Microfabrication of Hepatocyte/Fibroblast Co-cultures: Role of Homotypic Cell Interactions

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Cell−cell interactions are important in embryogenesis, in adult physiology and pathophysiology of many disease processes. Co-cultivation of parenchymal and mesenchymal cells has been widely utilized as a paradigm for the study of cell−cell interactions in vitro. In addition, co-cultures of two cell types provide highly functional tissue constructs for use in therapeutic or investigational applications. The inherent complexity of such co-cultures creates difficulty in characterization of cell−cell interactions and their effects on function. In the present study, we utilize conventional "randomly distributed" co-cultures of primary rat hepatocytes and murine 3T3-J2 fibroblasts to investigate the role of increasing fibroblast density on hepatic function. In addition, we utilize microfabrication techniques to localize both cell populations in patterned configurations on rigid substrates. This technique allowed the isolation of fibroblast number as an independent variable in hepatic function. Notably, homotypic hepatocyte interactions were held constant by utilization of similar hepatocyte patterns in all conditions, and the heterotypic interface (region of contact between cell populations) was also held constant. Co-cultures were probed for synthetic and metabolic markers of liver-specific function. The data suggest that fibroblast number plays a role in modulation of hepatocellular response through homotypic fibroblast interactions. The response to changes in fibroblast number are distinct from those attributed to increased contact between hepatocytes and fibroblasts. This approach will allow further elucidation of the complex interplay between two cell types as they form a functional model tissue in vitro or as they interact in vivo to form a functional organ.

Introduction

Tissue formation and function in vivo are influenced by many factors, including cytokines, cell−matrix interactions, topology, mechanical forces, and cell−cell interactions. In particular, the importance of cell−cell interactions has been noted extensively in embryogenesis and also in the function of many adult organs such as the gut (Simon-Assmann et al., 1988), kidney (Aufderheide et al., 1987), liver (Houssaint, 1980), testis (Gerard et al., 1995), lung (Slavkin et al., 1984), and bone marrow (Zsebo et al., 1990). Attempts to reconstruct functional tissues in vitro for therapeutic applications have involved exploration of many of these modulators of cellular function, including cell−cell interactions. Specifically, co-cultivation of parenchymal cells with mesenchymal cells has been attempted in the area of liver (Guguen-Guillouzo, 1983), skin (Rheinwald and Green, 1975), urothelium (Howlett et al., 1986), myocardium (Nishida et al., 1993), and vasculature (Fillinger et al., 1993) to improve tissue function. While these studies have demonstrated improved viability, function, and tissue stability, often, the specific mechanisms by which co-cultivation of two cell types modulates tissue function have remained unclear.

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In tissue engineering of the liver, for therapeutic or investigational purposes, co-cultivation has been attempted with primary hepatocytes and many different cell types (Guguen-Guillouzo et al., 1983; Rojkind et al., 1995; Donato et al., 1990; Matsuo et al., 1992). The influence of co-culture on hepatocyte function has typically been measured using "all or nothing"-type outcomes. For example, hepatocytes in co-culture have been compared to hepatocytes cultured alone (Mesnil et al., 1987), hepatocytes in a transwell separated by a porous filter from the second population (Donato et al., 1994), or hepatocytes exposed to media "conditioned" by the second cell type (Kuri-Harcuch and Mendoza-Figueroa, 1989; Donato et al., 1990; Shimaoka et al., 1987). In general, these studies have indicated that viability and function of hepatocytes is stabilized by the presence of a second cell type in a co-culture configuration, and not otherwise. These data, however, provide little insight into the mechanisms by which two cell populations interact in co-culture to produce stable hepatic function.

Hepatocellular function in co-cultures may be influenced by three cellular variables: homotypic hepatocyte interactions (the effects of hepatocytes on one another), the heterotypic interface (the amount of contact between cell populations), and the homotypic interactions of the nonhepatocyte population (which may produce secondary effects on hepatocellular response). Previous studies on homotypic hepatocyte interactions have been performed

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in pure (hepatocyte only) cultures. The role of homotypic interactions has primarily been investigated using variations in seeding density (Ben-Ze'ev et al., 1988; Ichihara, 1991; Moghe et al., 1997). Also, multicellular aggregates have been compared to monolayer cultures (Koide et al., 1990), suggesting some role for cell−cell interactions in induction of liver-specific function. The utility of these data in predicting the influence of homotypic hepatocyte interactions in co-cultures is limited since "randomly distributed", conventional co-cultures will suffer from both a diversity of homotypic interactions and variable local contact with another cell type.

To explore the role of cell−cell interactions in the hepatocellular response in co-cultures, we previously utilized microfabrication techniques to create micropatterned co-cultures of primary rat hepatocytes and murine 3T3-J2 fibroblasts as a model of parenchymal/mesenchymal interactions (Bhatia et al., 1997). These techniques allowed spatial localization of both cell types, allowing control over homotypic hepatocyte interactions, homotypic fibroblast interactions, and the extent of the heterotypic interface. Previous studies of cellular function using microfabrication had been limited to the study of a single cell type (Hammarback et al., 1988; Clark et al., 1993; Bousse et al., 1994; Chen et al., 1997; Turner et al., 1997). Furthermore, our methodology allowed markedly improved control of cell−cell interactions over that previously achieved in conventional, "randomly distributed" co-cultures. In our recent study, we showed that the total initial heterotypic interface in co-cultures modulated long-term hepatocellular function (Bhatia et al., 1998b). Specifically, these experiments demonstrated that the use of similar cell numbers in various configurations of increasing heterotypic interface had increased levels of production of markers of liver-specific function. In addition, hepatocytes close to the heterotypic interface had increased levels of intracellular albumin as determined by immunostaining. Finally, our data indicated that signal(s) for induction of liver-specific function may be localized to the heterotypic interface. Taken together, these data suggested that the initial heterotypic interface was an independent modulator of hepatocellular response in co-cultures. In the present study, we set out to use conventional techniques as well as microfabricated co-cultures to extend previous investigations to the role of fibroblast number in homotypic fibroblast interactions. Specifically, we studied whether changes in fibroblast number would modulate the nature of the fibroblast "signal" through homotypic fibroblast interactions. Microfabricated co-cultures were configured to create variations in fibroblast number in the setting of both a fixed heterotypic interface and fixed homotypic hepatocyte interactions. Using this approach, we hope to elucidate some key aspects of the complex interplay between two cell types in a model tissue.

