Exploring Interactions Between Rat Hepatocytes and Nonparenchymal Cells Using Gene Expression Profiling

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Cocultivation of primary hepatocytes with a plethora of nonparenchymal cells (from within and outside the liver) has been shown to support hepatic functions *in vitro*. Despite significant investigation into this phenomenon, the molecular mechanism underlying epithelial–nonparenchymal interactions in hepatocyte cocultures remains poorly understood. In this study, we present a functional genomic approach utilizing gene expression profiling to isolate molecular mediators potentially involved in induction of liver-specific functions by nonparenchymal cells. Specifically, primary rat hepatocytes were cocultivated with closely related murine fibroblast cell types (3T3-J2, NIH-3T3, mouse embryonic fibroblasts) to allow their classification as "high," "medium," or "low" inducers of hepatic functions. These functional responses were correlated with fibroblast gene expression profiles obtained using Affymetrix GeneChips. Microarray data analysis provided us with 17 functionally characterized candidate genes in the cell communication category (cell surface, extracellular matrix, secreted factors) that may be involved in induction of hepatic functions. Further analysis using various databases (*i.e.,* PubMed, GenBank) facilitated prioritization of the candidates for functional characterization. We experimentally validated the potential role of two candidates in our coculture model. The cell surface protein, neural cadherin (N-cadherin), was localized to hepatocyte–fibroblast junctions, while adsorbed decorin up-regulated hepatic functions in pure cultures as well as cocultures with low-inducing fibroblasts. In the future, identifying mediators of hepatocyte differentiation may have implications for both fundamental hepatology and cell-based therapies (*e.g.,* bioartificial liver devices). In conclusion, the functional genomic approach presented in this study may be utilized to investigate mechanisms of cell–cell interaction in a variety of tissues and disease states. (HEPATOLOGY 2004;40:545–554.)

The development and function of tissues depend on interactions between nonparenchymal and epithelial cells to modulate differentiation, proliferation, and migration. Specifically, epithelial–nonparenchymal interactions are important in physiology,[1] pathophysiology,[2] cancer,[3] development,[4] and in attempts to replace tissue function through "tissue engineering."[5] Although the functional importance of such cell–cell interactions is well established, in many cases the underlying molecular mechanisms remain elusive. Investigation of these phenomena is further confounded by the diversity of supportive cell types found in organs. For example, fibroblasts are often classified together based on their morphology, mesenchymal markers, and adherence to tissue culture plastic; however, even fibroblasts in a single organ can vary significantly in their transcriptional profiles.[6] Even though such dramatic transcriptional variations in nonparenchymal cells (NPCs) would be expected to impact their interaction with surrounding epithelia, a correlation of nonparenchymal gene expression with epithelial function has not been systematically explored. Such correlative data should offer insight into

Abbreviations: NPC, nonparenchymal cell; E-cadherin, epithelial cadherin; MEF, mouse embryonic fibroblasts; N-cadherin, neural cadherin; VEGF, vascular endothelial growth factor.

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the underlying mechanisms of cell–cell interaction in a given tissue. In this study, a robust hepatic model of epithelial–nonparenchymal interactions was so examined.

In vivo, liver development requires interaction of the endodermal hepatic bud with the surrounding mesenchyme, with soluble signals fibroblast growth factor 2 and bone morphogenetic protein 4 being essential for early specification.[7] However, cell–cell contact through unknown mediators is also required for further liver development.[8] *In vitro*, cocultivation of primary hepatocytes with a plethora of distinct NPC types from different species and organs has been shown to support differentiated hepatocyte function in a manner reminiscent of hepatic organogenesis.[9,10] These hepatocyte cocultures have been used to study various aspects of liver physiology and pathophysiology such as lipid metabolism[11] and induction of the acute phase response.[12] This area of investigation has gained particular interest because of its relevance to both hepatic tissue engineering[13] and development of *in vitro* models for pharmaceutical drug screening.[14]

Despite significant investigation, a complete picture of the molecular mediators of epithelial–nonparenchymal interaction in hepatocyte cocultures is unavailable. To date, the data suggest that both matrix deposition[15,16] and direct cell–cell contact play a role[10,17,18] in the "coculture effect," whereas soluble factors have proven to be largely ineffective.[14,19,20] Two promising candidate cell surface proteins are epithelial cadherin (E-cadherin)[17] and liver-regulating protein[9]; however, NPCs lacking E-cadherin and liver-regulating protein retain the ability to support hepatic functions, suggesting that neither is the sole mediator of the "coculture effect."[21] Indeed, it is likely that several distinct mechanisms cooperate to modulate hepatocyte function in cocultures. Nevertheless, at least some of these multifactorial mechanisms appear to be highly conserved in mammals as primary hepatocytes from a variety of species (*e.g.*, human, murine, porcine) are stabilized to different extents by NPCs from different species, tissues, or embryological origin (*i.e.*, epithelial or mesenchymal).[10,11,22,23] Thus, identification of a set of nonparenchymal-derived signals that support hepatocyte differentiation would have broad fundamental and technological implications.

