CRN 12-mo progress update

Gene transfer studies for cystinosis

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Initial specific aims

I) Validate *in vitro* gene transfer results on primary murine hepatocytes by *in vivo* gene transfer to the liver

II) Generate clinically relevant vectors (HD CAV-2 and AAV8) expressing *CTNS*

III) Conduct *in vivo* corneal-targeted gene transfer studies

IV) Refine characterisation of the CNS anomalies in *Ctns*$^{-/-}$ mice and determine whether cysteamine can cross the blood-brain barrier

Our initial proposal was to obtain a salary for technical help. We hired Sandy Ibanes (a research assistant) in December 2007. Here, we report the advancement of our project over the last 6 months.

Abbreviations

AAV8	adeno-associated virus serotype 8
AAV-CIG	adeno-associated virus vector containing the CTNS-IRES-GFP expression cassette
AAV-GFP	adeno-associated virus vector containing the gene *GFP*
CAV-2	canine adenovirus serotype 2
CAV-CIG	canine adenovirus vector containing the CTNS-IRES-GFP expression cassette
CAV-GFP	canine adenovirus vector expressing the gene *GFP*
HD CAV-2	helper-dependent canine adenovirus vector (devoid of all viral genes)
E1	early 1 region of the adenoviral genome that encodes trans-activating factors
E3	early 3 region of the adenoviral genome that encodes immune-modulating factors
GFP	green fluorescent protein
IRES	internal ribosomal entry site
CTNS-IRES-GFP	expression cassette containing *CTNS* and *GFP* separated by an IRES sequence
CTNS$^{-/-}$	homozygous deletion of the human *CTNS* gene
Ctns$^{-/-}$	homozygous deletion of the mouse *Ctns* gene
pp/ml	number of physical viral particles per ml of stock (titre terminology for CAV vectors)
vg/ml	number of viral genomes per ml of stock (titre terminology for AAV vectors)

I) *In vivo* gene transfer studies

We reported the final results of this project in our 6-mo update report and at that time the corresponding publication was under review. This study representing the first *in vitro* and *in vivo* viral vector-mediated gene transfer studies for cystinosis has now been published:

Hippert, C., Dubois, G., Morin, C., Disson, O., Ibanes, S., Jacquet, C., Schwendener, R., Antignac, C., Kremer, E.J. & Kalatzis, V. (**2008**) Gene transfer may be preventive but not curative for lysosomal storage due to a defective transporter. ***Mol. Ther.*** 16: 1372-1381.

Thus this project, as it was initially defined, is now completed.

II) Generation of clinically relevant vectors (HD CAV-2 and AAV8) expressing *CTNS*

Background:

At our 6-mo report, Sandy Ibanes was finalising the production and purification of a control GFP-expressing HD CAV-2 vector (a CAV-2 vector devoid of all viral genes). This was the first time in two years that we advanced so far with HD CAV-2 vector production (bearing in mind that we are the only laboratory in the world with the technology to produce this vector). We subcontracted AAV8 vector production to a specialised platform.

Results:

Over the last 6 months**:**

i) We finalised the production of the control HD CAV-2 vector (referred to here as HD CAV-GFP). The titre of the HD vector was 5.4 x 10^{11} pp/ml and the contamination with the helper vector, which provides the viral proteins necessary for HD production in *trans*, was < 0.01%. We showed that HD CAV-GFP was functional *in vitro* and *in vivo*.

ii) We began production of the HD CAV-2 vector expressing a CTNS-IRES-GFP cassette (HD CAV-CIG). The production of HD CAV-CIG was less straightforward. The size of HD CAV-CIG (32 kb) is similar to that of the helper vector (33 kb) thus precluding an efficient separation between the two vectors by cesium chloride (CsCl) gradient. By comparison the size of HD CAV-GFP is 30 kb, which allows a better separation from the helper and hence resulted in the low contamination rate. We produced an initial stock of HD CAV-CIG with a titre of 2.5 x 10^{11} pp/ml but with a high percentage of helper contamination (the exact percentage has not yet been calculated because we are in the process of setting up a quantitative PCR (qPCR) protocol to perform the quantification rather than counting transduced cells). Thus we began the production of a second vector and varied the centrifuge times of the CsCl gradient to enhance separation. This second stock of HD CAV-CIG has a titre of 5.4 x 10^{11} pp/ml and a lower contamination rate (still to be quantified).

iii) We verified that the expression cassette is functional *in vitro*: we detected both cystinosin and GFP expression by immuno- and epifluorescence studies of transduced cells, and reduced cystine levels by 70% in *CTNS*$^{-/-}$ fibroblasts. Thus, we will now begin *in vivo* testing of HD CAV-CIG (see section IV).

iv) In parallel, we returned to our initial 30-kb HD CAV-CIG plasmid to remove 2 kb of sequence by restriction enzyme digestion. We will begin production of the smaller 30-kb HD CAV-CIG vector, which should separate more efficiently from the helper vector thus further minimising the contamination rate.

v) AAV vector production: we received a second (in June) and third (in October) stock of AAV8-CTNS-IRES-GFP and AAV8-GFP (vehicle control) from the Vector Production Platform at the Centre of Biotechnology and Animal Gene Therapy (Barcelona, Spain). Due to the higher titres of these new stocks, we now have enough material for *in vivo* experiments (see sections III and IV).

