The Role of Hepatic Progenitor cells in Human Liver Regeneration

Prof Dr Tania Roskams, MD, PhD
Department of Morphology and Molecular Pathology
University of Leuven
Belgium
A stem cell is an undifferentiated cell capable throughout life of renewing itself as well as of generating one or more type of differentiated cells.

- Embryonic stem cells: **totipotential**
- Fetus and adult: **multi/pluripotential**

Closer to final differentiation: **Progenitor**
- Committed stem cells, **committed**
- Transit cells, **transit**
Stem cells: easy to find in lining epithelia
Liver progenitor cells
Liver progenitor cells: CK7, CK19, OV6, OV1, Chrom A, NCAM
subpopul: CD34, c-kit, flt-3, thy-1, sca1, CD133
CD133 in chronic hepatitis C pt
Liver Regeneration with preserved parenchyma

Normal Liver  Partial hepatectomy

Bile duct ep  Bile duct ep

Progenitor cell

Hepatocyte  Hepatocyte

PHx, transplantation, living-related donor
Progenitor Cell Activation

Hepatocyte/ bile duct damage
inhibition of replication

Bile duct ep

Progenitor cell

Hepatocyte

CDAAF, galactosamine intoxication in rat
Majority of human liver diseases
Control Mechanisms of Progenitor Cell Activation and Differentiation
Oval cells/progenitor cells in rodent models are activated when hepatocyte replication is inhibited:

- CDAAAF model
- Modified Solt Farber Model
- Animal models with alcoholic liver disease
- Mouse models of fatty liver disease
- Ethionine intoxication in mice
- ...
Human **acute and chronic** liver diseases are characterized by replicative senescence of hepatocytes

- **Acute (sub)massive liver necrosis**
  (Katoonizadeh Liver Int 2007)

- **Alcoholic liver disease and viral hepatitis**
  (Crary Hepatol 98, Paradis Hum Pathol 01, Falkowski J Hepatol 03, Roskams Am J Pathol 03, Eleazar J hepatol 04, Marshall Gastroenterology 05)

- are associated with **inhibition of hepatocyte replication**
Human chronic liver diseases are characterized by replicative senescence of hepatocytes.

- Telomere shortening and replicative senescence of mature hepatocytes (and not of hepatic stellate cells or lymphocytes) is a general feature of the cirrhotic stage of a variety of chronic liver diseases. (Wiemann FASEB J 03, Falkowski J Hepatol 03, Rudolph Science 00, Fausto Hepatology 2004)

- This inhibition of replication is associated with progenitor cell activation in human liver diseases. (De Vos Am J Pathol 92, Roskams J Hepatol 98, Lowes Am J Pathol 99, Libbrecht J Pathol 00, Roskams Am J Pathol 03, Katoonizadeh Liver Int in press...
HPC in Chronic Liver Diseases

- Normal
- Stage I
- Stage II-III HCV hepatitis
- Stage IV

Stage I-II (N)ASH
Stage IV CK7 staining
# Acute Severe Liver Impairment

**Histopathological correlations**

<table>
<thead>
<tr>
<th>Severity of hepatocyte loss</th>
<th>No. of patients</th>
<th>No. of HPCs</th>
<th>No. of mib-1+ hep</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild (&lt; 30%)</td>
<td>7</td>
<td>54 ± 34</td>
<td>11.5 ± 6.7</td>
</tr>
<tr>
<td>Moderate (30- 50%)</td>
<td>17</td>
<td>76 ± 40</td>
<td>12.7 ± 10</td>
</tr>
<tr>
<td>Severe (50-75 %)</td>
<td>22</td>
<td>150.2 ± 68</td>
<td>8.3 ± 9.2</td>
</tr>
<tr>
<td>Very severe (&gt; 75 %)</td>
<td>28</td>
<td>138 ± 53.7</td>
<td>4.1 ± 5.8</td>
</tr>
</tbody>
</table>

Classification of patients according to the severity of hepatocyte loss and the number of HPCs or proliferating (mib-1 positive nuclei) hepatocytes for each group.

