Liver Regeneration

ASIP Rous-Whipple Award Lecture
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Department of Pathology
The liver of sacrificial animals in ancient times was used as a tool to “divine” the omens for the forecast of the future. The “signs” were the ubiquitous scars and their location on the liver surface. The presence of such scars and other signs of “divination” on the surface of the liver of sacrificial animals shows the high frequency of injuries to the liver sustained by animals in the wild.

Babylon, 1900-1600 BCE. Inscribed model of sheep’s liver, used in a “divination” school for instructing pupils in training.
Why does liver need to be able to regenerate?

1. Liver performs irreplaceable vital functions:
   g. Synthesis of plasma proteins and coagulation factors.
   h. Secretion of bile.
   i. Metabolism of complex chemicals
   j. Processing and biotransformation of absorbed food components.
   k. Lipid and cholesterol regulation.
   l. Regulation of glucose storage and release.

2. Liver is exposed to dangerous xenobiotics ingested with food. Most absorbed food ingredients (including xenobiotics) proceed to liver where they become altered into useful metabolites. Many xenobiotics become highly reactive electrophiles after biotransformation in the liver and cause massive death of hepatocytes. Massive hepatic damage as a commonplace event in the wild created the evolutionary pressure for liver regeneration.
Growth of baboon liver transplanted into human: Is there a “hepatostat”?
In a typical clinically overt case of viral hepatitis, 90% of the hepatocytes die. They are replenished as fast as they die until the hepatocytes carrying active virus are eliminated.
Fulminant Hepatic Failure.
If the proliferative capacity of either of the two epithelial cell types of liver, Hepatocytes and Biliary cells, fails, then they can function as “facultative stem cells” for each other! In humans, this does not always “save the day”.
Rat Liver Regeneration After 70% Partial Hepatectomy (PHx)

Compensatory hyperplasia of remnant lobes restores liver mass lost due to 70% PHx

FIG. 3. Liver regeneration after partial hepatectomy. Liver of a normal rat at operation (the excised lobes are outlined) and at 1 to 4 weeks after partial hepatectomy.

Restoration of hepatic mass after partial hepatectomy in the rat.

Wet weight of remnant liver. Regenerated rate of the hepatic remnant is shown as percent of the calculated preoperative whole liver weight.
DNA synthesis cycles after partial hepatectomy.

One-hour incorporation of $^3$H-thymidine into hepatic DNA in 200 g male Sprague Dawley rats at intervals after partial hepatectomy. Vertical lines indicate the standard error of the mean, numbers the number of rats per point. From Bucher, Patel and Cohen, with kind permission of Pergamon Press.
DNA synthesis of different hepatic cell types after partial hepatectomy.

Modified from original work by Dr. Joe Grisham
Liver regeneration
George K. Michalopoulos
Journal of Cellular Physiology
From the 60s and 70s: Mitotic stimuli for hepatocytes circulate in the blood after partial hepatectomy

- Partial hepatectomy stimulates DNA synthesis in hepatic fragments grafted in any site of the body (Grisham et al.).
- In rats pairs with joined (parabiotic) circulation, partial hepatectomy of one member of the pair stimulates DNA synthesis in the liver of the unoperated members of the pair. The regenerative activity is proportionate the percent of the tissue removed (Bucher, Fischer)
- Isolated hepatocytes transplanted in the adipose tissue of rats respond to DNA synthesis after partial hepatectomy of the rat liver (Jirtle and Michalopoulos)
Thymidine Autoradiography of Proliferating Hepatocytes
Hepatocyte Growth Factor (HGF)

Synthesized in inactive form as a single continuous polypeptide.

Activated by urokinase plasminogen activator (uPA) and by a Factor XII homologous protein known HGFA, by cleavage at an RVV site to the mature two-chain heterodimeric form.
Receptor Tyrosine Kinase structures
Met expression in different tissues.
Extracellular Signals implicated in liver regeneration

**COMPLETE MITOGENS:**

1. Mitogenic in hepatocyte cultures in chemically defined (serum-free) media.
2. Cause liver enlargement and hepatocyte DNA synthesis when injected into whole animals:
   
   - Hepatocyte Growth Factor (HGF) and receptor c-Met
   - Ligands of the EGF R (EGF, TGFα, HB-EGF, Amphiregulin)

**AUXILIARY MITOGENS.**

1. Ablation of their signaling pathways causes delay but does not abolish liver regeneration.
2. They are not mitogenic in hepatocyte cultures and when injected in vivo do not cause hepatocyte DNA synthesis and liver enlargement.
   