Conventional approaches to investigate mechanisms of cell–cell interaction have included conditioned media and transwell culture. In hepatocyte cocultures, these techniques have recently been supplemented with microfabrication-based patterning tools that provide additional insight into mechanisms of cell–cell communication.[10] Despite the progress in available technology to study epithelial–nonparenchymal interactions, examination of potential molecular mediators in hepatocyte cocultures has generally progressed through serial investigation of individual candidates. In the era of functional genomics, the opportunity now exists to correlate global patterns of gene expression with functional responses resulting from cell–cell interaction. As has been widely demonstrated, DNA microarrays coupled with bioinformatic tools offer the ability to perform quantitative, parallel measurements of gene expression.[6,24] In this study, we present a gene expression profiling approach to identify nonparenchymal genes that may modulate hepatocyte function. First, a "functional profile" of cell–cell interaction was established via measurement of liver-specific functions in hepatocytes upon cocultivation with several closely related murine fibroblasts, which were subsequently scored as high, medium, or low inducers of hepatic function. Finally, fibroblast gene expression profiles obtained via Affymetrix GeneChips (Affymetrix, Inc. Santa Clara, CA) were correlated with the hepatocyte functional profile. Using microarray data analysis, we reduced the list of approximately 12,000 fibroblast genes and expressed sequence tags to a handful of candidate genes that may modulate hepatocyte function in cocultures. Of the candidates we identified, two were subsequently shown to play a role in epithelial–nonparenchymal interaction in our model system, thereby validating our approach. Ultimately, the functional genomic approach presented here may serve as a general tool to facilitate mechanistic study of cell–cell interactions in diverse fields such as tissue engineering, stem cell biology, and cancer.

Materials and Methods

Cell Culture

Hepatocyte Isolation. Hepatocytes were isolated from 2- to 3-month-old adult female Lewis rats (Charles River Laboratories, Wilmington, MA) weighing 180–200 g using a modified procedure of Seglen.[25] Detailed procedures for hepatocyte isolation and purification have been previously described.[26] Routinely, 200–300 million cells were isolated with 85%–95% viability, as judged by trypan-blue exclusion. Nonparenchymal cells, as judged by their size (<10 μm diameter) and morphology (nonpolygonal), were less than 1%. Hepatocyte culture medium consisted of Dulbecco's Modified Eagle Medium with high glucose, 10% (v/v) fetal bovine serum, 0.5 U/mL insulin, 7 ng/mL glucagon, 7.5 μg/mL hydrocortisone, and 1% (v/v) penicillin-streptomycin.

Fibroblast Culture. 3T3-J2 fibroblasts were the gift of Howard Green (Harvard Medical School, Cambridge, MA).[27] Mouse embryonic fibroblasts (MEFs) were the gift of James Thomson (University of Wisconsin–Madison), and NIH-3T3 cells were purchased from the Amer-

ican Type Culture Collection. 3T3 fibroblast culture medium consisted of Dulbecco's Modified Eagle Medium with high glucose, 10% bovine calf serum, and 1% penicillin-streptomycin. MEF media consisted of 10% fetal bovine serum instead of calf serum and was supplemented with 1% (v/v) nonessential amino acids.

Hepatocyte–Fibroblast Coculture. Six-well plates were coated via adsorption of 0.13 mg/mL collagen-I in water for 1 hour at 37°C. Purification of collagen from rat-tail tendons has been described previously.[26] Briefly, rat-tail tendons were denatured in acetic acid, salt-precipitated, dialyzed against HCl, and sterilized with chloroform. Pure hepatic cultures and cocultures on decorin utilized coadsorption of 0.13 mg/mL collagen-I and different concentrations of bovine decorin (Sigma, St. Louis, MO). Protein-coated culture dishes were seeded with 125,000 hepatocytes in 1 mL of hepatocyte medium. After 24 hours, 125,000 fibroblasts were added in 1 mL of fibroblast medium. For coculture experiments involving three different cell types, fibroblasts were growth-arrested via incubation in media supplemented with mitomycin-C (Sigma; 10 μg/mL) for 2 hours at 37°C. Each of the fibroblast types was then added to hepatocyte cultures at 350,000 cells per mL of its respective medium. For all cocultures, the medium was replaced with hepatocyte medium 24 hours after fibroblast seeding and subsequently replaced daily.

Analytical Assays

Spent media was stored at −20°C. Urea concentration was assayed using a colorimetric endpoint assay utilizing diacetylmonoxime with acid and heat (Stanbio Labs, Boerne, TX). Albumin content was measured using enzyme linked immunosorbent assays (MP Biomedicals, Irvine, CA) with horseradish peroxidase detection and o-phenylenediamine (Sigma) as a substrate.[26]

Microscopy

Specimens were observed and recorded using a Nikon Diaphot microscope equipped with a SPOT digital camera (SPOT Diagnostic Equipment, Sterling Heights, MI), and MetaMorph Image Analysis System (Universal Imaging, Westchester, PA) for digital image acquisition.

Gene Expression Profiling

Fibroblast RNA Isolation and Microarray Hybridization. Pure fibroblast cultures were grown in duplicate on collagen-coated polystyrene in their respective media for 24 hours, after which the media was replaced to hepatocyte media to mimic coculture conditions to the extent possible. After an additional 24 hours, fibroblast RNA was extracted at approximately 80% preconfluency using TRIzol-LS (Gibco, Gaithersburg, MD). Each one of the duplicate fibroblast RNA samples was labeled, hybridized to an Affymetrix MG-U74Av2 microarray, and scanned as described previously.[28] Briefly, double-strand complementary DNA was synthesized using a T7- $(dt)_{24}$ primer (Oligo) and reverse transcription (Gibco). Complementary DNA was then purified with phenol/chloroform/isoamyl alcohol in phase lock gels, extracted with ammonium acetate, and precipitated using ethanol. Biotin-labeled complementary RNA was synthesized using the BioArray HighYield RNA Transcript Labeling Kit (Enzo Life Sciences, Farmingdale, NY), purified over RNeasy columns (Qiagen, Valencia, CA), eluted, and then fragmented. 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. The gene expression data is available at http://mtel.ucsd.edu/gene_expression/fibroblasts as a community resource.

