Effects of Retinoic Acid on Proliferation and Differentiation of HepG2 Cells

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Abstract: Hepatoma cell lines have characteristics derived from both their hepatic lineage and their transformation. We examined the potential of retinoic acid to enhance the function of a hepatoma cell line, HepG2, under conditions of reduced serum as are desired for bioreactor cultivation. We found that retinoic acid drastically slows the growth of HepG2 cells and induces a more spread morphology. Retinoic acid also augments the differentiated function of the cells, as measured by albumin and urea secretion rates. Expression levels of a panel of liver-enriched transcription factor were increased from retinoic acid exposure. Overall, we demonstrate that retinoic acid has significant effects on HepG2 growth and differentiated function. These results have implications for the use of retinoic acid both as a chemotherapeutic agent and as a medium component for cells of hepatic origin.

Keywords: Hepatocyte culture, differentiated function, liver-enriched transcription factors, gene expression.

INTRODUCTION

Hepatoma cells are useful in understanding both phenotypes of liver cancer and phenotypes of hepatocytes relevant to healthy and diseased states. Among human hepatoma cell lines, HepG2 is probably the best characterized. HepG2 cells serve a variety of functions in the laboratory, including assessment of the toxicology of compounds, such as antiviral drugs, genotoxicants and antigenotoxic agents [1-4]. HepG2 cells are also employed to study a range of physiological and pathophysiological conditions, including liver disease, gene expression and transcription in cancer, inflammatory response, and blood coagulation [5-8]. Furthermore, HepG2 cells are being developed as a potential cell source for bioartificial liver devices [9]. Their versatility and function make them extremely useful as a model system for liver function.

The liver is one of a few organs in the body capable of proliferation and differentiation when necessary in response to trauma, stress, or during homeostasis. There is often a balance between differentiation and proliferation, with cells ending their proliferation cycle through differentiation. However, when taken from an animal and placed into culture, primary hepatocytes do not proliferate and tend to exhibit decreased levels of differentiated functions. In contrast, HepG2 cells are capable of both proliferation and differentiation, which enhances their utility.

In the present study, we investigated the potential of retinoic acid to influence HepG2 growth, morphology, function, and gene expression. Retinoic acid, a derivative of vitamin A, plays many roles in cell differentiation, proliferation, and apoptosis. Retinoic acid has been shown to cause growth arrest and differentiation in several cancer cell lines [9-14]. Retinoic acid has previously been determined to slow HepG2 growth [15, 16], and high concentrations (166 μM), induced

apoptosis [17]. We show that significantly lower concentrations (1 – 10 μM) are sufficient to quantitatively alter morphology, proliferation, gene expression, and levels of hepatic differentiation markers under conditions of reduced serum in the culture medium, which is particularly advantageous for HepG2 cell cultivation in bioreactors.

MATERIALS AND METHODOLOGY

Cell culture. Human HepG2 cells (ATCC, Rockville, Maryland) were maintained in MEM medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 200 U/ml penicillin, 200 μg/ml streptomycin, 100 μM non-essential amino acids, 1 mM MEM sodium pyruvate, and 2 mM L-glutamine (Invitrogen) in a humidified 37ºC, 5% CO_2 incubator. Cells were plated at an initial density of 1×10^5 cells/cm^2 in 10% FBS media overnight. Cells were then grown for 14 days in MEM media supplemented with 2% FBS, with an equivalent volume of serum replacement solution containing 3 mg/ml bovine serum albumin (Sigma) and 315 mOsm/kg NaCl (Sigma), in the presence or absence of 10, 5, 2.5, or 1 μM trans-retinoic acid (tRA) (Sigma). The tRA was diluted from a stock concentration of 100 mM in DMSO (Sigma).

Image analysis. Images were taken on an Olympus CK40 at 20X magnification. Areas and aspect ratios were calculated using Olympus MicroSuite program with a sample size of 50 cells.

