PREDICTOMICS

Short-term in vitro assays for long term toxicity

Contract n° LSHB-CT-2004-504761

A specific targeted research project (STREP) within the VI EU Framework Research Program
(Thematic priority: LifeScieHealth LSH-2002-1.2.3-2)

With the participación of groups from:

Austria, Belgium, France, Germany, Ireland, Italy, The Netherlands, Spain and Switzerland

Clinical relevance of drug organ toxicity

- Liver and kidney are among the most frequently affected organs by compounds (drugs) acting as chronic toxins.
- It is consequence of their active involvement in the metabolism and clearance of xenobiotics.
- Both organs possess a tissue structure resulting in significant gradient concentrations of xenobiotics and, hence, differential toxicity.
- Their functional integrity is essential for the homeostasis, being involved in the regulation of the energetic metabolism, water balance, acid-base balance etc.

End-point parameters and their relationship to the mechanism of toxicity

Toxin →  → 

changes

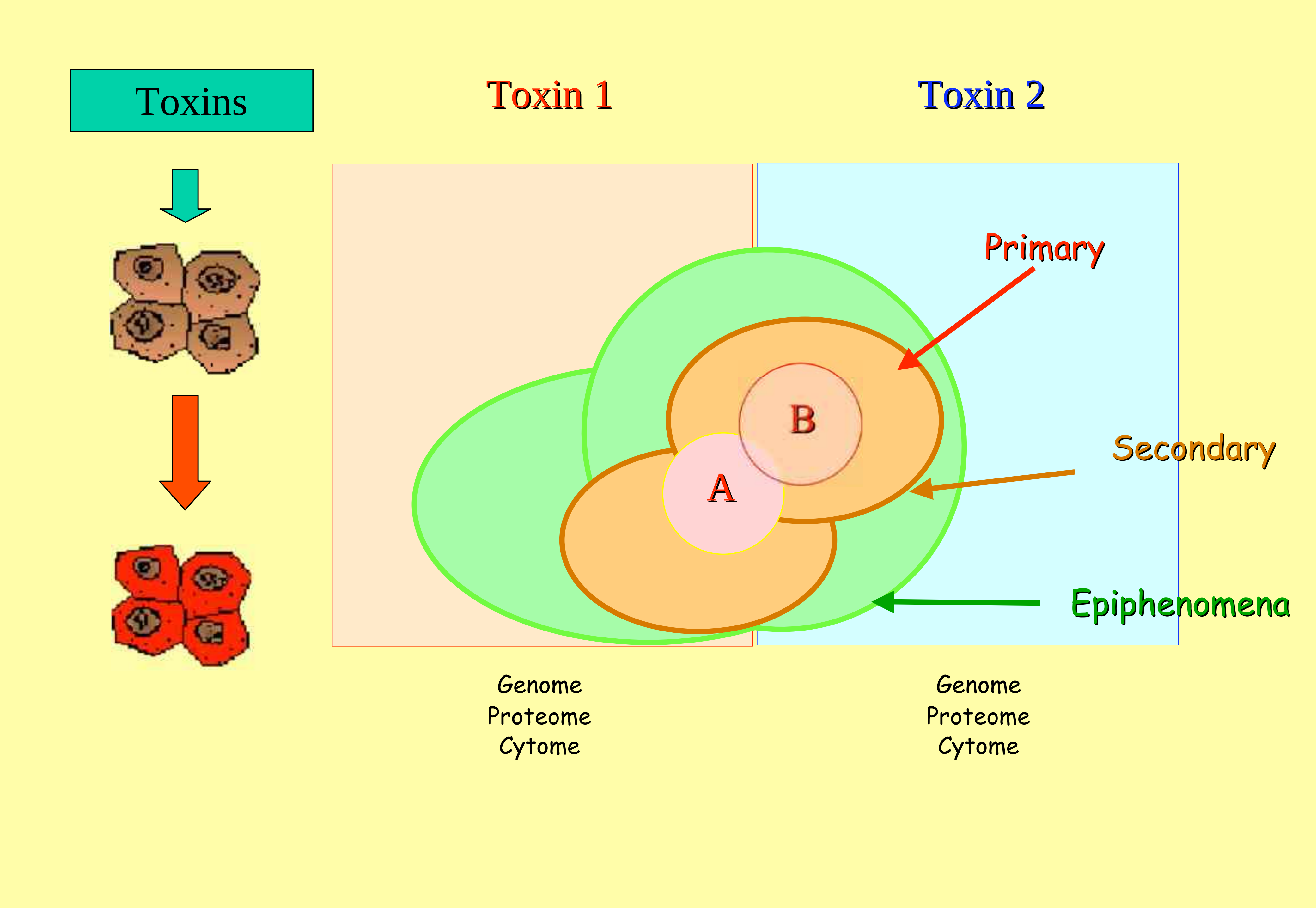
Relationship with the mechanism of toxicity

A) **Directly related to the mechanism of damage:** Functional inhibition of a transporter, inhibition of an enzyme, transcription blocking

B) **Defence against injury:** Changes in gene expression such as SOD, stress proteins, inflammatory response.

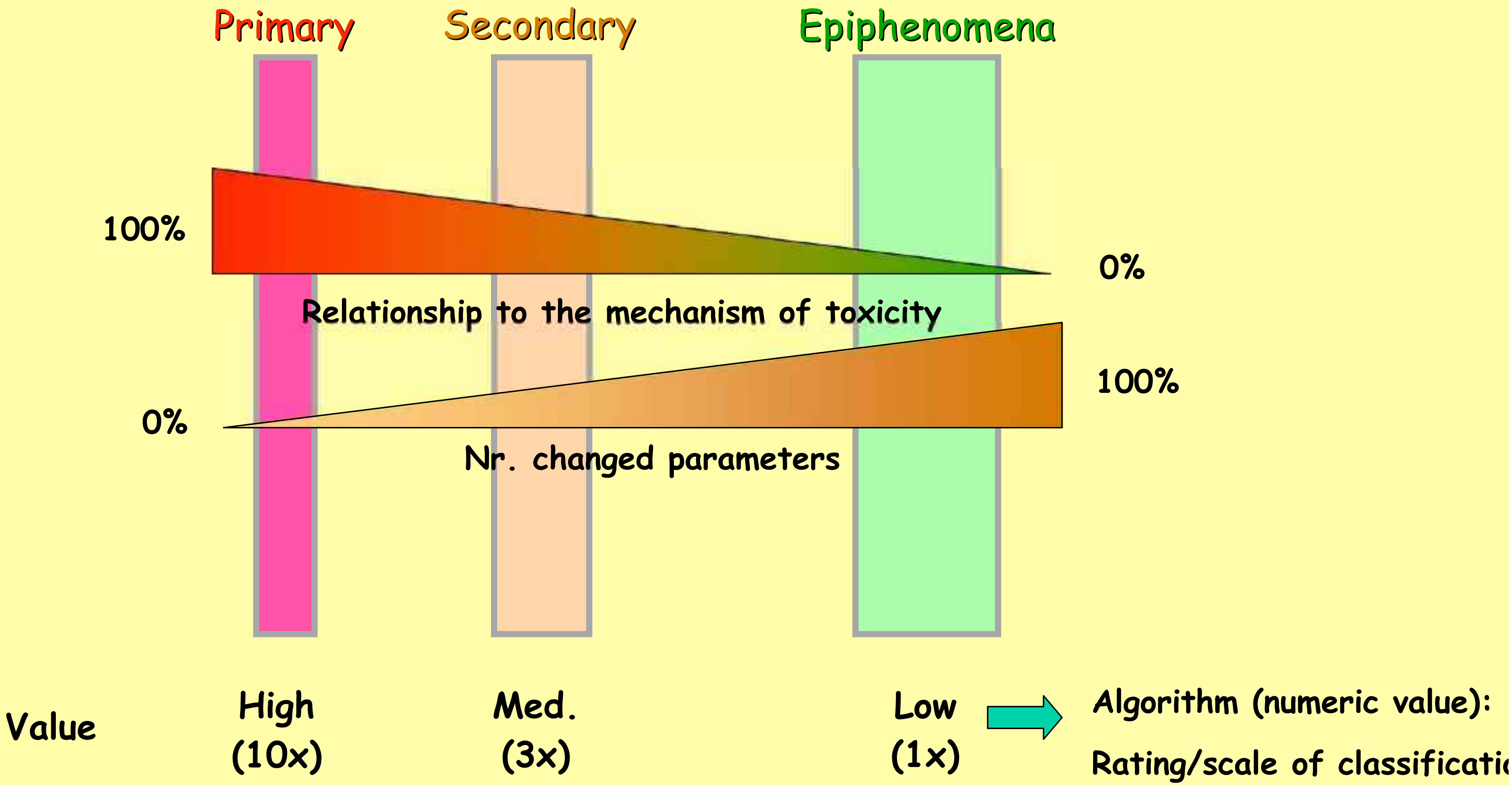
C) **Adaptative metabolic changes:** Re-routing of energetic metabolism, *service* functions.

D) **Epiphenomena:** Changes (in some cases very striking), but without a clear relation to the mechanisms of toxicity.



Toxins
Toxin 1
Toxin 2
Primary
B
A
Secondary
Epiphenomena
Genome
Proteome
Cytome
Genome
Proteome
Cytome

Parameters affected in the course of toxic phenomena and their relevance



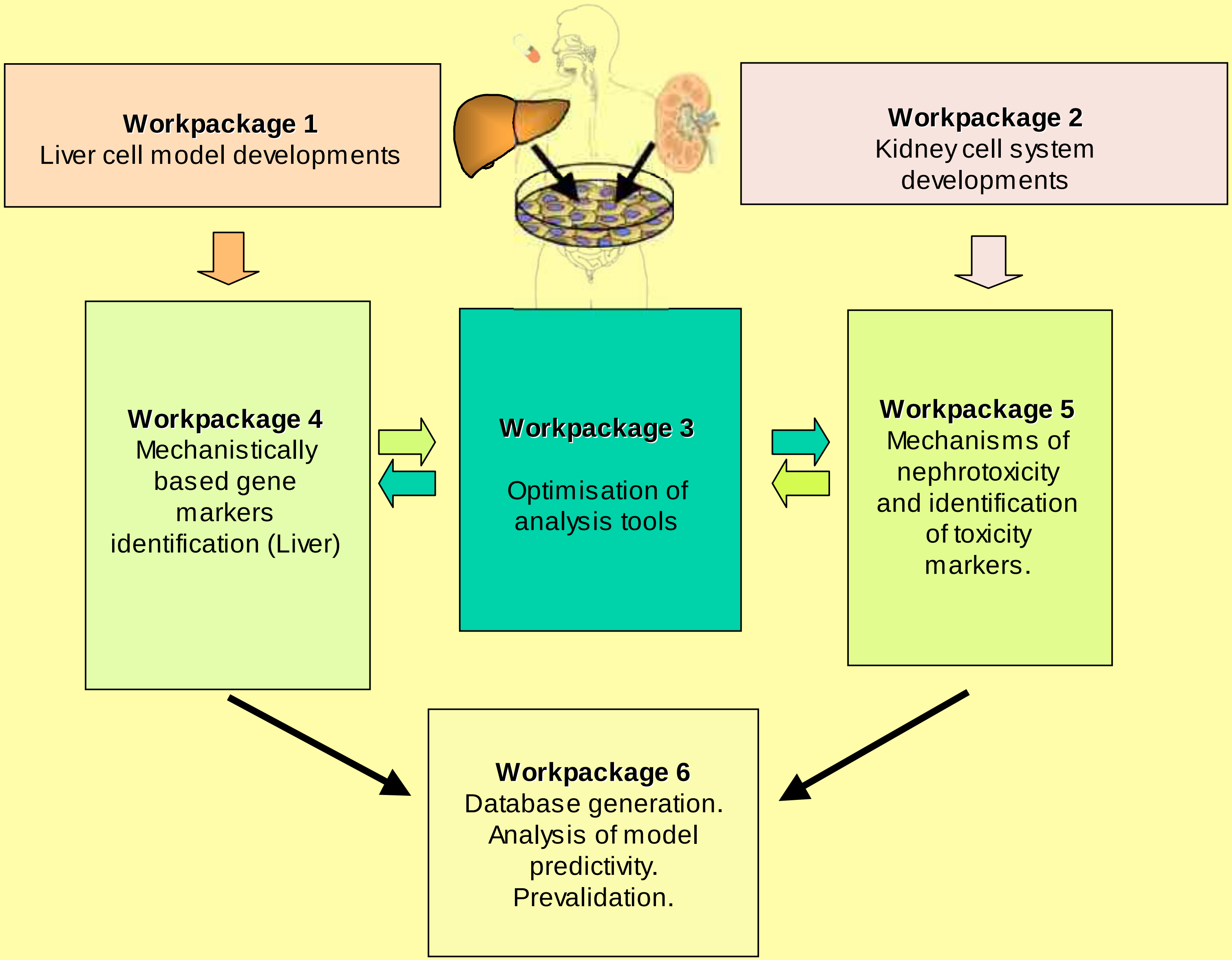
PREDICTOMICS

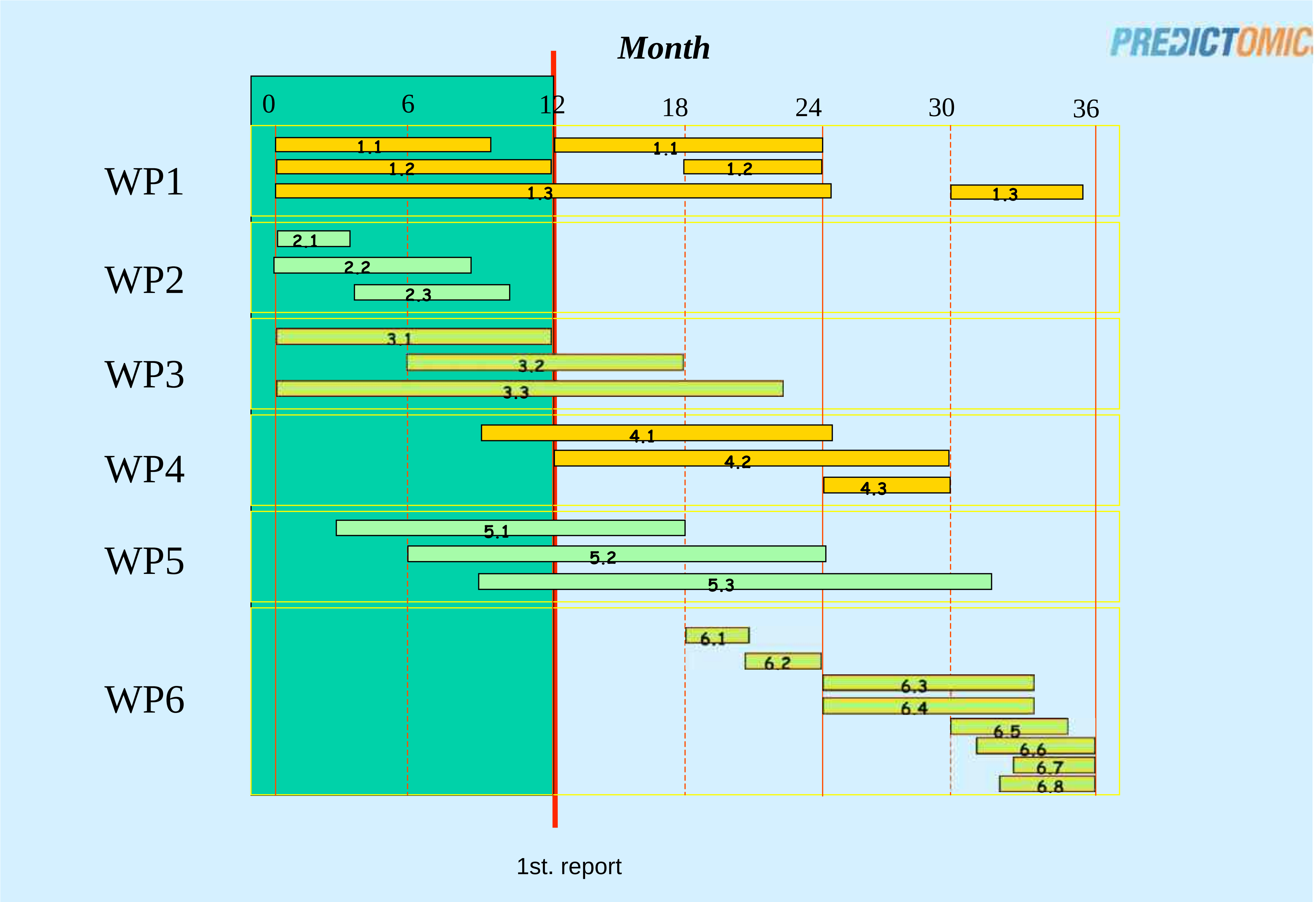
Final objective:

