Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biomaterials 26 (2005) 6762–6770
Biomaterials
www.elsevier.com/locate/biomaterials

Engineered spatial patterns of FGF-2 immobilized on fibrin direct cell organization ☆

Phil G. Campbell[a,*], Eric D. Miller[b], Gregory W. Fisher[c], Lynn M. Walker[d], Lee E. Weiss[e]

[a]Institute for Complex Engineered Systems, Carnegie Mellon University, 1213 Hamburg Hall, 5000 Forbes Avenue, Pittsburgh, PA 15213, USA
[b]Department of Biomedical Engineering, Carnegie Mellon University, 5000 Forbes Avenue, Pittsburgh, PA 15213, USA
[c]Molecular Biosensor and Imaging Center, Carnegie Mellon University, 5000 Forbes Avenue, Pittsburgh, PA 15213, USA
[d]Department of Chemical Engineering, Carnegie Mellon University, 5000 Forbes Avenue, Pittsburgh, PA 15213, USA
[e]The Robotics Institute, Carnegie Mellon University, 5000 Forbes Avenue, Pittsburgh, PA 15213, USA

Received 21 January 2005; accepted 12 April 2005
Available online 6 June 2005

Abstract

The purpose of this study was to initiate the exploration of cell behavioral responses to inkjet printed spatial patterns of hormones biologically immobilized on biomimetic substrates. This approach was investigated using the example of preosteoblastic cell response in vitro to fibroblast growth factor-2 (FGF-2) printed on fibrin films. Concentration modulated patterns of FGF-2, including continuous concentration gradients, were created by overprinting dilute FGF-2 bioinks with a custom inkjet printer. The immobilized FGF-2 was biologically active and the printed patterns persisted up to 10 days under cell culture conditions. Cell numbers increased in register to printed patterns from an initial random uniform cell distribution across the patterned and non-patterned fibrin substrate. Patterned immobilized FGF-2, not cell attachment directed cell organization because the fibrin substrate was homogeneous. The capability to engineer arbitrary and persistent hormone patterns is relevant to basic studies across various fields including developmental biology and tissue regeneration. Furthermore, since this hormone inkjet printing methodology is extensible to create complex three-dimensional structures, this methodology has potential to create therapies for tissue engineering using spatial patterned delivery of exogenous hormones.

Keywords: Tissue engineering; Inkjet technology; Solid freeform fabrication; Growth factor; Printing technology; Biological patterning

1. Introduction

The work reported here demonstrates that printed two-dimensional (2D) hormone spatial patterns, including continuous concentration gradients, immobilized to extracellular matrix (ECM) substrates, persist under cell culture conditions, and that cells organize in register with these patterns in vitro. This work is motivated by hormonal control in biological systems where spatial patterning of hormones, including morphogens and growth factors, directs cell organizational behavior by providing positional information required for embryonic morphogenesis [1,2] and post-embryonic tissue repair or regeneration [3]. Positional information can occur via the diffusion of soluble hormones down concentration gradients directing cell behavior based on differential cell perception of hormone concentrations within the gradient, or via the formation of more complex, discrete periodic shapes [4,5].

Experimental approaches to quantify cellular response to individual gradients of soluble (liquid-phase)

☆ Our paper introduces an enabling technology to begin a systematic experimental approach to understand and eventually control biological patterning. Beyond understanding the mechanisms behind biological patterning is the application of such knowledge to developing new medical technologies, especially related to tissue engineering or regeneration.

*Corresponding author. Tel.: +1 412 268 4126; fax: +1 412 268 5229.
E-mail address: pcampbel@cs.cmu.edu (P.G. Campbell).

0142-9612/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2005.04.032

hormones range from in vitro cell migration [6,7] to complex in vivo cell differentiation responses [8–10]. Theoretical models postulate how more complex stationary periodic hormone patterns might form based on reaction–diffusion kinetics [4,5,11], but these models remain to be experimentally verified. One of the challenges of using liquid-phase hormones to study pattern responses, or for use in therapeutic delivery systems, is to maintain pattern persistence until targeted cells can access the hormones. Our methodology addresses this issue by using hormones immobilized (solid-phase) to a substrate or matrix.

Solid-phase hormones are biologically relevant, and endogenous solid-phase extracellular hormone gradients have been reported [9]. Solid-phase hormones occur because many hormones exhibit inherent binding properties to ECM molecules directly or through specific binding protein intermediaries [12]. Hormonal sequestration in the ECM can mediate spatial control by locking-in hormones at specific locations within the ECM to create persistent patterns. Proteoglycans represent a class of cell surface and ECM molecules that can sequester hormones within the extracellular environment [13]. For example, heparin-binding domains contained within a hormone's structure can control formation of extracellular hormone patterns [14,15], which are essential for spatial patterning during vascular morphogenesis [14,16]. However, the exact role of ECM sequestration of hormones in spatial patterning remains unclear.

