Experimental Cell Research 356 (2017) 48–56

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

# Deriving and testing of dysplastic murine hepatocytes: A new platform in liver cancer research

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ARTICLE INFO

*Keywords:*
Dysplastic
Pre-neoplastic
Primary culture
Transformed

ABSTRACT

Dysplastic hepatocytes (DH) represent altered hepatocytes with potential for malignant transformation. To date, most research on pathways to hepatocarcinogenesis has focused on use of "hepatoma" cell lines derived from hepatocellular carcinoma (HCC). We describe a novel technique for deriving/culturing DH and demonstrate their utility for functional studies *in vitro*, compared to primary hepatocytes (PH) and HCC. PH and DH were prepared by portal vein collagenase perfusion from C57BL/6J mice. DH were subsequently subjected to FACS. HCC from diethylnitrosamine (DEN)-injected mice were mechanically isolated. Cell cycle analyses were performed by flow cytometry and PCNA immunohistochemistry. To establish utility of DH, we studied pathways of p53 turnover, apoptosis and cell proliferation using pfithrin-α (PFT) and nutlin-3. Like PH, DH were minimally proliferative compared to HCC. Only 30 ± 0.03% of DH were in $G_2$/M phase versus 51 ± 0.01% of HCC; this difference corroborated with PCNA-immunostaining of dysplastic nodules from DEN-injected mice. In DH and HCC, nutlin-3 suppressed *p53* mRNA, induced p53 and mdm2 activation but paradoxically resulted in increased anti-apoptotic and proliferative activity. Primary murine DH display distinctive biological characteristics compared with PH and HCC. As an intermediate cell type to HCC, they offer a new pathobiologically relevant primary cell culture system with which to interrogate the molecular changes in hepatocarcinogenesis.

## 1. Introduction

Most studies in liver carcinogenesis utilize human or rodent hepatoma cell lines [1,2], which are immortalized, have been repeatedly passaged and some derived from tumors other than hepatocellular carcinoma (HCC, eg. hepatoblastoma for HepG2) [3]. It is therefore likely that these cells do not always reflect the properties of hepatocytes committed to hepatocarcinogenesis, and during their multiple lineages may have confounding somatic mutations [4]. HCC cell lines exhibit highly variable and contrastingly different transcript expression profiles of cytochrome P450 enzymes [5]. A recent in-depth comparative and quantitative proteomic analyses demonstrate considerable differences between primary human adult hepatocytes and HepG2 cells, especially in drug uptake, excretion transporters and Phase I /II metabolism enzymes [6]. These findings add further evidence that data derived from hepatoma cell lines in *in vitro* drug metabolism studies should be interpreted with caution.

Primary hepatocytes (PH) are readily isolated by collagenase perfusion of the portal vein and can be grown on collagen-coated surfaces in minimal media. However, their viability decreases rapidly with time in culture; 25% of cells are in apoptosis by day 2 [7]. For decades, hepatocarcinogenesis has been shown to proceed experimentally and in clinical samples via formation of dysplastic cells (dysplastic hepatocytes, DH), which are altered, self-replicating cells that have not yet taken on the invasive characteristics of HCC [8,9]. Preneoplastic alterations in gene

*Abbreviations:* HCC, Hepatocellular carcinoma; DH, Dysplastic hepatocytes; Mdm2, Mouse double minute 2; PFT, Pfithrin-α; PH, Primary hepatocytes; HBSS, Hank's balanced salt solution; EDTA, Ethylenediaminetetraacetic acid; BSA, Bovine serum albumin; FACS, Fluorescence activated cell sorting; DEN, Diethylnitrosamine; GPC-3, Glypican-3; PBS, Phosphate buffered saline; FBS, Fetal bovine serum; GST-pi, Glutathione S-transferase pi form; PE, Phycoerythrin; , AX488, Alexa Fluor® 488; EpCAM, Epithelial cell adhesion molecule; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, Dimethyl sulfoxide; PI, Propidium iodide; RNase A, Ribonuclease A; PCNA, Proliferating cell nuclear antigen; RT-PCR, Reverse transcriptase-polymerase chain reaction; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase

