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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

RESEARCH LETTER

Epigenetic Reprogramming of Human Hepatoma Cells: A Low-Cost Option for Drug Metabolism Assessment

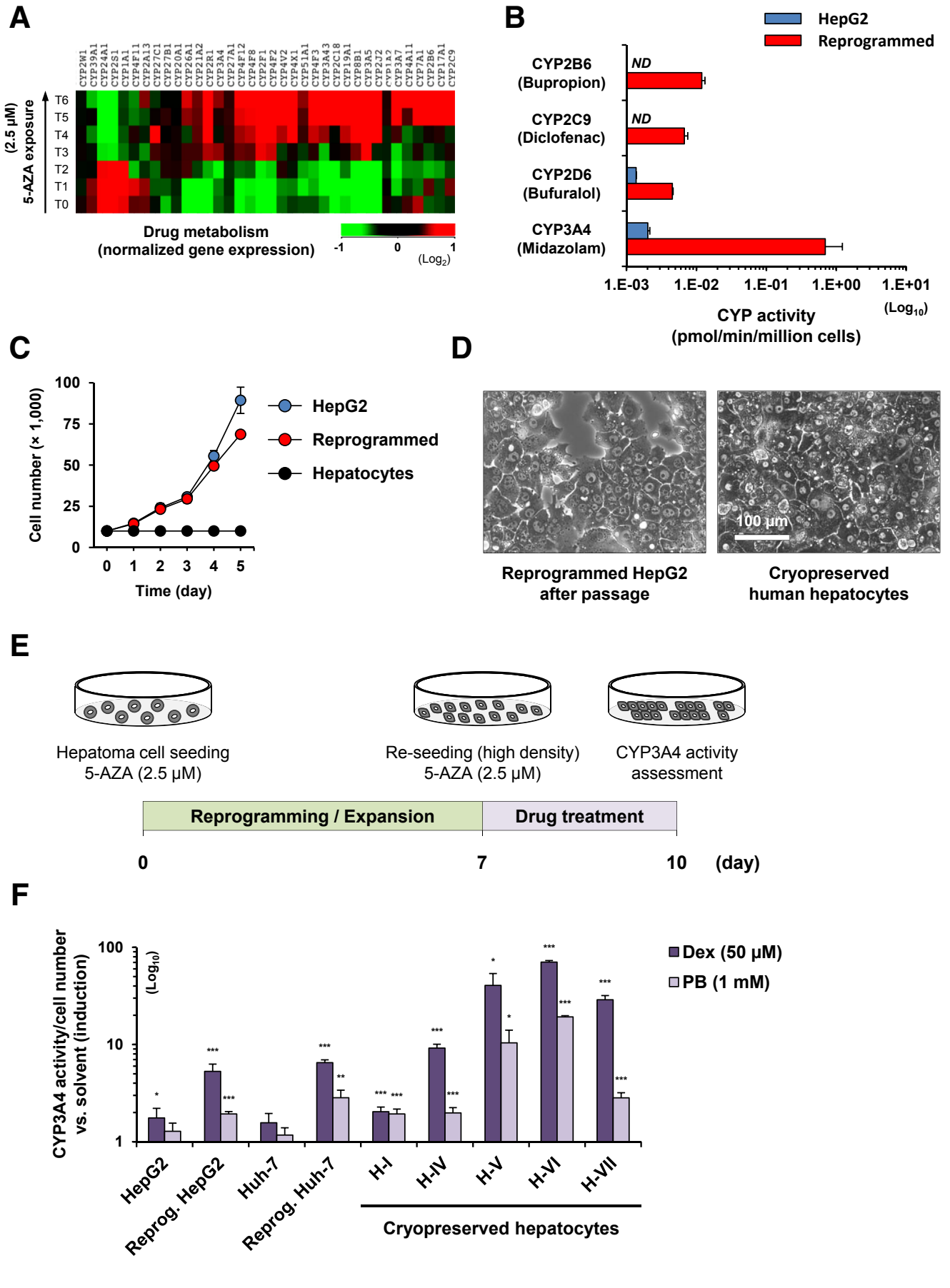
Primary cultures of hepatocytes are widely considered the gold standard for evaluating the hepatic metabolism of pharmacologic molecules.[1] However, limited access to human hepatocytes has led to the development of various alternative models.[2–5] The procedures used to generate these cells remain complex and time-consuming. In addition, the resulting hepatocytes frequently display heterogeneous hepatic gene expression, rapidly dedifferentiate, and lose their metabolic functions, which are essential for biological, pharmacologic, and toxicologic studies. Strategies that reverse epigenetic alterations in malignant cells offer a unique opportunity for cellular reprogramming,[6–11] which is valuable for the bioengineering of surrogate hepatic models. In this study, we aimed to develop a robust method that enables the recovery of a differentiated phenotype in hepatoma cells and significant drug-metabolizing features for drug metabolism studies. The procedure applied is based on a continued exposure to the demethylating agent 5-azacytidine (5-AZA).

