FULL PAPER

Bioactive Photodegradable Hydrogel for Cultivation and Retrieval of Embryonic Stem Cells

Jungmok You, Amranul Haque, Dong-Sik Shin, Kyung Jin Son, Christian Siltanen, and Alexander Revzin**

The development of a novel photodegradable heparin-based hydrogel for cultivation and retrieval of embryonic stem cells is described. Mouse embryonic stem cells cultured atop the gel with encapsulated growth factors (GFs) express higher levels of differentiation markers compared to a standard protocol employing soluble GFs. Beyond improving differentiation of stem cells, the novel hydrogels can be used to release specific stem cell colonies without disturbing neighboring cells. This way, stem cell colonies can be retrieved at different time points and from different locations of the culture surface for polymerase chain reaction (PCR) analysis without the loss of the microenvironment context. The ability to retrieve some stem cell colonies without disturbing neighboring colonies will open possibilities for characterizing in-dish heterogeneity of stem cell phenotype and will also allow to conserve cells/reagents. Overall, the bioactive photodegradable hydrogel developed in this study may offer new possibilities for cultivation and analysis of stem cells as well as other cell types.

1. Introduction

The ability to control and promote the differentiation of embryonic stem cell (ESC) toward a renewable source of cell-based therapies has great potential in the field of regenerative medicine.[1,2] ESCs may be differentiated into any adult cell type and therefore offer tremendous promise for tissue engineering and regenerative medicine.[3] Some of the challenges of working with ESCs include difficulty in driving efficient differentiation of pluripotent stem cells along the desired lineage and the cost associated with differentiation protocols. The cost factor comes from adding expensive recombinant growth factors in relatively high concentrations into media and then changing media daily. One way to decrease consumption and cost of cultivation is to immobilize growth factors (GFs) on surfaces. A number of studies have shown that immobilization or tethering to surfaces enhances GF signaling through a combination of improved stability against proteolysis and increased receptor-ligand interactions.[4–8] There have been some reports describing cultivation of embryonic stem cells on surfaces containing covalently attached or physisorbed GFs.[4,9–11] Our laboratory has been interested in leveraging natural affinity of ECM components to GF molecules in designing surfaces for cell cultivation.[12–16] In particular, we have been interested in heparin-based hydrogels, materials composed of diacrylated poly(ethylene glycol) (PEG) and thiolated heparin. A number of proteins, including many GFs, express heparin binding domains and interact via secondary forces with heparin.[17,18] Heparin-based hydrogels have been shown by our group and others to sequester and release, in a controlled manner, a range of GFs including human growth hormone (hGH), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF).[19–22]

Recently, Anseth and co-workers developed a photodegradable PEG hydrogel and demonstrated its use for designing 3D cellular microenvironment.[23,24] Our lab has previously described simplified synthesis of photodegradable hydrogels composed of PEG with photolabile orthonitrobenzyl moieties and demonstrated the use of such photodegradable gel substrates for capture and release of cells.[25,26] However, photodegradable hydrogels developed to date have either been inert or modified with short peptides promoting cell adhesion. The goal of this study was to develop novel hydrogels that are bioactive—capable of inducing cellular differentiation—and photodegradable.

This novel biomaterial, termed "heparin photogel," was employed to differentiate mouse (m) ESCs into definitive endoderm—a germ layer of origin for several organs including liver, pancreas, and gut. We demonstrate that such heparin photogels allow to improve stem cell differentiation, decrease the usage of expensive growth factors used for differentiation protocols, and enable on-cue release of specific stem cell colonies for analysis