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http://dx.doi.org/10.1016/j.yexcr.2017.04.009
Received 1 March 2017; Received in revised form 6 April 2017; Accepted 8 April 2017
Available online 10 April 2017
0014-4827/ 

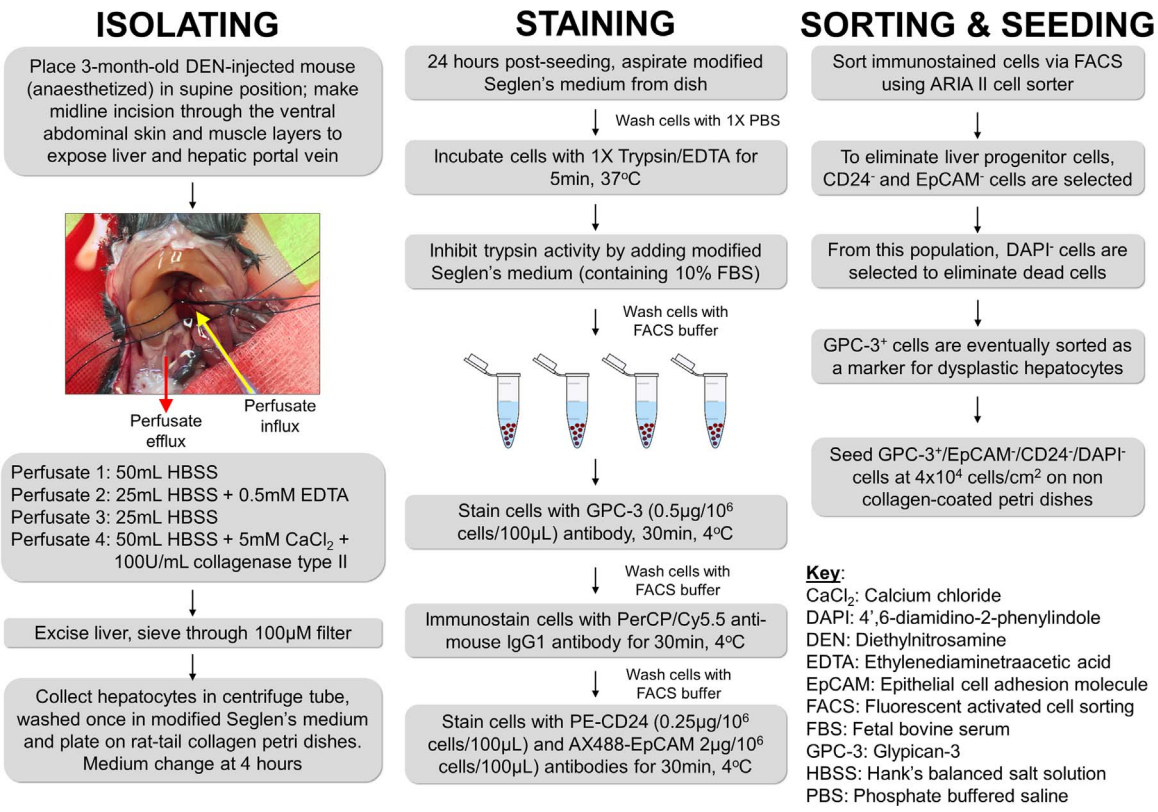


**Fig. 1. Schematic of derivation and isolation of primary dysplastic hepatocytes.** Hepatic portal vein collagenase perfusion was performed on anaesthetized 3mth DEN-injected mice. A 22G catheter was inserted via the hepatic portal vein and perfusate delivered to the liver at a rate of 6 mL/min. Perfusate was flushed via the inferior vena cava. The liver was excised and filtered through a 100 μM filter. Hepatocytes were washed with modified Seglen's medium, centrifuged at 50 *g* for 2 min at 4 °C and plated on uncoated petri dishes. Medium change was undertaken 4 h post-seeding. Twenty-four hours post-seeding, hepatocytes were trypsinized and stained with GPC-3 antibody, followed by a PerP/Cy5.5-conjugated secondary antibody. The cells were subsequently stained with fluorophore-conjugated CD24, EpCAM and DAPI before subjecting to FACS. Cells bearing GPC-3$^+$/CD24$^-$/EpCAM$^-$/DAPI$^-$ were selected and seeded at $4\times10^4$ cells/cm$^2$ on non collagen-coated petri dishes. Abbreviations: $CaCl_2$, calcium chloride; DAPI, 4′,6-diamidino-2-phenylindole; DEN, diethylnitrosamine; EDTA, ethylenediaminetraacetic acid; EpCAM, epithelial cell adhesion molecule; FBS, fetal bovine serum; GPC-3, glypican-3; HBSS, Hank's balanced salt solution; PBS, phosphate buffered saline.

