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Hepatocyte Differentiation

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Abstract

Increasingly, research suggests that for certain systems, animal models are insufficient for human toxicology testing. The development of robust, in vitro models of human toxicity is required to decrease our dependence on potentially misleading in vivo animal studies. A critical development in human toxicology testing is the use of human primary hepatocytes to model processes that occur in the intact liver. However, in order to serve as an appropriate model, primary hepatocytes must be maintained in such a way that they persist in their differentiated state. While many hepatocyte culture methods exist, the two-dimensional collagen “sandwich” system combined with a serum-free medium, supplemented with physiological glucocorticoid concentrations, appears to robustly maintain hepatocyte character Studies in rat and human hepatocytes have shown that when cultured under these conditions, hepatocytes maintain many markers of differentiation including morphology, expression of plasma proteins, hepatic nuclear factors, phase I and II metabolic enzymes. Functionally, these culture conditions also preserve hepatic stress response pathways, such as the SAPK and MAPK pathways, as well as prototypical xcnobiotic induction responses. This chapter will briefly review culture methodologies but will primarily focus on hallmark hepatocyte structural, expression and functional markers that characterize the differentiation status of the hepatocyte.

Keywords

Primary hepatocytes; hepatocyte differentiation; cell culture; hepatocyte morphology; xenobiotic responsiveness; hepatic nuclear factors; cytochrome P-450; extracellular matrix; dexamethasone; phenobarbital

1. Introduction

The adult liver is the largest glandular organ in mammals and carries out critical life functions involving both endocrine and exocrine pathways. Hepatocytes comprise ~85% of the liver mass (1) and are the predominant contributors to liver physiology. Hepatocyte functions include glycogen storage, lipid and serum protein biosynthesis, biotransformation of a diverse array of dietary substances, and the detoxification of a large variety of xenobiotic compounds. Of the available in vitro hepatic models, primary hepatocytes offer substantial advantages, including conserved uptake and excretion functions, the integration

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of phase I and phase II metabolic pathways, and the presence of cofactors necessary for enzyme activity. Although in practice since the 1950s, early methods, involving perfusion of rodent livers under pressure, resulted in grossly damaged hepatocytes. Isolation methods were vastly improved by Berry and Friend (2) through the introduction of collagenase as a means to enzymatically disperse cells and by Seglen's introduction of the two-step method (3). This two-step method, now considered the standard isolation method, consists of an initial perfusion with a calcium-free buffer to disrupt desmosomes that make up the tight junctions between cells followed by a second perfusion with a calcium-rich buffer containing collagenase to further digest cell junctions. Another breakthrough in hepatocyte isolation methods was the modification of the procedure to use only segments of the liver, rather than the entire organ, allowing cost-efficient scale-up of the procedure to use larger livers, such as human (4–6). Despite the improvement in methods, hepatocytes from these early isolation experiments dedifferentiated quickly in culture, within a few hours losing hallmark features of in vivo liver function, such as albumin secretion and biotransformation activity (7–9).

This dediffetentiation phenomenon has sparked investigation both of the culture conditions that preserve the differentiated phenotype and of the mechanisms responsible for differentiation status. In general, an inverse relationship has been described between a well-differentiated, growth-arrested phenotype and a pioliferative one, marked by a G0/G1 transition that is triggered by the isolation process itself as defined by upregulated protooncogenes such as *c-fos, c-jun*, and *c-myc* (10, 11). This prolifeiative state in vitro has been further characterized by activation of cell cycle-stimulating and stress-related proteins, such as AP-1 (11–13) and NFKB (12, 14), and by loss of liver-enriched nuclear factors such as C/EBPα and the hepatocyte nuclear factor (HNF) family members (11, 12, 15, 16). While the induction of a proliferative state is advantageous for investigations of liver regeneration mechanisms, studies of xenobiotic metabolism require hepatocytes that respond with the fidelity of the in vivo fiver. Thus, considerable effort has been put forth to identify conditions in which hepatocytes remain well differentiated. Unfortunately, many investigators continue to use sub-optimal culture methodologies.

2. Cell Culture

2.1. Three-Dimensional Bioreactors

Although hepatocyte culture variations are abundant, for and include the culturing of hepatocytes as spheroids (17, 18) and in various co-culture configurations (17, 19), two of the most prevalent culture methodologies, when implemented appropriately, preserve a well-differentiated hepatocyte phenotype, namely the use of three-dimensional bioreactors or two-dimensional sandwich culture configurations. The former methodology embeds hepatocytes within complex three-dimensional chambers, most commonly hollow fiber membrane bioreactors (Fig. 6.1A). The hollow fibers, woven into a three-dimensional scaffold for hepatocyte attachment, act as capillaries through which defined culture medium is perfused, providing a continuous supply of oxygen and nutrients to the cells, efficient removal of waste products, and controlled fluid dynamics designed to mimic in vivo shear stress and interstitial flow (20–24), Under ideal bioreactor conditions, hepatocytes tend to

exhibit a differentiated phenotype, over several weeks in culture, with cuboidal morphology, extensive cell-cell contacts (22, 25), and specialized structures such as bile canaliculi (26, 27). Additionally, certain functional hallmarks are preserved, as hepatocytes in bioreactors synthesize both albumin and urea (21, 22, 25–28), excrete galactose (26, 28), and demonstrate various drug biotransformation activities (22, 23, 25).

Nonetheless, the continuous perfusion inherent to this model has some associated difficulties, as components derived from cells or present in the media can clog pores on the membranes, subsequently altering the flow and possibly resulting in gradients of nutrients or oxygen through the chamber (20, 26, 29). Additionally, even though the rate of perfusion is controlled, the flow of fluid may introduce excess mechanical stress that may disrupt normal hepatocyte dynamics (30–32).

