Int J Clin Exp Med 2018;11(9):10183-10190
www.ijcem.com /ISSN:1940-5901/IJCEM0073535

Original Article

Differentiation of HepaRG cells into hepatocytes based on substrate elasticity

Jiazhi Li[1*], Zhi Zhang[2*], Zhiyong Dong[3], Lu Zhu[4], Jing Xu[5], Dan Wu[6], Wanqiang Liang[1]

[1]Department of Surgery, The Third Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi, China; [2]Department of Hepatobiliary Surgery, The First People's Hospital of Nanning, Nanning, Guangxi, China; [3]Department of Surgery, The Affiliated Hospital of Putian University, Putian, Fujian, China; [4]Institute of Medical Equipment, Academy of Military Medical Science, Tianjin, China; [5]Department of Hepatobiliary Surgery, The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi, China; [6]Department of Pediatrics, The People's Hospital of Guangxi Zhuang Autonomous Reglon, Nanning, Guangxi, China. [*]Equal contributors.

Received January 27, 2018; Accepted July 6, 2018; Epub September 15, 2018; Published September 30, 2018

Abstract: Objective: HepaRG cells have been used for many hepatic studies, but there isn't a most convenient way to control differentiation of HepaRG cells. The present study aimed to investigate a rapid and convenient way without changing the traditional medium for differentiation of HepaRG cells into hepatocytes and provide seed cells for a bioartificial liver. Methods: HepaRG cells were cultured on polymeric hydrogel substrates with altered mechanical properties. Protein levels of albumin (ALB) were measured by albumin-green fluorescent protein-reporter system (ALB-GFP-reporter system) and image J software. Immunofluorescence staining was used for detection of cytokeratin 19 (CK19) expression. Cell Titer-Blue cell viability assay kit (alamar blue) was used to quantify numbers of live cells. Results: Cells cultured on softer gels grew and proliferated in a spheroid phenotype. Levels of ALB were higher than the cells on stiffer gels along the cell culture time ($P<0.05$). CK19 expression was also higher on the softer substrate. Even on softer extracellular matrix (ECM), cells proliferate and have viability similar to cells on stiffer ECM ($P>0.05$). Conclusion: This work offers new insight concerning soft substrate elasticity which regulates differentiation of HepaRG cells, providing a potential method for achieving highly efficient and convenient differentiation of HepaRG cells into hepatocytes without changing the traditional culture medium. This will ensure cell-based researches and therapies for hepatic diseases.

Keywords: Bioartificial liver, dimethyl sulfoxide, HepaRG cells, hepatocytes, substrate elasticity

Introduction

Acute liver failure is a serious disease, with hepatocyte necrosis resulting in loss of liver function within weeks and high mortality [1, 2]. Bioartificial liver is a promising alternative for liver transplantation in the treatment of acute liver failure. However, its use is limited by appropriate hepatocytes [3, 4]. Normal human hepatocytes are the gold standard model *in vitro* for basic hepatic studies (including physiological function, hepatitis B virus infection, drug metabolism/toxicity, etc.) and clinical studies (3D printing model, bioartificial liver). Their use has some limitations, including scarcity, unpredictable availability, phenotypic variability, limited life-span, and growth activity [5]. Hepatocytes, derived from liver progenitor cell line HepaRG (isolated from hepatic tumor), fail to give rise to tumors and express a large panel of liver-specific functions, including albumin, transferrin, glycolytic enzyme aldolase B, cytochrome P450 (CYP)-related enzymes (CYP 2E1, CYP 3A4), phase I and phase II drug metabolism enzymes, and glutathione transferase (GST). These closely resemble normal human hepatocytes and are significantly higher than in HepG2 cells [6-8]. Thus, hepatocytes derived from HepaRG cells are a promising surrogate model to primary human hepatocytes. These are susceptible to bioartificial liver for the treatment of acute liver failure [9].

