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Transcriptional Profiling of Bipotential Embryonic Liver Cells to Identify Liver Progenitor Cell Surface Markers

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Abstract

The ability to purify to homogeneity a population of hepatic progenitor cells from adult liver is critical for their characterization prior to any therapeutic application. As a step in this direction, we have used a bipotential liver cell line from 14 days postcoitum mouse embryonic liver to compile a list of cell surface markers expressed specifically by liver progenitor cells. These cells, known as bipotential mouse embryonic liver (BMEL) cells, proliferate in an undifferentiated state and are capable of differentiating into hepatocyte-like and cholangiocyte-like cells in vitro. Upon transplantation, BMEL cells are capable of differentiating into hepatocytes and cholangiocytes in vivo. Microarray and Gene Ontology (GO) analysis of gene expression in the 9A1 and 14B3 BMEL cell lines grown under proliferating and differentiating conditions was used to identify cell surface markers preferentially expressed in the bipotential undifferentiated state. This analysis revealed that proliferating BMEL cells express many genes involved in cell cycle regulation, whereas differentiation of BMEL cells by cell aggregation causes a switch in gene expression to functions characteristic of mature hepatocytes. In addition, microarray data and protein analysis indicated that the Notch signaling pathway could be involved in maintaining BMEL cells in an undifferentiated stem cell state. Using GO annotation, a list of cell surface markers preferentially expressed on undifferentiated BMEL cells was generated. One marker, Cd24a, is specifically expressed on progenitor oval cells in livers of diethyl 1,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate-treated animals. We therefore consider Cd24a expression a candidate molecule for purification of hepatic progenitor cells.

Keywords

Hepatic stem cells; Hepatocyte differentiation; Dicarbethoxydihydrocollidine; Cd24a; Notch1

Introduction

That adult murine liver contains an experimentally inducible resident pool of progenitor cells capable of differentiating into hepatocytes and cholangiocytes has been well established. Three of the most common methods to induce hepatic progenitor cell or oval cell expansion in adult

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Disclosure of Potential Conflicts of Interest

G. Darlington has served as an officer or member of the Board for ATCC (Manassas, VA).

rodents are 2-*N*-acetylamino-fluorene intoxication followed by 70% hepatectomy, choline-deficient diets supplemented with ethionine, and diethyl 1,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate (DDC) supplemented diets. Each of these methods has in common the ability to cause parenchymal damage while inhibiting the normal hepatocyte proliferative response. The end result is the expansion and proliferation of a resident hepatic progenitor cell population originating from the terminal ends of bile ductules [1,2]. In order to understand their biology, the efficient isolation of these liver progenitor cells will be necessary. A set of progenitor cell-specific surface markers, similar to those used in hematopoietic stem cell purification, would tremendously aid in this effort. Adult hepatic progenitors have been reported to express cell surface markers used in the purification of hematopoietic stem cells (e.g., Sca1, cKit, Thy1, Cd34). However, these molecules would be difficult to use alone in any purification method, as they are also expressed by several types of cells within the liver such as cholangiocytes and resident hematopoietic cells [3–6]. In addition, a number of proteins have been characterized as being expressed by oval cells, an adult liver progenitor cell population (e.g., various glutathione S-transferase proteins, Pkm2, c-met, Krt1–19, Alb, Rab3b, Ear2, Arf4, Ltb, Cyp4a14, Crot, Nes, Psmd10, Afp) [7–17]. These gene products cannot be used for isolation of a pure homogenous progenitor cell population because they are either secreted, not expressed on the cell surface, or are expressed by other cells in the liver. This is not to say that liver progenitor cell surface markers have not been identified. A good example of this is the expression of Dlk1 on oval cells [18,19]. However, in the case of bipotential mouse embryonic liver (BMEL) cells, Dlk1 is probably not a viable marker, as expression analysis reveals that Dlk1 is not differentially expressed among BMEL culture conditions. Other progenitor cell isolation methods such as dye efflux and differential centrifugation have only provided a semipure heterogeneous population of progenitor cells [20–22]. However, these methods would prove useful if combined with a set of specific cell surface markers.

The present study used untransformed BMEL cell lines. These cells express genes characteristic of both hepatocytes and cholangiocytes and can readily be propagated in an undifferentiated state [23]. When cultured as aggregates, BMEL cells differentiated into hepatocyte-like cells expressing genes characteristic of mature hepatocytes. In contrast, BMEL cells cultured in Matrigel formed mixed cell populations, some of which went on to form ductular structures and express genes characteristic of cholangiocytes and hepatocytes. Importantly, BMEL cells were capable of contributing to new hepatocyte and new cholangiocyte growth after engrafting into damaged livers of the Alb-uPA/severe combined immunodeficient mouse [24,25]. Engraftment of BMEL cells did not involve cell fusion, a characteristic of hematopoietic stem cell liver engraftment [26], showing that BMEL cells serve as hepatic bipotential progenitor cells *in vivo* [25]. To identify hepatic progenitor cell surface markers, we analyzed the BMEL cell transcriptome from cells grown in the proliferating (bipotential) and differentiated states. Gene expression analysis was accomplished using Affymetrix (Santa Clara, CA, <http://www.affymetrix.com>) GeneChip arrays. After identifying differentially expressed genes, Gene Ontology analysis was used to extract potential progenitor cell surface markers from the cohort of genes, which demonstrated a higher relative expression in BMEL cells grown under bipotential conditions compared with expression levels in differentiated, hepatocyte-like BMEL cells.

Materials and Methods

Lipopolysaccharide and DDC Treatment

For lipopolysaccharide (LPS) treatment, CBA newborn mice were injected with 5 mg/kg body weight of LPS (Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>) intraperitoneally to induce a generalized inflammatory response. Livers were harvested 1 hour after LPS injection. For DDC treatment, C57BL/6 mice were fed a DDC diet (0.1% wt/wt in PicoLab 5LJ5 chow)

for 3 weeks. DDC was purchased from Sigma, and the DDC formulated chow was generated by the TestDiet division of LabDiet (Purina Mills, St. Louis, <http://www.purinamills.com>). Animals were maintained according to the NIH Guide for Care and Use of Laboratory Animals.

Cell Culture

Culture of BMEL cell lines is described in detail in [23]. Briefly, cells were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>) containing 10% fetal bovine serum, 50 ng/ml epidermal growth factor (EGF), 30 ng/ml insulin-like growth factor-2, 10 μ g/ml Insulin, and 50 U of penicillin/streptomycin. Under basal culture conditions, cells were grown on BD BioCoat Collagen I coated flasks (BD Biosciences, San Diego, <http://www.bdbiosciences.com>). For aggregate cultures, cells approaching confluence were removed from collagen-coated flasks using Trypsin/EDTA (Invitrogen) and resuspended in the above medium. Cell suspensions were then transferred to sterile, uncoated, bacterial 100-mm Petri dishes. Cell aggregates floating in suspension were collected 24 hours (Day 1) and 5 days (Day 5) after transfer to bacterial Petri dishes. Matrigel cultures were done as described in [23].