2.2. Two-Dimensional Sandwich Culture

A relatively simple, but nonetheless, robust methodology is the sandwich culture system, where hepatocytes are embedded between a substratum of collagen and an overlay of either collagen or a commercially available extracellular matrix (ECM), such as Matrigel, a derivative of the Swarm-Engelbreth-Holm carcinoma (Fig. 6.1B). When adopted in the appropriate context, the sandwich culture method is capable of achieving prolonged hepatocyte viability (33, 34) and differentiated morphology, such that hepatocytes remain cuboidal in structure and form closely associated cellular networks (33, 35–37). Functional capacity is also improved, displaying appropriately polarized membrane domains (38–40), enhanced biotransformation activity (33, 41–43), and long-term albumin secretion (34, 36, 40). This configuration mimics the in vivo microenvironment, where, as shown in Fig. 6.2, hepatocytes are anchored to two opposing surfaces, even though the precise signaling pathways that this configuration preserves have not been dearly defined.

ECM components present in die space of Disse, in particular laminin and collagen, are thought to not only provide anchorage for hepatocytes in vivo, but also to promote differentiation. These matrix components participate in the preservation of normal cytoskeletal organization (35, 44) and regulate the expression of HNF family members (45–47) and albumin (48, 49), highlighting the importance of ECM in the maintenance of hepatocyte differentiation. Since extracellular signals are often communicated to the cytoskeleton via the integrin family of cell surface receptors, it has been suggested that integrin signaling is crucial for maintenance of differentiation (35, 50); αt3βl integrin, in particular, facilitates hepatocyte attachment to collagen (51, 52) and fibronectin (53) and overall preservation of a differentiated morphology (54).

Recently, phosphatidylinositol signaling has been identified as a potential link between integrins and cytoskeletal rearrangement, as ECM/Matrigel attachment causes an increase in phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) phosphatase mRNA, with a subsequent decrease in PI(4,5)P2 levels and actin polymerization (46). Furthermore, integrin-linked kinase (ILK) has recently been shown to play a critical role in matrix-induced hepatocyte differentiation (55). These studies demonstrated that ILK is present in the cell-ECM adhesion sites of cultured hepatocytes. Furthermore, hepatocytes isolated from ILK knockout mice appeared less differentiated in culture than hepatocytes from wild-type mice.

2.3. Defined Media Conditions

In addition to culture configuration, defined media conditions are critical for the maintenance of differentiated hepatocyte phenotype, in particular the presence of physiological, nanomolar levels of glucocorticoids, for example, in the form of the synthetic hormone dexamethasone, when coupled with the absence of serum in the culture medium. Dexamethasone is a potent activator of the glucocorticoid receptor (GR), a member of the nuclear hormone receptor superfamily that, prior to ligand binding, is complexed in the cytosol with HSP90, p23, and one of several tetratricopeptide repeat proteins (56–59). Ligand binding causes a conformational change in GR, revealing nuclear localization signals that stimulate nuclear translocation of the receptor (60, 61). Once in the nucleus, GR binds to specific response elements, acting as an anti-inflammatory and an immunosuppressant, largely through repression of the NFKB and AP-1 pathways (62–64).

In primary hepatocyte culture, dexamethasone additions promote a cuboidal phenotypic architecture, facilitate the expression of liver-enriched transcription factors, such as C/EBPα, HFN-4α, and RXRα (13, 36, 65, 66), and suppress the hepatocyte proliferative state otherwise stimulated by growth factors such as EGF (67). Although high doses of dexamethasone may stimulate proliferation (68), low concentrations are often included in culture media designed to induce hepatic lineage differentiation for embryonic stem cells derived from human (69, 70), monkey (71), and mouse (72). Importantly, inclusion of nanomolar concentrations in the hepatocyte culture media serves to inhibit the induction of stress signaling pathways, such as MAPK and SAPK/JNK (13).

In these respects, for human hepatocyte culture, our laboratory has adopted a highly defined, serum-free, two-dimensional sandwich system that configures hepatocytes with collagen I as the substratum and a dilute overlay of ECM, combined with serum-free medium containing nanomolar levels of dexamethasone (13, 36, 73). This sandwich system is appropriate for rat and human hepatocytes, and our protocol for human hepatocytes is briefly outlined below. In our human studies, primary hepatocytes were obtained from the Liver Tissue Cell Distribution System (reference NIH Contract –#N01-DK-7–0004/HHSN267200700004C). Hepatocytes are isolated according to a three-step collagenase perfusion protocol (74). Preparations enriched for hepatocytes are received plated in collagen-coated, tissue culture plastic flasks, or dishes. The culture media consists of William's Media E supplemented with 1% penicillin/streptomycin, 10 mM HEPES, 20 μM glutamine, 25 nM dexamethasone, 10 nM insulin, 30 mM linoleic acid, 1 mg/ml BSA, 5 ng/ml selenious acid, and 5 μg/ml transferrin. Within 4–16 h, an ECM overlay is added. A 10 mg/ml stock solution of Matrigei (BD Biosciences, San Jose, CA) is added dropwise to the culture media and evenly distributed by gentle swirling such that the final concentration is 225 μg/ml. Matrigei is a liquid at 4°C temperatures and rapidly gels at room temperature or at 37°C; therefore the additions of Matrigei need to be made rapidly, and typically using pipette tips that are pre-chilled in the freezer. The media is subsequently changed every 48 h until cells are harvested for RNA extraction. The cells are maintained at 37°C under 5% CO_2.Under these conditions, the hepatocytes are non-proliferative and are stable in culture for extended periods of culture, e.g., >2 weeks. *See* also Chapter 3 and 23 of the present volume.