Materials and Methods

Microfabrication techniques and conventional methods were utilized to probe cell−cell interactions. Conventional, or "randomly distributed", co-cultures were examined with increasing numbers of fibroblasts and a constant population of hepatocytes. In addition, microfabricated co-cultures were generated to control cell−cell interactions by localization of two distinct cell types on collagen-patterned borosilicate substrates. This allowed increasing fibroblast numbers to be assessed without variation of heterotypic interface.[1]

Substrate Preparation. All co-cultures were performed on 2 in. diameter × 0.02 in. borosilicate wafers (Erie Scientific, Portsmouth, NH). "Randomly-distributed" co-cultures were performed on uniform, collagen-modified wafers, whereas "microfabricated" co-cultures were performed on collagen-patterned substrates.

Detailed procedures for microfabrication of substrates and subsequent modification were previously described (Bhatia et al., 1997). Briefly, wafers were spin-coated with positive photoresist (OCG 825-20cSt). Wafers were baked and then exposed to ultraviolet light in a Bottom Side Mask Aligner (Karl Suss, Waterbury Center, VT) through chrome masks of the desired dimensions (Advance Reproductions, N. Andover, MA). Exposed photoresist was then developed (OCG 934:water, 1:1) and rinsed in deionized (DI) water. Disks were baked and exposed to oxygen plasma at a base vacuum of 50 mTorr and O_2 pressure of 100 mTorr at a power of 100 W for 2−4 min.

Substrates were modified using experimental methods similar to those developed by Lom et al. (1993) and Britland et al. (1992) Silane immobilization onto exposed glass was performed by immersion into 2% v/v solution of [3-[(2-aminoethyl)amino]propyl]trimethoxysilane (AS, Huls America, Piscataway, NJ) in water followed by two rinses in DI water. Wafers were dried with nitrogen gas, baked, and then immersed in 2.5% v/v glutaraldehyde in phosphate-buffered saline (PBS, pH 7.4) for 1 h. Substrates were rinsed with PBS and immersed in a 1:1 solution of 1 mg/mL collagen I (preparation described in detail elsewhere, Dunn et al., 1991): DI water, pH 5.0 for 30 min at 37 °C. Discs were subsequently sonicated in acetone for 3 min to remove residual photoresist (Bransonic). Wafers were rinsed twice in DI water and stored dry at 4 °C for up to 1 week.

Uniform collagen-modified wafers to be used for "randomly distributed" co-cultures were generated by washing borosilicate wafers in Chem-Solv Detergent (Mallinckrodt, Paris, KY) prepared as directed by the manufacturer, followed by two rinses in water, and exposed to [[(aminoethyl)amino]propyl]trimethoxysilane, glutaraldehyde and collagen I as described above. To treat the immobilized collagen in the same fashion as collagen on the micropatterned wafers, discs were subsequently sonicated in acetone for 3 min, rinsed in water, and stored dry at 4 °C for up to 1 week.

Hepatocyte Isolation and Culture. Hepatocyte were isolated from 2−3-month-old adult female Lewis rats (Charles River Laboratories, Wilmington, MA) weighing 180−200 g, by a modified procedure of Seglen (1976). Detailed procedures for isolation and purification of hepatocytes were previously described by Dunn et al. (1989). Routinely, 200−300 million cells were isolated

[1] Heterotypic interface: The spatial dimension over which fibroblasts and hepatocytes undergo coplanar cell−cell contact. For example, in a circular island of hepatocytes surrounded by confluent fibroblasts, this would correspond to the perimeter of the circle. Homotypic fibroblast interactions: Fibroblast interactions with each other via humoral factors, extracellular matrix, or membrane-bound receptor/ligands; may be influenced by total fibroblast number, local fibroblast density, media composition and volume, etc. Homotypic hepatocyte interactions: Hepatocyte interactions with each other via humoral factors, extracellular matrix, or membrane-bound receptor/ligands; may be influenced by total hepatocyte number, local hepatocyte density, media composition, etc.

with viabilities between 85% and 95%, as judged by trypan blue exclusion. Nonparenchymal cells, as judged by their size (<10 μm in diameter) and morphology (nonpolygonal or stellate), were less than 1%. Hepatocyte culture medium was Dulbecco's Modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO), 0.5 U/mL insulin, 7 ng/mL glucagon, 20 ng/mL epidermal growth factor, 7.5 μg/mL hydrocortisone, 200 U/mL penicillin, and 200 μg/mL streptomycin. Serum-free culture medium was identical except for the exclusion of FBS. Cultures were performed in P-60 tissue culture polystyrene dishes. In some cases, hepatocytes were visualized with fluorescent dye CMFTR (chloromethylbenzoylaminotetramethyl rhodamine, C-2927, Molecular Probes). Cells were loaded by incubation in 25 μM dye in media for 45 min, rinsed, and incubated for 30 min prior to visualization.

NIH 3T3-J2 Culture. NIH 3T3-J2 cells were the gift of Howard Green, Harvard Medical School. Cells grown to preconfluence were passaged by trypsinization in 0.01% trypsin (ICN Biomedicals, Costa Mesa, CA)/0.01% EDTA (Boehringer Mannheim, Indianapolis, IN) solution in PBS for 5 min, diluted, and then inoculated into a fresh tissue culture flask. Cells were passaged at pre-confluency no more than 10 times. Cells were cultured in 175 cm^2 flasks (Fisher, Springfield, NJ) at 10% CO_2, balance moist air. Fibroblast culture medium consisted of DMEM (Gibco, Grand Island, NY) with high glucose, supplemented with 10% bovine calf serum (BCS, JRH Biosciences, Lenexa, KS), 200 U/mL penicillin, and 200 μg/mL streptomycin. In some cases, growth-arrested cells were obtained by incubation with 10 μg/mL mitomycin C (Boehringer Mannheim) in media for 2 h.