The human HepG2 cell line was used for the first part of this study. Despite their low metabolic capacity, HepG2 cells are frequently used for *in vitro* studies as a replacement for human hepatocytes.[12] Characterization of HepG2 cells in response to the 5-AZA reprogramming regimen showed extensive storage of lipids and glycogen compared with the control cells (Supplementary Figure 1). Moreover, the treated cells showed a gradual increase in the expression of genes associated with lipid metabolism, carbohydrate metabolism, and amino acid metabolic processes. Interestingly, our data also indicated that the demethylating treatment led to global enrichment in the expression of the cytochrome P450 (*CYP*) genes (Figure 1*A*). We analyzed the activity of 4 of the most important phase I drug-metabolizing enzymes before and after epigenetic reprogramming. The data showed that CYP activity was induced dramatically in reprogrammed HepG2 cells (Figure 1*B*). Notably, metabolism of midazolam by CYP3A4 showed the strongest induction in reprogrammed cells. Of the various members of the CYP superfamily, CYP3A4 certainly plays the most important role in the metabolism of marketed drugs,[13] supporting the relevance of epigenetically reprogrammed hepatic cells for the study of xenobiotic detoxification.

Next, we established a turnkey protocol for the epigenetic reprogramming of human hepatoma cells and CYP activity assessment. In contrast to hepatocyte cultures, the 5-AZA–treated cells showed persistent proliferation rates (Figure 1*C*), thereby allowing their expansion by serial passaging (Figure 1*D*). The reprogramming method was based on a 2-step procedure as shown in Figure 1*E*. First, hepatoma cells were exposed to the demethylating agent 5-AZA (2.5 μmol/L) for 7 days. Next, reprogrammed cells were re-seeded at a high density, maintained in 5-AZA, and treated with CYP inducers for 72 hours. We evaluated dexamethasone (Dex)- and phenobarbital sodium (PB)-mediated CYP3A4 metabolic capacity using reprogrammed HepG2 and Huh-7 cells. Cultures of cryopreserved hepatocytes were used as a reference. CYP3A4 activity was not obviously induced in HepG2 and Huh-7 cells before reprogramming (Figure 1*F*). Conversely, Dex and PB stimulation induced CYP3A4 activity by 5.29 ± 1.00-fold and 1.93 ± 0.11-fold, respectively, in reprogrammed HepG2 cells and by 6.51 ± 0.45-fold and 2.84 ± 0.55-fold, respectively, in reprogrammed Huh-7 cells ($P < .001$, t test vs solvent-treated control cells). The CYP3A4 activities measured in the 5 lots of human hepatocytes were relatively heterogeneous and ranged from 2.04 ± 0.24-fold to 70.45 ± 2.66-fold for Dex and ranged from 1.93 ± 0.23-fold to 19.25 ± 0.54-fold for PB, respectively. Notably, the CYP induction values obtained in reprogrammed hepatoma cells were relatively close to the average activities of the 5 lots of human hepatocytes after Dex and PB induction (30.24-fold and 7.28-fold, respectively). Furthermore, we found that CYP3A4 functions were more stable in reprogrammed hepatoma cells because the cryopreserved hepatocytes tended to extensively dedifferentiate over time (Supplementary Figure 2). Indisputably, the use of demethylating compounds for differentiating liver progenitors and cancer cells has been reported extensively in the literature.[10,14–16] However, none of these studies considered the actual functionality and drug-metabolizing capability of the treated cells, which supports the significance and novelty of our finding.