expression in DH result from epigenetic changes, chromosomal instability, and point mutations or loss of heterozygosity in specific cellular genes [10]. Some investigators have defined the high grade dysplastic nodule as a small tumor mass lacking invasive growth properties such as vascular invasion or intrahepatic metastasis [11]. However, study of DH has generally been limited to tissue studies as they have proved challenging to isolate and culture separately.

In the present work, we describe a technique that selects for and increases the yield of DH, further enabling them to thrive on "uncoated" plastic surfaces in culture for more than 40 days. We then compared their cell cycle phase distribution detected *in vitro* by fluorescence activated cell sorting (FACS) and tissue expression of cell cycle markers. Finally, to establish whether DH have already developed dysregulation of p53, a hallmark of HCC, we modulated p53 and mdm2 (mouse double minute 2) expression using pfithrin-α (PFT) and nutlin-3.

## 2. Materials and methods

### 2.1. Animal procedures

Male C57BL/6 J mice were maintained in a specific pathogen-free facility, fed on a commercial diet pellet (Gordon's Specialty Stockfeeds, NSW, Australia) and allowed water *ad libitum*. Mice were injected once with diethylnitrosamine (DEN, 10 mg/kg body weight intraperitoneally [ip], day 12–15 of life, Sigma-Aldrich, MO) or with vehicle (0.9% saline) to controls. All animal procedures were approved by The Australian National University's Animal Experimentation Ethics Committee (protocol A2012/19).

### 2.2. Isolation of primary murine hepatocytes

Hepatic portal vein perfusion was performed in 6- to 8-week-old control mice using Hank's buffered salt solution (HBSS, Sigma-Aldrich, MO), 0.5 mM EDTA (Sigma-Aldrich, MO), 5 mM of $CaCl_2$ (Sigma-Aldrich, MO) and 100 U/mL collagenase type II (Worthington, NJ). Livers were excised, hepatocytes isolated and seeded at a density of $6.5\times10^4$ cells/cm$^2$ on rat-tail collagen coated plates (5 μg/cm$^2$, Gibco, CA). PH were maintained in culture in William's E medium (Gibco, CA) containing 1% bovine serum albumin (BSA, Sigma-Aldrich, MO), 10 mM HEPES (Sigma-Aldrich, MO), 10 mM nicotinamide (Sigma-Aldrich, MO) at 37 °C with 5% $CO_2$.

### 2.3. Fluorescence activated cell sorting (FACS) of pure populations of dysplastic hepatocytes (DH) and their maintenance in culture

Dysplastic hepatocytes (DH) were isolated by hepatic portal vein collagenase perfusion from 3 month (mth) DEN-injected mice. Cells were plated on plastic petri dishes, media changed at 4 h. At 24 h, primary cells in culture were re-suspended in 1X Trypsin/EDTA (Sigma-Aldrich, MO), stained with Glypican-3 (GPC-3, 0.5 μg/$10^6$ cells/100 μL, LifeSpan Bioscience, WA) antibody, then washed with FACS buffer (phosphate buffered saline [PBS, Amresco, OH] containing 0.1% fetal bovine serum [FBS, Hyclone, TX]) prior to incubation with PerCP/Cy5.5 anti-mouse IgG1 antibody (0.25 μg/$10^6$ cells/100 μL, Biolegend, CA).

Cells were also stained with phycoerythrin (PE)-conjugated CD24 (0.25 μg/$10^6$ cells/100 μL, Biolegend, CA), Alexa Fluor® 488 (AX488)-conjugated epithelial cell adhesion molecule (EpCAM) antibodies