RNA Isolation

BMEL cells grown under basal, Matrigel, and aggregate conditions were homogenized, and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany, <http://www1.qiagen.com>). DNA digestion using RNase-Free DNase (Qiagen) was performed on-column according to the manufacturer's recommendations. RNA was quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, <http://www.nanodrop.com>). For Affymetrix GeneChip analysis, initial RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, <http://www.agilent.com>) and the NanoDrop ND-1000 Spectrophotometer by the Baylor College of Medicine Microarray Core Facility. In order to be used for GeneChip analysis, RNA had to have an 18S/28S ratio greater than 1.7, a 260/230 ratio greater than 1.5, and a lack of visual RNA degradation on Bioanalyzer electropherograms. All RNA was stored at -80°C until use.

cDNA Synthesis and Reverse Transcription-Polymerase Chain Reaction

Two μ g of total RNA isolated from BMEL cells cultured under basal and aggregate conditions was transcribed into cDNA using the SuperScript II RNase H Reverse Transcriptase Kit (Invitrogen). Polymerase chain reaction (PCR) was performed using a 1:20 dilution of sample cDNA, gene-specific primers, and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, <http://www.appliedbiosystems.com>) according to the manufacturer's protocol. PCR was run using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) as described in [27]. Briefly, thermal cycling conditions consisted of an initial step at 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds, 50°C for 60 seconds, and 72°C for 60 seconds. Data analysis was performed using the ABI Prism 7000 SDS Software (Applied Biosystems). Gene-specific PCR primers were designed using Primer Express (Applied Biosystems) and are listed in supplemental online Table 1. In all cases, experiments were done in triplicate. PCR analysis was performed according to a comparative C_t method with the geNorm VBA applet for Microsoft Excel utilizing the method detailed by Vandesompele [28]. The C_t value for each biological replicate represents the average of three technical replicates. β -Actin, γ -tubulin, and 18S were used as the normalizers for this method.

Affymetrix GeneChip Analysis

Gene expression values were determined using Affymetrix moe430a and mouse4302 arrays. The experiment conducted with moe430a arrays consisted of BMEL cell lines 9A1 and 14B3

cultured under basal, Matrigel, and Day 5 aggregate conditions. The experiment conducted with mouse4302 arrays consisted of BMEL cell lines 9A1 and 14B3 cultured under basal, Day 1 aggregate, and Day 5 aggregate conditions. Each cell line/culture condition combination was represented by two biological replicates. The Baylor College of Medicine Microarray Core Facility performed cRNA labeling, GeneChip hybridization, and biotinylated cRNA detection according to standard Affymetrix protocols. Hybridized arrays were scanned using an Affymetrix GeneChip Scanner 3000. The image files were analyzed for probe intensities and converted to tabular formats (CEL files) using the Microarray Suite Expression Analysis software from Affymetrix. Initial GeneChip quality control was done using DNA-Chip Analyzer (Dchip) [29] and the affy [30] and affycoretools analysis packages from the Bioconductor (BioC) [31] project in R [32]. Probe set normalization and expression value calculation were done via the robust multiarray average (RMA) method [33] using the affy BioC package. Prior to statistical analysis for differential probe set expression, the data were filtered to exclude probe sets with low variability by selecting those probe sets whose expression values had an interquartile range (IQR) greater than 0.5. The filtered data were used in unsupervised hierarchical clustering and principal component analysis. Hierarchical clustering was done using a distance measure of 1 minus the Pearson correlation coefficient between samples coupled to complete agglomeration using the R stats package. Principal component analysis was done using the prcomp function from the R stats package. A heat map of gene expression was done with Dchip using Euclidean distance and average linkage options. To compare probe set expression among BMEL culture conditions, we used the linear modeling functions from the BioC limma package [34]. Initially, a linear model was fit to a group-means parameterization design matrix defining the BMEL culture conditions under study. A contrast matrix defining all of the pairwise comparisons was subsequently fit, which utilized an empirical Bayes method to moderate the standard errors of the estimated log-fold changes as described [35]. The F statistic and derived F p values from the second fit combine the pairwise comparisons into a single F test equivalent to a one-way analysis of variance (ANOVA). Controlling the false discovery rate to 0.001 was used to correct for multiple testing across thousands of probe sets. Differentially expressed probe sets were defined as having an fdr corrected F p value less than .001. The resulting list of differentially expressed probe sets was used in downstream Gene Ontology (GO) [36] analysis. GO analysis was accomplished using the hyperGTest function within the BioC GStats package. Unique Entrez Gene identifiers were evaluated for GO biological process category over representation within a total GO universe defined by BioC mouse4302 annotation. GO categories with a test p value less than .05 and containing at least 20 probe sets are reported in supplemental online Tables 3 and 4.

Protein Isolation

Nonconfluent BMEL cells cultured under basal conditions were detached from collagen-coated flasks by Trypsin/EDTA (Invitrogen) treatment or directly lysed on the dish in RIPA buffer (for Notch analysis). BMEL aggregate cultures were collected by centrifuging culture medium containing cell aggregates at 700 rpm for 5 minutes. For whole cell extracts, BMEL cell pellets or homogenized liver tissue from LPS injected animals were lysed in RIPA Lysis Buffer (Santa Cruz Biotechnology Inc., Santa Cruz, CA, <http://www.scbt.com>) containing phenylmethylsulfonyl fluoride, sodium orthovanadate, and protease inhibitor cocktail according to manufacturer's protocol. Lysate was centrifuged at 10,000 rpm for 10 minutes at 4°C to remove cellular debris. For nuclear extracts, BMEL cell pellets or homogenized liver tissue from LPS-treated animals were resuspended in hypotonic buffer (10 mM Tris-HCl, pH 7.6, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol [DTT], and protease inhibitors) and incubated on ice for 5 minutes. Cells were sheared using a 28-gauge needle followed by 10 minutes of incubation on ice. Nuclei were pelleted by centrifugation at 10,000 rpm for 10 minutes at 4°C. The supernatant was collected as the cytoplasmic extract. Nuclei pellets were lysed in a high-salt buffer (20 mM Tris-HCl, pH 7.6, 25% sucrose, 0.42 M NaCl, 1.5 mM

MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, and protease inhibitors) and incubated on ice for 20 minutes. Extracts were centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was collected as the nuclear extract. Sample protein concentrations were determined using Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA, <http://www.bio-rad.com>). All protein extracts were stored at -80°C until use.