"a rating algorithm to identify potential chronic toxins..."

$$\sum_{1}^{n} P1xV1 + \sum_{1}^{m} P2xV2 + \sum_{1}^{l} P3xV3 = RatingValue$$

PREDICTOMICS




PREDICTOMICS
Month
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WP1
1.1
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WP2
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WP3
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WP4
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WP5
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WP6
6.1
6.2
6.3
6.4
6.5
6.6
6.7
6.8
1st. report

WP 1: *Liver Cell Model Developments*

Objectives:

1. ***To improve phenotype stability in 3D-collagen cultures***. Counteracting dedifferentiation of organotypic 3D-cultures of (rat/human) hepatocytes by molecules acting on chromatin structure and/or by key liver-enriched transcription factors.
2. ***Generation of stable differentiated human hepatocellular cell lines*** by transfection with key liver-enriched transcription factors and nuclear factors
3. ***Generation of functional human hepatocytes through adult stem cell technology***.

Research conducted

Primary aim:

to find a functional long-term hepatocyte culture system

Collagen cultures

transfected HepG2

HDAC-I TSA/Jung-1

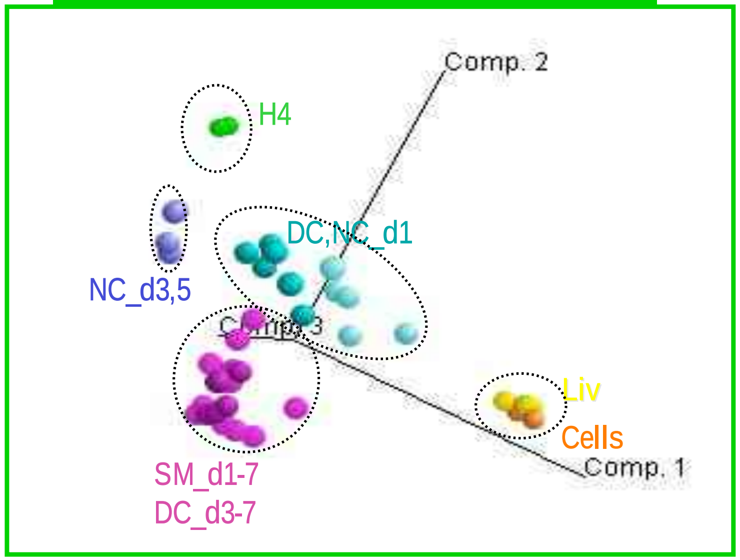
1. Problems with cytomic analyses concerning penetration of substrates (FFA) into the gel (partner 1)
2. Drastic genotypic changes (H.J. Ahr)

Gene expression in in vitro cultures – in vivo (Liver)

Bayer HealthCare - H.J. Ahr

Temporal stability of hepatocyte culture models

PCA - RG_U34A - QS p0.06



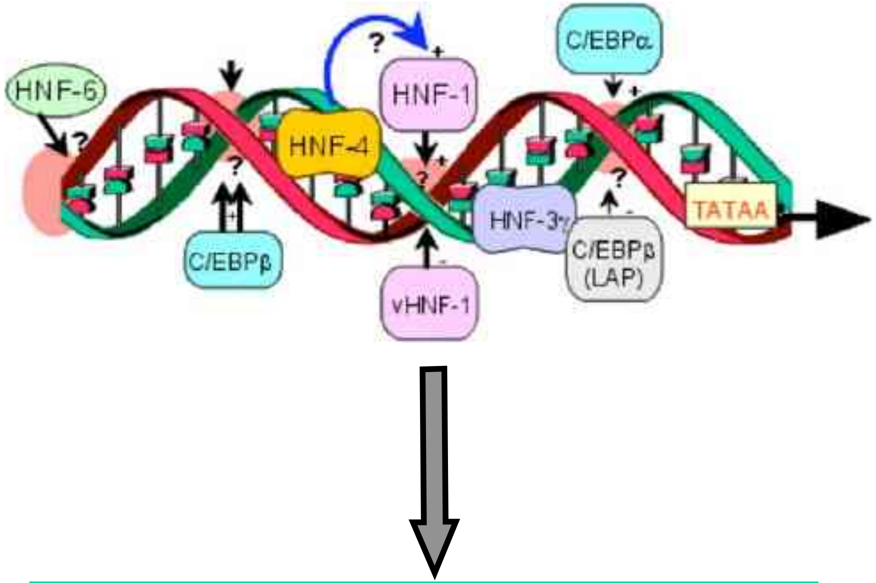
Analysis of gene expression profile (present genes) in rat hepatocytes:

- **liver in vivo**
- **freshly isolated hepatocytes (cells)**
- **culture on uncoated plates (NC)**
- **dry collagen culture (DC)**
- **sandwich culture (SM)**
- **H4 hepatoma cells**

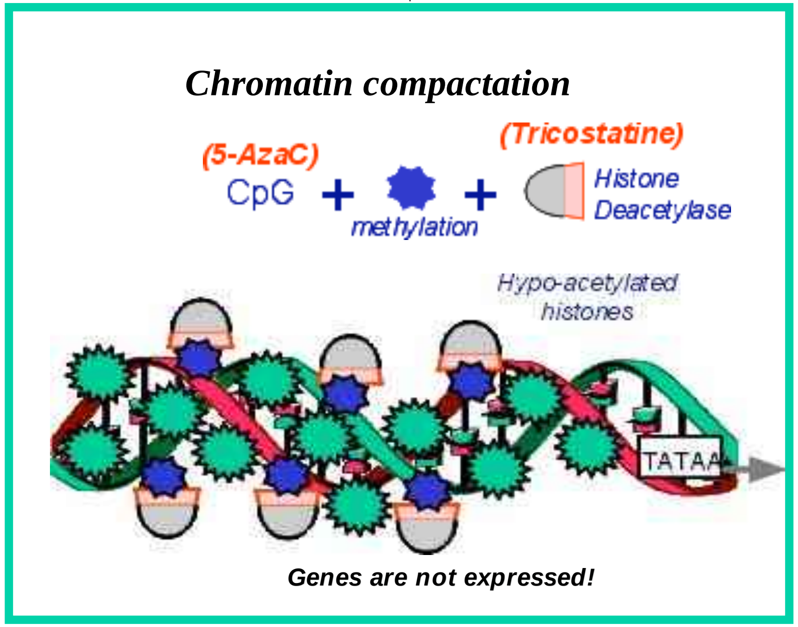
Some culture models tend to *"stabilize"* , but on a very different expression pattern as compared to the liver in vivo

- **Explore possibility of 1 layer dry collagen cultures**

Rationale

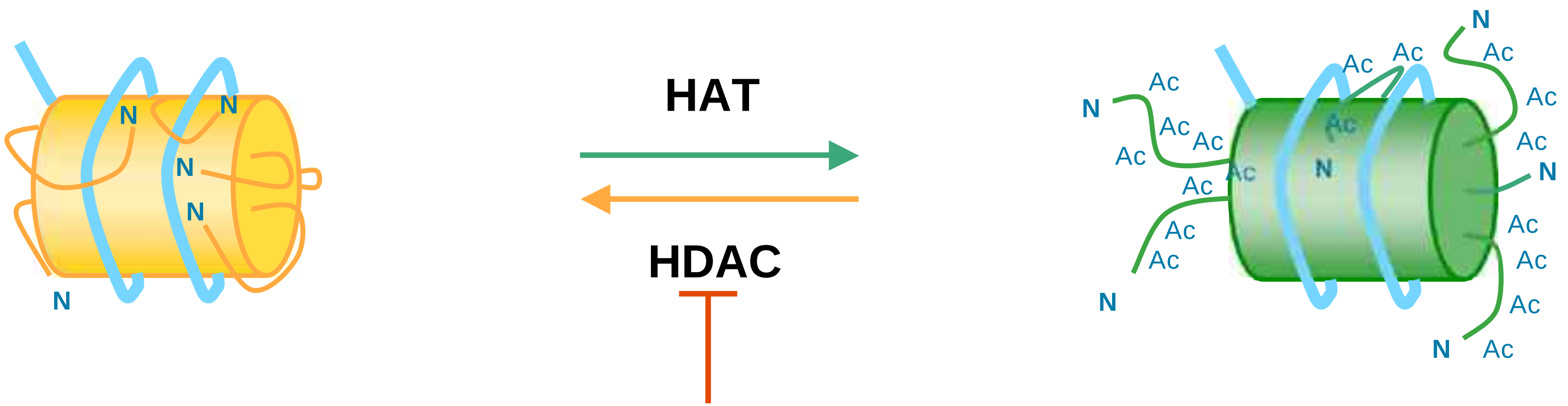


LIVER



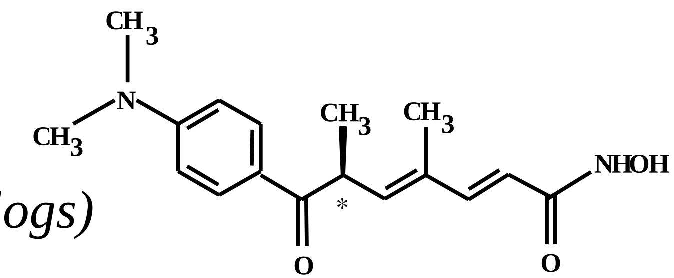
Deacetylation of histones results in a greater compactation of chromatin and gene repression

➢**Strategy:**



Transcriptional repression HDAC-I **Transcriptional activation**

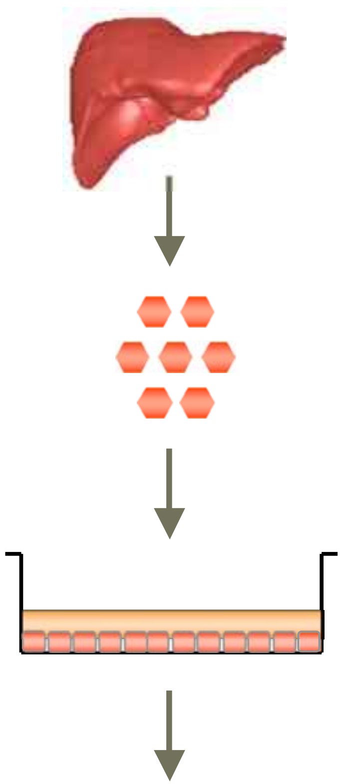
TSA
(and analogs)



HDAC-I Treatment – Experimental Setup

Time of onset of treatment:

+ 1 µM TSA		- TSA
C2	C1	C0



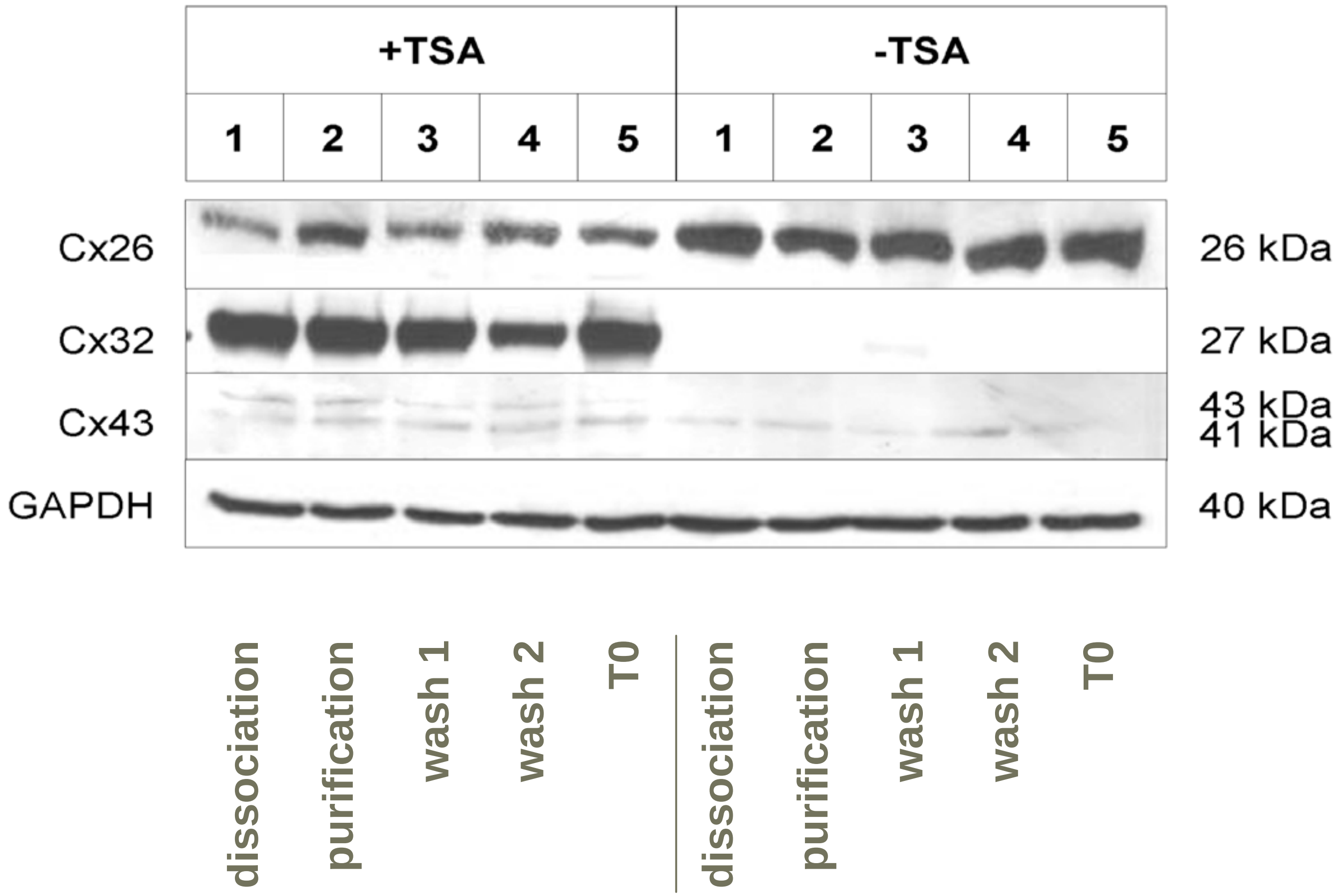
ISOLATION:
two-step collagenase perfusion