To address whether 5-AZA acts by directly influencing the methylation state of the *CYP3A4* gene, the correlation between DNA methylation and *CYP3A4* expression levels was evaluated in reprogrammed and control hepatoma cells. Gene expression measurement showed that *CYP3A4* expression showed a marked augmentation in the reprogrammed cells (Figure 2*A*). In silico genomic analysis showed that *CYP3A4* contained a CpG-rich region in its promoter (Supplementary Figure 3). We performed combined bisulfite restriction analysis to examine the methylation status of the identified CpG sites and found prominent hypermethylation of the *CYP3A4* promoter in Huh-7, Hep3B, and HepG2 cells compared with human hepatocytes (Figure 2*B*). By contrast, reprogrammed hepatoma cells showed significant demethylation of the analyzed CpG sites (Figure 2*C*), which was consistent with the re-expression of the *CYP3A4* gene observed after 5-AZA treatment.

In conclusion, the epigenetic reprogramming method offers several advantages. First, reprogrammed hepatic cells can be expanded without


A
CYP2W1 CYP39A1 CYP24A1 CYP2S1 CYP1A1 CYP4F11 CYP2A13 CYP27C1 CYP27B1 CYP20A1 CYP26A1 CYP21A2 CYP2R1 CYP3A4 CYP27A1 CYP4F12 CYP4F8 CYP2F1 CYP4F2 CYP4V2 CYP4X1 CYP51A1 CYP4F3 CYP3A43 CYP2C18 CYP19A1 CYP8B1 CYP3A5 CYP2J2 CYP1A2 CYP3A7 CYP4A11 CYP7A1 CYP2B6 CYP17A1 CYP2C9
(2.5 μM) 5-AZA exposure
T6
T5
T4
T3
T2
T1
T0
Drug metabolism (normalized gene expression)
-1
0
1
(Log2)
B
HepG2
Reprogrammed
CYP2B6 (Bupropion)
CYP2C9 (Diclofenac)
CYP2D6 (Bufuralol)
CYP3A4 (Midazolam)
ND
ND
1.E-03
1.E-02
1.E-01
1.E+00
1.E+01
(Log10)
CYP activity (pmol/min/million cells)
C
Cell number (× 1,000)
100
75
50
25
0
0 1 2 3 4 5
Time (day)
HepG2
Reprogrammed
Hepatocytes
D
100 μm
Reprogrammed HepG2 after passage
Cryopreserved human hepatocytes
E
Hepatoma cell seeding 5-AZA (2.5 μM)
Re-seeding (high density) 5-AZA (2.5 μM)
CYP3A4 activity assessment
Reprogramming / Expansion
Drug treatment
0
7
10
(day)
F
CYP3A4 activity/cell number vs. solvent (induction)
100
10
1
(Log10)
Dex (50 μM)
PB (1 mM)
HepG2
Reprog. HepG2
Huh-7
Reprog. Huh-7
H-I
H-IV
H-V
H-VI
H-VII
Cryopreserved hepatocytes