3. Markers of a Differentiated Hepatocyte

3.1. Morphology

An often overlooked aspect of the differentiated hepatocyte is the status of the plasma membrane, namely that the membrane retains polarized domains, forms junctions between cells to facilitate cell-cell communication, and contains specialized structures like bile canaliculi. In vivo, hepatocytes are arranged in plate-like arrays, focing the sinusoids on one side and bile ductules on the other. The plasma membrane is functionally compartmentalized based on these interactions, such that the basolateral, or sinusoidal, membrane is specialized for exchange of metabolites with circulating blood (Fig. 6.2). Similarly, the apical, or canalicular, membrane is specialized for bile secretion, and the lateral membrane, joining adjacent hepatocytes, is specialized for intercellular communication (35, 75). Functional polarity in vitro is demonstrated by marker proteins specific for lateral domains, such as connexins 26 and 32; basolateral domains, like epidermal growth factor receptor; and apical domains, such as dipeptidyl peptidase IV (40, 76–79). Alternatively, hepatobiliary transport, shown by the appropriate accumulation and excretion of bile acids and other organic anions (38, 39, 80–82), and gap junctional intercellular communication between adjacent hepatocytes (78, 79) demonstrate the compartmentalization of these specialized functions. As dedifferentiation occurs, the cuboidal networks of cells often flatten and lose expression of specialized structures such as bile canaliculi, as well as distinct cell–cell contacts (35, 40, 45, 83, 84).

Microscopically, in optimally cultured hepatocyte preparations, many of the morphological features of hepatocytes are visible. Figure 6.3 shows examples of primary human hepatocytes cultured in the absence and presence of Matrigel. The cells cultured in the presence of Matrigel (Fig. 6.3B, D and F) exhibited characteristic cuboidal, three-dimensional structure, and enhanced cell border definition. In contrast, cells cultured without Matrigel (Fig. 6.3A, C and E) exhibit a more flattened appearance, weakly defined borders, and evolve fibroblast-like spinous processes, indicative of dedifferentiation. A further example of the morphological features is illustrated in a previous study of the effect of culture conditions on rat hepatocytes (13), as presented in Fig. 6.4. These rat hepatocyte studies serve to illustrate the importance of low concentrations of glucocorticoid additions. In Fig. 6.4, hepatocytes were cultured in the sandwich configuration as described above along with varying concentrations of dexamethasone. Omission of dexamethasone resulted in perturbation of the cuboidal networks, with cells exhibiting condensed cytoplasm, abnormal rounding of cell structure, and formation of fibroblast-like protrusions. Further, as a measure of hepatocyte toxicity associated with morphological disruption, lactate dehydrogenase (LDH) leakage from the cells was assessed. In addition to protecting morphological integrity, nanomolar additions of dexamethasone protected against cytotoxicity, attenuating LDH leakage (Fig. 6.4).

3.2. Immunofluorescence

Expression of cytokeratins 18 and 19 is a widely recognized feature of differentiated hepatocytes, therefore its detection in cells via immunofluorescence is a useful marker of the mature phenotype. For example, investigators assessing the progression of embryonic stem

cells down the hepatic lineage often assess these markers (85–87). As indicated previously, expression and localization of connexin 32 is a hallmark feature of hepatocyte gap junctions. Our studies have shown that in the presence of Matrigel, hepatocytes exhibit enhanced gap-junctional formation, as assessed by immunofluorescence detection of connexin 32, when compared with hepatocytes cultured without Matrigel (88). ILK, a key factor in matrix-induced hepatocyte differentiation (55), is another hepatocyte marker that can be assessed using immunofluorescence. This marker is visible at the ECM adhesion sites of hepatocytes in culture.

3.3. Plasma Proteins

The most frequently assessed markers of hepatocyte differentiation include expression of plasma proteins such albumin, transferrin, transthyretin, and α−1-antitrypsin (45, 80, 84, 89–91), in that this organ is the dominant site of plasma protein synthesis (92, 93). On the other hand, hepatocyte dedifferentiated is reflected typically by the up regulation of alpha-fetoprotein (AFP) and glutathione-*S*-transferase P1 (GSTP1; GSTπ) (94, 95). AFP is normally silenced in adult livers and therefore an increase in its expression within primary hepatocyte cultures is indicative of a dedifferentiation process toward a fetal lineage (95). Similarly, GSTP1 is expressed selectively in fetal liver and silenced in the mature hepatocyte (94). Therefore, both of these markers are particularly useful indicators of cultured hepatocyte dedifferentiation status, largely repressed in differentiated cells but augmented in hepatocytes undergoing dedifferentiation processes. Quantitative RT-PCR (qRTPCR) analyses are convenient assays to conduct in this regard and assays for literally any human or mouse gene transcript are available commercially from sources such as Applied Biosystems (Carlsbad, CA). Figure 6.5 shows results of qRTPCR analyses for markers of differentiation and dedifferentiation on total RNA isolated from primary human hepatocytes maintained in defined culture media containing dexamethasone at physiological levels, in the absence and presence of ECM/Matrigel. When comparing expression profiles of selected markers between human liver, human hepatocytes cultured with Matrigel, and a commonly used human hepatoma cell line, hepatocytes cultured in the presence of a Matrigel overlay most closely resemble the expression profile of the human liver, while HepG2 cells, although expressing certain markers, differed from the expression levels of the liver by at least 10-fold and as much as 200-fold (Fig. 6.5). In other studies (data not shown), further comparisons to additional human liver tissues, from six different donors, were also conducted, with similar conclusions derived as that for the representative HL#154 liver presented here. Therefore, the cumulative evidence indicated that a Matrigel overlay was a positive regulator of differentiation status of primary human hepatocytes, facilitating the up regulation of differentiation makers, down regulation of de differentiation markers.

3.4. Cytochromes P450 and Hepatic-Enriched Nuclear Factors

Another hallmark feature of the liver is its biotransformation activity; thus, cytochrome P450 (CYP) monooxygenase and phase II enzyme expression and activity (36, 41, 90, 91, 96) are commonly used markers of hepatocyte differentiation. In addition, a number of liver-enriched nuclear factors, including HNF family members, CAAT/enhancer binding protein α (C/EBPα), and nuclear hormone receptor superfamily members, are prominently expressed in the mature liver and are engaged in critical regulatory roles underlying the maintenance of

biotransformation enzyme function as well as many other differentiated features of the hepatocyte. For example, the expression of C/EBPα has been noted to decline both as expression of protooncogenes increase and as normal morphology is altered (11–13,84), whereas the HNF4 family members play a role in liver-specific gene expression; targeted knockdown of this transcription factor results in decreased expression of the plasma proteins albumin and transthyretin (45, 46).