Randomly Distributed Co−cultures. To explore the effects of fibroblast number on hepatocyte function, randomly distributed cultures were performed with varying fibroblast:hepatocyte ratios. Reduction in fibroblast number was achieved by seeding progressively fewer fibroblasts on each set of wafers. Uniform, collagen-modified, glass wafers were sterilized in 70% ethanol in water for 45 min, rinsed in sterile water, treated with 0.05% sterile bovine serum albumin (BSA) in water, and finally rinsed in water followed by serum-free media. Since the number of attached hepatocytes is dictated by the number of viable seeded cells, 250 000 viable hepatocytes were seeded in serum-containing media to maximize efficiency of attachment. The next day, growth-arrested fibroblasts (0 to 2 million, depending on experimental condition) were trypsinized, counted with a hemocytometer, and plated in 3 mL of fibroblast culture medium (Figure 1A). After 24 h, the medium was replaced with 2 mL of hepatocyte culture medium and subsequently changed daily.

Microfabricated Co−cultures. Microfabricated co-cultures were generated as previously described (Bhatia et al., 1997). Briefly, hepatocytes were seeded in serum-free media on collagen-patterned wafers, resulting in a hepatocyte pattern due to selective cell adhesion. Addition of fibroblasts resulted in generation of micropatterned co-cultures. Reduction in fibroblast number (and thus, reduction of homotypic fibroblast interactions) with preservation of heterotypic interface was achieved by seeding cells on microfabricated substrates. Surface area dedicated to fibroblasts was varied while surface area dedicated to hepatocytes was kept constant between conditions. This was accomplished by reduction of the total patterned area through use of a polymer mask to cover regions of the wafer with simultaneous reduction of hepatocyte island spacing (Figure 2A−C). The number of fibroblasts intercalated between hepatocyte islands, therefore, varied across conditions, producing modification in homotypic fibroblast interactions. In this manner, the total number of hepatocyte islands (as well as the heterotypic interface) was preserved among various

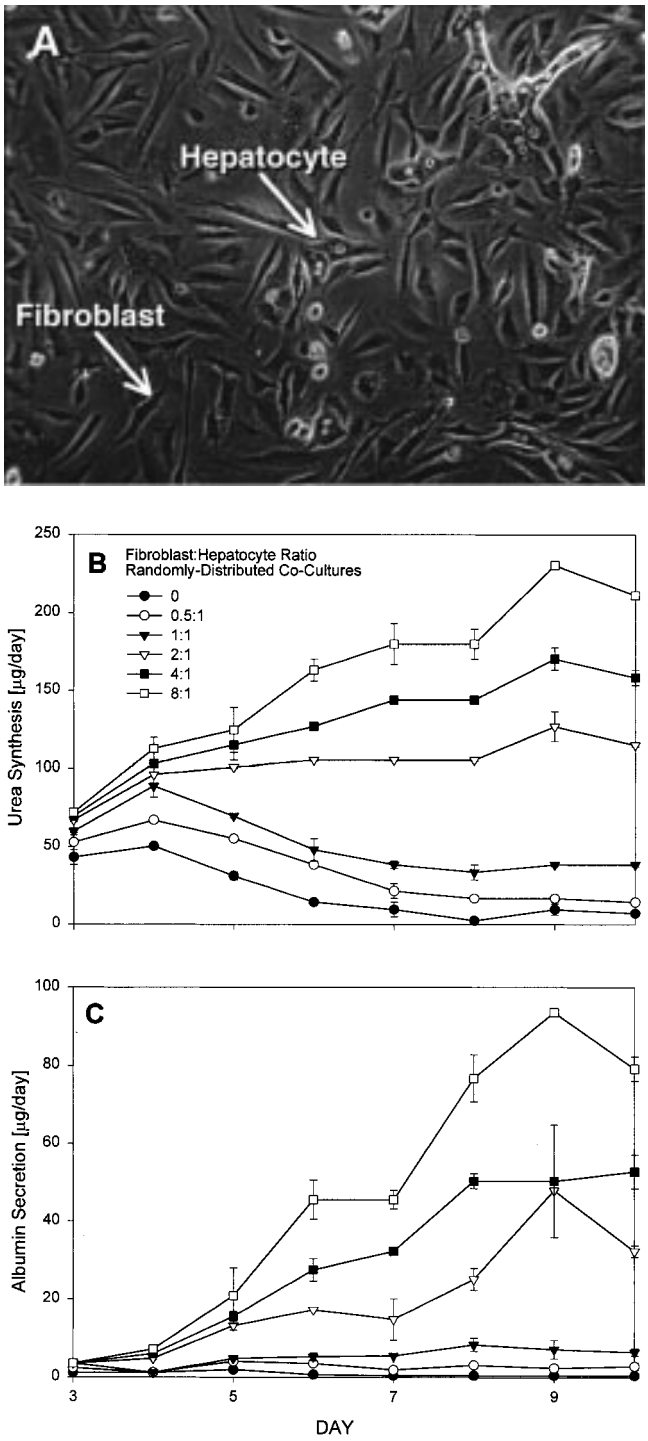

Figure 1. Randomly-distributed co-cultures. (A) Phase contrast micrograph of randomly distributed co-culture of 250 000 hepatocytes (30 h after seeding) and 2 million 3T3-J2 fibroblasts (6 h after seeding) depicting diversity of cell−cell interactions. (B) Urea synthesis in randomly distributed hepatocyte/fibroblast co-cultures with 250 000 hepatocytes as a function of increasing fibroblast number. Fibroblasts were growth-arrested prior to initiation of co-culture by incubation with mitomycin C. Legend indicates the ratio of growth-arrested 3T3 fibroblasts to the number of seeded hepatocytes. (C) Albumin secretion in randomly distributed co-cultures as in B. Both markers of liver-specific function increased with increasing fibroblast number.