The need for simple, controllable methods to study cellular responses to solid-phase hormone patterns is thus borne from examples in biology and ultimately for developing advanced therapies that might use patterned delivery of exogenous hormones for applications such as tissue engineering. Related patterning technologies include microcontact printing, photolithography, and inkjet printing. Microcontact printing, while not used for hormone patterning, has been used to create 2D patterns of fixed concentrations of ECM cell attachment molecules to control cell attachment and function [17–21], whereby cells can only attach to the printed ECM molecules. In contrast, there has been some work reported on cell responses to photolithographic patterning of solid-phase hormones. For example, epidermal growth factor (EGF) patterned on polystyrene with photolithography has been demonstrated to direct the growth of engineered cells overexpressing EGF receptors in response to discrete printed lines of EGF [22] and to a discontinuous density gradient of EGF [23]. The gradient pattern consisted of lines of fixed concentration of EGF with decreasing spacing between lines. Inkjet printing has also been used to create solid-phase patterns of insulin, insulin-like growth factor-I (IGF-I), and fibroblast growth factor-2 (FGF-2) on polystyrene or silicone films [24]. However, immobilization of these printed hormones required photoreactive chemical modification of the hormones to immobilize the printed hormones onto the non-biological substrates.

In the present study, we used a custom-built inkjet deposition system to synthesize 2D patterns of FGF-2 *naturally immobilized* onto uniform fibrin substrates using native binding affinities. This approach thus creates solid-phase patterns without prior chemical modification of hormones. Also, fibrin, not the hormones, controls cell attachment. Cell attachment is uniform across the substrate because, upon cell seeding, cells exhibit equal propensity to bind on or off the FGF-2 patterns. Therefore, the underlying uniform fibrin substrate removes the ECM as a variable affecting cell behavior. This approach is different than 2D patterning of cell attachment molecules [17–21] because in our approach cell patterns develop from initial homogenous cell dispersions *independent of cell attachment*. We show that FGF-2 patterns persist and direct the behavior of MG-63 human preosteoblastic cells in direct register with these solid-phase patterns in vitro.

2. Materials and methods

2.1. Inkjet patterning

FGF-2 was deposited with a custom inkjet system that we developed for precision deposition applications. This system permits complete control over the jetting parameters, including the shape of the inkjet drive waveform firing frequency, and printed pattern parameters such as drop overlap. This versatility is useful for optimizing jetting parameters for different biological inks (bioinks). The system also incorporates computer vision feedback to enable targeted drop deposition relative to predefined locations on the substrate when necessary.

Printing substrates are mounted on a set of direct-drive X-Y servo stages (Model ALS130, Aerotech, Inc., Pittsburgh, PA) that move the substrates relative to a fixed inkjet printhead located 100 μm above the substrate. The inkjet is a MicroJet^{TM} piezoelectric drop-on-demand device with a 30 μm diameter nozzle (MicroFab Technologies, Inc., Plano, TX), which produces ~25 μm diameter drops (18 pl) yielding 75 μm diameter deposited spots. The system operator fine-tunes the jetting parameters by observing jetted drops imaged with a CCD camera illuminated with a strobe synced to the jet triggering pulses. A Unidex 500 motion controller (Aerotech, Inc., Pittsburgh, PA) coordinates deposition with motion control, on-the-fly, by providing a triggering pulse to the inkjet at specified increments along the motion trajectories.

The system modulates deposited FGF-2 concentrations by overprinting individual substrate locations with a dilute bioink of FGF-2 in an aqueous solvent. For example, Fig. 1A shows the overprinting strategy to make a continuous concentration gradient. Gradient patterns, such as shown in Fig. 1B, were printed on fibrin films using a bioink consisting of 4 μg/ml biotinylated FGF-2 (biFGF2) (ProChon Biotech, Ltd.) in