Studies from our laboratory have also used whole genome expression profiling in human liver samples and in the commonly used HepG2 and Huh7 human hepatoma cell lines to determine mRNA expression levels coding for biotransformation enzymes and hepatic nuclear factors. When cultured in a two-dimensional Matrigel sandwich configuration, the transcription factors were tightly regulated in hepatocytes obtained from various human donors, as expression of the genes was maintained at levels less than 4-fold changed from liver (Fig. 6.6A). Among the two hepatoma cell lines studied, the expression profiles of the various transcription factors varied considerably compared to that of liver or primary hepatocytes, and there were notable differences in expression character even between the two cell lines. For example, mRNAs for *NR1I2* (pregnane X receptor (PXR)) and *NR1I3* (constitutive androstane receptor (CAR)) were undetectable in Huh7 cells and were >6- and 42-fold decreased in HepG2 cells, respectively (Fig. 6.6B). The expression levels for the retinoid X receptor-α (RXROA) were reduced ~5-fold in both of the respective cell lines, compared to liver. Generally, mRNAs for *CYP450* family members were expressed in hepatocytes at levels comparable to those detected directly in liver, with the exception of *CYP1A2* and *CYP2E1*, which were decreased (Fig. 6.6C). In contrast, in the hepatoma lines expression of *CYP450* isoforms is dramatically decreased or non-existent (Fig. 6.6D). These studies demonstrated that in vitro hepatocytes, in a sandwich culture with defined medium, are reasonably representative of in vivo liver, while the HepG2 and Huh7 ceils exhibited markedly deviant, dedifferentiated phenotype. When considering these comparative studies, one should also keep in mind that liver itself is comprised of ~80% hepatocytes, with the remainder of the tissue consisting of other types of cells, such as endothelial, biliary, and stellate cells. In tills regard, the measured comparisons refered to here between primary hepatocyte cultures and actual liver are likely even closer then otherwise indicated in these studies (88).

4. Stress Pathways and Hepatocyte Integrity

The importance of appropriate culture conditions on hepatocyte differentiation has been outlined above, but to further illustrate this point, previous studies from our laboratory demonstrating the interaction of culture conditions and stress pathways are presented. A compromised differentiation status is associated with the activation of stress-associated pathways in cultured hepatocytes, including the MAPK, SAPK/JNK, and c-Jun signaling pathways. For these studies, rat hepatocytes were cultured in a serum-free, highly defined medium in the absence and presence of Matrigel/ECM and with varying concentrations of dexamethasone. Cells cultured in the absence of dexamethasone exhibited a marked stimulation of p42/44 MAPIC, SAPK/JNK, and c-Jun phosphorylation (Fig. 6.7). The presence of Ma trigel served to attenuate the activation of these pathways, even at the 1 nM dexamethasone dose. The stress activation responses were blunted completely with 5 nM

dexamethasone. In contrast, cells cultured in the absence of a Matrigel overlay exhibited stress pathway activation responses that could only be attenuated modestly, even at the highest concentrations of dexamethasone tested. Thus, there is an apparent synergy between the effects of Matrigel and dexamethasone in providing attenuation of the stress cascades. It is interesting to note that omission of dexamethasone or Matrigel only had minimal impact on the phosphorylation status of PKB, a critical and positive effector of cell survival and death (Fig. 6.7). This latter result suggests that the cell survival stimulus associated with dexamethasone is independent of a PI3 kinase pathway. Consistent with the activation of the MAPK, SAPK/JNK, and c-Jun signaling pathways, limiting dexamethasone concentration also resulted in increased nuclear accumulation of the AP-1 complex ((13); data not shown). These results are consistent with a loss of control of the signaling machinery regulating cell cycle progression and mitogen-activated growth. Thus, it appears that dexamethasone and Matrigel prevent proliferative signals at the level of AP-1 activation and cell cycle progression, thus preserving the differentiated hepatocyte phenotype.

5. Functional Assessment of Hepatic Phenotype

An array of additional functional end points can offer insight into the degree of differentiation, due to the wealth of physiological functions in which the in vivo liver plays a role, including the synthesis of urea, clotting factors, and acute phase proteins (25, 26, 28, 91), synthesis of glucose and subsequent glycogen storage (26, 28, 80), excretion of bilirubin (39), and lipid and cholesterol transport (84). Use of the periodic acid Schiffs staining technique (American Master Tech Scientific Inc., Lodi, CA) is a useful method for detection of intracellular glycogen (85). Hepatic glutamine metabolism in connection with urea synthesis is required for systemic ammonia detoxication and pH regulation. Due to the important role of the liver in maintaining ammonia and bicarbonate homeostasis under physiologic and pathologic conditions, ammonia metabolism is often used as a functional marker of hepatic phenotype (97, 98).

5.1. Xenobiotic/Drug Induction Responses

A primary function of the liver is to conduct the metabolism of endogenous, dietary, and xenobiotic substances. Typically, the xenobiotic biotransformation process is typified by both phase I monooxygenation reactions, followed by phase II synthetic processes. The phase I process trends toward detoxification, with the resulting metabolites being more water soluble and exhibiting increased likelihood to undergo further reactions via phase II conjugation pathways. However, a large number of procarcinogens and other environmental toxins are bioactivated by the xenobiotic metabolizing CYPs. Several classes of environmental and therapeutic substances are recognized for their capacity to markedly modulate the transcriptional status of mammalian biotransformation enzymes. There are several prototypical inducing agents, including the polyaromatic and polychlorinated hydrocarbons, ethanol and organic solvents, peroxisome proliferator compounds such as the phthalate esters, dexamethasone, and several sedative-hypnotic medications. These substances tend to regulate their corresponding biotransformation enzyme pathways via the interplay of an array of soluble and nuclear receptors (99). Therefore, based on the complex series of events leading to xenobiotic induction of hepatic gene function, the ability of

