

Hepatic differentiation of human embryonic stem cells on growth factor-containing surfaces

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Abstract

Embryonic stem cells (ESCs) hold considerable promise in tissue engineering and regenerative medicine as a source of tissue-specific cells. Hepatocytes derived from ESCs will be useful for therapies, bioartificial liver assistance devices and drug discovery. In traditional stem cell cultivation/differentiation experiments, growth factors (GFs) are added in soluble form in order to provide signals for tissue-specific differentiation. In contrast, we investigated differentiation of hESCs cultured on top of GFs. In these experiments, glass substrates were imprinted with a mixture of ECM and GF molecules to form 500 μm diameter spots. hESCs were cultured onto these GF-containing ECM spots for up to 12 days to induce differentiation towards the hepatic lineage. The dynamics of differentiation were examined by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) and immunocytochemistry. Stem cells cultured on GF-containing surfaces stained positive for the endoderm markers *SOX17* and *FOXA2*, as well as early liver markers such as α -fetoprotein and albumin. qRT-PCR confirmed that pluripotency, endoderm and liver gene expression of hESCs cultured on GF-containing surfaces was consistent with hepatic differentiation. In comparison, hESCs cultured on ECM spots without GFs showed less pronounced loss of pluripotency and lower levels of liver gene expression. In summary, our study demonstrates that hESCs receive differentiation-inducing signals from GF-containing surfaces and may be pushed along the hepatic lineage when cultured on such surfaces. Compared to traditional approaches, the advantages of GF immobilization include reduction in the cost of experiments, stronger and longer stimulation and the possibility of screening GF-stem cell interactions in a multiplexed manner. Copyright © 2012 John Wiley & Sons, Ltd.

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1. Introduction

Liver failure affects hundreds of thousands of people per year around the globe and is a major health problem (Tilles *et al.*, 2002). Liver transplantation, while being the most effective therapy for acute or chronic liver diseases, is limited by the availability of donor livers and, as a result, many patients die each year while on a waiting list (Murray and Carithers, 2005). Transplantation of hepatocytes is emerging as an alternative to whole-organ

transplantation (Haridass *et al.*, 2008); however, this approach is also limited by the scarcity of human hepatocytes. Therefore, a significant emphasis is being placed on identifying a reliable source of functional hepatocytes to be used in liver-related therapies (Agarwal *et al.*, 2008; Lee, 2001; Min and Theise, 2004).

Pluripotent human embryonic stem cells (hESCs) are expected to improve accessibility to a variety of human cell types (Brolén *et al.*, 2010; Duan *et al.*, 2010). Because these cells may be expanded in culture and then differentiated into the desired cell type, they hold exceptional promise for serving as the source of functional hepatocytes to be used in liver-related cell therapies, bioartificial liver-assist devices and *in vitro* drug discovery and toxicity (Agarwal *et al.*, 2008; Min and Theise, 2004; Synnergren *et al.*, 2010).

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Many existing hESC lines have been cultured using mouse embryonic fibroblast (MEF) feeder cells and serum and other animal-sourced medium components. MEFs support self-renewal of hESCs but the continued use of feeders will hinder the development of clinical applications, due to possible immunogenicity and difficulties in quality control of serum-containing media. Therefore, it is important to define culture conditions that support the proliferation and differentiation of hESCs without the need for feeders or animal-derived components (Xu *et al.*, 2001; Yoon *et al.*, 2010).

While multiple factors can contribute to stem cell differentiation, growth factors (GFs) and ECM proteins represent some of the most important inducers (Ishii *et al.*, 2008). Several studies have described protocols employing GFs to induce ESC differentiation into hepatocyte-like cells (Jefferson *et al.*, 1985). In contrast to *in vitro* cell cultures employing soluble GF molecules, in the native tissue GFs interact non-covalently with ECM proteins and are released by cell-initiated proteolytic degradation of the matrix (Schuppan *et al.*, 1998). Binding of GF molecules to matrix components has been shown to enhance both the strength and duration of GF signalling to cells *in vitro* (Benezra *et al.*, 2002; Paralkar *et al.*, 1991). Therefore, non-covalently binding GFs to ECM in solid form may mimic a more physiological way of providing GF signals to cells.

Moreover, the need to add GFs into culture media makes stem cell differentiation experiments costly and limits the possibilities for multifactorial experiments tethering GF molecules to the surface; co-immobilizing these molecules with ECM proteins may be a more cost-effective way of utilizing expensive GFs. Therefore, immobilizing GF molecules on surfaces not only conserves expensive reagents but may also be a more effective way of delivering GF signals to cells. There is a growing interest in the tissue-engineering community in creating cell culture surfaces for solid-phase presentation of GFs (Alberti *et al.*, 2008; Fan *et al.*, 2007; Kuhl and Griffith-Cima, 1996; Mann *et al.*, 2001).

This laboratory has previously reported on the use of GF and ECM arrays for cultivation/differentiation of primary rat hepatocytes (Jones *et al.*, 2009), mouse embryonic stem cells (Lee *et al.*, 2009; Tuleuova *et al.*, 2010) and mesenchymal stem cells (Ghaedi *et al.*, 2011). In a separate set of studies, Zern and co-workers determined that addition of BMP-2, BMP-4, acidic (a)FGF and HGF into culture media promotes hepatic differentiation of hESCs (Duan *et al.*, 2007, 2010). In the present study, growth factors (BMP-2, BMP-4, α FGF and HGF) as well as carrier ECM molecules (collagen I, fibronectin and laminin) were immobilized onto surfaces and used for solid-phase signalling to the hESC cells cultured on top. Immunostaining and RT-PCR analysis revealed that GF-containing surfaces promoted hepatic differentiation of hESCs over the course of 12 days in culture. A more pronounced hepatic phenotype was observed in the case where hESCs were first differentiated towards definitive endoderm (DE) and then cultured on GF-containing surfaces. This study was one of the first to employ GF-containing surfaces for hepatic differentiation in human ESCs and is highly relevant, as

it offers a possible solution to the challenge of establishing cost-effective and scalable approaches for tissue-specific differentiation of hESCs.