   - Norepinephrine and the α1 adrenergic receptor.
   - TNF and TNFR1.
   - IL6
   - **Notch and Jagged (recombinant Jagged causes DNA synthesis in hepatocyte cultures)**
   - VEGF and receptors I and II.
   - Bile acids
   - Serotonin
   - Complement proteins
   - Leptin
   - Insulin
   - PPAR gamma
Mitotic stimuli for hepatocytes circulate in the blood after partial hepatectomy: As we understand it today:

- Within 1-2 hours after hepatectomy there is massive increase in the peripheral blood of HGF, Norepinephrine, TNFa, IL6, TGFβ1 and hyaluronic acid.
- HGF is a complete mitogen.
- Norepinephrine, TNFa, IL6, TGFβ1 are auxiliary mitogens. They contribute to enhancement and optimization of the effect of growth factors (HGF, EGF) by mobilization and activation of transcription factors (an effect known as “priming” (Fausto).
- While some of the effects of these substances maybe mediated from the peripheral blood, most of the effects are probably equally mediated by local release and availability in the liver tissue following matrix remodeling and local release of cytokines and effects of the massive increase in portal blood flow.
Chronology of **concurrent** early (first 1 hour) signaling events after PHx

- Multiple signaling pathways involving both growth factors, cytokines, paracrine signals (Notch/Jagged) and neuroendocrine factors (Norepinephrine) occur simultaneously within the first 60 minutes after PHx. Examples:
  - Increase in urokinase activity (first 5 minutes)
  - Translocation of N(otch)ICD to the nucleus (15 minutes)
  - Translocation of beta-catenin to the nucleus (5-10 minutes to 6 hours)
  - Decrease in HGF biomatrix stores (30 minutes to 3 hours)
  - Activation of the HGF receptor (within 30-60 minutes)
  - Activation of the EGF receptor (within 30-60 minutes)
  - Increase of HGF, Norepinephrine, IL6, TNFa, TGFb1 and hyaluronic acid in the plasma.
  - Activation of AP1, NFkB and STAT3
  - Extensive gene expression reprogramming of hepatocytes within 30 minutes after PHx (Taub et al.).
Increased translocation of residual β-catenin
**Notch/Jagged pathway**

**nucleus**

- **stable co-activator complex**
- **target genes**
- **NotchICD**
- **CBF1/RBPJk**
- **activation**
- **HAT** (Mastermind/Lag3)

**intracellular**

- **co-repressor complex**
- **SMRT**
- **HDAC complex**
- **NotchICD**

**extracellular**

- **Notch**
- **Delta**
- **activation**
- **Jagged**

**activation**

- **S-2 cleavage** (metalloproteases)
- **Presenilin** (S3-cleavage)

**+ co-activators:**
- Mastermind
- GCN4 and PCAF
- Histone acetylases (HATs)

**Diagram notes:**
- **Red** = Notch + Lin-12 repeats
- **Light blue** = EGF 11-12 of Notch
- **Dark blue** = Pest sequence
- **Orange** = EGF repeats
- **Yellow** = DSL domain (Delta:Serrate:LAG-2 domain)
- **Brown** = six ankyrin repeats
Fig. 5. Detection of cytoplasmic domain of Notch (NICD) in nuclear protein (NP) extracts by Western blot analysis (rabbit polyclonal antibody, Upstate). (A) Densitometric analysis of Western blots for NICD in nuclear protein (NP). Data are shown as mean ± SEM (n = 3). (B) Representative Western blot of NICD detection in NP of rat liver. Ponceau-S stain of a band at 176 kDa are used as loading control. Numbers indicate time elapsed after operation in minutes, hours, and days.

