BrainVectors Newsletter n° 3
Marie Curie Industry-Academia Partnerships and Pathways - Contract n°286071

BrainVectors dissemination: Madrid meeting

The BrainVectors meeting was decided to hold jointly with the annual meeting of the ESGCT that occurred in Madrid, in order to amplify the dissemination impact with the >300 participants.

Although at the time of this meeting the BrainVectors project just started with a secondment from UniRM to IBET, the meeting in Madrid was extremely useful allowing all partners to discuss further the scientific issues and exchange with scientists outside the consortium.

This NL contains the program and the abstracts of the scientific presentations

Program

day	time	speaker	Institution, city	title
24 / 10	14:00	Liliane TENENBAUM	CHUV Lausanne, CH	*Welcome remarks*
	14:05			*Optimization of AAV-tetON vectors for GDNF gene delivery in the rat striatum*
	14:45	Ludivine BREGER	LUNI Lund, S	*Development of lentiviral vectors with Tet-On system*
	15:25	Atze DAS	amc Amsterdam, NL	*Improved Tet-On systems for regulated gene expression*
	16:15	*coffee break*		
	16:45	Isabella SAGGIO	Sapienza Rome, I	*Transcriptomic analysis of the efficiency, specificity, and molecular toxicity of viral vector transduction in neurons*
	17:25	Gloria GONZALES	Universidad de Navarra Pamplona, SP	*Development of central nervous specific tetracycline inducible expression systems for AAV vectors.*
	18:15	*End of the session*		
25 / 10	9:30	Hueseyin FIRAT	FIRALIS Huningue, F	*Expected immune responses to viral vectors and their transgenes*
	10:10	Joost VERHAAGEN	Netherlands Institute for Neuroscience Amsterdam, NL	*Developing a potential immune-inert tetracycline regulatable lentiviral vector.*
	10:50	*coffee break*		
	11:20	Manuel CARRONDO	IBET Oeiras, PT	*IBET competences for viral vectors and vaccines*
	11:50	Miguel CHILLON	UAB Bellaterra, SP	*Production, administration and in vivo analysis of viral vectors to peripheral and central nervous systems*
	12:30	*BrainVectors* internal issues		
	13:00	*End of the meeting*		

Optimalization of AAV-tetON vectors for GDNF gene delivery in the rat striatum

A. Chtarto[1], M. Humbert-Claude[2], O. Bockstael[1], A.Das[3], T. Tshibangu[2], S. Boutry[4], P. Koch[5,] B.Berkhout[3], L. Tenenbaum[2].

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We have previously designed a tetracycline-inducible AAV vector (AAV-tetON$_{bidi}$) configured to avoid interference between tet system components and with the AAV ITRs (1).Using the rtTA2^{S}-M2 mutant tetON transactivator (2), we have demonstrated a tightly-regulatable expression of GDNF as well as control of neuroregeneration (3) and undesired effects (4). in a PD rodent model. However, the doxycycline (dox) dose required to observe these effects was approx.50-fold higher than the recommended dose for patients and precluded preclinical testing in non human primates.

To address this limitation, a dox-sensitive rtTA mutant V16 provided by Atse Das(6) was introduced in AAV-tetON. The new vector responds to a significantly lower dox dose *in vivo* in the brain and in the retina. The lowest dox dose providing neuroprotective effect is currently being evaluated in a partial 6-OHDA model (Marie Humbert-Claude).

Another limitation of the vector is the immunogenicity of the rtTA transactivator evidenced after intramuscular injections (7). No rtTA-induced humoral or cellular immune response has however been described after intracerebral injection of tet-inducible viral vectors so far (8). In addition, it has to be taken into account that a large proportion of the human population (60%) is seropositive for HSV-1, thus possibly have memory antibodies against the HSV1 VP16-derived portion of the rtTA. Whether immune responses will be a concern for clinical applications use of the AAV-tetON$_{bidi}$ vector , in diseased brain remains to be determined.

. In order to limit the immune response, more efficient vectors and expression cassettes allowing to use a reduced amount of rAAV have been successfully implemented in an hemophilia clinical trial (9). In order to administer therapeutically-relevant doses of GDNF with a reduced viral load and rtTA amount, we have codon-optimized the GDNF sequence and obtained higher GDNF levels in the striatum with titer matched rAAV1 batches. Other potential means of reducing the immunogenic risk:efficacy ratio are using more efficient serotypes and tissue-specific tetON promoters developed by Gloria Aseguinolaza. Finally, Another important factor to reliably evaluate the immune response is the purity of the viral preparations.