cultured hepatocytes to respond to xenobiotic inducers is insightful and potentially a uniquely specific indicator of their differentiated state. Studies from our laboratory (13, 36, 73, 88) and others (83, 100–102) have shown that under proper maintenance conditions, hepatocytes will respond appropriately and robustly to a given xenobiotic-inducing agents. Several of the induction pathways are rather robust and are maintained in both established cell lines and even in hepatocytes that are maintained sub-optimally in culture. An exception is phenobarbital (PB). Although used in humans as a sedative and anti-seizure agent without serious long-term adverse effects (103), PB promotes rodent tumorigenesis through mechanisms including inhibition of apoptosis (104), activation of β-catenin (105), selective promotion of cells with low TGFβ receptor expression (106), reduction in G1 checkpoint efficiency (107), and alteration of DNA methylation (108). Mechanistically, PB mediates these effects through activation of the constitutive androstane receptor (NR1I3, or CAR), a member of the nuclear hormone receptor super family of transcription factors (reviewed in (109–112)), In vivo, CAR is retained in the cytoplasm complexed with HSP90 and die tetratricopeptide repeat-containing protein cytoplasmic CAR retention protein (CCRP), until activation by xenobiotics such as PB induces nuclear translocation (113–116). Once in die nucleus, CAR forms a dimer with RXRα (117) and recruits coactivator proteins, such as steroid receptor coactivator 1 (SRC-1) (118), GR-interacting protein 1 (GRIP-1) (119), and peroxisomal proliferator-activated receptor-γ coactivator 1α (PGC1α) (120), to drive transcription of genes, notably *CYP2B* and *CYP3A* family members, containing PB-responsive enhancer modules (PBREMs) within their promoter regions (121, 122). The PB induction response is typically lost in hepatoma-derived cells or in primary hepatocytes cultured in sub-optimal conditions. An example of the PB induction response that is obtainable in primary cultures of human hepatocytes, and not apparent in most human hepatoma cell lines, is shown in Fig. 6.8. The, authors contend that assessment of the PB induction response in particular appears to serve as a uniquely sensitive and important marker of hepatocyte differentiation status (13).

6. Species-Specific Considerations

Even though there are noted differences across species, the vast majority of validation studies have been carried out in hepatocytes of rodent origin due to limitations in the availability of human hepatocytes. Although further experiments with human hepatocytes may only confirm current culture methodologies, past experience has shown that there are inherent species-specific phenotypic differences in hepatocytes. For instance, early isolation studies reported significantly lower viability in rat and hamster hepatocytes vs. those from mouse and rabbit under the same conditions, as well as a steep decline in cytochrome P450 content in mouse and rat hepatocytes vs. nearly unchanged concentrations in those from rabbit (9). Time-course discrepancies have also been noted for membrane repolarization, in that co-localization of canalicular transport proteins with canalicular markers occurs faster in hepatocytes from rats compared to those from humans (76). Fur titer, while a sandwich culture configuration was demonstrated as critical for the induction of biotransformation enzymes in rat hepatocytes (36, 83), some studies have concluded that a collagen or Matrigel overlay is not vital for enzyme induction in primary human hepatocytes, despite improved morphology and cytoarchitecture in sandwich culture (123). Considering these species-

specific responses to in vitro conditions, thorough evaluation of any primary hepatocyte culture systems is warranted in order to secure confidence in its use as a model for liver biology or as predictive tool for in risk assessment.

7. Conclusion

This chapter summarizes an otherwise large body of available information relating to hepatocyte function and provides the reader with an overview of appropriate experimental methodology that can be applied to assess the biological character of primary hepatocytes in culture. It is not intended to be a complete compilation of these issues; rather, this chapter strives to delineate and discuss several important considerations of hepatocyte biology that should be considered in the evaluation of a given primary culture system. Careful attention to criteria such as morphology, functional end points, and expression of appropriate differentiation/dedifferentiation markers are required in any in vitro hepatocyte model system in order to validate its use and robustness as accurate model of hepatocyte phenotype as it exists in vivo.

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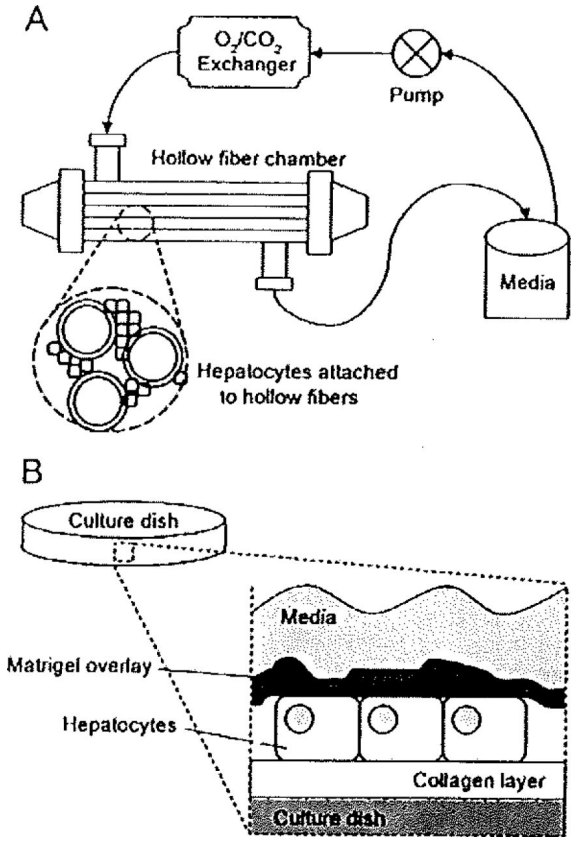

Fig. 6.1.

