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In Vitro Liver Tissue Model Established from Transgenic Mice: Role of HIF-1alpha on Hypoxic Gene Expression

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ABSTRACT

The instability of the hepatocyte phenotype *in vitro* has limited the ability to quantitatively investigate regulation of stress responses of the liver. Here, we adopt a tissue-engineering approach to form stable liver tissue *in vitro* by forming collagen "sandwich" cultures of transgenic murine hepatocytes harboring a regulatory gene of interest flanked by loxP sites. The floxed gene is excised in a subset of cultures by transfection with adenovirus carrying the gene for Cre-recombinase, thereby generating wild-type and null liver tissues from a single animal. In this study, we specifically investigated the role of hypoxia inducible factor 1 alpha (HIF-1α) in the hepatocellular response to hypoxia. Using high-density oligonucleotide arrays, we examined genome-wide gene expression after 8 h of hypoxia in wild-type and HIF-1α null hepatocyte cultures. We identified more than 130 genes differentially expressed under hypoxia involved in metabolic adaptation, angiogenic signaling, immediate early response, and cell cycle regulation. Real-time polymerase chain reaction analysis verified that known hypoxia-responsive genes such as glucose transporter-1 and vascular endothelial growth factor were induced in a HIF-1α-dependent manner under hypoxia. Our results demonstrate the potential to integrate *in vitro* tissue models with transgenic and microarray technologies for the study of physiologic stress responses.

INTRODUCTION

OXYGEN, in addition to being an essential substrate of energy production, is a critical regulator of cellular function. In particular, it is known that physiologic oxygen gradients in the liver sinusoids regulate the diverse functions of the liver, including synthesis, metabolism, and biotransformation.[1] Furthermore, extremes in the dynamic hepatocyte microenvironment presented by normal and disease states may yield hypoxic conditions, inducing stress response and adaptation. Although oxygen regulates cellular processes at all levels from transcription to post-translational modification, the alterations in gene expression under hypoxic conditions are often the most critical to cell survival.

Hypoxia inducible factor 1 alpha (HIF-1α) is a key regulator of functional hypoxic responses in mammalian cells and contributes to upregulation of genes involved in glycolytic metabolism (phosphoglycerate kinase (PGK), glucose transporter (GLUT-1), angiogenesis (vascular endothelial growth factor (VEGF)), or hematopoiesis (erythropoietin (EPO)).[2] HIF-1α, a basic helix-loop-helix transcription factor, is post-translationally stabilized under hypoxia,

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permitting dimerization with aryl hydrocarbon receptor nuclear translocator (ARNT) and subsequent targeting of hypoxic responsive promoter regions.[3] Hepatic tumor-derived cell lines (HepG2, Hep3B) have been important tools in assessing the regulation of EPO production and the glycolytic switch by HIF-1α.[4,5] Furthermore, although HIF-1α has been studied in liver zonation[6] and implicated in glucokinase and plasminogen activator inhibitor-1 up-regulation in primary hepatocytes,[7,8] the potential role of HIF-1α in regulating liver-specific processes has not been fully investigated.

In vitro evaluation of HIF-1α regulation in hepatocytes is limited by two factors: rapid loss of function of isolated hepatocytes in culture and embryonic lethality of HIF-1α null mice on E10.5.[9] Tissue engineering approaches such as 3-dimensional extracellular matrix presentation, homotypic cell aggregation, or spatial-constrained co-cultures have greatly improved *in vitro* function of primary hepatocytes, advancing the prospects for cell-based therapies as well as physiological model systems. In particular, collagen sandwich culture, which extends liver-specific function in rat hepatocytes to several weeks, is well suited for molecular studies because of its pure cell populations and uniform control of the oxygen microenvironment.[10] Second, investigation of the HIF system in differentiated cells has been achieved with conditional HIF-1α knockout models implementing tissue-promoter-driven Cre/loxP systems. Although successfully applied to elucidate the role of HIF-1α in neurons, chondrocytes, and macrophages,[11–13] HIF knockout in hepatocytes has not been reported.

Deoxyribonucleic acid (DNA) microarray analysis is particularly well suited to evaluate complex adaptive responses to hypoxia. Recent studies have used oligonucleotide arrays to characterize human hepatocyte cell lines under hypoxia, inflammatory response in rat hepatocytes, and HIF-1α null embryonic fibroblasts.[14–17] This study establishes a long-term, transgenic hepatocyte culture system to investigate HIF-1α regulation of gene expression after hypoxia. Collagen sandwich culture maintains differentiated liver function and permits adenovirus-mediated HIF-1α gene deletion *in vitro*. Gene expression in wild-type and HIF-1α null cultures was analyzed using Affymetrix Genechips containing more than 12,000 murine expressed sequences and validated using real-time PCR. These findings demonstrate successful integration of tissue-engineering strategies with transgenic technology to elucidate hypoxic adaptations not easily addressable using conventional culture systems.

METHODS

Hepatocyte isolation and culture

Primary mouse hepatocytes were isolated and purified using a modified procedure of Seglen.[18] Conditional HIF-1α knockout mice, described previously, at 8–12 weeks of age, weighing 30 to 50 g were anesthetized before *in situ* perfusion of the portal vein.[19] Following a 2-step perfusion of Krebs Ringer Buffer and collagenase, dissociated cells were passed through nylon mesh and purified on a Percoll gradient. Cell viability typically exceeded 90% as assessed using Trypan blue exclusion. Hepatocytes were seeded on 0.1% rat-tail collagen gel and allowed to attach for 2 h, at which point medium was replaced. Twenty-four hours post-isolation, cultures were overlaid with another layer of 0.1% collagen gel.[10] Cultures were maintained in Dulbecco's modified Eagle medium (DMEM, GibcoBRL, Rockville, MD) with 10% fetal bovine serum supplemented with insulin, hydrocortisone, and antibiotics. Images were obtained using a Nikon Eclipse TE300 inverted microscope, CCD camera (CoolSnap HQ, Roper Scientific, Tucson, AZ), and Metamorph Image Analysis System (Universal Imaging, Downingtown, PA). Medium was changed every 48 h until day 6, when adenoviral treatment was initiated.

Functional assays

Medium was collected from sandwich cultures for analysis of albumin secretion and urea production. Functional studies were conducted independent of adenoviral and hypoxic treatments described below. Albumin levels were determined using enzyme-linked immunosorbent assay using anti-mouse albumin primary (RDI, Flanders, NJ) and horseradish peroxidase–conjugated secondary antibodies (Sigma, St. Louis, MO) with o-phenylenediamine substrate development. Urea was measured using colorimetric endpoint analysis using blood urea nitrogen reagents (Sigma).

