression could be evidenced in plasma from mice injected intramuscularly with the vectors. Two injection methods were tried: electroporation using a method kindly transferred by a member of the Nicolas Mermod's group (EPFL, Lausanne) or using chemically-assisted (PEI) *in vivo* transfection (a method mastered by members of the mAbsolys company, FIRALIS associate). This work is still on-going in order to find out why MuSeAP expression was not detected in the plasma of intramuscularly-injected mice (Injection failure, cellular immune response to MuSeAP, too low expression level, plasma interference with MuSeAP detection, etc..).
Due to the cancellation of 3 x 2 months secondments (François Legeux at CHUV (2 months instead of 4 months), UAB to FIRALIS, FIRALIS to FIMA) and the rejection of our prolongation request, this project is currently not being pursued.

(ii) Development of a sensitive proprietary ELISA assay for GDNF.

Transfer of material, know-how and protocols from CHUV to FIRALIS concerning GDNF tools (Western blots and ELISA), samples (supernatants of transfected cells and brain extracts from injected rats). Dr Chtarto, seconded from ULB to FIRALIS, has generated a vector which efficiently expresses GDNF fused to a histidine tag after *in vitro* transfection of HEK293T cells. GDNF was detected using a commercially-available GDNF ELISA. Monoclonal antibodies against GDNF were generated by intramuscular injection of commercially-available recombinant GDNF protein. In order to test these antibodies and design a ELISA kit, Western blots of transfected cells were incubated with the antibodies generated by FIRALIS as well as with commercially-available GDNF antibodies provided by CHUV and successfully used for immunohistochemistry (as described in Chtarto et al., 2016). This work was performed by Georgia Zanetti (UniRoma) during her secondment at FIRALIS under the supervision of François Legeux, Unfortunately no signal was obtained so far, neither with the new antibodies, nor with the commercially-available antibodies. Experiments are going-on to understand the reasons for the lack of detection of a GDNF band at the expected size in cell supernatants (formation of GDNF dimmers, too low amounts of GDNF, stability of his-tagged GDNF, etc...).

Dissemination:
Writing and submission of a common article. Subject of the collaboration: Set-up of conditions for IL1-□ ELISA assay by Dr François Legeux (FIRALIS) during his secondment at CHUV-comparison with mRNA quantification (data obtained using RT-qPCR by J.Sandström and D.Duc, CHUV) in a LPS-induced model of Parkinson's disease.
This work has been accepted for publication in December 2016:

Humbert-Claude M, Duc D , Dwir D, Thieren L, Sandström von Tobel J, Begka C, Legeux F, Velin D, Maillard MH , Do KQ, Monnet-Tschudi F, Tenenbaum L. (2016). Tollip, an early

regulator of the acute inflammatory response in the substantia nigra. J Neuroinflammation: 13(1):303.

Training

-Seminar *at FIRALIS*: "New tools for dose-dependent drug-inducible gene therapy in the brain." 30 June 2016 with an introduction on Gene therapy, Gene therapy in the CNS and description of the on-going clinical trials and market situation.

-Organization of weekly lab meetings and when necessary telephone conferences with members of the FIRALIS group (including current and previous seconded researchers: Abdelwahed Chtarto, Atze Das, Diego Pignataro) and when necessary other partners (AMC, CHUV, UniRoma) or FIRALIS associate (mAbsolys). Planning of the secondment of Georgia Zanetti from UniRoma in September and October 2016.

-Scientific organization of the final BrainVectors workshop: Neurotrophic factor gene delivery for neurodegenerative diseases: still a promising clinical paradigm ? Lausanne, CHUV, 12-14 Sept 2016.

Publications mentioning the BrainVectors contract

See the online list at http://www.brainvectors.org → "*Publications*"

4. CNRS → IBET (Eric KREMER, July-August 2015)

Seconded researcher:
Eric J Kremer

Project manager:
Catarina BRITO

Project: ***Learn the details involved in the initiation and maintenance of 3D cultures of human neural precursor cells in bioreactors.***

Advances in the fundamental nature of neurological disorders have been slow due to the lack of powerful *in vitro* models. Three dimensional cellular models have high biological relevance, however, their lack of robustness and scarcity of analytical tools adapted to 3D hampers their widespread implementation. Brito and colleagues developed human midbrain-derived neural progenitor cells (hmNPC), cultured as 3D neurospheres in stirred culture systems that differentiate into complex tissue-like structures containing functional dopaminergic neurons, as well as astrocytes and oligodendrocytes.
During my stay I learned the technical challenges involved in seeding and maintaining hmNPC 3D cultures in bioreactors. In addition, we incubated these cells with CAV-2 vectors to further characterize the functionality of transduced neurons. This was particularly important concerning the effect of coxsackievirus adenovirus receptor engagement and degradation. In these short-term assays we did not detect a significant impact on neuron homeostasis

A. Project objectives for the period

The Brainvector project aims to determine the efficacy of different vectors for gene therapy in neurodegenerative diseases. In this context, our main objective during this first period has been to generate doxycycline-inducible canine adenovirus type 2 (CAV-2) vectors expressing eGFP, luciferase or GDNF and test their efficacy *in vitro*.

B. Work progress and achievements during the period

Firstly the cassettes of interest from pAC1-V16-rTA-eGFP, pAC1-V16-rTA-luciferase and pAC1-V16-rTA-GDNF were cloned in a shuttle vector (pTCAV-12a). After, the different cassettes where cloned in CAV-2 vector backbone (pCAVdeltaE3SceI) by homologous recombination in BJ5381 cells. When we had the pCAV-rTA-eGFP, pCAV-rTA-luciferase and pCAV-rTA-GDNF, we transfected them in DKZeoSceI cells to generate the CAV vectors as previously described[1].
The efficacy of the three different vectors has been tested in 293T cells plated in 6 well plates. 293T cells were infected with 2500 pp/cell and after 1 hour we treated them with different concentration of doxycycline or without doxycycline. After 24 hours the expression of eGFP, luciferase or GDNF was tested.
Cells infected with CAV-rtTA-eGFP showed eGFP expression when they were treated with doxycycline in dose-dependent manner whereas cells infected with CAV vector and non-treated with doxycycline showed low basal levels of expression (Figure 1). The expression of eGFP was analyzed with a fluorescent microscopy (Figure 1A) and by FACS (Figure 1B).

[1] Ibanes S and Kremer E. (2013) Canine adenovirus type 2 vector generation via I-Sce-mediated intracellular genome release. PLoS One 8(8):e71032

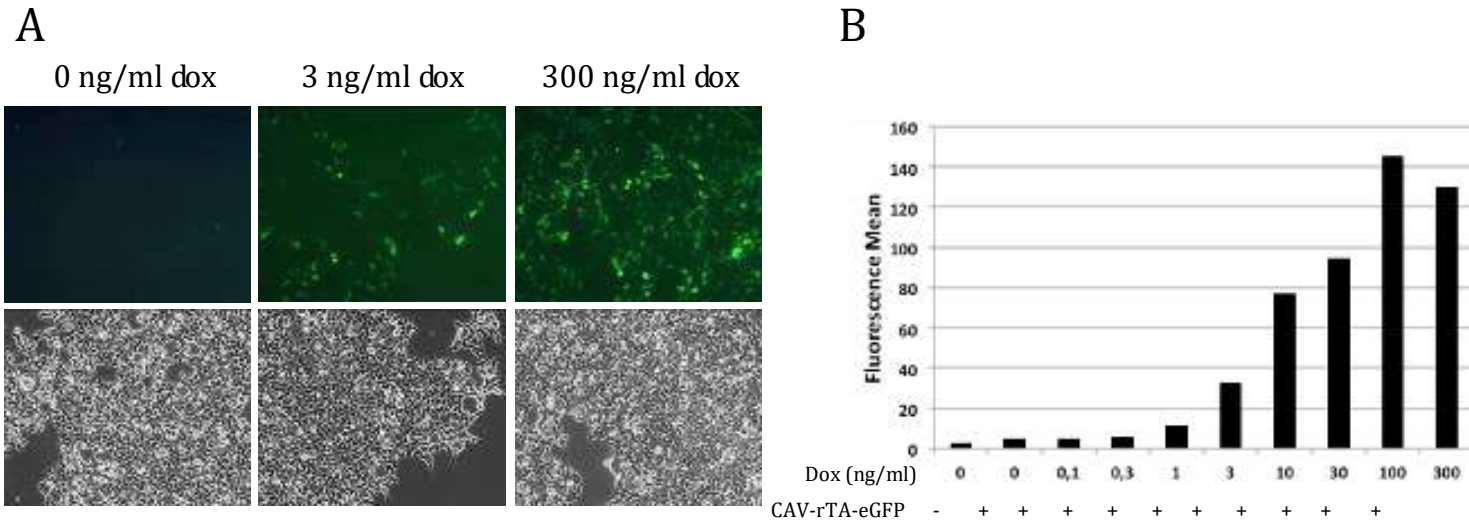


Figure 1. Expression of eGFP in 293T cells infected with CAV-rTA-eGFP. A, Images showing the expression of eGFP at different concentrations of doxycycline and their corresponding phase contrast images. B, Levels of eGFP analyzed by FACS.

To determine the expression of luciferase in 293T cells infected with CAV-rTA-luciferase we performed an assay to measure the lucierase acivity (Figure 2). As was observed with the expression of eGFP, luciferase activity increases with doxycycline treatment and in a dose-dependent manner. As happened with CAV-rTA-eGFP, cells infected with CAV vector and non-treated with doxycycline showed low basal levels of luciferase activity.

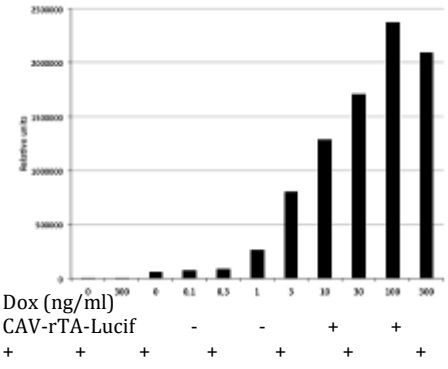


Figure 2. Luciferase activity from 293T cells infected with CAV-rTA-Luciferase.

To assay the efficacy of CAV-rTA-GDNF we collected the supernatants after 24 hours of infection and doxycycline treatment. The levels of GDNF were determined by ELISA (Figure 3A). Moreover, the functionality of GDNF was tested. For that, the supernatant from 293T cells infected with CAV-rTA-GDNF treated with 300 ng/ml of doxycycline or non-treated was added to TGW cells and protein extracts were collected after 24 hours to determine the relative levels of Tyrosine Hydroxylase.
The results obtained showed that the GDNF produced by 293T cells infected with CAV-rTA-GDNF and treated with doxycycline was capable to induce an increase of this protein in TGW cells (Figure 3B). Thus, we can conclude that the GDNF expressed by CAV-rTA-GDNF vector is functional.

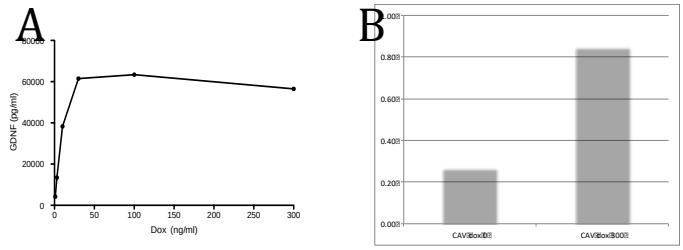


Figure 3. A, Levels of GNDF in 293T cells infected with CAV-rTA-GDNF treated with different concentrations of doxycycline. B, Bar Graph showing the relative quantification of Tyrosine Hydroxylase determined by western blot from TGW cells incubated with conditioned media from 293T cells infected with CAV-rTA-GDNF treated with 300 ng/ml of doxycycline or non-treated.

Scheduled deliverables

3.1 CAV-dox-reporter: a CAV-2 vector with reporter gene under control of dox-regulated promoter - Finished

3.2 Report describing CAV-dox-reporter efficacy in vitro and in vivo

3.3 CAV-dox-GDNF: a CAV-2 vector with GDNF under control of dox-regulated promoter - Finished

3.4 Report describing CAV-dox-GDNF: efficacy *in vitro* and *in vivo*

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5. IBET → CNRS (Catarina BRITO, Sept - Nov 2015 and June – Aug 2016)

Seconded researcher :
Catarina BRITO

Project manager:
Eric KREMER

Project: ***Human stem cell derived 3D neural cultures to study the impact of CAV2 viral vector transduction on neuronal functionality.***

Gene therapy is a promising approach with enormous potential for treatment of neurodegenerative disorders. During viral vector development, preclinical testing is crucial to evaluate both efficacy and safety, while understanding vector–cell interactions. Although the traditional primary cultures of rodent brain cells and animal models provide valuable data, these models too often diverge considerably from the human phenotype, thus not accurately predicting the outcome of clinical trials. In this context, stem cell-derived human neural cells, along with three dimensional (3D) culture systems, have great potential as complementary tools in preclinical research, bridging the gap between human clinical studies and animal models. Previously, we have developed a strategy for 3D culture and differentiation of human midbrain-derived neural progenitor cells (hmNPC), in stirred culture systems and applied this to the preclinical evaluation of hdCAV2 (Brito et al 2012, Simão et al 2015, Simão et al 2016). Recently, and within the scope of BrainVectors, we adapted the technology to human NSC derived from human induced pluripotent stem cells (iPSC-NSC). The main objective of this secondment, aligned with the secondment of Dr Eric J Kremer to iBET, was the implementation of these 3D cultures using iPSC-NSC generated in the Dr Kremer lab. iPSC-NSC cells were cultured as 3D neurospheres in shake flasks. Untargeted neural differentiation was induced by including insulin, progesterone, and sodium selenium in a basal neural medium formulation (Androutsellis-Theotokis et al 2008). After 1 month of culture, cells differentiated into complex tissue-like structures. Characterization by confocal

microscopy and live fluorescent assays showed that these tissues contained functional neurons, as well as cells of the glial lineages. Additionally, the models generated were transduced with several CAV-2 constructions generated in the Kremer Lab expressing gfp. hd-CAV-2 vectors preferentially transduced neurons and not glial cells and transduction led to stable long-term gfp expression with minimal toxicity. These results corroborated previously data obtained using hmNPC.