A frequently used 2-step procedure for HepaRG cell differentiation has been described in previous studies [6]. Briefly, cells are cultured for 2

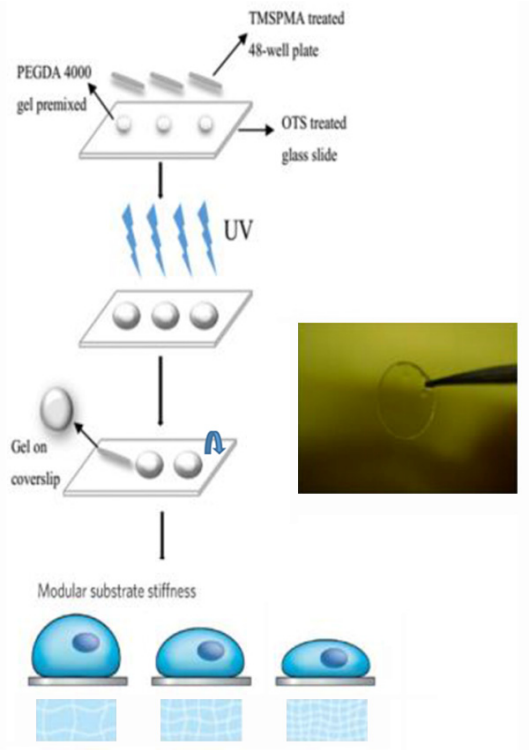

Figure 1. Schematic of the PEGDA hydrogel fabrication protocol. Prepolymer solutions of PEGDA (0%, 2%, 5%, 10%, respectively) were polymerized for stiffness gradient hydrogel substrates system.

weeks in the growth medium and then in the presence of 2% dimethyl sulfoxide (DMSO) for 2 more weeks. Although the presence of DMSO significantly increases expression levels of most drug-metabolizing genes (CYP1A2, CYP2B6, and CYP3A4) and maintains cell differentiation status, DMSO seems ineffective in upregulating the synthesis of hepatic functions in cluster-neighboring regions (ALB and ammonia elimination) and induces cell death [9-11].

Therefore, a friendly, rapid, and DMSO-free culture of cell differentiation is needed in research using HepaRG cell lines as a tool. It has been found that mechanical force balance generated by cadherin adhesions to neighboring cells, integrin adhesions to the extracellular matrix (ECM), and internal cytoskeletal struts establish cytoskeletal prestress stabilizing cells, tissue, and organ shape and structure [12, 13]. Alterations in the tensegrity-based mechanical force balance between neighboring cells, the ECM, and opposing cytoskeletal elements take control of cell fate switching due to biochemical responses caused by mechanical stresses or through channeling directly to the nucleus along cytoskeletal struts (filaments and molecules) connecting the cytoskeleton to the nucleus [14-17]. In addition, it has been found that soft ECM could promote muscle stem cells (MSC) differentiation into adipocyte and cell apoptosis by inactive YAP/TAZ located in the cytoplasm. Stiff ECM could promote MSC differentiation into osteoblast and endothelial or epithelial cells proliferation by active YAP/TAZ located in the nuclear [16]. However, Gilbert PM, et al. found that pliant hydrogel promoted MSC regenerative potential, increased the total number of cells, and reduced cell death [18]. This present study hypothesized that substrate elasticity can regulate rapid differentiation of HepaRG cells without changing the traditional culture medium. This study explored how alterations in mechanical forces generated by substrate elasticity influence differentiation of HepaRG cells, which are controlled by soluble chemical factors and genes.

Materials and methods

Materials

An incubator was purchased from Thermo (USA). Ultraviolet (UV) exposure was purchased from Excelitas (OmniCure SERIES 1500, Canada, 20 mW cm^{-2}). Fluorescence microscope was purchased from Nikon (Nikon Eclipse Ti-S microscope, Japan). HepaRG cell was a gift from Prof. Yanan Du, Tsinghua University. Cell Titer-Blue cell viability assay kit (alamar blue) was purchased from Promega (Wisconsin, USA). The photoinitiator, 2-hydroxy-1-[4-(hydroxyethoxy)-phenyl]-2-methyl-1-propanone (Irgacure D2959, I2959), was purchased from Insight High Technology Co. LTD (China). N-Acryloxysuccinimide (NAS) was purchased from J&K (China) and Williams' E was purchased from Gibico (USA). Additionally, 5 ug/mL insulin was purchased from Aladdin (China) and 1% penicillin-streptomycin solution was purchased from Wisent (Canada). Polyethylene glycol diacrylate (PEGDA) was purchased from Monomer Polymer & Dajac Labs. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated.