2. Material and methods

2.1. Chemicals and materials

Glass slides (75 × 25 mm²) were obtained from VWR (West Chester, PA, USA). (3-Acryloxypropyl)trichlorosilane was purchased from Gelest (Morrisville, PA, USA). Sulphuric acid, hydrogen peroxide, ethanol, collagenase, collagen from rat tail (type I), hepatocyte growth factor (HGF), gelatin, laminin and fibronectin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Concentrated phosphate-buffered saline (PBS; 10×) was purchased from Lonza (Walkersville, MD, USA). The following reagents were obtained from Invitrogen (Carlsbad, CA, USA): Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F-12), minimal essential medium (MEM), non-essential amino acids, L-glutamine sodium pyruvate, 2-mercaptoethanol, knock-out serum replacement (KOSR) fetal bovine serum (FBS), basic fibroblast growth factor (bFGF), acidic α FGF, bone morphogenetic protein (BMP)-2, BMP-4 and activin A. Polypropylene microarray plates (384-well) were obtained from Genetix (New Milton, NH, USA). The following antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA): goat anti-human Oct4, goat anti-human Sox17, goat anti-human FoxA2, goat anti-human ALB, rabbit anti-human α -fetoprotein (α FP), Cy3-conjugated rabbit anti-goat IgG and Cy3-conjugated mouse anti-rabbit IgG. Formalin was purchased from Fisher (Pittsburgh, PA, USA). Slide-A-Lyzer Mini Dialysis Units were purchased from Pierce (Rockford, IL, USA).

2.2. Cultivation of hESCs

The hESC line H9 was purchased from WiCell Research Institute (Madison, WI, USA; www.WiCell.org) and was expanded according to the provider's instructions. Briefly, hESCs were propagated on irradiated mouse embryonic fibroblast (MEF) feeder layers in DMEM-F12 media and 20% knock-out serum replacement supplemented with 4 ng/ml bFGF, 1 mM glutamine, 1% mM non-essential amino acids and 0.1 mM β -mercapthoethanol at 37°C, 5% CO₂ and 90–95% humidity, with a medium change every day. Undifferentiated hESCs were passaged every 4–5 days onto fresh feeders by mechanical dissociation, using a Stem Cell Cutting Tool (VWR).

2.3. Preparation of glass substrates for ESC cultivation

Glass slides were cleaned by immersion in 'piranha' solution, consisting of a 3:1 ratio of aqueous solutions of

50% v/v sulphuric acid and 30% w/v hydrogen peroxide for 30 min. The glass slides were thoroughly rinsed with distilled water, dried under nitrogen and kept in a Class 10 000 cleanroom prior to use. For silane modification, the glass slides were exposed to oxygen plasma for 5 min at 300 W (YES3, Yield Engineering Systems, Livermore, CA, USA) and then placed for 10 min in 2 mM solution of (3-acrylopropyl) trichlorosilane diluted in anhydrous toluene. The reaction was performed in a glove bag under a nitrogen blanket to avoid exposure to atmospheric moisture. After silanization, the slides were rinsed with fresh toluene, dried under nitrogen and cured at 100°C for 4 h. The silane quality was assessed using a contact angle goniometer (Rame-Hart, Netcong, NJ, USA). The silane-modified glass slides were stored in a desiccator before use.

2.4. Printing of protein spots on glass substrates

Prior to printing, ECM proteins (laminin 200 µg/ml; collagen I 100 µg/ml; and fibronectin 50 µg/ml) were dissolved in 1× PBS containing 0.005% v/v Tween. Subsequently, GFs were added to ECM solution to create the following concentrations: 500 ng/ml HGF, 100 ng/ml BMP-4, 500 ng/ml αFGF, and 100 ng/ml BMP-2 (final volumes of 10 µl). GFs were allowed to interact with ECM proteins in solution for 30 min at room temperature prior to printing. Protein microarrays were contact-printed under ambient conditions on 75 × 25 mm² silane-modified glass slides, using a MicroCaster hand-held microarrayer system (Schleicher and Schuell). The protein arrays consisted of 6 × 12 spots with an individual spot diameter of ~500 µm. Glass substrates with imprinted arrays could be kept in a refrigerator for at least 1 week prior to use.

2.5. Cultivation of hESCs on GF-containing surfaces

For cell-seeding experiments, the glass slides with imprinted protein spots were diced into 1 × 1 inch pieces, placed into wells of a conventional six-well plate and sterilized by immersion in 70% ethanol (Jones *et al.*, 2009). hESC colonies were excised from MEF-based cultures and seeded onto the protein spots. Two culture protocols were tested in this study:

- *Method I.* Following enzymatic dissociation using collagenase IV, colonies of undifferentiated hESCs were cut into small pieces, using a Stem Cell Cutting Tool (VWR). This preparation contained stem cells and MEFs. In order to separate the two cell populations, the cell suspension was incubated on gelatin-coated microwells of a six-well plate for 30 min. During this step MEFs adhered to the tissue culture plate, so that stem cells could be aspirated out and seeded onto glass

substrates containing protein spots. These cells were cultured for 16 h in RPMI 1640 medium supplemented with 5% FBS and 100 ng/ml activin A. Subsequently, the hESCs were maintained in the differentiation medium, consisting of Iscove's Modified Dulbecco's Media (IMDM) with 20% FBS, 2 mM L-glutamine, 1 mM non-essential amino acids, 1% antibiotic-antimycotic, 0.126 U/ml human insulin, 14 ng/ml glucagon, 100 nM dexamethasone and 0.5% DMSO for 10–14 days.

- *Method II.* hESCs were first differentiated towards definitive endoderm (DE) and then cultured on GF-containing surfaces to induce hepatic differentiation. DE was initiated under conditions described previously (Duan *et al.*, 2010; Kubo *et al.*, 2004). Briefly, hESCs were cultured in RPMI 1640 medium supplemented with 100 ng/ml activin A, 2 mM L-glutamine and 1% antibiotic-antimycotic for 48 h. Then, 1× B27 supplement and 0.5 mM sodium butyrate were added into the same medium and hESCs were cultured in this medium for 6 days, with daily medium changes. The DE cells were then removed by trypsinization and reseeded onto glass substrates containing GF spots at a concentration of 1 × 10⁶ cells/ml. After 12 h incubation at 37°C, unbound cells were removed by washing with warm 1× PBS, leaving behind clusters of stem cells adhering to the protein spots. For the first day, DE-differentiated stem cells were cultured in the differentiation medium described above, but without DMSO. This reagent was added on the second day of culture and stem cells were cultured for 10–14 days. The medium was changed daily during stem cell cultivation experiments. In a parallel experiment to compare differentiation on GF-containing surfaces with the hepatic differentiation protocol involving soluble GFs developed by the Zern group previously (Duan *et al.*, 2010), DE stem cells were cultured on ECM spots in the culture medium supplemented with αFGF (20 ng/ml), HGF (20 ng/ml), BMP2 and BMP4 (10 ng/ml each).