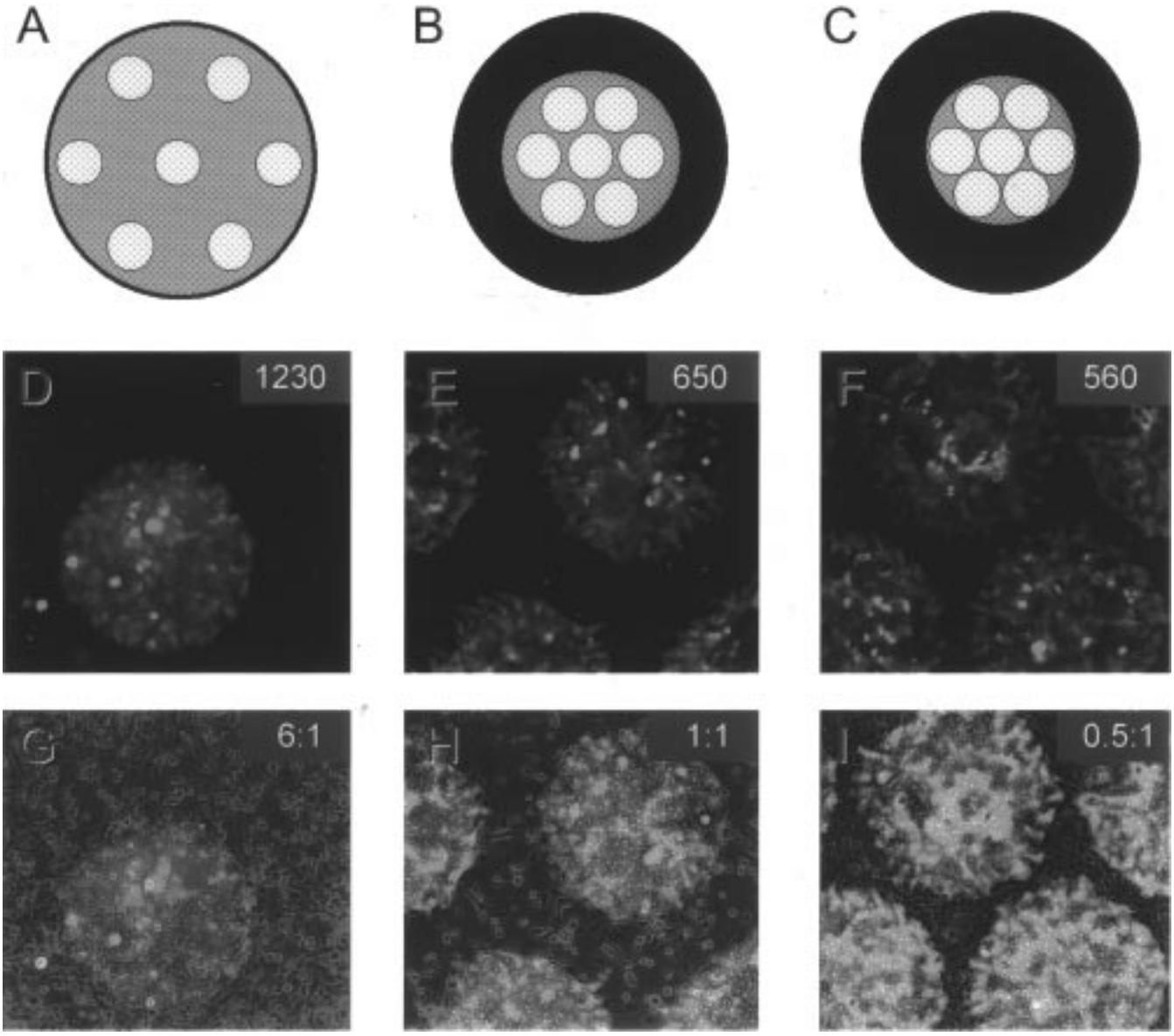

Figure 2. Microfabricated co-cultures. (A−C) Schematic representation of three different co-culture conditions which allowed preservation of the initial heterotypic interface while reducing the fibroblast number. Black represents bare glass, gray circles represent hepatocyte islands, and the remainder of surface is dedicated to fibroblasts. Therefore, compaction of the patterned area allowed preservation of the number of hepatocyte islands (and thus, heterotypic interface) with progressively less fibroblasts. (D−F) Fluorescent micrographs of 490 μm hepatocyte islands in hepatocyte/fibroblast co-cultures with center-to-center spacing as follows: (D) 1230, (E) 650, and (F) 560 mm. (G−I) Phase contrast images of hepatocyte/fibroblast co-cultures overlayed with fluorescent micrographs of labeled hepatocytes with the following fibroblast:hepatocytes ratios: (G) 6:1, (H) 1:1, and (I) 0.5:1.

cultures with decreasing numbers of fibroblasts. Therefore, hepatocyte number was also held constant (estimated at 250 000−300 000). On the basis of previous data suggesting morphologic stability of a 490 μm diameter hepatocyte island, microfabricated substrates utilized a hepatocyte island diameter of 490 μm in all cases, with variations in center-to-center spacing. Patterns were hexagonally packed islands of 490 mm diameter with center-to-center spacing as follows: 1230, 930 (not pictured), 650, and 560 μm.

Reduction of total patterned wafer area was achieved by masking portions of wafers with polydimethysiloxane (PDMS, Sylgard 184, Dow Corning, Lansing, MI) elastomer prepared as directed by the manufacturer. PDMS films of <1 mm thickness were then cut into annuli of the following dimensions (inner diameter, outer diameter): 0.94 in., 2 in.; 1.06 in., 2 in.; 1.5 in., 2 in., corresponding to patterns with center-to-center spacings of 560, 650, and 930 μm, respectively. The pattern with the largest center-to-center spacing (1230 μm) did not require a polymer mask because the entire wafer was utilized for cell adhesion. Annuli were then sterilized in 70% ethanol, dried, and mounted on appropriate collagen-patterned wafers.

Composite PDMS/collagen-patterned glass wafers were then utilized for cell culture as described above−sterilized in 70% ethanol, rinsed, treated with (0.05%) BSA to deter nonspecific cell attachment, rinsed, and seeded repeatedly with hepatocytes in serum-free media. In each case, the total collagen-patterned surface area was equivalent to that necessary for adhesion and spreading of 250 000 hepatocytes. The following day, growth-arrested fibroblasts were seeded as follows: 1.5 $\times$ 106 for 1230 μm spacing, 1.3 $\times$ 106 for 930 μm, and 0.88 $\times$ 10^6 for 650 μm, and 0.59 $\times$ 10^6 for 560 μm spacing. Since the fibroblasts attached with equal efficiency to both PDMS and exposed glass, these seeding densities corresponded to fibroblast number in the central patterned region of

1.5×10^6, 0.75×10^6, 0.25×10^6, and 0.125×10^6. Finally, the next day, PDMS annuli (with adherent fibroblasts) were removed under sterile conditions, resulting in microfabricated co-cultures with fibroblast: hepatocyte ratios of 6:1, 3:1, 1:1, and 0.5:1, while maintaining constant hepatocyte numbers and heterotypic interfaces. Control cultures for no added fibroblasts were performed for a single, representative pattern (1230 μm spacing).

