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

Nb: In grey our goals completed at the 12-month time-point.

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

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AAV8</td>
<td>adeno-associated virus serotype 8</td>
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<tr>
<td>AAV-CIG</td>
<td>adeno-associated virus vector containing the CTNS-IRES-GFP expression cassette</td>
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<tr>
<td>AAV-GFP</td>
<td>adeno-associated virus vector containing the gene GFP</td>
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<tr>
<td>CAV-2</td>
<td>canine adenovirus serotype 2</td>
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<tr>
<td>CAV-CIG</td>
<td>canine adenovirus vector containing the CTNS-IRES-GFP expression cassette</td>
</tr>
<tr>
<td>CAV-GFP</td>
<td>canine adenovirus vector expressing the gene GFP</td>
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<tr>
<td>HD CAV-2</td>
<td>helper-dependent canine adenovirus vector (devoid of all viral genes)</td>
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<tr>
<td>E1</td>
<td>early 1 region of the adenoviral genome that encodes trans-activating factors</td>
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<tr>
<td>E3</td>
<td>early 3 region of the adenoviral genome that encodes immune-modulating factors</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>IRES</td>
<td>internal ribosomal entry site</td>
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<tr>
<td>CTNS-IRES-GFP</td>
<td>expression cassette containing CTNS and GFP separated by an IRES sequence</td>
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<tr>
<td>Ctns−/−</td>
<td>homozygous deletion of the mouse Ctns gene</td>
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II) Generation of clinically relevant vectors (HD CAV-2 and AAV8) expressing CTNS

Background:
At the time of our 12-mo report, Sandy Ibanes had finished the production of a helper-dependent (HD) CAV-2 vector (devoid of all viral genes) containing a CTNS-IRES-GFP cassette (HD CAV-CIG). We had also received the AAV8 vectors expressing CTNS-IRES-GFP (AAV-CIG) or GFP (AAV-GFP).

Results:
HD CAV-CIG has a size of 32 kb. The helper vector, which provides the viral proteins necessary for HD production in trans, is 33 kb. Therefore, it was difficult to obtain a clear separation of these vectors on a caesium chloride (CsCl) gradient (Fig. 1). To address this technical problem, we reproduced HD CAV-CIG by varying the centrifugation times to improve the separation from the helper vector. After production, we estimated a helper contamination of ~6% by quantitative PCR (qPCR). This contamination rate, although acceptable for in vivo experiments in mice, needed to be further reduced. Thus, we returned to our initial 32-kb HD CAV-CIG plasmid to remove 2 kb of sequence by restriction enzyme digestion. Our rationale was that this smaller vector (which I will refer to as HD CAV-CIG-30 kb) would separate more efficiently from the helper than HD CAV-CIG-32 kb. In parallel, Sandy Ibanes produced a new helper vector (CAV-Cherry) that has a delayed packing time as compared to the HD vector, which should thus decrease the rate of helper contamination. Furthermore, CAV-Cherry expresses a red fluorescent protein, as opposed to the original helper that expressed a non-fluorescent protein, which will allow us to more efficiently sort and collect cells, thus further minimising the rate of helper contamination. The sorting parameters have now been established and we will begin production of HD CAV-CIG-30 kb within the next two weeks.

Figure 1: CsCl separation of HD CAV-CIG from the helper vector. The banded vectors are too close to allow retrieval of only HD CAV-CIG.
III) In vivo corneal-targeted gene transfer studies

Background:

Our gene transfer studies using E1/E3-deleted adenovirus vectors to the liver provided the proof-of-concept that viral vector-mediated gene transfer could reduce lysosomal cystine levels in vivo. Our next goal was to perform gene transfer studies to the cornea, a tissue that is more clinically relevant for cystinosis, using the more stable HD CAV and AAV viral vectors.

Results:

Our first results (produced by Nicolas Serratrice, a PhD student in our lab) showed that intra-stromal injection of HD CAV vectors ex vivo in human cornea and in vivo in mouse cornea resulted in a strong transgene expression from 24 h, which was short-lived (4 weeks ex vivo, 2 weeks in vivo). The non-integrating HD CAV vectors are likely eliminated from the cornea due to an apoptosis/proliferation repair mechanism following injection.