Fig. 6. Detection of localization of the intracytoplasmic domain of Notch (NICD) in normal liver (A-a and A-b), liver at 15 minutes after partial hepatectomy (B-a and B-b), or sham operation (C-a and C-b). In normal liver and in sham-operated animals, NICD is localized only on the cytoplasm or the plasma membrane. There is no green fluorescence in the nuclei. Green fluorescence is seen in the nuclei at 15 minutes after partial hepatectomy. The nuclei were counter-stained with Hoechst dye shown in Fig. A-b, B-b, and C-b, to serve as comparison with the corresponding (a) figures in order to facilitate visual localization of the nuclei. Cytoplasmic and membrane localization of NICD is shown by long arrows. Nuclear localization (seen only in B-a) is shown by short arrows.
HGF, Hepatic Extracellular Matrix, Urokinase, Matrix Metalloproteinases

• Previous work from our laboratory has shown the following:
  – HGF protein is localized in hepatic extracellular matrix.
  – Urokinase activates single chain HGF to the active heterodimer in regenerating liver homogenates (anti-uPA antibody blocks activation)
  – Liver urokinase activity rises dramatically within minutes after PHx.
  – As expected, this leads rapidly to activation of plasminogen to plasmin.
  – Plasmin activated MMP9 is expressed by proliferating hepatocytes as a wave from periportal to centrilobular areas from 3 hours to 48 hours after PHx.
HGF was isolated from plasma of hepatectomized rats as the mitogenic substance rising in the blood after partial hepatectomy.

Fig. 3. Form of endogenous HGF/SF in the blood after PHX. Blood was harvested from two animals at indicated times after PHX and plasma was prepared as described in the Materials and Methods. Plasma totaling 13 mL for each time point was loaded on a heparinagarose column. The bound protein was eluted with 1.2 mol/L NaCl and extracted as described in the Materials and Methods. The sample was then resolved on a 10% Tris-tricine acrylamide gel and blotted to a polyvinylidene difluoride membrane. The reference lane consists of purified rat telHGF/SF and the telHGF/SF is labeled.

HGF in plasma increases 20-fold within 1 hour.
HGF mRNA expression in liver and lung starts at 3 hours peaking at 20-30 hours.
Where does the plasma HGF come from?
Proteolytic cascades involved in matrix remodeling.

uPA initiates matrix remodeling and activates HGF!
uPA activity increases rapidly following partial hepatectomy.

Normal Liver

One minute after PHx

-anti uPA

+ anti uPA
Urokinase activates scHGF to tcHGF after hepatectomy.
Effect of portal hemodynamics on liver regeneration studied in a novel portohepatic shunt rat model.

Marubashi et al., *Surgery*, 136:1028-1037, 2004
Activation of plasminogen to plasmin after PHx

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Plasminogen → Plasmin
scHGF and tcHGF during liver regeneration after partial hepatectomy.

Consumption from pre-existing stores

Synthesis of new HGF

- PHx
- Sham

[Graph showing HGF mRNA fold increase and DNA synthesis over time post hepatectomy]
Tyrosine phosphorylation of Met and EGFR after PHx.
Decrease in c-met after ShMet Treatment

Real Time PCR

Phospho Met WB

mRNA & protein levels in ShMet treatment
Suppression of Cell Division after shMet treatment

Complete absence Of mitoses at 24 hours
Mitotic rate at 48 hrs Surpassed the rate of The scrambled RNA Treated rats.

ShRNA: 4%.
Scr RNA: 80%.
Changes in expression of cell cycle and growth regulating genes following treatment with Met Silencing RNA

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<td>cyclin H</td>
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Changes in expression of cell cycle and growth regulating genes following treatment with Met Silencing RNA

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Caspase 3 activation after treatment with ShMet RNA.
Figure 9. Mitotic index was estimated as described in Methods Section. At day 1, suppression of mitosis was seen in shEGFR treated rats, compared to controls. For comparison Data for shMet treated rats is also shown.

Pro-apoptotic genes upregulated by treatment with Sh-EGFR.

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<tr>
<td>Caspase 7</td>
<td>293</td>
<td>156</td>
<td>1.87</td>
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What would happen if both EGFR and MET were blocked?

- “Knock-down” of either EGFR or MET leads to arrest of proliferation and increase in pro-apoptotic gene expression.
- There is extensive literature from other cell types documenting pro-survival effects of MET, EGFR and other receptor tyrosine kinases.
- Decreased levels of MET lead to increased availability of death receptor Fas for trimerization and ligand-dependent or ligand-less activation (Zarnegar et al.)
- When each of these receptors is subjected to “knock-down” by itself, there is no apoptosis of hepatocytes, perhaps due to redundancy of signaling and compensation by the other receptor.
- What would happen if both mitogenic receptor tyrosine kinases were subjected to “knock-down”? 
A