Table 1. Criteria Used in Bullfrog Software to Detect Differentially Expressed Genes Between Fibroblasts

Criterion	Value
Difference call of	I, MI, D, MD
Fold change ≥	1.8
Average difference change ≥	50
Absolute call	P
Directional stringency?	Yes

NOTE. Directional stringency implies that the sign of a change is same in all comparisons.

Abbreviations: I, increase; MI, marginal increase; D, decrease; MD, marginal decrease; P, present.

Selection of Differentially Expressed Genes. All expression data was scaled to a target intensity of 200 (Affymetrix MAS 4.0 software), which corresponds to approximately 3–5 transcripts per cell.[28] Six microarray experiments were performed, which included 3 fibroblast cell lines that were prepared and hybridized in duplicate. These data were used to generate pairwise comparison files for every cell type combination (*i.e.*, 3T3-J2 replicate-1 vs. NIH-3T3 replicate-1; 12 files total). These comparison files were then filtered using BullFrog filtering software[29] to detect genes that were consistently differentially expressed. Criteria used for filtering were selected (Table 1) based on their ability to yield false-positive rates of less than 1% (*i.e.*, the number of genes differentially expressed in replicates divided by total genes). These criteria[30] had to be consistent in at least ten of twelve comparisons.

Microarray Data Analysis. Filtered data was exported to GeneSpring software (Silicon Genetics, Redwood City, CA), and hierarchical clustering was employed with vector-angle distance metric to generate

clusters of specific expression profiles. Other unsupervised (statistically driven) analysis methods (self-organizing maps and k-means clustering) yielded similar results to those obtained using hierarchical clustering. Clusters whose average expression profiles correlated with hepatocyte functional profiles (*e.g.*, high–medium–low albumin and urea secretion) were selected as candidate genes for further analysis, which included functional annotation via the NetAffx analysis portal (Affymetrix), which integrates information from various public databases such as GenBank and Swissprot.

Western Blot Analysis and Immunofluorescence

Fibroblasts grown on collagen-coated surfaces in hepatocyte medium were lysed in radioimmunoprecipitation (RIPA) buffer (Upstate Biotechnology, Waltham, MA) with protease inhibitor cocktail (Roche, Indianapolis, IN). Lysates were separated using polyacrylamide gel electrophoresis and transferred onto a polyvinylidene flouride membrane, blocked, incubated with primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and Horseradish peroxidase conjugated secondary antibody (Sigma), and visualized using chemiluminescence (Pierce SuperSignal, Pierce Biotechnology, Rockford, IL). For indirect immunofluorescence, samples were fixed with paraformaldehyde, permeabilized with Triton-X100, stained with primary and fluorescein isothiocyanate-(FITC)–conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.), and counterstained with Hoechst stain.

Statistical Analysis

Experiments were repeated 2–3 times with duplicate or triplicate samples for each condition. For functional assays, one representative outcome is presented where the same trends were observed in multiple trials. Statistical significance was determined using one-way ANOVA or Student's *t* test and Tukey's *post hoc* test on Prism (GraphPad, CA).

Results

Differential Induction of Liver-Specific Functions in Hepatocytes by Murine Fibroblasts

To categorize NPCs by their ability to induce hepatic functions, we cocultured primary rat hepatocytes with three closely related murine fibroblasts: 3T3-J2 and NIH-3T3 cell lines and primary MEFs. Induction of hepatic functions was scored using measurements of urea and albumin synthesis as markers of liver metabolic and synthetic function, respectively. Figure 1A compares functions of hepatocytes in the 3 cocultures with hepatocytes

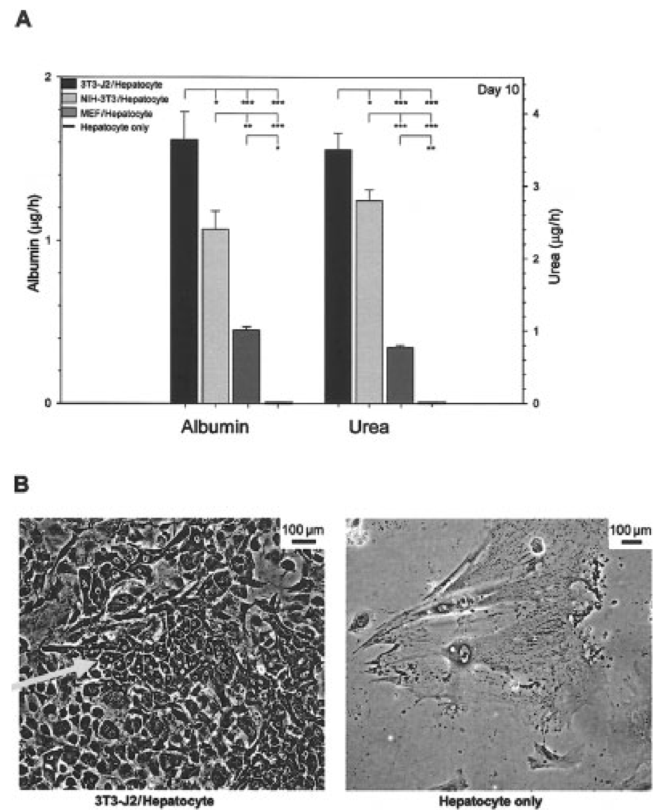


Fig. 1. Differential induction of liver-specific functions in rat hepatocytes upon cocultivation with murine fibroblasts. (A) Rate of albumin and urea production by hepatocytes on day 10 of coculture with 3 different murine fibroblasts. These trends were observed over many days. Inductive capacity of fibroblasts was therefore scored as follows: 3T3-J2 > NIH-3T3 > Mouse Embryonic Fibroblasts (MEFs). (B) In all cocultures, hepatocytes exhibited polygonal morphology (**arrow**), distinct nuclei, and visible bile canaliculi, whereas hepatocyte morphology deteriorated in pure cultures. $*P < .05$; $**P < .01$; $***P < .001$ (one-way ANOVA and Tukey's *post hoc* test). Error bars represent standard error of the mean ($n = 4$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

