MICROGRAVITY MAINTAINS STEMNESS AND ENHANCES GLYCOLYTIC METABOLISM IN HUMAN HEPATIC AND BILIARY TREE STEM/PROGENITOR CELLS.

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The global burden of chronic liver diseases

Total deaths worldwide from cirrhosis and liver cancer rose by 50 million per year over 2 decades,

according to the first-ever for World Health Organization (WHO) study of liver disease mortality
The main causes of chronic liver diseases

Viral:  HCV, HBV, HDV
Alcol:  
NAFLD: non alcoholic fatty liver diseases  (Metabolic Syndrome)!
HBV prevalence in European population!

Very effective treatment! (entecavir, tenofovir...)

HCV prevalence in european population!

Very effective treatment! (DAA)
Alcol and liver diseases

N. of pts. consuming doses of alcol responsible of progressive liver diseases.

Unresolved problem!
Fatty Liver
The epidemic of obesity in western countries

Unresolved problem!
Etiology of HCC in Italy: observed and expected temporal trends (23 centres: 1733 HCC)
NAFLD: natural history

- Steatosis
- SteatoHepatitis
- SteatoHepatitis+fibrosis

Steatosis to SteatoHepatitis: 10-15%
SteatoHepatitis to Cirrhosis: 30-35%
Cirrhosis to Cirrhosis+hepatocellular carcinoma (HCC): 10-15%
LIVER CIRRHOSIS: CLINICAL FEATURES

COMPENSATED CIRRHOSIS

DECOMPENSATED CIRRHOSIS:
- Ascitis and (PBS, HRS..)
- Bleeding (varices.....)
- Hepatic Hencefalophaty
- Portal Trombosis
- HCC
The treatment of chronic liver diseases

Viral: DAA, anti-HBV (very effective!)

Alcol: stop drinking!?

NAFLD: No effective treatment!

Liver Transplantation

the only option for untreatable liver diseases!
Liver Transplantation (OLT)

However ............... 

--The number of donated livers is limited !
--OLT can OLT in Italy, year 2015 be very adv very adv 
contrain contrain 
--Post-surgery complications and rejection are still significant 
--High costs: typically ~$150,000 to $180,000 for transplant and first-year medical follow-up !

Fig. 1. Primary diseases leading to liver transplantation in Europe (01/1988–12/2011) [40]. *Others: Budd-Chiari: 792, Bening liver tumours or polycystic diseases: 1228, Parasitic diseases: 80, Other liver diseases: 1304.
Alternative to OLT

CELL THERAPY and LIVER DISEASES

The cell therapy for treatment of liver
diseases is the object of extensive
investigations but,

the _ideal cell sources_ still represent an
unresolved issue!
Cell Sources:
Adult Hepatocytes

Stem/progenitor-cells:
1. Fetal stem cells
2. Adult hepatic stem cells
3. Mesenchymal stem cells
4. Amniotic fluid-derived stem cells
5. Induced pluripotent stem cells (iPS)
**The biliary tree—a reservoir of multipotent stem cells**

**Alvaro and Gaudio Hepatology 2016**

**The Human Biliary Tree Stem Cells (hBTSCs)**

**PBGs in Hepato-pancreatic ampulla**
- Pluripotency genes +
- Hepato-biliary markers +
- Pancreatic markers +

**PBGs in Intra-Hepatic Bile Ducts**
- Pluripotency genes +
- Hepato-biliary markers +++
- Pancreatic markers -

**PBGs in Pancreatic Ducts**
- Pluripotency genes -
- Hepato-biliary markers -
- Pancreatic markers +++

**Radial axis of maturation**
- Proximal to distal axis
- 1, 4, 5

**Mature cells**
- Primary cilium, CFTR, SR
- Mucin
- Insulin-producing cells

**Transit-amplifying cells**
- SOX9, SOX17, EpCAM, LGR5

**BTSCs/GSCs**
- SOX9, SOX17, PDX1, EpCAM, CD133, LGR5, SALL4, Pluripotency

**PBGs**
- BTSC niche
- hBTSCs

**Radial axis of maturation**
- 2, 1
1. Wide availability and easy supplying (specially from extrahepatic sources)

2. *in vitro*: sufficient amount of cells for transplantation

human biliary tree stem cells (hBTSCs)
Adult hBTSCs: Multipotency in vitro

Differentiation towards

Hepatocytes

Pancreatic islets

Cholangiocytes

Phenotype and functions were evaluated under **conditioned media** for hepatocytes, cholangiocytes or pancreatic islets. Transfer into differentiation conditions resulted in distinct mature fates.