Androutsellis-Theotokis A et al (2008) Generating neurons from stem cells. In: Weiner LP (ed) Neural stem cells. Humana, New York, pp 31–38

Brito C et al (2012) 3D cultures of human neural progenitor cells: dopaminergic differentiation and genetic modification. Methods 56:452–460

Simão D, Pinto C, Piersanti S, Weston A, Peddie CJ, Bastos AEP, Licursi V, Schwarz SC, Collison LM, Salinas S, Serra M, Teixeira AP, Saggio I, Lima PA, Kremer EJ, Schiavo G, Brito C, Alves PM (2015) "Modelling human neural functionality in vitro: 3D culture for dopaminergic differentiation", Tissue Engineering – Part A 21, 654-68.

Simão D, Pinto C, Fernandes P, Peddie CJ Piersanti S, Collison LM, Salinas S, Saggio I, Schiavo G, Kremer EJ, Brito C, Alves PM (2016) "Evaluation of helper-dependent canine adenovirus vectors in a 3D human CNS model", Gene Therapy 23, 86-94. http://dx.doi.org/10.1038/gt.2015.75

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6. CNRS → IBET (Bertrand BEUCHER, Aug - Sept 2015 and Oct 2016)

Project managers:

Seconded researcher:
Bertrand BEUCHER,
Research assistant, IGMM
bertrand.beucher@igmm.cnrs.fr

Ana Sophia COROADINHA
(IBET)

Eric KREMER
(CNRS)

Project: ***Evaluation of CAV-2 production in Hyperflask system to increase yield and efficacy***

Report: CAV-2 vectors have high capacity cloning, low innate and induced immunogenicity, which makes them promising candidates for clinical gene transfer, Because CAV-2 vector preferentially transduce neurons, with an efficient axonal transport to afferent regions, they are particularly powerful for neurodegenerative disorders.

Ana Sophia COROADINHA's lab can produce CAV-2 vector at large scale, with a high quality process using classical T-flask. The aim of my secondment was to evaluate use of Hyperflask vessel to produce CAV-2 vector. Hyperflasks allows a much greater cell growth surface area than traditional T-flasks. The 10-layer Hyperflask has a total growth area 10 times of a standard T175 flask. This means that this vessel saves time and practical work.

After growing E1-transcomplementing cells in Hyperflask for 24 h, we infected cells with CAV-GFP and incubate them for 48 h. After discarding supernatant, CAV-GFP was recovered by Triton lysis. After filtration, the vectors were purified by anion exchange chromatography and then by size exclusion chromatography. For each step, a quality control was done to evaluate number of particles (NanoSight, Turbidimetry assay), protein (BCA assay) and DNA (QuantIt) concentration.

We found that a high quantity of particles was present before chromatography. Compared to T-flask production, Hyperflasks give a higher productivity. Unfortunately, a very low quantity of virus was recovered after anion exchange chromatography due to porosity of the new tested column. It seems that higher porosity of this new column did not allow virus to well attach on the resin.

We titrated CAV-GFP particles before and after chromatography by flow cytometry. The ratio of infectious versus physical viral particles was excellent.

To conclude, Hyperflask vessel can be used to produce CAV-2 vector in large quantity. This system saves time compared to T-flask production for a similar production of viral vector.

<u>Secondment update</u> August – October 2016

Project manager**: Ana Sophia COROADINHA**

<u>Report:</u> CAV-2 vectors have high cloning capacity, low innate and induced immunogenicity, which makes them promising candidates for clinical gene transfer, Because CAV-2 vector preferentially transduce neurons, with an efficient axonal transport to afferent regions, they are particularly powerful for neurodegenerative disorders.

Ana Sophia COROADINHA's lab can produce CAV-2 vector at large scale, with a high quality process using classical T-flasks. The aim of my secondment was to evaluate use of Hyperflask vessel to produce CAV-2 vector. Hyperflasks allows a much greater cell growth surface area than traditional T-flask. The 10-layer Hyperflask has a total growth area 10 times of a standard T175 flask. This means that this vessel saves time and practical work.

After growing E1-transcomplementing cells in Hyperflask for 24 h, we infected cells with CAV-GFP and incubate them for 48 h. After discarding supernatant, CAV-GFP was recovered by Triton lysis. After filtration, the vectors were purified by anion exchange chromatography and then by size exclusion chromatography. For each step, a quality control was done to evaluate number of particles (NanoSight, Turbidimetry assay), protein (BCA assay) and DNA (QuantIt) concentration.

We found that a high quantity of particles was present before chromatography. Compared to T-flask production, Hyperflasks give a higher productivity. Unfortunately, a very low quantity of virus was recovered after anion exchange chromatography due to porosity of the new tested column. It seems that higher porosity of this new column did not allow virus to well attach on the resin.

We titrated CAV-GFP particles before and after chromatography by flow cytometry. The ratio of infectious versus physical viral particles was excellent.

To conclude, Hyperflask vessel can be used to produce CAV-2 vector in large quantity. This system saves time compared to T-flask production for a similar production of viral vector.

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7. iBET – CNRS (Daniel SIMÃO, April – August 2016)

Seconded researcher:
Daniel SIMÃO

Project manager:
Eric J. KREMER

Optimizing CAV-2 vectors for gene transfer to the CNS

Report:

Gene therapy is a promising approach with enormous potential for treatment of neurodegenerative disorders. Viral vectors derived from canine adenovirus type 2 (CAV-2) present attractive features for gene delivery strategies in the human brain, by preferentially transducing neurons, are capable of efficient axonal transport to afferent brain structures, have a 30-kb cloning capacity and have low innate and induced immunogenicity in preclinical tests.

The Kremer lab created replication-defective and helper-dependent CAV-2 vectors. During my secondment I learned and followed the technical challenges related to CAV-2 construction and production.

For CAV-2 vector construction, different molecular biology methodologies were followed. SLiCE (Seamless Ligation Cloning Extract) method was tested as an alternative method to the traditional homologous recombination strategies. Protocol optimizations were pursued, namely comparing different transformation times and bacteria strains. Overall, this method revealed efficient cloning of different transgenes. Moreover, SLiCE significantly reduces the time needed for transgene cloning, as compared to homologous recombination method. Additionally, modifications in CAV-2 plasmid design were performed to increase the versatility and ease for SLiCE cloning of novel transgenes in the deleted E1 and E3 regions, via the introduction of unique restriction sites.

The new CAV-2 vector cloning plasmids were validated by analyzing restriction enzyme profiles. Transgene expression was confirmed by transfection of DK cells, followed by immunostaining (for non-fluorescent transgenes) and fluorescence microscopy analysis. After these validations, CAV-2 vector production was initiated by transfecting DK cells expressing SceI. Cells and supernatant were harvested 48 hours post-transfection and cell lysis was performed by three freeze-thaw cycles. The cleared lysate was used for the next round of amplification. Successive amplification steps were performed and vector production was confirmed at each step by visual inspection of cytopathic effect (CPE). CAV-2 vectors were purified by triple banding on cesium chloride density gradients.

8. CNRS – iBET (Eric J KREMER, April 2015, Jul-Aug 2015, October 2015, Feb-April 2016, June-July 2016, Sept-Oct 2016. 6 months in total)

Seconded researcher:
Eric J. KREMER

Project managers:
Catarina BRITO and **Paula ALVES**

Projects:

1) *Initiation, maintenance and testing of 3D cultures of human neural precursor cells in bioreactors.*

Advances in the fundamental nature of neurological disorders have been slow due to the lack of powerful *in vitro* models. Traditional primary cultures of rodent brain cells and animal models provide valuable data, but these models often diverge considerably from the human phenotype, and do not accurately help predict the outcome of clinical trials. Three dimensional cellular models have high biological relevance, however, their lack of robustness and scarcity of analytical tools adapted to 3D hampers their widespread implementation.

Brito and colleagues developed human neural progenitor cells (hmNPC), cultured as 3D neurospheres in stirred culture systems that differentiate into complex tissue-like structures containing functional dopaminergic neurons, as well as astrocytes and oligodendrocytes. During my stay I learned the technical challenges involved in seeding and maintaining hmNPC 3D cultures in bioreactors. iPSC-NSC cells were cultured as 3D neurospheres in shaker flasks where neural differentiation was induced. The cells differentiated into complex brain-like structures containing functional neurons and glia.

Gene transfer to neurons has enormous potential for treatment of neurodegenerative disorders. We incubated these cells with CAV-2 vectors to further characterize the functionality of transduced neurons. This was particularly important concerning the effect of coxsackievirus adenovirus receptor engagement and degradation. In these short-term assays we did not detect a significant impact on neuron homeostasis.

Summary: During my secondment, I learned how to adapt the 3D technology to human NSC derived from human induced pluripotent stem cells (iPSC-NSC) and test CAV-2 vectors transduction, which led to stable expression with minimal toxicity.

2) *Large scale production and purification of CAV-2 vectors*

Helper-dependent (HD) CAV-2 vector development, production, purification and preclinical testing is crucial to evaluate cost-effective production and feasibility. HD CAV-2 vector are safe and efficient tools for gene transfer to neurons with high cloning capacity. During my secondment, I was involved in the characterization of the production profile of HD CAV-2 vectors in spinner flasks and in cell factories.

The importance of Cre recombinase to minimize helper vector contamination during HD vector production is well documented for human Ads. Cre recombinase induces double-strand breaks in DNA that cause genotoxic effects in cultured cells. The effect of Cre-expression on cell line stability, co-infection and their relation to amplification/helper contamination were tested during production protocol for HD CAV-2 vector production. We found that Cre levels and MOI ratio impact vectors yields and infectivity, allowing us to improve HD manufacturing.

Secondly, the multiple amplification steps needed to produce HD CAV-2 vectors is a bottleneck in the production process and vector availability. To characterize the factors limiting productivity, I was involved in the analyses of the progression of HD propagation. When compared with E1-deleted (ΔE1) vectors, HD vectors had a faster replication. While genome packaging was equal between the two, more immature capsids were produced during HD production, which decreased the physical-to-infectious particles ratio. We found that an increase in empty capsids and cell death contribute to the lower efficacy. Finally, to prepare to meet the needs for clinical uses, we expanded our analyses of a GMP-compliant bioprocess. MDCK-E1 cell lines, grown in stirred tank bioreactors using serum-free medium, were used to produce HD CAV-2 vectors - that were purified using state of the art column chromatographic steps. While still lower yields than the time-consuming approach of CsCl purification, our results constituted a step toward a scalable process for CAV-2 vector production compliant with clinical material specifications.

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9. iBET – CNRS (Paula ALVES, August – October 2016)

Seconded researcher:
Paula ALVES

Project manager :
Eric J. KREMER

Projects:
(i) ***Cav-2 vector construction***
(ii) ***Scalable production and purification of CAV-2 Vectors***
(iii) ***3D cultures of human neural precursors cells***

REPORT:

Gene therapy is a promising approach with enormous potential for treatment of neurodegenerative disorders. Canine adenovirus type 2 (CAV-2) vectors overcome many of the clinical immunogenic concerns related to viral vectors, commonly used for gene therapy trials, that are often derived from human adenoviruses (hAdVs). In addition, CAV-2 vectors preferentially transduce neurons with an efficient traffic via axons to afferent regions when injected into the brain.

The main objectives of my secondment, aligned with the visits of Eric Kremer, Sara Salinas and Bertrand Beucher to several labs of iBET Animal Cell Technology Unit, that I coordinate, and the visits of Catarina Brito and Daniel Simão to Kremer lab at CNRS, were to ensure the execution of three projects defined within the scope of BRAINVECTORS and to warranty the transfer of methodologies and technologies developed and/or implemented in the laboratories of both participant institutions (iBET and CNRS).

The projects aimed at establish at IBET and CNRS harmonized methodologies and robust protocols for (i) Cav-2 vector construction; (ii) scalable production,

purification and characterization of CAV-2 Vectors and (iii) 3D cultures of human neural precursors cells to test CAV-2 transduction.

Overall the outcome of the collaboration between iBET and CNRS, done within the scope of BRAINVECTORS was very positive: we published several joint papers (see list below) and shared our complementary skills allowing us to establish in both labs methodologies for the scalable and GMP compliant production and characterization of CAV vectors and for testing CAV transduction in vitro using human neural cell models derived from human induced pluripotent stem cells.

Projects (i) and(ii): Production and characterization of CAV-2

During my 8 weeks secondment at CNRS I learned how to construct CAV-2 vectors (replication defective and helper dependent CAV-2). For that purpose different molecular biology methodologies, well established at Kremer lab, were used. CAV-2 cloning plasmids were prepared using traditional homologous recombination and SLiCE protocols. DK cells were transfected using the cloning plasmids obtained using the two different approaches. Cell supernatants were harvested and viral vectors purified by ultracentrifugation using CsCl gradients. The presence of CAV vectors was confirmed by Electron Microscopy.

Using a well characterized viral vector CAV-2 master viral bank produced at iBET, I successful validated at CNRS the SOPs (Standard Operating Procedures) for the scalable production and purification of CAV-2 that Eric Kremer and his collaborators learned in my lab at iBET and implemented at CNRS, namely the use of stirred tanks and hyperflasks for production and column chromatographic steps for purification. Troubleshooting was performed, during the last two weeks of my secondment, in the bioprocess steps but also in defining the optimum MOI (Multiplicity of Infection), CCI (Cell Concentration at Infection) and HPI (Harvesting time POs Infection) both in DK and MDCK cultures grown as monolayers (both cell types) and in suspension (MDCK cells). The use of serum free formulations and supplementation strategies at the time of infection were tested. While still lower yields than the serum containing medium bioprocesses the results obtained constituted a step towards a safer and GMP compliant manufacturing bioprocess.

