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Using Growth Factor Arrays and Micropatterned Co-Cultures to Induce Hepatic Differentiation of Embryonic Stem Cells

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Abstract

The success in driving embryonic stem cells towards hepatic lineage has been confounded by the complexity and cost of differentiation protocols that employ large quantities of expensive growth factors (GFs). Instead of supplementing culture media with soluble GFs, we investigated cultivation and differentiation of mouse embryonic stem cells (mESCs) on printed arrays of GFs. Hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF) and bone morphogenetic protein (BMP4) were mixed in solution with fibronectin and collagen (I) and then printed onto silane-modified glass slides to form 500 μ m diameter protein spots. Mouse ESCs were cultured on top of GF spots for up to 12 days and analyzed by RT-PCR and immunostaining at different time points. The stem cells residing on HGF-containing combinations of GFs exhibited requisite features of hepatic differentiation including pronounced loss in pluripotency (Oct4), transient (up and down) expression of endoderm (Sox17) and upregulation of early hepatic markers –albumin and alpha-fetoprotein. The hepatic differentiation was enhanced further by adding hepatic stellate cells to surfaces that already contained mESCs on GF spots. A combination of co-culture with non-parenchymal liver cells and the optimal GF stimulation were found to induce endoderm and hepatic phenotype earlier and to a much greater extent than the GF arrays or micropatterned co-cultures used individually. While this paper investigated hepatic differentiation of mouse ESCs, our findings and stem cell culture approaches are likely to be relevant for human ESC cultivation. Overall, the platform combining printed GF arrays and heterotypic co-cultures will be broadly applicable for identifying the composition of the microenvironment niche for ESC differentiation into various tissue types.

INTRODUCTION

The liver performs many complex functions including carbohydrate metabolism, urea and lipid metabolism, storage of essential nutrients, and the production/secretion of bile acids.[1] Therefore, hepatic failure, end-stage cirrhosis and infections targeting the liver present a major health problem. Given the shortage of organs for liver transplantation, an increasing emphasis is being placed on cell-based liver therapies.[2] However, primary human hepatocytes are in

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short supply and can not be expanded in vitro. Embryonic stem cells (ESCs) on the other hand are capable of both unlimited proliferation and differentiation into tissue-specific cells. ESCs therefore represent a very attractive source of hepatocytes for liver-related cell therapies.[3, 4] A number of reports have been dedicated to identifying in vitro culture conditions required for hepatic differentiation of ESCs. These differentiation protocols aim at recapitulating aspects of in vivo microenvironment by introducing into culture dish growth factors (GFs), extracellular matrix (ECM) proteins and adult cells present in the liver.[5–10]

GF signaling is particularly important for hepatic differentiation of stem cells. The liver arises from the endoderm germ layer which is generated during the gastrulation stage of embryogenesis.[11–13] The same endoderm layer is thought to be the origin of tissues other than liver, including pancreas, lung and thyroid; therefore, provision of appropriate cues is critical to the development of the desired tissue type. Growth factors are the signals that drive ESCs to foregut endoderm and further, toward the hepatocyte lineage.[11–13] The growth factors that are significant in the liver development are numerous, including: hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor- α (TGF- α), members of the TGF- β superfamily (including activin and bone morphogenetic proteins (BMPs)) and fibroblast growth factor (FGF).[11,14]

Current protocols of differentiating ESCs towards endoderm and hepatic lineage rely heavily on supplementing culture media with GFs such as activin, BMP4 and HGF.[15–18] The need to add GF molecules into solution, coupled to the need to change media frequently (daily) makes these protocols very expensive. Cost and complexity of experiments are also roadblocks in identifying and optimizing GF formulations required for tissue-specific differentiation of ESCs. Adding GFs in solution may not be the most physiological way of presenting these molecules to cells since in vivo GF molecules associate with ECM components and are released by cell-initiated proteolytic degradation of the matrix.[19] Binding of GF molecules to matrix components has been shown to enhance and prolong GF stimulation of cells in vitro.[20–22] Therefore, immobilizing GF molecules on surfaces does not only conserve expensive reagents, but may also be a more effective way of delivering GF signals to cells. Given these advantages, a number of groups have been exploring solid-phase presentation of GF molecules for in vitro maintenance/differentiation of stem cells.[23–26]

Surface immobilization also makes it possible to design strategies for high-throughput screening of stem cell - GF interactions. Printing signaling molecules in a microarray format has been proposed as a way to expedite discovery of differentiation inducers. A number of studies have used arrays of ECM proteins,[27,28] small molecules[29] and biomaterials[30, 31] to identify inducers of stem cell differentiation. A considerably smaller number of reports have focused on printed GF arrays as surfaces for stem cell differentiation,[28,32,33]. We are not aware of studies investigating the use of GF arrays for guiding hepatic differentiation of ESCs.

Our laboratory has recently reported that HGF molecules co-printed with ECM proteins (e.g. collagen (I), (IV), laminin) are retained on printed spots for several days under culture conditions.[34] Importantly, primary hepatocytes cultivated on top of the printed HGF/ECM arrays remained functional after 10 days in culture. In a separate study, our group has demonstrated that hepatocytes injured with ethanol while residing on top of HGF and BMP7 arrays were protected against apoptosis and fibrosis by bottom-up stimulation from GF-containing spots.[35] Given the promising results involving maintenance/differentiation of primary hepatocytes, we wanted to explore the utility of GF arrays for guiding hepatic differentiation of ESCs. In the present paper, printed GF array format was used to investigate the effects of HGF, BMP4 and bFGF on hepatic lineage selection of mouse (m)ESCs. Specifically, the paper explores the following questions: 1) can solid-phase presentation of GF

molecules be used to push mESCs towards hepatic lineage? 2) will there be differences in hepatic phenotype expression of mESCs residing on different types of GF spots but bathed in the same media? 3) will adding stellate cells (a major source of GFs in the liver) to surfaces already containing GF arrays further enhance hepatic phenotype of mESCs?