Analytical Assays. Media samples were collected daily and stored at 4 °C for subsequent analysis for urea and albumin content. Urea synthesis was assayed using a commercially available kit (Sigma Chemical Co., Kit No. 535-A). Albumin content was measured by enzyme-linked immunosorbent assays (ELISA) as described previously (Dunn et al., 1991). Rat albumin and anti-rat albumin antibodies were purchased from Cappel Laboratories (Cochranville, PA). Urea and albumin production were utilized as markers of liver metabolic and synthetic function, respectively. Fibroblast production of these markers was determined to be negligible (data not shown). DNA analysis was adapted from a method of MacDonald and Pitt (1991). Cells were incubated with 0.05% (w/v) type 4 collagenase (Sigma) in Kreb's Ringer Buffer at 37 °C for 30 min, to release the cell layer from the underlying substrate, and detached mechanically with a rubber policeman. Cells were pelleted, resuspended in PBS, freeze−thawed, and probe-sonicated. Samples were mixed with salt/dye buffer (2 M NaCl, 10 mM Tris, 1 mM EDTA, and 1.6 μM Hoechst 33258 (Molecular Probes, Eugene, OR)) at room temperature in the dark for 30 min before reading on a spectrofluorometer (Millipore, Bedford, MA): excitation 360 nm, $^1/_2$ bandwidth 40 nm; emission 460 nm, $^1/_2$ bandwidth 40 nm.

Statistics and Data Analysis. Experiments were conducted with duplicate or triplicate culture plates for each condition. Two duplicate wells were measured for biochemical analysis. One representative experiment is presented where the same trends were seen in multiple trials but absolute rates of production varied with each animal isolation. Each data point represents the mean, and error bars represent standard errors of the mean. Nonlinear regression was performed using Sigma Plot (Jandel Scientific, Corte Madera, CA).

Results

Randomly-Distributed Co−cultures. Conventional "randomly distributed" co-cultures were generated to investigate the influence of increasing fibroblast number on a fixed population of hepatocytes. Co−cultures were obtained by seeding 250 000 primary rat hepatocytes on uniform, collagen I-modified glass wafers followed by seeding of growth-arrested murine 3T3-J2 fibroblasts. Figure 1A displays a phase contrast micrograph of approximately 2 million fibroblasts and 250 000 hepatocytes (8:1 ratio). Hepatocytes were clearly visible with distinct nuclei and bright intercellular borders as compared to fibroblasts which displayed a more elongated morphology with less distinct nuclei. Notably, even within a single dish, individual hepatocytes and fibroblasts were exposed to a variety of local cell−cell interactions. For example, homotypic hepatocyte interactions varied markedly-single hepatocytes, doublets, and multihepatocellular aggregates were all noted in these co-cultures. Similarly, the heterotypic interface was dictated by hepatocyte distribution.

The effects of increasing fibroblast number on biochemical function were probed using markers of metabolic and synthetic liver-specific function. Urea synthesis, a marker of metabolic function, was measured in randomly distributed hepatocyte/fibroblast co-cultures with 250 000 hepatocytes as a function of increasing (growth-arrested) fibroblast number (Figure 1B). As fibroblast:hepatocyte ratio increased from 0.5:1 to 8:1, steady-state urea synthesis increased as well. Fibroblast: hepatocyte ratios of greater than 2:1 (inclusive) produced 100−200 μg/day by day 9. In contrast, lower fibroblast: hepatocyte ratios yielded a decline in urea synthesis to background levels, similar to hepatocyte cultured alone (data not shown). The apparent bifurcation in hepatocyte response occurred between 250 000 and 500 000 fibroblasts.

Albumin secretion, a marker of synthetic liver-specific function, displayed similar behavior, increasing progressively as the fibroblast:hepatocyte ratio was varied from 0.5:1 to 8:1. Ratios of greater than 2:1 (inclusive) produced steady-state levels between 30 and 80 μg/day, whereas cultures with fewer fibroblasts produced negligible amounts of albumin by day 9. As with urea synthesis, the transition from relatively poor albumin production to stable, highly functional co-cultures occurred between 250 000 and 500 000 fibroblasts. Thus, under these "randomly distributed" co-culture conditions, reduction of fibroblasts below a threshold level led to significant loss of liver-specific functions.

Microfabricated Co−cultures. "Randomly distributed" cultures, though useful, suffer from a number of shortcomings: (1) homotypic hepatocyte interactions are highly variable and are dictated by seeding density, cell aggregation, and migration and (2) increasing fibroblast number causes a simultaneous increase in both homotypic fibroblast interactions and the heterotypic interface between cell types. To address these issues, microfabricated co-cultures were performed which allowed homotypic hepatocyte interactions to be precisely controlled and the total heterotypic interface to be held constant. The experimental design is schematically represented in Figure 2A−C. All co-cultures were performed with 490 μm diameter hepatocyte islands with progressive reduction in both center-to-center spacing and surface area dedicated to fibroblast adhesion. In this way, all co-cultures contained similar numbers of hepatocyte islands, degrees of homotypic hepatocyte interactions, and total heterotypic interface length, but progressively fewer fibroblasts. We chose this particular island size due to previous data suggesting that patterns composed of hepatocyte islands of this size are highly functional; however, microfabrication techniques would easily allow the use of other dimensions and configurations (Bhatia et al., 1998b).