Moreover, during my secondment at CNRS, I organized a one day training were I introduced to Kremer Lab members basic and introductory concepts of GMP compliant bioprocesses (Good Manufacturing Practices). A special focus was done on GMP procedures for viral vectors production and characterization.

Projects (iii): Human stem cell derived 3D neural models to evaluate CAV-2 transduction

Three-dimensional stem cell-derived human neural *in vitro* models have great potential in preclinical research. At iBET we developed a strategy for 3D culture and differentiation of human midbrain-derived neural progenitor cells (hmNPC), in stirred tank bioreactors systems that allow the maintenance of the neural cells phenotype for several months. During BRAINVECTORS we applied similar methodologies to obtain 3D neural models derived from human induced Pluripotent Stem cells (iPSC-NSC) generated in the Kremer lab. The transfer of 3D culture technologies to CNRS started with a secondment of Eric Kremer to iBET where he learned the protocols to adapt 3D culture procedures to human cell cultures followed by a secondment of Catarina Brito to CNRS were she implemented these

methodologies and started several 3D cultures that were transduced with several CAV-2 vectors produced at iBET and CNRS, namely CAV expressing gfp. During my secondment I follow up on Brito and Kremer long term cultures and experiments and evaluated the long term expression of gfp. I did some troubleshooting on the 3D models SOPs transferred to Kremer lab and trained his team on exquisite techniques used for sample preparation for the characterization of 3D cell structures by fluorescence microscopy.

In summary during my secondment at CNRS I verified and discussed and did troubleshooting of several SOPs for viral vectors production and for the establishment of 3D neural in vitro models and adapted iBET optimized conditions of the methodologies and technologies transferred to the infrastructure existing at CNRS.

We also used the BRAINVECTORS secondments of Eric Kremer at iBET and mine at CNRS to discuss and plan new collaborative project proposals that we intend to submit to Horizon2020 future calls.

Publications:

1. Fernandes P, Almeida AI, Kremer EJ, Alves PM, Coroadinha AS. Canine helper-dependent vectors production: implications of Cre activity and co-infection on adenovirus propagation. Sci Rep. 2015 Mar 16;5:9135. doi: 10.1038/srep09135. PubMed PMID: 25774853; PubMed Central PMCID: PMC4360735.
2. Castro R, Fernandes P, Laske T, Sousa MF, Genzel Y, Scharfenberg K, Alves PM, Coroadinha AS. Production of canine adenovirus type 2 in serum-free suspension cultures of MDCK cells. Appl Microbiol Biotechnol. 2015 Sep;99 (17):7059-68. doi: 10.1007/s00253-015-6636-8. PubMed PMID: 25994255.
3. Fernandes P, Simão D, Guerreiro MR, Kremer EJ, Coroadinha AS, Alves PM. Impact of adenovirus life cycle progression on the generation of canine helper-dependent vectors. Gene Ther. 2015 Jan;22(1):40-9. doi: 10.1038/gt.2014.92. PubMed PMID: 25338917
4. Simão D, Pinto C, Piersanti S, Weston A, Peddie CJ, Bastos AEP, Licursi V, Schwarz SC, Collison LM, Salinas S, Serra M, Teixeira AP, Saggio I, Lima PA, Kremer EJ, Schiavo G, Brito C, Alves PM (2015) "Modelling human neural functionality in vitro: 3D culture for dopaminergic differentiation", Tissue Engineering – Part A 21, 654-68.
5. Simão D, Pinto C, Fernandes P, Peddie CJ Piersanti S, Collison LM, Salinas S, Saggio I, Schiavo G, Kremer EJ, Brito C, Alves PM (2016) "Evaluation of helper-dependent canine adenovirus vectors in a 3D human CNS model", Gene Therapy 23, 86-94. http://dx.doi.org/10.1038/gt.2015.75
6. Piersanti S, Burla R, Licursi V, Brito C, La Torre M, Alves PM, Simao D,Mottini C, Salinas S, Negri R, Tagliafico E, Kremer EJ, Saggio I. Transcriptional Response of Human Neurospheres to Helper-Dependent CAV-2 Vectors Involves the Modulation of DNA Damage Response, Microtubule and Centromere Gene Groups. PLoS One. 2015 Jul 24;10(7):e0133607. doi: 10.1371/journal.pone.0133607. PubMed PMID: 26207738; PubMed Central PMCID: PMC4514711.

10. LUND → GENIBET (Luis QUINTINO, Sept 2014 and Aug 2016)

Seconded researcher:
Luis QUINTINO

Vector production methodologies under Good Manufacturing Practices (GMP)

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 was 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 collaborate in short-term projects to exchange scientific expertise and generate synergies for future collaborations.
- Step 2 (to be accomplished within the last 24 months of the BRAINVECTOR project)- Implementation of defined tasks in step 1

B. Work progress and achievements during the period.

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 at GENIBET. This step was performed on step 2 of the secondment. Nevertheless, the researcher was introduced to the basic upstream and downstream virus/vector production methodologies, namely production in bioreactors and chromatography purification techniques.
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 exchanged 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 scalable downstream methods optimal for CNS gene delivery using Lentiviral Vectors.

The second part of the secondment between Lund University and GenIbet was performed in October 2016. In this secondment, Luis Quintino visited the GenIbet state-of the art facility in Oeiras, Portugal.

The visit started with an overview of GenIbet organization and introduction to the concepts of good laboratory practices (GLP) and good manufacturing practices (GMP). These two key words are the cornerstone of today's pharmacological product development. GenIbet has permits since 2014 from the European Medicines Agency (EMA) and Infarmed, its representative in Portugal, to produce a wide range of biopharmaceutical Active Pharmaceutical Ingredients (biologicals) under GMP conditions for clinical trials. This fact made this Secondment a unique opportunity to increase the knowledge on how to translate an innovative research finding into a biomedical product for clinical trial.

Tiago Ferreira introduced the 1000 square meters of GMP upstream and downstream manufacturing such as the Bacterial Unit, Viral Unit for gene therapies and vaccines, Animal Cell Culture Unit and Fill and Finish Unit for small scale semi-automatic aseptic filling.

Tiago Ferreira and GenIbet members also discussed in depth several factors affecting the creation and more importantly the validation of every single step of the method to produce a novel biological. Such expert knowledge is very seldom available for researchers doing research and development on novel therapies. This was a very timely Secondment as the Gene Therapy Field is experiencing a period of exponential growth due to the increased safety and efficiency of novel viral vector based biologicals.

One of discussed aspects was the financial consideration of developing a novel biological. The sheer amount of funding needed is in orders of magnitude greater than the funding obtained by research laboratories in academic institutions. Therefore, industrial partnerships are paramount to develop novel products.

Another aspect is the logistics of validating the method to develop a novel biological. The experts at the analytics laboratory at GenIbet were able to show how the validation of all tests was performed and what were the timeframes for validating a novel biological for GMP production.

It became quite patent that there is a gap between the research performed in academic institutions and the development of novel biologicals needed for clinical trials. The focus of academic institutions on novel discoveries/methods/therapies needs to be complemented with higher requirements in terms of reproducibility and GLP mindset from the start to enable the creation and validation of novel biologicals that can help patients and ultimately the society at large. It is also very desirable to increase industry-academia partnerships to enable faster and safer product development.

11. IBET → CHUV (Vanessa BANDEIRAS, May – November , 2014)

Project manager:
Liliane TENENBAUM

Seconded researcher:
Vanessa BANDEIRAS

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

Aim of the secondment Transfer of knowledge for the development of a robust mid-scalable method for production and purification of Adeno-Associated viral vectors for treatment of Parkinson's disease.

Document Scope This document briefly reports the procedures and results obtained at IBET and CHUV under the scope of the BrainVectors secondment that occurred between May and November 2014.

Exchange of biologicals, materials and protocols

IBET received from CHUV:

1- Crude lysate from 60 x 10 cm plates of rAAV2/1-V16-hGDNF virus (12 tubes with 5 mL/each);
2- Two cryovials of HEK 293T cells (P15);
3- 5 µl of AAV RSS2 (standard virus from ATCC for qPCR titrations at $3x10^{10}$ copies/µl);
4- SV40 qPCR primers at 10 µM (250 µl/each);
5- FAM-TAMRA probe (undiluted, 100 nM/mL, 15 µl);
6- pDPrs1 (incapsidation plasmid for AAV1): 3 x 1 mL at 1 mg/mL and pAAV-V16-imESC1-GFP (vector plasmid): 2 x 0.2 mL at 1.9 mg/mL;
7- Doxycycline hyclate solution at 1 mg/mL (Sigma, D9891) in ethanol 100%;
8- Etoposide (Sigma, E1383);
9- PEI powder (PolySciences, 23966-2) around 100 mg;
10- Production/purification/analytics SOPs (in Annex 1-4). SOPs from IBET, UAB and UNAV (other seconded researchers) were also exchanged between institutions at the same period.

Documentation All data generated during the project were documented and stored in lab books, SOPs and data files.

Materials and Reagents were described in each of the SOPs performed and can be find in attachment (Annex 1-4).

Overall Summary of the secondment

Mid-scale production of GFP and GDNF batches of AAVs produced in IBET and CHUV (Annex 1)

Five batches of AAVs were produced in the scope of this project. Two of them were produced at the CHUV in a 60 plates system – AAV2/1-V16-GDNF and AAV2/1-V16-

imESC1-GDNF – another two were produced at IBET in a cell factory of 10 layers (CF10) – AAV2/1-V16-imESC1-GFP (batch #1 and batch #2) - and a fifth production of AAVs encoding GDNF was lately performed at the CHUV in a CF10 for transfer of knowledge (IBET to CHUV), according to the SOP already used in IBET.

Purification of the GFP and GDNF batches of AAVs produced in IBET and CHUV (Annex 2)

AAV2/1-V16-imESC1-GFP batch #1 produced in IBET was purified according to the SOP in attachment (Annex 2). For the remaining batches some modifications* were performed but also respecting the protocol used at IBET and that is resumed below:

- Freeze and thaw cycles (3) of the lysate;
- Benzonase treatment;
- PEG precipitation;
- Iodixanol gradient (using the Fraction Collector System for recovery of viral fractions);
- Concentration and exchange of buffer of the pure fractions with Amicon Centriplus 100;

*The modifications performed to the initial protocol were the addition of an 11 000 rpm centrifugation after benzonase treatment and the use of the double concentration of DNA for transfection (5.2 µg DNA/10^6 cells).

Analytical assays and quality control For the four batches of AAVs, produced and purified during this secondment, analysis of the viral productivity and quality was performed in order to screen these vectors as the ideal agents for use in the treatment of neurodegenerative diseases. The concentration and quality of the final AAVs preparation was analyzed by qRT-PCR (Annex 3) and SDS-Page Silver Staining (Annex 4) at the CHUV. Quantification of total protein, capsid proteins, endotoxins and DNA contaminants were analyzed at IBET. The results, comparing the three vectors (GFP, GDNF and immESC1-GDNF) and 2 different systems of production used (60 plates and CF10) are described below.

i. Viral Productivity measured by qRT-PCR

AAV1-V16-imESC1-GFP (CF10)						
Step	VG/mL		VG total		VG/cell	
Batches	#1	#2	#1	#2	#1	#2
Lysate	8.0E+11	n.a.	3.8E+13	n.a.	9.1E+04	n.a.
After PEG	n.a.	2.1E+12	n.a.	6.4E+13	n.a.	1.5E+05
Iodixanol (total of fractions)	-	-	2.1E+13	6.5E+11	5.0E+04	1.6E+03
Joined fraction 1	2.3E+12	2.1E+11	9.3E+12	2.1E+11	-	-
Joined fraction 2	2.9E+12	2.3E+11	1.2E+13	2.3E+11	-	-
After Amicon (Final)	**9.4E+12**	**1.1E+12**	7.5E+12	4.2E+11	1.8E+04	1.0E+03

n.a. not available; batch #1 and batch #2 were produced by transfection of a total of 2.6 µg and 5.2 µg/10^6 cells, respectively.