MATERIALS AND METHODS

Chemicals and materials

Glass slides (75×25 mm) were obtained from VWR international (West Chester, PA). 3-Acryloxypropyl trichlorosilane was purchased from Gelest, Inc (Morrisville, PA). Sulfuric acid, hydrogen peroxide, ethanol, collagen from rat tail (type I), laminin, bovine serum albumin, dexamethasone and Tween 20 were obtained from Sigma-Aldrich (St. Louis, MO). Phosphatebuffered saline (PBS) 10× was purchased from Cambrex (Charles City, IA). Dulbecco's modified Eagles' medium (DMEM), minimal essential medium (MEM), Iscove's Modified Dulbecco's Medium (IMDM), sodium pyruvate, non-essential amino acids, L-glutamine, ES-qualified fetal bovine serum (FBS), certified FBS, 2-mercaptoethanol and phenylindole, diacetate (DAPI) were purchased from Invitrogen Life Technologies (Carlsbad, CA). Total mRNA isolation kit, QuantiTect Reverse Transcription Kit and FastStart Universal SYBR Master Mix were purchased from Roche (Indianapolis, IN). Glucagon and insulin were obtained from Eli-Lilly (Indianapolis, IN). ESGRO (leukemia inhibitory factor: LIF), fibronectin, primary mouse embryonic fibroblasts (MEF), and ES cell characterization kit were obtained from Millipore (Temecula, CA). Monoclonal anti-human/mouse α -fetoprotein antibody was purchased from R&D Systems (Minneapolis, MN). Anti-mouse IgG FITC, anti-rabbit IgG-FITC, anti-donkey IgG-TexasRed and monoclonal anti-mouse Sox17 and Oct4 antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Mouse ESC (D3) were purchased from ATCC (Manassas, VA).

Surface modification and microarray printing

Glass slides were cleaned by immersion in piranha solution consisting of a 3:1 mixture of concentrated sulfuric acid and 35% w/v of hydrogen peroxide for 10 min, and subsequently were silanized using protocols described by us previously.[36,37] The silane-modified glass slides were stored in a desiccator before use. Collagen (I) and fibronectin were dissolved at 0.1 mg/mL concentration in 1× PBS with 0.005% (v/v) Tween 20. GFs (HGF, BMP4, bFGF) were added to solution of ECM proteins to create 200 ng/mL concentration per GF. The protein microarrays were contact-printed under ambient conditions on silane-modified glass slides using MicroCaster hand-held microarrayer system (Schleicher & Schuell). The protein arrays consisted of 6 × 12 spots with individual spot diameter of ~500 μ m and center-to-center distance of 1250 μ m. The glass slides with the protein microarrays were stored in a refrigerator for at least one month without detriment to arrays. Different conditions tested were: ECM only, ECM/HGF, ECM/BMP4, ECM/bFGF, ECM/HGF/BMP4 (denoted as ECM/GF mix 1), ECM/HGF/BMP4/bFGF (denoted as ECM/GF mix2). Solution concentrations for all GFs were held constant at 200 ng/mL. Two types of arrays were printed: 1) "redundant arrays" where all spots were of the same composition and 2) "combinatorial arrays" where all of the conditions were present in the same array. Comparison of stem cell differentiation on the two types of arrays was performed to assess the possibility of endocrine signals masking the effects of surface-printed GFs.

Micropatterning of mESCs on printed GF arrays

MESCs (D3 cells) were expanded by cultivating on growth-arrested murine embryonic fibroblast (MEF) feeder cells in gelatin-coated tissue culture plates at 37 °C and 5% CO₂. The culture medium consisted of DMEM supplemented with 15% ES-qualified FBS, 200 U/mL penicillin, 200 mg/mL streptomycin, 2 mM L-glutamine, 1 mM nonessential amino acids, 100

nM 2-mercaptoethanol, and 1000 U/mL LIF. For cell seeding experiments, the glass slides containing protein microarray were cut into 0.5 by 0.5 in. squares and placed into wells of a conventional six-well plate. The samples with imprinted protein arrays were sterilized with 70% ethanol, and washed twice with 1× PBS. The cell seeding was carried out by incubating D3 cell suspension with glass slides in culture medium at a concentration of 1×10^6 cells/mL. After 1 h of incubation at 37 °C, unbound cells were removed by washing with warm 1× PBS, leaving behind clusters of stem cells adhering on 500 µm diameter protein spots. Stem cell arrays were maintained in differentiation medium consisting of IMDM supplemented with 20% FBS, 200 U mL⁻¹ penicillin, 200 mg mL⁻¹ streptomycin, 1 mM nonessential amino acids, 0.5 U mL⁻¹ insulin, 14 ng mL⁻¹ glucagon, and 100 nM dexamethasone. Cells on glass substrates were imaged using a brightfield microscope (Carl Zeiss Inc., Thornwood, NJ).

Creating micropatterned co-cultures of mESCs and stellate cells

To investigate possible synergy between surface-bound GFs and heterotypic cellular signals, stem cells residing on GF arrays were co-cultured with hepatic stellate cells. A human hepatic stellate cell line used in our experiments[38] was maintained in DMEM supplemented with 10% FBS, 200 units/mL penicillin, and 200 µg/mL streptomycin at 37 °C in a humidified 5% CO₂ atmosphere. In creating micropatterned co-cultures we took advantage of the fact that mESCs required ligands like fibronectin for adhesion and became confined to protein spots upon seeding. This left glass regions around the stem cell islands unoccupied and available for stellate cell attachment. Given that stellate cells are robust mesenchymal cells secreting abundant ECM proteins, they were able to adhere on glass substrate, around but not on top of the stem cell clusters. Stellate cells were seeded on the surface at 0.25×10^6 cells/mL and were incubated for 30 min, after which unbound stellate cells were removed by washing with warm 1× PBS. The stem cell – stellate cell co-cultures were maintained in stem cell differentiation medium for the duration of experiment.