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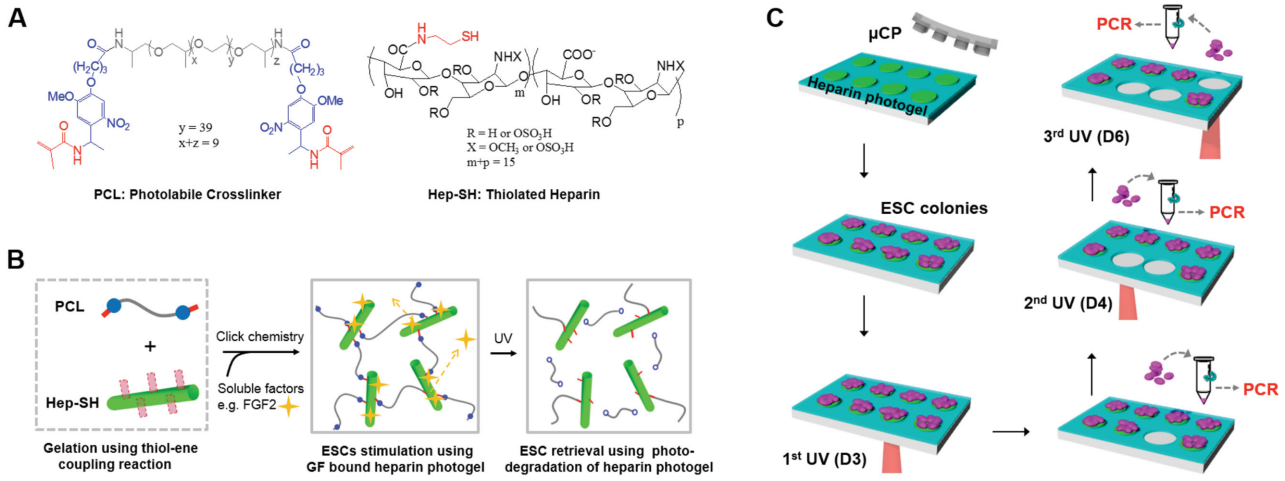

Figure 1. A) Components of photodegradable bioactive hydrogel terms heparin photogel: o-nitrobenzyl groups (photolabile crosslinker–PCL) and thiolated heparin (Hep-SH). B) Polymerization and degradation of heparin photogels. PCL and Hep-SH crosslinking is achieved by chemically initiated free radica thiol-ene coupling reaction. Heparin photogel is able to release growth factors in controlled manner and can be degraded by exposure to 365 nm UV light. C) Process flow for micropatterning colonies of embryonic stem cells on heparin photogel. Specific colonies may be retrieved from the surface at different days of culture for RT-PCR analysis assessing differentiation.

by reverse transcription polymerase chain reaction (RT-PCR). Such smart biofunctional hydrogels will be broadly applicable for cultivation of stem cells and other reagent-intensive and expensive cell types.

2. Results and Discussion

2.1. Fabricating and Characterizing Heparin Photogel

In this study, as shown in **Figure 1**A, heparin photogel consisted of thiolated heparin molecules and custom synthesized PEG molecules with bifunctional acryl terminated photolabile orthonitrobenzyl moieties.[25,26] Hydrogel was formed by chemically initiated thiol-ene reaction between thiolated heparin and diacrylated photolabile PEG linker (Figure 1B). While gelation occurred by the use of chemical initiator, gel degradation happened by illumination with 365 nm UV light. As shown in Figure 1B,C, we imagined loading inductive GFs into heparin photogel to affect stem cell differentiation and then utilizing degradability of the gel to retrieve stem cells at different time points during culture to assess differentiation.

First we wanted to demonstrate that heparin photogel is both photodegradable and bioactive. **Figure 2**A shows a micropattern of bicycle (symbol of Davis, CA) created by exposure of rodamine-containing photogel to UV through a photomask. This gel acts as a positive tone resist; regions exposed to UV become water soluble and appear dark in the image. Importantly, the gel contains heparin, as evidenced by staining with Toluidine blue O – a stain that interacts with negatively charged molecules such as heparin (Figure 2B). As another proof of bioactivity, heparin photogel retained and released fibroblast growth factor 2 (FGF2) slower than pure PEG gel of comparable molecular weight and polymer mesh. Less than 60% of FGF2 was released from heparin photogels within 3 d, whereas 90% of FGF2 was released from pure PEG photogels in 3 d. FGF2 along with Activin A are morphogens most commonly used to drive differentiation of mESCs toward endoderm.[27,28]

Figure 2. Characterizing photodegradability and bioactivity of heparin photogel. A) Rodamine-labeled heparin photogel exposed to UV light through a photomask. Regions affected by light become water soluble and appear black in the image. B) Staining of heparin photogel pattern with toluidine blue O—a dye that binds to negatively charge molecules such as heparin (Scale bar = 200 μm for (A) and (B)). C) Profile of bFGF release from heparin photogel. More than 40% of bFGF was retained in heparin photogels after 3 d while most of bFGF was released from PEG hydrogels in the same period of time.