PEGDA hydrogel substrate elasticity fabrication

Hydrogel fabrication was performed as described in (**Figure 1**) [19]: First, PEGDA (0%

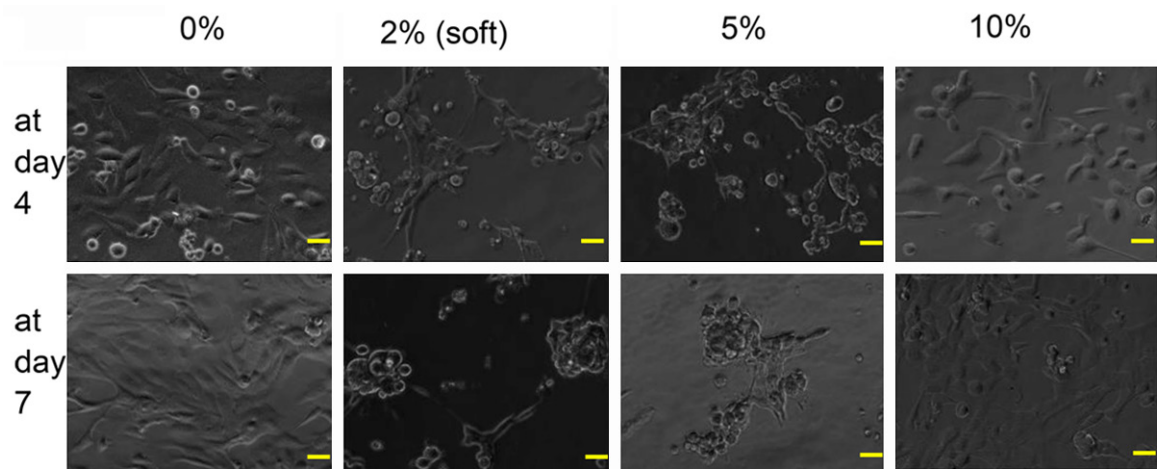

Figure 2. Morphology of HepaRG cells. Bright field images of fluorescent analysis for HepaRG cells, showing cells on softer gels grew in a spheroid phenotype (10×).

wv^{-1}, 2% wv^{-1}, 5% wv^{-1}, 10% wv^{-1}, respectively), I2959 (0.5% wv^{-1}), and NAS (1% wv^{-1}) were dissolved in cold phosphate-buffered-saline (PBS) solution to prepare PEGDA precursor solution. Second, the premixed solution was photo-crosslinked to form PEGDA hydrogel on a 3-(trimethoxysilyl) propyl methacrylate (TMSPMA)-treated 48-well plate by UV exposure for 4 seconds, with an octadecyltrichlorosilane (OTS)-treated glass slide placed on top of the solution to keep the surface flat. Stiffness gradient of hydrogel substrates was determined by PEGDA concentrations and gel thickness was controlled by premixed solution volume. Third, the hydrogel system was completely infused in 75% ethanol for 1 hour to sterilize and wash off the un-crosslinked molecules. Gels were incubated overnight at 4°C with 0.1% gelatin dissolved in distilled water for cell seeding.

Cell culture

HepaRG cells seeded into matrigel pre-coated (4°C overnight) hydrogel platform (48-well plate) at 2×10^4 cells/cm^2, were maintained for 7 days in William's E medium supplemented with 10% fetal bovine serum, 5 μg/mL insulin, 5×10^{-5} M hydrocortisone hemisuccinate, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2% DMSO. Cells were maintained at 37°C in a humidified 5% CO_2 incubator (Thermo) and fresh media were added every day.

Assay for ALB expression

Plasmid constructs expressing albumin-green fluorescent protein (ALB-GFP) were transfected into HepaRG cells on the first day to establish an ALB-GFP -reporter system. Afterward, at days 4 and 7 of induction, ALB-GFP-positive cells were observed and photographed using fluorescence microscope. The green FITC and blue DAPI channel signals were simultaneously collected to ensure that the green signal was observed from inside the cell. Subsequently, Image J software (NIH, USA) was used to extract cells with green threshold area over 20 in RGB color mode and automatically perform quantification of cell numbers with high-fluorescence expression representing ALB-GFP-reporter gene expression levels (same effect as FACS analysis).

Immunofluorescence staining for cytokeratin19 (CK19)

On day 7, cells were fixed with 4% paraformaldehyde, permeabilized using 0.5% triton, and blocked with 1% bull serum albumin (BSA). Next, cells were incubated with TRITC-coupled secondary antibody after overnight incubation at 4°C with primary antibody and counter-stained with 4',6-diamidino-2-phenylindole (DAPI). They were then observed using Nikon fluorescence microscopy. Blue fluorescence demonstrated positive CK19-staining.