Intracellular immunostaining of stem cells cultured on GF arrays

Immunofluorescent staining was used to assess stem cell phenotype and the extent of differentiation towards liver. For immunostaining, cells at different time points were fixed and permeabilized with 4% paraformaldehyde in 1× PBS containing 0.3% Triton-X100 for 20 min. The cells were then incubated in blocking solution (1% BSA in 1× PBS with 0.3% Triton-X100) for 1 h at room temperature and then incubated with primary antibodies overnight at 4° C. Rabbit anti-Oct4 and goat anti-Sox17 were diluted 1:100, and mouse anti-AFP was diluted 1: 50 in 1× PBS containing 1% BSA and 0.3% Triton-X100 prior use. The following day, the cells were washed 3 times with 1× PBS and incubated with secondary antibodies diluted in the same buffer as primary antibodies for 1 hour. The following dilutions were used: 1: 200 for anti-rabbit IgG-FITC, 1:1000 for anti-goat IgG-Alexa488 and 1:50 for anti-mouse IgG-Texas Red. After washing 3 times in 1× PBS samples then were counterstained with DAPI. Unless specified otherwise all incubations were performed at room temperature. The stained cells were visualized and imaged using confocal microscope (Zeiss LSM Pascal).

Stem cells retrieval and RT-PCR analysis

Stem cells on GF arrays were fixed with ice-cold 70% ethanol, and dried under nitrogen. mESCs cultured on “redundant arrays” (all spots of the same composition) were trypsinized, resuspended in 200 mL of lysis buffer (Roche) and then stored at –80 °C. The trypsinization was also employed for collection of cells and isolation of nucleic acids from stem cell-stellate cell co-cultures. This was possible because the two cell types originated from different species, stem cells were of mouse origin whereas stellate cells were of human origin. PCR primers, discussed below, were designed for mouse gene analysis and were not cross-reactive with human genes.

Stem cells on “combinatorial arrays” containing several GF combinations were scraped off from the desired locations using a sterile razor blade. Stem cell colonies were large enough to permit manual picking (500 μ m diameter spots with 1250 μ m center-to-center distance). Total RNA was extracted from the cell lysates using Total mRNA isolation kit (Roche) according to the manufacturer's instructions.

cDNA was synthesized using Quantitest Reverse Transcription Kit (Roche) according to manufacturer's instructions. Briefly, 9 μ L of master mix containing reaction buffer, dNTPs, random hexamer primers and Reverse Transcriptase was added to the 11 μ L of RNA sample (20 mL) and the sample was incubated at 25°C for 10 min, 42 °C for 50 min; then the reaction was terminated by heating the sample at 85 °C for 3 min.

The oligonucleotide sequences for Oct4, Sox17, Afp, Albumin and β -Actin (house keeping gene) as well as quantitative Real-Time PCR protocol have been described by us in detail elsewhere.[39] All PCR reactions were done in duplicate. The relative expression level of each gene was calculated using the comparative threshold cycle (Ct) method with β -actin as a housekeeping gene and an internal standard. Median Ct values of duplicate samples were used to calculate Δ Ct value from the housekeeping gene for the same sample. A denaturing curve for each gene was used to confirm homogeneity of the PCR product.

One-tailed two-sample t-tests with equal and unequal variances were performed for gene expression associated with various surface types/culture conditions tested. F-tests were used to determine variance equality between data sets. T-test results with p-values < 0.05 were considered significant. The number of samples (culture surfaces) used for statistical analysis of PCR data was n = 3 for all conditions.

RESULTS AND DISCUSSION

In this study mESCs were cultured on printed spots containing a mixture of ECM proteins and liver-related GFs including: HGF, BMP4 and bFGF (see Figure 1A). HGF-containing surfaces were found to be most effective for inducing endodermal and early hepatic differentiation of mESCs. Moreover, adding stellate cells to stem cells residing on GF-containing spots further enhanced hepatic phenotype expression. Beyond hepatic tissue engineering, the approach described here should be applicable for stem cell differentiation towards other tissue types.

Culturing stem cells on printed GF spots

Prior to printing protein spots, glass substrates were modified using an acrylated silane. Introducing acrylate functional groups on the surface has been used by us in the past for covalent attachment of hydrogel structures to glass.[36] More recently we have been employing this silane coating for printing proteins arrays[37] and forming micropatterned co-cultures on glass.[39,40] Silanization made glass substrates somewhat hydrophobic (contact angle ~ 50°), thus, improving the quality of printed protein spots. Silane coating also served an important function as a partially non-fouling layer. When seeded onto silanized glass slides containing protein arrays, mESCs preferentially attached to protein spots but not to silane-modified glass regions (see Figure 1B for example of stem cell patterning). The silanized glass regions were only moderately non-fouling and allowed stem cell colonies to expand over time in culture. Significantly, glass regions were also permissive to attachment of stellate cells – this fact is not surprising given the role of the stellate cells play in secretion of ECM proteins in the liver. The adhesiveness of stellate cells could be leveraged in construction of micropatterned co-cultures by adding stellate cells to surfaces that already contained arrays of stem cell colonies (see Figure 1C for example of a micropatterned stem cell-stellate cell co-culture). Creation and analysis of micropatterned co-cultures are described in greater detail in the latter part of this paper.