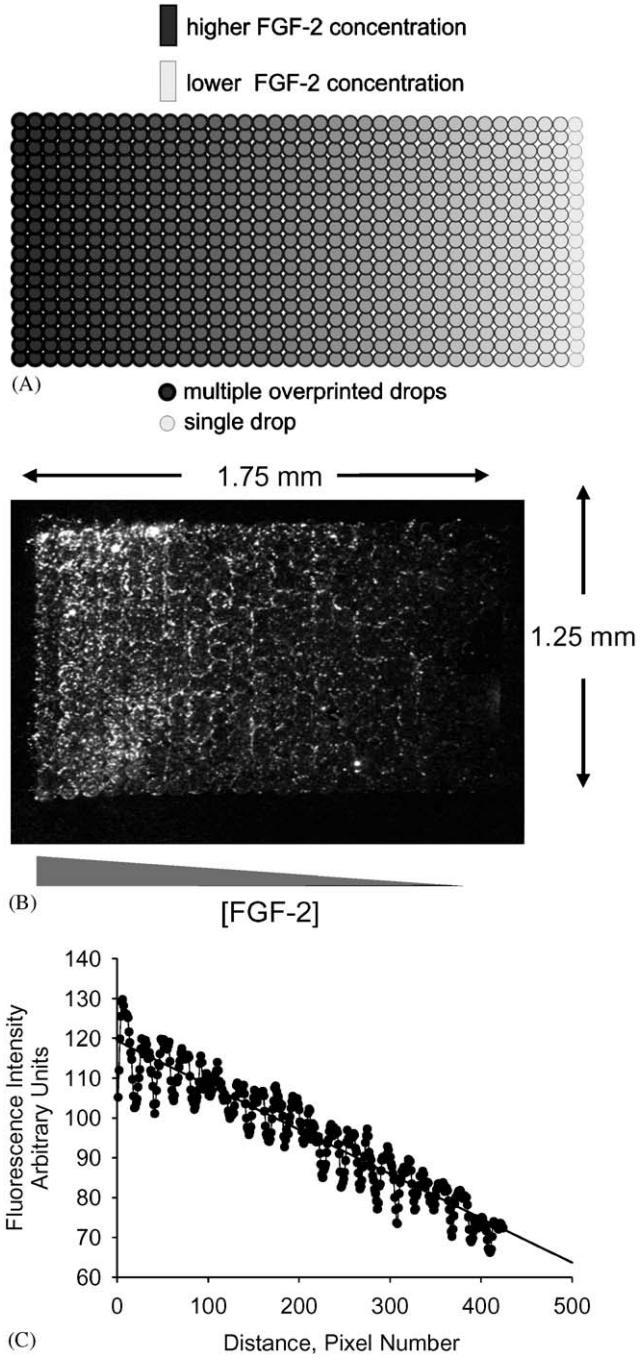

Fig. 1. Concentration gradient of FGF-2 created using inkjet printing. (A) Overprinting strategy for printing concentration gradients. (B) Example of an FGF-2 gradient printed with the overprinting strategy. The gradient was printed using biotinylated FGF-2 and then labeled with streptavidin conjugate quantum dots. (C) FGF-2 concentration gradient depicted by fluorescent intensity scan of printed gradient in Fig. 1B.

10 mM sodium phosphate buffer, pH 7.4. Patterns were 1.25 mm wide, 1.75 mm long with a center-to-center drop distance of 75 μm. After printing, the gradient patterns were sterilized in 70% ethanol for 10 min, rinsed three times in phosphate-buffered saline (PBS), pH 7.4, and incubated overnight in serum-free minimal essential medium (Invitrogen Corp., Carlsbad, CA) supplemented with Ham's F-12 (Invitrogen Corp., Carlsbad, CA) (MEM-F12) to remove unbound FGF-2 prior to seeding with cells.

Discrete patterned arrays of uniform FGF-2 concentration were also printed on fibrin films. Each FGF-2 pattern array consisted of four 0.750 mm squares that were spaced 1.75 mm apart and had a center-to-center drop distance of 75 μm. Patterns shown in Fig. 2 were overprinted 20 times with a bioink consisting of 50 μg/ml FGF-2 (Chiron, Emeryville, CA) and 50 μg/ml cyanine3 (Amersham, Piscataway, NJ) labeled FGF-2 (Cy3 FGF-2) in 10 mM sodium phosphate, pH 7.4. For all of the printed patterns that were going to be seeded with cells and the cell number measured, the locations of the patterns were indicated by scratching fiducial marks into the glass (Figs. 3 and 5) rather than using fluorescently labeled FGF-2. Patterns shown in Fig. 3 were overprinted 2, 12, 22, and 32 times, respectively, with a bioink consisting of 4 μg/ml FGF-2 in 10 mM sodium phosphate buffer, pH 7.4. As with the concentration gradients, after printing, the patterns were sterilized in 70% ethanol for 10 min, rinsed three times in PBS, pH 7.4, and incubated overnight in serum-free medium to remove unbound FGF-2. Preliminary cell growth studies compared ethanol pretreated patterns to non-treated patterns and found that ethanol did not impact the biological activity of solid-phase FGF-2.

Fluorescence images of the printed FGF-2 patterns were obtained using a Zeiss IM35 Axiovert microscope equipped with a 5X, 0.15 N.A. objective and a Photometrics C-250 cooled CCD camera. Fluorescent filters were from Chroma Technology Corp. (Rockingham, VT). The biFGF-2 patterns were labeled with 655 nm streptavidin conjugate quantum dots (sAvQdot) (Quantum Dot Corporation, Hayward, CA) according to the manufacturer's protocol followed by epifluorescence imaging using a 470/30 nm excitation and 665/45 nm emission filter set. Printed pattern arrays of Cy3 FGF-2 were visualized with epifluorescence using a Cy3 filter set.

2.2. Preparation of fibrin-coated slide printing substrates

Homogenous fibrin films were prepared essentially as described by Lorthois et al. [25]. Briefly, glass slides (Corning Glassworks, Corning, NY) were cut into 8 mm squares. Squares were cleaned in a sulfuric acid bath with NOCHROMIX for 2 h, rinsed 10 times with deionized water, and dried under nitrogen gas. Cleaned squares were then incubated in a 95% acetone solution containing 1% 3-aminopropyltriethoxysilane (Gelest, Inc., Morrisville, PA) for 10 min at 23 °C to functionalize the glass surfaces with amine groups. Subsequently, squares were rinsed 3 times respectively in acetone, ethanol, and deionized water to hydrolyze the silane and quench the surface reaction.

Silanized squares were dried at 120 °C for 45 min and incubated in a 3% glutaraldehyde solution (Electron Microscopy Sciences, Hatfield, PA) in PBS, pH 7.4, for 2 h at 37 °C to react with the amines on the glass surfaces and expose reactive aldehyde groups. Squares were then rinsed twice with methanol and deionized water, respectively.