Immunostaining experiments performed to track hepatocytes in co-cultures over time indicated relatively limited reorganization and subsequent disruption of the heterotypic interface due to cell motility (for one representative pattern over 9 days, data not shown). Figure 2D−F depicts fluorescent micrographs of 490 μm hepatocyte islands in hepatocyte/fibroblast co-cultures. Hepatocytes had been previously labeled with a fluorescent vital dye to visually demonstrate center-to-center spacing of 1230, 930 (not pictured), 650, and 560 μm. Hepatocytes spreading was noted to be comparable on all four patterns; therefore, the number of hepatocytes was approximately equal on all surfaces. Phase contrast micrographs of microfabricated co-cultures were then overlayed with fluorescent micrographs of fluorescently labeled hepatocytes to demonstrate similarity in heterotypic interfaces across co-culture conditions despite dif-

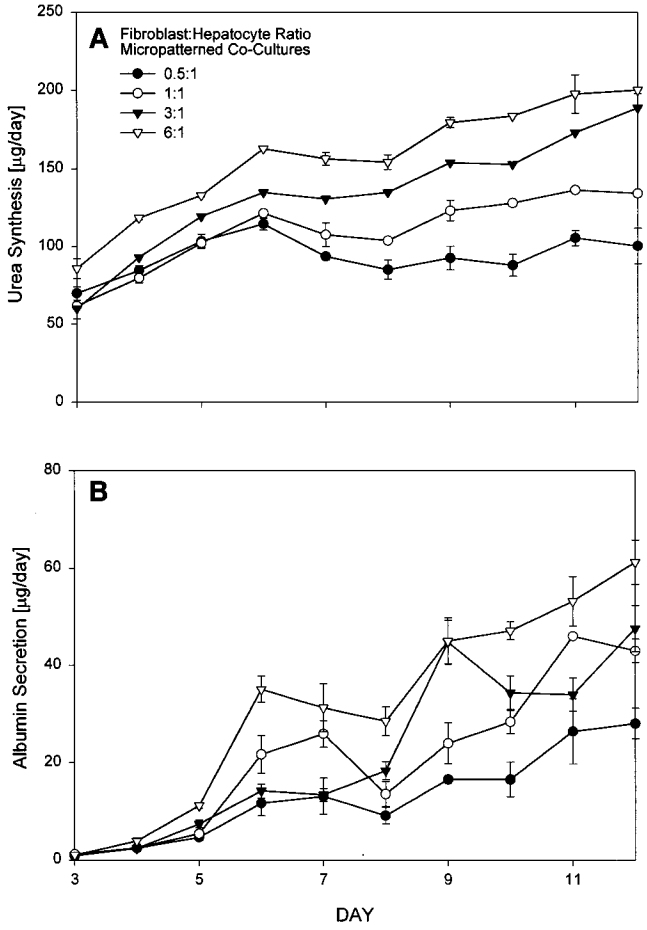

Figure 3. Liver-specific function of microfabricated co-cultures. (A) Urea synthesis as a function of decreasing fibroblast number but constant heterotypic interface, as shown in Figure 2. Legend indicates fibroblast:hepatocyte ratios of 6:1, 3:1, 1:1, and 0.5:1 corresponding to 490 μm hepatocyte islands with center-to-center spacings of 1230, 930, 650, and 560 μm. (B) Albumin secretion in microfabricated co-cultures as in A.

ferences in fibroblast numbers (Figure 2G−I). The resulting fibroblast:hepatocyte ratios were 6:1, 3:1 (not pictured), 1:1, and 0.5:1. Notably, fibroblasts and hepatocytes remained confined to patterned areas, i.e. did not spread into outlying bare glass regions, remained attached and, well-spread, and retained ability to retain a fluorescent vital dye for up to 3 weeks (data not shown).

We probed the level of function of these microfabricated co-cultures by measurement of liver-specific functions—urea synthesis as a marker of metabolic function and albumin secretion as a marker of protein synthesis. Figure 3A shows urea synthesis as a function of increasing fibroblast number in microfabricated co-cultures with constant heterotypic interface. In contrast with "randomly distributed" co-cultures, stable urea production of 100−200 μg/day was achieved by day 6 under all microfabricated co-culture conditions. In general, an increase in fibroblast number corresponded to an increase in steady-state urea production. Specifically, a 12-fold increase in fibroblast number resulted in an approximately 2-fold increase in steady-state urea synthesis.

Similarly, albumin synthesis in microfabricated co-cultures with comparable heterotypic interfaces was measured (Figure 3B). All cultures demonstrated induction of this liver-specific function, as evidenced by the increase in albumin secretion to 20−60 μg/day over 11 days of culture. A similar trend in functional dependence on fibroblasts was observed wherein increase of the fibroblast:hepatocyte ratio by 12-fold led to approximately a 2-fold increase in daily albumin secretion.

Microfabricated co-cultures allowed isolation of fibroblast number as an independent variable influencing hepatocyte function. Data on production of urea and albumin on day 11 of co-cultivation was fit by nonlinear regression using the following form:

$$P = \frac{P_{\max} N}{K_{0.5} + N} + P_0 \qquad (1)$$

when P indicates the production rate of biochemical, N represents the seeded fibroblast number, $P_{\max}$ indicates the theoretical maximal production rate, P_o represents the production rate in the absence of fibroblasts, and $K_{0.5}$ represents the number of fibroblasts required for a half-maximal response. The physical basis of this model is derived from a Michaelis−Menten model of receptor−ligand interactions at the hepatocyte surface that are likely to lie at the root of these cell−cell interactions. Suppose that fibroblast-derived ligand, L, interacts with a hepatocyte receptor on the hepatocyte surface, R, to reversibly form the receptor/ligand complex, RL, which can then irreversibly activate some second messenger, M:

$$[R] + [L] \overset{k_1/k_2}{\longleftrightarrow} [RL] \xrightarrow{k_3} [R] + [M] \qquad (2)$$

Further, if we relate fibroblast number, N, linearly to fibroblast-derived ligand concentration, L, and we relate the second messenger concentration, M, linearly to a biochemical hepatocyte product, P, we obtain eq 1.

Figure 4 depicts the dependence of urea synthesis and albumin secretion on fibroblast number. Notably, data for urea and albumin production rates in the absence of fibroblasts (i.e., fibroblast number = 0) were drawn from a previous study (Bhatia et al., 1998a). The theoretical maximal urea synthesis was found to be approximately 230 μg/day with a half-maximal value achieved at approximately 466 000 ± 76 000 fibroblasts. Albumin secretion demonstrated a similar behavior with maximal theoretical albumin production of 78.5 μg/day with a half-maximal production rate occurring at approximately 458 000 ± 51 000 fibroblasts. Note the striking similarity both in the shape of the curve and in the absolute value for $K_{0.5}$ despite use of two distinct markers of liver-specific function.

