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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 μm diameter protein spots. mESCs 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 was 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.

1. 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 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 give rise to other tissues 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].

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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 mESCs. 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?

2. Materials and methods

2.1. 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). Phosphate-buffered 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 alpha-fetoprotein (AFP) antibody was purchased from R&D Systems (Minneapolis, MN). Anti-mouse IgG-FITC, anti-rabbit IgG-FITC, anti-donkey IgG-Texas Red and monoclonal anti-mouse Sox17 and Oct4 antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Mouse ESC (D3) was purchased from ATCC (Manassas, VA).

2.2. 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 μm and center-to-center distance of 1250 μm. 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.

2.3. 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% CO_2. 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 non-essential 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 penicillin, 200 mg/mL streptomycin, 1 mM non-essential amino acids, 0.5 U/mL insulin, 14 ng/mL glucagon, and 100 nM dexamethasone. Cells on glass substrates were imaged using a bright-field microscope (Carl Zeiss Inc., Thornwood, NJ).

2.4. 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 U/mL penicillin, and 200 μg/mL streptomycin at 37 °C in a humidified 5% CO_2 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.

2.5. 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