Glutaraldehyde prepared squares were coated with fibrinogen by incubation in 0.1 mg/ml fibrinogen (Aventis Behring, King of Prussia, PA) contained in 10 mM sodium phosphate

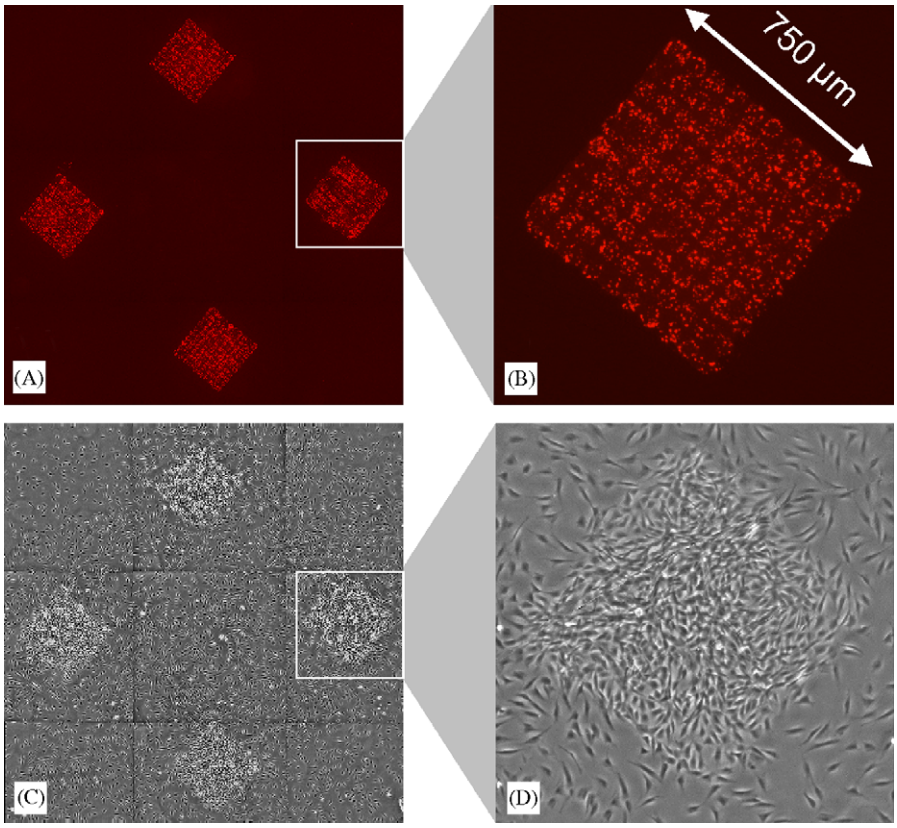

Fig. 2. Periodic 2D FGF-2 patterns and registered cell organizational response to FGF-2 patterns. (A) Tiled fluorescence image of FGF-2 square pattern array printed on fibrin films using a bioink consisting of native and Cy3-labeled FGF-2. (B) Higher magnification of single square pattern of FGF-2. (C) Tiled phase contrast image of cell organizational response in direct register to pattern array after 5 days in culture. (D) Higher magnification of cell organizational response.

buffer, pH 7.4 for 18 h at 4 °C. Excess unbound fibrinogen was removed by aspiration and the remaining active sites blocked with 0.3 M glycine (Bio-Rad Laboratories, Hercules, CA), pH 7.4 for 2 h at 4 °C followed by three rinses with PBS. Immobilized fibrinogen was converted into fibrin by incubating squares in 4 U/ml thrombin (Aventis Behring, King of Prussia, PA) contained in 10 mM sodium phosphate buffer with 1 mM calcium chloride at 37 °C for 2 h, then rinsed 3 times with PBS and stored in PBS at 4 °C for up to 2 weeks. Prior to patterning, squares were rinsed 3 times with sterile deionized water and air-dried in a laminar flow hood. The presence and uniformity of the fibrin coating was verified using transmission and scanning electron microscopy. The thickness of the fibrin films was estimated to be approximately 20 nm.

2.3. Cell culture

Human MG-63 osteosarcoma cells (American Type Culture Collection, Manassas, VA) were cultured at standard conditions (37 °C, 5% CO_2) in MEM-F12 supplemented with 10% fetal calf serum (Invitrogen Corp., Carlsbad, CA) and 1% penicillin/streptomycin (Invitrogen Corp., Carlsbad, CA). For all experiments, the cells used were between passages 100 and 120. To seed a patterned slide with cells, the cells were detached from the culture flasks with trypsin–EDTA (Invitrogen Corp., Carlsbad, CA) and resuspended in MEM-F12 with 10% serum, centrifuged at 50*g*, and washed three times with serum-free media. The cells were resuspended to ~20,000 cells/ml in serum-free medium with 1 μg/ml aprotinin (Bayer Corp., Pittsburgh, PA) and 2 ml were added to each patterned slide placed in a 12-well plate. After allowing the cells to plate for 3 h, the patterned square was removed from the 12-well plate and fixed to the bottom of a 60 mm Petri dish with sterile vacuum grease. The dish was filled with 8 ml of serum-free medium with 1 μg/ml aprotinin. The culture dishes were then placed in a standard incubator and removed every 24 h for imaging.