Immunoblot Analysis

Prior to loading polyacrylamide gels, protein extracts were boiled at 100°C for 5 minutes; 50–80 µg of protein extract was separated in 7.5% Tris-HCl Ready Gel Precast Gels (Bio-Rad) and transferred to Immobilon-P nylon membranes (Millipore, Billerica, MA, <http://www.millipore.com>) or, in the case of Notch detection, to a nitro-cellulose membrane (GE Healthcare, Piscataway, NJ, <http://www1.gelifesciences.com>). Membranes were blocked with 5% nonfat dry milk in 1× Tris-buffered saline/Tween 20 (TBS/T) for 1 hour at room temperature. Primary antibodies were added in 5% nonfat dry milk in 1× TBS/T and incubated overnight at 4°C with the exception of β-actin (1 hour at room temperature). The primary antibodies used were as follows: rabbit anti-mouse Stat5a/b (Santa Cruz Bio-technology; sc-835, 1:100), rabbit anti-human phospho-Stat5a/b (Upstate, Charlottesville, VA, <http://www.upstate.com>; 05-886, 1:500), rabbit anti-mouse Stat3 (Santa Cruz Biotechnology; sc-482, 1:100), rabbit anti-mouse P-727 Stat3 (Santa Cruz Biotechnology; sc-8001-R, 1:100), goat anti-human Notch1 (Santa Cruz Biotechnology; sc-6014, 1:500), goat anti-human Jag1 (Santa Cruz Bio-technology; sc-6011, 1:500), rabbit anti-human cleaved Notch1 (val1744) (Cell Signaling Technology, Beverly, MA, <http://www.cellsignal.com>; 2421, 1:2,000), mouse anti-chicken α-tubulin (MP Biomedicals, Irvine, CA, <http://www.mpbio.com>; 691251, 1:500), and mouse anti-human β-actin (Sigma; A-5136, 1:5,000). Membranes were then washed 3× 10 minutes in 1× TBS/T followed by addition of secondary antibody. The secondary antibodies used were as follows: goat anti-rabbit horseradish peroxidase (HRP) (Jackson ImmunoResearch Laboratories, West Grove, PA, <http://www.jack-sonimmuno.com>; 111-035-003, 1:1,000), goat anti-mouse HRP (Santa Cruz Biotechnology; sc-2005, 1:2,000), and swine anti-goat HRP (Caltag Laboratories, Burlingame, CA, <http://www.caltag.com>; G50007). Membranes were then washed 3× 10 minutes in 1× TBS/T followed by specific signal detection using ECL Plus (GE Healthcare) for Notch1, cleaved Notch1, Jag1, and α-tubulin. SuperSignal chemiluminescent detection reagents (Pierce, Rockford, IL, <http://www.piercenet.com>) were used for the remaining antibodies. Treated membranes were exposed to X-OMAT AR film (Kodak, Rochester, NY, <http://www.kodak.com>) to visualize antibody binding.

Electromobility Shift Assay

Prior to labeling, oligonucleotide probes were gel purified through a 15% polyacrylamide gel electrophoresis gel. Oligonucleotide probes were 3' end-labeled using Klenow and [α^{32} P]dCTP (MP Biomedicals) to a specific activity of approximately 1× 10⁹ dpm/µg; 100,000 cpm of probe was incubated with 15 µg of whole cell or nuclear extract in binding buffer (100 mM KCl, 25 mM Tris-HCL, pH 7.6, 5 mM DTT, 2 mM MgCl₂, and 10% glycerol) with 2 µg of poly(dI-dC) for 30 minutes at room temperature in a 30-µl reaction. Unlabeled competitor oligonucleotide, Stat3 antibody (Santa Cruz Biotechnologies; sc482X), and Stat5a/b antibody (Santa Cruz Biotechnology; sc835X) were incubated for 10 minutes with cell extract on ice prior to addition of labeled probe. Reaction products were visualized by separation on 5% polyacrylamide gels, dried, and exposed to X-OMAT AR film. A mouse intercellular adhesion molecule 1 interferon-gamma-activated site was used to assay for Stat3 binding (5'-AGGAGGTTTCCCGGAAAGTGG-3'). A rat casein beta (Csn2) Stat5 binding site was used to assay for Stat5 binding (5'-GGACTTCTTGGAATTAAGGGA-3').

In Situ Hybridization

Using cDNA generated from cells cultured under basal conditions, DNA riboprobe templates were generated using PCR with Cd24a specific primers (forward: GAAATTCGACGGGATTAAAGGA; reverse: GAACCAAGCCCCCTTTCAG). These primers were designed with extensions at their 5' ends with either T7 or SP6 promoter sequences (T7: GCGTAATACGACTCACTATAGGG; SP6: GCGATTTAGGTGACACTATAG). Digoxigenin labeled RNA riboprobes were generated from DNA riboprobe templates using the Riboprobe Combination kit (Promega, Madison, WI, <http://www.promega.com>) and Digoxigenin-11-dUTP (Roche Diagnostics, Basel, Switzerland, <http://www.roche-applied-science.com>). Before use in situ hybridization, labeled RNA riboprobes were diluted in hybridization mix (Ambion, Austin, TX, <http://www.ambion.com>) to a final concentration of 150–400 ng/ml. Labeled RNA riboprobes were hybridized to fixed adult liver sections by the Baylor College of Medicine In-Situ Core as described in [37].

Immunohistochemistry

Livers from adult animals treated with and without DDC were removed, frozen in Tissue-Tek OCT Compound, and sectioned to a thickness of 5–7 microns. All sections were stored at –80°C until use. Upon use, frozen sections were air-dried and fixed in 100% acetone at 4°C for 10 minutes. Fixed sections were air-dried and washed in 1× phosphate-buffered saline (PBS) for 10 minutes at room temperature (RT). When using biotinylated secondary antibodies, slides were blocked for endogenous biotin using the Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, CA, <http://www.vectorlabs.com>). After washing in 1× PBS, slides were blocked in 20% normal serum diluted in 1× PBS for 1 hour at RT. For the detection of cytokeratin 19 alone, rat anti-mouse monoclonal antibodies (TROMA III clone, a generous gift of Rolf Kemler at the Max-Planck Institute) were incubated at a 1:250 dilution in 1× PBS for 1 hour at RT. Secondary antibodies were either a fluorescein isothiocyanate (FITC) conjugated polyclonal goat anti-rat IgG (Pierce; 31629, 1:50) or a biotinylated polyclonal rabbit anti-rat IgG (Vector Laboratories; BA-4000, 1:50). Biotinylated antibodies were detected using Texas Red Avidin D (Vector Laboratories; A1100, 1:50) for 30 minutes at RT. For double Cd24a and cytokeratin 19 immunostaining, a rat anti-mouse Cd24a antibody (BD Biosciences; 557436, 1:50) was incubated with sections overnight at 4°C. Cd24a antibody was detected using a biotinylated mouse anti-rat IgG_{2b} specific antibody (Serotec Ltd., Oxford, U.K., <http://www.serotec.com>; MCA1294B, 1:50) for 1 hour at RT followed by Texas Red Avidin D (A1100, 1:50) for 30 minutes at RT. Cytokeratin 19 was subsequently detected using primary antibody as described above coupled with a FITC conjugated mouse anti-rat IgG_{2a} specific antibody (Serotec; MCA278F, 1:50) for 1 hour at RT. For double Cd24a and Cd45 immunostaining, a FITC conjugated rat-anti-mouse Cd24a antibody (BD Biosciences; 553261, 1:50) was incubated with sections for 1 hour at RT. Cd45 was subsequently detected using a phycoerythrin (PE) conjugated rat anti-mouse Cd45 antibody (BD Biosciences; 553081, 1:50) incubated for 1 hour at RT. All images were captured using a Hamamatsu C5810 color chilled 3ccd camera (Hamamatsu Photonics, Hamamatsu City, Japan, <http://www.hamamatsu.com>) and an Olympus IX70 microscope (Olympus, Tokyo, <http://www.olympus-global.com>).