We should note that heparin photogel did not appear to interact with Activin A as it was released at the same rate from PEG gel control and heparin photogel (Figure S1, Supporting Information). However, this may be explained by the fact that the active form of Activin A available to us for these experiments lacks heparin binding domains.[29]

2.2. Cultivating and Retrieving ESCs from Heparin Photogel

Stem cells do not readily attach to heparin hydrogel surfaces. To promote attachment of mESCs, these surfaces were imprinted with circular fibronectin (Fn) domains using microcontact printing.[12] As shown in a diagram of Figure S2, Supporting Information, printing of Fn spots was followed by seeding of mESCs and formation of stem cell colonies corresponding in size to individual spot diameter (500 μm). Forming stem cell colonies has biological significance–it is important to create high local concentration of cells for differentiation to proceed efficiently, and also enables retrieval of individual colonies at different time points for in-dish assessment of stem cell phenotype.

Figure 3A demonstrates formation of mESC colonies on heparin photogel containing 500 μm diameter islands for Fn. Importantly, individual colonies could be retrieved by UV-initiated degradation of the underlying gel. The area of the gel to be degraded is first determined by adjusting diaphragm of a microscope while exposing to light that does not cause cleavage. The region of the substrate to be degraded is subsequently illuminated with UV light through a 4′ 6-diamidino-2-phenylindole (DAPI) filter of a standard fluorescence microscope. Upon brief (3 min) exposure to 365 nm UV light gel began degrading and stem cells detached from the surface over the course of 1 h. It should be noted that such brief exposure to low intensity 365 nm light has been shown to be benign to a variety of cell types, including stem cells.[23,24,30] In our case, retrieved stem cells were >95% viable, as shown in Figure 3B, and, upon seeding on gelatin-coated surfaces, proliferated to confluence over 5 d in culture (Figure 3C). The proliferative behavior of retrieved stem cells was similar to mESCs that were never exposed to UV.

2.3. Differentiating ESCs into Endodermal Cells on Heparin Photogel Substrates

Upon demonstrating attachment and cultivation of mESCs, we wanted to apply novel heparin photogels for inducing differentiation of stem cells. We chose to focus on differentiation toward endoderm—a germ layer that gives rise to liver, lung, gut, and pancreas tissues. Endodermal differentiation protocols typically take 6 d to complete—this fact was used to set the timeframe of our experiments. The goal of first set of experiments was to compare endodermal differentiation of mESCs on heparin photogel versus regular heparin gel versus glass control surfaces. In all three scenarios surfaces were imprinted with 500 μm diameter Fn islands for cell attachment. As shown in **Figure 4**A, stem cells on gel surfaces remained in more compact colonies and, while growing upward, did not expand laterally. By contrast, stem cells were able to migrate more easily out of colonies formed on glass substrates. Figure 4B compares expression of pluripotency (Rex1, Nanog) and endoderm markers (Sox 17, Foxa2) in mESCs after 6 d of differentiation in the same culture media on three substrates—heparin photogel, regular heparin gel, and glass control. This comparison revealed that mESCs on gel substrates in the presence of soluble Activin A and FGF2 experienced more pronounced loss of pluripotency markers and greater gain of endoderm markers than on glass controls. For example, Sox17 expression was 2.5 and 4 times higher on heparin gel and heparin photogel substrates, respectively, compared to glass control. The second conclusion from Figure 4B is that comparable loss of pluripotency and gain of