2.4. Image acquisition and processing

Images of the cells on the gradient and uniform patterns were acquired with a Zeiss IM35 Axiovert microscope using a 5 ×, 0.15 N.A. phase 1 objective and phase optics (Carl Zeiss, Inc., Thornwood, NY) with BDS-Image software (Biological Detection Systems, Pittsburgh, PA). The approximate locations of the patterns were indicated using fiducial marks which were scored onto the glass slides immediately after printing. The exact locations of the patterns were determined by their cell response. Once a cell response was visible on one of the printed features, the locations of the other patterns could be located and imaged since the exact distance between all of the printed features was known. For example, the cell response to

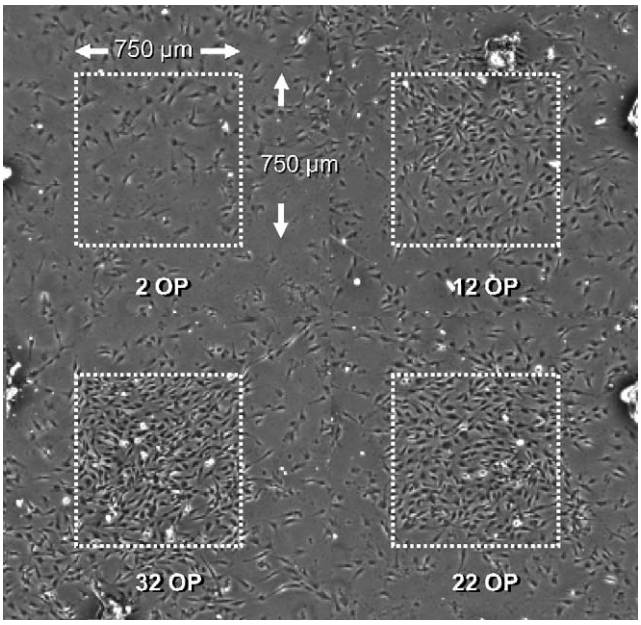

Fig. 3. Dose responsiveness of cell behavior to periodic 2D FGF-2 patterns. Patterns of varying FGF-2 solid-phase concentration were created as described in Fig. 2 except that the number of overprints of FGF-2 bioink was varied between individual 750 × 750 μm square patterns (0.563 mm^2) within a printed array. The numbers of overprints (OP) were 2, 12, 22, and 32, corresponding to 0.14, 0.86, 1.54, and 2.24 pg of deposited FGF-2 per jetted droplet (with 100 total drops per 0.563 mm^2).

a 22 or 32 overprint square was visible earlier than a 12 overprint square. Since there was 1.75 mm between all of the printed squares, the 2 and 12 overprint squares could be located after the 22 or 32 overprint cell response had formed. After the images of the cell responses to the patterns were collected, the cell numbers on the patterns were determined using BDS-Image software and custom software that runs within the BDS-image environment.

Initially, cell counting on the printed FGF-2 squares and the concentration gradients was performed both manually using NIH image and in an automated fashion using BDS software and the results compared. After proving that the two methods gave identical results, all cell counting was automated using the following protocol. First, background images were generated by applying a smoothing filter to the cell images, and the respective background images were used to flatfield cell images. This greatly reduced shading in the corners of images and provided uniform contrast across the images. Background pixel values were then determined from histograms, and image backgrounds were adjusted so that the image means were identical. Subsequently, cells were gray-level thresholded and either counted or rejected based on the number of pixels belonging to the cell masks. Generally cell masks did not overlap, so more complicated watershed [26] and image processing algorithms [27] were deemed not necessary for the studies reported here. Cell counts were performed on the square patterns of FGF-2 with varying overprints and on the concentration gradients. The gradient patterns were divided into three equal areas and the cell number on each area was determined.

3. Results

Spatial patterns of fluorescently labeled FGF-2 on planar homogeneous fibrin films were printed to confirm the accuracy and persistence of the patterns. An example of a printed FGF-2 concentration gradient is shown in Fig. 1B. An intensity scan of the epifluorescence image along the gradient is shown in Fig. 1C, demonstrating the resolution and control of our printing approach. Using a bioink with 4 μg/ml of FGF-2, each droplet contains a physiologically relevant FGF-2 dose of 0.07 pg. Quantification of the final bound FGF-2 surface concentration remains to be determined.

Square patterns printed using 50 μg/ml FGF-2 and 50 μg/ml Cy3 FGF-2 are shown in Figs. 2A and B. We purposefully selected a square cornered pattern (750 μm × 750 μm) for experiments based on the assumption that if significant FGF-2 desorption occurs, diffusion and reabsorption of desorbed FGF-2 would likely produce a rounding of corners and be reflected in cell response. This would not have been as obvious using circles, other round edged shapes, or straight lines. Persistence of FGF-2 patterns is shown through repeated epifluorescence imaging over time which established retention of printed FGF-2 patterns with less than 1–2% loss in fluorescence intensity occurring during cell culture for up to 10 days. FGF-2 release from fibrin can occur via proteolysis [28]. Therefore, the protease inhibitor aprotinin was added to the cell culture medium to block fibrin proteolysis.

The observed cellular response to printed patterns is determined through measurements of local cell density with time after seeding of cells. Printed FGF-2 on fibrin slides were seeded with MG-63 cells in serum-free medium. Location of cells with time was probed using microscopy. The observed cell organizational response at 5 days in register to the square patterns (Figs. 2C and D) confirms retention of biologically active FGF-2 within the patterned configuration. These patterned responses continued to be observed over 10 days in culture. Cell behavioral responses were determined visually once every 24 h; therefore, the type of cell organizational response, cell migration, or proliferation, or a combination of both could not be clearly determined from these particular experiments.