Adenoviral transduction

LoxP sites flanked the HIF-1α gene on both alleles of the conditional knockout mouse, which enables gene excision in the presence of Cre recombinase. Cultures were treated with adenovirus-containing (type 5) genes encoding Cre or b-galactosidase (control) at multiplicity of infection (MOI) ranging from 10 to 200 on day 6. Virus was added to DMEM without serum and incubated at 37°C for 24 h, after which medium was replaced. Cellular viability was assessed using dimethylthiazol-diphenyltetrazolium bromide (MTT, Sigma) staining, precipitate extraction with 1:1 isopropanol: dimethylsulfoxide, and spectrophotometric measurement at 24, 48, and 96 h post-infection. Infection with control adenovirus was determined by fixing cultures with 4% paraformaldehyde and staining with x-gal reagent. After viability and gene deletion analysis, MOI of 50 was found to be optimal in further experiments.

Determination of HIF-1α excision and gene expression using quantitative PCR

Hepatocytes were removed from collagen gel sandwich culture by incubating with 0.5 mg/mL collagenase in

DMEM without serum for 45 min followed by gentle scraping. To isolate genomic DNA, cells were pelleted using centrifugation at 100 g for 3 min and digested in 10 mM Tris-hydrochloric acid pH 8.0, 100 mM sodium chloride, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium dodecyl sulphate with 0.25 mg/ml proteinase K (Roche, Indianapolis, IN) using agitation for 5 h at 37°C. DNA was extracted using a mixture of phenol:chloroform: isoamylalcohol (25:24:1) and precipitated with ethanol. After pelleting, the DNA was washed in 70% ethanol then resuspended in 10 mM Tris and 1 mM EDTA pH 8.0 and stored at −80° C. Primer express software was used to design forward, reverse, and fluorescein dye-tagged oligonucleotides for use with quantitative PCR (Applied Biosystems, Foster City, CA). Excision of HIF-1α was quantified by normalizing cycle threshold values to the control gene, c-jun. The primers and probes used for evaluating HIF-1α excision are listed in Table 1.

Gene expression analysis was carried out using real-time analysis of PCR on *in vitro* transcripted complementary DNA (cDNA). Total ribonucleic acid (RNA) was isolated from cultures using total RNA isolation (TRI)-Reagent (Molecular Research Center, Cincinnati, OH), after collagen digestion as described above. Purified RNA pellet was washed with 70% ethanol, resuspended in double distilled water and spectrophotometrically quantified for reverse transcription. cDNA synthesis was carried out using random hexamers with the Superscript II First Strand Synthesis kit (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed using ABI Prism 7700 with primer and probe sequences listed in Table 1. In the case of activating transcription factor 3 and c-Fos gene expression analysis, commercial primer/probe reagents were used (Assays-on-Demand, Applied Biosystems).

Experimental design

The primary aim of microarray analysis was to evaluate the changes in hepatocyte gene expression under short-term hypoxic conditions in wild-type (WT) and HIF-1α null cultures. To this end, hypoxia-treated cultures were subjected to incubation with 1% oxygen (O_2)/5% carbon dioxide/94% nitrogen at 37°C for 8 h on culture day 7 and before RNA isolation. Duplicate normoxic WT controls were established from separate animals wherein cultures were untreated and treated with Adβgal. WT (Adβgal treated) and HIF-1α null (AdCre treated) cultures from 3 separate animals were placed under hypoxic conditions and subsequently processed for microarray analysis. A total of 10 microarray hybridizations were performed: normoxia untreated WT (n = 2), normoxia adenovirus treated WT (n = 2), hypoxia WT (n = 3), and hypoxia HIF-1α null (n = 3). Fig. 1 depicts the experimental design and timeline of this study.

Sample preparation and array hybridization

Total RNA from murine hepatocyte cultures was isolated using TRI-Reagent and purified using isopropanol precipitation. Reverse transcription of total RNA was carried

Table 1. Primer and Probe Sequences

Primer	*5′ to 3′ Sequence*
HIF-1α fwd	CTATGGAGGCCAGAAGAGGGTAT
HIF-1α rev	CCCACATCAGGTGGCTCATAA
HIF-1α probe	6FAM-AGATCCCTTGAAGCTAG-BHQ
c-jun fwd	TGCATGCTATCATTGGCTCATAC
c-jun rev	CACACCATCTTCTGGTGTACAGTCT
c-jun probe	VIC-CCCGGCAACACACA-BHQ
PGK fwd	CAAATTTGATGAGAATGCCAAGACT
PGK rev	TTCTTGCTGCTCTCAGTACCACA
PGK probe	6FAM-TATACCTGCTGGCTGGATGGGCTTGGACT-BHQ
VEGF fwd	AGTCCCATGAAGTGATCAAGTTCA
VEGF rev	ATCCGCATGATCTGCATGG
VEGF probe	6FAM-TGCCCACGTCAGAGAGCAACATCAC-BHQ
GLUT-1 fwd	GGGCATGTGCTTCCAGTATGT
GLUT-1 rev	ACGAGGAGCACCGTGAAGAT
GLUT-1 probe	6FAM-CAACTGTGCGGCCCCTACGTCTTC-BHQ
β-Actin fwd	AGGCCCAGAGCAAGAGAGG
β-Actin rev	TACATGGCTGGGGTGTTGAA

HIF, hypoxia inducible factor; PGK, phosphoglycerate kinase; VEGF, vascular endothelial growth factor; GLUT-1, glucose transporter 1.

Abbreviations: HIF-1α , -1alpha; EPO, erythropoietin; ARNT, aryp hydrocarbon nuclear translocator; MOI; multiplicity of infection; WT, Wild type; MAS, Microarray Analysis Suite; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ATF, activating transcription factor; PDGF, platelet derived growth factor; PLGF, placental growth factor; ADM, adrenomedullin; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; GADD45, growth arrest and DNA damage inducible 45; AP-1, activator protein-1; LRF-1, liver regenerating factor-1; NFκB, nuclear factor κB; JNK, Jun N-terminal kinase.

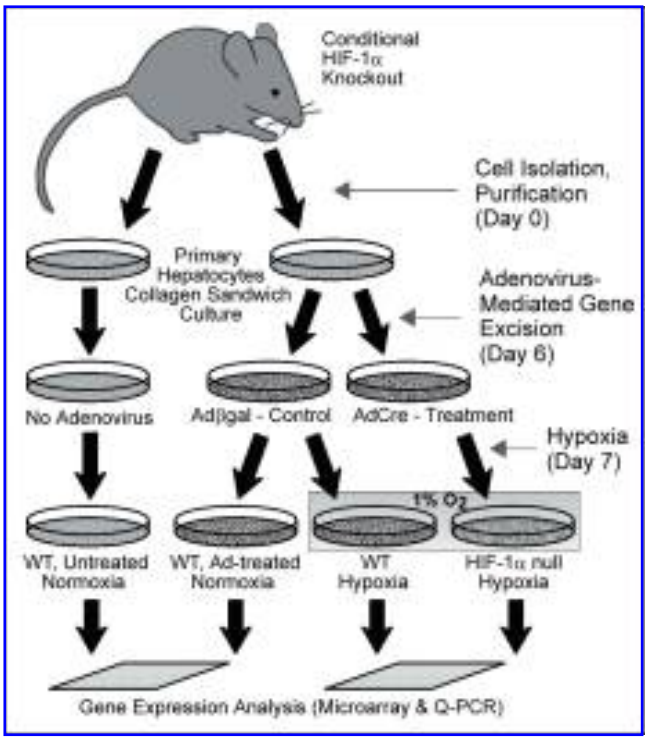

FIG. 1. Experimental design. All cultures were established from mice containing floxed hypoxia inducible factor 1 alpha (HIF-1α) genes on both alleles. Resultant cultures were exposed to Adβgal, AdCre, or no vector on day 6. Wild-type (WT) (Adβgal treated) and HIF-1α null mice were further exposed to 1% oxygen for 8 h on day 7 and compared with normoxic WT controls in terms of gene expression. Q-PCR, quantitative polymerase chain reaction.