CULTIVATION:
Cp, DMEM
(1 week)

SAMPLES:
days 1, 4 and 7
(D1, D4, D7)

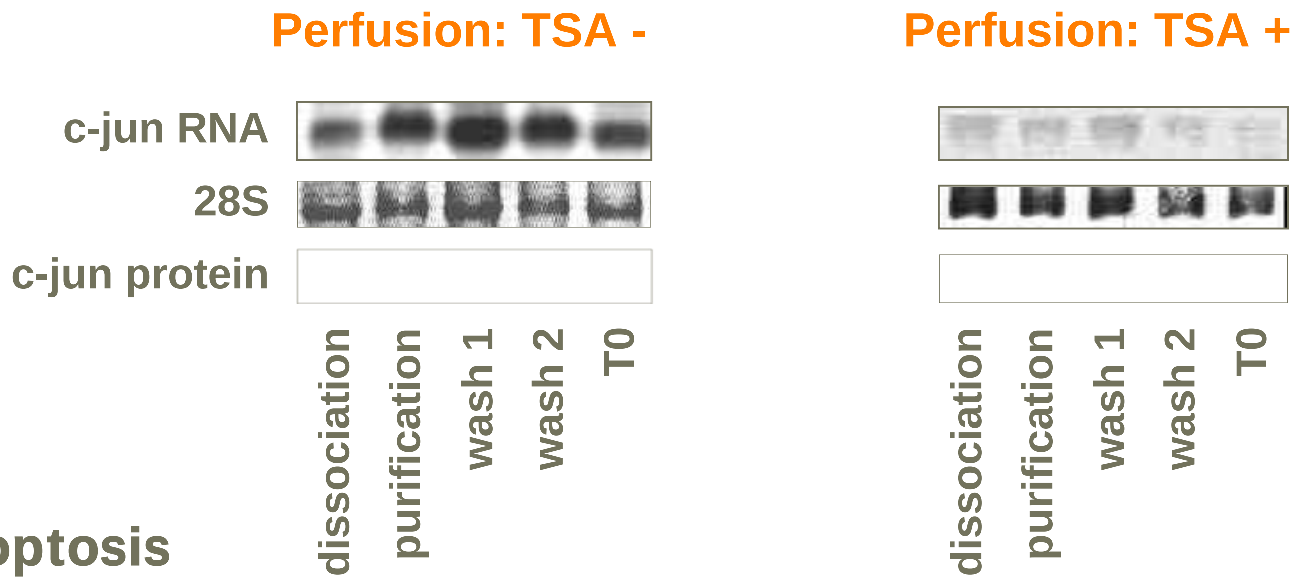
HDAC-I Treatment – Mechanisms Beyond

1. Connexin expression during hepatocyte isolation

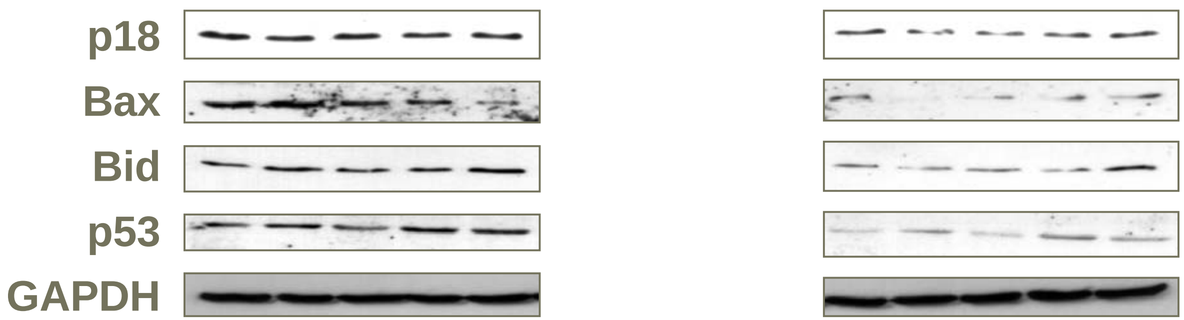


HDAC-I Treatment – Mechanisms Beyond

2. *c-jun* expression during hepatocyte isolation

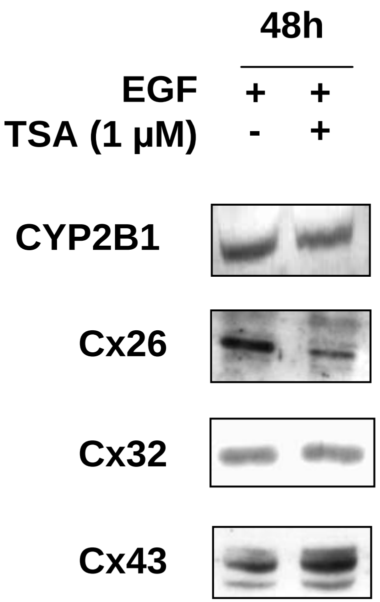


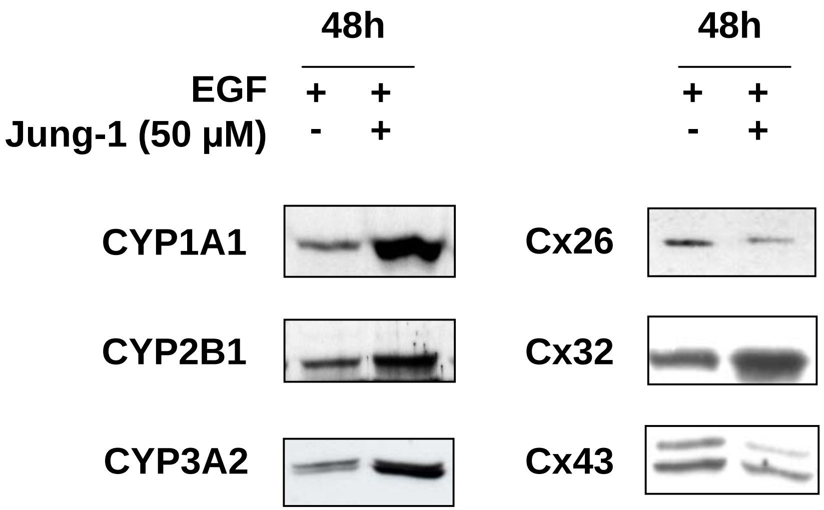
3. Apoptosis



Choice of HDAC-I: Jung-1

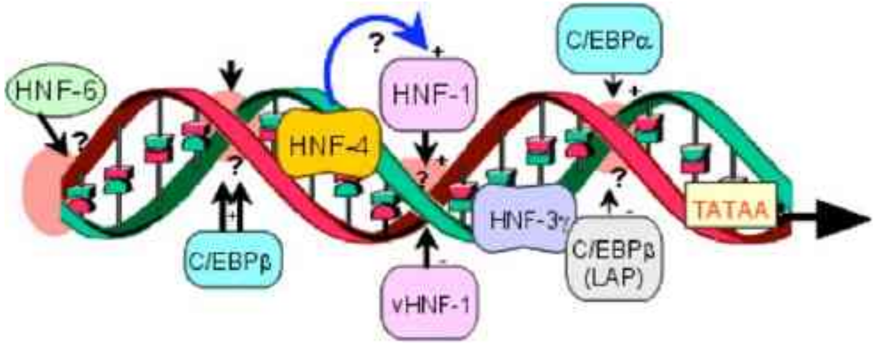
Connexin and CYP expression





Jung-1 > TSA

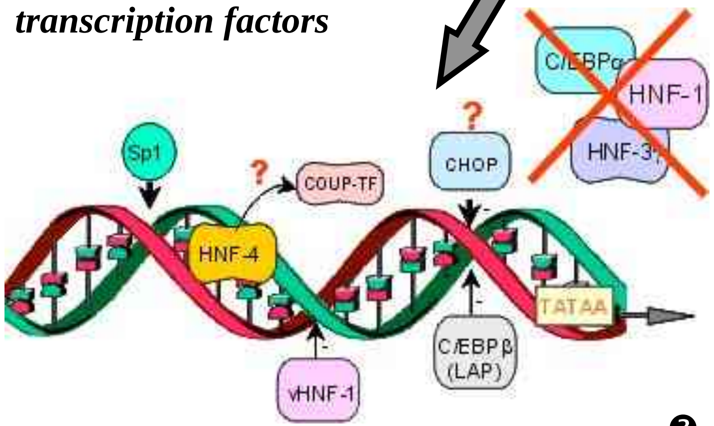
Rationale



LIVER

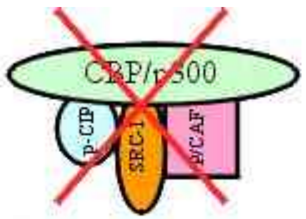
Hepatic cell lines

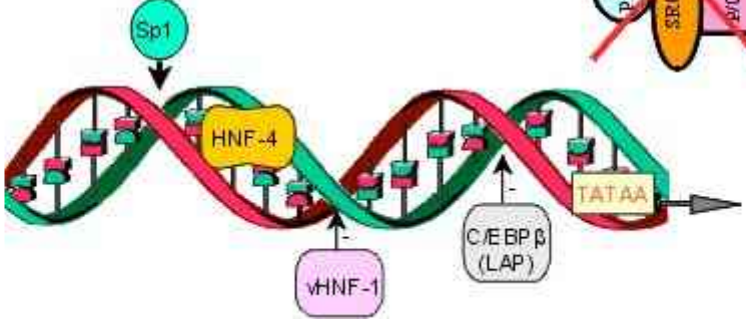
Œ *Lack of expression and/or non-functional transcription factors*



Genes are not expressed!

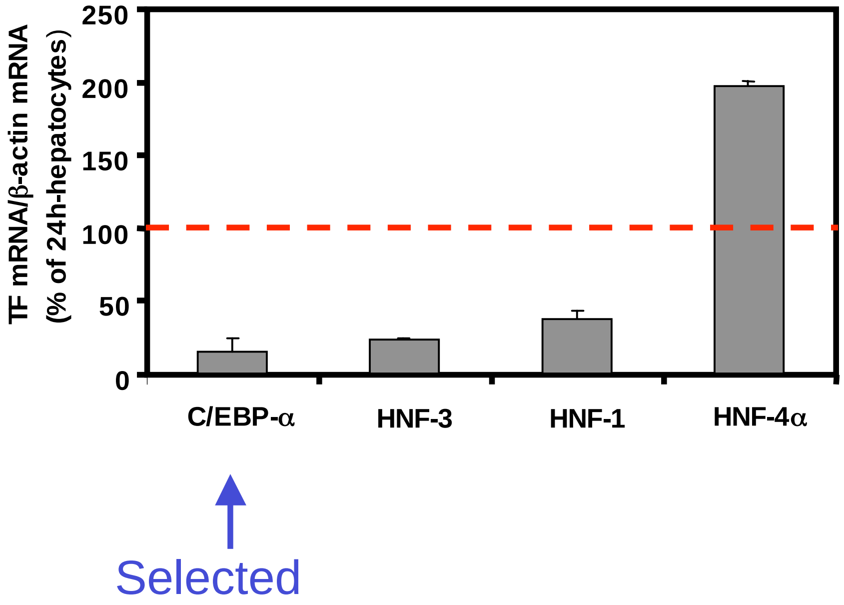
❸ *Altered expression of key coregulators*

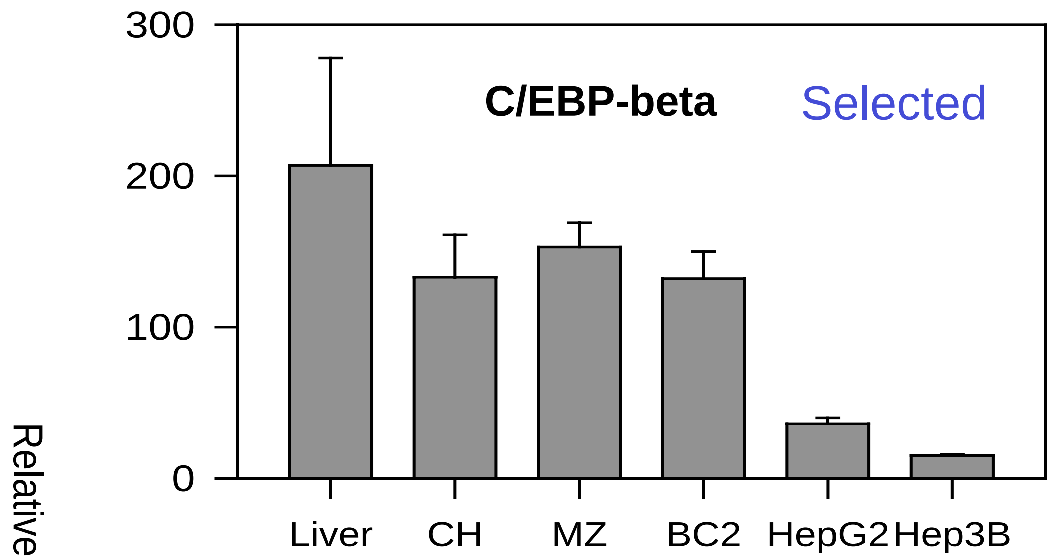


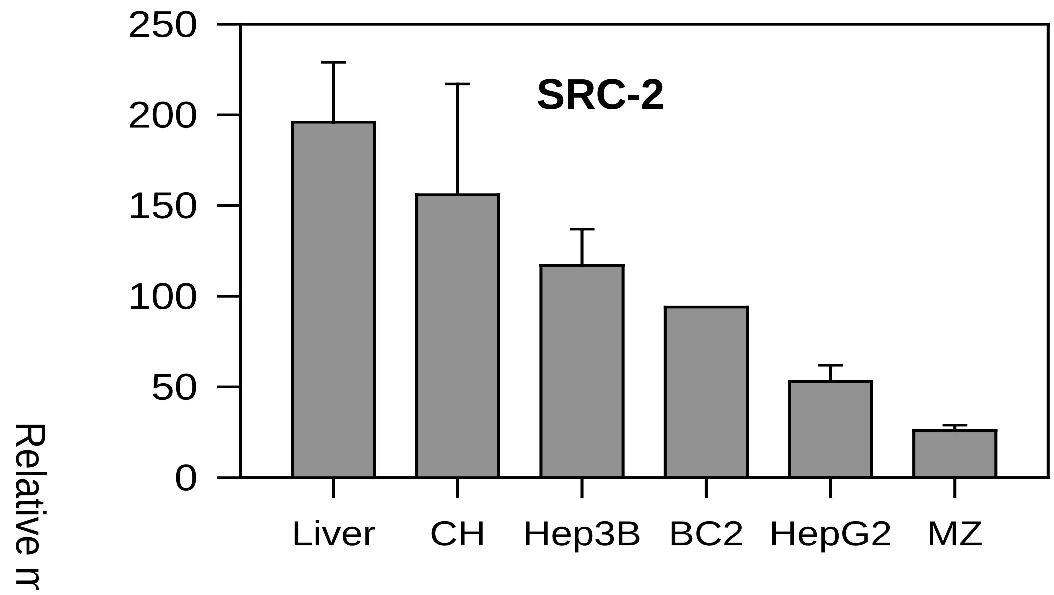


Genes are not expressed!