AAV1-V16-GDNF (60 plates)			
Step	VG/mL	VG total	VG/cell
Lysate	n.a.	n.a.	n.a.
After PEG	n.a.	n.a.	n.a.
Iodixanol (total of fractions)	-	4.2E+11	1.4E+03

Joined fraction 1	2.0E+09	1.0E+10	-
Joined fraction 2	2.7E+11	2.7E+11	-
After Amicon (Final)	**3.3E+11**	6.5E+10	2.2E+02

n.a. not available

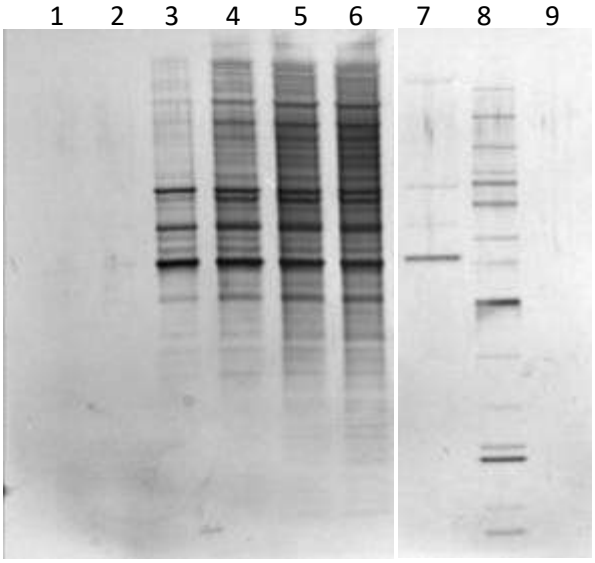
B. AAV1-V16-immESC1-GDNF						
	60 plates			CF10		
Step	VG/mL	VG total	VG/cell	VG/mL	VG total	VG/cell
Lysate	n.a.	n.a.	n.a.	3.2E+11	1.6E+13	3.8E+04
After PEG	n.a.	n.a.	n.a.	4.0E+10	1.2E+12	2.9E+03
Iodixanol (total of fractions)	-	1.7E+11	5.7E+02	-	4.6E+11	1.1E+03
Joined fraction 1	2.1E+10	1.4E+11	-	1.2E+11	3.7E+11	-
Joined fraction 2	2.8E+10	2.8E+10	-	4.5E+10	9.0E+10	-
After Amicon (Final)	**8.4E+11**	1.7E+11	5.6E+02	**8.1E+11**	3.2E+11	7.7E+02

n.a. not available

Purity by SDS-Page Silver Staining

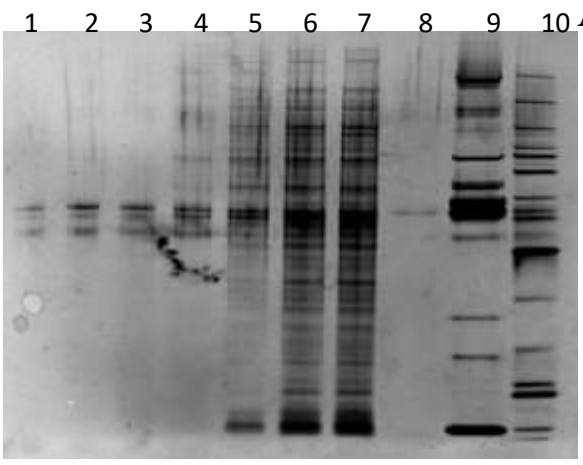
AAV-V16-imESC-GFP (CF10) batch #1
Not available; with several contaminants

AAV-V16-imESC-GFP (CF10) batch #2



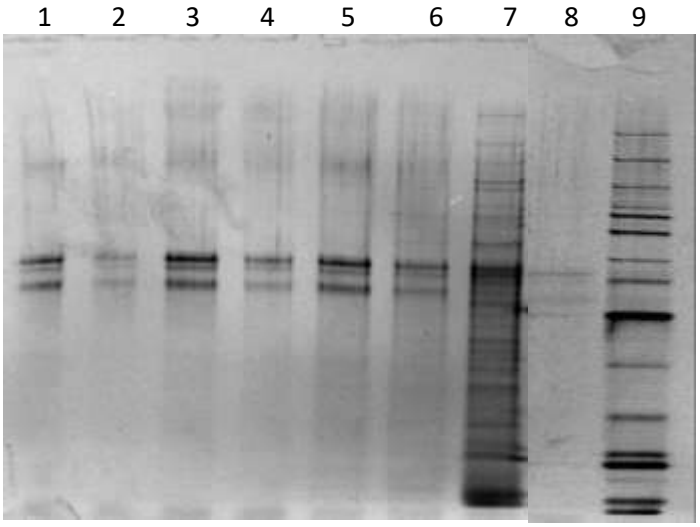
Legend:
1 - Fraction F2
2 - Fraction F3
3 – Fraction F4
4 – Fraction F5
5 – Fraction F6
6 – Fraction F7
7 - RSS AAV8 (positive control)
8 – BenchMark Protein Ladder
9 – DPBS-CMF (negative control)

AAV-V16-GDNF (60 plates)



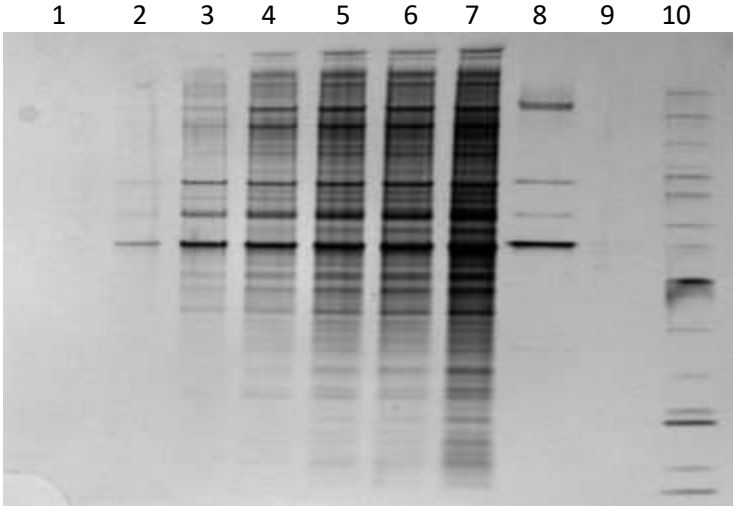
Legend:
1 - Fraction F2
2 - Fraction F3
3 – Fraction F4
4 – Fraction F5
5 – Fraction F6
6 – Fraction F7
7 - Fraction F8
8 – DPBS-CMF (negative control)
9 – AAV 29 (positive control)
10 - BenchMark Protein Ladder

AAV-V16-imESC1-GDNF (60 plates)



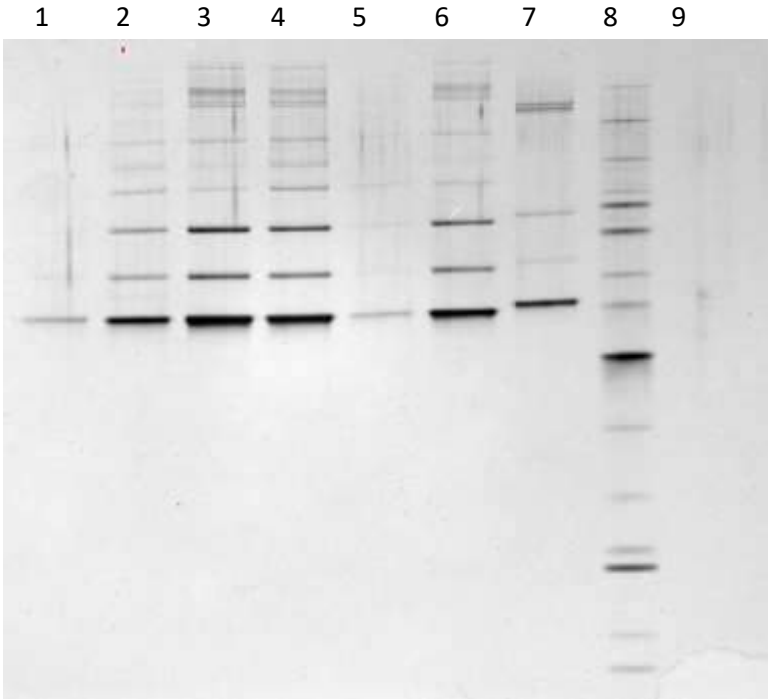
Legend:
1 - Fraction F2
2 - Fraction F3
3 – Fraction F4
4 – Fraction F5
5 – Fraction F6
6 – Fraction F7
7 - Fraction F8
8 – DPBS-CMF (negative control)
9 – BenchMark Protein Ladder

AAV-V16-imESC1-GDNF (CF10)



Legend:
1 - Fraction F2
2 - Fraction F3
3 – Fraction F4
4 – Fraction F5
5 – Fraction F6
6 – Fraction F7
7 - Fraction F8
8 - AAV 29 1:2 (positive control)
9 – DPBS-CMF (negative control)
10 – BenchMark Protein Ladder

Final vector preparations after Amicon 100 buffer exchange



Legend:
1 – AAV-V16-GDNF F2-F6
2 – AAV-V16-imESC1-GDNF F2-F7
3 – AAV-V16-imESC1-GFP CF10 F4-F5
4 – AAV-V16-imESC1-GFP CF10 F6-F7
5 – AAV-V16-imESC1-GDNF CF10 F3-F5
6 – AAV-V16-imESC1-GDNF CF10 F6-F7
7 - AAV 29 1:2 (positive control)
8 - BenchMark Protein Ladder
9 - DPBS-CMF (negative control)

	Total DNA (Pico green assay) (ng/ml) Note – detection limit of 8 ng/ml	Host cell protein (HEK kit from Cygnus) (ng/ml)	Titration AAV1 -Kit from Progen Note - the samples over the calibration curve should be repeated if necessary	Endotoxins -Endosafe assay, Charles River (EU/ml)	Total protein (BCA kit) (µg/ml) Note – all the samples are below detection limit of the method. Should be repeat with Micro BCA kit
Sample 1-AAV1-V16-GDNF 2-6=3,2E11 VG/mL	Below detection	<4 ng/ml	2,12E+12	<10	<8 µg/ml
Sample 2- AAV1-V16-imESC-GDNF 2-7=8,3E11 VG/mL	Below detection	<4 ng/ml	Over detection	<10	<8 µg/ml
Sample 3- AAV-V16-imESC-GFP CF 4-5=1,3E12 VG/mL	Below detection	<4 ng/ml	Over detection	<10	<8 µg/ml
Sample 4- AAV-V16-imESC-GFP CF 6-7=8,1E11 VG/mL	Below detection	<4 ng/ml	Over detection	n.d.	<8 µg/ml
Sample 5- AAV-V16-imESC-GDNF CF 3-5=2,2E11 VG/mL	Below detection	54 ng/ml	1,88E+12	<10	<8 µg/ml
Sample 6- AAV-V16-imESC-GDNF CF 6-7=1,4E12 VG/mL	Below detection	30 ng/ml	1,90E+13	n.d	<8 µg/ml
Sample 7- AAV-V16-GDNF #29 (dil1:2),	Below detection	20 ng/ml	Over detection	n.d.	<8 µg/ml

In vivo experiments with mice

Transfer of knowledge from CHUV to IBET was attained by learning in vivo protocols for injection of the AAV vectors produced in mice brain for treatment of neurodegenerative diseases as Parkinson's disease. Assays as stereotaxy, behavioural studies, perfusion, fixation and imuno-staining were learned and can be found in Annexes 5, 6 and 7.

Annex 1. Adeno-Associated Virus (AAVs) production in Cell factory (CF10)

Equipment and materials

Laminar flow cabinet
Eppendorf benchtop centrifuge
Vacuum pump with fluid aspiration system
Freezer -80 ºC
50 mL Falcon tubes
Serological pipettes.
T-flasks of 175 cm^2
ThermoScientific Cell factory of 10 layers

Reagents

HEK 293T cell line
DMEM 4.5g/l Glucose, L-Glutamine, Pyruvate, Life Technologies # 41966-052
Fetal Bovine Serum, Life Technologies #
Trypsin, Life Technologies
DPBS, Life Technologies

1. Cell Seeding

1.1. Count the cells of 10 to 12 T175 flasks and prepare the cell suspension with an innocullum of $6.7x10^4$ cells/cm^2 in a total of 800 mL DMEM 10% FBS ($6.7x10^4$ cells/cm^2 x 6320 cm^2 = ~420 x 10^6 cells); Do not forget to prepare also a cell suspension for a T175 flasks as a control ($6.7x10^4$ cells/cm^2 x 150 cm^2 = 10 x 10^6 cells/T150).
1.2. Confirm the cell concentration of the innocullum;
1.3. Inoculate the cell suspension in the CF10 and T150 flasks according to the manufacturer guidelines and incubate for 24 hours in humidified atmosphere of 5-7% CO_2.

2. Transfection

2.1. Twenty-four hours after seeding the culture medium is exchanged and replaced with new culture medium containing the transfection mix. As referred previously in the remaining batches the DNA concentration was doubled to 5.2 µg/10^6 cells.

CF10	Concentration (µg/µl)	M (µg)	Volume (mL)
DMEM w/o FBS	-	-	44 mL
PAC1-v16imESC1-hGDNF	0.9	0.6 µg /10^6 cells x 420 = 252 µg	280 µl
pDP1rs	1.0	2 µg /10^6 cells x 420 = 840 µg	840 µl
DMEM w/o FBS	-	-	42 mL
PEI	1 mg/ml	3 x 2.6 µg/10^6 cells x 420 = 3.3 mL	3.5 mL
T150 flasks	Concentration (µg/µl)	M (µg)	Volume (mL)
DMEM w/o FBS	-	-	973 µl
PAC1-v16imESC1-hGDNF	0.9	0.6 µg /10^6 cells x 10 = 6 µg	6.7 µl
pDP1rs	1.0	2 µg /10^6 cells x 10 = 20 µg	20 µl
DMEM w/o FBS	-	-	922 µl
PEI	1 mg/ml	3 x 2.6 µg/10^6 cells x 10 = 78 µg	78 µl

2.2 Prepare the DNA and the PEI mixture. Vortex. Filter (0.2 µm) the DNA solution into the PEI mixture;
2.3. Incubate at RT for 15 minutes;
2.3. Add the transfection mix in a 1L Schott bottle with 800 mL of DMEM 10% FBS. Shake the schott for efficient mixing;
2.4. Change the culture medium of the CF10 carefully, avoiding bubble formation;
2.5. Incubate for 48 hours in humidified atmosphere of 5-7% CO_2.

3. Observation of cells and addition of doxycycline to one of the T150 flasks

3.1. Doxycycline (1 mg/mL) was added 24h after transfection at a final concentration of 1 µg/mL to the T175 flask to induce expression (TetON system) of the GFP gene, so 30 µl of doxycycline was added to the 30 mL of culture media.

4. Vector harvesting

4.1 Forty-hours post-transfection, GFP fluorescence of the T150 was observed at a fluorescence microscope to estimate the percentage of the transfected cells. GFP expression can also be analysed in FACS, after fixation of the cells.
4.2. The cell factory was harvested by removing the supernatant (which can be saved at 4ºC for further analysis). 300 mL of citric saline was used to release the cells from the surface and the CF10 agitated back and forward to detach the cells. CF10 was washed with another 200 mL of citric saline and all volumes of cell suspension was recovered and centrifuged at 200-300g for 10min (RT). After centrifugation, supernatant with citric saline was stored at 4ºC, for future analysis (if needed) and cell pellet was ressuspended with AAV ressuspension buffer (containing 50 mM Tris, 150 mM NaCl and 2 mM MgCl2 pH 8.5) in a final volume of 50 mL. Two falcons, containing ~ 24 mL/each of the cell lysate were stored at -85ºC until further analysis.