out using T7-Oligo(dT) primers and Superscript II. The cDNA strand was created by an addition of DNA polymerase and DNA ligase (Invitrogen) and incubation at 16°C. Double-stranded cDNA was purified using the Genechip Sample cleanup module (Affymetrix, Santa Clara, CA) and resuspended in diethylpyrocarbonate-treated water. *In vitro* transcription of cDNA was performed using the ENZO Bioarray Transcript labeling kit (Affymetrix), and biotin-labeled transcripts were then purified with the Sample Cleanup module. Samples were hybridized to the Affymetrix Murine U74Av2 array following the manufacturer's recommendations. Briefly, fragmented cRNA samples were spiked with bioB, bioC, bioD, and cre controls in hybridization buffer and incubated with arrays for 16 h at 45°C. After hybridization, chips were washed, stained, and scanned using the Affymetrix Genechip System with integrated Microarray Analysis Suite 5.0 (MAS).

DNA microarray expression analysis

Raw genechip images were quantified and scaled to an intensity of 200 using MAS. The quality of expression data was assessed using the manufacturer's instructions, which included criteria such as low background values and 3′/5′ actin and glyceraldehyde-3-phosphate dehydrogenase ratios below 2. Pairwise comparison files were first generated between all treatment conditions (i.e., normoxia–sample 1 versus hypoxia–sample 1) and then filtered using BullFrog software (TeraGenomics, Reston, VA) to detect genes that were consistently differentially expressed.[20] Criteria used for filtering were selected based on their ability to yield false-positive rates of less than 1.6% (number of genes differentially expressed in replicates/total genes). These criteria included a fold change of 1.7 or greater and directional consistency, which refers to the sign of a change being the same in all comparisons. In addition, criteria had to be consistent in 4 of 6 comparisons.

The filtered gene list (Table 2) was functionally annotated using reports from the literature and via the NetAffx analysis portal (Affymetrix), which integrates information from various public databases such as Genbank and Swissprot. A complete list of differentially expressed genes is available as supplementary data. Complete data sets of hybridizations were submitted to GEO bank (http://www.ncbi.nlm.nih.gov/geo/ [Series accession # GSE1321]).

RESULTS

Phenotypic stability of murine hepatocytes in sandwich culture

To assess the feasibility of using long-term primary cultures for gene expression studies, hepatocytes from mice with an intact, double-floxed HIF-1α gene were cultured in a collagen sandwich configuration for 15 days. Cultures were evaluated over this period for maintenance of liver-specific function as indicated by hepatocyte morphology, albumin synthesis, and urea production. The phase-contrast photomicrograph in Fig. 2A displays multinucleation, characteristic polygonal morphology, and marked bile canaliculi at cell borders on day 15 of culture. Albumin secretion was determined every other day and compared with hepatocyte cultured on collagen alone (Fig. 2B). Cultures on absorbed collagen showed a rapid loss of albumin secretion by day 5, which correlated with cell death as assessed using microscopy (data not shown). In contrast, albumin levels in sandwich culture were maximal at day 5 and were maintained at high levels out to day 15. Similar trends were noted with urea production with a time-dependent decrease in hepatocytes on absorbed collagen and stable levels of approximately 8 μg/hr per 10^6 cells in sandwich culture through day 15 (Fig. 2C).

Characterization of adenovirus-mediated gene excision

Establishment of HIF-1α null cultures *in vitro* was accomplished by transducing stable hepatocytes with Cre-expressing adenovirus. Transient expression of control

Table 2. Differentially Expressed Genes in Hypoxia-Treated Murine Hepatocytes

Function *Gene Title*	*Gene Bank Accession No.*	*Wild Type* Mean FC expression	*HIF-1α-Null* Mean FC expression	*Change in Signal Intensity %*
Cell Cycle Regulation				
Growth arrest and DNA-damage-inducible 45 alpha	U00937	4.3	5.6	18
FBJ osteosarcoma oncogene	V00727	4.1	2.9	−34**
DNA-damage inducible transcript 3	X67083	3.0	3.6	26
Cyclin-dependent kinase inhibitor 1A (P21)	U09507	2.4	1.8	−18
Jumonji domain containing 1	AW049513	2.4	1.5	−33**
Wee 1 homolog	D30743	2.3	2.5	23
Jun-B oncogene	U20735	2.1	2.1	−4
Epidermal growth factor receptor	AW049716	2.1	1.7	−26*
Myelocytomatosis oncogene (Myc)	L00039	1.8	2.2	15
N-myc downstream regulated-like	U52073	3.0	2.1	−30**
N-myc downstream regulated 1	U60593	2.7	2.0	−24*
Max interacting protein 1	L38822	1.8	−1.1	−45***
Dual specificity phosphatase 1	X61940	1.7	1.8	2
Cell Structure Components				
Caudin 4	AB000713	5.5	6.4	18
Claudin 1	AF072127	4.1	4.4	8
A kinase anchor protein 2	AF033275	1.7	1.8	10
Cortactin	U03184	−2.2	−1.3	76†
Claudin 2	AF072128	−7.1	−3.1	113†
Coagulation				
Coagulation factor III (tissue factor)	M26071	5.2	5.7	18
Coagulation factor II receptor-like 1	Z48043	2.2	2.0	−6
Serine proteinase inhibitor, clade E1 (PAI-1)	M33960	2.0	1.5	−7
Growth Factors and Signaling				
Adrenomedullin	U77630	20.9	18.7	−14
Inhibin beta-B	X69620	8.3	10.9	79†
Vascular endothelial growth factor A	M95200	5.7	5.1	−8
Amphiregulin	L41352	5.2	8.0	43†
Placental growth factor	X80171	4.9	5.4	7
Ephrin A1	U90662	3.1	2.7	−14
Parathyroid hormone-like peptide	M60057	2.5	2.2	−17
Platelet derived growth factor, alpha	M29464	2.1	1.9	−4
Angiopoietin-like 4	AA797604	−2.0	−2.4	−20
Fibroblast growth factor 1	M30641	−2.7	−1.9	43†
Metabolism and Biosynthesis				
Cytochrome P450, family 2S1	AW123273	4.9	3.9	−9
Hexokinase 2	Y11666	4.3	2.1	−62***
ERO1-like	AW120614	3.9	2.5	−36**
UDP-glucose ceramide glucosyltransferase	AI853172	3.1	3.5	31
Glycogen synthase 1, muscle	U53218	2.3	1.1	−51***
Cholesterol 25-hydroxylase	AF059213	2.2	2.1	2
Glucosaminyltransferase, I-branching enzyme	U68182	2.1	2.1	10
Spermidine/spermine N1-acetyl transferase 1	L10244	2.0	2.1	−3
EGL nine homolog 1	AI850202	2.0	−1.1	−56***
Sterol O-acyltransferase 1	L42293	1.9	1.4	−19
Phosphofructokinase, platelet	AI853802	1.9	1.4	−18
Phosphatidylinositol glycan, class A	AV308550	1.9	1.5	−12
Procollagen-proline, 2-oxoglutarate 4-dioxygenase, alpha II polypeptide	AV370017	1.9	1.3	−30**
S-adenosylmethionine decarboxylase 1	D12780	−1.8	−1.5	13
Hydroxyprostaglandin dehydrogenase 15	U44389	−2.0	−2.3	−25*
Hydroxysteroid (17-beta) dehydrogenase 4	X89998	−2.0	−2.4	−19
Methionine adenosyltransferase II, alpha	AW124835	−2.0	−1.7	11