2.6. Analysis of hESC differentiation by immunofluorescence

Differentiation to DE and the hepatic phenotype was assessed by immunofluorescence staining for OCT4, SOX17, FOXA2, albumin (ALB) and α-fetoprotein (αFP). In these experiments, stem cells cultured on protein spots were washed twice with PBS, fixed for 30 min in a 4% paraformaldehyde solution at room temperature and then permeabilized with 0.4% Triton X-100 for 20 min. The cells were incubated overnight at 4°C with one of the following primary antibodies: goat anti-human OCT4 (1:100), goat anti-human SOX17 (1:100), goat anti-human FOXA2 (1:100), goat anti-human ALB (1:100) or rabbit anti-human αFP (1:300). The next day, the stem cells were washed three times with PBS and incubated with Cy3-rabbit anti-goat IgG or Cy3-mouse anti-rabbit IgG (1:1000) for 1 h at 37°C in the dark. After washing with 1× PBS, the cells were incubated with 4,6-diamidino-

2-phenylindole (DAPI; 1:1000) to stain the nuclei. The stained cells were visualized and imaged using a confocal microscope (Zeiss LSM, Pascal, Thornwood, NJ, USA).

2.7. Analysis of hESC differentiation by RT-PCR

Real-time quantitative RT-PCR (qRT-PCR) was used to determine the expression of *OCT4*, *SOX17*, albumin and α FP in cultured cells on the ECM and ECM/GFs. Total cellular RNA was prepared using the RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. Single-stranded cDNA analysis was performed using the RT-PCR protocol of the First Strand cDNA Synthesis Kit (Qiagen). For quantitative analysis of samples, first-strand cDNA was pre-amplified using a gene-specific protocol. Briefly, a 1/10 volume of first-strand cDNA was pre-amplified using a 1:120 dilution of Assay-on-Demand (Applied Biosystems, Foster City, CA, USA; <http://www.appliedbiosystems.com>) in a multiplex PCR for 20 PCR cycles. The number of PCR cycles for the pre-amplification was determined in a validation experiment and adjusted to prevent any pre-amplified gene from entering the PCR plateau phase to prevent PCR competition and alteration of amplification efficiencies. Amplification efficiencies of the multiplex pre-amplification reaction were assessed by running parallel reactions of the pre-amplification for different numbers of pre-amplification cycles. Subsequently, the pre-amplified materials were analysed by TaqMan PCR, determining the expression of *OCT4*, *SOX17*, *ALB*, α FP and *GAPDH*, employing Applied Biosystems Assay-on-Demand primers. The standard curves were plotted and, from the slope of the standard curves generated on these parallel reactions, the amplification efficiencies were calculated. Concentrations of all primers were optimized before use and all PCR reactions were performed in duplicate. The comparative C_T value method, using *GAPDH* as the housekeeping gene for an internal standard, was employed to determine relative levels of gene expression. Average threshold cycle (C_T) values from the triplicate PCR reactions for a gene of interest (GOI) were normalized against the average C_T values for *GAPDH* from the same cDNA sample. The fold-change of GOI transcript levels between sample X and sample Y = 2^{-C_T} , where $C_T = C_{T(GOI)} - C_{T(GAPDH)}$ and $C_T = C_{T(X)} - C_{T(Y)}$.

2.8. Statistics

All experiments were repeated for at least three times and each condition was analysed in triplicate. Data are presented as mean \pm SEM for quantitative variables. An unpaired Student's *t*-test was used to analyse the data; $p \leq 0.05$ (two-tailed) was considered statistically significant.

3. Results and discussion

In this study, hESCs were cultured on surfaces containing a mixture of four GFs implicated in liver development:

HGF, BMP-2, BMP-4 and α FGF. Immunostaining and RT-PCR analysis revealed that stem cells received liver-inductive signals from GF-containing surfaces and differentiated towards the hepatic lineage. Our studies demonstrated that bottom-up stimulation may be used to induce tissue-specific differentiation of hESCs.

3.1. Cultivation of hESCs on GF spots

Mouse feeder cells are typically used during differentiation of hESCs. Feeder-dependent cultures of hESCs are problematic because: (a) the physiology of the feeder cells is perturbed by the drug treatments or genetic modifications; and (b) mouse cells are immunogenic and will hinder the clinical utility of hESCs (Ishii *et al.*, 2008; Xu *et al.*, 2001; Yoon *et al.*, 2010). However, hESCs can attach and function well on substrates coated with collagen (I, IV), fibronectin or laminin (Ishii *et al.*, 2008). The choice of ECM proteins in our study was driven by the need to promote both the attachment of stem cells and retention of GF molecules. Our previous studies showed that GF molecules can be printed on cell culture surfaces in combination with collagen I and laminin (Jones *et al.*, 2009). Herein, we tested adhesion of hESCs and determined that stem cells attach well to fibronectin and collagen I. Although laminin is a principal component of matrigel, hESCs did not attach well to laminin-coated slides. However when cultured on surfaces containing a mixture of laminin (200 μ g/ml), collagen I (100 μ g/ml) and fibronectin (50 μ g/ml), hESCs attached faster and remained adherent longer than when cultured on individual ECM proteins. Based on these observations, we chose to culture stem cells on protein spots, each spot containing the same mixture of three ECM proteins and four GFs.

To test the ability of surface-immobilized GFs to promote hepatic differentiation of hESCs, ECM/GF spots were printed onto silane-modified glass substrates. Glass slides with ECM spots but without GFs were used as controls. Based on the protocol established in the Zern laboratory, the DE cells were differentiated toward the hepatocyte lineage using culture medium supplemented with FGF-4, HGF, BMP2 and BMP4.