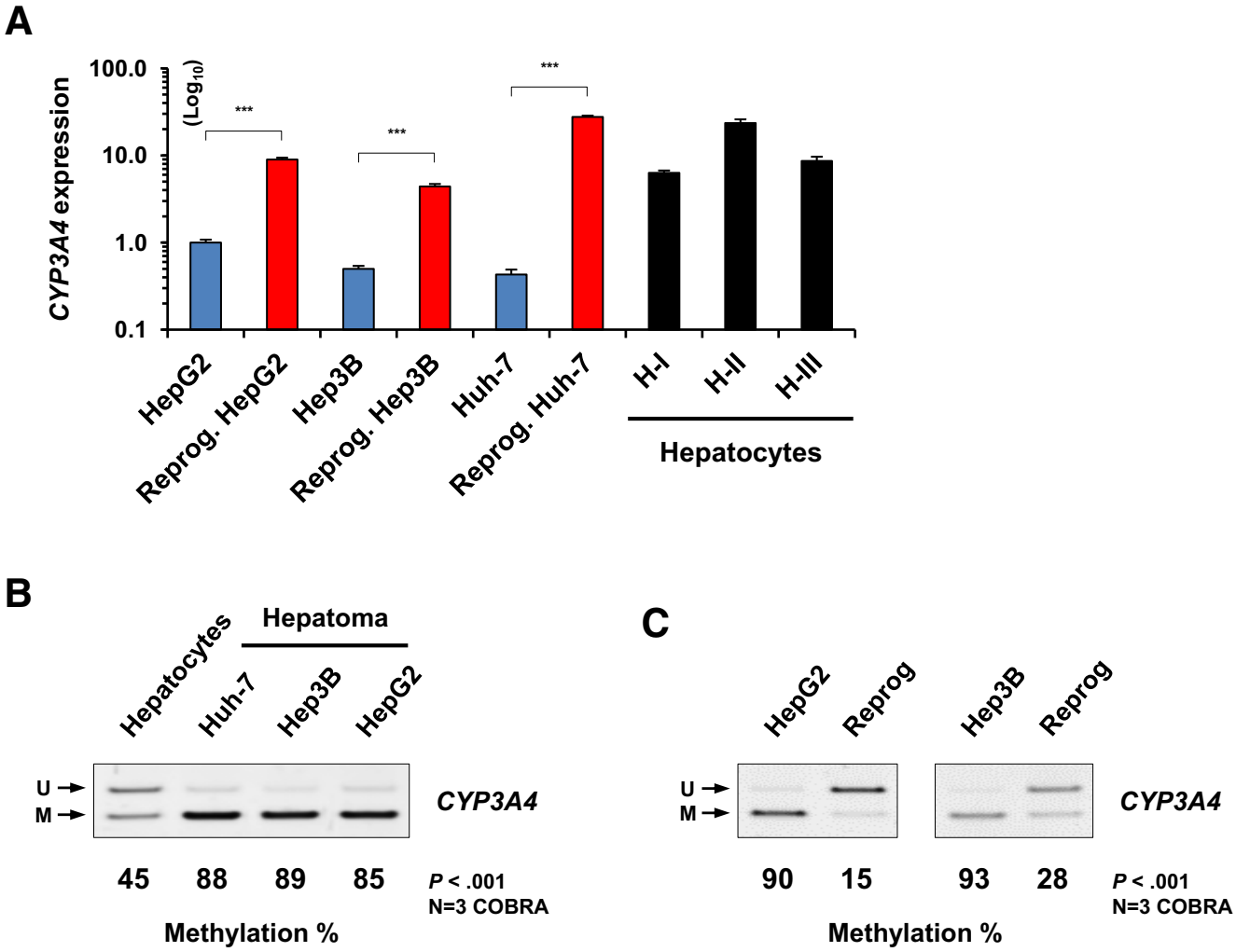


Figure 2. Expression levels and methylation profiles of *CYP3A4* after epigenetic reprogramming. (*A*) Relative expression levels of the *CYP3A4* gene in reprogrammed hepatic cells and human hepatocytes. The histograms show the means ± SD and statistical significance (reprogrammed vs control) was ***$P < .001$ (*t* test). (*B*) Comparison of human hepatocytes and hepatoma cells with regard to the methylation levels (%) of the *CYP3A4* promoter by combined bisulfite restriction analysis (COBRA). (*C*) *CYP3A4* methylation after epigenetic reprogramming. Reprogrammed HepG2 and Hep3B cells were treated with 5-AZA (2.5 μmol/L) for 10 days before genomic DNA extraction. Representative gels for 3 distinct COBRA experiments are shown. Statistical significance: ***$P < .001$ (*t* test). H, hepatocytes; M, methylated; Reprog, reprogrammed; U, unmethylated.

deleterious effects on metabolic functions, thus eliminating the issue of scarcity. Another advantage of the procedure is its ease of use. Although alternative hepatic models require the introduction of reprogramming factors via retrovirus integration,[5,17] 5-AZA–based reprogramming is a rapid and low-cost solution to generate large amounts of human cells with significant CYP activities. The patented HepaRG model (Saint-Grégoire, France) is also an interesting substitute for human hepatocytes.[18] However, these cells differentiate into a mixture of bile