TABLE 2. (CONTINUED)

Function *Gene Title*	*Gene Bank Accession No.*	*Wild Type* Mean FC expression	*HIF-1α-Null* Mean FC expression	*Change in Signal Intensity %*
Carbonyl reductase 3	AI324801	−2.0	−1.8	9
Bile acid-CoA: amino acid N-acyltransferase	U95215	−2.1	−2.6	−28*
Tyrosine aminotransferase	AI255353	−2.7	−2.9	−12
2,3-bisphosphoglycerate mutase	X13586	−3.2	−3.3	−6
Tryptophan 2,3-dioxygenase	AI194855	−3.2	−2.8	2
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1	X98848	−9.1	−9.0	8
Signal Transduction				
Arginine vasopressin receptor 1A	AV240013	2.8	2.7	2
Bone morphogenic protein 2 inducible kinase	AA673486	2.7	3.0	14
Cytoplasmic tyrosine kinase, Dscr28C related	X55663	2.5	2.5	3
Interferon-related developmental regulator 1	V00756	2.4	2.6	3
v-ral simian leukemia viral oncogene homolog B (ras related)	AI847236	1.9	2.2	17
Induced in fatty liver dystrophy 2	AA770736	1.9	1.6	−19
Adenylate kinase 4	AW061337	1.8	−1.2	−56***
Regulator of G-protein signaling 16	U94828	1.7	1.9	16
Serine/threonine kinase 16	AF062076	−1.7	−1.6	14
Ceramide kinase	AW125333	−3.0	−1.7	141†
Transcriptional Regulation				
Nuclear receptor co-activator 6	AA414339	9.0	8.5	−4
Activating transcription factor 3 (LRF-1)	U19118	7.9	7.2	−2
Nuclear receptor subfamily 3, group C, member 1	X04435	3.9	4.5	36
Retinoic acid receptor, alpha	M60909	2.9	2.7	−12
Kruppel-like factor 4 (gut)	U20344	2.6	3.5	14
TG interacting factor	X89749	2.5	3.3	22
High mobility group box 2	X67668	2.4	2.0	−17
Kruppel-like factor 3 (basic)	U36340	2.2	2.6	26
Nuclear factor, interleukin 3, regulated	U83148	2.1	1.5	−36**
Basic helix-loop-helix domain containing, B2	Y07836	2.1	1.9	−10
TGFB inducible early growth response 1	AF064088	1.7	1.7	11
Activating transcription factor 5	AB012276	−1.8	−1.9	−14
Inhibitor of kappaB kinase gamma	AW122931	−2.6	−2.8	−16
Transport				
Solute carrier family 2 (glucose), member 1	M22998	8.3	5.2	−37**
Solute carrier family 41, member 1	AI837116	6.6	3.7	−45***
Chloride channel 3	AF029347	2.6	−1.2	−54***
Solute carrier family 20 (phosphate), member 1	M73696	2.6	3.7	33
Solute carrier family 30 (zinc), member 1	U17132	2.5	2.7	−16
Testis derived transcript	X78989	2.0	2.1	8
SEC14-like 2	AI172965	−1.9	−1.6	7

Key

*Signal intensity decrease 20–29%; **Signal intensity decrease 30–40%; ***Signal intensity decrease >40%; †Signal intensity increase >40%
DNA, deoxyribonucleic acid.

cultures treated with Adβgal was evaluated using X-gal staining of Adβgal-infected cells across a range of MOI from 10 to 100 (Fig. 3A). Because increasing doses of viral particles may produce deleterious effects on culture conditions, cell viability at various MOIs was assessed according to MTT activity. Neither the Adβgal nor AdCre vectors produced significant loss of cell viability out to 96 h post-infection at 100 MOI (Fig. 3B). Furthermore, the effect of adenoviral exposure on gene expression was assessed using microarray analysis, comparing and filtering untreated WT cultures with Adβgal-treated cultures. Approximately 35 differentially expressed genes were found, of which the known genes are shown in Fig. 4.

To assess efficiency of transient Cre recombinase expression necessary for Cre-loxP-mediated gene excision, the content of the HIF-1α gene was quantified using PCR. Percentage deletion of HIF-1α increased in a dose-dependent manner (Fig. 3C), reaching a maximum of 90% at an