Annex 2. Adeno-Associated Virus (AAVs) purification

1. Treatment of lysate

1.1. For the purification the recovered AAV lysates were thawed in the water bath at 37ºC and two cycles of freeze (dry ice with ethanol 96%) and thaw were performed. After each thaw the tubes were vortex/agitated until a more homogenous suspension was formed. After the last thawing the suspension was incubated with 50U/mL of benzonase (250U/µl) at 37ºC for 60 min (5 µl/tube). During the incubation period the lysate should be agitated once in a while.
1.2. After incubation, the falcon tubes were centrifuged at maximal velocity (5000g) for 30 minutes and the supernatant containing the AAVs retrieved, benzonase was inactivated with EDTA 5 mM (2.3 mL of a 50 mM stock/tube) and stored at -80ºC for further analysis. As referred previously, the centrifugation of 5000g was exchanged by a centrifugation of 11 000 rpm during the purifications performed at the CHUV to further eliminate the remaining cell debris.

2. PEG Precipitation

Equipment and materials

Laminar flow cabinet
Refrigerated benchtop centrifuge
Water bath at 37 ° C
Vortex
Oak Ridge centrifuge tubes

Reagents

5M NaCl
40% PEG-8000
Resuspension Buffer AAV (50 mM Tris, 150 mM NaCl, 2 mM $MgCl_2$, pH 8.5)

2.1. For performing the PEG precipitation protocol 1 mL of NaCl 5M was added to each tube containing 24 mL of the crude lysate, in order to adjust the final concentration of NaCl to 200 mM.
2.2. 1mL of PEG-8000 (40 %) was also added *per* 4 mL of crude lysate (6 mL of PEG *per* tube) in order to achieve a concentration of PEG of 8%.
2.3. The lysates were then transferred to two Oak Ridge tubes and incubated overnight at 4 ºC.
2.4. On the next day, centrifugation of the lysates for 15 min at 8000xg and 4 ºC was performed.
2.5. Supernatant was discarded by aspiration; the precipitate formed was washed with 1 mL of AAV re-suspension buffer and ressupended in final volume needed to perform the iodixanol gradient (between 12.5 to 15 mL depending on the tubes used). Ressuspension of the precipitate is time-consuming and difficult (up and down should be around 100 times *per* tube until complete dissociation of the precipitate). At the end the tubes should be vortexed during one minute.
2.6. Re-suspension is ready for the iodixanol gradient and can be stored up to 48h at 4ºC (may improve further dissolution of the precipitate).

3. Purification by iodixanol gradient

Equipment and materials

- Falcon tubes
- Serological pipets
- Eppendorf tubes 1.5 ml
- Ultracentrifuge OptiSeal Tubes
- OptiSeal Stoppers
- Adapters for Opti or Quick Seal tubes
- Syringes of 20 mL
- Rallonge pompe-seringue
- Infusion needles 135x2, 0mm
- Push syringe pump
- Recovery Fraction Collector
- Rotor 70Ti
- Laminar flow cabinet
- Ultracentrifuge

Reagents

- Optiprep (60% iodixanol) (Sol. A)
- Phenol Red 0.5%
- 10x PBS
- PBS CMF
- 10x PBS-MK w/ 10 mM MgCl2 and 25 mM KCl pH 7.2 (Sol. B)
- 1x PBS-MK w/ 1 mM MgCl2 and 2.5 mM KCl pH 7.2 (Sol. C)
- PBS-MK w/ 2M NaCl (Sol. D)
- 54% iodixanol working solution (Sol. E)

3.1. Before starting the following solutions should be prepared by dilution of Solution A (60% iodixanol, Optiprep):
- 60%: 40 mL of Sol. A with 20 µl of Phenol Red
- 40%: 20 mL of Sol. E and 7 mL of Sol. C
- 25%: 12.5 mL of Sol. E, 14.5 mL of Sol. C and 13.5 µl of Phenol Red 0.5%
- 15%: 15 mL of Sol. E, 27 mL of Sol. D and 12 mL of Sol. C

3.2. Deposit each solution, to the bottom of the ultracentrifuge tube slowly and carefully (using syringes), in the following order with the volumes depending on the tubes used:

Solution/Volume used	OptiSeal (30 mL)	QuickSeal (40 mL)
Lysate	**12.5**	**15**
15% iodixanol	**4.5**	**7.5**
25% iodixanol (red)	**4**	**5**
40% iodixanol	**4**	**4**
60% iodixanol (yellow)	**4**	**4**

3.3. When using a syringe pump put at 90 mL/hour. At the end adjust the volume with PBS-MK until the top of the tube, if necessary.
3.4. Place the non-reusable black cap on top of the tube following the instructions of the OptiSeal tubes. Add the spacer/cap of the tube to prevent the collapse of the tubes within the rotor.
3.5. Equilibrate tubes and centrifuge them in a 70 Ti rotor 69.000rpm for 1h30min at 18 ºC.
3.6. After centrifugation, place the tube in the holder manifold (Fraction Recovery System) (the needle enters through the bottom of the tube and should be washed with water). Discard the first 2-3 mL (F1) and collect fractions of 500µl each, F2 to F8.
7. Take 20 µl aliquots of each fraction from F2 to F6 for the quantification and analysis of the purity of AAV vectors (RT-PCR and SDS-PAGE).
8. Store all fractions at -80 ° until use.

4. Micro-concentration and buffer exchange

4.1. For microconcentration using Amicon Centriplus 100 of 15 ml tubes (Millipore), the fraction to be concentrated should be diluted 1:1 or more with DPBS Ca^{2+} Mg^{2+}.
4.2. Reduce the volume in the Amicon by centrifugating at 5000g for 2 minutes.
4.3 Add more DPBS Ca^{2+} Mg^{2+} up to 15 mL until volume is reduced and then perform 3 washes with DPBS Ca^{2+} Mg^{2+} to replace the buffer of the viral preparation.
4.4. Make aliquots of the virus in siliconized 1.5 mL tubes and store at -80ºC.

<u>Annex 3.</u> Real-Time PCR for Adeno-Associated Virus (AAVs) titration

Equipment and materials
- LightCycler instrument (Roche)
- LightCycler DNA Master SYBR Green I (Roche)
- Eppendorf miniSpin centrifuge
- Nuclease free water
- Disposable pipette tips with filter
- Sterile 1.5 ml Eppendorf tubes
- PAC1-V16imESC1-GFP plasmid
- Universal ITR forward primer (GGAACCCCTAGTGATGGAGTT)
- Universal ITR reverse primer (CGGCCTCAGTGAGCGA)

1. Preparation of standard plasmid

1.1. Standard reference was made by digestion of PAC1-V16imESC1-GFP plasmid with BamHI enzyme. Plasmid was then purified and quantified.
1.2. Calculations on the amount of copies that a given quantity of DNA in ng/µl corresponds in terms of amount of viral copies/µl were performed.
1.3. Plasmid was diluted to 1E9 copies/µl and 1:10 serial dilutions were perfomed form 1E9 to 1E2 copies/µl to be used as a standart curve.

2. RT-PCR
2.1. In a clean laminar flow pipette the water necessary for the mix and samples to avoid contaminations.
2.2. Perform 1:10 dilutions of the samples (1:10, 1:100, 1:1000, 1:10000).
2.3. Prepare enough mix for reactions of each standart curve, samples and controls (PCR grade water, SDB):

	Volume (µl)/sample		Conc.
Sybrgreen (2x)	**10**	µl	1x
Fw primer (5 µM)	**1.2**	µl	0.3 µM
Rv primer (5 µM)	**1.2**	µl	0.3 µM
Nuclease free water	**0.6**	µl	-
Sample	**7**	µl	-

2.4. Vórtex and spin-down master mix and distribute 13 µl *per* tube.
2.5. Add 7 µl of controls, samples and standart dilutions to the tubes (from the most diluted to the most concentrated) by order.
2.6. Transfer the tubes to the instrument and start run using the following conditions:

Program	Temperature (°C)	Hold Time (sec.)	Cycles
Pretreatment	95	600	1
Amplification			
Denaturation	95	15	45
Annealing	59	10	
Elongation	72	10	
Melting	95	0	1
	65	15*	
	95	0	
Cooling	40	30	1

*slope of 0.1°C/sec in this step. All remaining steps were performed at a slope of 20°C/sec.

2.7. When run finishes analyze the results using "Second Derivative Maximum" and "Melting Curve". Slope should be between -3.3 and -3.9 and error below 0.1.
2.8. Initial concentration of samples is calculated based on the following equation:

Viral Titer (VG/mL) = Titer (PCR) x dilution x 1000 (mL)

For the RT-PCR assays performed at the CHUV, optimization of the PCR conditions needed to be optimized for QIAGEN equipment. Removal of the last amplification step (elongation at 72°C) and dilution of samples in sample dilution buffer (200 µg/mL ssDNA, 10% PF68 and H_20) was successful for achieving good results.

Annex 4. Identity and Purity assay of Adeno-Associated Virus samples

Equipment and materials

- 1.5 ml tubes
- Dry-bath at 70°C
- Microcentrifuge
- Electrophoresis Apparatus
- Shaker
- Stir plate
- UV imaging system
- Imaging software to determine band densities
- 10 wells NuPage 4-12% Bis-Tris gel

Reagents

- Milli-Q water
- 1x DPBS-CMF
- 20x MOPS-SDS NuPage
- 1x MOPS running buffer
- NuPage Reducing Agent
- NuPage LDS Sample buffer (4x)
- BenchMark Protein Ladder
- SilverXpress staining kit
- Methanol
- Acetic Acid 100%

1. Safety

- Wear gloves, safety glasses, and protective clothing while preparing and working with all the silver stain solutions.
- The image Development Reagent should be used only in areas with good ventilation. Avoid breathing vapors. Avoid contact with skin.

2. Polyacrilamide Gel Procedure

2.1. Prepare the reducing buffer by mixing 1 ml of LDS Sample buffer (4x) and 400 ml Reducing Agent (10x) (all solutions are at 4°C in the door of the fridge)
2.2. Prepare reduced samples:
- Prepare 1 aliquot of the virus containing 13 µl of pure virus;
- Make 1 negative control using an equivalent volume of DPBS-CMF;
- Add 7 µl of LDS reducing mix (5 µl of LDS sample buffer and 2 µl of reducing agent) to each sample;
- Heat samples for 10 minutes to 70°C;
- Allow samples to cool at room temperature, approximately 5-10 minutes and spin-down;

2.3. Prepare protein ladder standards for Gel (Silver Stain):
- In a micro centrifuge tube labeled "Protein Ladder Dilution", add 38 ml of 1x DPBS-CMF and 2 ml of protein ladder standard;
- In a microcentrifuge tube labeled "Ladder A, Reduced", add 1 ml of "Protein Ladder Dilution" and 9 ml of 1x DPBS-CMF and 4 ml of reducing buffer;

2.4. Assemble the gel apparatus:
- Prepare 1 polyacrylamide gel and the electrophoresis unit according to manufacturer's directions;
- Fill the upper buffer chamber with 1x running buffer until the wells are completely covered and with 2 cm of buffer at the bottom;

2.5. Load the samples, protein ladders and controls in the gel;
2.6. Carefully place the lid on the gel box. Avoid disturbing samples. Lid must be attached so that red and black power jacks on the safety lid and base line up.
2.7. Allow the gels to run for approximately 90-120 minutes at 120 volts or until dye front has reached the bottom of the gel;
2.8. When electrophoresis is complete, open the gel cassette with a gel knife, cut an identifying mark in the bottom right corner of the gel under lane 10.

3. Silver Staining Procedure

3.1. Prepare Fixing Solution by combining reagents in the order shown below:

Reagent	Volume
Milli-Q Water	90 ml
Methanol	100 ml
Acetic Acid	20 ml

3.2. Pour the Fixing solution into a clean bowl in glass on a shaker and shake at 50 RPM;
3.3. Invert the gel and plate under fixative solution in the tray and gently agitate until the gel separates from the plate (can cut gel from the cassette with a razor) and for 10 minutes;
3.4. Remove fixative solution and waste it;
3.5. Wash gel in 100 ml of Sensitizing solution, twice for 30 minutes:

Reagent	Volume
Milli-Q Water	105 ml
Methanol	100 ml
Sensitizer	5 ml

3.6. Rinse the gel in 200 ml of Milli-Q water, twice for 10 minutes with gentle agitation;
3.7. Stain and Develop the gel by preparing the staining solution as listed below and staining with gentle agitation for 15 minutes (do not prepare more than 5 minutes prior to use):

Reagent	Volume
Milli-Q Water	90 ml
Stainer A	5 ml
Stainer B	5 ml

3.8. Wash the gel twice in Milli-Q water, 5 minutes each;
3.9. Develop the gel with 95 ml Milli-Q water and 5 ml of Developer;
3.10. When bands are well defined add 5 ml of Stopper and continue shaking for 10 minutes (developing of bands usually occurs quickly, but it may take up to 15 minutes);
3.11. Rinse the gel A in 200 ml of Milli-Q water for 5 minutes to overnight;
3.12. Remove carefully the gel from the bowl to a transparent plastic box of petri dish and put some water;
3.13. View the gel using the BioRad system, normal light (in some cases conditions of ethidium bromide/gel red detection also works).

Annex 5. Manipulation dose of AAV2/1-V16-GDNF – Behavior studies overview

Animals: rat wistar female
N=6 to 8 *per* group
N= 38 in total

Groups :
5 different doses of AAV1-V16-GDNF: dox 0 (n=8), 0.025 (n=6), 0.075 (n=6), 0.2 (n=6), 1 (n=6) g/kg of diet and 1 control group of AAV1-V16-GFP: dox 1g/kg of diet (n=6).