Cardinale V, Wang Y, ..., Gaudio E, Alvaro D, Reid L. Hepatology. 2011
Fetal liver (18th-22nd week) or marginal livers

Liver + Extrahepatic Biliary tree + Gallbladder

Filtering up to 30 µm

Mechanical dissection

Digestion buffer: Type I Collagenase for 20-30 min at 37°C

Human Ab anti-EpCAM

EpCAM- cells

EpCAM+ cells

FLOW CYTOMETRY ANALYSIS

MICROBIOLOGICAL TESTS
Phase I/II study on advanced cirrhosis transplanted with fetal hBTSCs. 2° Paz.

Pz. A.P., 71 yrs, Cirrhosis-HCV,  Child-Pugh 12, Meld 21

60 millions freshly isolated fetal BTScs, EpCAM+ (50% Lgr5+) injected via hepatic artery

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<tr>
<th></th>
<th>0’</th>
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<td>Child-Pugh score</td>
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<td>10</td>
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<tr>
<td>Meld score</td>
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<td>17</td>
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<tr>
<td>Bilirubin</td>
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<td>2.08 mg/dl</td>
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<tr>
<td>Albumin</td>
<td>3.2</td>
<td>3.44 g/dl</td>
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<td>INR</td>
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<tr>
<td>Creatinine</td>
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However ..... 

Only 7-10% of cells infused as suspension engraft the liver!

How to improve the benefits of hBTSC transplantation?
Ongoing projects:

1. Enhance the engraftment efficiency by infusing tridimensional cell clusters!
2. Cryopreservation of hBTSCs and HA-hBTSCs, the generation of a cell bank;
3. Identify additional subpopulations of hBTSCs possessing the properties of the ideal stem cell and suitable for clinical programs?
Organoids: A New Paradigm for Organ Modeling, Drug Efficacy Testing, and Organ Replacement Therapy

Madeline A. L. Hertogs, Laura C. M. Koning, and Megan J. E. M. van Dijk

Science 2014
Tridimensional cultures and organoids

Model systems in life sciences
- Monolayer cell culture
- Spheroid
- Organoid
- Tissue explant
- Multiplexed models “on-a-chip”

Bioengeneering strategies to obtain 3D cultures
- In vitro specific conditions
- Bioprinting
- Biomimetic scaffolds
- Microfluid devices
- Microgravity based

Complexity of culture

Biochemical tools

Stem Cell Organoid Engineering, Yin et al. (2016)

Thyroid Organoid Formation in Simulated Microgravity: Influence of Keratinocyte Growth Factor

To cite this article:

Published in Volume: 10 Issue 6: January 30, 2009
Microgravity exerts different effects on the gravity-evolved organisms

- Systemic effects on the human health
- Research focused mainly on musculoskeletal apparatus, cerebellum and cardiovascular system

Alteration of carbohydrate and lipidic metabolism and reduction of the number of macrophages. (Racine et al. 1992)

Changes in the content and activity of CYP450 in rats during spatial flights. (Rabot et al. 2000)
AIMs

1. To evaluate whether microgravity may help the development of tridimensional cultures of human biliary tree stem cells (hBTSCs), to be used for the regenerative medicine of liver diseases and for development of liver devices;

2. To evaluate the effects of microgravity on biological properties and functions of isolated hBTSCs;
Isolation of human Biliary Tree Stem Cells

normogravity and microgravity cultures

The hBTSC were phenotypically characterized (CK-7, CK-19, NCAM, EpCAM, CLDN-3, PTc, CKIT, alpha-fetoprotein, Thy1, albumin), plated and cultured in basal and differentiation medium in both normogravity and microgravity.
Rotary Cell Culture System (RCCS)