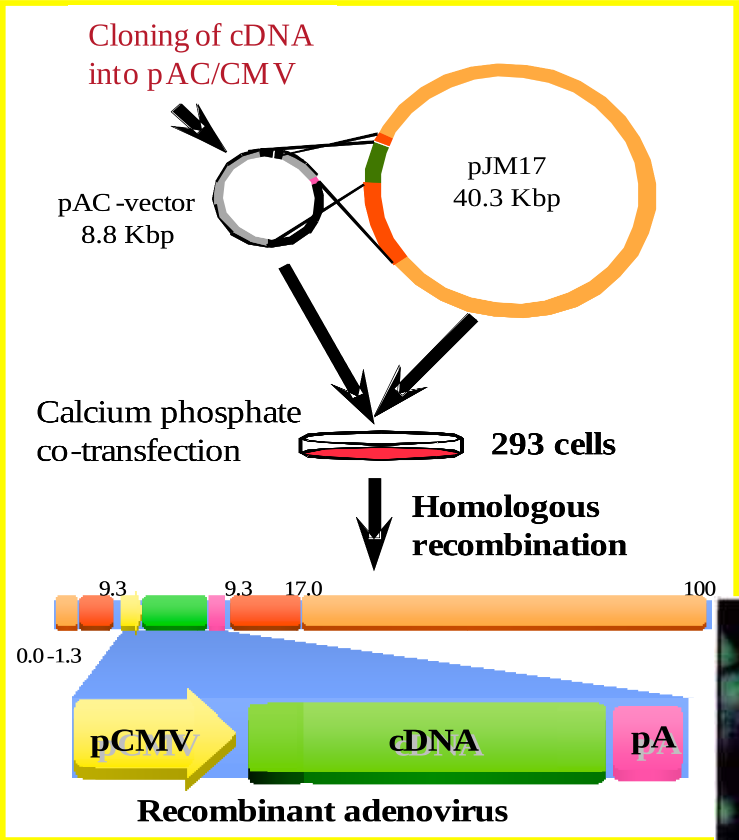
Activatinf factors needed for hepatocytes to express their adult phenotype, which are lacking in hepatoma of de-differentiated cells





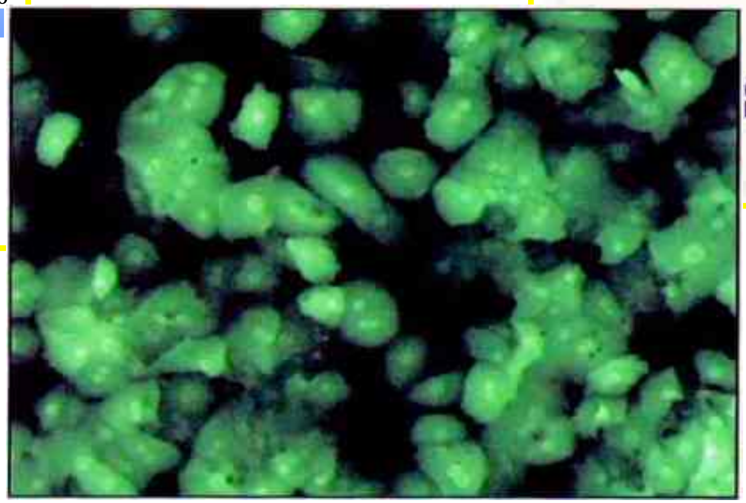


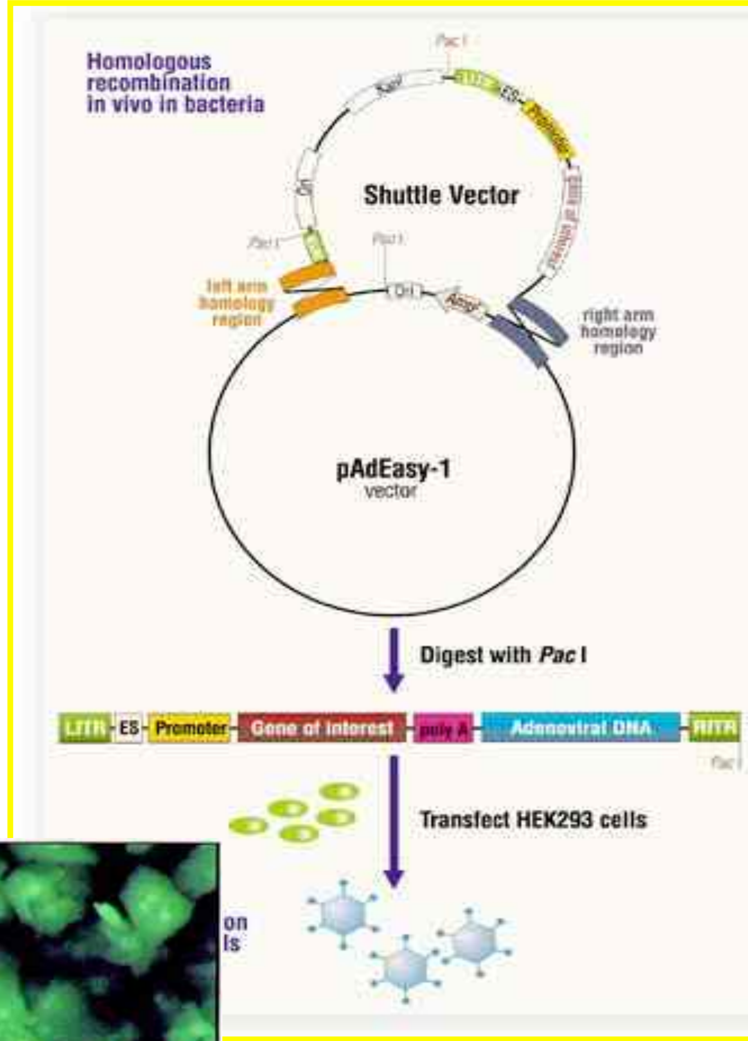
Generation of recombinant adenoviruses for highly efficient transfection of cultured cells