Prior to cell-seeding experiments, glass slides with imprinted protein spots were sterilized by immersion in 70% ethanol. This protocol did not have detrimental effects on the function of primary rat hepatocytes and mESCs, as described in our previous studies (Jones *et al.*, 2009; Tuleuova *et al.*, 2010). When seeded on these glass substrates, hESCs (Figure 1A, B) or DE cells (Figure 1D, E) selectively attached onto printed protein spots, forming colonies corresponding in size to the underlying spots. Cell attachment was defined by the presence of ECM protein on the surface and happened equally well regardless of the presence of GFs. Stem cell organization on protein arrays occurred with high fidelity and minimal non-specific attachment on silanized glass regions. hESCs or DE clusters started to roll up, forming spheroids after 10 days of culture on the ECM/GF spots (Figure 1C, F),

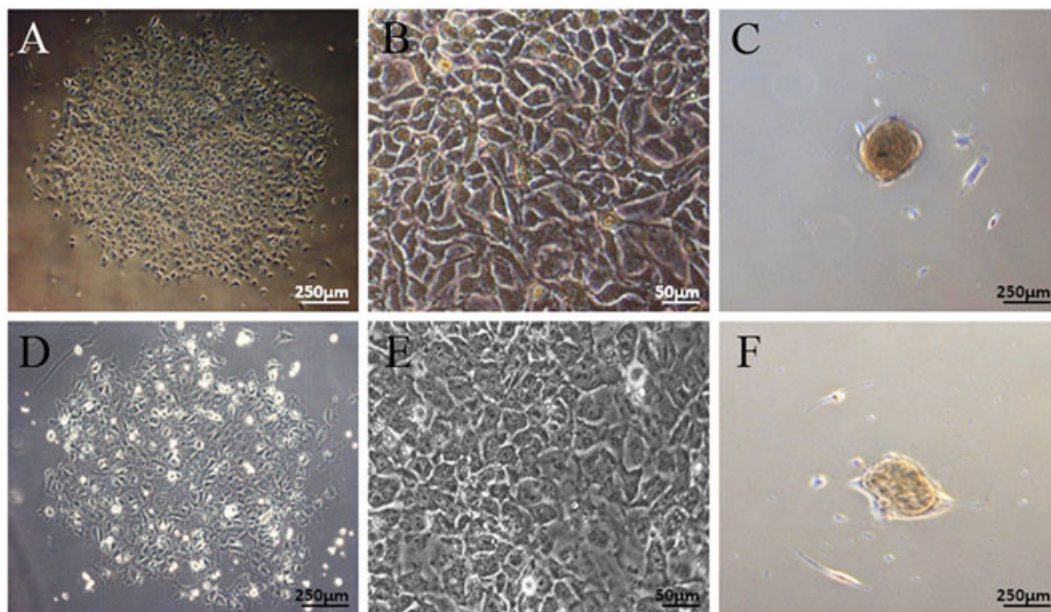


Figure 1. Human embryonic stem cells (hESCs) cultured on 500 μm diameter ECM/GFs arrays: (A) hESCs on ECM spots without GFs at 4 days in culture; (B) hESCs on ECM/GFs spots at day 8 in culture; (C) hESCs spheroids formed on ECM/GFs on day 12; (D) DE after transferring to ECM/GFs spot at day 1; (E) DE on ECM/GFs spots at day 8 in culture; (F) DE spheroids formed on ECM/GFs spots at day 12 in culture

so that by day 12 approximately 90% of the clusters were in spheroid form. In comparison, hESCs or DE cells cultured on ECM arrays without GFs retained a two-dimensional (2D) monolayer morphology (data not shown). The organization of hESCs into spheroids on GF spots may be one reason for the enhanced hepatic function, as a number of recent studies have suggested that hepatocytes in three-dimensional (3D) culture are more functional than those in standard monolayer cultures (Chang and Hughes-Fulford, 2009; Curcio *et al.*, 2007; Nakazawa *et al.*, 2006). We previously observed a similar transition from monolayer to a spheroid configuration in primary hepatocytes cultured on HGF spots (Jones *et al.*, 2009), and hypothesize that spheroid formation may be triggered by a high localized concentration of HGF, which is known to promote cell motility.

3.2. Differentiation of hESCs cultured on GF-containing surfaces

Given the central role played by GF signalling in liver development and regeneration (Rossi *et al.*, 2001), we hypothesized that surface-immobilized GFs will affect the differentiation of hESCs towards the hepatocyte lineage. Based on previous differentiation protocols established in the Zern laboratory, we chose to focus on αFGF , HGF, BMP4 and BMP2. FGFs and BMPs are implicated in providing signals during embryogenesis for differentiation from transversum mesenchyme into endoderm (Rossi *et al.*, 2001). HGF is also critical to the signalling pathway that controls differentiation, proliferation and maturation of fetal liver hepatocytes (Rossi *et al.*, 2001; Soto-Gutierrez *et al.*, 2008).

Unlike stem cell differentiation protocols that utilize soluble GFs, our goal was to present GFs in solid-phase, in association with ECM proteins. Several studies have shown that association with ECM may enhance the stability and function of GFs (Benezra *et al.*, 2002; Ishii *et al.*, 2008). In order to assess the effects of ECM-bound GFs on endoderm expression and hepatic differentiation of hESCs, genes and proteins indicative of pluripotency, definitive endoderm (DE) and hepatic phenotype were analysed. These markers were OCT4 (indicator of pluripotency), SOX17 and FOXA2 (DE markers), αFP (endoderm/early liver marker immature hepatocytes) and albumin (early liver marker).