Discussion

Previous studies on the role of cell−cell interactions in modulating responses of complex tissues have been attempted using co-cultivation of two cell types in vitro (Rheinwald and Green, 1975; Guguen-Guillouzo et al., 1983; Howlett et al., 1986; Nishida et al., 1993; Fillinger, 1993). In these co-cultures, as in vivo, the influence of one cell population on the other comes about as a result of a complex interplay. The present study initially utilized conventional "randomly distributed" co-cultures to systematically study how the function of a model tissue is affected by these factors.

In particular, hepatocytes were co-cultured with increasing densities of fibroblasts in "randomly distributed" configurations. Using this technique, we demonstrated that increasing fibroblast number (and, thus, heterotypic interface) in conventional, "randomly distributed" co-cultures produced a "dose-dependent" increase in liver-specific function of the neighboring hepatocytes. Our data indicated that two distinct markers of liver function,

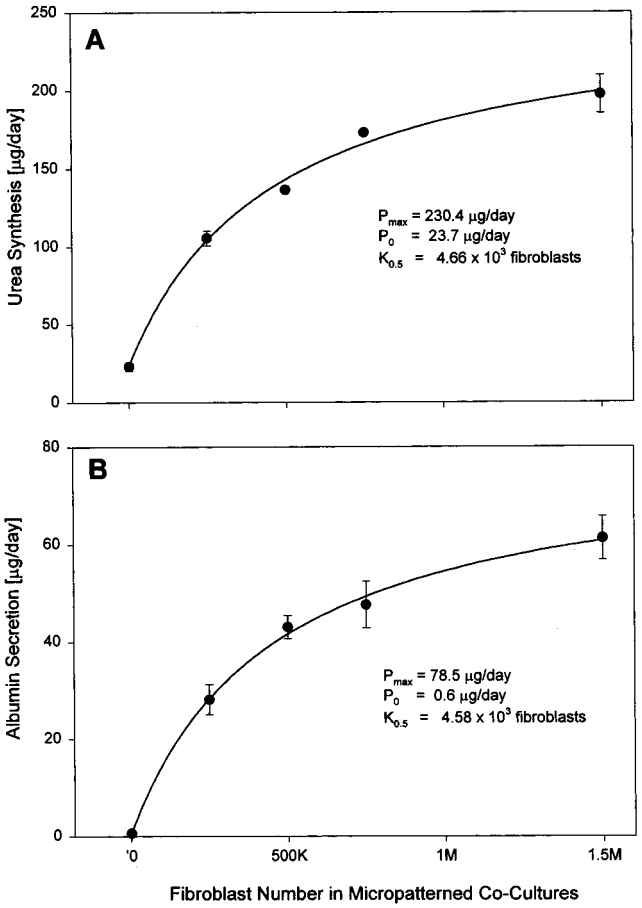

Figure 4. Dose-response of (A) urea synthesis and (B) albumin secretion as a function of fibroblast number. Microfabricated co-cultures allowed isolation of fibroblast number as an independent variable influencing hepatocyte function. Data on production of urea and albumin on day 11 of co-cultivation was fit using eq 1. Note the similarity in $K_{0.5}$ for two distinct markers of liver-specific function.

urea synthesis a marker of metabolic function and albumin secretion a marker of synthetic function, increased dramatically with fibroblast number. Fibroblast: hepatocyte ratios of greater than 2:1 produced significant induction and stabilization of two liver-specific functions, urea and albumin production. In contrast, hepatocytes in co-cultures with fewer fibroblasts, lost liver-specific function over 1 week, as do hepatocytes when cultured alone (data not shown; Dunn et al., 1992). We concluded that the changes in the level of hepatocellular function in these co-cultures were primarily due to variations in the level of function rather than the hepatocyte number by examination of the total DNA of these co-cultures over time. Total DNA of co-cultures of growth-arrested fibroblasts co-cultures showed that hepatocyte DNA increased by less than 30% over 10 days (data not shown), indicating that the measured urea and albumin were produced by a relatively invariant hepatocyte population.

Previously, others have reported the use of "randomly distributed" 3T3 fibroblasts in co-culture with primary hepatocytes to induce the stabilization of liver-specific function in vitro. These studies, utilized a fibroblast: hepatocyte ratio of 1:1 for various applications: stabilization of P450 enzymatic detoxification activity (Utesch et al., 1991; Donato et al., 1990), lipid metabolism (Rincon-Sanchez et al., 1992), triglyceride accumulation in response to polychloride biphenyls (Kuri-Harcuch and Mendoza-Figueroa, 1989), albumin secretion (Morin et al., 1988). Some groups have co-cultured hepatocytes with other cell types such as fat-storing cells (Rojkind et al., 1995), Kupffer cells (Matsuo et al., 1992), and a mixed population of liver nonparenchymal cells (Shimaoka et al., 1987). These studies have utilized empirically determined ratios of ~1:6, 1:10, and 10:1, respectively. Thus, our demonstration that fibroblast density modulates hepatocyte function in a dose-dependent manner is the first systematic examination of the influence of cell number on tissue function.

This aspect of our study demonstrated that increases in heterotypic interface achieved by increasing fibroblast number can alter the phenotype of hepatocytes in vitro from noncuboidal, poorly demarcated cells with poor liver-specific function and viability to cuboidal cells with bright intercellular borders, distinct nuclei, and high rates of production of liver-specific markers, as seen in vivo. However, under these "randomly distributed" conditions, homotypic hepatocyte interactions are highly variable throughout each co-culture (Figure 1A). They depend on cell attachment, aggregation, and migration—all relatively uncontrolled. Furthermore, homotypic fibroblast interactions increase in parallel with fibroblast number in "randomly distributed" co-cultures, confounding our ability to discern the relative roles of these variables from that of the heterotypic interface. To characterize the influence of each of these mechanisms more quantitatively, we evaluated function in microfabricated co-cultures which allowed control of homotypic hepatocyte interactions, homotypic fibroblast interactions, and the heterotypic interface.