Fluorescence-Activated Cell Sorting

Untreated and DDC-treated adult mouse livers were perfused with 0.28 mg/ml collagenase IV (Sigma; C-5138). Cells were passed through a 70-μm filter to remove undigested tissue debris and centrifuged at 10g for 5 minutes to pellet hepatocytes. The supernatant was centrifuged at 350g for 10 minutes and resuspended in 90 μl of running buffer (1× PBS, pH 7.2, 2 mM EDTA, 2% bovine serum albumin) per 10⁷ total cells. Resuspended cells were mixed with 10 μl of anti-mouse Ter119 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany,

<http://www.miltenyibiotec.com>; 130-049-901) per 10^7 total cells and incubated at 4°C for 15 minutes. The labeled cell suspension was applied to an autoMACS separator (Miltenyi Biotec) to deplete Ter119 positive cells. The depleted cell suspension was then incubated with fluorescently conjugated antibodies on ice for 10 minutes followed by resuspension to 10^7 cells per milliliter for fluorescence-activated cell sorting (FACS) analysis. Data were acquired using a Dako (Glostrup, Denmark, <http://www.dako.com>) Cytometer. The antibodies used for FACS analysis were FITC-conjugated rat anti-mouse CD45 monoclonal antibody (mAb) (BD Biosciences; 553080), FITC-conjugated rat anti-mouse Ter119 (BD Biosciences; 557915), and PE-conjugated rat anti-mouse CD24 mAb (BD Biosciences; 553262).

Results

BMEL Cells Grown Under Basal, Matrigel, and Aggregate Conditions Represent Three Distinct States of Differentiation

To characterize the effects of three different culture conditions on BMEL cell gene transcription, Affymetrix moe430a Gene-Chips were used to measure transcript levels. Array probe set expression values were normalized and modeled using the RMA method of Irizarry et al. [33]. Array probe sets with little expression variability across the culture conditions were removed from the data set to eliminate uninformative ones. This was accomplished using an IQR filter applied to the RMA normalized expression values as described in the supplemental online materials. Principal component analysis (PCA) of samples using the filtered data set was performed to visualize the relationship between the three culture conditions as defined by their probe set expression patterns and to classify the highest sources of variance. As seen in the screeplot from Figure 1A, the first three principal components (PC1–PC3) account for approximately 91% of the total variance inherent to the experiment. PC1, at 74% of the total variance, represented the largest source of variance. PC2, the second largest variance component, contributed only 9% to the total variance. One can conclude from this that the experimental parameters that make up PC1 are responsible for the majority of the experimental variance and constitute the main treatment effect. PC1 defined the difference among the three BMEL cell culture conditions: basal (undifferentiated), Matrigel (cholangiocyte-like), and aggregate (hepatocyte-like), which was evident by graphing the two largest principal components against each other (PC1 vs. PC2) (Fig. 1B). In contrast, PC2 defined the small amount of variance present between biological replicates of the 9A1 and 14B3 cell lines (Fig. 1B). The fact that replicate samples of the 9A1 and 14B3 BMEL cell lines primarily grouped together based on culture condition and not cell line demonstrated that these two independently isolated BMEL cell lines have a similar phenotype and respond in a similar fashion to the experimental culture conditions. As judged by PC1, the largest difference in BMEL culture conditions occurred between the basal and Day 5 aggregate samples. Using Pearson's correlation coefficient between samples as a distance measure, hierarchical clustering analysis (Fig. 1C) showed that the Matrigel and aggregate differentiated samples group together on a branch separate from the basal undifferentiated samples. That two different methods for BMEL differentiation grouped together on the same branch is not too surprising, as earlier studies showed that they both express genes characteristic of mature hepatocytes, exit the cell cycle upon differentiation, and differ from basal cultures by absence of a collagen adherence substrate [23].

One of the principal goals of our study was to identify a list of differentially expressed cell surface markers/proteins to isolate and enrich hepatic progenitor cells from adult livers. Because results from PCA and clustering analysis indicated that the number of differentially expressed probe sets was greatest between the basal and aggregate cultures, we narrowed analysis to the differential expression between undifferentiated BMEL cells in basal cultures and the differentiated BMEL cells from aggregate cultures. In addition, the tight grouping of

the two different BMEL cell lines based on culture condition (Fig. 1B) permitted us to treat the four samples (two for each cell line) within each culture condition as biological replicates.

Differentially Expressed Genes in Undifferentiated (Basal) Versus Hepatocyte-Like (Aggregate) Culture Conditions

Cells were analyzed 5 days after aggregation (Day 5 [D5] aggregate) to characterize the gene expression profile of hepatocyte-like cells in relation to undifferentiated BMEL cells as initially studied by Strick-Marchand et al. [23]. To put the hepatocyte-like gene expression profile into context, gene expression changes occurring earlier after BMEL cell aggregation were also investigated. An earlier time point was added, and cells were analyzed 24 hours (Day 1 [D1] aggregate) after aggregation. These samples were hybridized to Affymetrix mouse4302 GeneChips, which approximately doubled the number of probe sets available for analysis. As before, RMA normalization and model expression, IQR expression filtering, and PCA were used to visualize the relationship among the culture conditions. As with the moe430a experiment, the first three principal components accounted for the majority of the total variance. Graphing the first two principal components against each other revealed that PC1, at 62% of the total variance, primarily defined the difference among the three BMEL cell culture conditions: basal, D1 aggregate, and D5 aggregate (data not shown). PC2 again defined the small variance among biological replicates of the 9A1 and 14B3 cell lines in each culture condition (data not shown).

Differential probe set expression was determined using a linear model fit to a group-means parameterization design matrix defining the basal, D1, and D5 culture conditions. In this model, samples from both cell lines within each culture condition were grouped and treated as four biological replicates due to the low contribution of the cell line effect to the total variance. A contrast matrix detailing all the pairwise culture condition comparisons (D5 vs. basal, D1 vs. basal, and D5 vs. D1) was used in a second fit to extract log₂ fold changes among culture conditions. ANOVA *F* *p* values from the second fit were corrected for multiple testing by controlling the false discovery rate [38]. Probe sets with an adjusted *F* *p* value of less than .001 were considered to be significantly changing across culture conditions and were included in further analysis. In this way, probe sets not contributing information about the difference between basal and aggregate samples were removed; 34 genes (supplemental online Table 1) were selected for PCR validation of the array-determined fold changes. These genes showed a broad range of fold changes varying from strongly upregulated in basal samples to strongly upregulated in aggregate samples to no change among culture conditions. Each of the 34 genes was assayed in basal, D1, and D5 samples. Figure 2 shows array and PCR determined log₂ fold changes between the aggregate and basal samples plotted against each other. A least squares best-fit line had an *r*² value of .71, indicating good agreement among fold changes determined by the array experiment and PCR. Figure 2 also shows that the array data underestimate biological fold change as determined by PCR, reflecting the greater accuracy of PCR versus array detection [39].