Real-time PCR. On days 7 and 14 of the experiment, cell samples were taken and RNA extracted using the Roche total RNA extraction kit (Roche). The cDNA was created using specific primers and AMV reverse transcriptase (Promega) according to the manufacturer's directions. Real-time PCR was performed using a Lightcycler (Roche) and QuantiTech SYBR Green PCR kit (Qiagen). RNA levels were normalized using 18S rRNA as a stable housekeeping marker. For a particular gene, mRNA levels were calculated using the formula mRNA$_{gene}$=2^($C_{T, 18S} - C_{T, gene}$), where C_T is the "crossing point" or (interpolated) cycle at which the fluorescence reaches a predetermined value. The mRNA level for each

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gene in each sample was then normalized within each experiment to that gene in a control sample.

Metabolic Secretory Assays. Albumin secretion rates in culture supernatants were detected *via* competitive ELISA using purified human albumin standards (MP Biomedicals) and peroxidase-conjugated secondary antibody for detection (MP Biomedicals) in accordance with previous protocols [18]. Urea secretion rates were measured from supernatants using an enzymatic urea nitrogen kit (Stanbio). Data were obtained in triplicates and fit to standards using either a semi-logarithmic (albumin) or a linear (urea) curve. These values were then normalized to the numbers of viable cells for each sample.

Statistics. Samples were of three separate experiments with three replicates each. Samples from each set were normalized to those for control, and the average was taken across experiments. Errors represent the standard error of the mean, and comparisons among groups were made by ANOVA, followed by Tukey's test for individual comparisons.

RESULTS

In order to observe both growth and differentiation effects of retinoic acid, we seeded HepG2 cells at a moderate density of 1.0×10^5 cells/cm^2 on 6 well plates in 10% FBS media and allowed them to attach overnight. Cells were then grown for 14 days in reduced serum (2% FBS) media, containing 0-10 µM all-trans retinoic acid (tRA), DMSO (which is the solvent for tRA), or untreated as a control. We observed the morphological and functional characteristics over a 14 day period following onset of treatment. Retinoic acid treatment induces several morphological changes, with cells becoming larger, and in some cases, multinucleated, at all of the tRA concentrations tested (data not shown). In comparison, DMSO and control cells maintain small size with a single nucleus per cell. Quantitative analysis of the cells shows that tRA treated cells are statistically larger than the corresponding controls and that the enlargement is not due to elongation, since the cell aspect ratio is not significantly altered by tRA treatment (Table **1**).

Table 1. **Cell Morphological Characteristics**

Treatment	Aspect Ratio	Area (µm^2)
Untreated	1.64 ± 0.06	427 ± 21
DMSO	1.50 ± 0.05	419 ± 16
1 µM tRA	1.66 ± 0.06	970 ± 50 (*)
2.5 µM tRA	1.55 ± 0.04	1260 ± 178 (*)
5 µM tRA	1.59 ± 0..05	1061 ± 52 (*)
10 µM tRA	1.61 ± 0.05	1249 ± 81 (*)

*p < 0.05 *vs* untreated control.

We then examined the effect of tRA on growth. Consistent with behavior observed in 10% FBS [16], retinoic acid retards the growth of HepG2 cells (Fig. **1**) at all concentrations employed (1-10 µM) in reduced (2%) serum. However, growth in reduced serum is slowed markedly as compared to