Katoonizadeh Liver International 2007
### Histopathological correlations

<table>
<thead>
<tr>
<th>Severity of hepatocyte loss</th>
<th>No. of patients</th>
<th>No. of HPCs</th>
<th>No. of mib-1+ hep</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild (&lt; 30%)</td>
<td>7</td>
<td>54 ± 34</td>
<td>11.5 ± 6.7</td>
</tr>
<tr>
<td>Moderate (30-50%)</td>
<td>17</td>
<td>76 ± 40</td>
<td>12.7 ± 10</td>
</tr>
<tr>
<td>Severe (50-75 %)</td>
<td>22</td>
<td>150.2 ± 68</td>
<td>8.3 ± 9.2</td>
</tr>
<tr>
<td>Very severe (&gt; 75 %)</td>
<td>28</td>
<td>138 ± 53.7</td>
<td>4.1 ± 5.8</td>
</tr>
</tbody>
</table>

Classification of patients according to the severity of hepatocyte loss and the number of HPCs or proliferating (mib-1 positive nuclei) hepatocytes for each group.
CK7, CK19: progenitor cell activation/differentiation

< 30% necrosis  > 50% necrosis
Comparison of HPCs activation /differentiation based on the duration of disease

- <1 week: N=5
- 1-4 weeks: N=30
- > 4 weeks: N=39

- HPCs activation
- Presence of intermediate hepatocytes
Regeneration after (sub)massive necrosis

24h  1 week later

Roksams J hepatol 98
Katooni Zadeh Liver Int. 2006
**Clinicopathological correlations**

<table>
<thead>
<tr>
<th></th>
<th>(mib-1+) hepatocytes</th>
<th>HPCs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MELD score</strong></td>
<td>-0.59</td>
<td>0.40</td>
</tr>
<tr>
<td><strong>INR</strong></td>
<td>-0.60</td>
<td>0.38</td>
</tr>
<tr>
<td><strong>Bilirubin</strong></td>
<td>-0.36</td>
<td>0.40</td>
</tr>
</tbody>
</table>

Comparison of the number of proliferating (mib 1 positive nuclei) hepatocytes and HPCs with clinical parameters at the time of liver biopsy.
## Comparison of histopathological parameters and outcome

<table>
<thead>
<tr>
<th>Histopathological parameter</th>
<th>Group A Alive (n=24)</th>
<th>Group B Died (n=10)</th>
<th>Group C Transplanted (n=40)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;50 % hepatocyte loss</td>
<td>8% (2/24)</td>
<td>80% (8/10)</td>
<td>95% (38/40)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Number of proliferating hepatocytes/HPF</td>
<td>14.3 ± 9.3</td>
<td>2.5 ± 2.5</td>
<td>5.7 ± 7.3</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Number of HPCs/HPF</td>
<td>74 ± 55</td>
<td>138 ± 52</td>
<td>141 ± 58</td>
<td>0.003</td>
</tr>
</tbody>
</table>

> 50% hepatocyte loss, low proliferative activity of hepatocytes, high progenitor cell activation, independent factors for bad prognosis.
Survival of progenitor cells in disadvantageous environments
Stem cells: ability to exclude the Hoechst dye 33342

- Reflects possession of one of the ABC-binding cassette transporter pumps BCRP (Zhou 2001)

- On fluorescent-activated cell sorting: cells separate as ‘side-sorted’ population
BCRP in progenitor cells, giving these cells a multidrug-resistant phenotype. Putative side population in man.

Vander Borght et al. J Pathol 2006
Upregulation of ABC transporters MDR1 and MRP3 in progenitor cells, giving these cells a multidrug-resistant phenotype. Putative side population in man.

Ros et al. J Pathol 03, Gut 03
Side-population analysis in the adult liver

Bart Spee
Collaboration with Utrecht University
Experimental Setup

3-step liberase

isodensity centrifugation

pellet

supernatant

Parenchymal fraction

Non-parenchymal fraction
Side-population, cell sorting

Incubation with Hoechst33342

a.o. ABCG2/BCRP receptor

Fluorescent Activated Cell Sorting (FACS)
FACS analysis

1. **Hoechst**
   - Acquisition image side-population

2. **Hoechst + Fumetrimorgin-C**
   - (ABCG2 inhibitor)

3. **Hoechst + Reserpine**
   - (ABC-transporter inhibitor)

4. **Hoechst + Verapamil**
   - (ABC-transporter inhibitor)

5. **Hoechst + DMSO (1:1,000)**
   - Control dilutions

6. **Hoechst + CD45**
   - CD45 panleukocyte antigen (hematopoietic marker)
Hoechst  
- Side-population 3.2 %