In Figure 3, the Dchip array analysis program was used to display a heat map of the 2,656 probe sets whose expression changes were significant (*F* *p* value < .001) (supplemental online Table 2). As depicted in Figure 3, gene expression patterns fell into five main groups labeled A–E. Group A (995 probe sets) included probe sets that showed the highest relative expression in D5 samples. Group B (97 probe sets) included probe sets that showed a higher relative expression in D1 samples, which was maintained in D5 samples. Group C (117 probe sets) included probe sets that showed the largest relative expression exclusively after 24 hours of aggregation. Taken together, groups A, B, and C contained probe sets whose expression levels increase in response to aggregation relative to the undifferentiated BMEL cells. Also evident in Figure 3 are probe sets regulated in the opposite direction from those in groups A, B, and

C. Group D (965 probe sets) contained probe sets with a higher relative expression in the basal samples compared with cells cultured as aggregates. These probe sets were of particular interest because among them should be genes that are important for maintenance of the undifferentiated, bi-potential state. Probe sets from group E (482 probe sets) showed a higher relative expression in basal samples, which was maintained or slightly decreased after 24 hours of aggregation when compared with D5 of aggregation. Additionally, Figure 3 showed that for a large set of probe sets (groups A, D, and E) the expression differences between D1 and basal samples were in the same direction as those between D5 and basal samples. The main difference between these two contrasts was the magnitude of the probe set fold change values. In each case there was a greater difference between the D5 and basal values than the D1 and basal values. This indicated for the most part that differences in expression detected as early as D1 continue to increase through D5. This made sense because D1 is probably a snapshot of the differentiation process caused by aggregation ending at D5 in this experiment. However, after 24 hours of aggregation, it was not clear whether all of the cells had begun to differentiate uniformly. It is possible that Day1 samples represent a mixture of differentiated and undifferentiated cells.

Gene Ontology Biological Process Analysis

Probe sets from all groups were characterized according to GO annotation [36]. Over-representation of GO biological process terms, one of the three main GO classification schemes containing functional terms, was used to characterize the differences among BMEL culture conditions based on the 2,656 significantly changing genes. For this analysis, probe sets from groups A, B, and C were grouped together representing the differentiated cell state, and probe sets from groups D and E were grouped together representing the undifferentiated cell state. The enriched GO categories (p value $< .05$) identified from probe sets in groups A, B, and C represent classic hepatocyte functions and include processes such as acetyl-CoA metabolism, alcohol metabolism, lipid metabolism, and carbohydrate metabolism (supplemental online Table 3). GO biological process over-representation analysis of probe sets from groups D and E (supplemental online Table 4) showed a vastly different pattern of significant (p value $< .05$) GO terms. Probe sets from groups D and E did not show an over-representation of functional categories associated with differentiated hepatocytes, agreeing with analysis done by Strick-Marchand et al. [23]. Rather, they included cell cycle processes such as chromosome segregation, DNA replication, cell division, mRNA metabolism, and ribosome biogenesis and assembly. These categories indicate that BMEL cells in basal culture are actively proliferating. Of note, the BMEL lines were developed from 14 days postcoitum embryonic livers [23], a stage when liver contains dividing and differentiating hepatoblasts (embryonic liver progenitor cells) [40]. Thus, BMEL cells in basal culture may mimic proliferative mechanisms of early hepatoblasts and could be representative of an activated adult hepatic progenitor cell.

Gene Ontology “Transcription” Analysis

We also utilized GO annotation of the significantly changing probe sets to identify factors responsible for driving differentiation of BMEL cells and factors responsible for maintaining undifferentiated BMEL cells. We looked specifically at GO biological processes containing the word “transcription,” which include transcription factors (TFs) and factors regulating the transcriptional process.

We knew from GO biological process over-representation analysis that probe sets from groups A, B, and C of Figure 3 contributed to many aspects of liver function. The largest group of TFs influencing hepatocyte function came from within group A, those probe sets strongly induced by D5 of BMEL cell aggregation. Nr1i3 (Car), Nr1i2 (Pxr), Nr5a2 (Lrh), Hnf4g, Nfe2l2 (Nrf2), and Mlx1p (Wbcr14, ChREBP) were all highly expressed by D5 of aggregation. These genes

are involved in the induction of bile acid synthesis [41], xenobiotic gene induction [42–44], lipid synthesis [45], and carbohydrate metabolism [46], all of which are normal liver functions.

Within groups B and C, 24 hours after aggregation, we detected the modest induction of the TF Stat3, known to be critical for the liver acute phase response [47] and the transient induction of Stat5. Because Stat proteins are involved in many aspects of the acute phase response, and evidence exists that Stat3 is activated in rat oval cells [48], reverse transcription-PCR analysis was used to confirm Stat3 and Stat5 expression levels. A slight trend for higher expression in aggregate cultures was revealed, which did not reach statistical significance (supplemental online Table 1). Figure 4 shows Western analysis of Stat3 and Stat5 protein expression and phosphorylation. The second and fourth panels of Figure 4A show that both total Stat5 and total Stat3 protein expression did not change between basal, D1, and D5 samples. However, Stat3 seemed to be constitutively phosphorylated across culture conditions (Fig. 4A, panel 3), whereas little if any Stat5 phosphorylation was detected (Fig. 4A, panel 1). Stat3 is known to be a target of EGF in the liver; therefore, the use of EGF during BMEL cell culture could be contributing to constitutive Stat3 phosphorylation across basal and aggregate culture conditions. Figure 4B serves as a control for this Western blot and shows that the Stat3 and Stat5 bands detected in basal samples are the same size as bands expressed in adult livers before and after 1 hour of LPS treatment, which is known to induce the expression and phosphorylation of Stat proteins [49]. Electrophoretic mobility shift assay (EMSA) analysis of BMEL protein extracts did not detect any specific Stat5 or Stat3 binding to DNA consensus elements despite phosphorylation and apparent activation of Stat3 (Fig. 5).

Another TF implicated in liver function is Notch. Alagille syndrome in humans, characterized by a scarcity of bile ducts [50], is linked to mutations in the Jagged-Notch signaling pathway, demonstrating that Notch signaling is important in liver function. Interestingly, the GO category “Notch signaling pathway” (GO:0007219) was over-represented among probe sets compiled from groups A, B, and C (supplemental online Table 3). These groups contained those probe sets induced by aggregation. Microarray data indicated that regardless of culture condition, BMEL cells expressed mRNA for Notch ligands Jagged1 and Jagged2 as well as the Notch target gene Hes1 (data not shown). Microarray analysis showed a transient induction of Notch1 mRNA 24 hours after aggregation, which dropped back to below basal levels by D5 of aggregation (supplemental online Table 2). Furthermore, mRNA for two Notch signaling pathway inhibitors, Numb and Sel1h, was induced in aggregate samples. To confirm a role of Notch signaling during BMEL cell differentiation, we analyzed its protein expression pattern by Western blot. Both Jagged1 and full-length Notch1 proteins were present in undifferentiated and differentiated cells. However, the activated cytoplasmic domain of Notch, NICD, was only detected in undifferentiated cells (Fig. 6). This indicated that Notch signaling was active only in cycling, undifferentiated BMEL cells and that differentiation was accompanied by inhibition of the pathway. To our knowledge, these are the first cell lines in which endogenous NICD expression has been observed without the addition of overexpression vectors.