Printed spots were comprised of ECM proteins and GF molecules. The choice of ECM proteins was driven by the need to promote attachment of stem cells and retention of GF molecules. Our previous studies have shown that mESCs attached well to fibronectin-coated surfaces [39] whereas GF molecules bound well on collagen (I) or laminin-containing protein spots. [34,35] Therefore, in the present work, printed arrays contained fibronectin, for stem cell attachment, and collagen (I), for GF binding. These ECM proteins were mixed in solution with GF molecules and the mixture of ECM/GF proteins was then printed onto glass substrates. While GF retention experiments were not carried out here, we have previously demonstrated that HGF/Collagen (I) printed onto silanized glass substrates was present on the surface after 5 days of tissue culture conditions.[35] As discussed in the next section, stem cells exhibited striking differences in phenotype depending on the surface composition, providing additional evidence that functional GF molecules were indeed present on the surface.

Pluripotency and endodermal phenotype expression in stem cells cultured on GF arrays

A program of hepatic differentiation of mESCs should involve loss of pluripotency, transient expression of endoderm and rise of liver specific markers.[12] In our study, surface culture conditions were categorized as liver inductive if they exhibited patterns of phenotype expression described by a cartoon in Figure 2. The markers used to assess different stages of stem cell differentiation were Oct4 (pluripotency), Sox17 (definitive endoderm), albumin and AFP (both early hepatic phenotype).

Given the central role played by growth factor signaling in liver development, we hypothesized that surface immobilized GFs will affect stem cell differentiation towards liver. While a large number of GFs are implicated in early liver development, we chose to focus on HGF, BMP4 and bFGF because of the role these signaling molecules play in liver development/regeneration. [12] Unlike majority of other stem cell differentiation protocols that utilize soluble GFs, we wanted to immobilize GFs in solid-phase and in association with ECM proteins. Previous studies showed that GFs bound to ECM proteins and immobilized on the surface may be more functional and stable in comparison to soluble GFs added in culture media.[20] In addition, immobilizing GFs on surfaces opens possibilities for high-throughput screening of stem cell – GF interactions and discovery of inductive signals.

In the experiments described in this section stem cells were cultured on “redundant arrays” (all spots of the same composition) in order to first characterize stem cell differentiation on surface providing one type of stimulus and then compare differentiation trends to those obtained with “combinatorial arrays” (spots of different composition in the same array). It should be noted that hepatic differentiation media was the same in all experiments. For the sake of simplicity we chose to investigate only one GF concentration (200 ng/ml) for HGF, BMP4 and bFGF.

Figure 3A shows dynamics of Oct4 gene expression in mESCs cultured on top of different protein spot for 12 days. As can be seen from these data, mESCs residing on ECM (fibronectin/collagen (I)) spots without GFs showed only gradual decay in Oct4 gene expression (less desirable), whereas GF containing spots induced a much more rapid loss of Oct4 expression (more desirable). By day 12 of culture mESCs on ECM spots containing HGF, HGF/BMP4 (ECM/GF mix1) and HGF/BMP4/bFGF (ECM/GF mix2) expressed the lowest level of Oct4 transcripts. Compared to Oct4 levels of undifferentiated mESCs, Oct4 gene expression on the most optimal surface (ECM/GF mix 2) was lower by ~77 fold whereas Oct4 expression on suboptimal GF spots was only 7 to 10 fold lower. In addition, Oct4 gene expression was 5 times lower in stem cells residing on top of ECM/HGF/BMP4/bFGF compared to stem cells cultured on ECM spots and exposed to the same combination of GFs in solution (denoted as ECM + soluble GF in Figure 3). This result is significant, because GF molecules in arrays were printed one time at the beginning of the experiment whereas soluble GFs were replenished

daily with media changes. Over the course of a 12 day stem cell culture experiment, 60 times less of GF molecules were needed for solid-phase presentation.

Beyond loss of pluripotency, mESCs cultured on GF arrays underwent endodermal differentiation. Definitive endoderm is an intermediate stage in the development of hepatocytes, and is considered to be a pre-requisite to hepatic differentiation of ESCs.[12,16, 18] Importantly, because endoderm is an intermediate and not a terminal stage of differentiation, it should exhibit transient expression as described in Figure 2. Sox17 is a transcription factor that is commonly used as a marker of definitive endoderm.[16] Figure 3B shows Sox17 gene expression over the course of 12 days in mESCs cultured on surfaces of different GF composition. Stem cells cultured on ECM spots without imprinted GFs showed a slow increase in Sox17 expression, whereas stem cells on ECM/BMP4 and ECM/bFGF surfaces exhibited a more pronounced upregulation of Sox17. In contrast, ECM/HGF, ECM/HGF/BMP4 (ECM/GF mix1) and ECM/HGF/BMP4/bFGF (ECM/GF mix2) spots induced transient (up and down) expression of endoderm marker in mESCs over the course of 12 days. Cultivating mESCs on ECM spots with a mixture of soluble HGF/BMP4/bFGF (denoted as ECM/sol GF in Figure 3B) resulted in the same type of desirable endodermal differentiation. This again suggests that stem cells cultured on top of GF-containing surfaces receive signals of sufficient strength and duration to induce differentiation that is comparable to or better than traditional protocols involving soluble GF molecules.

Immunofluorescent staining was used to confirm RT-PCR analysis of stem cell differentiation. Figure 4 shows immunostaining results for mESCs cultured on ECM/HGF/BMP4/bFGF spots. As expected, expression of pluripotency marker Oct4 decreased from day 2 to day 8 in culture (Figure 4A), whereas endoderm marker Sox17 became upregulated over the same time period. These data corroborate RT-PCR analysis and point to loss of pluripotency and upregulation in endodermal differentiation in stem cells cultured on top of GF spots.