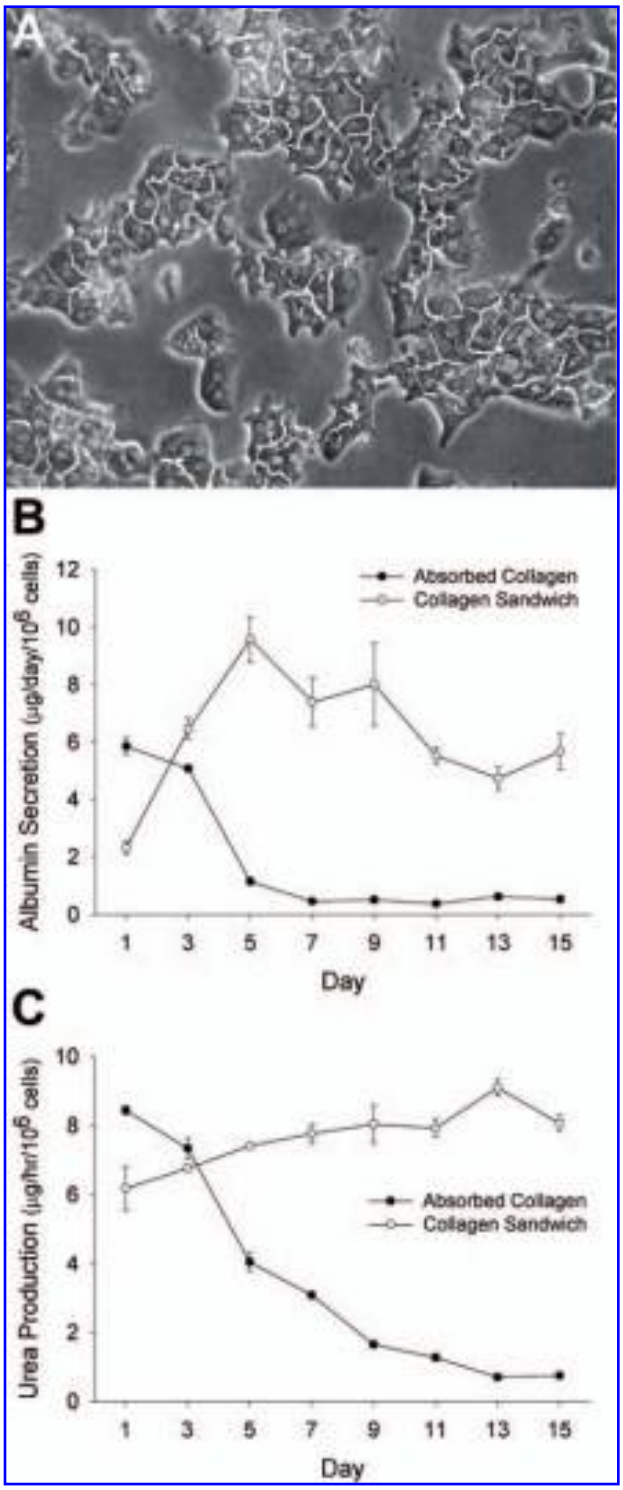

FIG. 2. Functional characteristics of murine hepatocytes in sandwich culture. (**A**) Phase-contrast photomicrograph of hepatocyte on day 15 post-isolation show polygonal morphology and distinct bile canaliculi. (**B**) Albumin synthesis from hepatocytes in sandwich culture showed greater levels over 15 days than hepatocytes on adsorbed collagen. (**C**) Likewise, high levels of urea production were maintained long term in sandwich culture. Data points represent means and standard errors (n = 3).

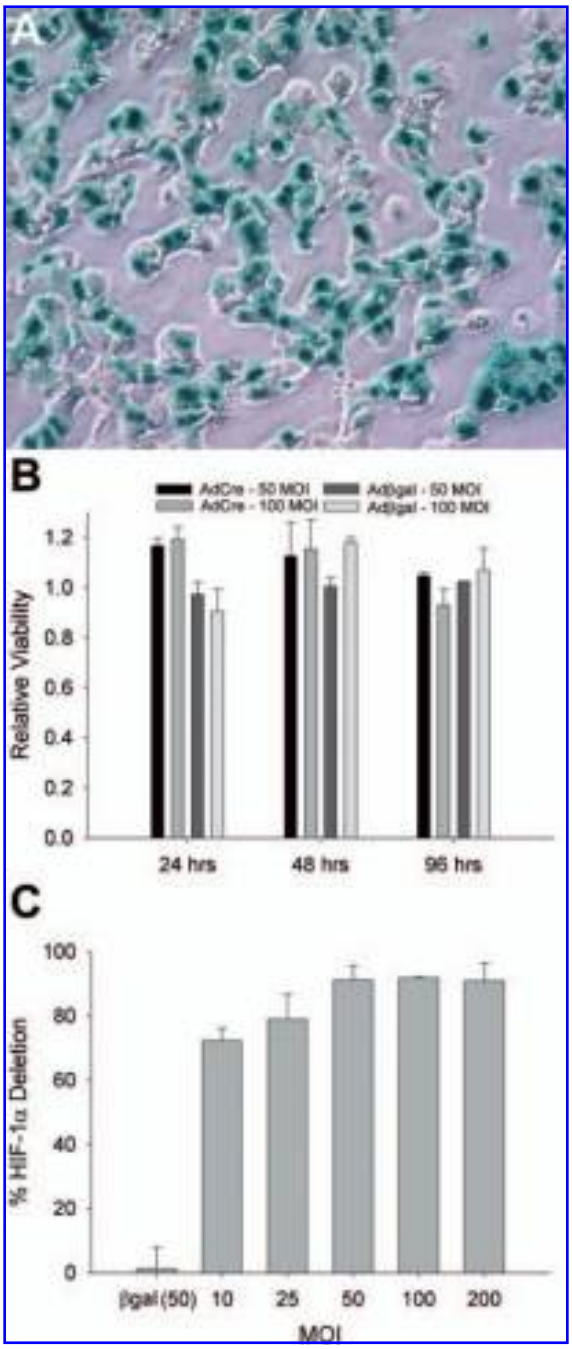

FIG. 3. Adenovirus-mediated gene excision in hepatocyte cultures. (**A**) Photomicrograph of hepatocytes transduced with Adβgal (50 multiplicity of infection (MOI)) on day 5. Twenty-four h later, cultures were fixed in 4% paraformaldehyde and stained with x-gal (1 mg/mL X-gal, 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, 2 mM magnesium chloride) for 4 h. (**B**) Viability of cultures was assessed using MTT staining 24, 48, and 96 h after adenoviral transduction with the indicated viral constructs and dose. (**C**) Deletion of hypoxia inducible factor 1 alpha (HIF-1α) was determined using quantitative polymerase chain reaction. Percentage excision of floxed HIF-1α according to transient AdCre expression increased in a dose-dependent manner. Data represent means and standard errors (n = 3). Color images available online at www.liebertpub.com/ten.

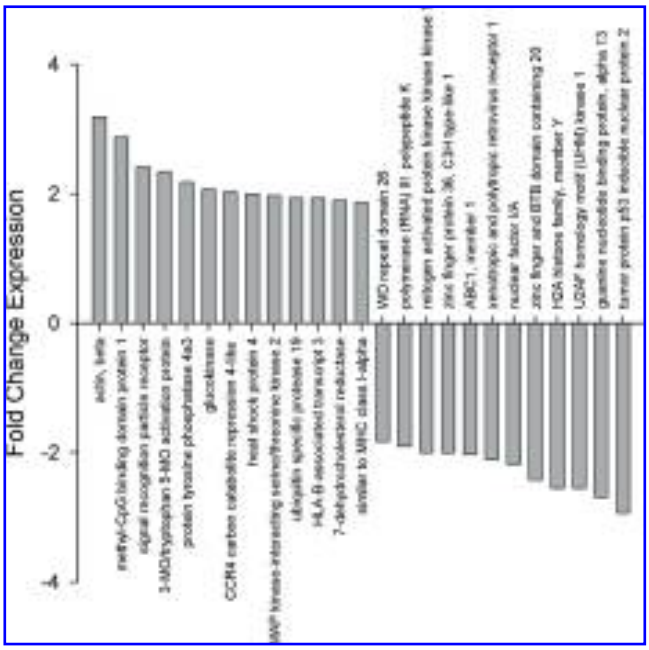

FIG. 4. Differentially expressed genes after exposure to adenovirus. Known genes that were upregulated or downregulated 1.8-fold or greater are listed. Values represent mean fold change expression from at least 3 of 4 comparisons based on the filter criteria.