In the near future, we are planning to analyze the purity of our rAAV batches in a secondment with Genibet and to inject purified batches in HLA2-2.1-DR1ko mice (10) provided by Prof Lemonnier. Humoral and cellular immune responses will be analyzed by FIRALIS.

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3. Yang 2009. Gene Med. 11, 899-912)
4. Chtarto 2007. Exp.Neurol. 189, 303-316.
5. Bockstael 2008.. Hum. Gene Ther. 19, 1293-1306.

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8. Xiong 2088, Mol Ther 16, 343-351
9. Natwani 2012 Mol Ther 19, 876-885
10. Pajot 2004 Eur. J. Immunol., 34: 3060.

Development of lentiviral vectors with Tet-On system

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1. Rationale and significance

Because of their ability to transduce non-dividing cell of various pseudotypes, lentiviral vectors (LV) provide a potent way to transfer genes in the central nervous system. The proof of concept that they can be successfully used to express glial cell derived neurotrophic factor (GDNF) and rescue dopaminergic neurons in animal model of Parkinson's disease has been for long established[1]. Moreover, clinical trials using LV have reported encouraging feasibility and tolerability[2,3]. The first aim of the future work to be presented at the meeting is to generate a bidirectional Tet-On regulated GDNF LV. The second main goal is to produce LV with cell-specific expression using a 2-vectors inducible system.

2. Experimental procedure

The bidirectional Tet-On regulated GDNF LV will be designed based on the construct chosen in Work Package 1. It will be synthesis with specific enzyme restriction sites for cloning in a LV backbone, as well as attR1-R2 gateway sites allowing the replacement of the GDNF transgene by the green fluorescent protein (GFP) or luciferase sequence. The second part of the project will use co-transfection of 2 LV: 1) rtTAV16 construct under control of a cell specific promoter, 2) GFP transgene controlled by the tetracycline responsive element (TRE). Part of this work will look at different promoters and comparing neuron specific enolase promoter (NSEp) and microtubule-associated protein 1 a promoter (MAP1Ap) for neuron specific expression. It will also assess glia specificity of GFP expression under glial fibrillary acidic protein promoter (GFAPp), with the addition of miR124 target sequence to reduce background expression in neuronal cells. Finally, GDNF LV will be produced with using the chosen constructs to ensure cell specific expression.

3. Expected results & Discussion

The bidirectional Tet-On regulated vector is expected to lead to GDNF expression in various cell types and off-target, in downstream nuclei. Contrarily, the dual vector system should result in cell-specific expression of GDNF, either in glial cells (GFAPp-rtTAV16-miR124T vector) or in neuronal cells (NSEp-rtTAV16 or MAP1Ap-rtTAV16 vector). Off target expression are expected when GDNF is expressed in neurons but not in glial cells.

4. References

1. Rosenblad C *et al.* 2000, Mol Cell Neurosci.15(2):199-214
2. Biffi A *et al*, Science. 2013 Aug 23;341(6148):1233158.
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Improved Tet-On systems for regulated gene expression

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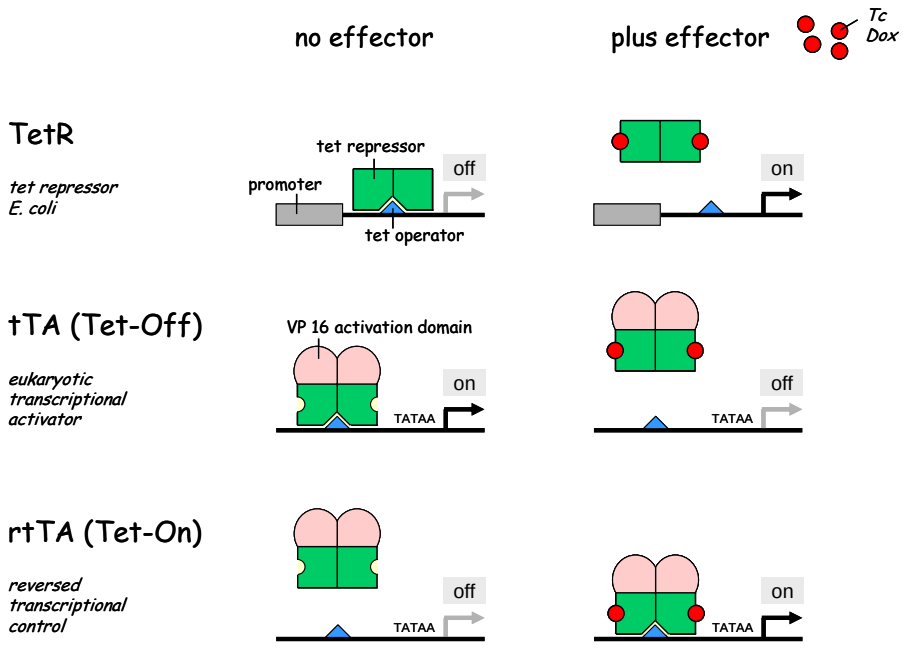
Technology for the regulation of gene expression in cells and tissues is essential for both functional genomic research and applications in medicine (gene therapy) and biotechnology. All these applications require that production of the protein-of-interest is controlled in both a quantitative and temporal way. For this purpose, artificial gene expression systems have been developed. The popular Tet-On system is based on the components of the E. coli tetracycline resistance operon. In this system, the rtTA transcriptional activator protein induces gene expression from tet Operator (tetO)-containing promoters exclusively when doxycycline (Dox; a tetracycline derivative) is administered. Because of its excellent regulation performance and the fact that it is controlled by a non-toxic, clinically approved drug, the Tet-On system is the most widely used regulatory circuit in mammalian cells.