III) *In vivo* corneal-targeted gene transfer studies

Background:

Our gene transfer studies to the liver provided the proof-of-concept that viral vector-mediated gene transfer could reduce lysosomal cystine levels *in vivo*. The next step was to perform gene transfer studies to the cornea, a tissue that is more clinically relevant for cystinosis. Although E1/E3-deleted adenovirus vectors were suitable for the proof-of-concept, for the corneal gene transfer studies we plan to use more stable, less immunogenic viral vectors (HD CAV and AAV) to be as clinically relevant as possible.

Results:

i) As mentioned in our 6-mo report, we performed numerous intrastromal injections with an E1/E3-deleted CAV-GFP vector and consistently observed a strong transgene expression *in vivo* from 24 h in the stromal keratocytes. However, expression diminished and was no longer detectable by 2 weeks. More recently, we observed the same expression kinetics with HD CAV-GFP. One possible hypothesis is that the intrastromal injections provoke an apoptosis/proliferation repair mechanism and thus the non-integrating CAV vectors are then eliminated from the cornea following repopulation by dividing keratoblasts. We tried two other routes of administration, intravitreal and intracamaral, to reach the corneal stroma. However, via these two routes GFP expression remains localised to the corneal endothelium. The size of the adenovirus vectors (70 to 90 nm) probably precludes these vectors from crossing this barrier and entering the stroma. A Ph.D. student in the lab, Nicolas Serratrice, is currently trying to address and circumvent the problem of the short duration of transgene expression from CAV vectors.

ii) Following discussion with our collaborators in Toulouse (the laboratory of François Malecaze), it appears that AAV expression is more stable in the cornea following intrastromal injection. Our colleagues detect GFP expression from 2 weeks (earliest time point studied) and it remains detectable up to 4 months by histological studies. As AAV vectors are theoretically non-integrating, a possible explanation could be that the lag time necessary for the single-stranded AAV genome to convert to double-stranded somehow helps these vectors avoid clearance by newly dividing cells. Thus we are currently concentrating on using AAV8 vectors in our *in vivo* eye study. Due to the high titre of our latest stocks, we are able to inject small volumes (< 2 µl) into the mouse cornea making injections more practicable. Our current experiment is promising: we detected GFP expression *in vivo* from AAV8-GFP in the keratocytes from 24 h. We will follow these mice daily for period of 2-4 weeks or until extinction of the signal. We will then screen for GFP expression by histological studies and qPCR. In parallel, we tested the intracamaral route of administration and observed GFP expression in the corneal stroma at 72 h post-injection. It is thus possible that AAV vectors, due to their smaller size (20 nm), are able to cross the endothelial barrier.

IV) Refine characterisation of the CNS anomalies in *Ctns*$^{-/-}$ mice

Background:

Our previous work suggested that *Ctns*$^{-/-}$ mice have age-related learning and memory defects likely due to cystine accumulation in the hippocampus. Having identified the brain regions affected, the next step is to identify the cell type(s). At the time of our 6-mo report, we had begun pilot experiments to dissociate the brain, label individual cell types with fluorescent-labelled cell markers and isolate these cells via fluorescence-activated cell sorting (FACS). The aim is to assay each cell type for their respective cystine levels. For our first experiments, we used an anti-CD11b antibody specific to microglial cells and we were able to clearly separate this cell population.

Results:

Over the last 6 months:

i) We extended our experiments to other cell types using antibodies to specific cell surface markers: the glutamate transporter EAAT1 for astrocytes and myelin oligodendrocyte glycoprotein for oligodendrocytes. We were able to isolate the corresponding cell populations for both of these markers. The choice of neuronal cell surface marker has been the most difficult. We found that our initial choice (neuronal growth factor or NGF) is not specific to neurons and our next choice (neurexin Iα) is localised to the axons that are cleaved during the dissociation process.

ii) Being unable to find a neuron-specific surface marker that is localised to the cell body, we are currently trying to separate neurons using an Optiprep gradient. An Optiprep gradient separates each cell type into different fractions, which can then be individually collected. In this way, we should be able to isolate neurons while avoiding a cell-labelling step.

iii) In parallel, we are trying to improve our protocol in order to increase the efficiency of cell sorting. Currently, the isolation of 10^5 astrocytes (the minimum number for a reliable cystine assay) takes ~1 h. Improving cell sorting efficiency will be essential for isolating minority cell types.

iv) Finally, we began stereotaxic injections of our HD CAV-CIG and AAV8-CIG vector stocks to target the hippocampus in mice. As a pilot experiment, we injected HD CAV-GFP and AAV8-GFP vectors in wild-type mice and sacrificed the mice 2- and 4-wk post-injection, respectively. We screened the brain sections of the HD CAV-injected animals and detected GFP expression in the hippocampus. We will sacrifice the AAV-injected mice next week. Our long-term goal is to express cystinosin in the hippocampus to determine whether we can restore learning and memory in *Ctns*$^{-/-}$ mice.