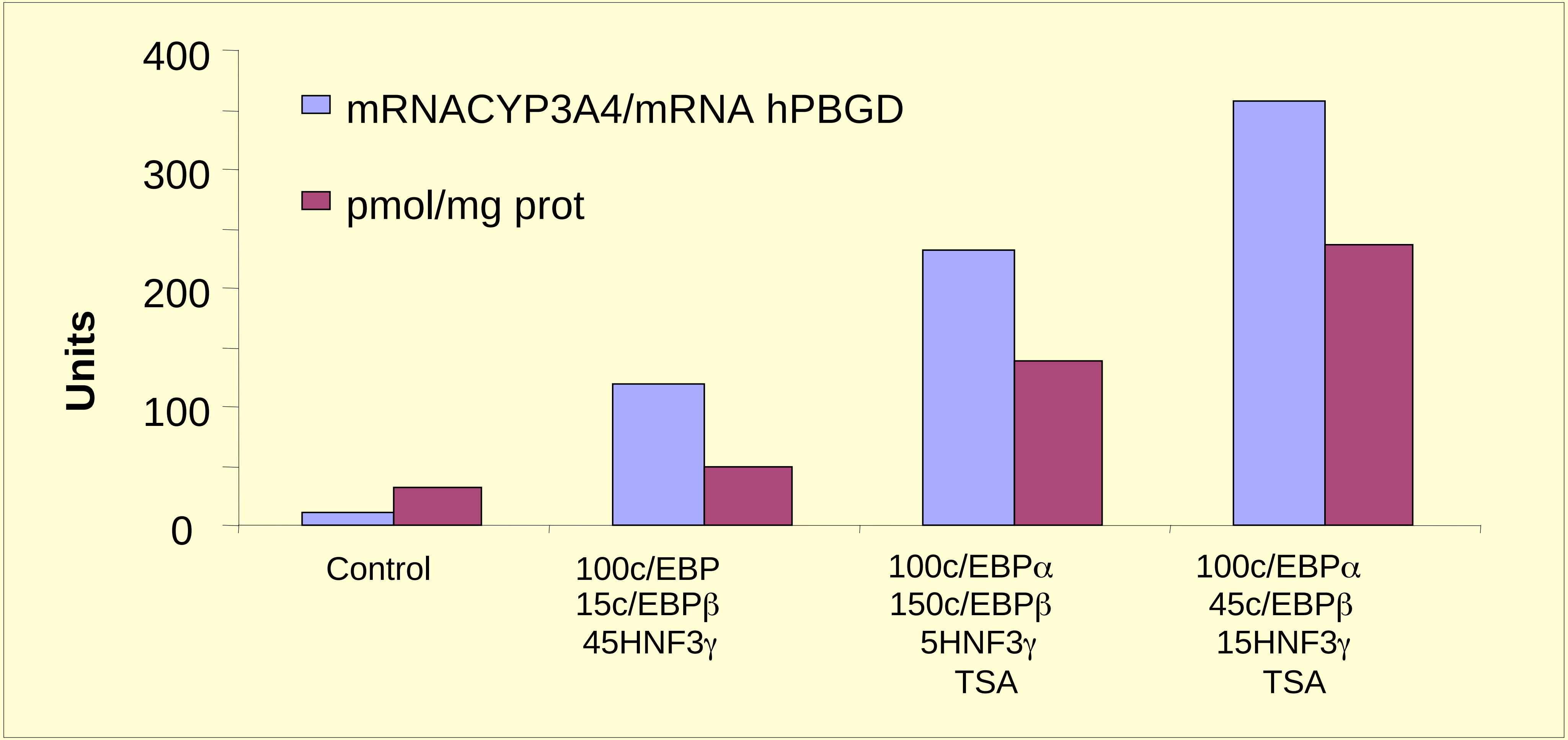
Ad-GFP

Human hepatocytes



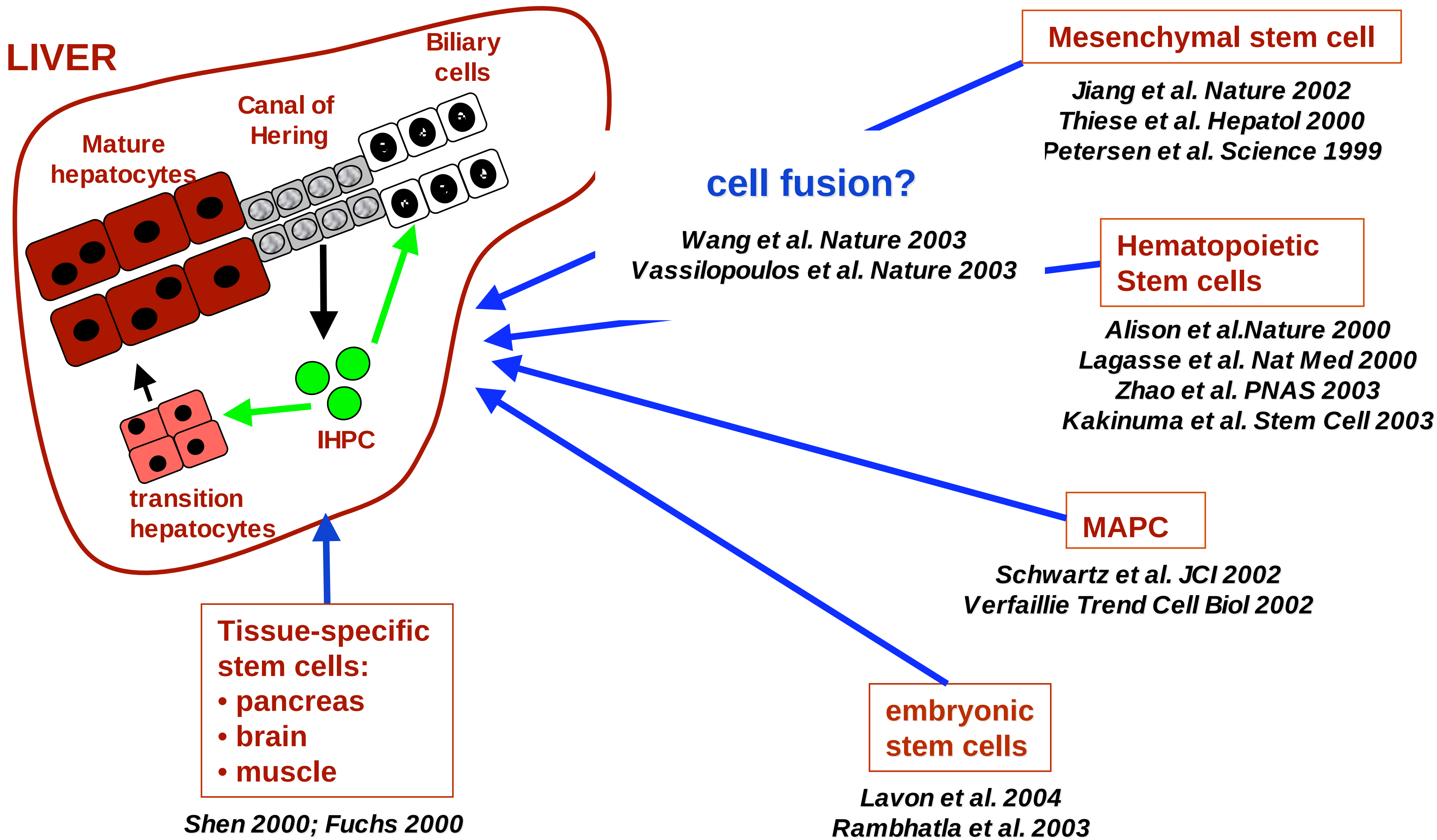


CYP 3A4 activity in transfected HepG2 cells



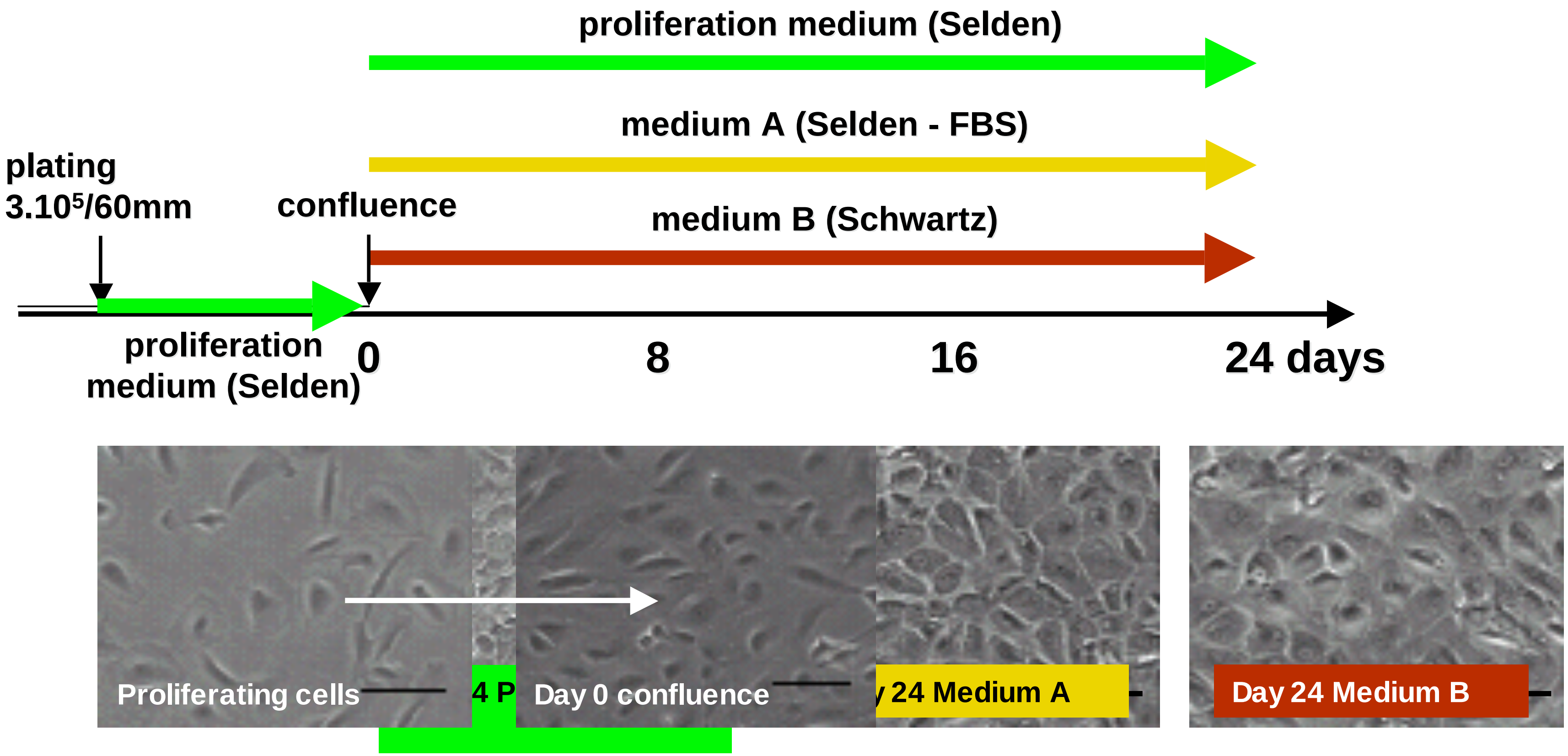
(CYP 3A4 substrate, midazolam)

Hepatocytes from stem cells: (too?) many possibilities



IHPC: intra hepatic progenitor cells (“oval cells”?)

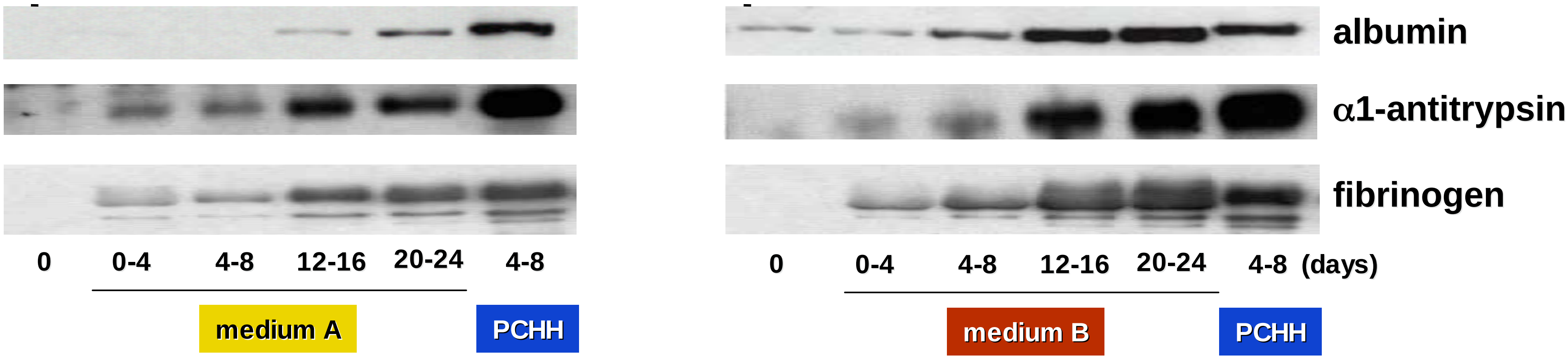
In vitro differentiation of IHPC



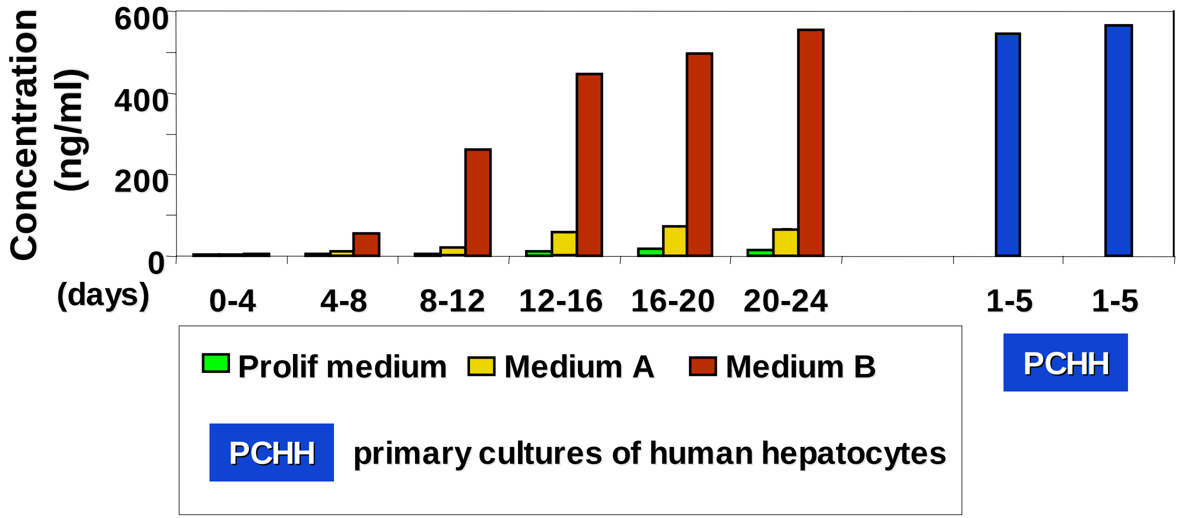
Selden medium: MEM 10%FBS, HGF, EGF, TRH, etc.
Schwartz medium: DMEM, FGF4, HGF, etc.

Plasma protein production

immunoblottings



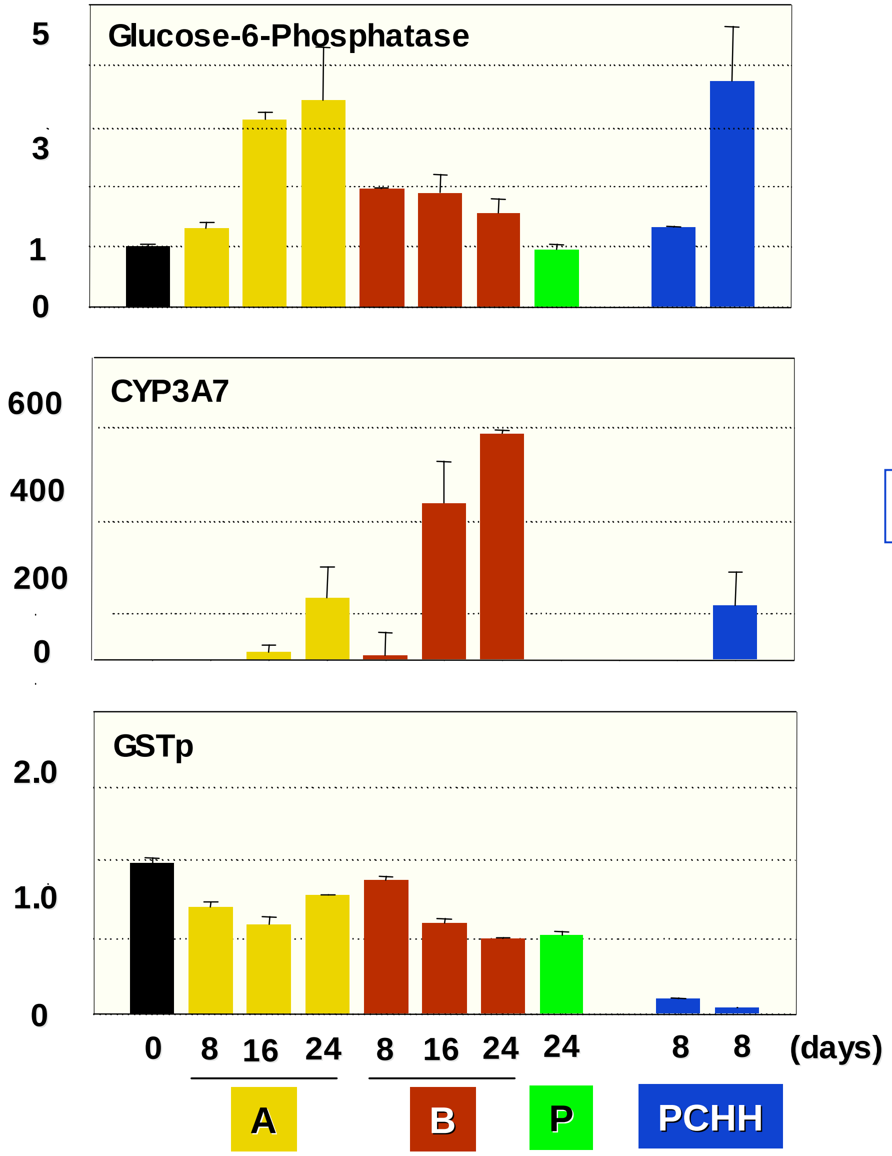
Albumin production



AFP not detectable

Metabolism enzyme mRNA expression

Real time Q-PCR

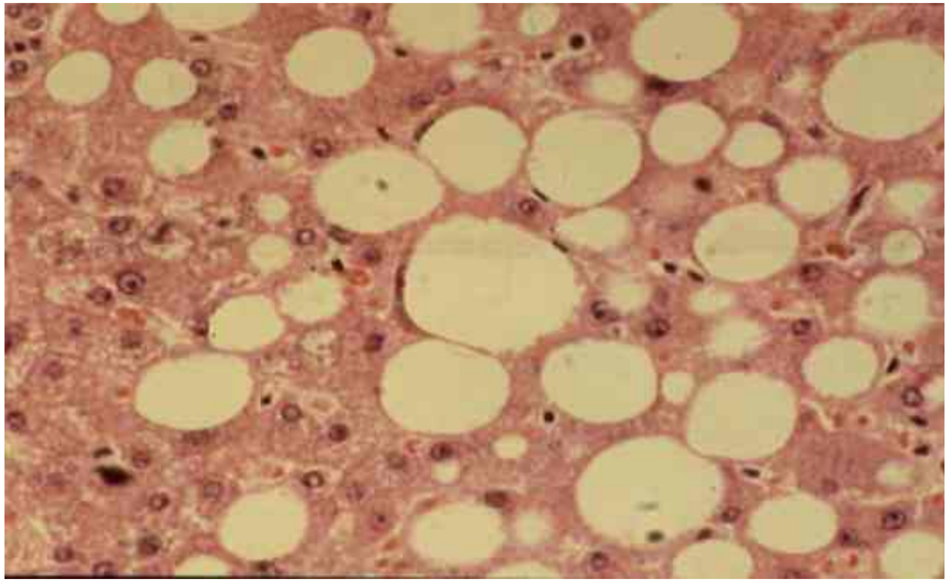


CYP3A4 not detectable

WP3: ***Optimisation of tools and analyses***

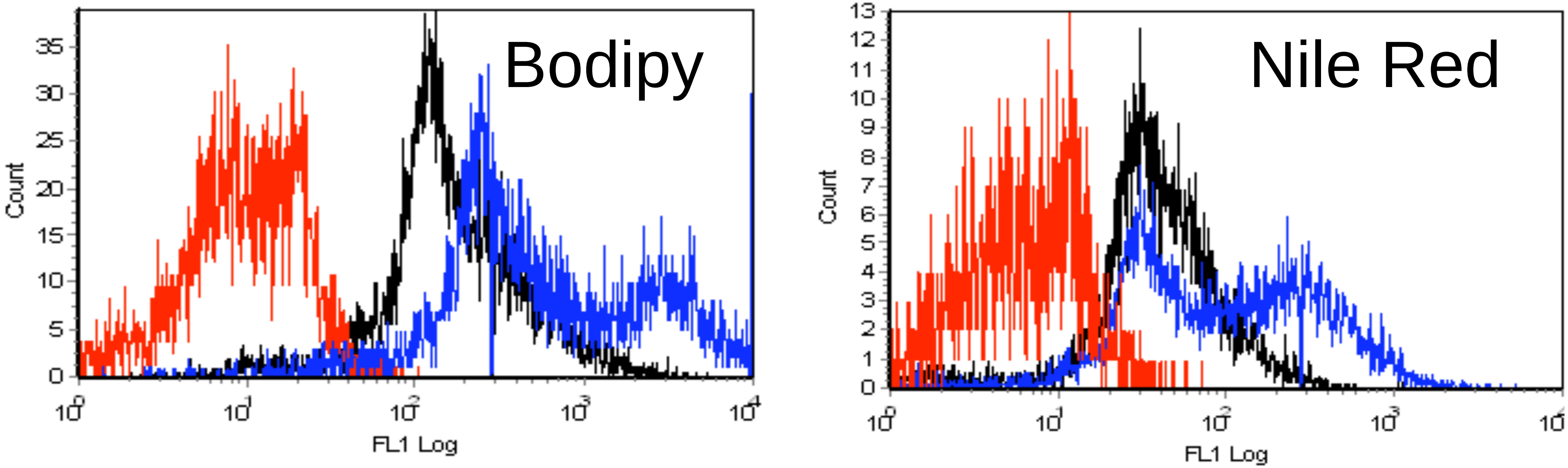
- ***Development of new cytomic assays:*** cell-based in vitro assays for steatosis

1. Objectives of the study
2. Characterization of cell models
3. Lipid loading
4. Fluorescent probes for lipid detection
5. Fluorescent probes for functional assays
6. Results