We previously constructed a genetically modified HIV-1 virus variant of which the gene expression and replication is controlled by the Tet-On system. This HIV-rtTA variant can be

turned on and off at will by the addition or removal of Dox, respectively, and was developed as a live-attenuated vaccine candidate (1). We demonstrated that spontaneous virus evolution selected for improved HIV-rtTA variants in which the introduced Tet-On system was optimized for its function in human cells (2). We thus generated improved Tet-On systems that were much more active (>7-fold) and dox-sensitive (>100-fold) than the regular Tet-On system (3). These optimized rtTA variants are particularly useful for in vivo applications that require a more sensitive or more active Tet-On system (4,5). The unique HIV-rtTA virus is also used to develop novel Tet-On systems that are responsive to new effectors, such as tetracycline-derivatives with low antibacterial activity or compounds that penetrate the blood-brain barrier more efficiently than Dox.

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Transcriptomic analysis of the efficiency, specificity, and molecular toxicity of viral vector transduction in neurons

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Advances in the use of viral vectors in clinical trials have raised the need for the complete knowledge of their impact on the host (cell). This is particularly crucial for the application of viral vectors to diseases affecting the central nervous system, where the physiology of the target cell, the neuron, is specific, complex, and highly sensitive to stressing agents. Although a significant body of information is now available on the toxicity of vectors at the organismal level, less is defined at the cellular level.

In the last years we have produced a set of data that has contributed to the definition of the effect of viral vectors on the host cell by means of transcriptome modulation studies ([1] [2] [3] and references therein). In the context of the current *Brainvector* project, with the goal of expanding the knowledge of the molecular toxicity of viral vectors in neurons, we have transcriptomically investigated the effect of lentiviral and adenoviral vectors on different neuronal systems.

We have taken into consideration two neuronal culture systems: 2D cultures of dopaminergically differentiated human neuronal midbrain progenitors (hmNPCs) and 3D cultures of these same cells. While the biological properties of 2D cultures are well assessed and described in the literature ([4]), the 3D system is not yet fully characterized. So, as a first step, we transcriptomically characterized 3D neurospheres, which were produced and differentiated by P.A. Alves and collaborators ([5]). We assessed that differentiated 3D hmNPCs displayed the activation of dopaminergic markers (400- and 16-fold increase at day 32 post differentiation, for TH, and DRD2 respectively). The cells showed positive modulation of neurotrophin receptors. Indeed TRK1 and TRK2, receptors respectively of NGF and BDNF, were upregulated upon differentiation, with a 2- and 28-fold increase at 32 days, respectively. In addition, we showed that the expression of different presynaptic markers, such as SYT-1 and SYP, gradually increased during differentiation, reaching up to 2- and 6-fold greater levels by the end of the differentiation process.

Next we evaluated the effect of vectors on differentiated neuronal cells by testing both third generation lentiviral vectors (LV) and last generation helper dependent canine adenovectors (HD-CAV-2). Both these vectors trandsuced 2D cultures with high efficacy (up to 90% when using 1000 VG/cell), conversely 3D hmNPCs were better transduced with HD-CAV-2 as compared to LV.

