Artificial liver in PKU

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Discovery of PKU

- Discovered in 1934 by Dr. Asbjørn Fölling
- Egeland family asked if children’s urine odor was related to their mental retardation
- Studied urine samples from children
- Turned dark green when mixed with ferric chloride
- Isolated phenylpyruvic acid

The Discovery of Phenylketonuria: The Story of a Young Couple, Two Retarded Children, and a Scientist
Siegried A. Centerwall and Willard R. Centerwall
*Pediatrics* 2000;105;89-103

Fig 2. Dr Asbjørn Fölling, the scientist, in mid-life, about the time of the discovery of PKU in 1934.
Discovery of PKU

• Tested 430 institutionalized children
• 8 had the same abnormality (2 sibling pairs)
• Dr. Penrose later named the disease phenylketonuria after the characteristic presence of the phenylketone, phenylpyruvic acid in the urine.
• 1950s diet food developed
• 1960s testing infants became common

Borgny & Harry Egeland and their 2 children.

The Discovery of Phenylketonuria: The Story of a Young Couple, Two Retarded Children, and a Scientist
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**Phenylketonuria (PKU)**

- Autosomal recessive disorder
- Classic form due to inactivation of *Pah*
  - Over 500 cataloged mutations
  - Major biosynthetic route to tyrosine
- Cannot metabolize phenylalanine
  - Phe makes up 5% of protein foods
  - Significant buildup of phenylketones in bloodstream
  - Mental retardation (IQ<30), skin rashes, seizures, excessive restlessness, irritable behavior
- **Dietary Therapy**
  - Formula foods
  - Stringent, foul tasting, expensive
Phenylalanine hydroxylase (PAH)

- PAH gene located on chromosome 12
- Converts Phenylalanine to tyrosine in the presence of BH4 co-factor
How do you cure PKU?

Liver Transplant
Hepatocyte transplantation using the domino concept in a child with Tetrabiopterin non-responsive phenylketonuria


- Infusion of transplanted liver cells using a transcutaneous portal catheter
  - Cells from explanted liver of 14mo old with type Ib glycogen storage disease
  - First series: 2 infusions over 2 days (viability: 82% day 1, 78% day 2)
  - Second series: donor cells from male cadaver (viability 82%)
- Effects lasted 3 months

Accepted 1/5/12 epub provisional 10/8/2011 Cell Transplantation
“We conclude that cell transplantation for PKU patients is an option that may correct severe hyperphenylalaninemia in poorly controlled patients being at high risk of neurologic and intellectual impairment.”
How to overcome the Immune System

Protect cells (native or genetically engineered) from ever being exposed to the immune system.

• Encapsulate cells within semi-permeable microspheres
Hypothesis

Injection of encapsulated cells which express Phenylalanine Hydroxylase (PAH) that can function as an artificial liver can reduce serum Phe levels and reverse disease characteristics.
Can a non-dietary therapy reverse phenotypic changes?

Goal: Develop an effective long acting non-dietary therapy.
Cell Encapsulation

Cells
- Allografts (Other Humans)
- Xenografts (Animals)

Biocompatible Material

- Tyrosine, Toxins
- Immune cells, Antibodies
- Nutrients, Phenylalanine

Biocompatible Material
Cell encapsulation for PKU

• Cells are washed 2x in PBS and resuspended in 1.5% alginate
• Infusion pump at 59 ml/hr
• Air pushes droplets off
• Spheres polymerize upon contact with CaCl$_2$ solution
• Spheres filtered through 70 micron filter
• Grown under normal cell culture conditions or transplanted
Encapsulated Cells

Optimize:
- Distance
- Cell concentration
- Air pressure
- Infuser pump speed
- Set up design

\[ 97.9 \pm 20.0 \text{ microns} \quad n=41 \]
Phe Measurement

- Plate cells and media
- Collect and freeze media samples
- Spot samples on Newborn Screening Cards
- Phe measured by Tandem Mass Spectrometry
Does cell encapsulation affect the ability of cells to reduce Phe?

50% reduction in Phe concentration (6 days post-transfection)
Do encapsulated cells remain viable over time?

- Cells encapsulated in microspheres dissolved in sodium citrate buffer to degrade alginate beads
- Cells stained with trypan blue and counted using hemocytometer
**Cell Viability results *in vitro***

<table>
<thead>
<tr>
<th>Days</th>
<th>293T cells</th>
<th>WRL68 cells</th>
<th>HepG2 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>92.33 ± 1.45</td>
<td>89.33 ± 1.15</td>
<td>91.53 ± 1.34</td>
</tr>
<tr>
<td>4</td>
<td>89 ± 1.56</td>
<td>87.67 ± 1.53</td>
<td>90.2 ± 0.75</td>
</tr>
<tr>
<td>7</td>
<td>88.43 ± 1.54</td>
<td>85.33 ± 1.17</td>
<td>88.45 ± 1.24</td>
</tr>
<tr>
<td>14</td>
<td>86.2 ± 1.51</td>
<td>84.2 ± 1.63</td>
<td>86.86 ± 1.43</td>
</tr>
<tr>
<td>21</td>
<td>83.67 ± 1.83</td>
<td>81.76 ± 1.74</td>
<td>84.52 ± 1.78</td>
</tr>
<tr>
<td>28</td>
<td>79.32 ± 1.92</td>
<td>78.42 ± 2.12</td>
<td>82.79 ± 1.49</td>
</tr>
</tbody>
</table>

Cell viability remains high throughout 28 days of cell encapsulation.
How does PAH expression vary between cell lines?