Summary of results

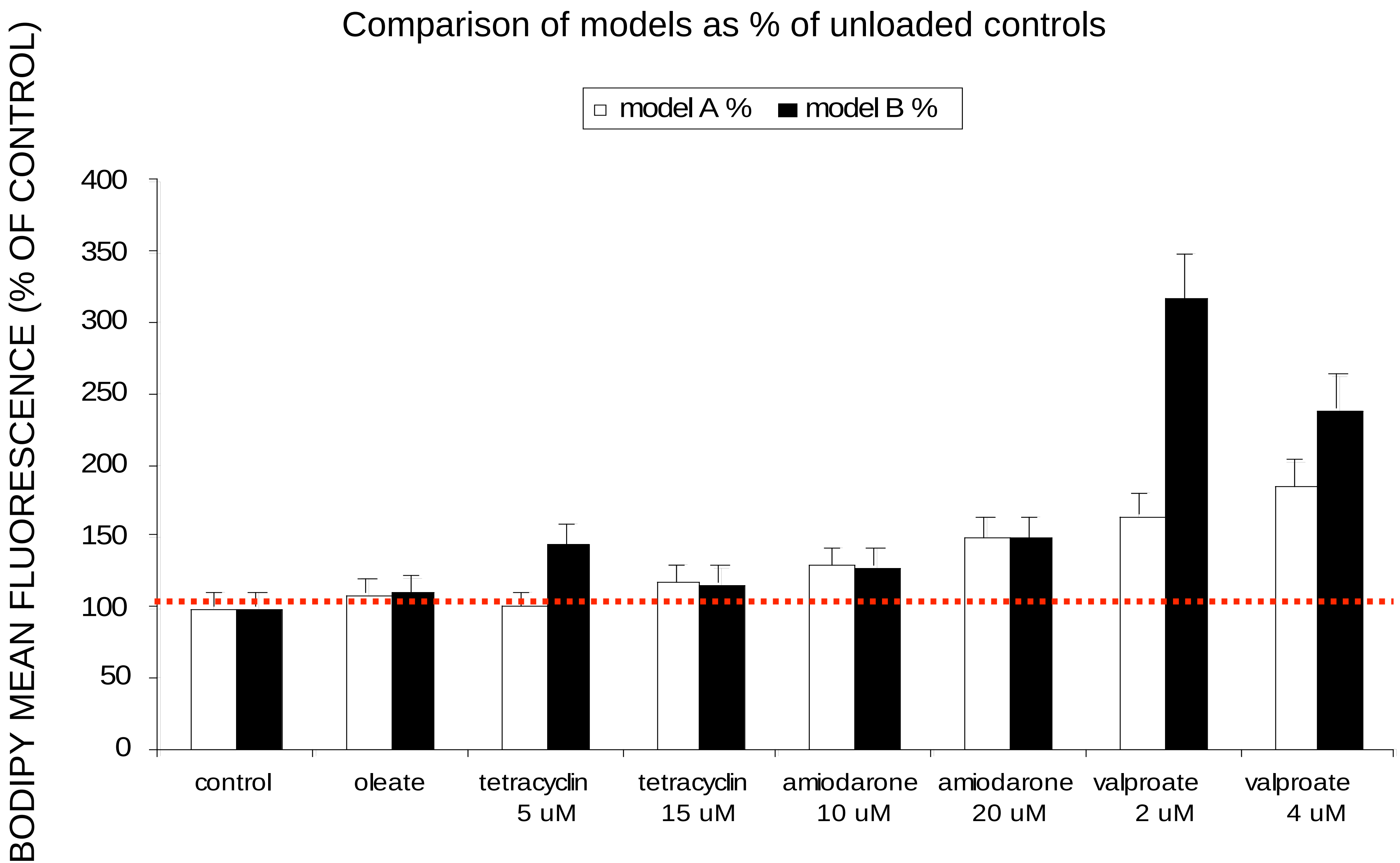
Human Hepatocytes



---- Autofluorescence ---- BSA ---- Lipids+BSA

Bodipy dye is more sensitive than Nile Red for the analysis of intracellular lipids

Summary of results



Kidney:

Workpackage 2: Kidney cell system development

Workpackage 3: Optimisation of tools and analysis

Workpackage 5: Mechanisms of nephrotoxicity and identification of toxicity markers

PREDICTOMICS

WP 2: Kidney cell system developments

2.1 Development of cultures of renal cells

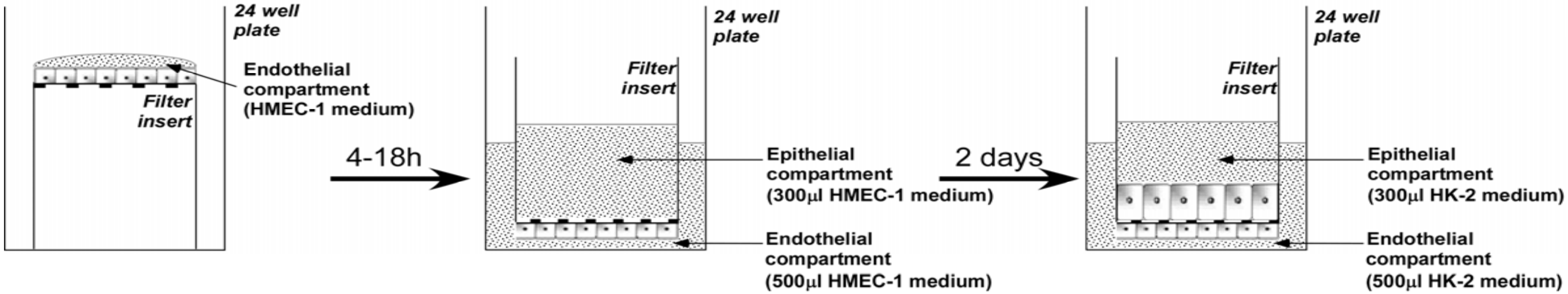
2.2 Advanced culture techniques: co-cultures, perfusion culture models

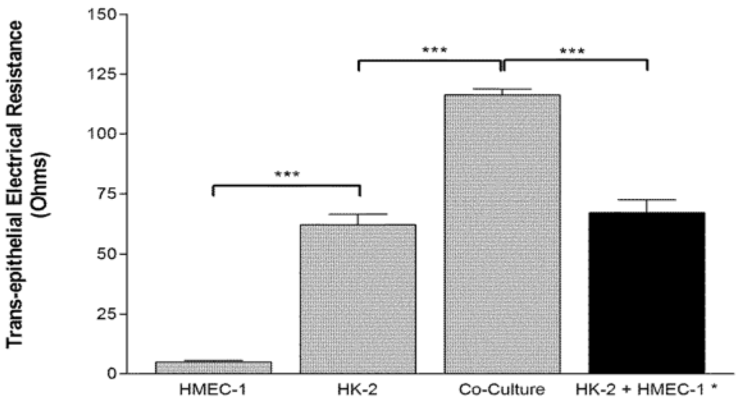
2.3 Molecular biology studies on kidney cell differentiation (P2, P5, P6, P7 and P8)

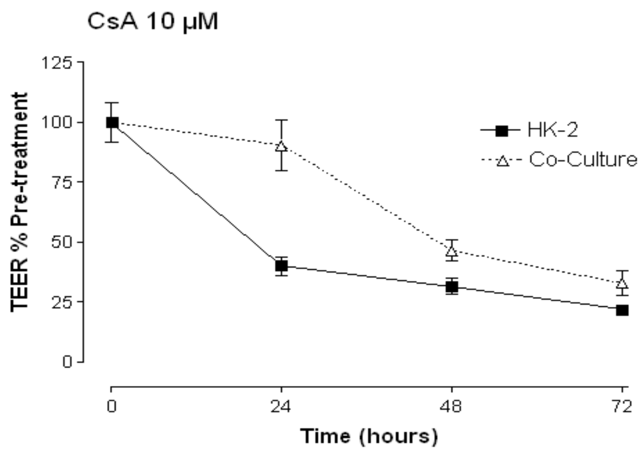
(WP 2)

Co-culture System Developed and Optimised

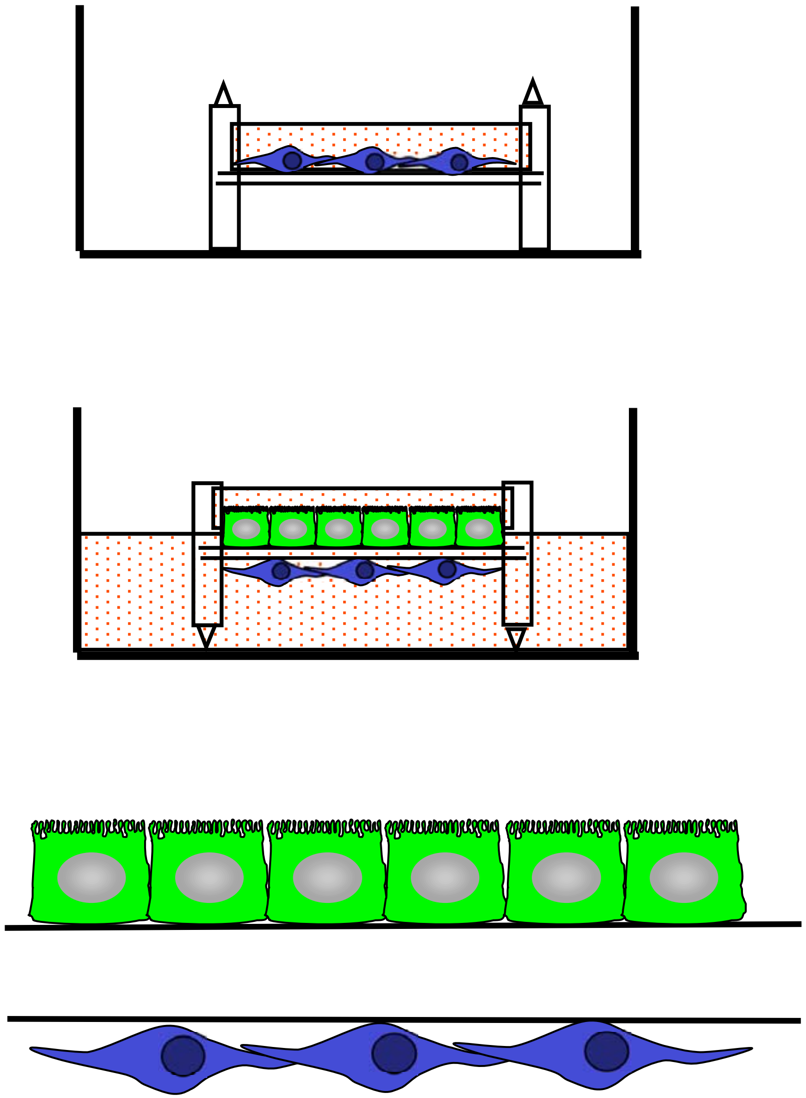
- HK-2 and human dermal microvascular endothelial cells (HMEC-1)
- Non-contact close proximity culture system – filter based

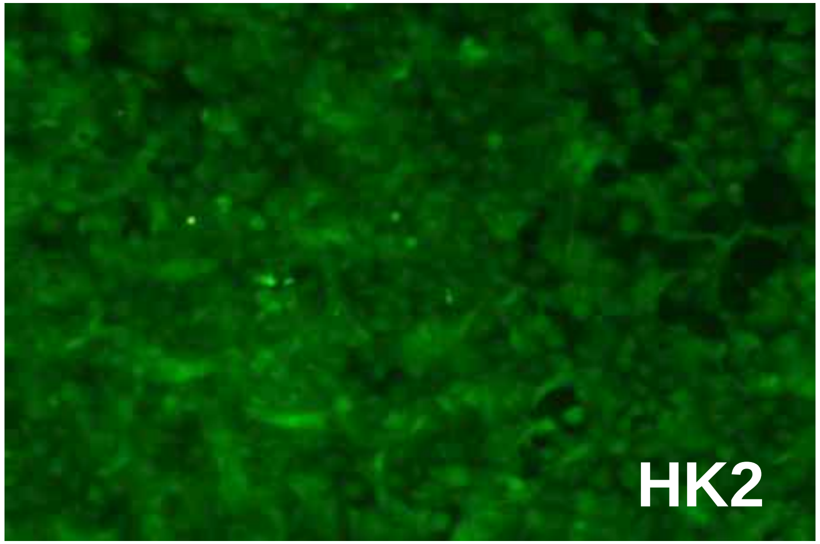


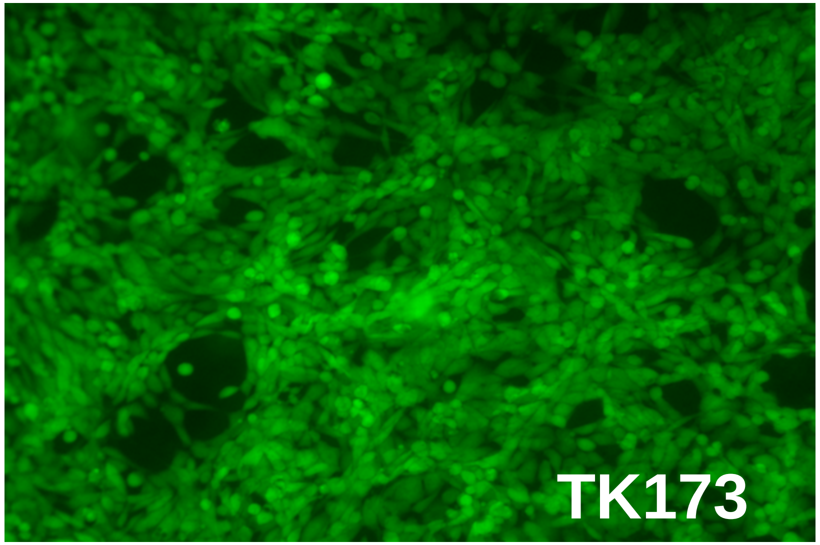




Co-culture of renal fibroblasts and tubular epithelial cells







PREDICTOMICS

(WP 2) **Morphological and phenotypical characterization of fibroblasts and tubular epithelial cells in monoculture**