The molecular signature of HD-CAV-2 on neuronal cultures included the significant modulation of DNA damage and cell cycle controlling genes, as well as the alteration of the microtubule system, particularly evident when the transcriptome of 3D cultures was analysed. We then performed a gene expression comparative study among *in vitro* data set and data obtained by the analysis of RNAs extracted from *Microcebus* brains injected with HD-CAV-2 (injections done in the context of the EU BrainCAV project by CNR and INSERM partners). Importantly, a common transcriptional pattern could be recognized in all systems, confirming that those that we had identified in 2D and 3D transduced neuronal cultures, were indeed the main traits of the biological response of primate neurons to HD-CAV-2.

The LV vector produced on the differentiated hmNPCs a different impact as compared to HD-CAV-2. This included the Interferon pathway response, that has been well described also in other transduction systems ([6]). LV transduction also impacted, in an intriguingly complex way, on the cell

DNA damage function. At first (24h post transduction), LV induced abundant DNA damage γH2AX foci in the cell nucleus, and, at later times (5 days post transduction), LV positive cells exhibited the down regulation of a set of genes implicated in the DNA damage response pathway.

Perspectively, we plan now to go more in depth in the biological pathways affected by the two vectors, exploiting both the comparative and absolute analysis of collected data.

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3. Simão D, Pinto C, Piersanti S, Collinson L, WestonA, Salinas S, Serra M, Teixeira AP, Saggio I, Kremer EJ, Schiavo G, Brito C, Alves PM. Modeling human neural functionality *in vitro*: 3D culture for dopaminergic differentiation. Submitted.

Developing an immunologically-inert tetracycline regulatable viral vector for gene therapy in the peripheral nerve.

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Viral vector mediated gene transfer of neurotrophic factors is an emerging and promising strategy to promote the regeneration of injured peripheral nerves. Unfortunately the chronic exposure to neurotrophic factors results in local trapping of regenerating axons or other unwanted side effects. Therefore, tight control of therapeutic gene expression is required. The tetracycline/doxycycline-inducible system is considered to be one of the most promising systems for regulating heterologous gene expression. However, an immune response directed against the transactivator protein rtTA hampers further translational studies. Immunogenic proteins fused with the Gly-Ala repeat of the Epstein-Barr virus Nuclear Antigen-1 protein have been shown to successfully evade the immune system. In this article we used this strategy to demonstrate that a chimeric transactivator, created by fusing the Gly-Ala repeat with rtTA and embedded in a lentiviral vector (i) retained its transactivator function *in vitro*, in muscle explants, and *in vivo* following injection into the rat peripheral nerve, (ii) exhibited a reduced leaky expression and (iii) had an immune-evasive advantage over rtTA as shown in a novel bioassay for human antigen presentation. The current findings are an important step towards creating a clinically applicable tetracycline-regulatable viral vector system.