Met & EGFR Real time PCR

Relative expression

0 200 400 600 800 1000 1200 1400 1600

Hours Post PHx

0 24 48

scr-met
shMet
scr-EGFR
shEGFR

B

TIME ZERO 24 HOURS AFTER PHx

c-met

Scrambled RNA
ShRNA against Met and EGFR
Scrambled RNA
ShRNA against Met and EGFR

EGFR
Massive hepatic necrosis induced by simultaneous blockade of EGFR and MET followed by 2/3 partial hepatectomy.
Massive hepatic necrosis induced by simultaneous blockade of EGFR and MET followed by 2/3 partial heptectomy.
Activated caspase 3 following combined silencing of both HGF and EGF receptors

With hepatectomy

Without hepatectomy
Proapoptotic genes

- Fold Regulation Treated/scr control

- Proapoptotic genes: Bak1, Bax, Bok, Bid, Bad, Casp3, Casp9, Fas, Tp53, Apaf1

- Treatments: shMet, shEGFR, shMet+EGFR, shScr
Hepatic Failure: A disequilibrium between Growth Factors and Inflammatory Cytokines?

- During regular liver regeneration, the simultaneous influence of growth factors and inflammatory cytokines (e.g. TNF) assures that the effect of the latter will result in enhanced mitogenic stimulus instead of an apoptotic one.

- When the effect of growth factors is blocked, the effect of inflammatory cytokines is diverted from pro-mitogenic (e.g. activation of NF-kB) to pro-apoptotic, leading to dysregulated cell function or cell death.

- Depletion of the levels of HGF receptor MET leads directly to increased availability of pro-apoptotic receptors (Fas).

- Fulminant hepatic failure, mediated by massive hepatocyte apoptosis, may be due to blockade of the growth factor receptor levels simultaneous to an increase in levels of inflammatory cytokines.

- Such growth factor receptor “blockade” could be due to receptor depletion because of lack of new receptor synthesis against an increase in receptor degradation.
What terminates liver Regeneration?

Candidate signals:

1. **TGF beta 1**: The protein inhibits hepatocyte proliferation in culture and delays regeneration in vivo. Transgenic expression of TGFβ1 in hepatocytes does not block regeneration. Deletion of TGF beta receptor 1 does not alter termination of regeneration (work by S. Thorgeirsson et al.).

2. **Activin**. Mitoinhibitory to hepatocytes in cultures

3. **Extracellular Matrix**. In hepatocyte cultures it maintains cell differentiation and inhibits cell proliferation. ECM is degraded early in regeneration and re-synthesized at the end of regeneration.
Extracellular Matrix and Hepatocyte Proliferation and Differentiation

- Hepatocytes in primary culture lose their characteristic gene expression patterns. They can be stimulated to proliferate under the influence of HGF and/or EGF.
- Addition of artificial extracellular matrix to hepatocytes in culture (e.g. Matrigel, Type I collagen gels) restores full differentiation and inhibits hepatocyte proliferation.
- Matrix breakdown and reconstitution are essential components of the processes associated with liver regeneration after partial hepatectomy.
- QUESTION: What is the role of hepatic extracellular matrix and the associated signaling through integrins in maintenance and regulation of hepatocyte proliferation and differentiation in the setting of an intact liver?
- STUDY: Eliminate matrix induced signaling from hepatocytes by liver-targeted genetic elimination of Integrin Linked Kinase. ILK $\text{loxP/loxP}$ mice were either treated with Adenovirus-Cre or mated with mice expressing Cre recombinase under hepatocyte specific promoters (AFP enhancer/Albumin promoter).
Injection of Adeno-Cre in mice with ILK^{loxP/loxP} results in massive hepatocyte apoptosis and necrosis (fulminant hepatitis)

**beta-gal**  **Cre**
Crossing of the ILK-Floxed mice with the Foxa3 Cre, AFP-albumin Cre, albumin-Cre mice yields conditional knock-out of ILK in the liver at different stages of development.
Hepatocyte Proliferation and Apoptosis

Proliferation of HNF1 pos. biliary epithelial cells in ILK-KO (Liver) at 16 weeks

Proliferation of stellate cells in ILK-KO (Liver) at 16 weeks
Increased Liver weight in ILK-KO mice due to higher proliferation

**PCNA Positive Cells**

- ILK-/-
- ILK +/-

**TUNNEL Positive Hepatocytes**

- ILK-/-
- ILK +/-

**Mitoses**

**Liver to body weight ratio**

- WT
- KO
Enhanced hepatocyte proliferation and loss of differentiation at the early stages of ILK removal resembles hepatocytes in culture in the absence of matrix.
PCNA positive cells at 10 Days after hepatectomy
Alterations in signaling pathways associated with excessive regeneration
Expression of the top 150 hepatocyte-associated genes in control and ILK-KO-Liver mice after partial hepatectomy
Is Glypican3 a “terminator” for liver regeneration?