GO transcription factor analysis of probe sets with higher expression under basal culture conditions (groups D and E) identified a different set of genes. Interestingly, Foxm1 was among the differentially expressed probe sets within group D (supplemental online Table 2). Foxm1 regulates many aspects of cell cycle progression within the hepatocyte [51]. Animals null for Foxm1 do not develop livers [52], indicating that this gene may be important for proliferation of hepatocytes in vivo. Therefore, Foxm1 may also be important for promoting proliferation and maintenance of the bipotential state of BMEL cells cultured under basal conditions. In group D we also observed the upregulation of Etv5 and Taf4b, two genes critical for sperm production. Spermatogonia from animals null for either the Sertoli cell expressed Etv5 [53] or the spermatogonia cell expressed Taf4b [54] lose the ability to undergo asymmetric division, a hallmark function key to stem cell self-renewal. The upregulation of Etv5 in basal cultures

was confirmed by PCR analysis (supplemental online Table 1). The expression of two critical stem cell associated genes, *Etv5* and *Taf4b*, in undifferentiated BMEL cells is consistent with their demonstrated progenitor cell capability. Also present in group D was the transcription factor *Myc*. Interestingly, *Mybl2* (group D), previously shown to positively regulate *Myc* transcription [55], and *Ruvbl1/Ruvbl2* (group D), previously shown to inhibit *Myc* mediated apoptosis [56], were also upregulated in basal cultures. Interestingly, Frye M et al. showed that activation of *Myc* within epidermal stem cells of the skin causes their differentiation and the subsequent depletion of the stem cell niche [57]. Taken together and in consideration of the high proliferative index of BMEL cells grown under basal conditions, we feel *Myc* expression supports the proliferation of undifferentiated BMEL cells under basal conditions.

Gene Ontology Cellular Component Analysis

A main goal of this analysis was to detect cell surface markers for purification of hepatic progenitor cells from the adult liver. Best candidates would be genes whose expression is higher in basal cultures relative to aggregate cultures. Of the 2,656 genes (supplemental online Table 2) shown to be differentially expressed, genes associated with the cell surface or the extracellular space were filtered based on GO cellular component terms “integral,” “plasma membrane,” or “extracellular” in their GO descriptions. Using D5 of aggregation as an approximate filter for genes expressed by hepatocytes, we eliminated probe sets not displaying at least a twofold greater expression in basal cultures from the D5 versus basal array contrast. This reduced our chances of selecting markers with a significant level of expression in hepatocytes. The list of cell surface markers can be found in supplemental online Table 5. It is encouraging that the TWEAK receptor, *Tnfrsf12a*, was identified as a cell surface progenitor cell candidate. Recently, Jakubowski et al. showed that mice null for *Tnfrsf12* fail to undergo a ductular reaction and oval cell proliferation in response to DDC treatment [58]. Up-regulation of *Tnfrsf12a* in undifferentiated BMEL samples could indicate that these cells use signaling mechanisms similar to oval cells, further supporting the use of the BMEL cells as an experimental model. Using PCR, the expression of candidates *Cmkor1*, *Vcam1*, *Fzd2*, *Fzd6*, *Epha4*, *Cd24a*, *Steap1*, *Cd9*, and *Gja1* was confirmed to be higher in basal samples than aggregate samples (supplemental online Table 1). In addition, PCR confirmed *Cmkor1*, *Steap1*, and *Gja1* were higher in basal samples relative to D1 aggregate samples. It was not surprising that some of the markers are still expressed in BMEL cells after 24 hours of aggregation. D1 of aggregation probably represents a transition from bipotential, undifferentiated cells to differentiated, hepatocyte-like cells and for this reason might display gene expression patterns of both stages.

To validate expression of our candidate cell surface markers in adult mouse liver, we used a chemical model of liver injury that causes oval cell proliferation [22,59]. Treatment with the porphyrinogenic agent DDC causes an atypical ductular proliferation in which progenitor cells (oval cells) emanate from terminal bile ductules known as the canals of Herring [1]. We determined which BMEL cell-derived candidate genes are expressed within the expanded progenitor/oval cell compartment after 3 weeks of DDC treatment while remaining absent from surrounding cell types. Figure 7A shows the effects of 3 weeks of DDC treatment on mouse liver. Ductular proliferation was readily apparent with routine H&E staining. In addition to progenitor cell proliferation and expansion, a significant inflammatory infiltration characteristic of all models of oval cell induction was observed [60]. Figure 7B shows cytokeratin 19, a bile epithelial and progenitor cell marker, staining in an untreated mouse liver highlighting the bile ducts. Compared with staining in Figure 7B, Figure 7C shows the expansion of cytokeratin 19 stained areas, marking the extent of ductular expansion and oval cell proliferation. Figure 7D–7F shows RNA localization of the candidate marker *Cd24a* using in situ hybridization. *Cd24a*, also known as the heat stable antigen, is a glycosylated GPI linked cell surface protein upregulated in basal samples (supplemental online Tables 1, 2). Arrows

point to regions of digoxigenin-labeled antisense probe/antibody precipitate at both low (Fig. 7E) and higher magnification (Fig. 7F) showing Cd24a mRNA localizing to cells surrounding bile ducts. The remainder of Figure 7 shows the localization of Cd24a protein in the DDC induced model of oval cell proliferation. Cytokeratin 19 was used as a general marker for progenitor cells, and the pan-hematopoietic marker Cd45 was used to label the DDC associated inflammatory cells. Figure panels 7G and 7H depict cytokeratin 19 and Cd24a immunostaining, respectively, in DDC treated animals. The merged image of these two micrographs (Fig. 7I) shows that the vast majority of the DDC induced ductular reaction that is positive for cytokeratin 19 protein is also positive for Cd24a protein. No clear Cd24a staining is visible in the surrounding parenchyma indicating that little if any Cd24a is expressed on hepatocytes. Figure 7J–7L offers a higher magnification image of cytokeratin 19 and Cd24a colocalization at sites of ductular proliferation. Although Cd24a immunostaining colocalizes with most regions positive for cytokeratin 19 (Fig. 7L), asterisks depict small regions of Cd24a immunostaining with little or no cytokeratin 19 colocalization. Because DDC treatment caused inflammation in and around the same areas where ductular proliferation and Cd24a staining are seen, we tested to see whether Cd24a positive cells were also Cd45 positive. If Cd24a and Cd45 colocalize it would indicate that the Cd24a positive cells may be of hematopoietic origin and therefore not hepatic progenitor cells. Figure panels 7M and 7N illustrate Cd24a and Cd45 immunostaining in and around a region of ductular proliferation. The merge of these photomicrographs (Fig. 7O) clearly shows that these two markers do not overlap, indicating that Cd24a positive cells are not derived from the DDC-induced inflammatory reaction. Consistent with the increase in immunostaining of Cd24a in livers from DDC-treated animals, FACS analysis (supplemental online Fig. 1) of livers from DDC-treated animals shows a 4.2-fold increase in Cd24a positive cells when compared with livers from untreated animals.