Hoechst + Fumetrimargin-C  
- Side-population 2.1 %

Hoechst + Reserpine  
- Side-population 1.2 %
Side-population, cell sorting CD45-PE labelled

- CD45 panleukocyte antigen (hematopoietic marker)

- 74.3 % of side-population is CD45 positive
- Outside side-population no CD45 positive cells
- 0.2 % of the total population is CD45 negative suggesting a hepatic lineage
Side-population, cell sorting

RNA isolation (RNAlater)

1. Bulk population: 200,000 cells, 33.3 ng/ul, RIN 6.3
2. CD45 positive cells: 6,500 cells, 5.7 ng/ul, RIN N/A
3. CD45 negative cells: 3,500 cells, 1.9 ng/ul, RIN N/A

Gene-expression analysis

<table>
<thead>
<tr>
<th>Gene Expression</th>
<th>CD45 Positive</th>
<th>CD45 Negative</th>
<th>Bulk Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Kit (hemato/prog)</td>
<td>+</td>
<td>+++</td>
<td>+/-</td>
</tr>
<tr>
<td>CD34 (mes/hema/prog)</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>CD133/Prominin (hemato)</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Thy-1/CD90 (mes/hema/prog)</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>CK7 (prog/bile)</td>
<td>+/-</td>
<td>++</td>
<td>+/-</td>
</tr>
<tr>
<td>CK19 (prog/bile)</td>
<td>+/-</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>CK18 (hepatocyte)</td>
<td>+/-</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>CSA (hepatocyte)</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>AFP (prog/hepato)</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>HNF4A (hepatocyte)</td>
<td>-</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>ABCG2/BCRP (hepato/prog)</td>
<td>-</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>FN14/TWEAKR (prog)</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
microscopic “niche” of stromal cells

- Nurse stem cell
- Give proper instructions
- Hair follicle of mouse: niche for melanocytic stem cells (Nature 02)
- Trabecular bone (haematopoietic stem cell niche: Nature 03)

Liver: PCs surrounded by HSCs.
Characterization of the progenitor cell niche

Mitogenic stimuli

Microdissection with Laser Capture Microscopy (LCM) of ck7+HPC
**Immunohistochemical technique:** Rapid IHC (< 25 min)
**Marker:** CK7

**Primary ab**  
Progenitor cell  
**Secondary ab (HRP labelled)**  
**Substrate**  
**Brown color**

**Example:** Primary Biliary Cirrhosis (PBC)

Before  
After
EXPERIMENTAL SETUP

Microdissected CK7+ HPC from

<table>
<thead>
<tr>
<th>Acute hepatitis (AH)</th>
<th>Hepatocytic diseases (HCV-C)</th>
<th>Billiary diseases (PBC)</th>
</tr>
</thead>
</table>

RNA isolation

RNA amplification

Gene-expression analysis
- Superarray (real-time PCR 84 genes)

IHC confirmation
## Superarray (84 genes of interest)