GPC3 is highly up-regulated in Hepatocellular Carcinoma (HCC) and hepatoblastoma, but not in normal liver or tissue adjacent to tumors.

Simpson-Golabi-Behmel (SGB) Syndrome

Introduction

X-linked disorder

Pre- and post-natal organ overgrowth

Increased risk of embryonic tumors during early childhood

Numerous visceral and skeletal anomalies

Caused by loss-of-function mutation in GPC3 gene

http://outlook.wustl.edu/winter2002/overgrowth.html
Is Glypican3 a “terminator” for liver regeneration?

GPC3 RNA and Protein Levels Change During Liver Regeneration

A

B

RT-PCR

C

Western
Glypican 3 increases in culture as hepatocytes stop replicating. Silencing RNA against Glypican 3 enhances hepatocyte proliferation.
Partners of GPC3 revealed by yeast-two-hybrid assay

**CD81**

**Other names:** TAPA1 (target of anti-proliferative antibody 1!!!); S5.7; TSPAN28.

**Location:** chromosome 11p15.5

**Expression:** widely expressed except for red blood cells and platelets.

**Transcription:** 17 kb DNA; 8 exons; 20 kb cDNA.

**Protein:** 236 amino acids; 26 kDa protein.

**Localization:** cell surface tetraspanin protein

**Function:** involved in many biological pathways and responses. plays a critical role in HCV attachment and/or cell entry by interacting with HCV E1/E2 glycoproteins heterodimer.
Co-immunofluorescence of GPC3 and CD81 at different times after partial hepatectomy.
Supporting evidence 2:

**HGF and EGF induce expression of TGFβ1 in organoid cultures**

**Figure 8.** Expression of albumin, TGF-1 and collagen type IV in cultures at different days, maintained in the presence of either HGF or EGF or both. Control cultures had neither HGF nor EGF supplementation. Hepatocyte pellet isolated at the end of collagenase perfusion as well as whole normal rat liver tissue (NRL) were also examined for comparison. Analysis of extracted RNA was conducted by Northern gels. The upper GAPDH is used as a normalizing control for albumin and TGF-1 whereas the lower GAPDH was used for the normalization of the data on collagen type IV, because the corresponding RNA were run on two separate gels. EGF was a stronger inducer of both TGF-1 and collagen type IV at day 8, compared to HGF.
Matrix-derived HGF and duodenum-derived EGF.

Stellate Cells

Enhanced production of TGFβ1.

Synthesis of extracellular matrix including GPC3 and proteins with similar effects.

Enhanced binding and inactivation of HGF and TGFβ1 in pericellular matrix surrounding hepatocytes.

ECM derived Integrin and ILK mediated signaling to hepatocytes

Hepatocytes return to G0

Synthesis of new HGF

Inhibition of expression of urokinase

Feedback loop between mitogens HGF and EGF, cytokine TGFβ1 and ECM leading to initiation and termination of liver regeneration

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- Shirish Paranjpe: Research Associate; HGF and Met silencing
- Bowen Liu: Graduate Student; Glypican 3
- Shashi Donthamsetty: Postdoctoral fellow; ILK Liver knockout
- Vishakha Bhave: Postdoctoral fellow; Hepatocytes as stem cells
- Ann Orr: Histologist
- Callie Norris: Research Technician
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Independent Collaborators

- Wendy Mars: IL6 synthesis, NFkB and HGF
- Paul Monga: beta Catenin
- Reza Zarnegar: Met and Fas
- Aaron Bell: Hepatocyte transcription factors, Matrix, PB and HNF4
- Cary Wu: Integrin Linked Kinase
- Jianhua Luo: HCC Gene Expression and Genomics
- George Tseng: Biostatistics
- A. Jake Demetris: Liver Pathology
- Mike Nalesnik and Erin Ochoa: hepatocyte < >biliary transdifferentiation
- Steve Strom: Human Hepatocytes
- Yuhua Liu: HGF Plasmids
- Marie C. DeFrances: Liver Regeneration
TGFβ1 levels in plasma after PHx.