• Cell growth with or without solid support (scaffold, microcarrier beads);
• Versatility - more than 50 cell types grown successfully;
• Spontaneous formation of 3D tissue;
• Propagation of mono- and co-cultures;

Cytodex 3 microcarrier

10-30 rpm: 10,000 fold less than g
Simulated microgravity favors development of tridimensional cultures of hBTSCs and HepG2 cells. Cluster Ø: 350 µm - 787.5 µm. β-actin staining at 40x and 20x magnification.
Tridimensional cultures in microgravity conditions and organoid-based technologies for research and medicine applications

- Disease modeling
- Drug efficacy testing
- Drug safety testing
- Disease modeling

HepG2
+Matrigel
+0.1% HA

Cells were able to readhere on plates to form colonies

![Graph showing organoid diameter (µm) over time (5 days, 7 days, 10 days, 12 days)]
Expression of Stem Cell Markers in human Biliary Tree Stem Cells cultured in hepatocyte differentiation medium, normogravity

Gravity

KM = Kubota’s medium (basal medium)
HM = Hepatocyte Differentiation Medium

Normogravity downregulated Stem Cell Markers in hBTSCs cultured in hepatocyte differentiation medium.
Expression of Stem Cell Markers in human Biliary Tree Stem Cells cultured in hepatocyte differentiation medium, microgravity

The main hBTSCs stem cell markers were surprisingly upregulated when cultured in hepatocyte differentiation medium in microgravity conditions.
Expression of typical genes of mature hepatocytes in human Biliary Tree Stem Cells cultured in hepatocyte differentiation media

**Normogravity vs Microgravity** (14 days in culture)

The expression of “mature hepatocytes” genes was significantly downregulated in microgravity conditions!
Expression of stemness genes and genes of “mature hepatocytes” in HepG2 cells

Normogravity vs Microgravity (14 days in culture)

Important stemness marker were upregulated in HepG2 cultures in microgravity compared to normogravity

Alb was upregulated - but not Cyp3A4 - in HepG2 cultures in microgravity compared to normogravity
**hBSTC: exometabolome analyses**

by Nuclear Magnetic Resonance

- **Microgravity:** no significant differences between the metabolisms of hBSTCs in basal vs differentiation medium;
- when compared with normogravity, hBTSCs in microgravity consumed more glucose and produced more lactate, acetate, glutamate;

![OPLS analysis](image)

- **1H-NMR spectrum**

**Area of metabolites (a.u.)**

- **Normogravity**
- **Microgravity**

<table>
<thead>
<tr>
<th>Beta-hydroxybutyrate</th>
<th>Lactate</th>
<th>Alanine</th>
<th>Acetate</th>
<th>Glutamate</th>
<th>Pyruvate</th>
<th>Glucose</th>
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*p < 0.05*
The metabolism of HepG2 that have been cultured in microgravity were significantly different from the metabolism of HepG2 in normogravity (p<0.05)

HepG2 in microgravity consumed more glucose and released a more amount of fermentation derivatives and glutammate when compared to the cells grown in normogravity.
Differentiation is associated to:
- a development of the mitochondrial network and cristae
- a metabolic shift to prevalent oxidative phosphorylation (OXPHOS)
RESULTS:
Summary

1. Microgravity favors the organization of hBTSCs in tridimensional clusters.

2. Microgravity favors the maintenance of stemness features and counteracts the differentiation of hBTSCs toward mature hepatocytes;

3. The effects of microgravity on hBTSCs are associated with a metabolic shift to glycolysis and to the detriment of OXPHOS.
Perspectives

Regenerative medicine:
--Microgravity could help the generation and maintainance of tridimensional cultures of pluripotent stem cells to be used for regenerative medicine;

Implications:
--Identifying molecular and biologic mechanisms associated with the maintenance of stemness in microgravity could help the identification of putative therapeutic target to modulate stem cell differentiation.
Microgravity maintains stemness and enhances glycolytic metabolism in human hepatic and biliary tree stem/progenitor cells.

Thank you for attention.
The progression of liver diseases

Normal  →  Cirrhosis

Hepatocellular carcinoma