OCT4 is a homeodomain factor responsible for maintaining the self-renewal and pluripotency of ESCs (Niwa *et al.*, 2000), which is commonly used as an indicator for the undifferentiated hESC phenotype. Loss of pluripotency and early differentiation of ESCs is characterized by a decrease in the expression of this gene. As shown in Figure 2A, the level of OCT4 expression relative to the housekeeping gene (*GAPDH*) decreased over the time in culture in both ECM and ECM/GFs; however, this decrease was more pronounced in the case of ECM/GFs. In comparison to undifferentiated hESC controls, OCT4 levels decreased to 7% on ECM/GF spots vs 13% on ECM-only spots after 8 days cultivation in differentiation medium. Figure 2B shows immunostaining for OCT4 in hESCs cultured on ECM/GFs spots and corroborates the decrease in pluripotency observed with RT-PCR. Overall, our data point to more pronounced pluripotency loss in hESCs cultured on GF/ECM spots compared to stem cells on ECM-only spots. Importantly, in both cases hESCs were exposed to the same hepatic differentiation culture medium.

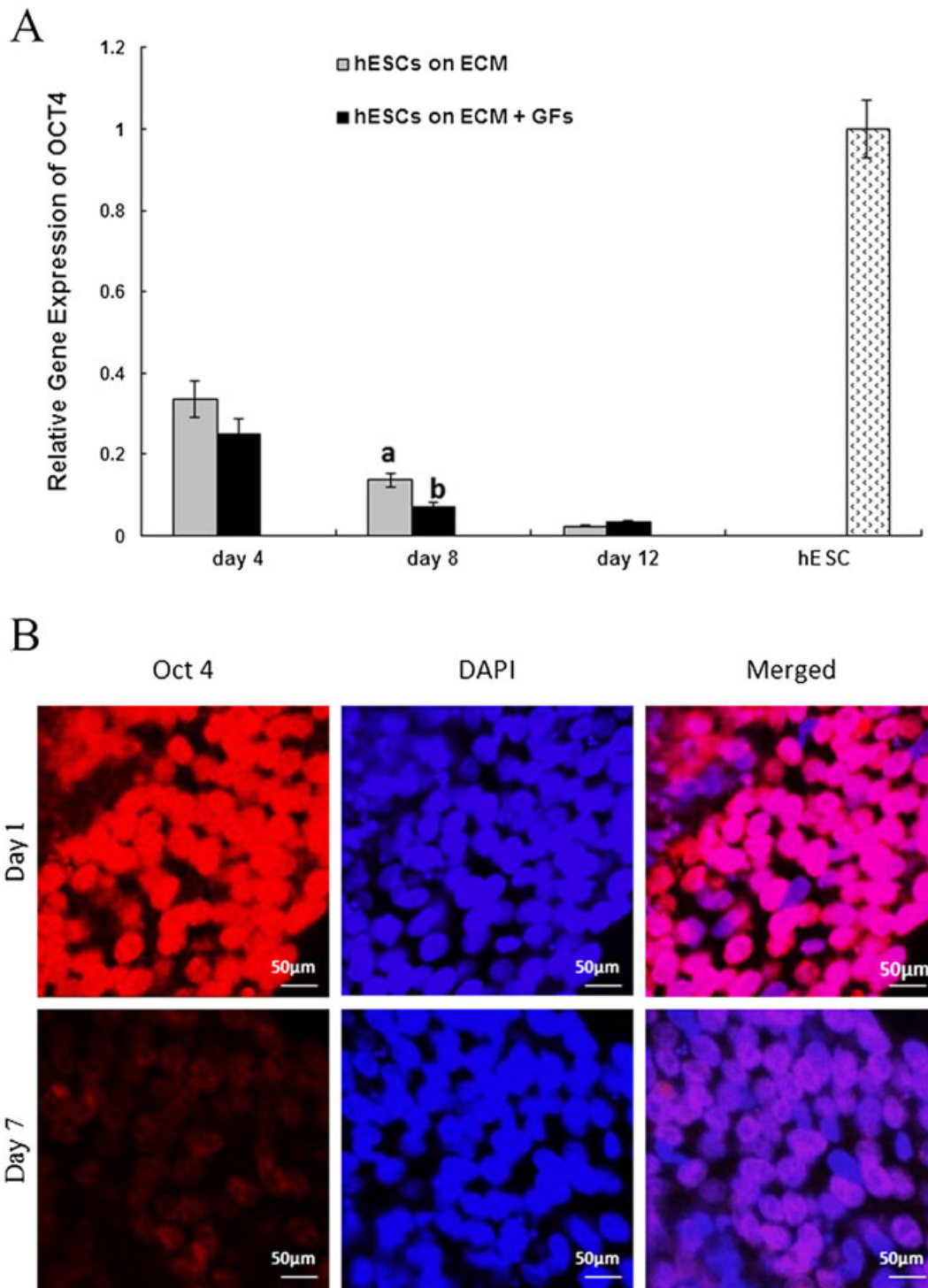


Figure 2. Immunostaining and RT-PCR analysis of pluripotency marker OCT4. (A) RT-PCR analysis of *Oct4* gene expression in hESCs after 4, 8 and 12 days of cultivation on ECM/GF and ECM-only protein spots. Gene expression values were normalized to the house-keeping gene *GAPDH*. hESCs not exposed to differentiation conditions were used as controls (right-most bar). The decrease in *OCT4* expression is greater in hESCs on ECM/GFs on days 0 and 8 when compared to cells on ECM alone. Moreover, the expression of *OCT4* in the culture medium significantly decreased on day 12 of differentiation when compared to expression on day 0. Error bars represent standard error of mean (SEM). a, statistically significant difference ($p < 0.05$) when comparing *Oct4* expression on ECM-only vs ECM/GF spots at day 4; b, statistically significant difference ($p < 0.05$) in *Oct4* expression at days 4 and 8 on ECM/GF spots. (B) Immunofluorescent staining for OCT4 in hESCs cultured on ECM/GF spots on days 1 and 7; these images show decrease in OCT4 expression over time

Embryonic liver arises from DE, therefore the appearance of markers associated with this germ layer is an important indicator of hepatic lineage selection (Brolén *et al.*, 2010; Synnergren *et al.*, 2010). In this study we

monitored two markers of endodermal differentiation, SOX17 and FOXA2. Both are transcription factors expressed in DE as well as in extraembryonic endoderm (Brolén *et al.*, 2010; Synnergren *et al.*, 2010). Figure 3