Expression of liver markers in stem cells cultured on printed GF arrays

In order to assess hepatic phenotype of mESCs cultured on various surface coatings we chose to monitor expression of AFP and albumin. Both markers appear at the early stages of liver development and may be associated with hepatoblast phenotype. As in the experiments described in the previous section, mESCs were cultured on “redundant arrays” (one type of spots per glass slide). The extent of hepatic gene expression in mESCs was compared to mouse hepatocyte cell line (Hepa 1–6). Figure 5 demonstrates expression of AFP and albumin on protein spots of different composition. As can be seen from Figure 5A, AFP gene expression was undetectable on protein arrays containing only ECM proteins (fibronectin/collagen (I)) and was only detectable at day 12 on ECM/BMP4, ECM/bFGF and ECM/HGF spots. In contrast, stem cells on ECM/HGF/BMP4 (ECM/GF mix1) and ECM/HGF/BMP4/bFGF (ECM/GF mix2) spots started expressing AFP at day 8 and were expressing 18% and 28% respectively of AFP levels in mouse hepatocyte control by day 12. Similarly, the highest level of albumin gene expression was observed on ECM/HGF/BMP4/bFGF (ECM/GF mix1) combination (see Figure 5B). mESCs residing on top of printed GFs expressed higher levels of albumin compared to stem cells residing on ECM spots and exposed to a mixture of soluble HGF, BMP4, bFGF (ECM/sol GF).

Differentiation of mESCs on “combinatorial arrays”

One of the objectives of this paper was to characterize differentiation of stem cells cultured on a surface presenting multiple signaling molecules. A common concern with printing multiple stimulatory factors on the same surface is that endocrine communications between the neighboring stem cell colonies receiving different stimuli will obscure the effects of imprinted signaling molecules. We therefore were interested in comparing differentiation program of

stem cells cultured on GF spots within a “combinatorial array” vs. stem cells cultured on a “redundant array”. A “combinatorial array” consisted of 6×12 array of 500 μm diameter spots with 1250 μm center-to-center distance printed onto a glass slide. The array contained 6 different spot types with 12 repeats per condition: ECM-only spots as well as 5 different types of GF-containing spots. The size of cell colonies and center-to-center spacing was large enough to allow manual picking of colonies from different locations within the array. This retrieval method was sufficient for characterizing an array with limited number of conditions. In the future, when the panel of tested conditions is expanded and the spot size/spacing is decreased we intend to use laser catapulting for automated collection of cells/nucleic acids from different regions of the cell array for downstream RT-PCR analysis.[39,40]

Figure 6 provides analysis of pluripotency (Oct4), endoderm (Sox17) and hepatic (albumin and AFP) gene expression in mESCs cultured on combinatorial GF arrays. These data once again point to HGF-containing arrays, particularly HGF/BMP4 (GF mix1) or HGF/BMP4/bFGF (GF mix2) combinations, as most conducive to hepatic differentiation of mESCs. At day 12 in culture, stem cells residing on these types of spots had the lowest levels of Oct4 expression (Figure 6A), undetectable levels of Sox17 (Figure 6B) and the highest levels of albumin/AFP (Figure 6C). This trend conforms to the desirable pattern of differentiation involving loss of pluripotency, transient expression of endoderm and upregulation of hepatic makers (see Figure 2). The data in Figure 6 are significant, as they demonstrate that the differences in phenotype of mESCs bathed in the same media but residing on spots of varying GF composition were caused largely by the composition of the underlying substrate. The fact that differentiation of mESCs on a surface imprinted with several GF types was in many respects similar to differentiation on surfaces containing one type of GF (see Figures 3,4) is also important as it suggests that combinatorial GF arrays may be used in long-term culture experiments and may be predictive of stem cell behavior in a dish containing only one stimulus.

We would like to note that Sox17 expression in stem cells residing on ECM, ECM/BMP4 and ECM/bFGF spots within a “combinatorial array” appeared to decay over the course of 12 days (Figure 6B) indicating desirable, transient endoderm expression. On the other hand, the results from “redundant arrays” presented in Figure 3B pointed to gradual rise in Sox17 expression on the same time scale i.e. a much slower rate of endodermal differentiation. This result may be attributed to a certain level of endocrine/paracrine interactions where stem cells on suboptimal surfaces are pushed towards endoderm differentiation by the neighboring stem cell colonies receiving optimal GF stimulation. However, on the whole, “combinatorial arrays” correctly identified combinations of GFs most suitable for hepatic differentiation of mESCs and closely followed patterns of stem cell differentiation observed with simple surfaces containing one stimulus.

Creating micropatterned co-cultures of stem cells and stellate cells

Co-cultivation has been used extensively for maintaining differentiated phenotype of adult hepatocytes in vitro.[41,42] Recently, Moghe and co-workers[43] as well as our laboratory [39] have described the co-cultures of mESCs with hepatic cells and demonstrated that presence of hepatocytes promoted hepatic lineage selection of mESCs. In the present study, we wanted to explore co-cultures of mESCs with another liver cell type – stellate cells. Stellate cells are non-parenchymal cells of mesenchymal origin that reside in the liver and regulate liver growth/repair through matrix remodeling, de novo ECM synthesis and GF release. We hypothesized that co-cultivation with stellate cells may induce liver-specific differentiation of mESCs. In addition, we were interested in exploring how the combination of GF stimulation from the printed arrays and heterotypic cell interactions affected hepatic differentiation of mESCs. As discussed before, stem cells reproducibly attached on protein spots and formed colonies corresponding in dimensions to the underlying spots (500 μm diameter). Stellate cells on the

other hand were capable of producing endogenous ECM proteins, thus modifying glass substrate with adhesive ligands. When seeded on the surface that already contained stem cell arrays, stellate cells attached on glass regions around the islands of mESCs as shown in Figure 3B. These micropatterned co-cultures were maintained in stem cell differentiation medium for 12 days and were analyzed at different time points by RT-PCR and immunostaining to assess hepatic phenotype (AFP and albumin markers). Because mouse ESCs were co-cultured with human stellate cells and primers used in our experiments did not have cross-species reactivity, stem cell analysis could be performed by trypsinizing the co-cultures and then extracting nucleic acids for RT-PCR analysis of mouse gene expression.