The measurements of cell response to printed hormone patterns also show that the development of a patterned cell density response is FGF-2 concentration dependent, which is also observed for liquid-phase FGF-2 [29]. Fig. 3 is a representative image which illustrates that cell density on printed areas increases as the concentration of solid-phase FGF-2 increases on discrete 750 μm × 750 μm patterns. Standard image processing and masking techniques were used to determine the cell number on each of the printed

squares. Plots from three separate experiments illustrating the relationship between cell number per area and overprints with time are shown in Fig. 4. An equivalent region on the slide adjacent to the patterns was imaged and served as a control (0 overprints). Cell number was not normalized to correct for any differences in initial cell number between squares. A FGF-2 dose-dependent increase in cell number was observed for up to 22 overprints where a leveling off of the curves occurred. An actual decrease in cell number in the control group occurred between 96 and 120 h. The relationship between the cell number and overprints corresponded to the representative images of the patterned responses shown in Fig. 3. An air bubble occurred over the 22 overprint square in Fig 4C; therefore, no data are available for that sample.

Having established concentration dose dependency upon discrete uniform FGF-2 solid-phase concentration patterns, we used the versatility of the printing approach to show that a concentration gradient of solid-phase FGF-2 will result in a graded cell response. Fig. 5A is a representative example of a graded cell organizational response to a solid-phase FGF-2 concentration gradient pattern. Similar concentration gradient dose-dependent cell growth responses are observed for all such gradient experiments performed. Images of the gradient were acquired every 24 h for 3 days. Fig. 5B illustrates the difference in cell number over time for each of the three assigned regions of equal area on the gradient pattern. As previously mentioned, it is unclear if the gradient cell response was formed primarily by migration or proliferation since the images were taken every 24 h. As seen in Fig. 5B, the cell number in each of the assigned regions was in register with the printed FGF-2 concentration gradient. Cell number was not normalized to correct for any differences in initial cell number between squares. Cell seeding was performed with the intent of providing uniform cell distribution; however, slight variability in initial cell number commonly occurs as is represented in Fig. 5B.

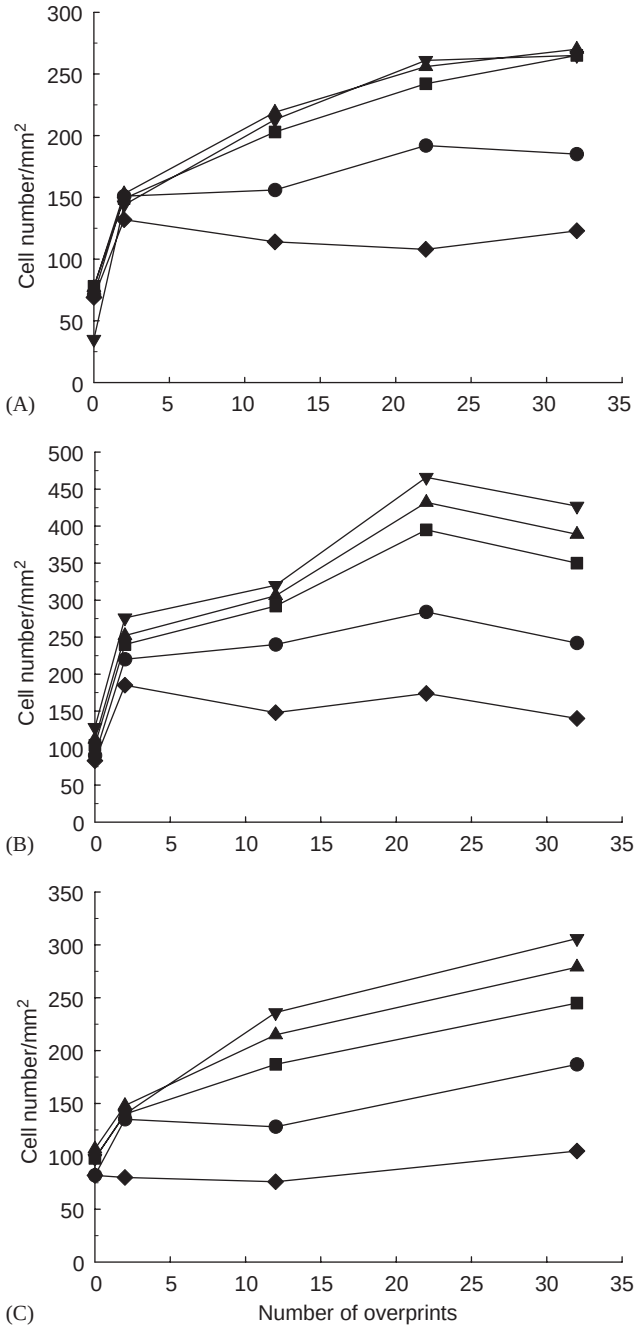

Fig. 4. Graphic plot of cell numbers derived from three separate experiments described in Fig. 3 (A, B, C represent experiments 1, 2, and 3, respectively) illustrating the relationship between cell number and number of overprints for printed FGF-2 patterns over time. Each curve represents a different time point: 24 h, ◆; 48 h, ●; 72 h, ■; 96 h, ▲; 120 h, ▼.