Illustrations of two primary hepatocyte culturing methodologies that preserve a differentiated phenotype. (**A**) Hollow fiber membrane bioreactors generally contain the following components: a reservoir containing defined media, a pump, a carbon dioxide/oxygen exchanger, and a chamber containing a complex network of hollow fibers enabling both media perfusion and sites of hepatocyte attachment. (**B**) In the sandwich culture system, hepatocytes are typically embedded between a collagen substratum and a dilute Matrigel overlay. Other forms of sandwich culture include the direct attachment of cells to either tissue culture plastic or poly-lysine-coated surfaces, followed by Matrigel overlay.

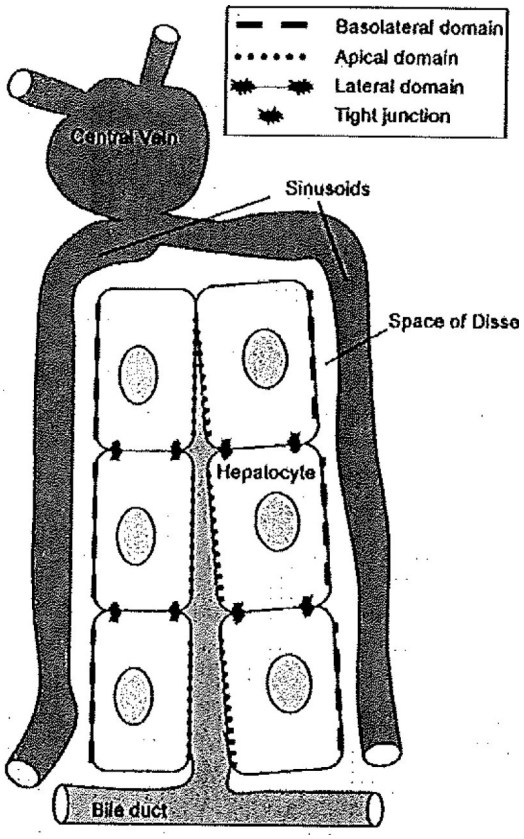

Fig. 6.2.

Illustration of hepatocyte plate structure in the liver. The circulatory blood vessels and polarity features of the hepatocyte are indicated. Hepatocytes in vivo have polarized membranes with specialized function based on location within the liver lobule. The basolateral, or sinusoidal, domain is specialized for exchange with blood, the apical, or canalicular, domain is specialized for bile secretion, and the lateral domain is specialized for intercellular communication. The various domains are separated by tight junctions.

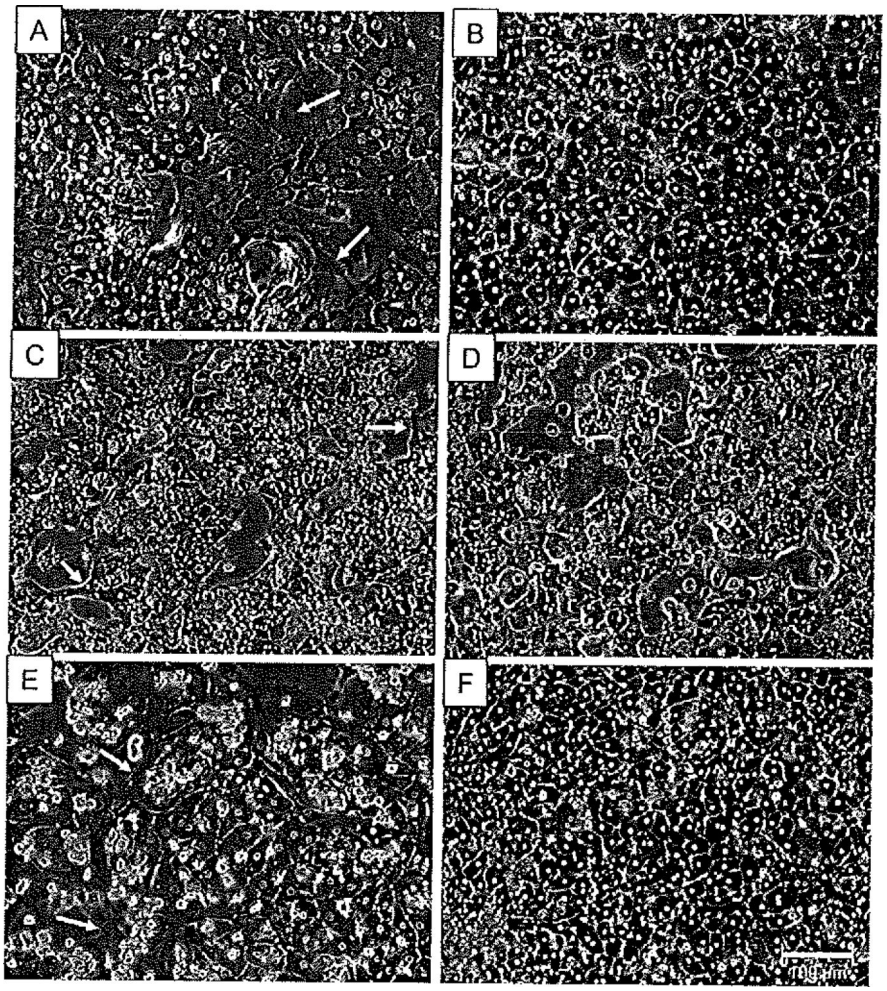

Fig. 6.3.

Matrigel enhances cellular morphology of primary human hepatocyte cultures. Primary human hepatocytes from Donor A (**A** and **B**), Donor B (**C** and **D**), and Donor C (**E** and **F**) were cultured in the presence (**B, D, F**) or absence (**A, C, E**) of a Matrigel overlay. Photomicrographs were taken under ×20 magnification using phase-contrast imaging. *Arrows* indicate compromised morphology in the absence of a Matrigel overlay. Reproduced from Toxicological Sciences, 2007 (73) with permission from Oxford University Press.