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CD133</td>
<td>WNT1</td>
<td>MYC</td>
<td>OCT4</td>
<td>ABCB1</td>
<td>ITGB1</td>
<td>HPRT1</td>
<td>85</td>
</tr>
<tr>
<td>2</td>
<td>KRT7</td>
<td>WNT3A</td>
<td>MMP-7</td>
<td>Nanog</td>
<td>PDGFA</td>
<td>CXCL12</td>
<td>RPL13A</td>
<td>86</td>
</tr>
<tr>
<td>3</td>
<td>ABCG2</td>
<td>WNT4</td>
<td>MMP-9</td>
<td>Sox2</td>
<td>PDGFR A</td>
<td>CXCR4</td>
<td>GAPD</td>
<td>87</td>
</tr>
<tr>
<td>4</td>
<td>KRT19</td>
<td>WNT5A</td>
<td>Axin2</td>
<td>LIF</td>
<td>BMP1</td>
<td>FGF4</td>
<td>ACTB</td>
<td>88</td>
</tr>
<tr>
<td>5</td>
<td>AFP</td>
<td>APC</td>
<td>CD44</td>
<td>LEF1</td>
<td>BMP2</td>
<td>KITLG</td>
<td>ACTB</td>
<td>89</td>
</tr>
<tr>
<td>6</td>
<td>ASMA</td>
<td>AXIN</td>
<td>DKK1</td>
<td>TCF3</td>
<td>BMP3</td>
<td>c-kit</td>
<td>ACTB</td>
<td>90</td>
</tr>
<tr>
<td>7</td>
<td>NCAM1</td>
<td>AXIN</td>
<td>BTRC</td>
<td>KLF4</td>
<td>VEGF</td>
<td>SHH</td>
<td>ACTB</td>
<td>91</td>
</tr>
<tr>
<td>8</td>
<td>GFAP</td>
<td>FZD1</td>
<td>FRAT1</td>
<td>Esrrb</td>
<td>IGF1</td>
<td>DHH</td>
<td>ACTB</td>
<td>92</td>
</tr>
<tr>
<td>9</td>
<td>CRYAB</td>
<td>DVL1</td>
<td>PPARD</td>
<td>Tbx3</td>
<td>BMI1</td>
<td>RB1</td>
<td>ACTB</td>
<td>93</td>
</tr>
<tr>
<td>10</td>
<td>DES</td>
<td>DVL2</td>
<td>DLL1</td>
<td>Tcl1A</td>
<td>NCAD</td>
<td>CCND1</td>
<td>ACTB</td>
<td>94</td>
</tr>
<tr>
<td>11</td>
<td>SYN</td>
<td>DVL3</td>
<td>DLL3</td>
<td>Dppa4</td>
<td>VCAM</td>
<td>HOXB4</td>
<td>ACTB-NR</td>
<td>A</td>
</tr>
<tr>
<td>12</td>
<td>TNFSF12</td>
<td>GSK3B</td>
<td>DLL4</td>
<td>ABCC1</td>
<td>Numb</td>
<td>18srRNA</td>
<td>ACTB-NTC</td>
<td>A</td>
</tr>
</tbody>
</table>

### Notes:
- The table includes 84 genes of interest.
- Each column represents a different gene family or category.
- The table is designed to show the correlation and expression of these genes in a superarray format.
CD133 and NCAM are up-regulated (67.46 folds and 30.29 folds) in Acute disease (AH) compared with chronic disease (HCV-C).
Jag-1 and Notch-4 are up-regulated (2.99 and 7.92 folds) in AH compared with HCV-C.
PRELIMINARY RESULTS: HPC markers

NCAM is up-regulated (19,47 folds) in PBC compared with HCV-C
Jag-1 and Notch-4 are up-regulated (18.87 and 10.47 folds) in PBC compared with HCV-C.
**PRELIMINARY RESULTS: WNT pathway**

LEF-1 is up-regulated (10,25) in AH and PBC compared with HCV-C

<table>
<thead>
<tr>
<th></th>
<th>AH</th>
<th>HCV-C</th>
<th>PBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Image</td>
<td>![Image of AH]</td>
<td>![Image of HCV-C]</td>
<td>![Image of PBC]</td>
</tr>
</tbody>
</table>
CONCLUSIONS

✓ Progenitor cells play role in human liver regeneration

✓ Control mechanisms are only partly understood

✓ Progenitor cells are in the ‘side population’: finally able to isolate progenitor cells

✓ Similar to the progenitor cell niche in other organs (gut, nervous system), wnt and notch pathways are implicated with HPC niche expansion and probably with the differentiation towards hepatocytes or cholangiocytes.
Acknowledgements

Prof. Em. Dr. V. Desmet  
B. Spee  
S. Vander Borght  
Dr. A. Katoonizadeh  
Dr. M. Komuta  
Dr. Guido Carpelli  
P. Aertsen  
Prof. Em Dr. Fevery  
Prof. Dr. Yap  
Prof. Dr. Van Steenbergen  
Prof. Dr Nevens  
Prof. Dr. C. Verslype  
Dr. C. Cassiman  
Prof. Dr. J. Pirenne  
Dr. R. Aerts  
Dr. D. Monballiu  
Dr Van Beckevoort  
Dr D. Bielen  
Dr S. Thorgeirsson  
Prof Dr C. Trautwein  
Dr. T. Luedde  
Dr. N Berazza  
Prof Dr A. Geerts  
Prof. Dr P. Jansen  
Prof. M. Strazzabosco  
Prof. L. Fabris  
Prof. Dr Pinzani  
Prof. Dr Rothuizen