4. Discussion

4.1. Directed cell response

In the work reported here, we focused on the general cell behavioral response in reference to inkjet printed concentration gradients and uniform patterns of FGF-2. Our experimental design consisted of a random uniform cell seeding distribution across the substrate, both on and off pattern. We did not observe differences in initial cell distributions on or off patterns at 3–4 h post cell-seeding. This represents the time of cell attachment prior to any migrational or proliferative response, and demonstrates that the printed FGF-2 does not impact cell attachment. The lack of significant desorption of Cy3 FGF-2 (Figs. 2A and B) and diffusion of soluble FGF-2 to direct migration argues that a cell surface receptor population must directly contact solid-phase FGF-2. Cells interacting with printed patterns will be limited to those within random walk lengths to pattern

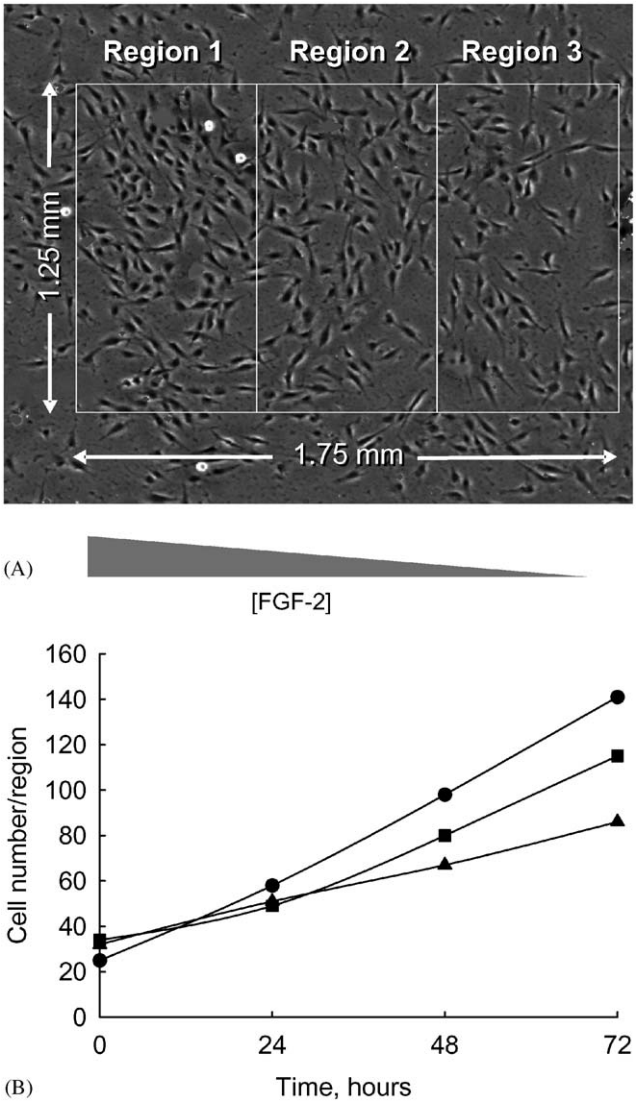

Fig. 5. Cell organizational response to solid-phase FGF-2 concentration gradient pattern (1.25 mm × 1.75 mm). Gradient patterns were created as described in Fig. 1. (A) Phase contrast micrograph taken on day 4 of culture. Gradient was divided into three designated concentration regions, from highest FGF-2 concentration (Region 1) to lowest (Region 3): 0–583, 583–1166, and 1166–1750 μm. (B) Cell number over time on each of the three regions: Region 1, ●; Region 2, ■; Region 3, ▲.

borders. Therefore, migration onto pattern by cells that contact the pattern through a random walk, retention of cells on pattern, and enhanced cell proliferation on pattern likely define the cell organizational response. Subsequent analysis using time-lapse imaging indicates that the primary cell response to printed patterns is cell proliferation on pattern while cell migration onto pattern is primarily random (Miller et al., 2005, Tissue Engineering, submitted for publication). Experiments are in progress to specifically address cell migration responses to printed hormonal gradients.

4.2. Extension to other hormones and substrates

We selected FGF-2 as a paradigm that represents a hormone critical in development, tissue maintenance, and wound repair [29]. FGF-2 is also a potent mitogen for osteoblast-like phenotypes [30]. Furthermore, FGF-2 readily binds with high specificity to ECM molecules such as fibrin [31] without chemical modification of hormone or substrate. And, fibrin provides the foundational substrate for wound healing supporting cell anchorage, hormone sequestration and localization, and cell differentiation [12,32]. Fibrin also supports osteoblast growth and differentiation [33]. This judicious choice of bioink and substrate facilitates pattern formation, predicted persistence of pattern, and biological response to pattern.

The FGF-2 printing methodology is readily applicable to other hormones [12] that contain heparin-binding domains, similar to FGF-2, which are responsible for fibrin solid-phase interaction [31]. For hormones such as IGF-I that do not contain a heparin-binding domain, IGF-binding proteins can be utilized as an intermediary to immobilize IGF-I to fibrin [34]. Furthermore, hormones can also be directly or indirectly engineered to induce persistent binding to not only fibrin but also to a wide array of relevant printing substrates whether native or synthetic [24,35–40].