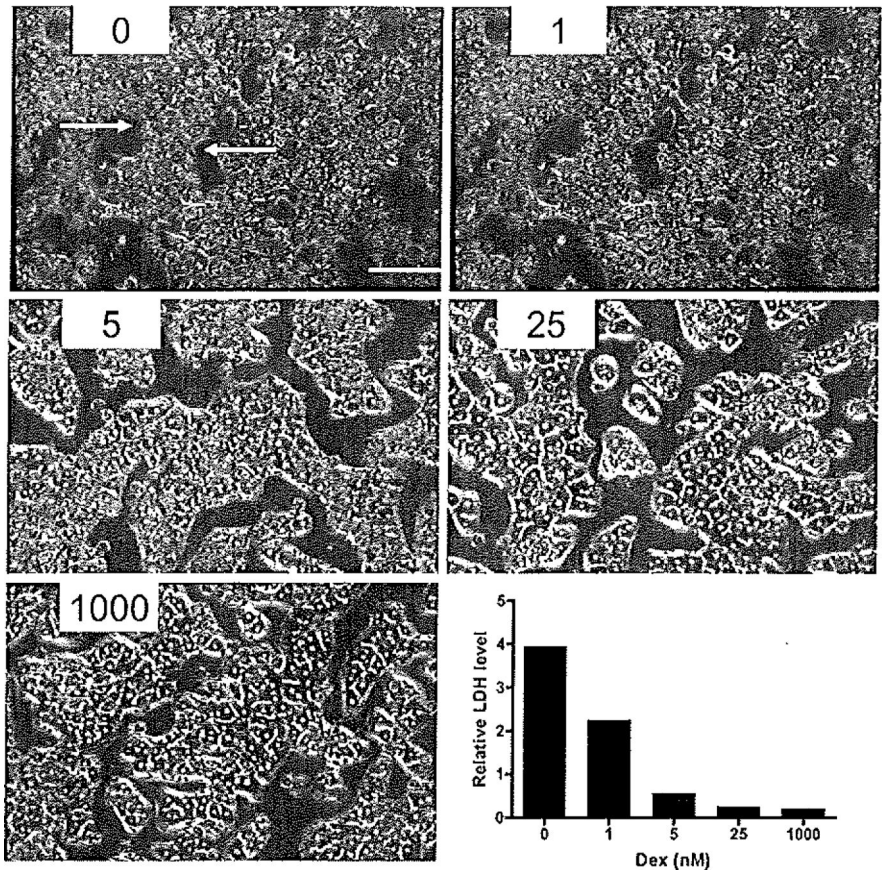

Fig. 6.4.

Effect of dexamethasone concentration dependency on hepatocyte morphology and viability. Primary rat hepatocytes were cultured for 96 h under the stated dexamethasone (Dex) concentrations (nM) in the presence of a Matrigel overlay (×20 magnification). *Arrows* identify evidence of perturbed morphology: condensed cytoplasm and rounded-up cells, attributed to cytotoxicity. The *tower right panel* shows the relative level of LDH leakage associated with each Dex concentration. Reproduced from Experimental Cell Research, 2004 (13) with permission from Elsevier

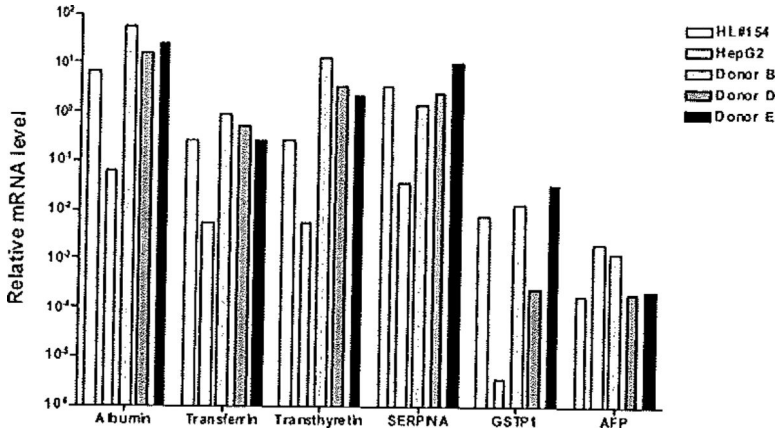

Fig. 6.5.

Effects of Matrigel addition on differentiation status of primary human hepatocyte cultures. Total RNA was isolated a section of human liver # 154, from HepG2 cells, as well as three different donor samples of primary human hepatocytes that were cultured for 5 days in the presence of a Matrigel overlay. Relative mRNA transcript expression levels were assessed using TaqMan qRTPCR analyses for a panel of differentiation markers, albumin, transferrin and transthyretin, and alpha-1-antitrypin (SERPINA), and de-differentiation markers GSTP1 and alpha fetoprotein (AFP). The ΔΔCt method was used for quantification (124). The results are graphically depicted, using a fog scale on the ordinate axis. Reproduced from Toxicological Sciences, 2007 (73) with permission from Oxford University Press.

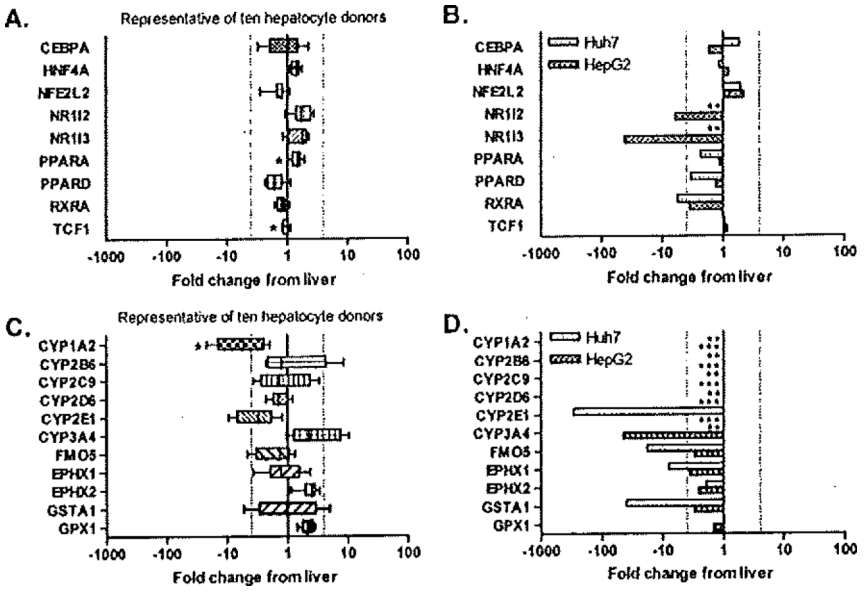

Fig. 6.6.