Discussion

The effective biological study of any cell population requires the ability to purify the cells from surrounding tissue to homogeneity. A proven isolation technique, widely used within the hematopoiesis field to isolate stem cells, is the use of fluorescent labeled antibodies to detect a combination of cell surface markers expressed on the cell of interest. Target cells can then be purified to relative homogeneity using FACS. The present work was aimed at discovering specific cell surface markers expressed by adult liver progenitor cells that might then be used for the isolation of a pure population of progenitor cells from the liver parenchyma.

As a model for liver progenitor cell gene expression, we studied two independently isolated BMEL cell lines known to contribute to both hepatocyte and cholangiocyte repopulation of damaged livers following transplantation. Principal component analysis of microarray expression data revealed little mRNA expression variation between the two independent BMEL cell lines, demonstrating the reproducibility of phenotypic profiles of BMEL cell lines isolated following the “plate and wait” isolation procedure. This reproducibility makes this isolation technique ideally suited for the production of liver progenitor cell lines as it enables reliable comparison between samples. PCA analysis and hierarchical clustering of unsupervised BMEL gene expression highlighted a clear distinction between cells differentiated in aggregate or Matrigel cultures and undifferentiated and heritably bipotential cells maintained in basal cultures. We have exploited this differential response of BMEL cells to culture condition to identify molecules showing enriched expression in liver progenitor cells and to search for regulatory pathways potentially involved in maintenance of their bipotentiality.

To define the initial gene expression changes occurring during differentiation, we examined two contrasts. The first was the difference between 24-hour aggregate and basal cultures to reveal genes involved in early actions of aggregate-induced differentiation as well as those

genes uniquely expressed in undifferentiated bipotential cells. The second contrast was the comparison between D5 aggregate and basal growth conditions to eliminate those genes expressed by hepatocytes.

Five main expression profile groups (A–E; Fig. 3) were identified that ranged from genes highly expressed in basal cultures to genes highly expressed in aggregate cultures. Gene Ontology analysis of these groups revealed a dramatic switch in functional profile as proliferating BMEL cells differentiated into hepatocyte-like cells. This analysis revealed that basal culture conditions, while maintaining bipotentiality, result in the active proliferation of BMEL cells and that aggregating culture conditions induce a mature liver differentiation program resulting in repression of cell cycle associated genes and induction of many genes characteristic of liver metabolism.

In addition to cell surface markers, we also wanted to identify transcription factors maintaining bipotentiality of BMEL cells as well as factors involved in early induction of hepatocyte specification. We identified not only transcription factors but also genes that regulate transcriptional processes. Three transcription factors (Stat3, Stat5, and Notch1) were chosen for further analysis due to their known importance for liver function. EMSA analysis of both Stat3 and Stat5 failed to reveal any binding to their respective consensus response elements within extracts of BMEL cells cultured under basal, D1 aggregate, or D5 aggregate conditions. This was surprising for Stat3 due to its high level of phosphorylation in all samples, indicating an apparent active state. However, another mechanism might be at play for Stat3. A study by De Miguel et al. showed that Stat3 has the ability to act as a coregulator for the steroid receptors Ar, Esr1, Nr3c1 (Gr), and Nr3c2 (Mr) [61]. According to microarray expression data, both Nr3c1 and Nr3c2 are expressed by BMEL cells, and, even though they were not differentially regulated across culture conditions (data not shown), availability of a coregulator could be limiting.

In murine fetal liver, Jagged1 is expressed in cells surrounding vessels, whereas Notch1 is expressed in all cells [62]. In the adult liver, Jagged1, Notch1, and the activated cytoplasmic domain of Notch, NICD, are all present in hepatocytes and bile duct cells. Partial hepatectomy, which causes proliferation of hepatocytes, is accompanied by an increase in NICD [63]. In BMEL cells, the activated Notch1 peptide NICD was present under basal conditions and not under aggregate conditions. In addition, array analysis indicated that both Numb and Sellh, two Notch signaling pathway inhibitors, were upregulated after 5 days of aggregation (supplemental online Table 2) [64,65]. This pattern of Notch1 activation in undifferentiated BMEL cells is consistent with findings by Tanimizu et al., which showed that Notch was expressed in hepatoblasts and that active Notch signaling blocked differentiation into hepatocytes [66]. BMEL cells grown under basal conditions may utilize Notch cell/cell signaling to maintain a niche required for the undifferentiated and bipotential states. Upon differentiation/aggregation, induction of Numb and/or Sellh may play a role in shutting down Notch signaling in differentiating BMEL cells.

Candidate progenitor cell surface genes were identified by filtering the 2,656 probe sets displaying significant differential expression for Gene Ontology terms associated with membrane localization and/or the extracellular space. From the basal versus D5 contrast, 64 genes were identified as having enriched expression in undifferentiated BMEL cells. In situ hybridization analysis showed Cd24a to be a promising cell surface candidate. In adult mouse livers, Cd24a mRNA was localized to cells surrounding bile ducts, the putative resting niche for adult liver progenitor cells. Immunofluorescent localization of Cd24a in DDC-treated livers showed the protein to localize to the oval cell compartment within regions of ductular proliferation and to bile epithelial cells within the same region. No Cd24a protein was detected on hepatocytes. From studies in other organisms, there is evidence that Cd24a may play a role

in progenitor cells. For example, it has been shown that mammary fat pad repopulation capacity segregates with epithelial cells showing Cd24a low expression [67]. In addition, Cd24a was identified as a potential marker for renal progenitor cells in an array study of embryonic and adult kidney [68]. Recent evidence has also suggested that Cd24a may serve as a prognostic marker for intrahepatic cholangiocarcinoma [69]. Liver cancer is one of many cancers thought to be a result of aberrant progenitor cell proliferation [70]. Double labeling with Cd45 and Cd24a antibodies showed these two markers to be mutually exclusive, indicating that cells expressing Cd24a are not of hematopoietic origin. In addition, FACS analysis of DDC-treated livers demonstrated that the Cd24a epitope remains intact after collagenase perfusion, indicating that the Cd24a antibody used in this study can be effectively used to isolate cells. Consequently, we conclude that Cd24a represents a genuine candidate cell surface marker for liver-derived progenitor cells.

When stem cells are rare, immortalized cell lines that undergo differentiation in vitro may serve to identify novel genes that can be subsequently verified in vivo for their utility. BMEL cells represent such a model system, and the data presented here have shown Cd24a to be a promising cell surface candidate for the isolation of progenitors from adult liver.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Principal component and hierarchical clustering analysis with interquartile range filtered expression data. Screeplot from principal component analysis analyzing basal, Matrigel, and Day 5 aggregate bipotential mouse embryonic liver (BMEL) samples (**A**). The relative contribution of each principal component to the total experimental variance is shown. Scatter plot of principal component 1 versus principal component 2 (**B**). The biological replicates of the three BMEL culture conditions group together in distinct clusters separated along the PC1 axis. In each case, filled objects are from the 9A1 BMEL cell line, and open objects are from the 14B3 BMEL cell line. Hierarchical cluster display of BMEL samples using a 1 – Pearson correlation coefficient among samples as a distance measure and complete linkage agglomeration (**C**). Abbreviations: A, aggregate; B, basal; M, Matrigel; PC, principal component.