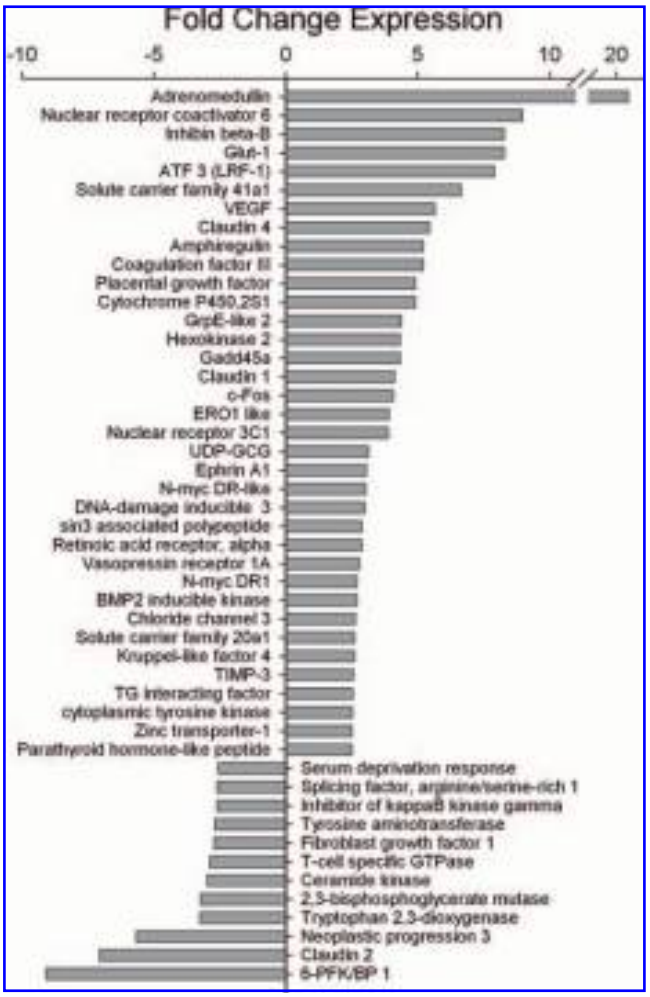

FIG. 5. Differentially expressed genes after hypoxic treatment of hepatocytes. Known genes that were upregulated or downregulated 2.5 fold or greater are listed. Values represent mean fold change expression from at least 4 of 6 comparisons based on the filter criteria. ATF, activating transcription factor; VEGF, vascular endothelial growth factor; GADD45, growth arrest and deoxyribonucleic acid (DNA) damage inducible 45; BMP2, Bone morphogenic protein 2.

MOI of 50, which was selected as the MOI for subsequent microarray and gene expression analysis.

Hypoxic gene expression in murine hepatocytes

Having established long-term murine cultures, gene expression analysis was performed using commercial oligonucleotide arrays, which allow parallel evaluation of more than 12,000 transcripts. First, cultures of WT and HIF-1α null hepatocytes were established and exposed to 1% O_2 for 8 h. After messenger RNA isolation, sample preparation, and hybridization, the data were processed to determine differentially expressed genes between normoxia and hypoxia. This list was filtered to exclude genes altered by adenovirus infection (Fig. 4) and those differentially expressed between biological replicates (i.e., false positives). A complete annotated list is provided as supplemental data (available on-line). Those genes whose expression was most dramatically changed by exposure to hypoxia are shown in Fig. 5. Finally, differentially expressed genes were functionally categorized using available data on gene function, regulation, and signaling (Table 2). Annotation of the most significant changes in signal intensity between WT and HIF-1α null is meant to facilitate identification of potential HIF-1α-regulated genes for which further investigation and validation are warranted. Our supervised analysis of differentially expressed genes focused on key hepatocyte stress responses, including metabolic adaptation, contributions to angiogenesis, and cell-cycle regulation, although many genes may be implicated in other important hepatocyte processes.

To validate results obtained using DNA microarray analysis, several genes differentially expressed after hypoxia were selected for validation using quantitative PCR. Expression of PGK, GLUT-1, and VEGF, all known to be hypoxia responsive and HIF-1α dependent, was evaluated and is shown in Fig. 6. PGK (Fig. 6A) did not meet our filtering criteria of 1.7-fold expression change but, according to PCR analysis, was induced 1.8-fold by hypoxia and was lower under normoxia and hypoxia in HIF-1α null cultures ($p < 0.01$). Similarly, validation of VEGF expression using quantitative PCR verified that hypoxic induction in hepatocytes was HIF-1α dependent (Fig. 6B, $p < 0.05$).