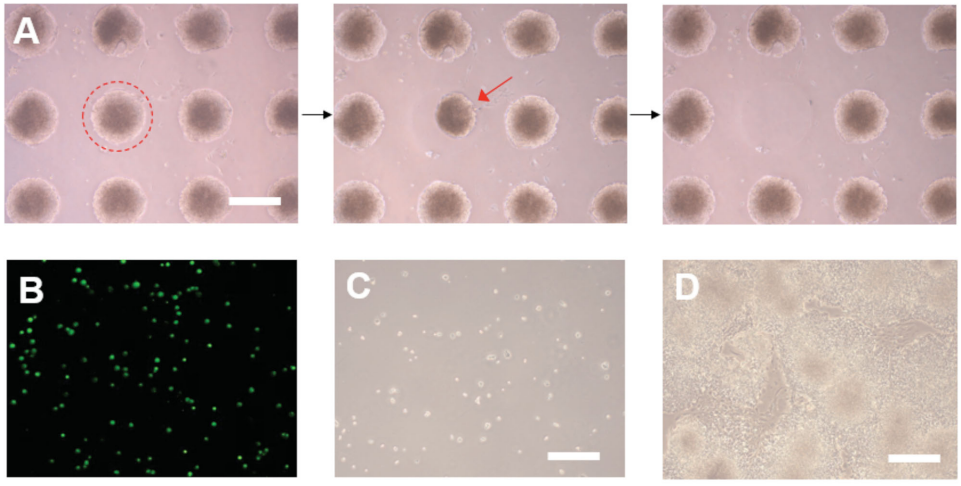

Figure 3. Selective retrieval and recultivation of mESCs. A) Images demonstrating a sequence of steps leading to release of one stem cell colony. Left image – focusing on the colony to be released, middle image – gel around the colony is affected by brief exposure to UV light through microscope objective, right image – the site from which colony was retrieved. Note that neighboring colonies (≈300 μm apart) remain undisturbed. B) Viability of mESCs retrieved from heparin photogel immediately after UV exposure. C,D) Cells proliferating 1 d (C) and 6 d after retrieval (D) (Scale bar = 500 μm).

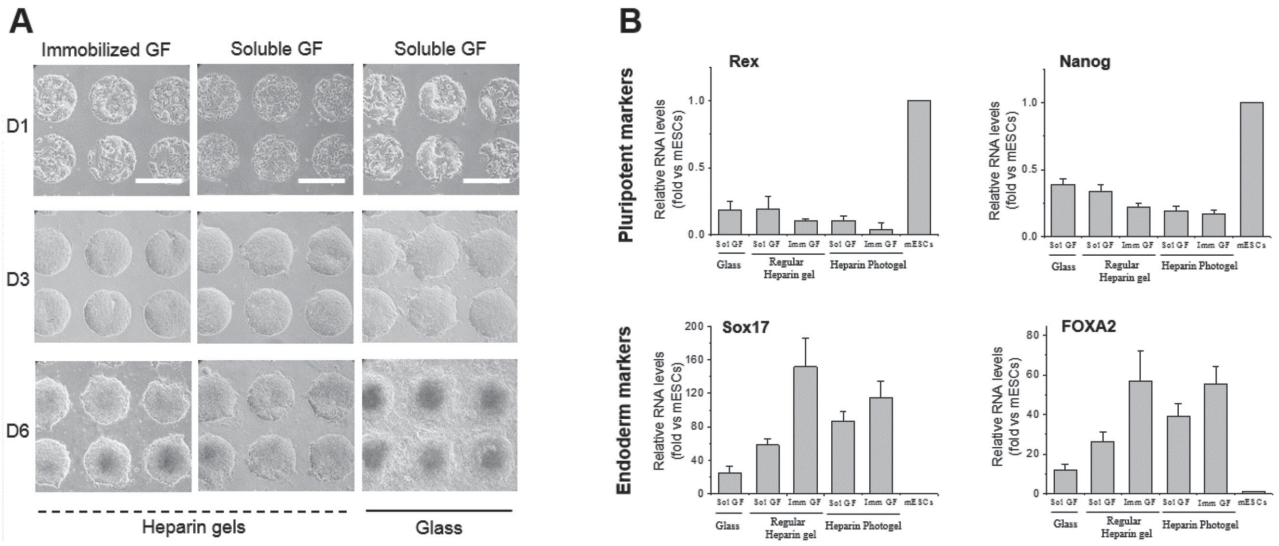

Figure 4. A) The differentiation of embryonic stem cells into endodermon on the heparin gel layers and glass substrate with GFs. B) RT-PCR analysis of pluripotency (Nanog, Rex1) and endoderm gene expression (Sox17, Foxa2). mESCs were cultured on regular heparin gel, heparin photogel, and glass substrates—all imprinted with the same pattern of 500 μm diameter Fn spot. Differentiation media was the same for all three surface types. For gel surfaces, GFs (Activin A and bFGF) were either encapsulated in the gel or added in soluble form. Undifferentiated mESCs were cultured on glass and used as controls. The data are normalized to mESC gene expression.

endoderm markers was observed for stem cells on regular heparin gel and heparin photogel, suggesting that incorporation of photolabile orthonitrobenzyl moieties into the gel did not affect its bioactivity. The third conclusion was that immobilization of endoderm-inducing GFs into the gel was as effective as adding GFs in solution in terms of driving differentiation of mESCs. Keeping in mind that amount of GFs incorporated into the gel was equivalent to the dose of GFs added into media and that in the latter case the media was changed daily for 6 d; immobilizing GFs in the heparin gel (regular or photo) decreased the cost of culture by a factor of six.