in pure culture. Hepatic functions were highest in the 3T3-J2 coculture, followed by the NIH-3T3 coculture and the MEF coculture, and were undetectable in hepatocytes cultivated alone. Furthermore, hepatocyte morphology deteriorated in pure cultures, whereas all cocultures were populated with polygonal hepatocytes with distinct nuclei and bright intercellular borders (Fig. 1B). Thus, 3T3-J2 cells were scored as "high inducers," NIH-3T3 cells as "medium inducers," and MEFs as "low inducers" of hepatic functions.

To explore whether or not poorly inductive fibroblasts (*i.e.*, MEFs) can inhibit hepatocyte function, we cocultivated hepatocytes with a 1:1 mixture of highly inductive (3T3-J2) and poorly inductive (MEF) fibroblasts. Fibroblasts were growth-arrested with mitomycin C to prevent confounding effects of proliferation of both fibroblast populations. Our results (Fig. 2) indicated that poorly

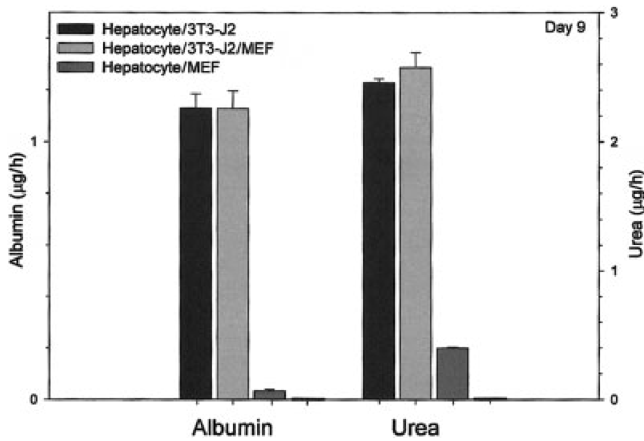


Fig. 2. Effect of poorly inductive mouse embryonic fibroblasts (MEFs) on hepatic functions in highly inductive 3T3-J2 cocultures. These experiments were conducted to explore the potential for MEFs to actively inhibit highly functional cocultures. Rate of albumin and urea production by hepatocytes on day 9 of coculture with a mixture of MEFs and 3T3-J2 fibroblasts is shown. These trends were observed over many days. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

inductive fibroblasts did not significantly diminish the function of hepatocytes in highly inductive cultures.

Gene Expression Profiling of Fibroblasts

To investigate the potential molecular mediators of epithelial–nonparenchymal interactions in hepatocyte cocultures, we used gene expression profiling. As part of this process (Fig. 3A), Affymetrix GeneChips were used to first quantify messenger RNA levels in pure fibroblast cultures grown on type I collagen in hepatocyte medium to mimic coculture conditions to the extent possible (Fig. 3B). The data was then filtered to detect genes that were consistently differentially expressed across fibroblast cell lines. Subsequently, we employed hierarchical clustering (Fig. 3C) to obtain genes whose expression profiles correlated positively (high–medium–low, Table 2) and negatively (low–medium–high, Table 3) with the pattern of hepatocyte induction observed. Finally, all candidate genes were functionally annotated using the NetAffx analysis portal. In conducting further analysis, we focused on proteins found on or around fibroblasts that may be involved with cell–cell communication, including cell surface proteins, extracellular matrix, and secreted factors. Below, we highlight key candi-