In contrast, our results with the AAV8 vectors are more encouraging as well as intriguing. We injected mouse corneas with AAV-GFP and followed expression by in vivo microscopy and histological studies:

i) Forty-eight h after AAV-GFP injection, we detected expression in the corneal epithelium likely due to the needle traversing this tissue (Figs. 2A and B). This expression disappeared by 1 wk post-injection (p.i.), probably due to the 7-day turnover of this tissue. We began to see GFP expression in the corneal stroma around 4 wk p.i. (data not shown) This expression persists until at least 6 mo p.i. (longest time point tested to date; Figs. 2C and D). Like CAV, AAV vectors are theoretically “non-integrating” thus we don’t know why they are able to escape the fate of CAV vectors following corneal repair. It is possible that AAV vectors do integrate into stromal keratocytes, or the vector genome remains encapsidated, or the extra-chromosomal >50 kb concatemerised vector genome is not lost during cell division.

ii) We made the interesting observation that if we re-injected mouse corneas with PBS 1 wk after the initial AAV-GFP injection (i.e. when GFP is not yet expressed), we provoked GFP expression in the stroma (Fig. 3). This expression rapidly decreases after 24 h. The same kinetics was observed when we re-injected with PBS 1 mo after the initial AAV-GFP injection (i.e. when GFP is already expressed). We will perform qPCR on injected corneas to determine whether PBS re-injection results in an increase in genome copies or mRNA expression.
iii) We performed similar experiments with AAV-CIG and detected the same tropism and profile of GFP expression. In a first experiment, we assayed cystine levels in Ctns<sup>-/-</sup> mice 2.5 mo after AAV-CIG injection. We did not detect a reduction in cystine levels. However, we did not detect a significant GFP expression in this experiment so it is possible that CTNS gene expression was also too low to allow cystine clearance. To increase transgene expression levels, we assayed cystine levels in mice that were re-injected with PBS 1 wk after the initial AAV injection. We detected a 50% decrease in cystine levels but with both AAV-GFP and AAV-CIG (Fig. 4). Thus, for an as yet unknown reason, injection of AAV caused a non-specific decrease in cystine levels. Finally, we will repeat this experiment (i.e. re-injection PBS 1 wk after AAV injection) but this time we will assay cystine levels 3 weeks later. In this way, we will test whether the non-specific reduction due to AAV-GFP disappears to reveal a specific CTNS effect similar to our recent observations in the liver (Hippert et al. 2008 Mol. Ther. 16: 1372-81).

Finally, to complement this study, we will compare the tropism of three other AAV vectors, serotypes AAV1, -2 and -5, to AAV8 in human corneas. In this way, we will evaluate the best vector for long-term expression following intra-stromal injection. Our rationale is as follows: if injection into the cornea allows gene expression for 1 year (or more), then corneal gene therapy may be a feasible alternative to the application of cysteamine eye drops every waking hour.
IV) Refine characterisation of the CNS anomalies in Ctns<sup>−/−</sup> mice

**Background:**

Our previous work suggested that Ctns<sup>−/−</sup> 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) as this will also dictate the choice of vector for subsequent gene transfer studies. Our strategy was to dissociate the brain, label individual cell types with fluorescent-labelled cell markers, isolate these cells via fluorescence-activated cell sorting (FACS), and assay each cell type for their respective cystine levels. In parallel, we also performed stereotaxic injections to target the hippocampus in mice.

**Results:**

This project is challenging. We first followed our initial strategy of isolating the different cell types by FACS to assay cystine levels but this resulted in two major problems: the lack of specificity of the antibodies and the recovery of only a small number of cells precluding a cystine assay. We tried using an Optiprep gradient to isolate cell types into different fractions, which were then individually collected. We performed multiple technical modifications to improve the purity of the fractions. Our preliminary results indicate that the microglia have the highest cystine content; the microglia are the resident macrophages of the brain. These results are thus consistent with our previous observations (Hippert et al. 2008) where we showed that the Kupffer cells, the macrophages of the liver, have the highest cystine content in this tissue. The high cystine levels in Ctns<sup>−/−</sup> macrophages are likely due to the high metabolic activity of this cell type.

![Figure 5: Assay of cystine levels in cell types of the brain of wild-type and Ctns<sup>−/−</sup> mice. The microglia have the highest cystine levels, followed by the oligodendrocytes and, lastly, neurons.](image)

Finally, in parallel, we have been performing stereotaxic injections in the brain of wild-type mice to determine the correct coordinates to consistently reach the hippocampus. Our next goal is to inject HD CAV-CIG (targets neurons) and AAV8-CIG (targets glial cells) in Ctns<sup>−/−</sup> mice and assay cystine levels post-transduction. This work should validate our *in vitro* data as to the cell type most affected in cystinosis.