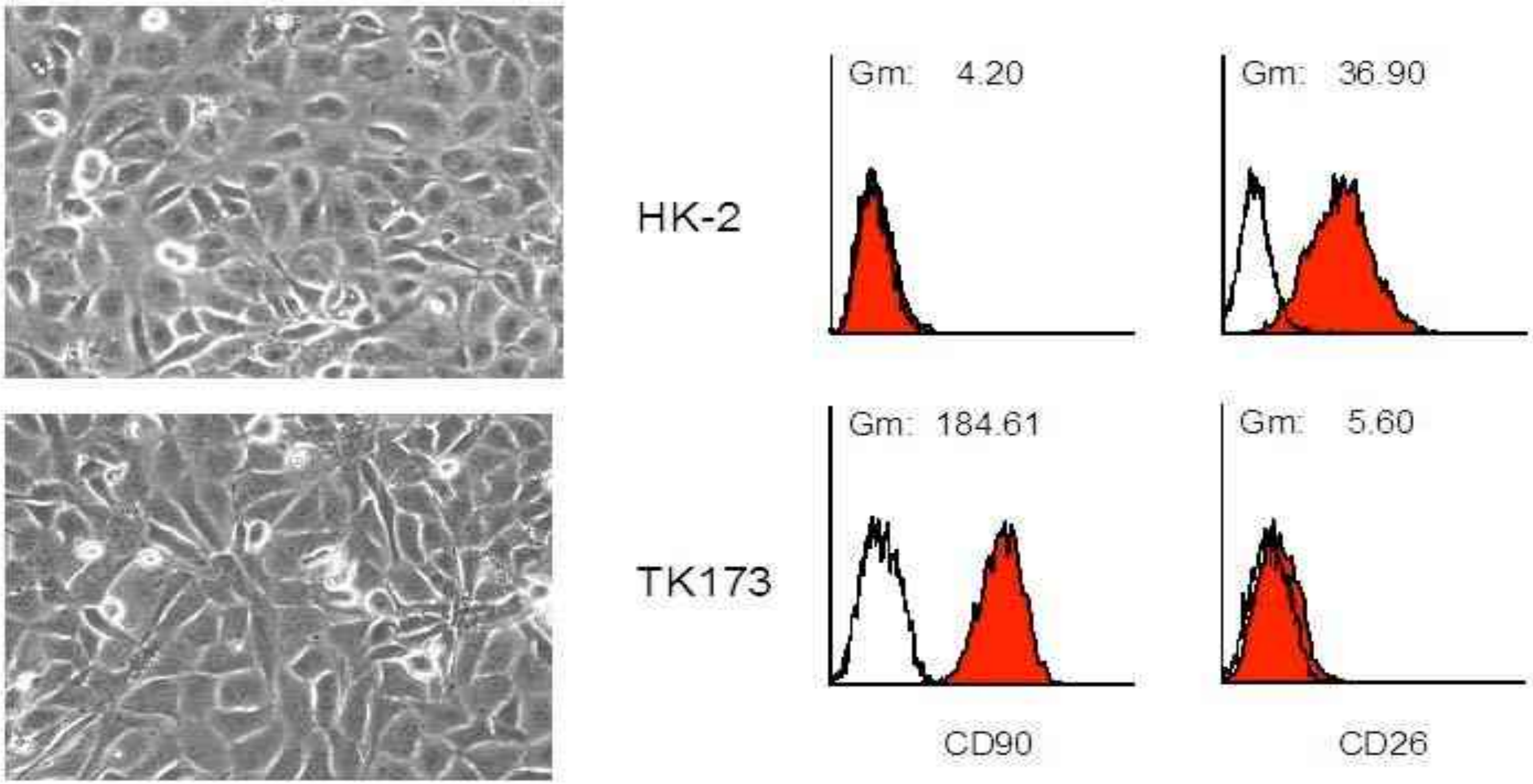


Figure 1: HK-2 and TK-173 were cultured on plastic in HK-medium and analyzed morphologically by light microscopy and phenotypically by flow cytometry using fibroblast and epithelial specific markers

PREDICTOMICS

RENAL GROUP

WP 3: Optimisation of tools and analyses

3.1 Improved genomic tools ✓

3.2 Assessment of protein profiles in cells

3.3 Development of cytomics assays

in progress

WP 5: Mechanisms of nephrotoxicity and identification of toxicity markers

Assays with model nephrotoxins:

- ***Cyclosporine***
- ***Rapamycin***
- ***FK506***
- ***Ochratoxin A***

PREDICTOMICS

(WP 2)

Deliverable 11 : Identification of changes in functionally relevant genes and comparison between mono and co-culture systems under static and perfusion culture conditions.

Plastic vs Filter

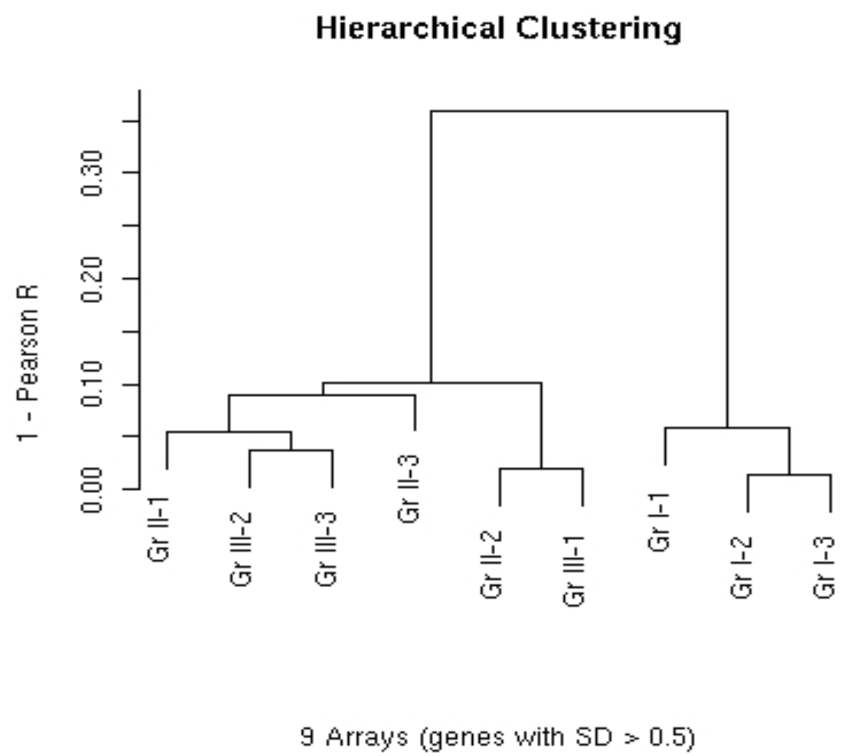
338 genes are down regulated 2-fold or higher
1159 genes up regulated 2-fold or higher

Plastic vs co-culture

380 genes down regulated 2-fold or higher 1190 genes up-regulated 2-fold or higher

Filter vs co-culture

28 genes down regulated 2-fold or higher
36 genes up regulated 2-fold or higher



PREDICTOMICS

WP 2: Overview: suitability of different kidney culture models

	Rat	Human
cell line OTA treatment	NRK-52E day 1 and day 3	HK-2 day 1 and day 3
primary cells time course OTA treatment	isolation by perfusion day 5, 7, 8, 10, 12 and 14 in culture vs rat kidney *in vivo* (only passage 0) day 1 and 3	proximal tubular fragments in vitro culture vs Human kidney in vivo (passage 1, cooperation with Innsbruck) day 1 and day 3
in vivo OTA treatment	rat kidney day 1, 3 and 7	

Black: planned
Red: WP2 in progress
Blue: WP5 in progress

OTA: ochratoxin A

PREDICTOMICS

Renal group: expansion to gene profiling

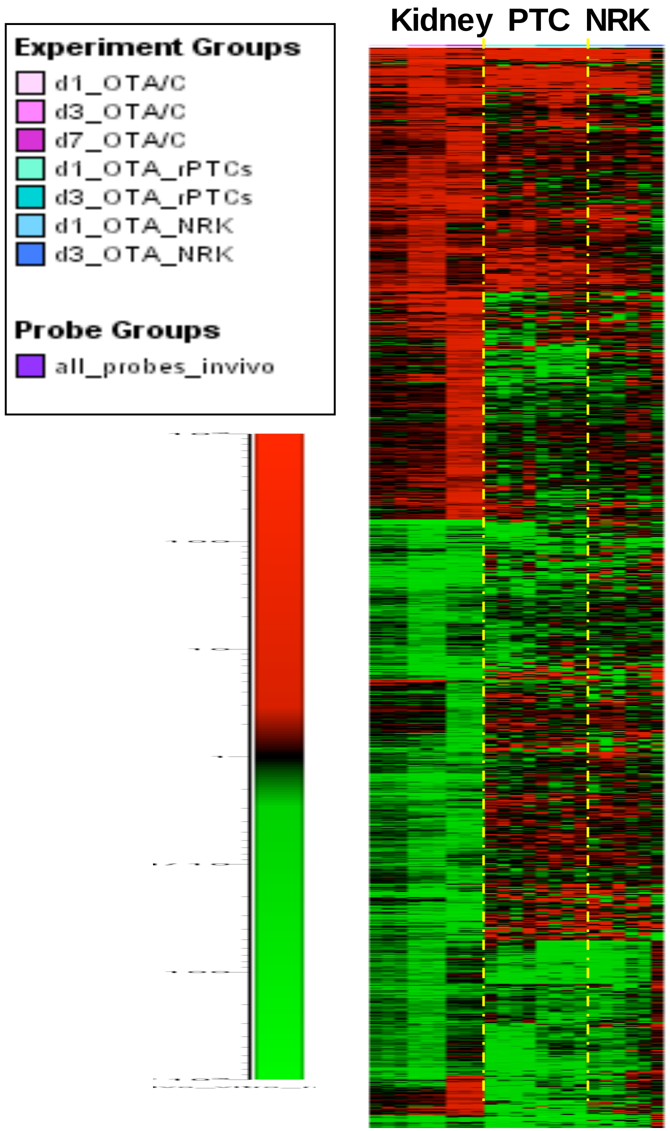
- Agreement of the renal group partners at the **6 month interim meeting** to compare **all the gene profiles identified in the advanced cell cuture models used in PREDICTOMICS** to gene profiles established for renal tissue (mostly comprised of renal proximal tubules) frome **live graft donors and renal biopsies** available from studies ongoing in other EU-FP 5 anf FP 6 projects responsible partners P5, P6

- This should subserve two purposes:

1. to establish something like a "gold standard" for human renal proximal tubular gene profiles

2. to identify genes relevant for risk assessment in nephrotoxicity

Expression profiling: kidney *in vivo* vs cell culture systems



Acute effects
Oxidative stress/DNA damage
Signaling
Immunity and defense

Proliferation, Regeneration
Apoptosis
Cell cycle control
DNA, RNA metabolism
Cytoskeleton, Cell adhesion

Dedifferentiation
Biotransformation
Membrane transport
Cellular metabolism

Proliferation
Cytoskeleton

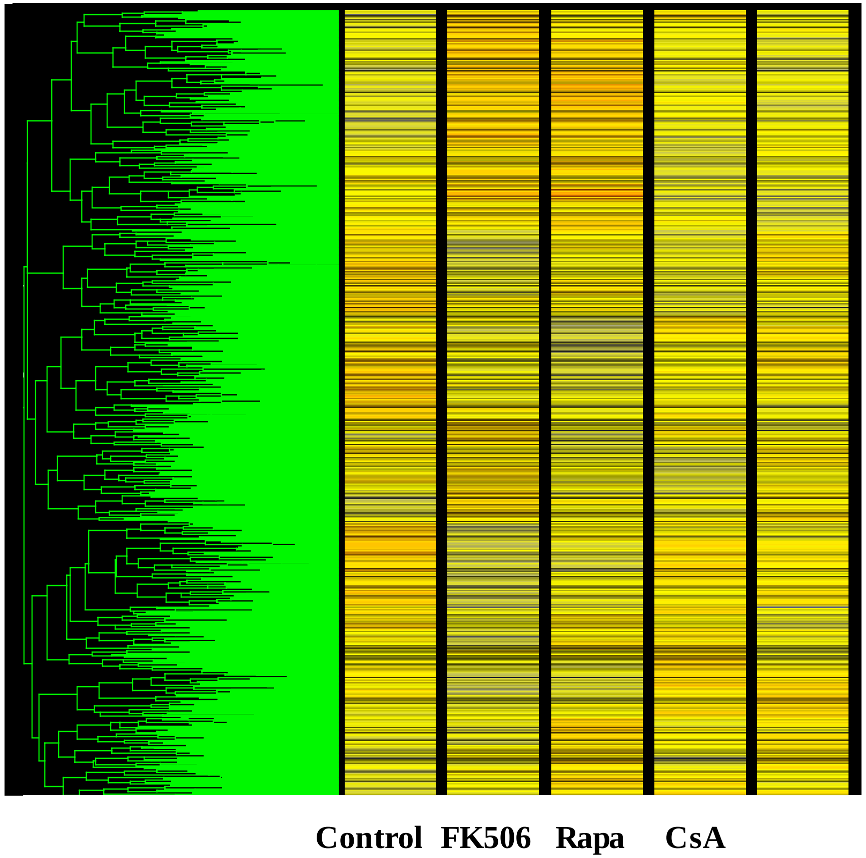
OTA: ochratoxin A

- **Genes:**
  Those significantly de-regulated by OTA in vivo
- **Kidney systems:**
  - **Rat kidney in vivo treated with OTA (3 mg/kg/day) for 1, 3, and 7 days**
  - **rPTCs treated with 20 µM OTA for 1 and 3 days.**
  - **NRK-52E treated with 20 µM OTA for 1 and 3 days**
- **All data are normalized to the time-matched and appropriate controls.**

Defined cell reactions are missing

(WP 5)

Effects of nephrotroxins on gene expression in kidney cells



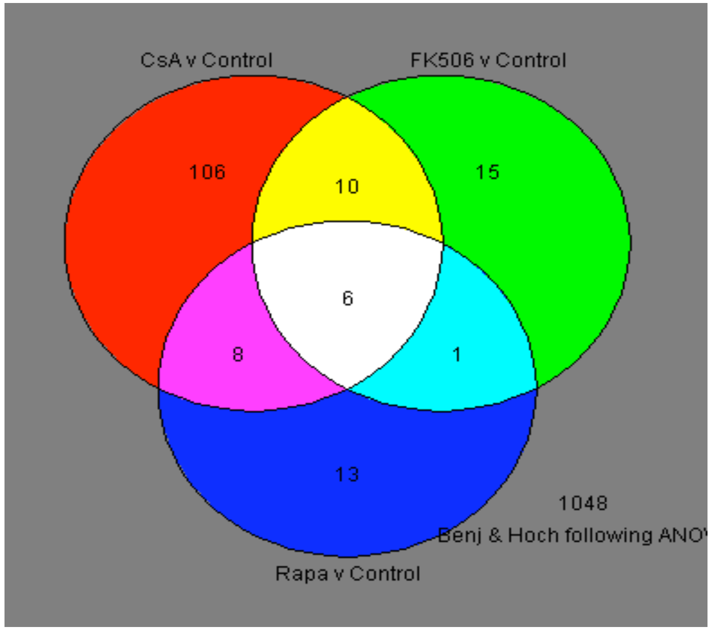
Hierarchical Clustering (Gene Tree) of GeneChip Microarray data

Data was analysed using GeneSpring Version 6.1. Samples were normalised, flags were present in 6 out of 10 samples, data was subject to statistical analysis, ANOVA, hierarchical clustering (gene tree) was performed using the Pearson correlation

(DELIVERABLE 21):