2.4. In-Dish Analysis by Retrieval of Stem Cell Colonies

The reverse transcription polymerase chain reaction (RT-PCR) analysis described in Figure 4B was carried out in a typical manner whereby a well of a multiwell plate was sacrificed to get one data point. Multiple wells containing hundreds of thousands of cells were sacrificed to characterize markers of stem cell differentiation over the course of a multiday experiment. Given the cost of stem cells and reagents, this traditional approach is wasteful. Plus, stem cells are destroyed in the process of PCR analysis; therefore, it is not possible to monitor differentiation in the same dish over time. Bioactive photodegradable hydrogel developed here allows to selectively retrieve desired stem cell colonies without perturbing neighboring stem cells. In a proof of concept experiment, described as a diagram in Figure 1C, mESCs were seeded on heparin photogel substrates forming colonies on 500 μm Fn islands. mESCs resided on heparin photogel and were exposed to differentiation media. To analyze extent of differentiation, stem cells were then collected from the surface/dish by illumination to UV through a microscope objective (using DAPI filter) at days 3, 5, and 6 of cultures for RT-PCR analysis.

Figure 5A shows a sequence of microscope images taken from the same stem cell culture surface after retrieval of stem cell colonies at different days of culture. As seen from this set of images, we can collect proximal or distant colonies from the same culture dish without perturbing neighboring cells. RT-PCR analysis of stem cells retrieved from heparin photogel (Figure 5B) could be used to monitor differentiation progression – loss of pluripotency markers Rex1 and Nanog, and gain of endoderm markers, Sox17 and Foxa2 – over time in culture. Importantly, the five fold increase in Sox 17 endoderm gene expression (vs glass control), obtained from analysis of stem cell colonies (Figure 5B), correlates with PCR analysis of large numbers of cells reported in Figure 4B.

3. Conclusion

To summarize, we report on the development of a novel photodegradable and bioactive heparin-based hydrogel. This heparin photogel was shown to enable stem cell cultivation and differentiation. Cultivation on hydrogels incorporating Activin A and FGF2 enhanced endodermal gene expression of ESCs five to six fold compared to glass controls. At the same time immobilization of GFs into the heparin photogel obviated the need for daily addition of these reagents into culture media and allowed to decrease the cost of endodermal differentiation by a factor of six. In other words, compared to standard differentiation protocol, our novel hydrogel allowed to decrease the usage of expensive recombinant proteins employed in a differentiation protocol while producing a better biological effect. In addition, we demonstrated that stem cell colonies may be retrieved from the same culture dish at different time points to enable in-dish analysis of stem cell differentiation. Typically RT-PCR analysis requires sacrificing the whole dish with hundreds of thousands