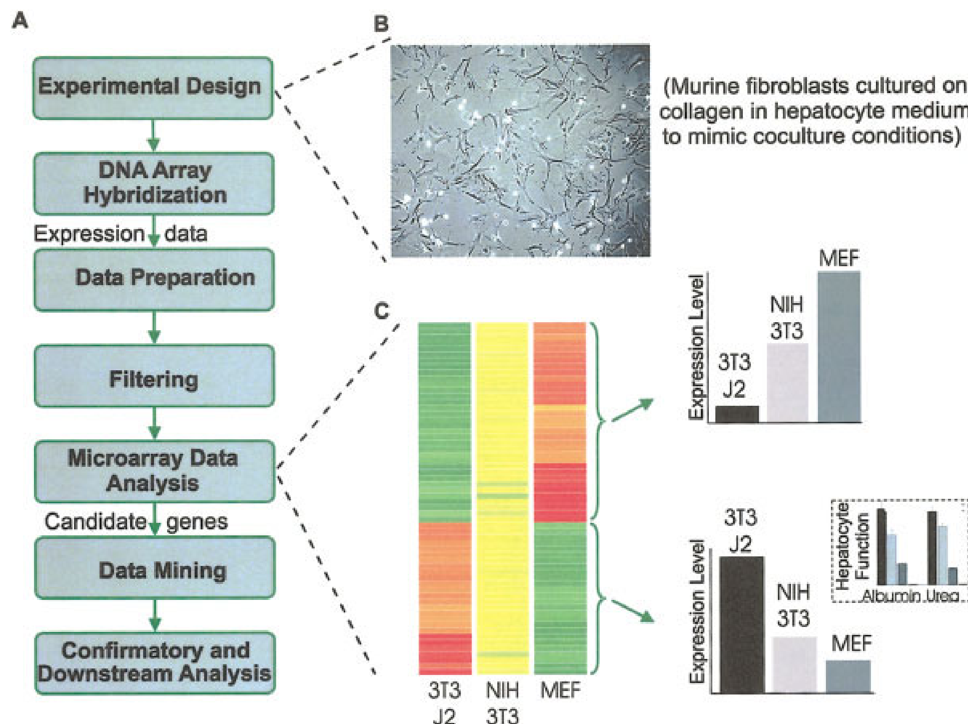


Fig. 3. Gene expression profiling of fibroblasts. (A) Flowchart depicting the use of DNA microarrays to obtain candidate genes involved with cell–cell interaction. Total RNA of fibroblasts (3T3-J2, NIH-3T3, Mouse Embryonic Fibroblasts [MEFs]) was harvested, labeled, and hybridized to Affymetrix GeneChips. Expression data was normalized, filtered, analyzed, and functionally annotated to obtain candidate genes. See Materials and Methods for additional details. (B) Phase contrast micrograph depicting fibroblast morphology (MEFs) on collagen-coated polystyrene in hepatocyte medium. (C) Clusterogram produced using hierarchical clustering with vector-angle distance metric is shown in which rows are gene expression values and columns represent different fibroblast cell types. In this clusterogram, genes with similar expression profiles across conditions are clustered together. High expression level is indicated with red, low expression with green, and medium expression with yellow. Average expression profiles of specific clusters that correlate positively and negatively with inductive profiles are shown.

Table 2. Fibroblast Candidate Genes Whose Expression Profiles Correlate Positively With the Inductive Profile Shown in Fig. 1A

Accession Number	Description
	Cell Surface
Z12171	Delta-like homolog 1 (*Drosophila*)
	Secreted
X99572	C-fos–induced growth factor (VEGF-D)
U49513	Small inducible cytokine A9
U49430	Ceruloplasmin
	Extracellular matrix or matrix remodeling
X53929	Decorin
	Transcription factors
M21065	Interferon regulatory factor 1
M31419	204 interferon-activatable protein
	Other
X53824	Splicing factor, arginine/serine-rich 3
	Heterogeneous nuclear ribonucleoprotein D-like protein
AB017020	JKTBP
L00993	Autoantigen La (SS-B)
U00431	High mobility group box 1
Z72486	DNA polymerase delta small subunit (pold2)
M86377	Esk kinase
J00388	Mouse dihydrofolate reductase gene: 3′ end
X07967	Pm1 protein
AW122347 (EST)	Rac GTPase-activating protein 1
AA655369 (EST)	Translocase of inner mitochondrial membrane 8 homolog a, yeast

NOTE. Unknown function EST accession numbers: AI037577, AI846197, AI841894, AI606951, AA940036, AI746846, AI551087, AA222883, AI848479.

dates that may be worthy of further investigation and present experimental data suggesting two candidates that may play a role in hepatocyte cocultures.

Cell Surface Proteins. Several studies have implicated cell surface proteins in epithelial-nonparenchymal interactions in hepatocyte cocultures.[17,18] Our gene expression profiling yielded Delta-like homolog 1, whose expression profile correlated positively (*i.e.*, high–medium–low) with the ability of fibroblasts to induce functions in hepatocytes. Delta-like homolog 1 belongs to the epidermal growth factor–like homeotic protein family that includes proteins such as the Notch receptor and its homologues (see Table 2).[31] Delta-like homolog 1 is strongly expressed in hepatoblasts from mouse fetal liver and has been implicated in differentiation of several nonhepatic cell types, suggesting it may play a functional role in hepatocyte cocultures.[31,32]

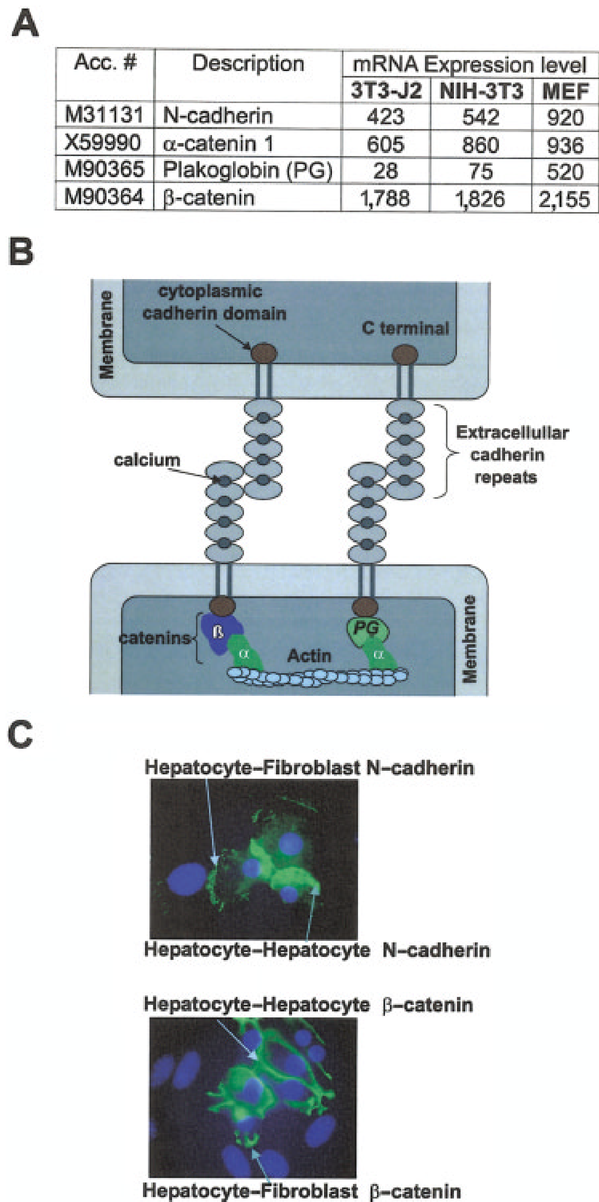
Further analysis of plakoglobin (γ-catenin) revealed that many of its interaction partners from the cadherin superfamily of cell adhesion molecules also had negative expression profiles (Fig. 4A). Classical cadherins, which are transmembrane proteins linked to the actin cytoskeleton via regulatory molecules such as catenins (Fig. 4B), may play roles in differentiation and heterotypic cell–cell interactions.[33,34] In the liver, cadherins are expressed in both hepatocytes and surrounding nonparenchyma under both physiological and pathophysiological conditions.[35,36] In cocultures of hepatocytes with L-929 chaperone cells, E-cadherin expression correlated positively with induced hepatocyte functions[17]; however, overexpression of E-cadherin in the developing liver prevents normal liver development.[37] In our cocultures, we verified protein expression and localization of neural cadherin (N-cadherin) and β-catenin at homotypic and heterotypic junctions using immunofluorescence (Fig. 4C). Thus, the negative correlation of cadherin-related molecules with hepatocyte function merits further investigation.