- To determine mRNA PAH expression levels in different cell lines by qRT-PCR
- RNA was isolated from frozen cell pellets
- mRNA expression levels of different cell lines were analyzed and compared

$$\Delta C_t = \text{expression}$$
HepG2 cells express the highest level of PAH.
Who’s Who of Mice

Homozygous C57PAH\textsuperscript{enu2} = PKU (grey fur)

Heterozygous C57PAH\textsuperscript{enu2} = no PKU (black fur)
Comparison of \textit{In Vivo} Phe levels post-treatment

P<0.05 denoted by *
## Analysis of survival

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>Survival at 28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>homozygous PAHenu2</td>
<td>empty spheres</td>
<td>5/5</td>
</tr>
<tr>
<td>heterozygous</td>
<td>empty spheres</td>
<td>5/5</td>
</tr>
<tr>
<td>BTBR</td>
<td>empty spheres</td>
<td>5/5</td>
</tr>
<tr>
<td>homozygous PAHenu2</td>
<td>encapsulated cells</td>
<td>5/5</td>
</tr>
</tbody>
</table>
**In vivo cell viability: Day 28**

Day 0 post-encapsulation: 88%

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Percent cell viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>83.3</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>72.9</td>
</tr>
<tr>
<td>4</td>
<td>85</td>
</tr>
<tr>
<td>5</td>
<td>79.4</td>
</tr>
<tr>
<td>6</td>
<td>74.2</td>
</tr>
<tr>
<td>7</td>
<td>84.7</td>
</tr>
</tbody>
</table>
Understanding the PKU mouse model is critical to testing effects of therapies.

• Coloring
• Fertility
• Metabolism
• Sleep
• Immune System
Hypothesis

Lowering Phe in mice will correct other characteristics found in homozygous (PKU) mice.

There is more to know than Phe levels!
Phenotypic Correction: HepG2

DAY 0

Treated male homozygous PKU mouse

Untreated male wild type mouse

DAY 28

Wild type mouse

Treated male homozygous PKU mouse

Female mouse: Delivered 5 pups
Day 1: 1.1 ± 0.06g
PKU mice are smaller than their counterparts.
What is the root cause of the difference?

INPUT
- Food Intake
- Food Composition
- Digestive Efficiency

OUTPUT
- Heat Production
- Physical Activity
**What are the ins & outs in PKU mice?**

<table>
<thead>
<tr>
<th>Group</th>
<th>Food Intake (grams/day)</th>
<th>Fluid Intake (ml/day)</th>
<th>Urine Output (grams/day)</th>
<th>Fecal Output (grams/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterozygous Female</td>
<td>3.72</td>
<td>2.97</td>
<td>0.35</td>
<td>1.16</td>
</tr>
<tr>
<td>(n=7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homozygous Female</td>
<td>4.47</td>
<td>5.39</td>
<td>1.72</td>
<td>1.46</td>
</tr>
<tr>
<td>(n=8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterozygous Male</td>
<td>3.28</td>
<td>2.95</td>
<td>0.33</td>
<td>1.00</td>
</tr>
<tr>
<td>(n=7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homozygous Male</td>
<td>4.41</td>
<td>4.37</td>
<td>1.13</td>
<td>1.41</td>
</tr>
<tr>
<td>(n=6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PKU mice eat more, drink more, make more urine and feces. PKU mice lose more calories through feces, but this is not made up for by the increased food ➔ smaller mice.
What are the ins & outs in treated PKU mice?

<table>
<thead>
<tr>
<th>Group</th>
<th>Food Intake (grams/day)</th>
<th>Fluid Intake (ml/day)</th>
<th>Urine Output (grams/day)</th>
<th>Fecal Output (grams/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterozygous Female (n=7)</td>
<td>3.72</td>
<td>2.97</td>
<td>0.35</td>
<td>1.16</td>
</tr>
<tr>
<td>Homozygous Female (n=8)</td>
<td>3.87</td>
<td>2.23</td>
<td>1.24</td>
<td>0.98</td>
</tr>
<tr>
<td>Heterozygous Male (n=7)</td>
<td>3.28</td>
<td>2.95</td>
<td>0.33</td>
<td>1.00</td>
</tr>
<tr>
<td>Homozygous Male (n=6)</td>
<td>2.89</td>
<td>1.8</td>
<td>0.93</td>
<td>0.835</td>
</tr>
</tbody>
</table>

PKU mice eat more, drink more, make more urine and feces ➔ *Corrected in treated PKU mice.*
The artificial liver reverses the increase in caloric intake and absorption.