Alamar blue detection for cell numbers

After 7 days of cultivation, Cell Titer-Blue cell viability assay kit (alamar blue) was added to mixed medium/well at 1:10. The reaction solution was extracted to a 96-well plate with 100 ul/well and incubated at 37°C for at least 2

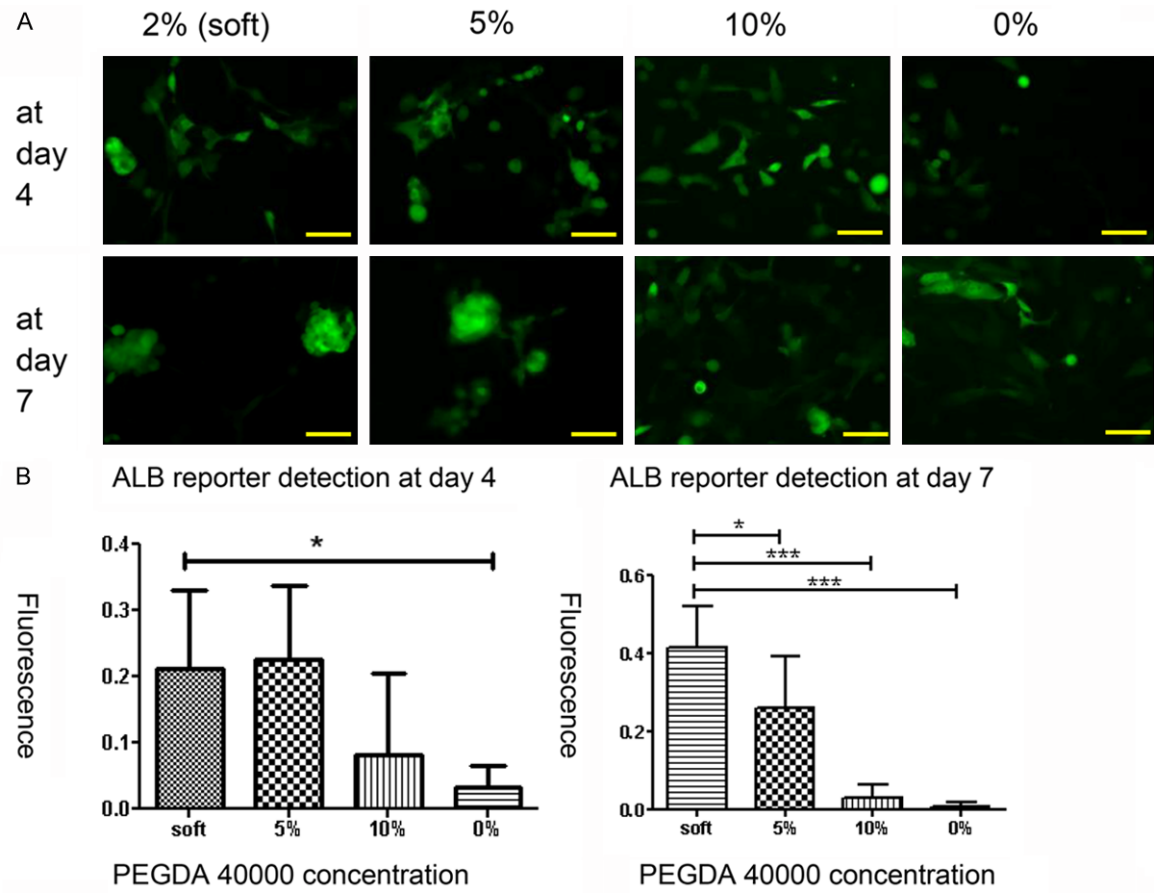

Figure 3. ALB-reporter gene-based fluorescence analysis. A. Fluorescent graphs for ALB reporter at day 4 and day 7 (20×); B. ALB levels were compared, respectively, at day 4 and day 7 (*; P<0.05, **; P<0.01, ***; P<0.001).

hours. A microplate reader (Molecular Devices, USA) was used to detect absorbance value (OD) reflecting relative levels of cell proliferation.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. One-way analysis of variance (ANOVA) was used to compare differences among groups. Differences are considered statistically significant when P<0.05.

Results

Stiffness gradient hydrogel substrates system

To investigate effects of different stiffness hydrogel substrates on HepaRG cell differentiation, this study framed a PEGDA-4000 hydrogel substrates platform with altered mechanical properties by controlling PEGDA concentrations. Substrate elasticity modulus (Young's modulus) from soft to stiff were 2% PEGDA (soft, <500 Pa), 5% PEGDA (500-800 Pa), 10% PEGDA (1.5-2 KPa), and 0% PEGDA (>5 KPa).