Table 3. Fibroblast Candidate Genes Whose Expression Profiles Correlate Negatively With the Hepatocyte Functional Profile Shown in Fig. 1A

Accession Number	Description
	Cell surface
L03529, AW046032 (EST)	Thrombin receptor (PAR-1)
X66084	Hyaluronic acid receptor (CD44)
M90365	Junction plakoglobin (cadherin-associated)
	Secreted
AJ243964	Dickkopf homolog 3 (*Xenopus laevis*)
X69619	Inhibin beta-A
M70642	FISP-12 protein
U77630	Adrenomedullin
	Extracellular matrix or matrix remodeling
X66976	Procollagen, type VIII, alpha 1
X62622	Tissue inhibitor of metalloproteinase 2
	Cytoskeletal-associated
M12347	Actin, alpha 1, skeletal muscle
U58513	Rho-associated coiled-coil forming kinase 2
U73199	Rho-interacting protein 2
	Cell cycle
U09507	Cyclin-dependent kinase inhibitor 1A (P21)
	Transcription factors
D76440	Necdin
AJ002366	General transcription factor IIH, polypeptide 1
	Intracellular signaling
U15784	Src homology 2 domain-containing transforming protein C1
AF053367	PDZ and LIM domain 1 (elfin)
U58883	SH3 domain protein 5
U58882	LIM and SH3 protein 1
	Other
X87817	Dihydropyrimidinase-like 3
Y13832	Maternally expressed gene 3
U41739, AI839950 (EST)	Four and a half LIM domains 1
AW125478 (EST)	Protease, serine, 11 (Igf binding)
AI183109 (EST)	Translin-associated factor X
AJ007376	DEAD box polypeptide, Y chromosome
D12713	SEC23A (*Saccharomyces cerevisiae*)
M31775, AW046124 (EST)	Cytochrome b-245, alpha polypeptide

NOTE. Unknown function EST accession numbers: AI848471, AI183109, AI648831.

Abbreviation: EST, expressed sequence tag.



Acc. #	Description	mRNA Expression level		
		3T3-J2	NIH-3T3	MEF
M31131	N-cadherin	423	542	920
X59990	α-catenin 1	605	860	936
M90365	Plakoglobin (PG)	28	75	520
M90364	β-catenin	1,788	1,826	2,155

Fig. 4. Analysis of the cadherin pathway suggests negative correlation with hepatocyte function. (A) Because the expression profile of plakoglobin–which interacts with cadherins–correlated negatively with inductive ability of fibroblasts in unsupervised analysis, we checked expression profiles of other constituents of the cadherin pathway and found them to be similar to that of plakoglobin. Values indicate average of duplicate samples, scaled to an intensity of 200. (B) Classical cadherins are transmembrane proteins whose cytoplasmic domains anchor to the actin cytoskeleton by interacting with various signaling molecules such as β-catenin, plakoglobin (γ-catenin), and α-catenin. (C) Immunofluorescent staining of N-cadherin (**top**) and β-catenin (**bottom**) demonstrate protein expression and localization at both homotypic and heterotypic cell–cell junctions in hepatocyte cocultures. Nuclei are counterstained with Hoechst nuclear dye. Representative staining is shown for 3T3 (medium inducers) cocultures, but protein localization was seen in all cocultures. mRNA, messenger RNA; MEF, mouse embryonic fibroblast; PG, plakoglobin.

Extracellular Matrix. Matrix deposition and remodeling have been implicated as key features of hepatocyte cocultures.[15,16] In our data, the gene expression of collagen-VIII correlated negatively with inductive ability of fibroblasts. This nonfibrillar, short-chain matrix protein is present in the arterioles and venules of normal liver[38] and may play an instructional role in differentiation of other cell types.[39] Even though the effect of collagen-VIII on hepatic functions has not been studied, other collagens (*e.g.*, collagen-I) are responsible for dramatic changes in hepatocellular phenotype.[26] Matrix remodeling via metalloproteinases and their inhibitors (*e.g.*, tissue inhibitor of metalloproteinase) may be an important feature of hepatocyte cocultures.[9] In our system, the expression of tissue inhibitor of metalloproteinase 2 correlated negatively with inductive ability of fibroblasts, suggesting that an imbalance in matrix remodeling may also underlie the hepatic dysfunction found in MEF cocultures.