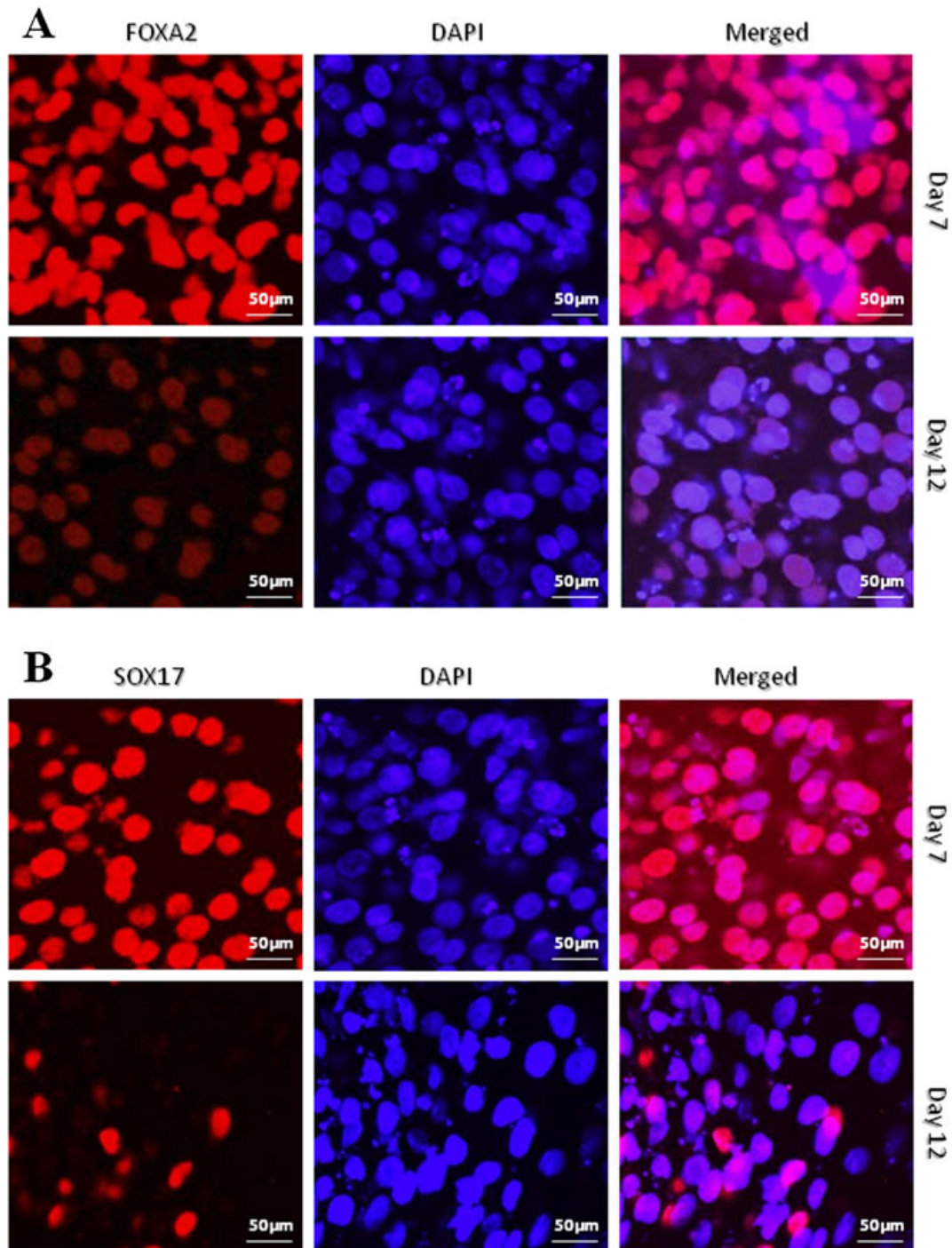


Figure 3. (A, B) Immunofluorescence analysis of endoderm markers Sox 17 (A) and FOXA2 (B) in hESCs cultured on ECM/GF spots. These images show a decrease in endoderm expression over time in culture. This trend is expected during hESC differentiation towards the hepatic lineage

shows immunostaining results for SOX17 and FOXA2 in hESCs cultured on ECM/GF spots. No expression of these markers was observed at day 0 (data not shown) and then endoderm expression decreased from day 7 to day 12. These results were expected, given that endoderm is a transient stage in liver development and is expected to peak and then fade as stem cells differentiate towards the early hepatic phenotype.

We also evaluated the expression of SOX17 by real time qRT-PCR. Figure 4A shows the expression level of Sox17 at days 4, 8 and 12. hESCs on ECM/GFs showed a

four-fold higher level of SOX17 expression compared to ECM-only spots by day 12 in culture. The results shown in Figure 4A confirm endodermal differentiation of hESCs on GF-containing culture surfaces. While some endodermal differentiation was observed in hESCs on ECM-only spots, it was considerably lower compared to hESCs on ECM/GFs.

α FP is widely used as a marker of DE as well as early hepatic differentiation. RT-PCR revealed that α FP expression was higher in stem cells residing on ECM/GF surfaces compared to stem cells cultured on ECM alone (Figure 4B).