Gene-level expression analysis of selected liver-specific categories in human hepatocyte donors and hepatoma-derived cell lines using microarray profiling. Distribution of fold change from the liver in 10 hepatocyte donors is shown for genes encoding select transcription factors (**A**) and drug-metabolizing enzymes (**C**). For comparison, the fold change for the same genes in HepG2 and Huh7 hepatoma cells are presented in panels **B** and **D**. Differential expression is defined as greater than 4-fold change from the human liver (*dotted lines*). * indicates the measured probe set is detected as absent in at least one human hepatocyte donor (PPARA: absent in two donors; TCF1: absent two donors; CYP1A2: absent in one donor). ** indicates the probe set is detected as absent in Huh7 cells (NR1I2, NR1I3, CYP1A2, CYP2B6, CYP2C9, CYP2D6, CYP3A4). *** indicates the probe set is detected as absent in HepG2 (CYP1A2, CYP286, CYP2C9, CYP2D6, CYP2E1). Reproduced from Toxicology and Applied Pharmacology (88) 2007, with permission from Elsevier.

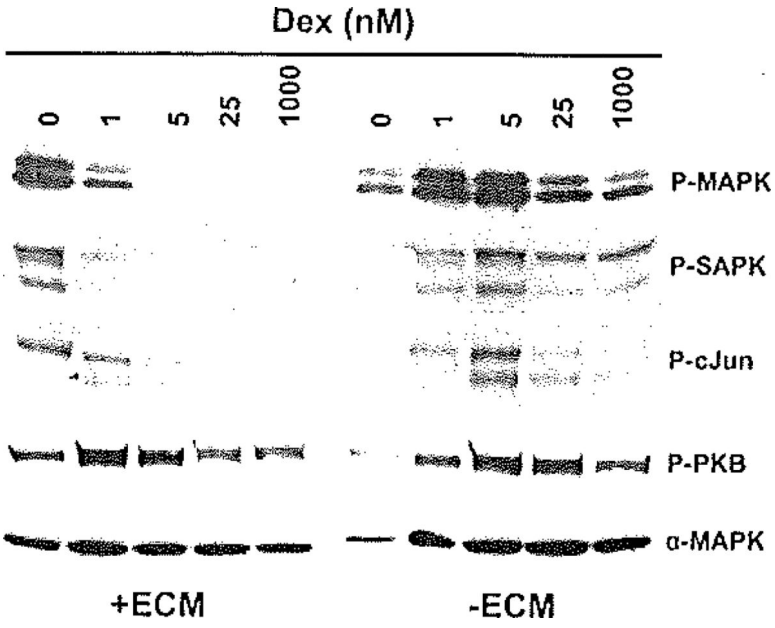

Fig. 6.7.

Effect of ECM overlay and dexamethasone concentration on the activation of stress signaling pathways in primary rat hepatocytes. Primary rat hepatocytes were cultured for 96 h under the variable concentrations of dexamethasone (Dex), as indicated, and in the presence (+ECM) or absence (−ECM) of an ECM/Matriget overlay. Total cell extracts were prepared and analyzed by Western blot analysis, Phospho-specific antibodies were used to discern the phosphorylation status of p42/44 MAPK (Thr202/Tyr204), SAPK/JNK (Thr183/Tyr185), c-Jun (Ser63), and Akt (Ser473). The levels of each targeted immunoreactive protein were assessed in parallel with phosphorylation-independent antibodies, as shown for αMAPK, Reproduced from Experimental Cell Research, 2004 (13), with permission from Elsevier.

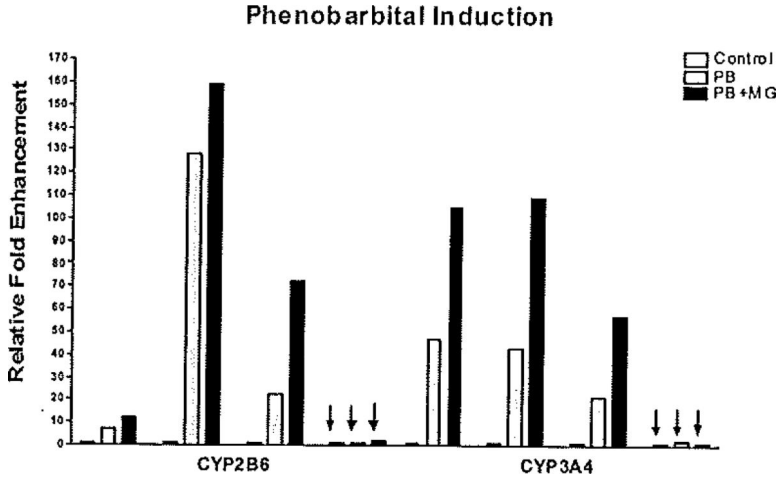

Fig. 6.8.

Effects of Matrigel addition on the phenobarbital induction activity primary human hepatocyte cultures. Primary human hepatocytes were cultured in the absence (control) or the presence of Matrigel (MG). Cultures of primary human hepatocytes and HepG2 hepatoma cells (indicated by *arrows*) were treated on day 4 with 0.5 mM phenobarbital (PB alone: PB; or PB in combination with MG, PB+MG) or DMSO (control, *leftmost bars* in each section of the graph) for 24 h prior to RNA isolation. Relative fold changes in transcript levels for the PB-inducible marker genes, CYP2B6 and CYP3A4, are indicated, normalized to OMSO control levels set (= 1). Reproduced from Toxicological Sciences, 2007 (73) with permission from Oxford University Press.