The only matrix protein whose expression profile correlated positively with fibroblast inductive ability was decorin, which is a chondroitin sulfate–dermatan sulfate proteoglycan that binds to collagen.[40] Decorin is a major liver proteoglycan that shows early and strong up-regulation during liver regeneration following partial hepatectomy, even though its role in this process is unclear.[41] To validate our functional genomic approach, we conducted preliminary studies to investigate decorin's effect on hepatocellular functions *in vitro*. Because of decorin's collagen-binding activity, hepatocyte function on collagen was compared with surfaces with coadsorbed collagen and decorin. In pure hepatocyte cultures, albumin production was up-regulated by 122% and urea secretion by 36% on decorin (Fig. 5A). In cocultures of hepatocytes and MEFs ("low inducers"), hepatic functions were up-regulated in a dose-dependent manner on adsorbed decorin compared with collagen alone, resulting in up to 40% of albumin secretion rate seen in coculture with "high inducers" (Fig. 5B).

Secreted Factors. Studies assessing the role of soluble factors in hepatocyte coculture models have yielded variable results. For example, treatment of hepatocytes with media "conditioned" by NPCs is typically ineffective.[14,19] Nonetheless, secreted factors that are labile or locally sequestered in matrix may play a role in cell–cell interaction. In our analysis, gene expression profile of vascular endothelial growth factor (VEGF) D correlated positively with induction of liver-specific functions. In addition to their role in angiogenesis, VEGFs play protective roles in liver regeneration (VEGF-A) and show dynamic pattern of expression in the developing liver (VEGF-D).[42,43] Besides VEGF-D, Dickkopf homolog 3 exhibited a negative expression profile. Found primarily in mesenchymal lineages, Dickkopfs are secreted proteins that have been implicated in modulating inductive epithelial–mesenchymal interactions.[44]

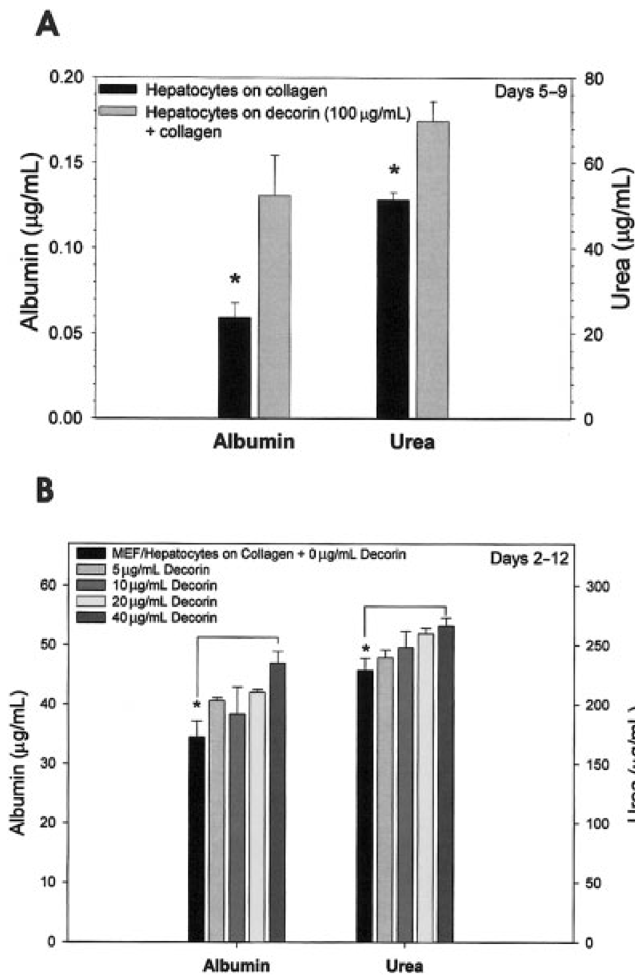


Fig. 5. Validation of extracellular matrix, decorin, as a potential mediator of cell–cell interaction. (A) Up-regulation of total urea and albumin production in hepatocytes plated alone on adsorbed decorin (summation over days 5-9). (B) Dose-dependent up-regulation of total hepatic functions in cocultures of hepatocytes and low function-inducing mouse embryonic fibroblasts (MEFs) (summation over days 2-12) on adsorbed decorin. $^*P < .05$. Error bars represent standard error of the mean (n = 3). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

Discussion

In the era of genomic medicine, biomedical phenomena may be investigated via global analyses rather than through the study of individual molecular species. This systems-level approach has been used to stratify clinical trials and predict the metastatic potential of tumors.[45] In this study, we present a functional genomic approach to explore the mechanisms of cell communication in epithelial–nonparenchymal interactions in a hepatic model system. Functional outcomes were scored and correlated with patterns of gene expression in a manner that can be generalized to the study of a variety of biological phenomena. Specifically, primary hepatocytes were cocultured with various NPC types to produce variable induction of liver-specific functions. Nonparenchymal genes whose expression correlated positively (*i.e.*, stimulatory) and negatively (*i.e.*, inhibitory) with the hepatic functional profile were catalogued for further experimental study. Using this approach, we identified 17 functionally characterized candidates in the relevant cell communication categories (cell surface, matrix, secreted factors), and produced the first global molecular definition of a hepatocyte-stabilizing nonparenchymal microenvironment. Finally, we experimentally validated the role of two candidates—decorin and N-cadherin—in hepatocyte–fibroblast interactions in our model system. These data provide a limited set of candidates that may be investigated for their role in induction of liver-specific functions, some of which may have technological implications (*e.g.*, stabilizing hepatocellular function in cell-based therapies).