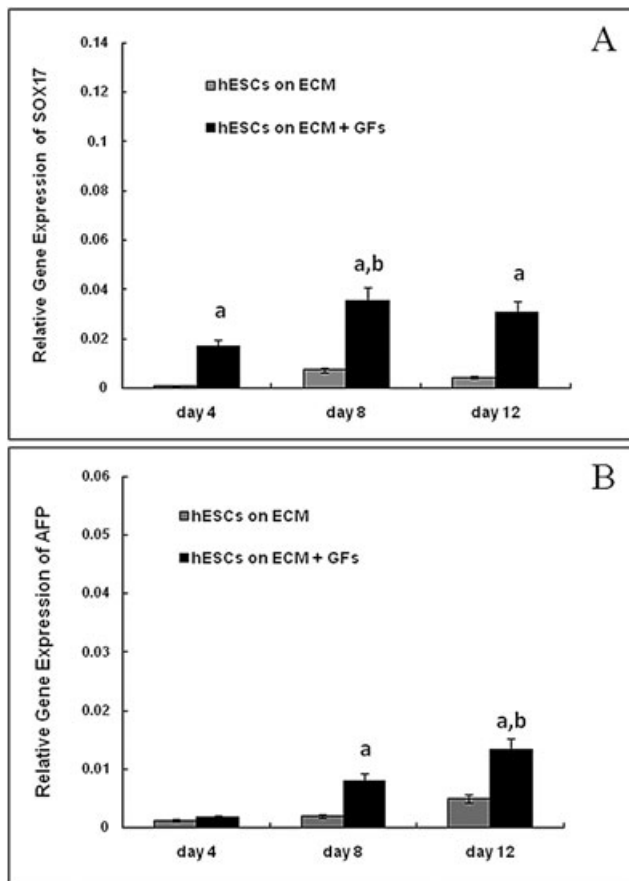


Figure 4. RT-PCR analysis of endoderm and early liver gene expression in hESCs cultured on protein spots. Levels of SOX17 (A) and α FP (B) expression were referenced to the housekeeping gene *GAPDH* ($2^{-\Delta Ct}$). hESCs on ECM/GFs showed a higher level of SOX17 and α FP expression compared to ECM-only spots by day 12 in culture. Moreover, the expression of SOX17 and α FP on ECM/GFs significantly increased on day 12 of differentiation when compared to expression on day 4. Error bars represent SEM. a, statistically significant difference ($p < 0.05$) when comparing hESC gene expression on ECM vs ECM/GF on days 4, 8, 12; b, statistically significant difference ($p < 0.05$) in hESCs on ECM/GF on day 4 vs day 8 for SOX17 and vs day 12 for α FP

The level of α FP transcripts on the ECM/GFs at day 12 was two-fold higher than hESCs on ECM alone. Moreover, the expression of α FP in the culture medium significantly increased on day 12 of differentiation in both hESCs on ECM and ECM/GFs when compared to gene expression at day 4. These results indicated that hESCs cultured on GF-containing surfaces did undergo differentiation towards the endoderm and early hepatic phenotypes. However, expression of markers associated with more mature hepatic phenotype (e.g. albumin) was not observed, suggesting that these stem cells remained at the early stages of the hepatic lineage.

3.3. Differentiation of DE stem cells cultured on GF-containing surfaces

In the early days of ESC research, it quickly became apparent that it was considerably harder to derive endodermal

lineages than those of the mesoderm and ectodermal germ layers. Endoderm is the last of the three germ layers to form during embryonic development *in vivo*; and the derivation of all endodermal lineages was proving technically difficult, including derivation of pancreatic β cells and hepatocytes as well as pulmonary epithelium. Therefore, attention turned to developing means by which endodermal differentiation could be specifically enhanced and the other two germ layers suppressed during the very early stages of ESC differentiation, before the emergence of mature somatic lineages. The most effective strategy yet developed is to mimic embryonic signalling events and expose ESCs to saturating concentrations of activin A, a member of the Nodal signalling family, during the first 7 days of differentiation. In 2004, a landmark paper clearly demonstrated that high levels of activin A promoted the sequential emergence of definitive endoderm, followed by the pancreatic β cell and hepatocyte lineages (Agarwal *et al.*, 2008; D'Amour *et al.*, 2005; Kubo *et al.*, 2004).

Several studies have shown that the hepatocyte-like cell population obtained via DE has many similarities with mature human hepatocytes, including the capacity to metabolize pharmaceutical compounds (Agarwal *et al.*, 2008; Brolén *et al.*, 2010; Duan *et al.*, 2010; Kubo *et al.*, 2004; Synnergren *et al.*, 2010). As described in the previous section of this paper, we did observe hepatic lineage selection in hESCs cultured on GF-containing surfaces; however, these cells did not express mature hepatic markers. We therefore wanted to derive DE stem cells from hESCs and then culture DE stem cells on GF-containing surfaces, reasoning that more mature stem cells would differentiate further along the hepatic lineage. Differentiation to DE was induced with activin A, using a protocol described previously (Duan *et al.*, 2010). Subsequently, DE cells were cultured on GF/ECM or ECM-only protein spots with differentiation monitored by RT-PCR and immunofluorescent staining.

Because DE is an intermediate (transient) stage of differentiation, the expression of DE-specific genes should first increase and then decrease during differentiation to hepatocytes. However, we pushed stem cells to DE prior to placing them onto GF-containing surfaces and in hepatic differentiation medium. Therefore, the results for SOX17 gene expression, shown in Figure 5A, capture only the decrease in endoderm expression over the course of 12 days in culture. Comparison of three conditions (ECM-only, ECM/solid-phase GF and ECM/soluble GF) shows that in the most pronounced loss of SOX17 expression occurs in the presence of GFs, with both soluble and solid-phase GFs having a similar effect at days 8 and 12. Importantly, loss in endoderm was concomitant with upregulation of hepatic markers such as α FP (Figure 5B) and albumin (Figure 6B). Hepatic gene expression was much stronger on ECM/GF-containing surfaces than on ECM alone (two-fold higher for α FP and albumin).

To corroborate PCR data, we carried out immunofluorescent staining for albumin. As shown in Figure 6A, a significant fraction of DE cells on an ECM/GF-containing