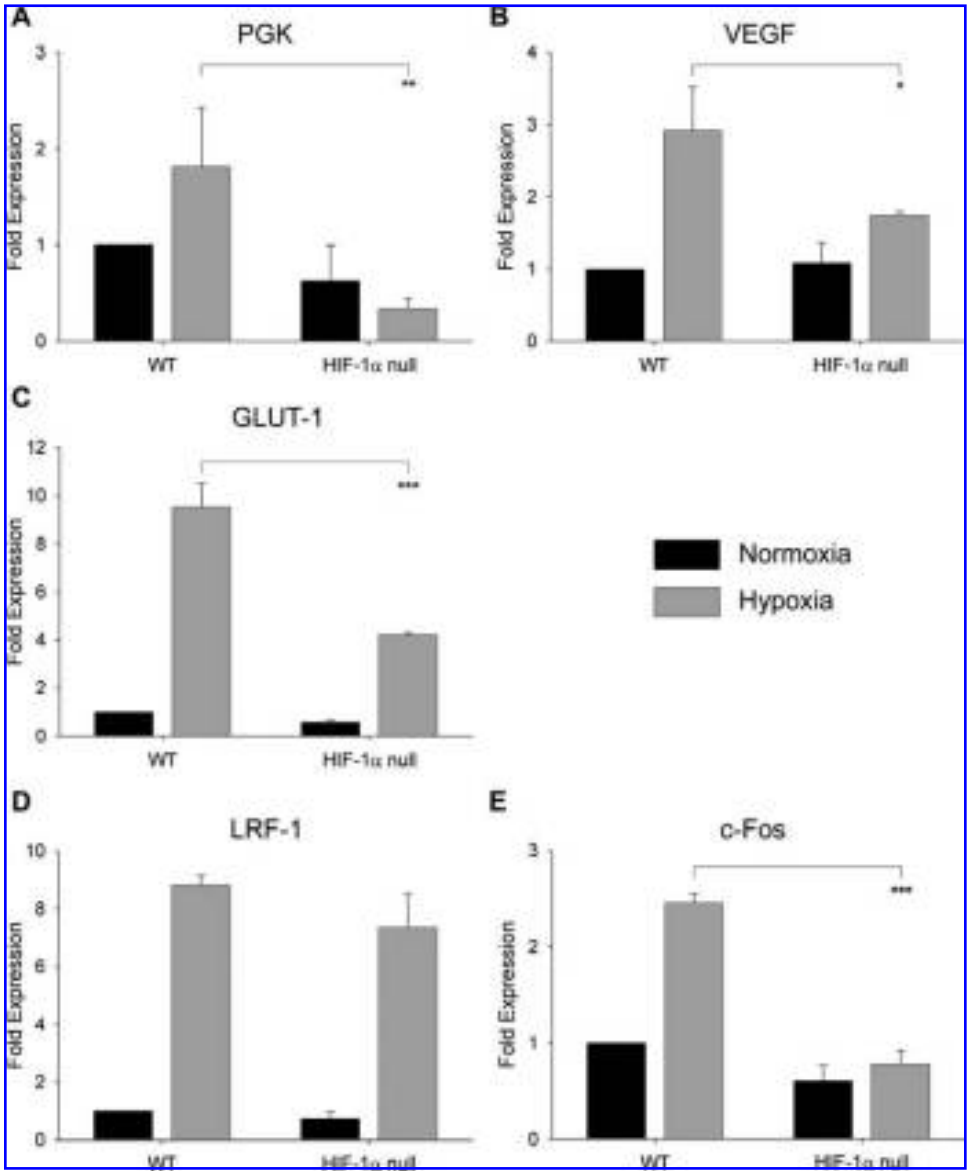

FIG. 6. Hypoxic expression of phosphoglycerate kinase (PGK), vascular endothelial growth factor (VEGF), glucose transporter 1 (GLUT-1), liver regenerating factor-1 (LRF-1), and c-FOS. Various genes were selected for verification of microarray data using quantitative polymerase chain reaction (PCR). Wild-type (WT) and HIF-1α null cultures were subjected to 1% oxygen for 8 h. Threshold values from PCR analysis of complementary deoxyribonucleic acid derived from total ribonucleic acid were normalized to normoxic WT controls and β-actin as a housekeeping gene. Means and standard errors from duplicate PCR reactions of 3 samples for each condition are shown. Statistical significance was determined using one-way analysis of variance (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Patterns in GLUT-1 expression (Fig. 6C) showed 9.5-fold induction under hypoxia, which was reduced to 4-fold in HIF-1α null hepatocytes ($p < 0.001$). Two additional genes were selected for validation using PCR: liver regenerating factor (LRF-1), which has been implicated in liver regeneration and whose expression appeared to be independent of HIF-1α; and c-Fos, an immediate early gene in stress response, for which microarray analysis suggested HIF-1α regulation. PCR analysis of LRF-1 expression (Fig. 6D) showed similar hypoxic upregulation of WT and HIF-1α null cultures, which corroborates microarray observations. HIF-1α appeared to regulate hypoxia-induced expression of c-Fos (Fig. 6E), as evidenced by a 3-fold decrease in expression between WT and null hepatocytes.

DISCUSSION

The primary objectives of this study were to establish long-term hepatocyte cultures in which transgenic technology

could be implemented and to extend that system to investigate hypoxic regulation of global gene expression. Stable albumin and urea production demonstrated maintenance of differentiated function of hepatocytes in sandwich culture, which has not been previously reported in a murine model.[10,21] Transgenic primary hepatocytes were manipulated *in vitro* to excise the HIF-1α gene, allowing investigation of hypoxic response in a controlled oxygen environment. Gene expression analysis of WT and HIF-1α null cultures provided a snapshot of genome-wide adaptation to a physiological stimulus important in regulating metabolism and cell survival. In a larger sense, the application of sophisticated molecular techniques such as Cre-mediated recombination and oligonucleotide arrays to tissue-engineered systems can facilitate better approximation of native organ function *in vitro*.

Convergence of tissue engineering, transgenics, and gene expression profiling

Historically, the clinical need for alternative therapy for hepatic failure has driven the development of tissue-engineered liver.[22] Fortunately, much of the supporting culture technology also lends itself to the establishment of phenotypically stable culture systems suitable for molecular analysis. Important applications of *in vitro* liver models include toxicology, drug development, and basic investigation of hepatocyte differentiation, for which various cell lines, co-culture systems, liver slices, and hepatocyte bioreactors have been proposed.[23–26] The advantages of our sandwich culture system include pure cell populations, long-term function and viability, and a uniform microenvironment for viral exposure and imposition of physiological stimuli such as hypoxia.

Although the majority of *in vitro* liver models are derived from rats, recombinant technologies in mice have transformed our approach to mechanistic studies. In particular, knockout mice, or conditional knockout models averting embryonic lethality, have been employed to study liver-specific processes. Knockout of key genes such as cytochrome P450 2E1,[27] transforming growth factor β receptor,[28] and constitutive androstane receptor[29] have yielded valuable insight into biotransformation, regeneration, and hepatotoxicity, respectively. Most investigations have been limited to *in vivo* methods, perhaps because of limitations in conventional culture methods. Other studies have reported deleterious effects of *in vitro* adenoviral transduction in hepatocytes, but Cre-mediated excision of HIF-1α in the current study did not result in loss of viability, thus permitting long-term (several weeks) knockout studies *in vitro*.[30] Less clear, however, is the effect of replication-deficient adenoviral infection on cellular functions and particularly, for this study, those involved in hypoxic response. A study of gene expression changes in Henrietta Lacks cells after adenovirus infection showed transcriptional changes in genes regulating cell cycle, cytokines, and stress response, likely triggered by viral E1a activity.[31] Even with the absence of viral regulatory proteins, adenoviral exposure in the present study altered expression of cellular metabolism genes (glucokinase and ABC-A1), transcriptional regulators (methyl-Cpg bdp1), and cell signaling (guanine nucleotide binding protein alpha 13 and mitogen activated protein 3 kinase 1), whose fold-change expression is shown in Fig. 4. Several of these genes are consistent with results from microarray analysis of E1-deficient adenoviral infection in other epithelial cells, but the extent to which the hepatocyte response differs remains unexplored.[32] Further studies to validate hepatocyte expression and function after adenovirus exposure and hypoxia are important, considering the high viral tropism for liver and emerging therapeutic technologies.[33]

Gene expression profiling has become a powerful method for screening and characterizing physiologic responses. Several studies have used gene expression analysis to study hypoxic response in hepatocyte cell lines and regeneration after hepatectomy.[14,17,34] However, cell lines may exhibit variable responses to hypoxia because of an incomplete repertoire of liver-specific functions, and whole liver extracts pool parenchymal and non-parenchymal fractions, potentially obfuscating small expression changes in hepatocytes. The present study introduces the advantage of pure hepatocyte cultures in which controlled physiologic stimuli may be interrogated. Ultimately, the stable murine hepatocyte culture system described here may better mimic physiological responses, thereby illuminating additional avenues for clinical studies involving hypoxic adaptation.