the corresponding conditions in 10% FBS [16]. The concentration of DMSO used as a solvent does not affect the morphology (Table **1**) or cell growth (Fig. **1**).
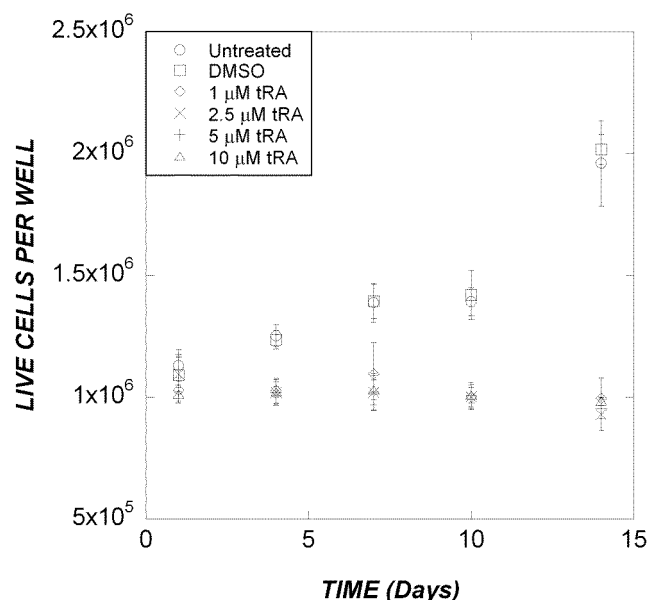

Fig. (1). Retinoic acid slows HepG2 cell growth. From days 4-14, all tRA treated samples are significantly different (p < 0.05) as compared to DMSO or untreated controls.

Since we observed differences in the morphology and growth, we examined whether these were accompanied by changes in differentiated function. Albumin and urea represent two prototypical markers of protein synthetic and metabolic differentiated function in hepatocytes, and the effect of tRA on these hepatic outputs has not been investigated previously. We find that albumin levels are not affected by tRA treatment until day 10, after which tRA treated cells produce markedly more albumin (Fig. **2**). By day 14 the effect of tRA on albumin secretion is dose-dependent, with a maximum in the range of 2.5 to 5 µM. We have observed a similar biphasic response in the albumin secretion of primary rat hepatocytes exposed to tRA (Burley and Roth. Submitted for publication). HepG2 cells also exhibit an increase in urea output with tRA treatment, with significant differences observed beginning at day 4 and continuing though the 14 day course of the experiment (Fig. **3**). The increase in urea production appeared to be weakly concentration dependent, with a slight maximum at 2.5 µM at 4 days that dissipated at later times.

The changes in morphology, growth, and function led us to examine the gene expression levels, using real-time PCR, of several liver-enriched transcription factors associated with hepatic differentiated function. We first examined the levels of albumin transcripts to determine if mRNA levels corresponded to secretion rates. Albumin mRNA levels are repressed slightly with DMSO treated cells and are increased for 5 and 10 µM tRA on days 7 and 14 respectively (Fig. **4A**). This corresponds with increased albumin protein secretion on those days (Fig. **2**). However, the magnitude of changes in albumin RNA levels were considerably less than the changes in secreted protein levels, suggesting that both transcriptional and post-translational processes may play a role in regulating increased albumin protein levels.
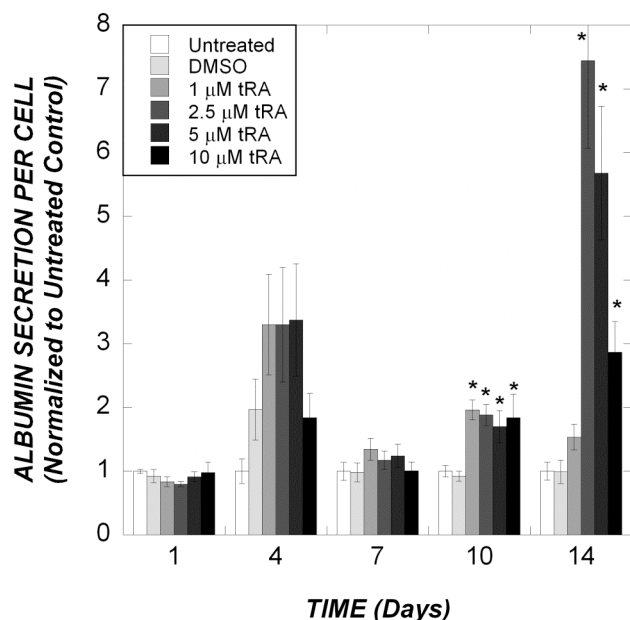
Fig. (2). Dose response effect of retinoic acid on albumin secretion. HepG2 cells were treated with varying concentrations of tRA over a 14 day period. Samples are normalized to untreated control. The symbols (*) represent $p < 0.05$ sample versus untreated control.