Microarray FC Validation

Figure 2.

Validation of microarray determined fold changes using reverse transcription (RT)-PCR. The expression of 34 genes (supplemental online Table 1) was assayed using RT-PCR in basal, D1 aggregate, and D5 aggregate bipotential mouse embryonic liver RNA samples. Log₂ fold change values were calculated for the D1 versus basal and D5 versus basal contrasts. The RT-PCR determined fold change values for the 34 genes were plotted against the corresponding microarray fold change values from the mouse4302 microarray data. A best-fit line through all of the data points was calculated using a linear least squares method (lm function from the R stats package) and had an r^2 value of .71; ● = D1 versus basal contrast; ○ = D5 versus basal

contrast. Abbreviations: B, basal; D1, day 1; D5, day 5; FC, fold change; PCR, polymerase chain reaction.

Figure 3.

Heat map visualization of the 2,656 differentially regulated probe sets. Probe sets with an F p value less than .001 from the linear differential expression analysis of basal, Day 1, and Day 5 samples were clustered using DNA-Chip Analyzer. Hierarchical clustering of probe sets was done using Euclidean distance and average linkage. Each row has been standardized by subtracting the mean expression and dividing by the standard deviation. Five main probe-set expression patterns labeled A–E. Red indicates a higher relative expression and blue a lower relative expression.

Figure 4.

Protein expression of Stat3 and Stat5 in bipotential mouse embryonic liver (BMEL) cells as determined by Western analysis. Nuclear extracts from the 9A1 BMEL cell line grown under basal, Day 1, and Day 5 culture conditions were assayed for both total and phosphorylated stat protein (**A**). Both Stat5 and Stat3 phosphorylation (**A**, panels 1 and 3) as well as total Stat5 and Stat3 protein levels are shown (**A**, panels 2 and 4). Each lane is an independent biological replicate. Nuclear liver extracts from newborn mice treated with LPS (5 mg/kg) or vehicle (untreated) for 1 hour serve as a control for the size and phosphorylation of Stat3 and Stat5 relative to the basal samples (**B**). Again, both Stat5 and Stat3 phosphorylation (**B**, panels 1 and 3) as well as total Stat5 and Stat3 protein levels are shown (**B**, panels 2 and 4). The sizes

of the Stat5 and Stat3 bands in the basal samples are the same as those induced by LPS in newborn mice. Abbreviation: LPS, lipopolysaccharide; MW, molecular weight; Unt, untreated.

Figure 5.

Electromobility shift assay (EMSA) for Stat3 and Stat5 proteins. Stat5 EMSA in bipotential mouse embryonic liver (BMEL) nuclear extracts (**A**). Lanes 4–12 do not show any detectable Stat5 binding to a rat Csn2 oligonucleotide probe in samples from BMEL cells cultured under basal, Day 1, or Day 5 aggregate conditions (**A**). Extracts from an adult untreated liver (**A**), lanes 1–3) serve as a control for Stat5 protein binding to the rat Csn2 oligonucleotide. Stat5 binding is specific as a Stat5 antibody causes a supershift (**A**, lane 2) and 100× nonlabeled rat Csn2 oligonucleotide competes (**A**, lane 3) with labeled rat Csn2 for protein binding. Stat3 EMSA in BMEL nuclear extracts (**B**). Lanes 7–15 do not show any detectable Stat3 binding to a mouse Icam1 oligonucleotide probe using the same BMEL cell extracts used in (**A**).

Extracts from newborn animals treated with LPS (5 mg/kg) ([B], lanes 4–6) serve as a control for Stat3 protein binding to the mouse Icam1 oligonucleotide. Stat3 binding is specific as a Stat3 antibody ([B], lane 5) causes a supershift and 100× nonlabeled mouse Icam1 oligonucleotide competes ([B], lane 6) with labeled mouse Icam1 for protein binding. Solid arrows point to specific Stat5 and Stat3 protein binding, asterisks show supershifted band(s), and a., b., and c. indicate unidentified specific protein binding to the mouse Icam1 oligonucleotide that does not supershift with the Stat3 antibody. Abbreviations: Ab., probe + antibody; Comp., probe + 100× nonlabeled oligonucleotide; Csn2, rat casein beta oligonucleotide; Icam, mouse intercellular adhesion molecule oligonucleotide; L:LPS, newborn animals treated with lipopolysaccharide; UL, untreated newborn animals.

Figure 6.

Protein expression of Notch1, Jagged 1, and cleaved Notch1 in bipotential mouse embryonic liver cells as determined by Western blot analysis. Protein extracts from the 9A1 and 14B3 cells lines were grown in undifferentiated (basal) culture and differentiated as aggregates for 5 days. Jag1 and Notch1 proteins were present in both undifferentiated and differentiated cultures. The cleaved active form of Notch1, NICD, was present only in undifferentiated cells. α -Tubulin was used to ensure equal protein loading. Abbreviations: α -tub, α -tubulin; Jag1, Jagged 1; NICD, cleaved Notch1.

Figure 7.

Detection of candidate cell surface markers in livers chemically induced to undergo progenitor cell proliferation. Mice were treated with DDC for 3 weeks to induce hepatic damage, atypical ductular reaction, and oval cell expansion. Routine H&E staining of liver sections taken from animals treated with DDC (A). Regions staining dark purple correspond to regions of DDC-induced ductular proliferation containing oval cells and hematopoietic infiltrate (solid arrows). The pink staining areas surrounding regions of ductular proliferation correspond to hepatocytes and endothelial-lined sinusoids. Cytokeratin 19 immunostaining of normal adult liver and DDC-treated liver, respectively, illustrates the extent of the DDC-induced ductular reaction (B, C). In situ localization of Cd24a mRNA using sense and antisense cDNA probes (D–F).

Hybridization using a control sense probe (**D**). Hybridization using an antisense probe at low and high magnifications where arrows point to regions of digoxigenin-labeled anti-sense probe/antibody precipitate (**E, F**). Low and high magnifications of cytokeratin 19 immunostaining in animals treated with DDC (**G, J**). Cd24a immunostaining of the same tissue sections from panel (**G**) and panel (**J**) (**H, K**). The merge of these photomicrographs illustrated a clear expression overlap between these two proteins in regions of ductular reaction (**I, L**). Cd24a and Cd45 immunostaining of a ductular reaction region from animals treated with DDC (**M–O**). Merging areas of CD24a and Cd45 staining showed that these two proteins are not expressed on the same cells, indicating that cells expressing Cd24a are not derived from hematopoietic cells (**O**);*, areas of Cd24a immunostaining that do not overlap with cytokeratin 19 immunostaining. Abbreviations: DDC, diethyl 1,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate; dr, diethyl 1,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate-induced ductular reaction; h, hepatocytes; wks, weeks.