Virus:

- AAV2/1-TetR V16-GDNF from Brussels of23/04/14. Titer of $5.03x10^{12}$ VG/mL. Diluted 2.5 times in DPBS -> $2x10^{12}$VG/mL.
- AAV2/1-TetR V16-EGFP stock 15. Titer of $7.8x10^{11}$VG/mL

Injections:
In the striatum: 4 injections of 1 µl (2 sites of injections, 2 levels of deposits)
Coordinats (Yang) :
AP : 0 +1
ML : -3.6 -2.8
DV : -4 et -5 -4.5 et -5.5
Except for rats 1 and 2:
AP : +0.2 +1
ML : -2.2 -2.8
DV : -4.5 et -6.0 -4.5 et +5.5

Chronology and Treatment:

Injection : from 11 to 15 August 2014
Treatment with dox: 18 August 2014
Replenishment every 2 days to avoid degradation of dox.
Rotations : 24-25-26-29 September 2014
Sacrifice : 13 October 2014 week = W9 post viral injection or W8 of treatment with dox.

Annex 6. Rat Brain Fixing by Paraformaldehyde Perfusion

Materials and reagents:

- Tube falcon 50 mL;
- Ice, PAF, NaCl and plastic bottles;
- Catheter Venflon with sleeve (1 per day), 3-way valve, dissection equipment, scalpel, 1 mL syringe, needles, balance;
- Bottle for PAF waste, transparent and yellow bags for animal disposal;
- Pentobarbital/ NaCl 0.9%, (extemporaneous dilution of ½, injection of 1mL/kg)

1. Preparation of PAF:

1.1. Prepare 0.4M phosphate buffer	For 1 liter:	
Sodium phosphate dibasic (Fluka-71642)	HNa_2PO_4	46 g
Sodium phosphate monobasic (Sigma 71507)	$H_2NaPO_4*H_2O$	10.5 g
H_2O bidistilled		up to 1 liter

1.2. Mix well by stirring and store at 4°C. Before use, warm to RT and stir to dissolve crystals.
1.3. To prepare 1 L of PF4 (Sigma, P6148), dissolve 40g of PAF powder in 625 ml of bidistilled water.
1.4. Add 40 drops of 1M NaOH and heat up to 60°C (use the stirrer). Dissolving takes about 20 min (if longer, add more NaOH).
1.5. Filter the fixative and add 375 ml phosphate buffer.
1.6. Cool the solution to RT and adjust the pH to 7.4.
1.7. Store at 4°C.

2. Preparation for perfusion protocol:
Perfusion solutions:

- NaCl 0.9% pH7.4, at RT, perfusion at 30 mL/min, ~ 100 mL (until discoloration of the liver).
- PAF 4%, 4°C (in ice), 30 ml/min, ~ 200mL.

2.1. Under hood purge the pump hoses with PAF and NaCl.
2.2. Connect the tubes to the PAF and NaCl bottles.
2.3. At the end of the pipe set a 3-way valve used to adapt the cath-Venflon. Fill the tubes with both solutions.
2.4. Perform animal incision at the bottom of the sternum, diaphragm and cut on side. Insert the bottom left ventricular catheter, go gently into aorta. Remove the needle and leave the sleeve, attach the 3-way valve connected to the tubing. Incision in the right atrium and begin immediately NaCl perfusion. Making a black mark on the end of the needle can help to check it its positioned in the aorta.

4. Post-fixation:
4.1. Put brain in 10 mL PAF 4% overnight;
4.2. Put brain in sucrose 20% until the brain falls to the bottom of the tube;
4.3. Put brain in 30% sucrose until the brain falls to the bottom of the tube.

Freezing:
2 flasks with isopentane (2-methylbutane, Sigma, #59070): one at -10°C and another at -20°C. Put the brain for 10 seconds at -10°C, for 30-60 seconds at -20°C and then wrap brains in aluminum foil and place them on dry ice and -80°C.

Annex 7. Immuno staining anti-GDNF / DAB on floating sections

Primary antibody:

Anti-hGDNF biotinilated IgG (R&D Systems, BAF212), at 0.2 mg/mL
Use at a dilution of 1:500.

First Day:
1. Cut with Cryostat (section of 50 um and stock at -20ºC in antigel;
2. Wash 3 times with TBS 1x for 10 min;
3. Block endogenous peroxidase with 10% methanol, 3% H2O2 in TBS for 30 min at RT;
4. Wash again 3 times in TBS 1x;
5. Block sections by incubating at RT for 30 min in 5% BSA in THST;
6. Incubate with primary antibody overnight at 4ºC in THST 1% BSA;
7. Wash 3 times with TBS 1x for 10 min;
8. Incubate at RT in the ABC mix;
9. Wash 3 times for 10 min in PBS 1x;
10. DAB labelling for 2 minutes until sections appear stained enough and keep the same time of incubation for all sections;
11. Wash on last time for 2 times 5 min in tap water;
12. Mount sections in a lamina;
13. Dehydrate for 30 seconds in EtOH 70%, EtOH 96%, EtOH 100% 2x, Xylol 2x;
14. Glue lamellae on top of the plate with 1 drop of Eukitt glue. Apply pressure and eliminate bubbles from lamina. Allow to dry for 12h at RT.

ABC Kit preparation:
1 drop of solution A + 2.5 mL of PBS 1x + 1 drop of solution B. Mix well and incubate for 30 min.

DAB kit (SK-4100):
2.5 mL of nanopure water + 1 drop of buffer stock. Mix well. Add 2 drops of DAB. Mix well. Add 1 drop of H_2O_2 and mix well.

THST:
Tris pH 7.6 at 50 mM + 0.5M NaCl + 0.5% Triton 100X

TBS:
10 mM Tris pH 7.6 + 0.9% NaCL

12. IBET → UAB (Cristina PEIXOTO, June – July , 2015)

Seconded researcher:
Cristina PEIXOTO,

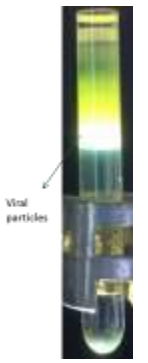
Title: ***Mid-scale production and purification of AAV vectors***

Overall Summary of the secondment

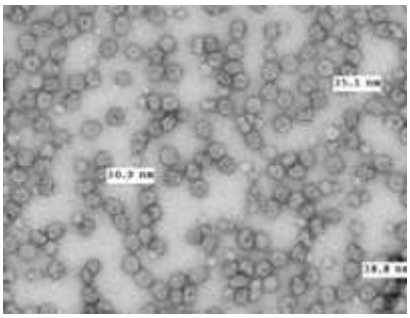
The main aim was the transfer of knowledge and technology for the development and implementation of a robust and scalable method for purification and characterization of adeno-associated viral vectors between iBET and UAB. This document briefly reports the procedures at UAB under the scope of the BrainVectors, which occurred during June and July of 2015.

Mid-scale production of AAVs was performed followed by the **Purification of AAVs batches** according to CsCl gradient Protocol in use at UAB. The purification steps included benzonase treatment, poly-ethylene-glycol-8000 precipitation, two rounds of CsCl gradient (Figure 1A) and final buffer exchange. However some modifications were performed. The concentration and exchange of buffer of the pure fractions was performed with Amicon plus 50 instead of using traditional dialysis cassettes and the ionic strength of the sample was increased up to 300mM of NaCl in order to improve the final recovery yield of virus particles. The purifications steps were discussed and compared with other methods for AAVs purification, namely purification by iododixanol gradient and affinity chromatography previously implemented at iBET.

Analytical assays and quality control
A rapid and robust rAAV titration method based on the quantitation of encapsidated DNA with the fluorescent dye PicoGreen® implemented by UAB was learned and transferred to iBET. Since this method allows detection from $3x10^{10}$ viral genome/mL up to $2,4x10^{13}$ viral genome/mL in a linear range. Contrasted with Dot-Blot or qPCR for example, the PicoGreen-based assay has less intra- and inter-assay variability**.** Analysis of the viral productivity and quality was performed in order to assess if these vectors can be used in the treatment of neurodegenerative diseases. The concentration of the final AAV preparation was determined and comparative analysis made and with iBET methodologies discussed. To evaluate the integrity and morphology (shape, size) of the virus particles, electron microscopy (TEM) was performed (Figure 1B).



A



B

Figure 1. A) 1st cesium chloride gradient. Virus particle are present in the white band. **B)** TEM analysis of AAVs produced and purified at UAB.

Finally, a seminar providing an overview on traditional and emerging technologies for AAV vectors characterization and quality control was performed. The advantages and drawbacks of different characterization techniques (ex. analytical ultracentrifugation, mass spectrometry analysis, dynamic light scattering) were assessed and discussed with UAB researchers.

13. FIMA - IBET (Diego PIGNATARO, September – October 2016)

Seconded researcher:
Diego PIGNATARO
jpignataro@alumni.unav

Activity 1. *Development of AAV vectors expressing eGFP constitutively under the control of astrocyte and neuronal specific promoters*

As the AAV has a small genome size, which limits its cloning capacity, the use of minimal specific promoters facilitates the expression of larger genes or co-expression of more than one gene from the same vector. FIMA has developed a series of astrocyte- and neuron-specific small promoters in the context of an AAV8 vector with the aim of using these vectors in 3D cultures and analyze their specificity. Astrocytes are one of the most abundant cell type in the vertebrate CNS and contribute to the pathogenesis of neurodegenerative disorders and may be an ideal cellular target for the delivery of therapeutic genes. Glial fibrillary acidic protein (GFAP) is the major intermediate filament protein in mature astrocytes and is essential for astrocyte maturation and for the response to CNS injury (Norenberg and Michael, 1994). Consequently, choosing the GFAP promoter could be a suitable alternative to drive widespread astrocyte-specific expression. First, at FIMA, we analyzed the expression pattern of eGFP under the control of a reduced version of the GFAP promoter developed by Lee et al. (2008) named gfaABC1D (hGFAP pr) and a modified version in which the D sequence of the promoter was removed (hGFAPΔD pr). The D region was shown to play an important role in the functionality of the promoter due to its interaction with the B region (Besnard et al., 1991). Furthermore, using the structure of the human gfaABC1D promoter and the sequence of the mouse GFAP promoter, a murine gfaABC1D promoter was constructed (mGFAP pr). With the aim of restricting the expression of the transgene to neuronal cells, the minimal promoters controlling the expression of the BM88 neuronal protein and the β2 subunit of the nicotinic acetylcholine receptor (CHNRB2) were used. These vectors have been tested in the HEK-293T.

Activity 2. *Training in the development of 3D cultured human neuroprogenitors*

During my stay at IBET, I was trained in the development of 3D in vitro models of the human CNS following an approach based on stirred culture systems. As cell source we used human midbrain-derived neural progenitor cells (hmNPC) as these can be expanded in vitro and differentiated into tyrosine hydroxylase (TH)-positive cells, in 2D culture conditions. The methodology is very amenable to be transferred to FIMA for the production of human differentiated neurospheres using the stirred culture systems. In the near future we will be allowed to culture and handle differentiated neurospheres for long periods of time, including overexpressing genes using our viral vectors. Although, the transduction efficiency must be optimized to our laboratory conditions. Long-term expression of the different transgenes is needed when the eGFP will be replaced by specific transcription factors to reprogram cultured cells in a different neuronal subtype.

14. ULB → FIRALIS (Abdelwahed CHTARTO, Oct 2015 - Oct 2016)

Seconded researcher :
Abdelwahed CHTARTO

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Project component: **Immunological aspects**

The interest of accomplishing this part at Firalis

Firalis is constituted of four companies including MABSOLYS, a SME specialized in the production of antibodies, their characterization and the setting up of Elisa Kits for commercialization, in addition to their expertise in the immunological study of a variety of antigens.

Development of ELISA Kit of Human GDNF

Interest: In the Brainvectors project, the Glial derivative neurotrophic factor (GDNF) constitutes the therapeutic gene to be transferred into Parkinson's disease models using the best viral vector tested and carrying the tetracycline cassette. In the other hand the commercially available GDNF ELISA Kit is expensive, has a low sensitivity and the time of realization is long. Thus it is important to try to develop an ELSA Kit more efficient for the determination of GDNF amount.

Procedures

Genetic immunization

Because the GDNF recombinant protein is very costly, we opted for gene immunization which consists on the intramuscular injection of the DNA vector expressing the GDNF by using JETPEI (Evita et al., 2012) a transfecting agent which acts, in addition, as adjuvant for an efficient reaction to the antigen.

Plasmids construction

In a first step I constructed a vector which expresses the GDNF under the control of the constitutive hCMV promoter. I also introduced a tag histidine to be able to purify the recombinant protein in an *in vitro* production system. Next, I wanted to increase the potential of secretion of GDNF for better results in immunization and purification of the recombinant protein. Thus I have replaced the signal of secretion of GDNF by the *Gaussia luciferase* signal shown as the best one when compared to those of a variety of proteins (Knappskog et al., 2007). (Figure 1)

Results

Comparison of pTT5-GDNF and pTT5-GDNF-GL per transient transfection in HEK

5x10E5 cells per well were seeded in a 6 well plate. The following day the cells were transfected by 200 ng of pTT5-GDNF and pTT5-GDNF-GL using the PEI method. 48 hours post-transfection the supernatant was replaced with fresh medium and the GDNF was measured in supernatant after 30 min, 60 min, 90 min and 48 h. The results represented in figure 2 show efficient secretion by the use of *Gaussia Luciferase* secretion

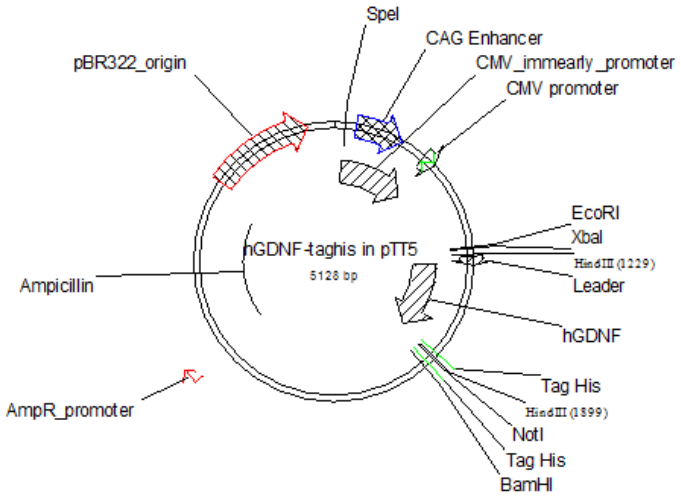
signal. Thus, the vector of choice that will be used for gene immunization is pTT5-GDNF-GL (*Gaussia Luciferase* secretion signal).