We reported elsewhere the use of microfabrication techniques to systematically vary the heterotypic interface in co-cultures with identical cellular constituents (i.e., fibroblast:hepatocytes ratio was held constant in all conditions) (Bhatia et al., 1998b). These studies demonstrated a significant role of the initial total heterotypic interface in modulation of the steady-state response of hepatocyte/fibroblast co-cultures with constant fibroblast number. Increased heterotypic interface correlated with increases in measured liver-specific functions. In contrast, the influences of homotypic hepatocyte interactions were investigated through studies of various sizes of hepatocyte islands (i.e., not in co-cultures) and found to have negligible effects on function (data not shown). Mechanistic studies to address the potential nature of the signal(s) responsible for induction of liver-specific function in co-cultured hepatocytes included in situ immunostaining for intracellular albumin, conditioned media using media incubated with fibroblasts, and spatial separation of cell populations. We concluded that the inductive signal(s), though yet unidentified, was likely to be fibroblast-associated rather than freely soluble (Bhatia et al., 1998b). Together, these results raised the following questions: If a fibroblast signal(s) arising from the heterotypic interface is responsible for modulating hepatocellular function, is this signal(s) influenced by surrounding fibroblasts? And if so, how?

To answer these questions, we constructed microfabricated co-cultures which allowed preservation of the heterotypic interface and homotypic hepatocyte interactions, while allowing reduction of fibroblast number and thus homotypic fibroblast interactions (Figure 2). Co-cultures consisted of hepatocyte islands of fixed diameter surrounded by fibroblasts with progressive reduction in island spacing and total patterned surface area. On these substrates, the level of liver-specific function, monitored by urea synthesis and albumin secretion, varied 2-fold with increasing fibroblast number (Figure 3). Thus, homotypic fibroblast interactions did indeed

Table 1. Effect of Fibroblast Number on Urea Production per unit Area

fibroblast: hepatocyte ratio	micro-patterned area (cm^2)	urea synthesis (μg/day)	urea synthesis/area (mg/(day·cm^2))
6:1	20.0	200 ± 2.1	10.0
3:1	11.4	188 ± 3.4	16.4
1:1	5.7	134 ± 5.0	23.6
0.5:1	4.2	100 ± 11.5	23.8

seem to be a critical determinant of the response of these complex tissues.

In addition to homotypic fibroblast interactions, the observed changes in hepatocellular function may be influenced by fibroblast adhesion on the top surface of hepatocytes. Previous functional and immunohistochemical studies of microfabricated co-cultures have not, however, been consistent with induction of liver-specific function due to potential overlying fibroblasts. Furthermore, fibroblast adhesion to hepatocyte islands was not noted under phase contrast microscopy, although this remains an active area of investigation. Future studies utilizing indirect immunofluorescent staining of both cell populations and confocal microscopy will allow us to examine this more fully.

The potential underlying mechanisms by which changes in fibroblast number could modify both urea synthesis and albumin secretion in the setting of preserved heterotypic interface include modification of homotypic fibroblast signaling via cytokines, secreted or cell-associated extracellular matrix, or membrane-bound receptors/ligands. Cytokines known to have homotypic fibroblast signaling capability include interleukin-1 (Fini et al., 1994; Bergsteindottir et al., 1991), basic fibroblast growth factor (Baird and Klagsburn, 1991), and transforming growth factor-β (Sporn and Roberts, 1990), among others. Similarly, mesenchymal matrix products such as collagen I and fibronectin may play a role in homotypic signaling. In addition, cell surface proteins such as connexins, the subunit which for gap junctions, have been implicated in functional fibroblast interactions (Ruch et al., 1995). These types of homotypic fibroblast signals could result in an altered expression of membrane-bound receptor/ligands or secondary secretion of cytokines or extracellular matrix which are presented to hepatocytes at the interface. Steady-state levels of urea and albumin production in microfabricated co-cultures had a characteristic dependence on fibroblast number (Figure 4). The similarities in the shape of the curves and the calculated half-maximal fibroblast numbers (466 000 ± 76 000 and 458 000 ± 51 000) for two distinct markers of hepatic function suggest that the induction of these functions may share a common mechanism.

The elucidation of how various cell−cell interactions modulate tissue function has a number of practical and scientific implications. For instance, in the case of hepatic tissue engineering, where functional liver tissue is intended to be harnessed for use on a liver failure patient (Sussman et al., 1992; Wu et al., 1995; Rozga et al., 1994), bioreactor design is influenced by surface area dedicated to hepatocellular culture. In this study, use of microfabricated co-cultures to alter fibroblast:hepatocyte ratio has demonstrated that a co-culture system of hepatocytes and fibroblasts may have optimal function per unit area at a 1:1 ratio, despite higher hepatocellular function at higher fibroblast:hepatocyte ratios (Table 1).

Furthermore, the ability to modulate hepatocyte function by both homotypic fibroblast interactions as well as extent of heterotypic interface may allow us to achieve supraphysiologic hepatocellular function in vitro, thereby reducing the cell mass necessary to form a temporary replacement of liver function. Indeed, previous work with hepatocytes in vitro have achieved levels of function comparable to physiologic hepatic function (hepatocytes cultured on matrigel, in a collagen sandwich, or in spheroids (Bissell et al., 1987; Dunn et al., 1992; Koide et al., 1990), whereas we have utilized co-culture of hepatocytes with fibroblasts to achieve variable levels of function, depending on the cell−cell interactions. We demonstrated elsewhere that heterotypic interactions modulate albumin secretion from supraphysiologic levels to physiologic levels (~2 μg/10^6 hepatocytes·h), Bhatia et al., 1998a). In the present study, we demonstrated that fibroblast number is also an independent modulator of this response from physiologic to twice physiologic levels. Notably, physiologic ratios of mesenchymal cells to parenchymal cells (0.5:1, Naughton, 1995) produced physiologic levels of both albumin and urea production in our model system, suggesting a potential control mechanism in vivo based on hepatocyte cellular microenvironment.

In summary, we have experimentally shown that fibroblast number influences hepatocellular function in a dose-dependent fashion. Furthermore, this effect is partially due to both the effects of increased heterotypic interface as seen in previous microfabrication studies and the effects of increased homotypic fibroblast interaction, as demonstrated here. In vivo, organs are composed of many individual cell types, together responsible for orchestrating tissue function. In attempts to mimic tissue function in vitro, a fundamental understanding of the role of each of these interactions will be crucial to progress in the field. Here, we have demonstrated that a model system of two distinct cell types can be useful in elucidating the importance of different types of interactions, both homotypic and heterotypic, in tissue function. This approach will have utility in the study of the role of cell−cell interactions in many other organ systems—both in normal physiology and in the pathophysiology of disease.

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