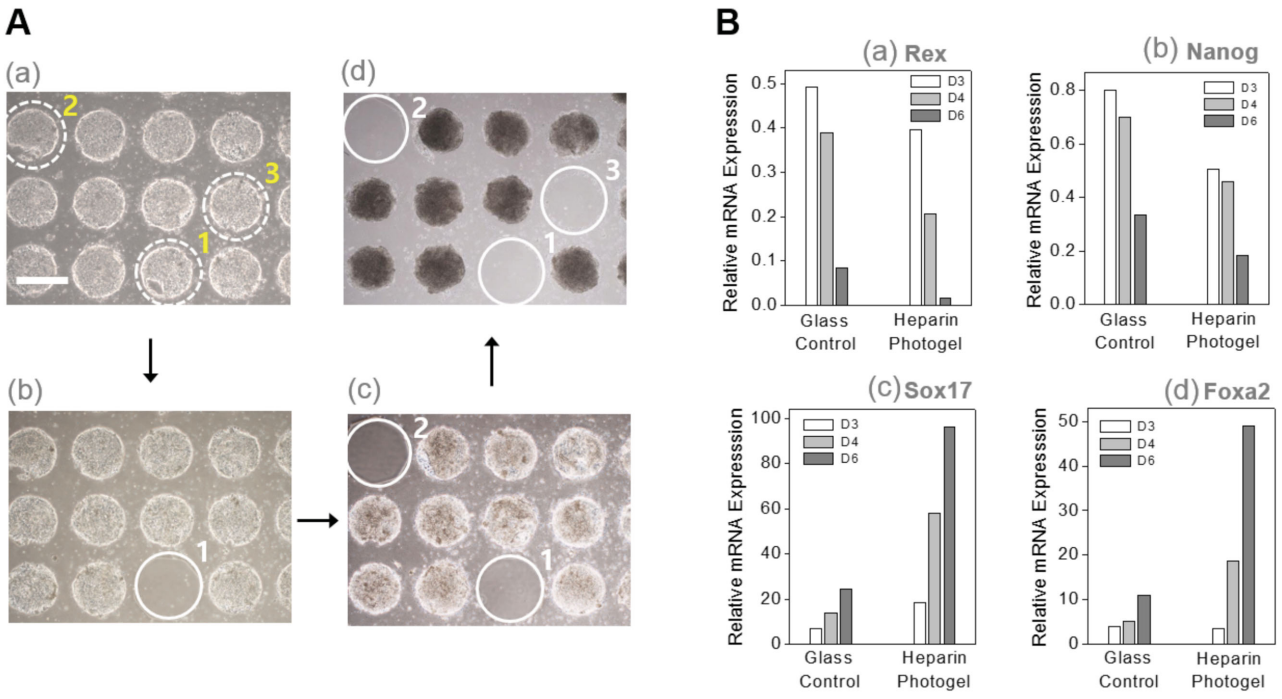

Figure 5. Retrieval of stem cell colonies for RT-PCR analysis. A) Optical microscopy images of mESC colonies retrieved from heparin photogel at day 3 (a and b), day 4 (c), and day 6 (d). Colonies were used to UV light for 1 min through DAPI objective of fluorescence microscope (Scale bar = 500 μm). B) RT-PCR analysis of pluripotency (Nanog, Rex1) and endodermal genes (Sox17, Foxa2) from stem cell colonies retrieved from heparin photogels.

of cells. The ability to retrieve some stem cell colonies without disturbing neighboring colonies will open possibilities for characterization of in-dish heterogeneity of stem cell phenotype and will also allow to conserve cells/reagents. Retrieval of cells demonstrated here is somewhat analogous to laser microdissection or catapulting in that it allows one to collect specific groups of cells from the culture surface.[31–34] However, methodology described here shifts the focus from expensive instrumentation (lasers for catapulting or microdissecting) toward inexpensive surface coatings that can be degraded by light from a microscope objective and can be used with live cells immersed in culture media. Overall, bioactive photodegradable hydrogel developed in this study offers new possibilities for cultivation and analysis of stem cells as well as other cell types.

4. Experimental Section

Chemicals and Materials: Glass slides (75 mm × 25 mm) were purchased from Fisher Scientific (Pittsburg, PA). (3-acryloxypropyl)-trichlorosilane were purchased from Gelest (Morrisville, PA). Heparin (sodium salt, from porcine intestinal mucosa) was purchased from Tocris Bioscience (Bristol, UK) and Sigma-Aldrich (St. Louis, MO, USA). Thiolated heparin (Hep-SH) was synthesized with the modification of carboxylic groups of heparin using carbodiimide chemistry, as previously reported.[35,36] Photolabile crosslinker (PCL) was synthesized and characterized by the method as described previously.[25] Methacryloxyethyl thiocarbamoyl rhodamine B (Acryl-Rho) was purchased from Polysciences (Warrington, PA). *N,N*-diisopropylethylamine (DIPEA), *N,N*-dimethylforamide (DMF), and piperidine were purchased from Aldrich Chemicals (Milwaukee, WI). LIVE/DEAD Viability/Cytotoxicity Kit was purchased from Invitrogen (Carlsbad, CA). Toluidine blue O was obtained from Sigma (St. Louis, MO). Phosphatebuffered saline (PBS) was purchased from Gibco (Grand Island, NY, USA). Dulbecco's modified Eagles' medium (DMEM), nonessential amino acids, L-glutamine, ES-qualified fetal bovine serum (FBS), and 2-mercaptoethanol 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, and primary mouse embryonic fibroblasts (MEF) were obtained from Millipore (Temecula, CA). UV illumination (365 nm, 18 W cm^{-2}) was carried out by a fiber optic light source (OmniCure Series 1000, Lumen Dynamics Group, Mississauga, Ontario, Canada). Fluorescence images were obtained with a confocal microscope (Zeiss LSM 5 Pascal, Carl Zeiss, Göttingen, Germany). B27 was obtained from Gibco and recombinant human activin A and recombinant human basic fibroblast growth factor (bFGF) were purchased from Peprotech.