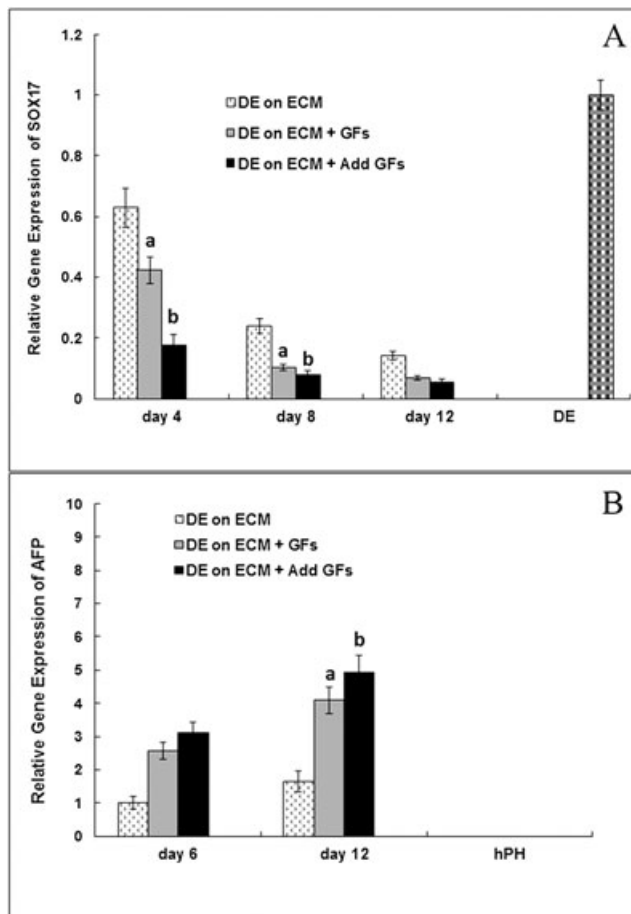


Figure 5. RT-PCR analysis of endoderm and early liver markers in DE stem cells cultured on ECM/GF or ECM-only spots or ECM spots with soluble GFs. (A) SOX17 gene expression in DE stem cells cultured on protein spots in the presence and absence of GFs. DE cells not exposed to hepatic differentiation conditions (right-most bar) were used as controls. DE stem cells interacting with surface-bound or soluble GFs and cultured in hepatic differentiation medium showed a dramatic decrease in SOX17 expression over 12 days in culture. Error bars indicate SEM; pairs for statistical significance comparison are as follows: a, stem cells on ECM/GF spots compared to ECM-only at days 4 and 8; b, hESCs on ECM spots adding GFs to the medium compared to ECM-only at days 4 and 8. No statistical significant difference was observed between stem cells on ECM/GF spots and ECM spots + soluble GF. In all cases $p < 0.05$ was tested. (B) Analysis of endoderm/early liver marker α FP showed upregulation in gene expression over 12 days in culture. Human primary hepatocytes (hPHs) were used as controls. α FP gene expression was significantly higher on ECM/GF at days 6 and 12 (a on graph, $p < 0.05$); b, hESCs on ECM spots adding GFs to media compared to ECM-only at days 6 and 12. No statistical significant difference was observed between gene expression on ECM/GF spots and ECM + soluble GF ($p < 0.05$)

surface stained positive for albumin after 12 days in culture. In contrast, no albumin signal was observed in DE cells cultured on ECM alone (data not shown).

We also wanted to compare differentiation on GF-containing surfaces with the hepatic differentiation protocol involving soluble GFs that was developed by the Zern group previously (Duan *et al.*, 2010). DE stem cells were cultured on ECM spots in the culture medium supplemented with α FGF (20 ng/ml), HGF (20 ng/ml), BMP2

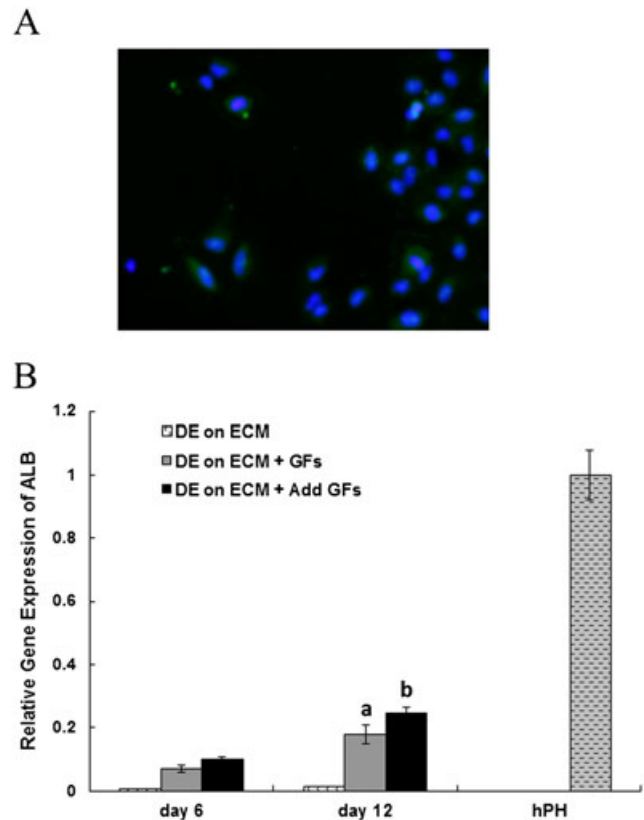


Figure 6. Immunostaining and RT-PCR analysis of the hepatic marker albumin. (A) Immunofluorescent staining for albumin in DE cells cultured on ECM/GF spots on day 12. (B) RT-PCR analysis shows upregulation of albumin gene expression in DE cells cultured on ECM/GF spots or in the presence of soluble GFs. DE cells express ~25% of the albumin gene expression in human primary hepatocytes (hPHs). Albumin expression on ECM/GF was significantly higher compared to ECM-only spots (a); b, hESCs on ECM spots adding GFs to media compared to ECM-only. No significant difference was observed between gene expression on ECM/GF and ECM + soluble GF

and BMP4 (10 ng/ml). Comparison of albumin and α FP gene expression in DE stem cells revealed no statistically significant difference between soluble and solid-phase presented GFs.

While the phenotype characterization presented here is somewhat limited, our results are quite promising and point to considerable hepatic differentiation on GF-containing surfaces. It should be noted that soluble GFs were added to the culture medium daily over the course of 12 days, whereas solid-phase presented GFs were printed once at the beginning of the experiment and were not supplemented during differentiation. We estimate that ca. 60 times less expensive GFs were required for stem cell differentiation on GF-containing surfaces.

4. Conclusions

Our study demonstrates that surface-bound GFs signal to hESCs cultured on top and drive hepatic lineage selection in these cells. While derivation of mature hepatocytes

from hESCs will require further protocol optimization, our approach and findings have significant implications for the field of stem cell research, where GFs are widely used for deriving somatic cell phenotypes. We believe that future clinical use of stem cells will depend in part on the ability to scale-up production of the desired cell types. Current stem cell differentiation protocols employ soluble GFs, require large amounts of these very expensive reagents and are therefore not scalable. Immobilizing GFs on surfaces allows the cost associated with stem cell

differentiation to be dramatically reduced and points towards a strategy for scale-up of stem cell cultures.

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