Genetic immunization

Three females 6 weeks aged BALB/cBYJRj mice were injected with the DNA complex (pTT5-GDNF-GL) / JetPEI (for the protocol see Evita et al., 2012). The mice were incubated for 3 weeks and were then re-injected with the same complex. 3 weeks after the second injection, the serum was harvested and tested for the presence of anti-GDNF antibodies. One mouse responded to immunization and was treated to find the best antibody pairs given a GDNF ELISA Kit with high-performance (production of the Kit in progress).

Figure 1

GDNF plasmid expression
Secretion signal: MKLWDVVAVCLVLLHTASA



GDNF plasmid expression with *Gaussia Luciferase* secretion signal
Secretion signal: MGVKVLFALICIAVAEA

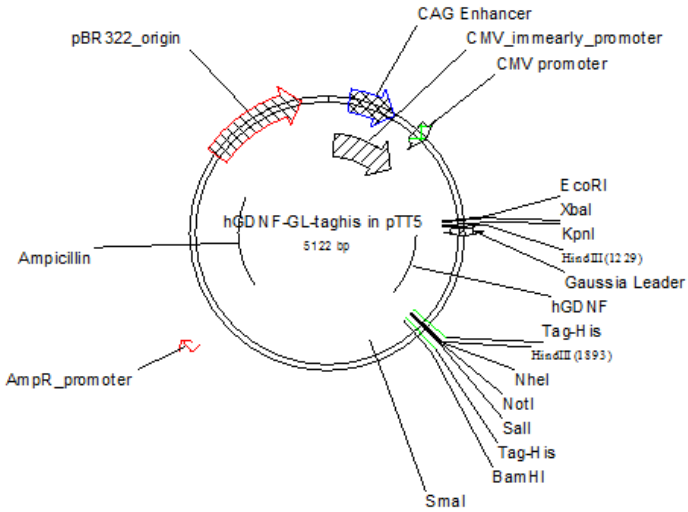
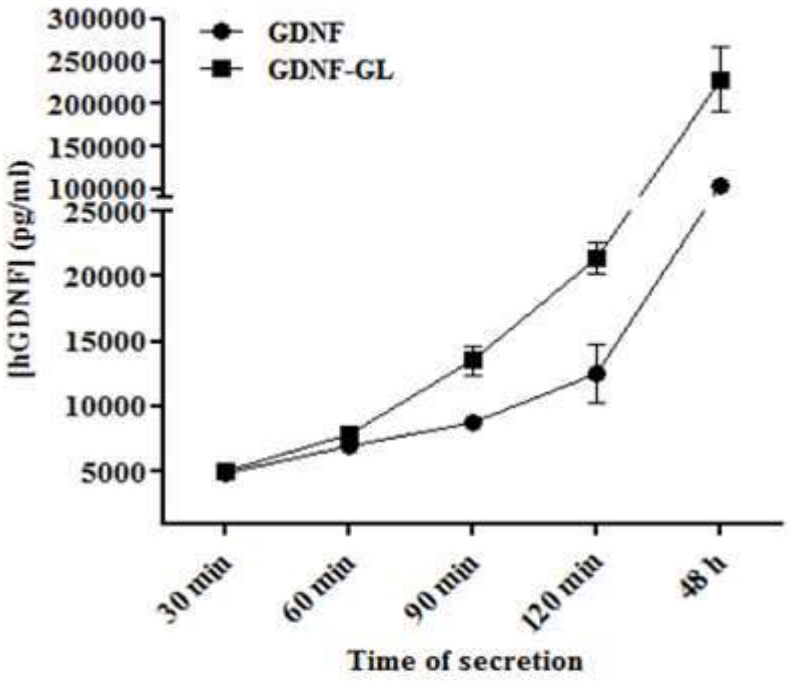


Figure 2



Secretion rate:

GDNF: 7211pg/ml/h ±1812

GDNF-GL: 9384pg/ml/h±1590

Immune response of rtTAV16 versus rtTAV16 immuno-escape (rtTAV16IS)

Although the tetracycline-inducible system remains among the best transgene regulatory systems for gene therapy of brain diseases, the immune response of the prokaryotic transactivator is a real obstacle to its use in humans. To overcome this disadvantage of the tetracycline-inducible system, Ben Berkhout's group developed a new, less immunogenic transactivator, rtTAV16is sensitive to a low level of inducer as rtTAV16.
The purpose of the second part of my secondment is to compare the immune response of rtTAV16 versus rtTAV16is after intramuscular injection in humanized mice. In order to avoid an immune response against the transgene, we chose the MUSAEP, a non-immunogenic transgene in humanized mice.

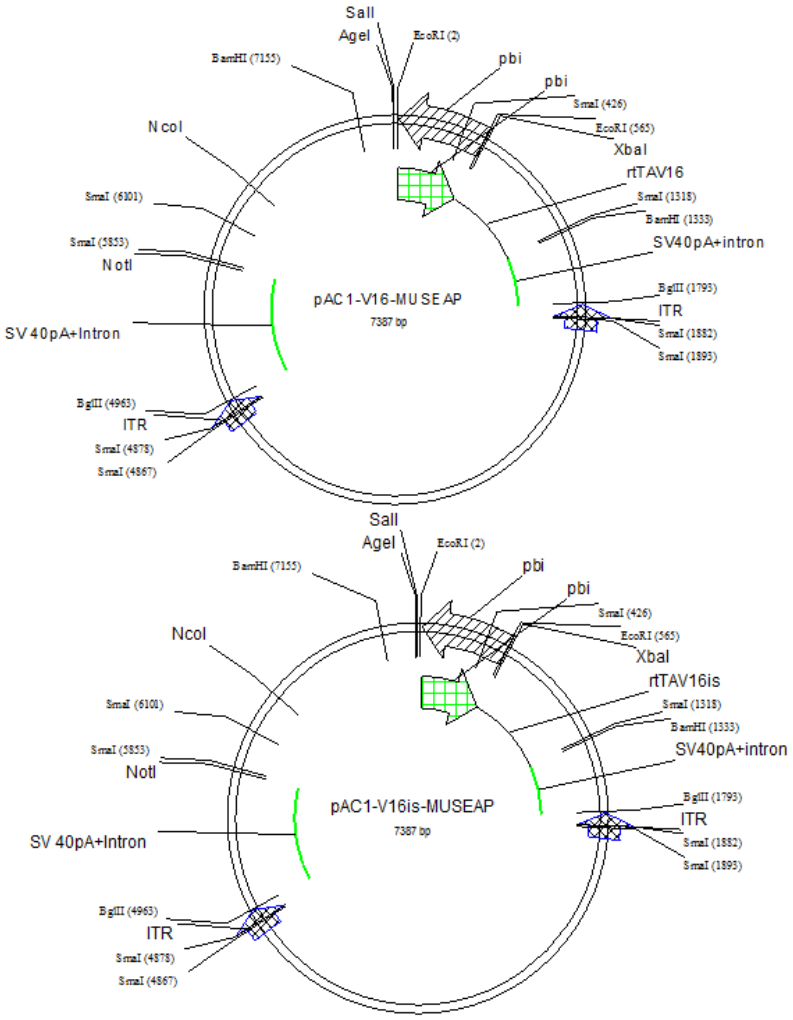
Plasmids construction and results

In pAC1-V16-EGFP and pAC1-V16is-EGFP I have replaced EGFP with MUSAEP (**Figure 3**).
A very important step in this part of the project is to perfect the test of detection of MUSEAP activity in the murine serum after intramuscular injection of the DNA / JetPEI complex.
Three females 6 weeks aged BALB/cBYJRj mice were injected with the DNA complex / JetPEI using a vector expressing constitutively MUSAEP transgene. The serum of injected mice was taken 3, 9, 18, and 34 days after immunization and tested for the

MUSEAP activity in comparison of the serum of non-injected mice as a group control. The result is represented in the secondment report of **Giorgia Zanetti**.

Given the inconclusive results of this experiment, it is necessary to invest more time in developing the MUSAEP detection test before evaluating the immune response of rtTAV16 versus rtTAV16is in humanized mice.

Figure 3



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Enhancement of plasmid DNA immunogenicity with linear polyethylenimine. Eur J Immunol. 2012 Nov;42(11):2937-48.

Knappskog S1, Ravneberg H, Gjerdrum C, Trösse C, Stern B, Pryme IF. The level of synthesis and secretion of Gaussia princeps luciferase in transfected CHO cells is heavily dependent on the choice of signal peptide. J Biotechnol. 2007 Mar 10;128(4):705-15

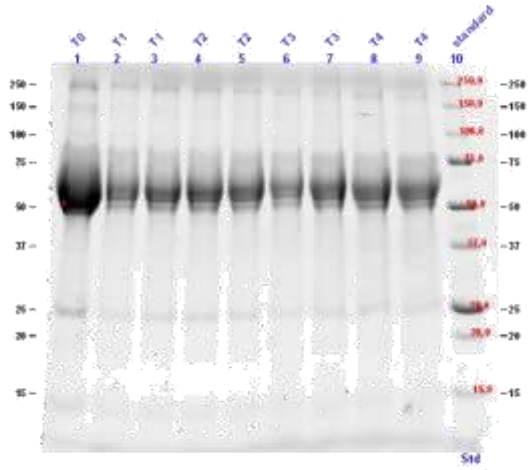
15. UniRoma → FIRALIS (Giorgia ZANETTI, September- October 2016)

Seconded researcher
Giorgia ZANETTI

GDNF production

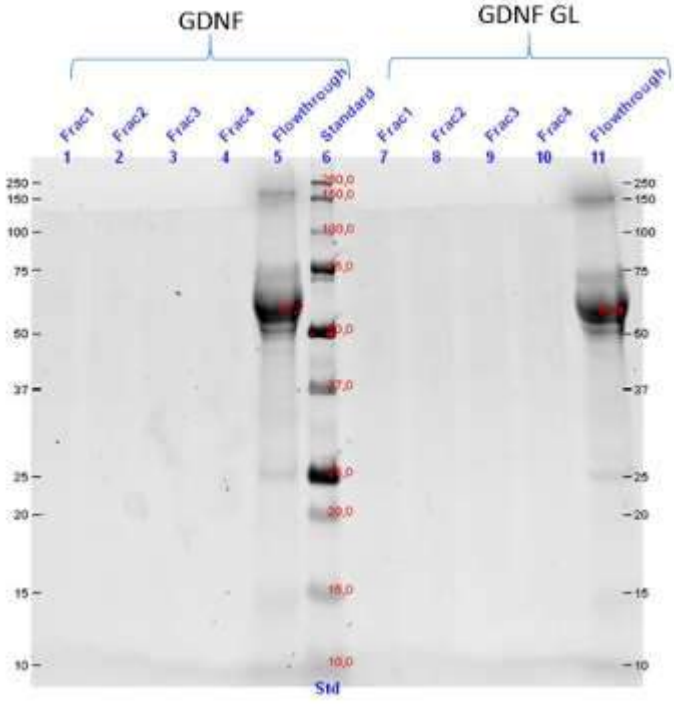
My goal was to purify human GDNF from cell culture medium. I transfected HEK 293T with 2 different plasmid, pTT5-GDNF and pTT5-GDNF-GL. The difference between them was only the presence of a small part of luciferase contained in the GDNF-GL plasmid. The transfection was carried out using Lipofectamine. I tried to follow the production of GDNF during time, picking an aliquot of the culture supernatant every 8 hours post transfection. In figure 1 the results of the acrylamide gel of the aliquots are shown: I could not detect GDNF, but it should migrate at the level of 30 kDa proteins.

Figure 1: Follow-up of GDNF production (the first lanes are GDNF, the second ones are GDNF GL)

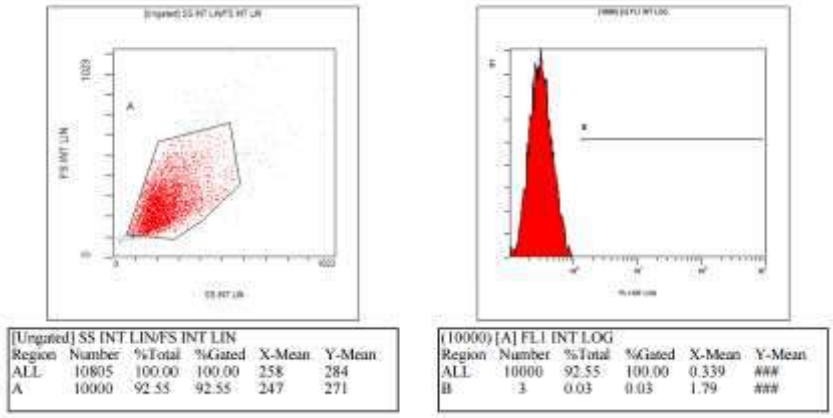


However, I proceeded with protein purification, using the Ni Sepharose 6 Fast Flow columns, because the GDNF had a histidine tag. Also after purification, I could not see any GDNF in the gel (fig. 2).

Figure 2: GDNF and GDNF-GL purificatio



I repeated the transfection using PEI and I used GDNF-GL plasmid and a GFP plasmid as control (because the GDNF and GDNF-GL plasmids don't have any selection gene). After 48 hours post transfection I analyzed the GFP transfected cells by FACS (Figure 3 and 4). I obtained a very high efficiency of transfection.