Cultivation of ESCs: The mouse ESC line D3 was purchased from ATCC (Manassas, VA, USA) and was expanded according to the provider's instructions. Briefly, ESCs were propagated on irradiated mouse embryonic fibroblast (CF-1 MEF, GlobalStem) feeder layers in self-renewal medium consisting of DMEM supplemented with 15% ES-qualified FBS, 2×10^{-3} M L-glutamine, 1×10^{-3} M nonessential amino acids, 100×10^{-9} M 2-mercaptoethanol, 200 U mL^{-1} penicillin, 200 μg mL^{-1} streptomycin, and 1000 U mL^{-1} LIF at 37° C, 5% CO_2, and 90%–95% humidity, with a medium change every day. Undifferentiated ESCs were passaged every 2 d onto fresh feeders using trypsin-EDTA (ethylenediaminetetraacetic acid).

Heparin Photogel Layer Fabrication: Glass surfaces were first functionalized with an acrylated silane to promote adhesion of photogel. Briefly, glass slides were exposed to O_2 plasma for 5 min at 300 W, placed into a nitrogen filled glovebag, and immersed in 0.1% (v/v) 3-acryloxypropyltrichlorosilane in anhydrous toluene for 1 h at room temperature. The silane-modified glass slides were stored in a desiccator before use. 200×10^{-3} M tretramethylethylenediamine (TEMED) in

distilled (DI) water (1.5 μL) was added to a mixture of 20% (v/v) PCL in DI water (5 L), 30% (v/v) thiolated heparin in PBS (1 μL), 20% (v/v) poly (ethylene glycol) methyl ether acrylate (PEG-A, M_n = 480) in DI water (1 μL) and 200 × 10^{-3} M ammonium persulfate in DI water (AP) (1.5 L) and mixed carefully to prevent the generation of bubbles inside the solution. The solution (5 L) was dropped onto the acrylated glass slides (cut to 1.25 cm × 1.25 cm sized pieces) and cover slips were placed over the solution. Cover slips were removed after 1 h and the photogel coated surfaces were washed with DI water and dried with nitrogen. For rhodamine incorporated heparin photogel, 20 × 10^{-3} M Acryl-Rho in DI water (0.2 L) was added to the mixture. For GFs incorporated heparin photogel, Activin A, and basic fibroblast growth factor (bFGF) solutions were added to heparin photogel precursor solution before gelation, resulting in the final concentration of 10 μg mL^{-1}, respectively.

Photodegradation of Heparin Photogel: The heparin photogel layer was selectively degraded by 365 nm UV light exposure through photomask. The photocleavage reaction was completed after 10 s exposure to UV (600 W) and the diffusion of the gel was observed after 1 h. A rhodamine incorporated photogel surface was exposed to UV light and fluorescence images were obtained from the fluorescence microscope after 1 h for diffusion of the photogel.

In Vitro GFs Release from Heparin Photogel: GFs release profiles from heparin-based and PEG photogels were compared. In these experiments, 10 μg mL^{-1} final concentrations of activin A and bFGF were mixed with gel precursor solutions. After gelation, stem cell culture media (800 mL) was added as a release buffer and samples were kept at 37 °C in humidified 5% CO_2 incubator to provide the same condition to cell culture. The release medium was replaced with fresh one every day and collected samples were immediately frozen at –20 °C prior to analysis. The Activin A and bFGF released at different time points were analyzed with human Activin A and bFGF ELISA kits from R&D systems. Absorbance was measured using a microplate reader with a 480 nm filter.