Morphology and ALB-reporter gene-based fluorescence analysis

Differentiation states of HepaRG cells were monitored by analyzing a representative level of ALB, only expressed in differentiated hepatocytes from HepaRG cells [6]. According to analysis of bright field images of HepaRG cells, cells on softer gels grew and proliferated in a spheroid phenotype (**Figure 2**). From ALB-reporter fluorescence analysis, fluorescent intensity on the softer hydrogel was stronger. At day 4, ALB expression in the soft group was significantly higher than the 0% (Glass) group, with statistical differences (P<0.05). There were no significant differences in comparison with the other groups (P>0.05). However, at day 7, ALB expression supported a favorable outcome for the soft group over all the other groups, according to pooled results of Image J software (P<0.05) (**Figure 3**).

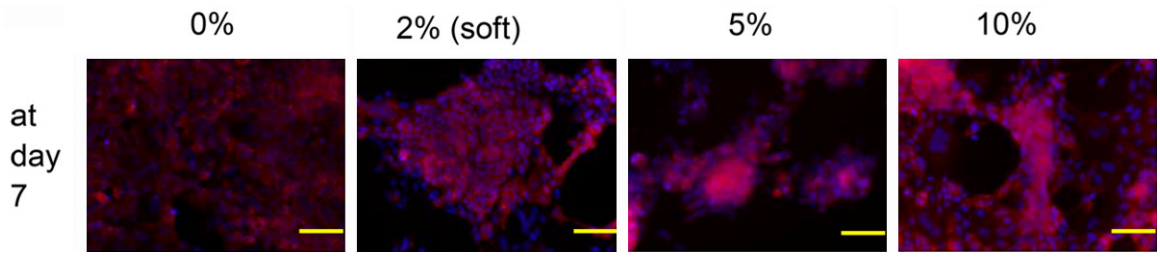

Figure 4. Expression of CK19 in HepaRG cells. Immunostaining of CK19 among groups at day 7 (20×).

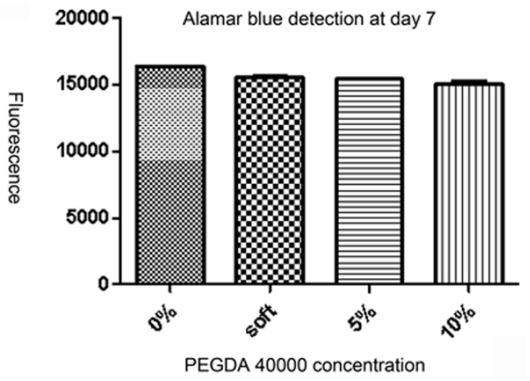

Figure 5. Cell viability. Cell numbers were estimated by alamar blue test at day 7, showing no statistical differences among the groups (P>0.05).

Expression of CK19

Whether CK19 expression is restricted to biliary-like cells is still debatable. However, Cerec, V. et al. found that CK19 expression was only in biliary-like cells by immunolocalization of CK19 in differentiated HepaRG cells, suggesting CK 19 as a marker for evaluating differentiation of HepaRG cells [7]. Thus, CK19 detection was performed with immunofluorescence staining at day 7. It was found that CK19 was expressed at all stages of HepaRG cell growth. However, on softer ECM, CK19 expression was higher (**Figure 4**).

Cell number

On day 7, cell viability quantification data supported the hypothesis that there were no statistically significant differences in cell numbers among the groups (P>0.05) (**Figure 5**).

Discussion

Biophysical properties, such as matrix rigidity, can alter cell fate involving differentiation and proliferation [18, 20, 21]. During a period of 7 days concerning stiffness gradient of hydrogel substrates, this study detected ALB-marker expression in cells on day 4 and 7. From fluorescence pictures and quantified data, ALB expression was higher on the softer gel than the stiffer gel for a longer time in culture, indicating that cells on softer gel can maintain the differentiated condition. Moreover, analysis of immunofluorescence staining showed that CK19 expression remained higher when culturing on the softer gel. These results suggest that HepaRG cells on softer hydrogels show better differentiation status. Cells grown in softer gels display a spheroid phenotype and may contribute to HepaRG cell differentiation [22].