Four stem cell culture conditions were compared to determine the levels of albumin and AFP gene expression: ECM/GF spots, ECM/GF/co-culture, ECM/sol GF/co-culture and ECM/co-culture where GF represents a mixture of HGF, BMP4 and bFGF. As can be seen from Figure 7(A,B) stem cells residing on GF spots and surrounded by stellate cells expressed much higher levels of albumin and AFP, ~53% and 85% respectively of mouse hepatocyte control when compared to stem cell monocultures formed on GF spots (25% and 50% of mouse hepatocyte expression of albumin and AFP). The differences in AFP protein levels in stem cell monocultures and co-cultures are highlighted by immunofluorescent staining results shown in Figure 7C.

Albumin gene expression was observed in stem cell-stellate cell co-cultures at day 8 whereas it appeared only at day 12 in stem cell monocultures. Also interestingly, stem cells receiving bottom-up GF stimulation in co-cultures had significantly higher level of albumin expression compared to stem cells in co-cultures with soluble GF. Finally, stem cell – stellate cell co-cultures formed on GF spots expressed significantly higher levels of albumin and AFP in comparison to co-cultures formed on ECM-only spots. These results indicate that a combination of co-cultures and bottom-up GF signaling is more effective in driving mESCs towards hepatic lineage than either one of these approaches individually.

CONCLUSIONS

Our laboratory has previously employed GF arrays for cultivation of primary hepatocytes and for screening anti-apoptotic effects of GFs in the context of model liver injury. In the present study we wanted to extend the use of printed GF arrays to stem cell differentiation and sought to explore the possibility of guiding hepatic lineages selection of mESCs cultured on HGF, BMP4 and bFGF arrays. Our studies revealed that the extent of hepatic differentiation in stem cells cultured in the same dish and bathed in the same media differed strikingly depending on the composition of the underlying protein spots. Spots containing HGF alone or in combination with BMP4 and bFGF were found optimal for hepatic differentiation of mESCs. Another important finding was that, over the course of 12 days, hepatic differentiation of stem cells cultured on printed GF spots was similar to or better than stem cell differentiation in media containing the same GFs in soluble form. This finding is significant because while soluble GFs were replenished with daily media exchanges, solid-phase presented GFs were printed only once at the beginning of a 12 day experiment. Thus, ECM/GF arrays may provide a more effective means of presenting functional GF molecules to stem cells as well as a more economical means of utilizing these expensive reagents. Finally we demonstrated that hepatic differentiation of mESCs residing on GF spots may be enhanced further by co-cultivating stellate cells on the same surface. In the future, the stem cell cultivation methods described here will be used for identifying inducers of human ESC differentiation towards liver or other tissue types.

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REFERENCES

- [1]. Kaplowitz, N. Structure and Function of Liver. In: Kaplowitz, N., editor. Liver and Biliary Diseases. Williams and Wilkins; Baltimore: 1992.
- [2]. Nussler A, Konig S, Ott M, Sokal E, Christ B, Thasler W, et al. Present status and perspectives of cell-based therapies for liver diseases. *J Hepat* 2006;45:144–59.
- [3]. Kisseleva T, Gigante E, Brenner DA. Recent advances in liver stem cell therapy. *Cur Opin Gastroenter* 2010;26:395–402.
- [4]. Wobus AM, Boheler KR. Embryonic stem cells: prospects for developmental biology and cell therapy. *Physiol Rev* 2005;85:635–78. [PubMed: 15788707]
- [5]. Baharvand H, Hashemi SM, Kazemi Ashtiani S, Farrokhi A. Differentiation of human embryonic stem cells into hepatocytes in 2D and 3D culture systems in vitro. *Int J Dev Biol* 2006;50:645–52. [PubMed: 16892178]
- [6]. Duan Y, Catana A, Meng Y, He S, Gupta S, Gambhir SS, et al. Differentiation and enrichment of hepatocyte-like cells from human embryonic stem cells in vitro and in vivo using lentivirus systems. *Stem Cells* 2007;25:3058–68. [PubMed: 17885076]
- [7]. Hamazaki T, Iiboshi Y, Oka M, Papst PJ, Meacham AM, Zon LI, et al. Hepatic maturation in differentiating embryonic stem cells in vitro. *Febs Letters* 2001;497:15–9. [PubMed: 11376655]
- [8]. Shirahashi H, Wu J, Yamamoto N, Catana A, Wege H, Wager B, et al. Differentiation of human and mouse embryonic stem cells along a hepatocyte lineage. *Cell Transplant* 2004;13:197–211. [PubMed: 15191158]
- [9]. Novik EI, Maguire TJ, Orlova K, Schloss RS, Yarmush ML. Embryoid body-mediated differentiation of mouse embryonic stem cells along a hepatocyte lineage: Insights from Gene Expression Profiles. *Tissue Engin* 2006;12:1515–25.
- [10]. Duan YY, Ma XC, Zou W, Wang C, Bahbahan IS, Ahuja TP, et al. Differentiation and characterization of metabolically functioning hepatocytes from human embryonic stem cells. *Stem Cells* 2010;28:674–86. [PubMed: 20135682]
- [11]. Jung J, Zheng M, Goldfarb M, Zaret KS. Initiation of mammalian liver development from endoderm by fibroblast growth factors. *Science* 1999;284:1998–2003. [PubMed: 10373120]
- [12]. Zaret KS. Hepatocyte differentiation: from the endoderm and beyond. *Curr Opin Genet Dev* 2001;11:568–74. [PubMed: 11532400]
- [13]. Rossi JM, Dunn NR, Hogan BL, Zaret KS. Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. *Gene Dev* 2001;15:1998–2009. [PubMed: 11485993]
- [14]. Matsumoto K, Nakamura T. Emerging multipotent aspects of hepatocyte growth factor. *J Biochem (Tokyo)* 1996;119:591–600. [PubMed: 8743556]
- [15]. Gouon-Evans V, Boussemart L, Gadue P, Nierhoff D, Koehler CI, Kubo A, et al. BMP-4 is required for hepatic specification of mouse embryonic stem cell-derived definitive endoderm. *Nature Biotech* 2006;24:1402–11.
- [16]. D'Amour KA, Agulnick AD, Eliazar S, Kelly OG, Kroon E, Baetge EE. Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nature Biotech* 2005;23:1534–41.
- [17]. D'Amour KA, Bang AG, Eliazar S, Kelly OG, Agulnick AD, Smart NG, et al. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nature Biotech* 2006;24:1392–401.
- [18]. McLean AB, D'Amour KA, Jones KL, Krishnamoorthy M, Kulik MJ, Reynolds DM, et al. Activin A efficiently specifies definitive endoderm from human embryonic stem cells only when phosphatidylinositol 3-kinase signaling is suppressed. *Stem Cells* 2007;25:29–38. [PubMed: 17204604]