Preparation of Fibronectin Micropatterned Heparin Photogel Substrate for ESCs: To promote ESC adhesion, we utilized microcontact printing (μCP) technique which relies on polydimethylsiloxane (PDMS) templates that were fabricated using traditional soft lithography approaches.[37] For fibronectin inking protocol, 0.2 mg mL^{-1} fibronectin solution was spread out onto a glass slide to create a thin liquid layer on the surface. When the array of PDMS pillars of posts 500 μm in diameter and 20 μm in height was brought in contact with the protein layer, only the tips of the pillars became functionalized with protein. Subsequently, when the PDMS template was pressed against heparin photogel layer, fibronectin was transferred into the regions of heparin photogel layer. As a control, fibronectin was also transferred into the glass substrates by μCP.

Differentiation of ESCs into Definitive Endoderm (DE): For cell seeding experiments, the glass slides with imprinted protein spots were diced into 1.2 cm × 1.2 cm pieces, placed into wells of a twelve-well tissue culture plate. The culture substrate was sterilized with absolute ethanol and washed twice with PBS. Following enzymatic dissociation of mESCs using trypsin-EDTA, the dissociated single cells were seeded onto 0.1% gelatin-coated microwells of six-well plate for 18 h in order to expand undifferentiated mESCs and reduced the fraction of growth-arrested MEF cells prior to differentiation. The cell seeding on imprinted protein spots was carried out by incubating dissociated ESCs on fibronection-micropatterned heparin photogels in ESC self-renewal medium without LIF. After 1 h of incubation at 37 ° C, unbound cells were removed by washing with warm PBS, leaving behind clusters ESCs adhering on 500 μm diameter protein spots inside each microwell of twelve-well plates. ESC arrays maintained in differentiation medium consisting of Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 1% FBS, 200 U mL^{-1} penicillin, 200 μg mL^{-1} streptomycin, 1 × 10^{-3} M nonessential amino acids, 0.5 U mL^{-1} insulin, 14 ng mL^{-1} glucagon, and 100 × 10^{-9} M dexamethasone for 4 d and then 1% FBS was replaced with 1% B27 after 4 d. In a parallel experiment mESCs were induced into DE on fibronectin-micropatterned glass substrates in the culture medium supplemented with Activin A (50 ng mL^{-1}) and bFGF (50 ng mL^{-1}) in order to compare differentiation on GF-containing surfaces with the differentiation protocol involving soluble GFs. The differentiation medium was changed every second day. Cells on heparin photogels were imaged using a bright-field microscope (Carl Zeiss Inc., Thornwood, NJ).

Table 1. List of primers for mouse ESCs used in qPCR.

Nanog-F	GAATTCTGGGAACGCCTCATC
Nanog-R	CCTTGTCAGCCTCAGGACTTG
Rex1-F	GCTCCTGCACACAGAAGAAA
Rex1-R	GTCTTAGCTGCTTCCTTCTTGA
Sox17-F	GGACACGACTGCGGAGTGAA
Sox17-R	GGTCGGCAACCGTCAAATG
FoxA2-F	GTCGTCCGAGCAGCAACATC
FoxA2-R	GGGTAGTGCATGACCTGTTCG
GAPDH-F	CCCCAATGTGTCCGTCGTG
GAPDH-R	GCCTGCTTCACCACCTTCT

Cell Viability Test: The mESC suspension was collected into a tube, centrifuged, and decanted. The cells were placed into a mixture of 4 × 10^{-3} M calcein AM and 2 × 10^{-3} M ethidium homodimer in PBS for 30 min. The stained cells were washed with PBS and fluorescence images were obtained by a fluorescent microscope.

Stem Cells Retrieval and Real-Time PCR: mESCs on heparin photogel were selectively exposed to 365 nm light projected from an epifluorescence microscope. The size of the exposed region could be controlled by changing the aperture equipped in the microscope (50–800 μm). After 1 h, mESCs were completely released out from heparin photogel layer due to photodegradation on UV exposed region. Total RNA was extracted from the cell lysates using Total mRNA isolation kit (Roche). cDNA was synthesized using ≈0.2 μg of total RNA in 20 μL reaction mixture using Quantitest Reverse Transcription Kit (Roche). The RNA extraction and cDNA synthesis was performed according to the manufacturer's instructions. Real-time PCR was performed with universal SYBR Green Master (Roche) and the relative expression level of each gene was calculated using the comparative threshold cycle (C_t) method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. All PCR reactions were done in duplicate. Primers used are listed in **Table 1**.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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