IBET COMPETENCES FOR VIRAL VECTORS AND VACCINES

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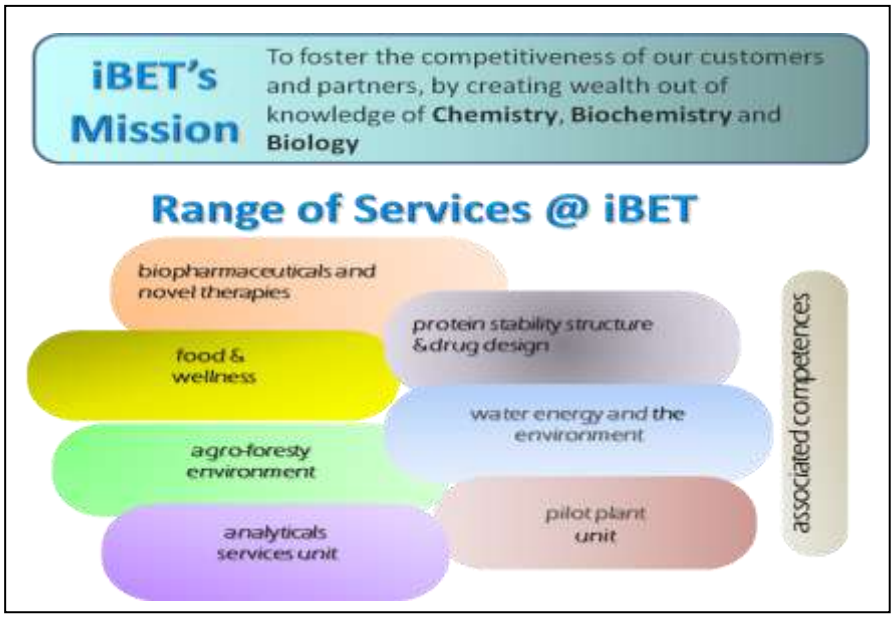
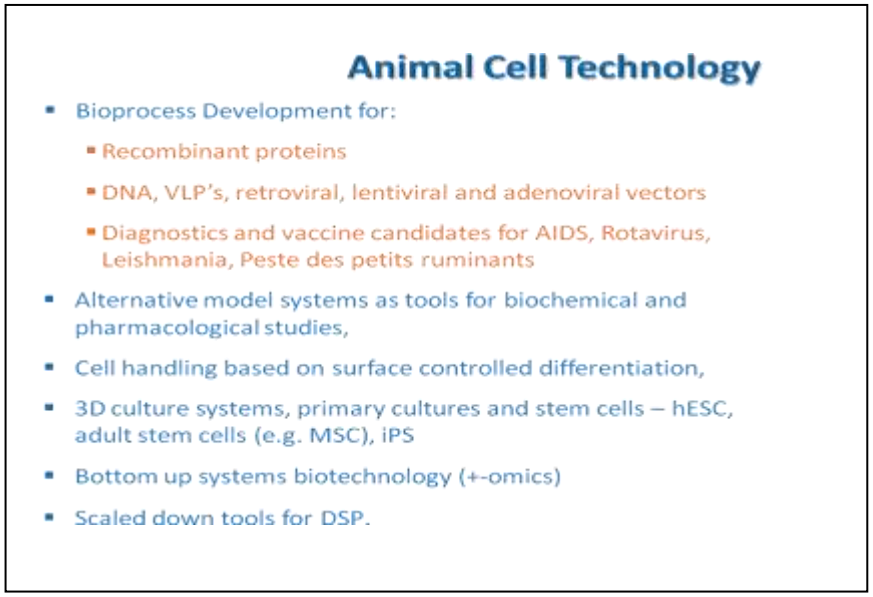
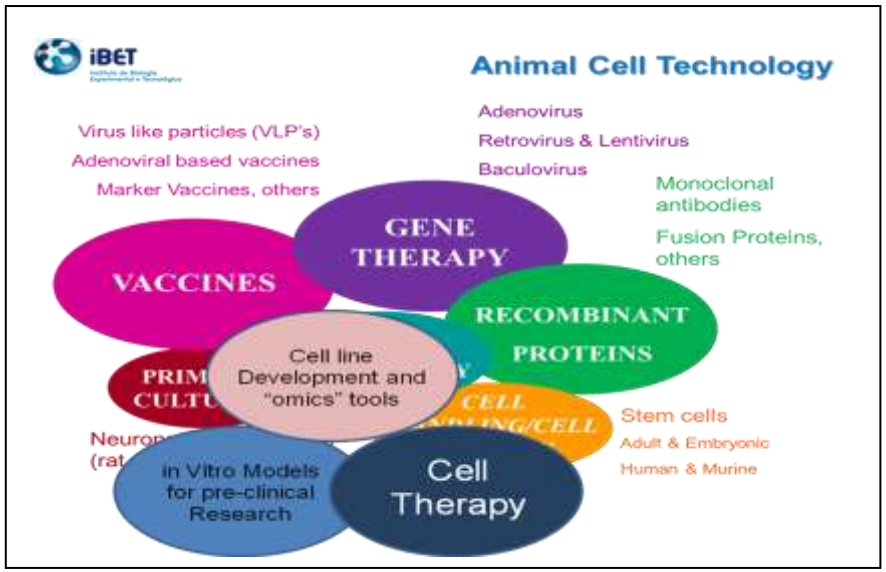
ABSTRACT