Fig. (3). Dose response effect of retinoic acid on urea secretion. HepG2 cells were treated with varying concentrations of tRA over a 14 day period. Samples are normalized to untreated control. The symbols (*) represent $p < 0.05$ sample versus untreated control.
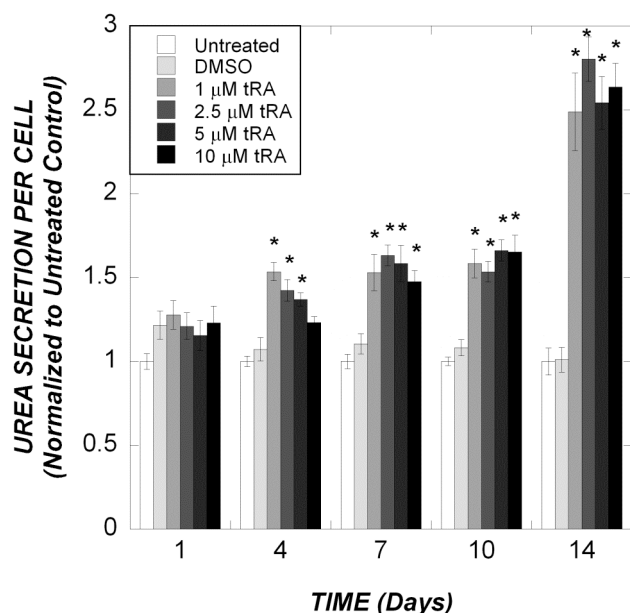
A panel of liver-enriched transcription factor genes that are expected to reflect the differentiated state of hepatocytes was profiled. Surprisingly, all of these genes were induced (upregulated) by tRA. However, their fold-change and dynamics varied considerably. The responses can be grouped into three categories: early, late, and continuous. The retinoic acid responsive genes that are induced early, as defined by high expression on day 7 and not day 14, are CEBPα (Fig.

4H), CEBPβ (Fig. **4I**) and HNF1α (Fig. **4B**). HNF4α (Fig. **4F**) exhibited a transient response to DMSO at day 7, masking tRA effects but was induced relative to DMSO or untreated controls for all tRA levels at day 14. The genes that are responsive to retinoic acid on both days 7 and 14 are HNF1β (Fig. **4C**), HNF3α (Fig. **4D**), HNF3β (Fig. **4E**), and HNF6α (Fig. **4G**). Some of the inductions were dramatic, including HNF6α with over a 70 fold increase for the 5 and 10 μM tRA conditions, CEBPα with a dramatic increase for all retinoic acid conditions, and CEBPβ with an over 30 fold increase for the 10 μM tRA condition.

DISCUSSION

The liver is an essential organ that regulates many metabolic processes. Liver diseases such as hepatitis A-C, liver cancer, and acute liver failure claim over 20,000 lives per year [19]. The best long-term solution for many of these patients is a liver transplant, but demand far outstrips supply. Liver assist devices, including bioartificial livers, can alleviate the problem, but there are many issues that limit development. One of the main issues is the type of cells being used in the bioartificial device. Primary human hepatocytes, the best cells for the task, are hard to come by and do not divide *in vitro*. Porcine hepatocytes and other xenogeneic cell lines, though relatively easy to acquire, are hampered by immunological problems and must be freshly isolated or cryopreserved with a concomitant loss in viablilty. Immortalized fetal or adult hepatocytes are another potential source, though they have limited function and division capability. Stem cells, from embryonic to hepatic, are an interesting alternative, but the ability to turn them into viable liver cells is still in progress.