In developing our gene expression profiling approach to study cell–cell interactions, we considered several experimental variables. First, we considered the possibility that NPCs receive reciprocal signals from surrounding epithelia. Therefore, the ideal source of NPCs for gene expression profiling would have been those that have undergone cocultivation with hepatocytes. However, due to the experimental difficulty of rapidly purifying nonparenchymal RNA from hepatocyte cocultures, we profiled pure fibroblast cultures as a first step toward identifying candidate genes involved with induction of liver functions. Previous experimental evidence as well as our own studies (data not shown) suggest that nonviable feeder layers (desiccated, fixed) elicit comparable responses to viable fibroblasts, lending support to the idea that at least some nonparenchymal-derived signals do not require reciprocal signaling.[10,46] To the extent possible, we mimicked other aspects of the coculture environment (media formulation and matrix coating) in pure fibroblast cultures to obtain a set of candidates that are involved with the functional outcomes we measured. Next, to address the possibility that hepatic function varied between cocultures because of variations in cell shape, average hepatocyte projected surface area was measured in each condition and found to be similar (data not shown).[47] Additionally, we noted that DNA microarrays report on messenger RNA levels rather than protein expression. We therefore verified select candidates at the protein level using Western blot analysis and immunofluorescence. Thus, our gene expression profiling approach is merely a necessary first step in dissecting the mechanisms of cell–cell interaction.

The choice of NPCs used in this study was another key parameter in the experimental design. A variety of both liver and non–liver-derived NPCs have been reported to induce hepatic function in coculture.[10,15,16] Furthermore,

induction has been reported by NPCs (both primary and immortalized) derived from a different species than the primary hepatocytes, suggesting possible conservation of underlying mechanisms.[11,14,19,22,23] The ready availability and ease of culture of immortalized murine fibroblasts has led to a resurgence of interest in their influence on hepatocyte functions for applications in bioartificial liver devices.[10,13,20] To broaden our findings in rat hepatocytes, we also demonstrated coculture-mediated stabilization of hepatocytes from the same species (mouse) and another species (human) as the fibroblasts (data not shown). Finally, because 3T3 and primary murine embryonic fibroblasts are commonly used as supporting feeder layers in other organ systems,[27,48] the gene expression data acquired in this study may be useful in a number of other applications. In the future, we aim to study the role of candidate genes elucidated in murine fibroblast cell lines in NPCs of the liver specifically (*e.g.*, sinusoidal endothelial cells).

The categorization of candidate genes into two groups (positive or negative correlations with induction profiles) was based on the premise that "low inducer" fibroblasts could, in fact, be actively inhibiting hepatocyte function. To test this hypothesis, we cocultivated hepatocytes with a mixture of MEFs ("low inducers") and 3T3-J2s ("high inducers"). Both fibroblast cell types were growth-arrested to prevent confounding effects of differential proliferation. Our results indicated no significant decrease in function due to the addition of MEFs compared with control cocultures (hepatocyte/3T3-J2), suggesting that either MEFs lack active inhibitory signals or that hepatic induction by 3T3-J2s dominates over any inhibition by MEFs. Nonetheless, these preliminary experiments cannot conclusively rule out the existence of inhibitory molecules. Indeed, one of our candidates, N-cadherin, was recently reported to inhibit chondrogenic differentiation of limb mesenchymal cells upon overexpression *in vitro*.[49]

Of the 17 functionally characterized candidates in the cell communication category obtained in our analysis, 2 were experimentally validated. Unsupervised analysis of the data showed that gene expression of decorin, an extracellular matrix proteoglycan, correlated positively with inductive activity. Experimentally, we confirmed that decorin did indeed induce liver functions in both pure hepatocyte cultures and in cocultures of hepatocytes with fibroblasts that had "low" inductive activity (see Fig. 5). Despite decorin's inductive ability, neither culture achieved maximal production rates of hepatic markers (as with 3T3-J2s) due to the addition of decorin alone. These data serve to validate the gene expression profiling approach and confirm the hypothesis that cell–cell interaction is likely to be multifactorial. Analysis of the cadherin pathway emerged from the identification of plakoglobin (γ-catenin) as a candidate. N-cadherin, β-catenin, and α-catenin expression profiles also correlated negatively with inductive activity. We confirmed the localization of N-cadherin and β-catenin at heterotypic (fibroblast/hepatocyte) junctions using immunofluorescence (see Fig. 4), providing our first evidence of functional communication between the cell types. In contrast, other groups have shown a positive inductive role for E-cadherin.[17] Interaction between N-cadherin and E-cadherin pathways has also been reported[50]; therefore, the interplay between various cadherins merits further investigation in hepatocyte cocultures.

In summary, we have developed a gene expression profiling approach to facilitate the study of cell–cell interactions. The nonparenchymal gene expression data provided online (http://mtel.ucsd.edu/gene_expression/fibroblasts) can also be used to identify candidate genes by other investigators in diverse areas such as the self-renewal of embryonic stem cells on nonparenchymal feeder layers[48] and differentiation of keratinocytes on fibroblasts.[27] In the future, we plan to evaluate the functional role of promising candidates in our cocultures using function-blocking antibodies, RNA interference, and overexpression of candidate genes. Identification of a set of critical proteins that mediate hepatocyte differentiation will have implications in both hepatocellular therapies and liver development. In addition, continued application of functional genomics and gene expression profiling to epithelial–nonparenchymal systems may provide fundamental insights into global and tissue-specific regulatory gene networks that control tissue development and function.

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