- [19]. Bashkin P, Doctrow S, Kagsbrun M, Svahn CM, Folkman J, Vlodavsky I. Basic fibroblast growth factor binds to subendothelial extracellular matrix and is released by heparitinase and heparin like molecules. *Biochemistry* 1989;28:1737–43. [PubMed: 2541764]
- [20]. Paralkar VM, Vukicevic S, Reddi AH. Transforming growth factor-beta binds collagen IV of basement membrane matrix: Implications for development. *Dev Biol* 1991;143:303–8. [PubMed: 1991553]
- [21]. Vlodavsky I, Fuks Z, Ishai-Michaeli R, Bashkin P, Levi E, Korner G, et al. Extracellular matrix-resident basic fibroblast growth factor: implications for the control of angiogenesis. *J Cell Biochem* 1991;45:167–76. [PubMed: 1711529]
- [22]. Vlodavsky I, Miao HQ, Medalion B, Danagher P, Ron D. Involvement of heparan sulfate and related molecules in sequestration and growth promoting activity of fibroblast growth factor. *Cancer Metastasis Rev* 1996;14:177–86. [PubMed: 8842489]
- [23]. Kuhl PR, Griffith-Cima LG. Tethered epidermal growth factor as a paradigm for growth factor-induced stimulation from the solid phase. *Nature Med* 1996;2:1022–7. [PubMed: 8782461]
- [24]. Alberti K, Davey RE, Onishi K, George S, Salchert K, Seib FP, et al. Functional immobilization of signaling proteins enables control of stem cell fate. *Nature Methods* 2008;5:645–50. [PubMed: 18552855]
- [25]. Marcantonio NA, Boehm CA, Rozic RJ, Au A, Wells A, Muschler GF, et al. The influence of tethered epidermal growth factor on connective tissue progenitor colony formation. *Biomaterials* 2009;30:4629–38. [PubMed: 19540579]
- [26]. Fan VH, Au A, Tamama K, Littrell R, Richardson LB, Wright JW, et al. Tethered epidermal growth factor provides a survival advantage to mesenchymal stem cells. *Stem Cells* 2007;25:1241–51. [PubMed: 17234993]
- [27]. Flaim CJ, Chien S, Bhatia SN. An extracellular matrix microarray for probing cellular differentiation. *Nature Methods* 2005;3:119. [PubMed: 15782209]
- [28]. LaBarge MA, Nelson CM, Villadsen R, Fridriksdottir A, Ruth JR, Stampfer MR, et al. Human mammary progenitor cell fate decisions are products of interactions with combinatorial microenvironments. *Integrative Biology* 2009;1:70–9. [PubMed: 20023793]
- [29]. Ding S, Schultz PG. A role of chemistry in stem cell biology. *Nature Biotech* 2004;22:833–49.
- [30]. Anderson DG, Levenberg S, Langer R. Nanoliter-scale synthesis of arrayed biomaterials and application to human embryonic stem cells. *Nature Biotech* 2004;22:863–6.
- [31]. Anderson DG, Putnam D, Lavik EB, Mahmood TA, Langer R. Biomaterial microarrays: rapid, microscale screening of polymer-cell interaction. *Biomaterials* 2005;26:4892–7. [PubMed: 15763269]
- [32]. Nakajima M, Ishimuro T, Kato K, Ko IK, Hirata I, Arima Y, et al. Combinatorial protein display for the cell-based screening of biomaterials that direct neural stem cell differentiation. *Biomaterials* 2007;28:1048–60. [PubMed: 17081602]
- [33]. Soen Y, Mori A, Palmer TD, Brown PO. Exploring the regulation of human neural precursor cell differentiation using arrays of signaling microenvironments. *Mol Syst Biol*. 2006
- [34]. Jones CN, Tuleuova N, Lee JY, Ramanculov E, Reddi AH, Zern MA, et al. Cultivation of liver cells on printed arrays of hepatocyte growth factor. *Biomaterials* 2009;30:3733–41. [PubMed: 19375794]
- [35]. Jones CN, Tuleuova N, Lee JY, Ramanculov E, Reddi AH, Zern MA, et al. Cultivating hepatocytes on printed arrays of HGF and BMP7 to characterize protective effects of these growth factors during in vitro alcohol injury. *Biomaterials* 2010;31:5936–44. [PubMed: 20488537]
- [36]. Revzin A, Tompkins RG, Toner M. Surface engineering with poly(ethylene glycol) photolithography to create high-density cell arrays on glass. *Langmuir* 2003;19:9855–62.
- [37]. Lee JY, Zimmer C, Liu GY, Revzin A. Use of photolithography to encode cell adhesive domains into protein microarrays. *Langmuir* 2008;24:2232–9. [PubMed: 18198912]
- [38]. Xu L, Hui AY, Albanis E, Arthur MJ, O'Byrne SM, Blaner WS, et al. Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis. *Gut* 2005;54:142–51. [PubMed: 15591520]
- [39]. Lee JY, Tuleuova N, Jones CN, Ramanculov E, Zern MA, Revzin A. Directing hepatic differentiation of embryonic stem cells with protein microarray-based co-cultures. *Integr Bio* 2009;1:460–8.