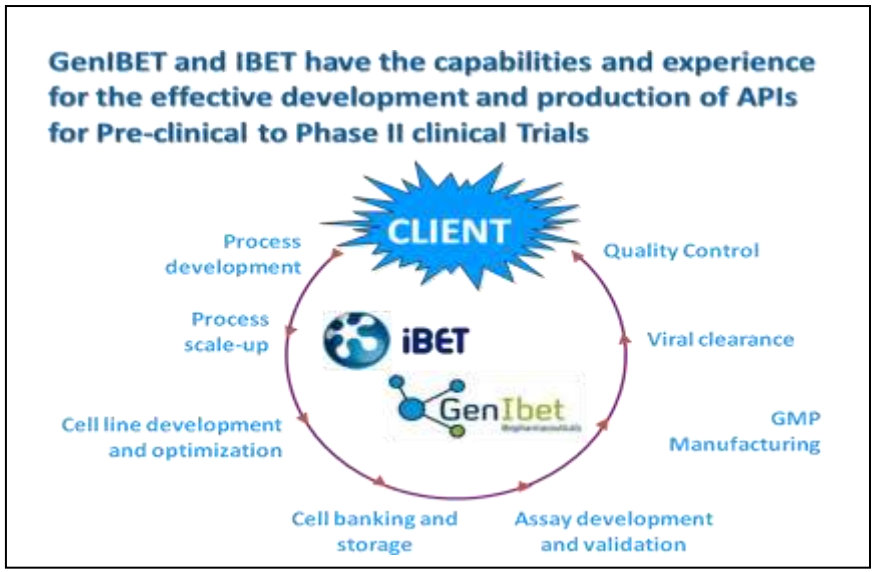
Over the years, IBET has dealt with many different biological systems to deliver and optimise viral vectors for gene therapy or viral vaccines, both at the up – as well as downstream levels.

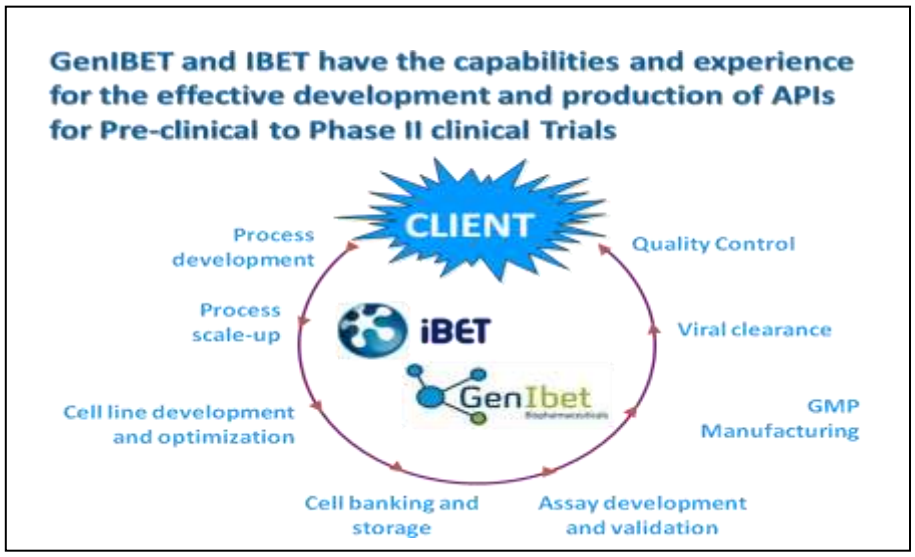
Within BRAINVECTORS, it is relevant to share this information so that the different assignments can be better targeted and more profitable for the research work the different partners are carrying out.











Summary Competences at iBET for BrainVectors

- Viral Vaccines and Viral GT Vectors;
- Cell Therapies (incl. SC) and Cancer Cell Vaccines;
- VLP (Model: Rotavirus) and Retro VLP (Hep C) Vaccines;
- Conjugate microbial Vaccines;
- Systems biotechnology + metabol-, prote-, secret-, -glyc –omics;
- cGMP Analytical services Unit (chem.-, biochem-, bio- assays);
- cGMP Production for Phase I/Phase II clinical trials at GenIBET;
- Seamless transition from customer "biological/vaccinology" knowledge to development optimization at IBET and cGMP production at GenIBET.

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Production, administration and in vivo analysis of viral vectors to peripheral and central nervous systems

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Viral vectors are widely used as tools for gene transfer and gene expression both in gene therapy studies and in gene function analysis in metabolic pathways, signal transduction or growth control. Their use is an attractive choice given the ease and flexibility to genetically express or inhibit one gene or a combination of genes in specific brain areas and periods of time, while avoiding compensation phenomena or other drawbacks associated with animal models. In this regard, our group at the Universitat Autonoma of Barcelona is able to produce and purify viral vectors at small scale (adherence cells and purification by ultracentrifugation) as well as medium-large scale (cells in suspension and purification by chromatography). The average productivity per cell is of 13.800 vg for adenovirus and 35.000 vg per AAV, regardless the serotype. In addition, our group has also optimized the intrathecal administration of viral vectors to different cell types of the peripheral nervous system, and has set-up procedures to transduce different brain areas of the central nervous system, as well as a wide battery of memory and behavioural tests to analyze the correction level of the pathological symptoms after the administration of gene therapy vectors to the animals.

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