Another approach is the use of human hepatoma cells lines, such as HepG2 [20]. One clinically tested liver assist device has used the C3A derivative of the HepG2 cell line [21]; however, this device did not result in improvements in overall survival or hepatic markers in phase I clinical trials. A major issue limiting the application of hepatoma cell lines is maintenance of differentiated function in liver assist devices. Furthermore, the ability to promote liver specific functions of HepG2 cells would aid in their application to toxicology screening and may be relevant in the context of hepatocarcinoma as well.

In the liver, there is a balance between differentiation and proliferation. Liver stem cells proliferate, then differentiate, in response to trauma, stress, or during normal development [22]. HepG2 cells recapitulate this balance, as they are capable of both proliferation and differentiation. There are several agents, such as trichostatin A, that may alter this balance by inducing cell cycle arrest and differentiation of HepG2 cells [23]. Such agents are of great interest due to their potential to prevent or slow tumorigenesis, but they could also be of great utility in the application of hepatoma cells for living cell devices, including bioartifical liver.

Derivatives of vitamin A, such as all-trans retinoic acid (tRA), play important roles in a variety of physiological processes, including vision, metabolism and differentiation [24]. Retinoic acid regulates the expression of many liver enriched genes, such as phosphoenolpyruvate carboxykinase (PEPCK), haptoglobin, and α-1 acid glycoprotein [25-27]. We found that tRA promotes the production of albumin and urea, two mark-
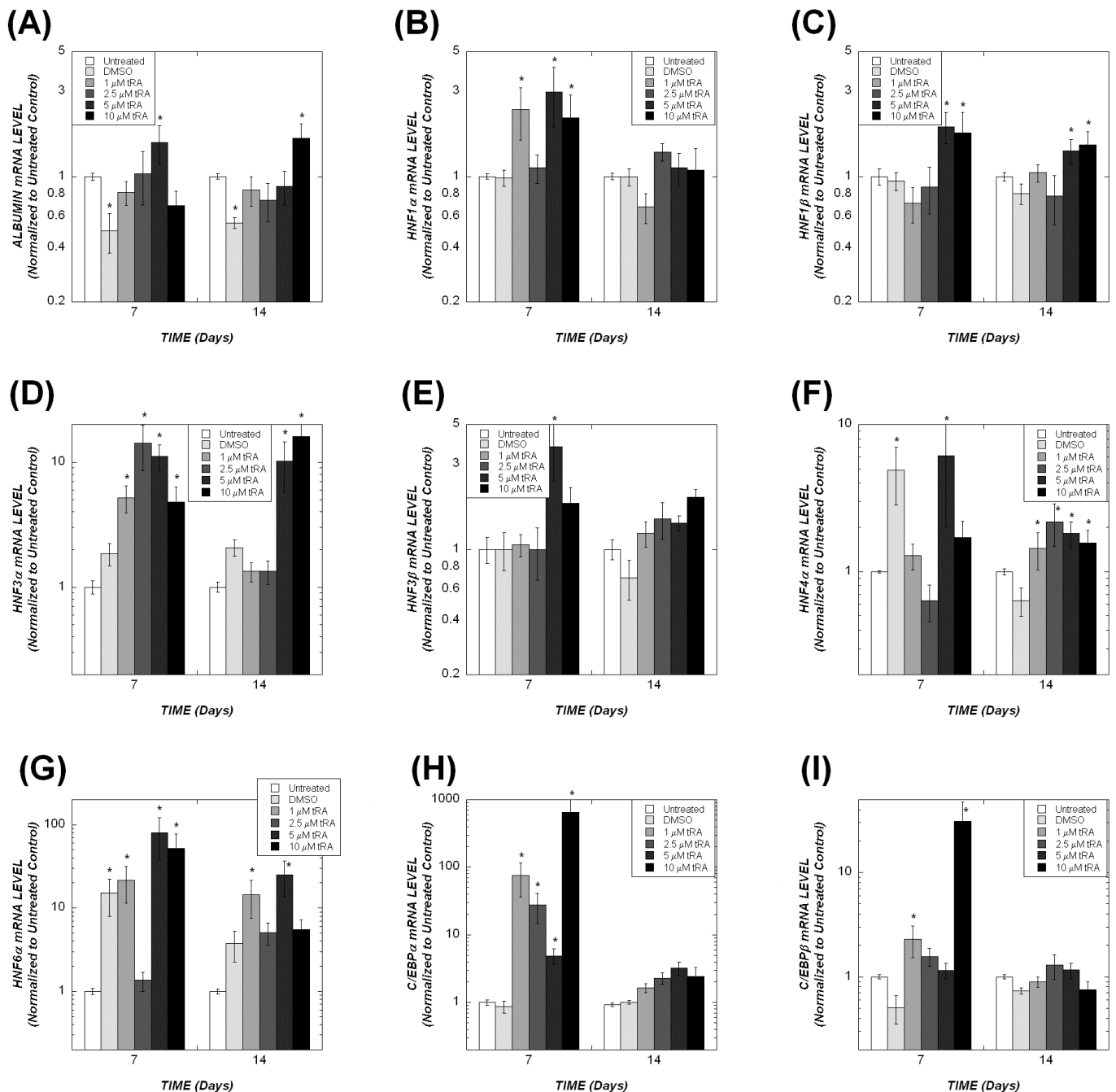
Fig. (4). Retinoic acid alters transcription factor levels. Albumin (**A**), HNF1α (**B**), HNF1β (**C**), HNF3α (**D**), HNF3β (**E**), HNF4α (**F**), HNF6α (**G**), CEBPα (**H**), CEBPβ (**I**). The symbols (*) represent $p<0.05$ sample versus untreated control.