- [40]. Lee JY, Jones CN, Zern MA, Revzin A. Analysis of local tissue-specific gene expression in cellular micropatterns. *Anal Chem* 2006;78:8305–12. [PubMed: 17165820]
- [41]. Bhatia SN, Balis UJ, Yarmush ML, Toner M. Effect of cell-cell interactions in preservation of cellular phenotype: cocultivation of hepatocytes and nonparenchymal cells. *FASEB J* 1999;13:1883–900. [PubMed: 10544172]
- [42]. Corlu A, Ilyin G, Cariou S, Lamy I, Loyer P, Guguen-Guillouzo C. The coculture: a system for studying the regulation of liver differentiation/proliferation and its control. *Cell Biol Toxicol* 1997;13:235–42. [PubMed: 9298244]
- [43]. Moore RN, Dasgupta A, Rajaei N, Yarmush ML, Toner M, Larue L, et al. Enhanced differentiation of embryonic stem cells using co-cultivation with hepatocytes. *Biotech Bioeng* 2008;101:1332–43.

Figure 1.

(A) Diagram of experiments performed in this paper. In scenario 1, arrays composed of HGF, BMP4 and bFGF were used for cultivation of mESCs. In scenario 2, stellate cells were added to surfaces containing stem cell arrays to create micropatterned co-cultures and to explore confluence of cell-cell and cell-GF interactions in driving hepatic differentiation of mESCs. (B) mESCs colonies on printed ECM/GF spots at day 1 in culture. Individual spots/colonies were 500 μm in diameter. (C) Micropatterned co-cultures of mESCs and stellate cells at day 6 in culture. When added on the surfaces containing stem cell micropatterns, stellate cells attached on glass regions around the stem cell clusters, forming co-cultures.

Figure 2.
Diagram describing desirable (ideal) pattern of embryonic stem cell differentiation towards hepatic lineage.

Figure 3.

RT-PCR analysis of pluripotency and endoderm gene expression. Experiments were performed on “redundant arrays” where all spots were identical. Gene expression was normalized to housekeeping gene β -actin. Error bars represent standard deviation of the mean for $n=4$ samples. Bars marked with (*) signify conditions tested for statistical significance ($p < 0.05$). Abbreviations: ECM/GF mix1 - spots containing ECM/HGF/BMP4; ECM/GF mix2 - spots containing ECM/HGF/BMP4/bFGF; ECM/sol GF means that mESC were cultured on ECM spots and exposed to soluble mixture of HGF, BMP4 and bFGF. (A) Surface-specific differences in downregulation of pluripotency (Oct4) gene expression. mESCs cultured on ECM/GF mix2 expressed lowest level of Oct4 – suggesting greatest loss of pluripotency. (B) Dynamics of endoderm (Sox17) gene expression were dramatically different depending on the underlying substrate. Suboptimal spots of ECM or ECM/BMP4 induced only a gradual increase in endoderm expression over 12 day period. In comparison, Sox 17 expression in mESC cultured on optimal surfaces containing HGF (e.g. ECM/GF mix2) rose and fell over the same time period. This transient expression was the desired pattern of endoderm expression.

Figure 4. Immunofluorescent staining for pluripotency (Oct4) and endoderm (Sox17) markers in stem cells cultured on GF arrays. This staining was performed in mESCs cultured on spots of ECM/HGF/BMP4/bFGF.

Figure 5.

RT-PCR analysis of early hepatic markers AFP and albumin in “redundant arrays”. Gene expression was normalized to housekeeping gene β -actin. Mouse hepatic cells (HEPA1–6) were used as a reference. Error bars represent standard deviation of a mean for $n=4$ samples. Bars marked with (*) denote t-test p -value < 0.05 . Abbreviations are as described above. **(A)** AFP expression appeared earlier (day 8) and was stronger in mESCs cultured on ECM/GF mix1 and GF mix2 or in mESCs exposed to a mix of soluble GFs. Note that AFP signal was not detected in mESCs cultured in differentiation media on ECM only spots. **(B)** Albumin gene expression was highest in mESCs on ECM/GF mix2 spots. mESCs on optimal GF combination expressed significantly more albumin than mESCs exposed to same GFs in soluble form.

Figure 6.

RT-PCR analysis of mESC differentiation on “combinatorial arrays” where different GF types were printed on the same glass substrate. These experiments were performed to assess differences in stem cell differentiation on simple surfaces providing one stimulus vs. complex surfaces providing multiple GF signals. GF mix1 and GF mix2 were once again optimal inducing greatest loss of pluripotency gene Oct 4(A), transient expression of endoderm gene Sox17 (B) and upregulation of hepatic markers AFP and albumin (C).

Figure 7.

Combining GF stimulation and co-cultivation of mESCs with stellate cell. (A–B) RT-PCR analysis of hepatic gene expression. Abbreviations are as follows: ECM/GF – printed spots of ECM/HGF/BMP4/bFGF, ECM/GF/co-cultures – same combination of printed GFs plus co-culture; ECM/sol GF/co-culture – printed ECM spots, soluble HGF, BMP4, bFGF plus co-culture, ECM/co-culture – printed ECM spots plus co-culture. As seen from these data by day 12 a combination of GF spots and co-cultures induced higher levels of AFP and albumin expression. T-tests showed that albumin gene expression of stem cells on ECM/GF/co-culture was significantly better than other cultivation conditions ($p < 0.05$, $n=3$). (C) Immunofluorescent staining for intracellular AFP in mESC mono- and co-cultures. Stronger AFP staining is seen in co-cultures – corroborating RT-PCR results.