Figure 1. (***See previous page***). **Drug metabolism assessment using epigenetically reprogrammed hepatic cells.** (*A*) Expression profiles of the *CYP* genes in 5-AZA–treated HepG2 cells (2.5 μmol/L for 12 days). Total RNA was collected every 2 days from T0 to T6 for the microarray analysis. See Supplementary Table 1 for the gene expression source data. (*B*) Measurement of CYP2B6, CYP2C9, CYP2D6, and CYP3A4 activities using a liquid chromatography and mass spectrometry–based system. (*C*) Proliferation rates of HepG2 cells before and after epigenetic reprogramming. (*D*) Morphology of reprogrammed HepG2 cells after expansion and passage. (*E*) Experimental design of the epigenetic reprogramming for CYP activity measurement using human hepatoma cells. (*F*) CYP3A4 induction level determined in reprogrammed HepG2 and Huh-7 cells using a CYP3A4 assay kit. Five lots of human hepatocytes were used as a reference. CYP3A4 activity was induced by treatment with Dex (50 μmol/L) or PB (1 mmol/L) for 72 hours. Statistically significant induction levels with respect to solvent-treated control cells were achieved at *$P < .05$, **$P < .01$, and ***$P < .001$ (*t* test). All data shown in the figure are means ± SD. H, hepatocytes; ND, not detected; Reprog, reprogrammed; T, time.

epithelial cells and hepatocytes and require several weeks of induction to show mature functions,[19] whereas reprogrammed hepatic cells can be obtained within 10 days. Obviously, cryopreserved hepatocytes that are commercially available represent the best model for scientists who have limited access to fresh liver tissues. However, the price of these cells can be substantial and their quality remains variable. Consequently, the epigenetic reprogramming method is a valuable alternative to generate human hepatic cell models for basic research and preclinical drug screening studies.

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Abbreviations used in this letter: **CYP, cytochrome P; Dex, dexamethasone; 5-AZA, 5-azacytidine; PB, phenobarbital sodium**

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Author contributions

Luc Gailhouste and Takahiro Ochiya conceived and designed the study; Luc Gailhouste, Lee Chuen Liew, Ken Yasukawa, Keitaro Hagiwara, and Izuho Hatada performed the experiments; Norihiko Iwazaki and Yasuhiro Yamada provided expertise for the drug-metabolizing enzyme activity analyses; Luc Gailhouste analyzed the data, performed statistical analyses, and wrote the manuscript; and Takahiro Ochiya obtained funding for the study.

Conflicts of interest

The authors disclose no conflicts.

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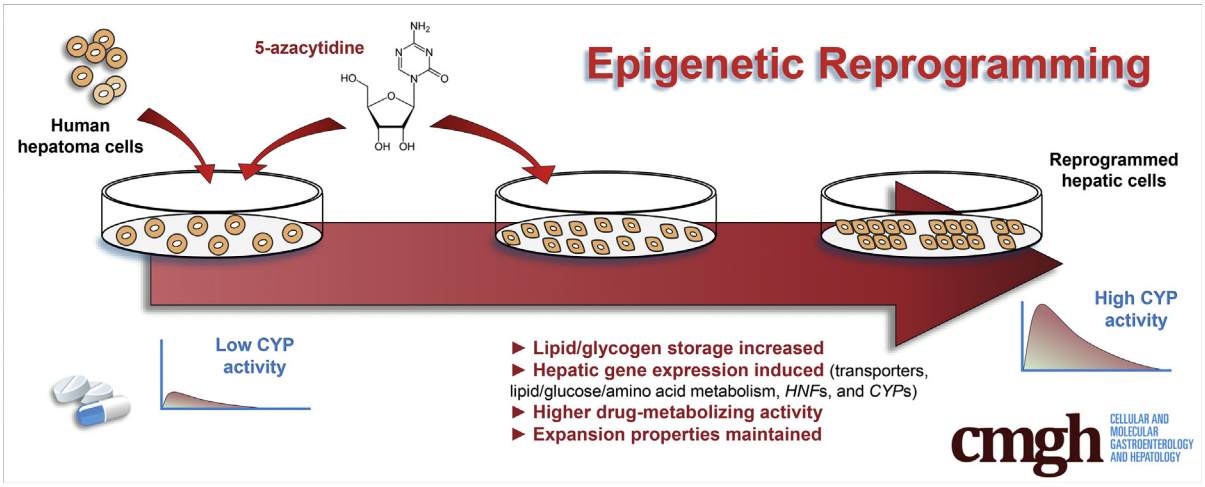

Supplemental Graphical Summary.