HIF-1α regulation of hypoxic response in hepatocytes

The analysis of the present study presented in Table 2 shows concordance with hypoxia-induced gene expression in HepG2 and Hep3B cells in terms of metabolic adaptation, growth factor signaling, and cell cycle regulation. However, stable cultures of HIF-1α null hepatocytes allow more-direct assessment of which changes in hypoxic gene expression the HIF system may regulat. This addendum to previous work elucidating HIF regulation of metabolism demonstrates the potential role of HIF-1α in liver-specific processes such as biosynthesis, biotransformation, and regeneration.[4,5]

Metabolic adaptation

Functional adaptation to hypoxia has previously been attributed to an increase in HIF-1α-mediated expression of glucose transporters and glycolytic enzymes to aid in maintaining adenosine triphosphate (ATP) production.[2] In these studies, as well as in ours, oxygen deprivation results in secondary changes in pH, ATP levels, and oxidative stress that may themselves affect gene expression; however, concordant changes in these variables likely reflects the physiologic, integrated response to hypoxia.[35] In our system,

expression of GLUT-1 showed HIF-1α-dependent regulation, although hypoxic induction in HIF-1α null was still 4 times as high as in normoxic controls, suggesting that other factors may modulate GLUT-1 expression under hypoxia.[9] glycolytic enzymes hexokinase 2 and phosphofructokinase, previously reported to have HIF-1α-dependent activity, were also greater under hypoxia with attenuated expression in HIF-1α null.[36] Glycolytic enzymes PGK, aldolase A, and lactate dehydrogenase, known to be highly inducible by hypoxia, were filtered out by our selection criteria but showed high baseline expression.[9] The observation that PGK expression was significantly lower in HIF-1α hypoxic cultures by PCR and not microarray analysis may highlight the limitations of microarray technologies and the need for further validation of candidate genes. Several other non-glycolytic metabolic pathways were altered under hypoxia, including those involving cholesterol 25-hydroxylase, sterol O-acyltransferase, hydroxysteroid (17-beta) dehydrogenase 4, and tryptophan 2,3-dioxygenase.[37,38]

Growth factor and cytokine signaling

The hypoxic upregulation of growth factors and other secreted signals primarily aimed at increasing oxygen delivery have been well studied.[2] We observed greater expression of VEGF, platelet-derived growth factor, placental growth factor, and ephrin A1, all of which are reportedly involved in angiogenesis.[39,40] Adrenomedullin, the most dramatically upregulated gene in our study, is involved in altering vasomotor tone, is known to be HIF-1α dependent under hypoxia, and has been reported to be elevated in serum of patients with cirrhosis.[41,42] Inhibin betaB, a gene whose hypoxic induction appear to be HIF-1α independent, is a member of the transforming growth factor beta superfamily and typically forms heterodimers with inhibin betaA to form activins.[43] The effect of activin dimers in the liver are diverse, including inhibition of DNA synthesis, promotion of matrix deposition, and initiation of apoptosis, phenomena commonly associated with fibrosis.[44]

Hypoxic determinants of cell fate

Related to the activator protein-1 system, a common pathway in many stress responses, we observed increased expression of c-Fos, JunB, and Myc, along with down-stream regulator elements of c-myc, with hypoxic treatment.[45] Expression of Myc is not likely regulated by HIF-1α but may be post-transcriptionally regulated, as has been proposed in liver regeneration studies.[46] The individual actions of immediate-early genes are not clearly elucidated, but factors including c-Fos and JunB have been identified as critical components of the G_1 phase of hepatocyte replication.[47] However, signals such as p21 and growth arrest and DNA damage inducible 45 (GADD45), which were upregulated in our study, regulate progression to replicative competence. Although HIF-1α is known to regulate the p21 gene, an inhibitor of cyclin-dependent kinase, p53 rather than HIF-1α may primarily regulate GADD45, which halts the cell cycle in response to DNA damage.[48,49] In addition, activating transcription factor 3, also called LRF-1, was significantly greater under hypoxia. LRF-1 is known to be highly expressed in liver regeneration, regulates cell cycle, and may alter glucose metabolism under oxidative stress.[47,50]

Many studies have demonstrated that hypoxia is an inhibitor of normal cell cycle, but *in vivo* stress response in hepatocytes is known to induce replication.[51,52] Ischemic liver injury or tumor necrosis factor–induced stress indicates that immediate-early genes or downstream nuclear factor kappa B or Jun N-terminal kinase activation may be common pathways from G_0 to apoptosis or mitosis.[53–55] In support of this paradigm, liver regeneration has been described as a multi-step process, regulated by distinct signals during hepatocyte priming and cell cycle progression.[56] It may be that hypoxia is among the other reported exogenous stress factors like sham surgery or collagenase perfusion that initiate hepatocyte priming to replication but are insufficient to induce growth. Owing to the incomplete understanding of the role of hypoxia and HIF-1α in cell cycle and apoptosis, further studies such as partial hepatectomy or chemical injury models in conditional liver knockout of HIF-1α might elucidate a role for hypoxia in priming, cell cycle progression, or regeneration kinetics.

CONCLUSION

We have established and characterized a long-term hepatocyte culture model suitable for transgenic studies. Evaluation of sandwich cultures showed sustained albumin and urea production over 2 weeks, as well as robust viability in response to adenoviral infection. In addition, high-efficiency Cre-mediated gene excision established the utility of tissue-engineered hepatocyte cultures as a transgenic model system. As proof of concept, we employed our culture system to profile hepatocyte gene expression after exposure to a controlled hypoxic environment. Analysis of high-density oligonucleotide arrays revealed differential expression of more than 130 genes, contributing to a range of cellular functions including metabolic adaptation to hypoxia (GLUT-1), promotion of angiogenesis (VEGF), and cell cycle regulation (LRF-1). Additional studies of hypoxic response and the role of HIF-1α in hepatocytes based on these gene expression studies will provide insight into oxygen as a regulator of normal and disease processes in the liver. Ultimately, the convergence of tissue engineering, transgenics, and microarray analysis demonstrated here represents an important advancement to investigate complex physiologic processes.

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