To further investigate the effects of substrate elasticity in regulating differentiation of HepaRG cells, total cell number and viable cells were quantified. It was found that, even in softer ECM, cells can proliferate and have a high viability, with no statistical differences compared to stiffer ECM. This indicates that softer gel doesn't restrain cell proliferation and viability in the process of HepaRG cell differentiation. These results are consistent with Liu J's finding [23].

By establishing the PEGDA-4000 hydrogel substrates platform with altered mechanical properties, it was proposed that: (1) Substrate elasticity can regulate rapid differentiation of HepaRG cells and differentiated cells have optimal function; (2) Soft substrate elasticity maintains proliferation of HepaRG-derived hepatocytes while preserving their differentiation status, without transdifferentiating into bipotent progenitor independent of cell density. To justify the use of substrate elasticity, this study performed a similar inducing experiment of different substrate elasticity without 2% DMSO. Similar results with respect to ALB expression and cell number as well as hepatocyte-like and

biliary-like cells were observed, suggesting substrate elasticity can regulate rapid differentiation of HepaRG cells even in DMSO-free cell culture [24]. Consequently, this methodology represents a promising alternative to obtain a large number of mature hepatocytes, offering several advantages over the 2-step inducting method: (1) HepaRG cells can differentiate into two morphologically different cell types, hepatocyte-like and biliary-like cells, and differentiated cells express ALB and maintain their differentiation status independent of cell density, consistent with previous researches [25]. However, the time for induction of differentiation by substrate elasticity was shorter compared to the traditional induction method; (2) Cell proliferation and viability were not restricted during the differentiation process of HepaRG cells induced by softer substrate, compared with the latter.

It has been proven that intracellular YAP/TAZ, a transcriptional factor, plays a pivotal role in mechanotransduction regulation of cell fate by substrate stiffness [26, 27]. In a previous study, it was found that on soft gel YAP was mostly present in the cytoplasm, indicating that softer substrate promoted HepaRG cell differentiation through YAP relocalization (unpublished data). Multiple cellular signal pathways may participate in YAP relocalization in cytoplasm, thus, regulating the fate of HepaRG cells from a proliferative progenitor/stem cell to a differentiated one: (1) Softer substrate activates hippo pathways through mechanically competent cytoskeleton. Afterward, downstream Lats1/2 kinase are phosphorylated, resulting in YAP localization in cytoplasm and its subsequent inactivation, thereby leading to cell differentiation by downstream transcription factors (PPXY, TEAD, TEF, etc.) [28, 29]; (2) Mechanical signals from substrates are transduced through Wnt/β-catenin signal (restriction) or GPCR signal pathways directly or via cytoskeleton [16, 30, 31]; (3) Mechanical signals transmitted by F-actin-capping/severing proteins (e.g. cofilin, capZ, gelsolin, as YAP inhibitors) or consumption of stress fiber promote YAP/TAZ localization in the cytoplasm and its inactivation, independently, of the phosphorylation by Lats1/2 pathways, resulting in HepaRG cell differentiation [16, 32]; (4) HepaRG cells feel substrate elasticity by baroreceptors and further transfer mechanical signals though E-cadherin/β-catenin signal pathways, thereby regulating YAP/TAZ localization [33].

In conclusion, the present study proves that softer substrate elasticity can regulate rapid and convenient differentiation of HepaRG cells without changing the traditional culture medium, providing a potential method for achieving adult hepatocytes and an alternative to primary human hepatocytes for cell-based studies and therapies of hepatic diseases. Further basic research should be conducted for other liver-specific functions (e.g. CYP, GST) and optimal substrate elasticity.

Acknowledgements

This research was supported by Science and Technology Project of Putian University (No. 2013035) and Youth Science Foundation of Guangxi Medical University (No. GXMUYSF201542), China. We would like to thank Prof. Yanan Du for providing HepaRG cells and Bingjie Wang for her kind assistance in cell experiments (School of Medicine, Tsinghua University, China). We also want to thank Dr. Priyanka Saini for language modification (Robert H. Lurie Comprehensive Cancer Center of Northwestern University, USA).

Disclosure of conflict of interest

None.

Address correspondence to: Wanqiang Liang, Department of Surgery, The Third Affiliated Hospital of Guangxi Medical University, 99 Jinxiang Avenue, Nanning 530219, Guangxi, China. Tel: +86-13978857033; Fax: +86-771-4828336; E-mail: drliangwq@163.com

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