<table>
<thead>
<tr>
<th>Group</th>
<th>Food Intake Σ kcal/day</th>
<th>Fecal Output Σ kcal/day</th>
<th>Absorbed Σ kcal/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterozygous</td>
<td>13.17</td>
<td>2.67</td>
<td>10.49</td>
</tr>
<tr>
<td>Homozygous</td>
<td>18.59</td>
<td>4.00</td>
<td>14.58</td>
</tr>
<tr>
<td>Homozygous - treated</td>
<td>15.05</td>
<td>3.04</td>
<td>12.01</td>
</tr>
</tbody>
</table>

![Absorbed Total kcal/day](chart.png)
Are there basic differences in resting metabolic rate in PKU mice?
Artificial liver starts to relieve the sleep disorder in females.
Immune system changes also begin to correct.

<table>
<thead>
<tr>
<th></th>
<th>Heterozygous</th>
<th>Homozygous</th>
<th>Treated Homozygous</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T Cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ Total</td>
<td>14.6 ±0.9</td>
<td>18.7 ±5.4</td>
<td>18.5 ±4.9</td>
</tr>
<tr>
<td>CD4+ Naïve</td>
<td>72.4 ±2.1</td>
<td>65.2 ±6.1</td>
<td>68.4 ±4</td>
</tr>
<tr>
<td>CD4+ Memory</td>
<td>9.4 ±4.3</td>
<td>9.8 ±3.6</td>
<td>10.7 ±2.8</td>
</tr>
<tr>
<td>CD8+ Total</td>
<td>10.1 ±1.5</td>
<td>12.7 ±2.6</td>
<td>8 ±4.1</td>
</tr>
<tr>
<td>CD8+ Naïve</td>
<td>32.6 ±3.7</td>
<td>28.8 ±4.1</td>
<td>27.7 ±2.5</td>
</tr>
<tr>
<td>CD8+ Memory</td>
<td>17.3 ±2.5</td>
<td>20 ±1.2</td>
<td>16.4 ±4.4</td>
</tr>
<tr>
<td><strong>NK Cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK1.1+</td>
<td>3.6 ±0.5</td>
<td>4.7 ±1.6</td>
<td>3.6 ±1.6</td>
</tr>
<tr>
<td><strong>B Cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B220+ Total</td>
<td>56.7 ±2.2</td>
<td>47.1 ±7.6</td>
<td>47.6 ±4.5</td>
</tr>
<tr>
<td><strong>B220+ CD80+</strong></td>
<td><strong>87.7 ±13.3</strong></td>
<td><strong>32.7 ±18.1</strong></td>
<td><strong>84.8 ±18</strong></td>
</tr>
<tr>
<td>B220+ CD86+</td>
<td>5.5 ±4.2</td>
<td>5.8 ±1.9</td>
<td>7.5 ±1.8</td>
</tr>
<tr>
<td>B220+ IgM+</td>
<td>47.9 ±15.2</td>
<td>56.9 ±4.3</td>
<td>59.7 ±6.7</td>
</tr>
<tr>
<td><strong>Dendritic Cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD11c+ Total</td>
<td>2.7 ±0.6</td>
<td>4.0 ±0.5</td>
<td>3.7 ±1.1</td>
</tr>
<tr>
<td><strong>CD11c+ CD80+</strong></td>
<td><strong>78.0 ±9.6</strong></td>
<td><strong>39.4 ±8.9</strong></td>
<td><strong>61.2 ±16.1</strong></td>
</tr>
<tr>
<td>CD11c+ CD86+</td>
<td>72.5 ±5.6</td>
<td>21.6 ±2.8</td>
<td>73.3 ±8.9</td>
</tr>
</tbody>
</table>
Who’s who in the immune system

B cells make antibodies and stimulate other immune system cells
Who’s who in the immune system

Dendritic cells process and present infections to stimulate other immune system cells
N=4
* = p<0.05, ** = p<0.01

** B Cells

- CD40
  - Heterozygous: 150
  - Homozygous: 50
  - Treated Homozygous: 100
  - p = 0.0640

- CD80
  - Heterozygous: 5000
  - Homozygous: 1000
  - Treated Homozygous: 2000
  - p = 0.0804

- CD86
  - Heterozygous: 150
  - Homozygous: 50
  - Treated Homozygous: 100

** Dendritic Cells

- CD40
  - Heterozygous: 150
  - Homozygous: 50
  - Treated Homozygous: 100
  - p = 0.0640

- CD80
  - Heterozygous: 3000
  - Homozygous: 1000
  - Treated Homozygous: 2000

- CD86
  - Heterozygous: 150
  - Homozygous: 50
  - Treated Homozygous: 100
  - * p<0.05, ** p<0.01
Summary

- Hepatocyte cell lines express high levels of PAH
- Encapsulating cells does not prevent processing of Phe
- Encapsulated cells reduce Phe in vitro and in vivo
- Phenotypic correction can be observed in treated PKU model mice
  - Change in fur color
  - Change in fertility
  - Changes in Food intake and absorbed calories
  - Changes in sleep
  - Changes in B cells and Dendritic cells
Cell transplantation with encapsulated PAH expressing cells holds potential has a non-dietary therapy for PKU syndrome.
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