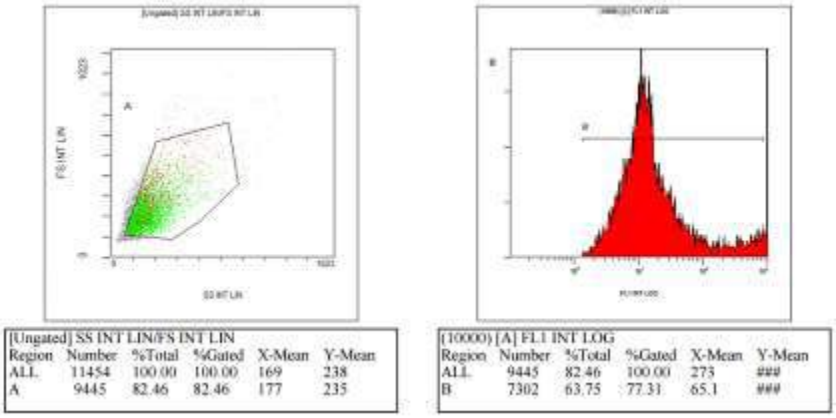
[Ungated] SS INT LIN/FS INT LIN

Region	Number	%Total	%Gated	X-Mean	Y-Mean
ALL	10805	100.00	100.00	258	284
A	10000	92.55	92.55	247	271

(10000) [A] FL1 INT LOG

Region	Number	%Total	%Gated	X-Mean	Y-Mean
ALL	10000	92.55	100.00	0.339	###
B	3	0.03	0.03	1.79	###

Figure 3: Non-transfected HEK 293T



[Ungated] SS INT LIN/FS INT LIN

Region	Number	%Total	%Gated	X-Mean	Y-Mean
ALL	11454	100.00	100.00	169	238
A	9445	82.46	82.46	177	235

(10000) [A] FL1 INT LOG

Region	Number	%Total	%Gated	X-Mean	Y-Mean
ALL	9445	82.46	100.00	273	###
B	7302	63.75	77.31	65.1	###

Figure 4: GFP-transfected HEK 293T

Even if the transfection worked, I could not detect the GDNF in the culture supernatant collected at 0, 24, 36 and 48 hours post transfection (Figure 5).

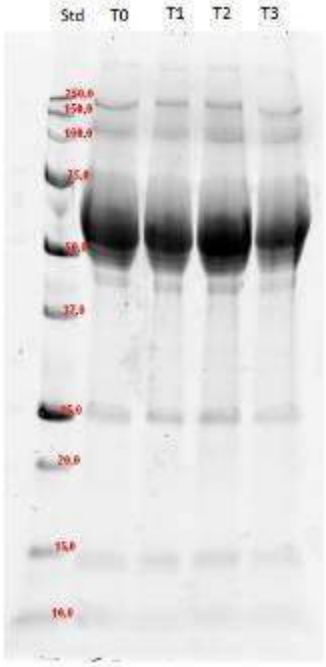


Figure 5: Follow-up of GDNF production

I did also the purification, but without obtaining any GDNF (Figure 6).

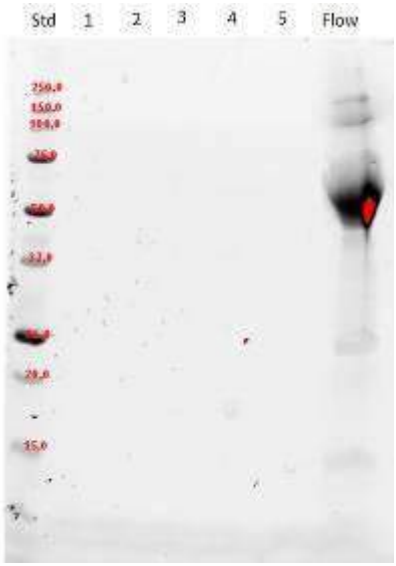


Figure 6: GDNF-GL purification

For this reason, I supposed that the secretion of the protein was insufficient. I decided to perform cell lysate, followed by protein purification (Figure 7), to check if GDNF was inside the cells. Also this time I could not detect the GDNF.
1p= elution 1
2p= elution 2
2f= 2ul of flow-through
5f= 5ul of flow-through
10f= 10ul of flow-through

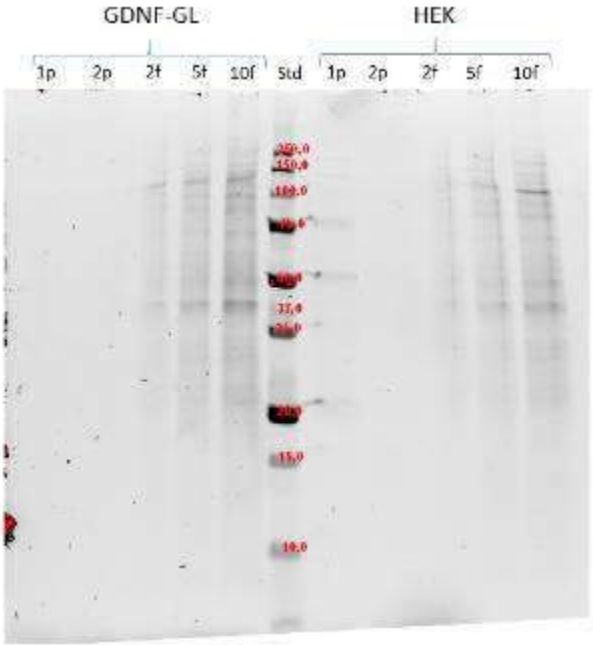


Figure 7 : GDNF-GL and control (NON TRANSFECTED HEK 293T) purification from cell lysate

In figure 7 you can see that there is a "contaminat
standard end up there, when I had loaded the well.

GDNF detection in mouse sera

Phospha-Light assay system is a chemiluminescent reporter gene assay for the detection of secreted human placental alkaline phosphatase used as a reporter protein (in this case to follow the expression of GDNF during time).
After performing an experiment with positive control samples to set up the experimental conditions of the luminometer, we decided to use 6 seconds as integration time. The

results of the mouse sera collected after 3, 9, 18 and 34 hours after immunization are shown in figure 8 and 9. As you can see, there is not significant difference between the samples and the negative controls.

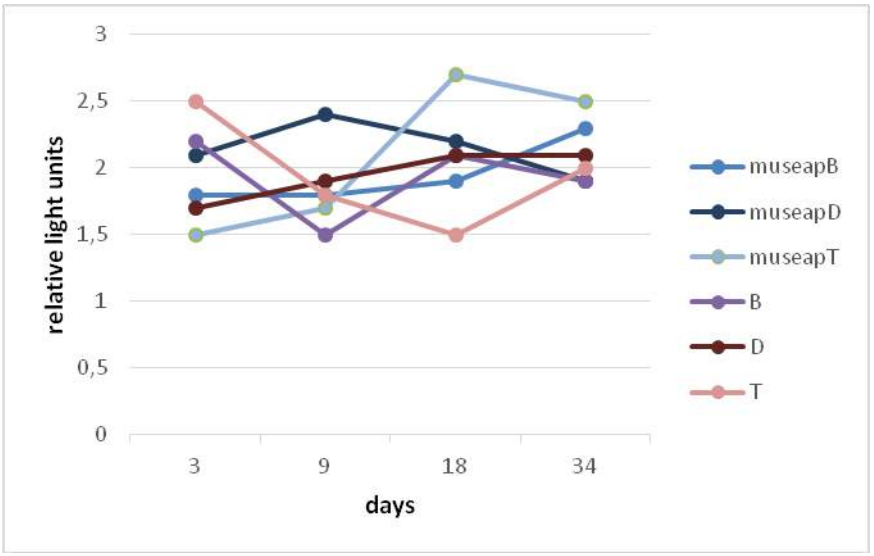


Figure 8: Light emission from mouse alkaline phosphatase (museap) and controls

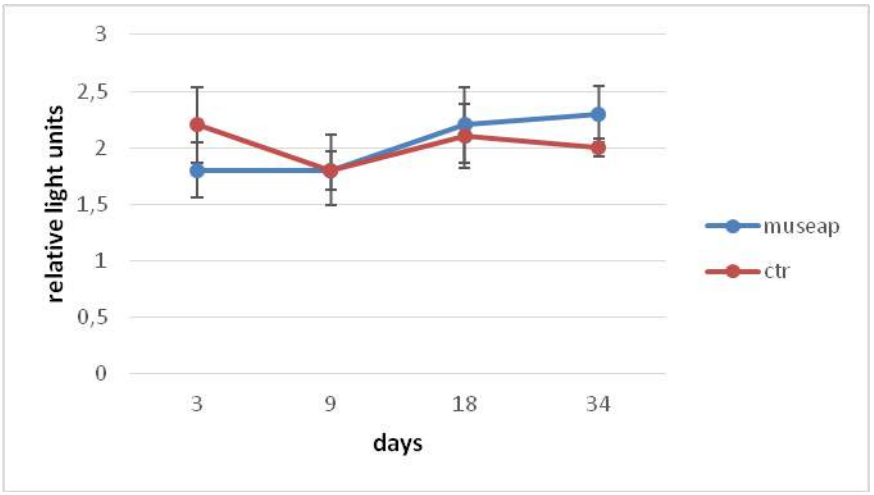


Figure 9: Mediated light emission from mouse alkaline phosphatase (museap) and controls

Tables of institutions and email of the contributors to this and all other newsletters (NL1-7)

SURNAME, Name, title	Institution, city, country	Email address	Contribution to newsletter(s)
ALVES Paula, CEO	iBET, Oeiras, PT	marques@ibet.pt	**NL1, 7**
ALVES Teresa, CEO	GENiBET, Oeiras, PT	teresa.alves@genibet.com	**NL1**
BANDEIRAS Vanessa, staff member	iBET, Oeiras, PT	vbandeira@itqb.unl.pt	**NL4, 7**
BERKHOUT Ben, head of laboratory	AMC, Amsterdam, NL	b.berkhout@amc.uva.nl	**NL1**
BEUCHER Bertrand, research assistant	CNRS, Montpellier, F	bertrand.beucher@igmm.cnrs.fr	**NL7**
BREGER Ludivine, PhD	Lund University, S	ludivine.breger@med.lu.se	**NL1, 3, 5**
BRITO Catarina, research assistant	iBET, Oeiras, PT	anabrito@ibet.pt	**NL7**
CARRONDO Manuel, head of laboratory	iBET, Oeiras, PT	mjtc@ibet.pt	**NL1, 3**
CHARTON Karine, team leader	GENETHON, Evry, F	charton@genethon.fr	**NL2**
CHILLON Miguel, team leader	UAB, Bellaterra, SP	miguel.chillon@uab.es	**NL1, 3**
CHTARTO Abdelwahed, staff member	ULB, Brussels, B	Abdelwahed.Chtarto@ulb.ac.be	**NL7**
DAS Atze, team leader	AMC, Amsterdam, NL	a.t.das@**amc**.uva.nl	**NL3, 5, 7**
FIRAT Hüseyin, CEO	FIRALIS, Huizinge, F	hueseyin.firat@firalis.com	**NL1, 5**
GONZALEZ Gloria, head of laboratory	FIMA, Pamplona, SP	ggasegui@unav.es	**NL1**
HOYNG Stefan A., head of laboratory	LUMC, Leiden, NL	s.hoyng@nin.knaw.nl	**NL3**
HUMBERT-CLAUDE Marie, PhD	CHUV, Lausanne, CH	marie.humbert-claude@chuv.ch	**NL1, 5**
KREMER Eric, team leader	CNRS, Montpellier, F	eric.kremer@igmm.cnrs.fr	**NL1, 7**
JUNYENT Felix, PhD	CNRS, Montpellier, F	felix.junyent@igmm.cnrs.fr	**NL1, 5**
LANCIEGO Jose Luis head of laboratory	FIMA, Pamplona, SP	jlanciego@unav.es	**NL1**
LEGUEUX François, staff member	FIRALIS, Huizinge, F	Francois.Legueux@firalis.com	**NL7**
LUNDBERG Cecilia, head of laboratory	Lund University, S	cecilia.lundberg@med.lu.se	**NL1**
MERTEN Otto-Wilhelm, team leader	EASCO, Evry, F	omerten@genethon.fr	**NL2, 5, 6, 7**
MEZZINA Mauro, EASCO administrator	EASCO, Evry, F	mezzina@easco.org	**NL1 - 7**
ONTIVEROS Maria, staff member	UAB, Bellaterra, SP	maria.ontiveros@uab.cat	**NL4**
PEIXOTO Cristina, team leader	iBET, Oeiras, PT	peixoto@ibet.pt	**NL5, 7**
PIERSANTI Stefania, PhD	UNIRoma, I	stefania.piersanti@uniroma1.it	**NL4**
PIGNATARO Diego, postdoc fellow	FIMA, Pamplona, SP	jpignataro@alumni.unav.es	**NL4, 5, 7**
QUINTINO Luis, staff member	Lund University, S	luis.quintino@med.lu.se	**NL4, 7**
SAGGIO Isabella, head of laboratory	UNIRoma, I	isabella.saggio@uniroma1.it	**NL1, 3**
SIMÃO Daniel, postdoc fellow	iBET, Oeiras, PT	dsimao@ibet.pt	**NL7**
TENENBAUM Liliane, team leader	CHUV, Lausanne, CH	Liliane.Tenenbaum@chuv.ch	**NL1, 3, 4, 5, 6, 7**
THIBAUT Martine, EASCO treasaurer	EASCO, Evry, F	marthibaut@yahoo.fr	**NL1 - 7**
ZANETTI Giorgia, PhD student	UNIRoma, I	giorgia.zanetti@uniroma1.it	**NL7**
ZHANG Wenwei, EASCO manager	EASCO, Evry, F	Wenwei.zhang@easco.org	**NL1 - 7**