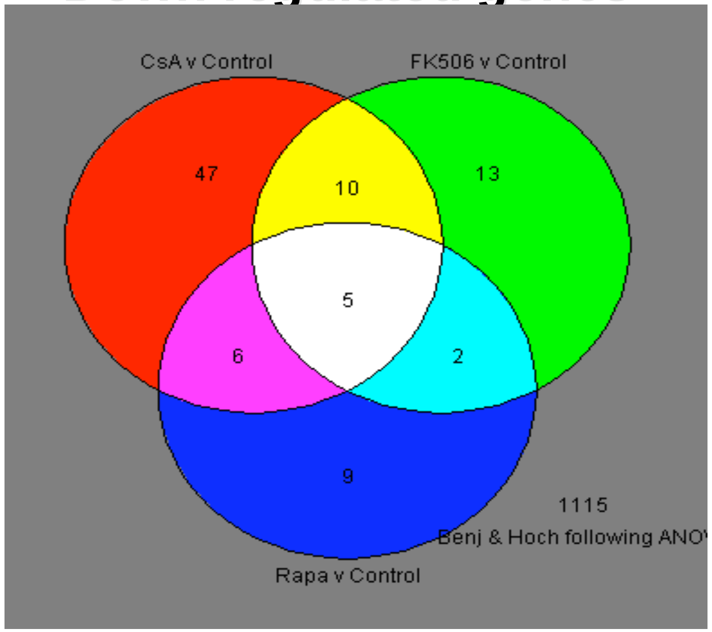
Control compared to cyclosporin A, FK506 and rapamycin- treated HK-2 cells

Up regulated genes



Up regulated genes (≥ 1.5 fold)

Down regulated genes

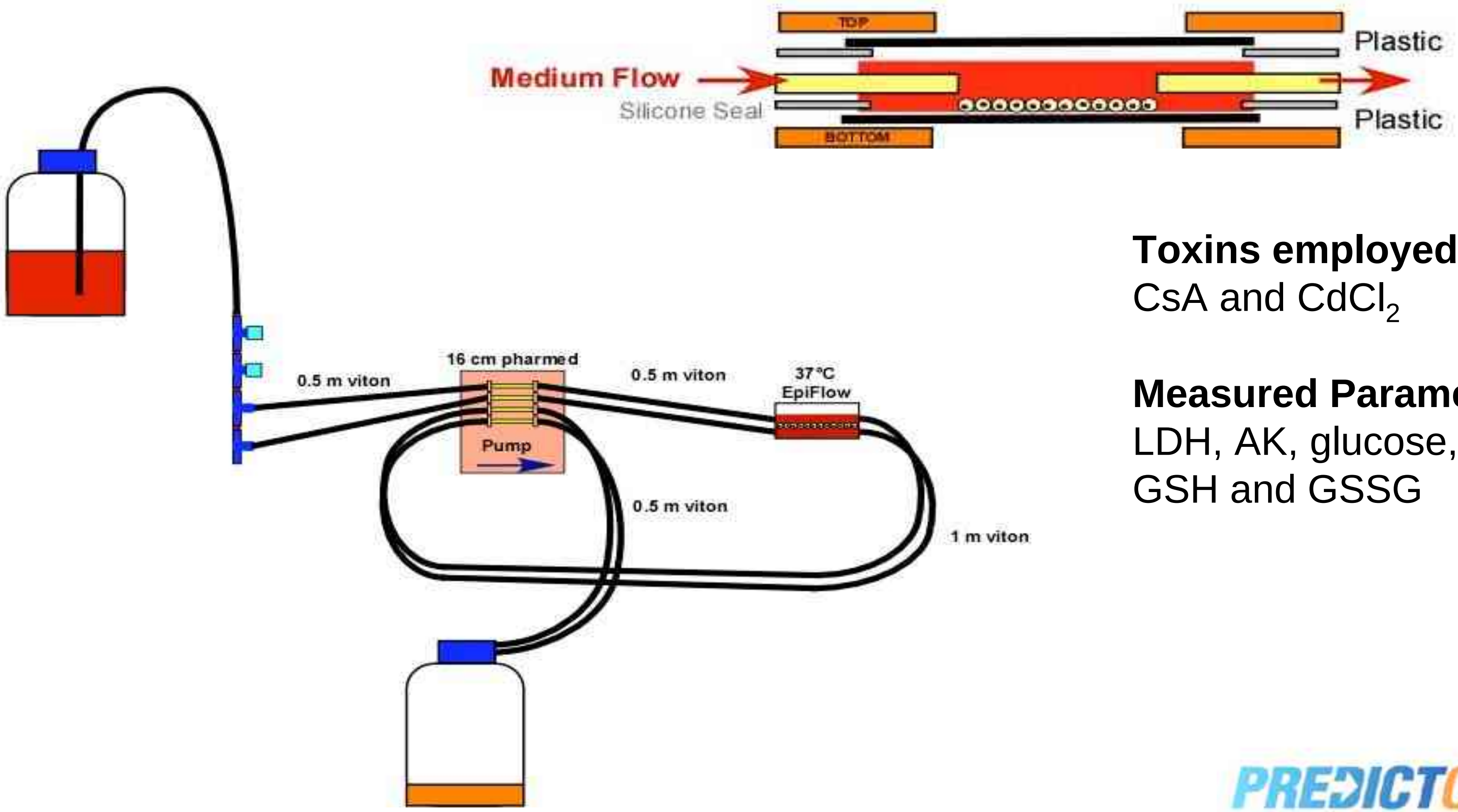


Down regulated genes (≥ 1.5 fold)

PREDICTOMICS

(WP 3)

Deliverable 23: Development of online monitoring procedures for measurement of epithelial solute transport and soluble renal cell injury markers from perfusion media.

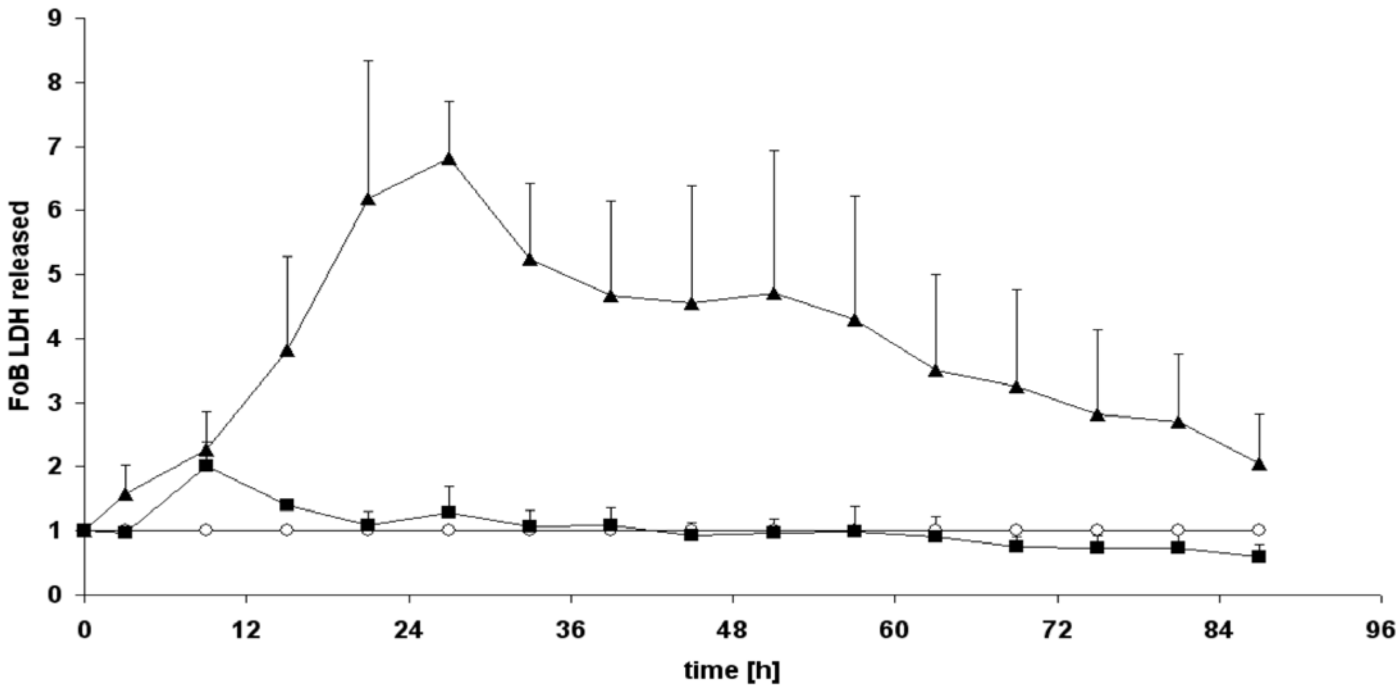


Toxins employed:
CsA and $CdCl_2$

Measured Parameters:
LDH, AK, glucose, lactate, GSH and GSSG

(WP 3)

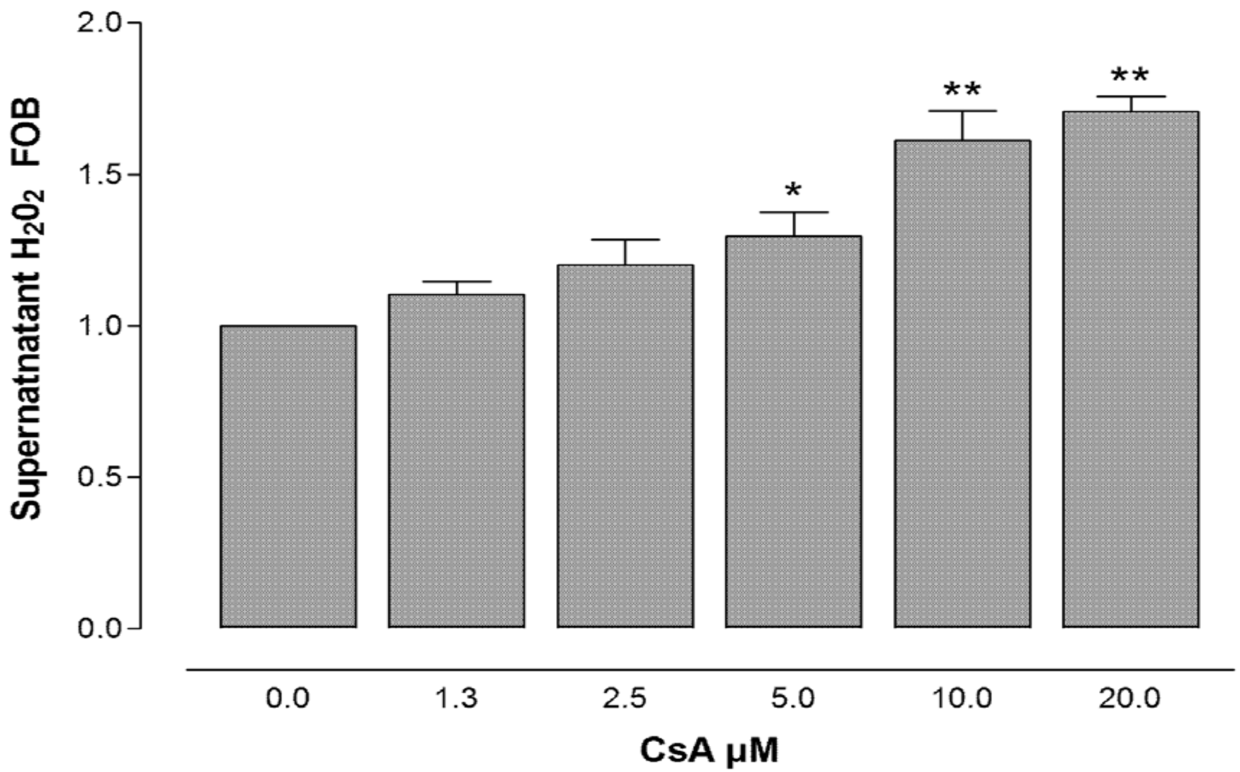
Deliverable 23: Development of online monitoring procedures for measurement of epithelial solute transport and soluble renal cell injury markers from perfusion media.



0 (O), 1 (■) and 5 µM (▲) $CdCl_2$

(WP 5)

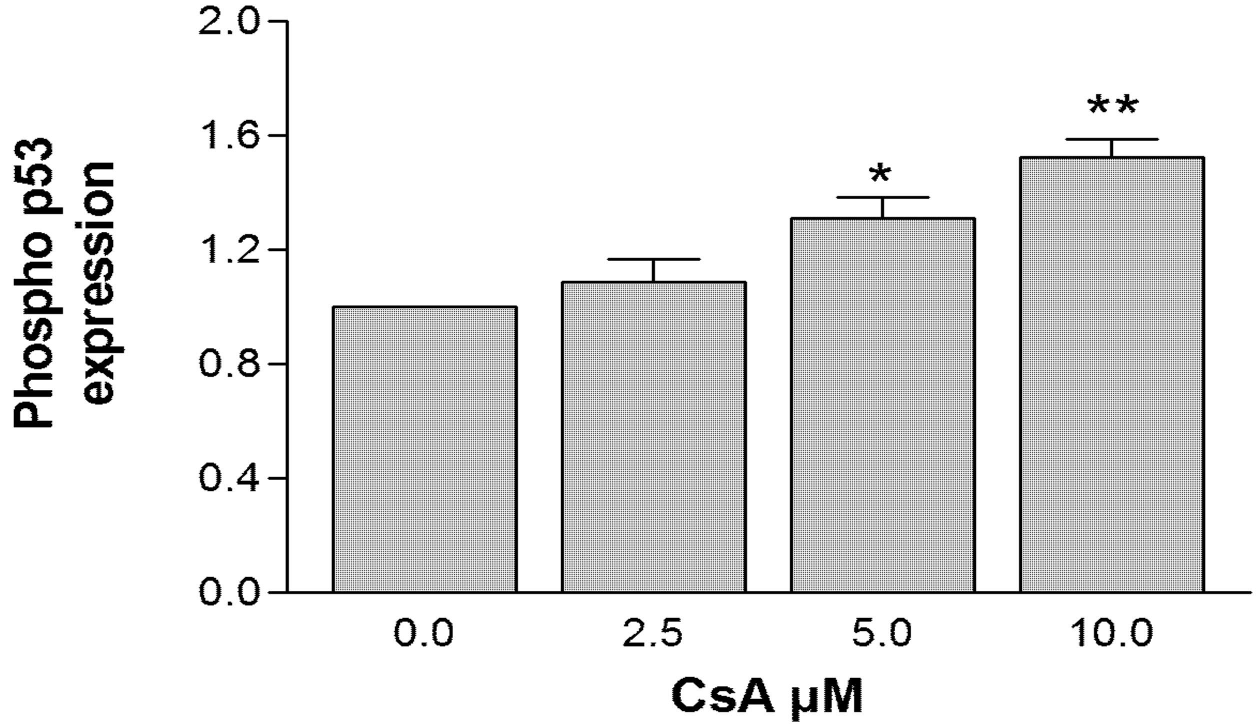
Cyclosporin A (CsA) induced hydrogen peroxide production indicative of ROS-mediated injury in HK-2 cells



PREDICTOMICS

(WP 5)

Cyclosporin A (CsA) induced increased expression of phosphorylated p53 in HK-2 cells



PREDICTOMICS

Management & Dissemination

WEB SITE: www.predictomics.org

Publically available information

Project Predictomics

- Abstract
- Summary of the project
- Partners
- Institutions involved
- Press releases
- Publications / Congresses

Restricted Area

(Only members of the consortium)

password

Documents

- SOPs
- Results
- Contract
- Technical annex I
- General conditions (Annex II)
- Partner address and participants
- Project Reporting
  Guidance notes
  Model of Financial Statement

Meetings

- Valencia
- Innsbruck
- Leiden
- Forthcoming

Appendix 1-4