ers of differentiation commonly used in the assessment of culture conditions for hepatocytes [28, 29]. Taken together, these results imply that retinoic acid activates signaling pathways and/or transcriptional programs that are fundamental to the differentiated hepatic phenotype.

It has been shown previously that tRA induces growth arrest and morphological changes in HepG2 cells [16]. The mechanisms by which this occurs are unclear. Retinoic acid induces growth arrest of HepG2 cells possibly by altering the activities of cyclin-cdk complexes involved in G1/S transition and S-phase progression [15]. Alternatively, the dramatic increase in cell area observed in this study under tRA treatment

implicates cell-matrix and cell-cell signaling as potential mechanisms that could lead to contact inhibition and alter the balance between proliferation and differentiation. For instance, tRA has been observed to reduce free pools of β-catenin [30], thus reducing proliferation signaling through the wnt pathway. Retinoic acid receptor α also has the capability to bind β-catenin, thus reducing the amount of TCF-4/ β-catenin complex, which is a proliferation signal [1]. Sequestration of β-catenin by E-cadherin has been shown to increase differentiated function of hepatocytes [31]; thus, tRA may work using a similar mechanism. This reduction in the proliferation signal may shift the balance towards differentiation.

However, RA influences a number of cellular pathways, and as such, its mechanism of action is probably multi-faceted. Retinoic acid may improve differentiation signals by increasing the amounts and phosphorylation of connexins, their stabilization in plasma membrane plaques and enhanced gap junctional intercellular communication [32]. Our results show that the mechanism of action involves induction of liver-enriched transcription factors. We observed dramatic increases in several liver-enriched transcription factors in response to tRA treatment, which appear to coordinately shift the balance toward differentiation. Transcription factor expression is often dynamic, and its influence on any particular target gene is combinatorial. Nonetheless, the fact that a cohort of liver-enriched transcription factors was unanimously induced is significant and indicative of a robust differentiation response. We have observed that differentiation function is induced by tRA both in HepG2 cells and in primary rat hepatocytes (Burley and Roth, submitted for publication). An agent such as tRA that can both control growth and promote differentiation is potentially beneficial for both anti-cancer applications and bioartifical liver devices.

ACKNOWLEDGEMENTS

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