4.3. Solid-phase patterning as a model for regenerative medicine and developmental biology

A major obstacle for tissue engineering of complex structures remains the inability to control spatial patterns of wound healing hormones [38]. We believe our technology enables such control. Our inkjet patterning approach is a maskless, programmable printing method that allows us to engineer persistent and arbitrary patterns of concentration-modulated solid-phase hormones on biologically relevant ECM-based substrates. In contrast to other patterning approaches, our approach uses native unmodified growth factors printed on native substrates with binding interactions dependent upon inherent binding kinetics between printed growth factors and substrate ECM. This capability, along with the ability to print additional hormones or other signaling molecules in complex arrays, mixed patterns, or complex gradient patterns, enables controlled systematic studies of hormonal spatial control of cell function. Establishing similar patterns is currently beyond the capabilities of methods based on soluble liquid-phase hormone delivery.

Inkjet based hormone patterning is an enabling technology which will provide an experimental basis to better study hormonal regulation of morphogenesis during development as well as the hormonal regulatory

events involved in tissue repair and regeneration. Such new experimental approaches hold promise in expanding our knowledge in both fields of developmental biology and tissue engineering.

While our current focus is on cell response to 2D patterns, solid-phase patterning methodology is extensible to 3D fibrin constructs. However, since gelled fibrin cannot be jetted, we are developing another printer that uses focused inkjet printheads for independent and concurrent deposition of fibrinogen, thrombin, and FGF-2 bioinks. 3D fibrin/FGF-2 structures are built-up, layer-by-layer, by jetted droplets mixing and gelling locally at the printed surface, resulting in specified spatial patterns of FGF-2 concentrations and defined fibrin microstructure [41]. The FGF-2 bioink consists of FGF-2 prebound to fibrinogen. The deposited concentration of FGF-2 is modulated by controlling the ratio of the FGF-2/fibrinogen bioink to the fibrinogen bioink. Our long-term goal is to use 3D printing for creating 3D patterned structures for therapeutic delivery of patterned hormones.

5. Conclusion

In conclusion, we have demonstrated a programmable printing methodology to create arbitrary and persistent hormone patterns on a biologically relevant substrate. This methodology is unique in its capability to quickly prototype complex 2D concentration-modulated hormone patterns. We show that such positional information directs cell behavior in near-perfect registration to these engineered patterns. This represents a significant advancement toward controlled engineered biological patterning.

Acknowledgments

This work was supported partially by the Office of Naval Research (Grant No. N000140110766), the National Science Foundation (Grants No. CTS-0210238 and DMI-9800565), the National Institutes of Health (Grant No. 1 R01 EB00 364-01), the Pennsylvania Infrastructure Technology Alliance (PITA) from the Pennsylvania Department of Community and Economic Development, the Health Resources and Services Administration (Grant No. 1C76 HF 00381-01), the Scaife Foundation, and the Philip and Marsha Dowd Engineering Seed Fund. We wish to thank Aventis Behring, L.L.C. (King of Prussia, PA) for their generous gift of lyophilized human fibrinogen and thrombin, and PROCHON (Rehovot, Israel) for biotinylated FGF-2. The authors declare that they have no competing financial interests.

References

[1] Wolpert L. Positional information and the spatial pattern of cellular differentiation. J Theor Biol 1969;25:1–47.
[2] Tickle C. Morphogen gradients in vertebrate limb development. Semin Cell Dev Biol 1999;10:345–51.
[3] Singer AJ, Clark RA. Cutaneous wound healing. N Engl J Med 1999;341:738–46.
[4] Gierer A, Meinhardt H. A theory of biological pattern formation. Kybernetik 1972;12:30–9.
[5] Meinhardt H, Gierer A. Applications of a theory of biological pattern formation based on lateral inhibition. J Cell Sci 1974; 15:321–46.
[6] Boyden S. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. J Exp Med 1962;115: 453–66.
[7] Whitesides GM, Ostuni E, Takayama S, Jiang X, Ingber DE. Soft lithography in biology and biochemistry. Annu Rev Biomed Eng 2001;3:335–73.
[8] Lawrence PA, Struhl G. Morphogens, compartments, and pattern: lessons from drosophila? Cell 1996;85:951–61.
[9] Gurdon JB, Bourillot PY. Morphogen gradient interpretation. Nature 2001;413:797–803.
[10] Teleman AA, Strigini M, Cohen SM. Shaping morphogen gradients. Cell 2001;105:559–62.
[11] Turing AM. The chemical basis of morphogenesis. Philos Trans R Soc Lond [B] 1952;237:37–72.
[12] Rifkin DB, Mazzieri R, Munger JS, Noguera I, Sung J. Proteolytic control of growth factor availability. Apmis 1999; 107:80–5.
[13] Bernfield M, Gotte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J, Zako M. Functions of cell surface heparan sulfate proteoglycans. Annu Rev Biochem 1999;68:729–77.
[14] Ruhrberg C, Gerhardt H, Golding M, Watson R, Ioannidou S, Fujisawa H, Betsholtz C, Shima DT. Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. Genes Dev 2002;16:2684–98.
[15] Ohkawara B, Iemura S, ten Dijke P, Ueno N. Action range of BMP is defined by its N-terminal basic amino acid core. Curr Biol 2002;12:205–9.
[16] Akeson AL, Greenberg JM, Cameron JE, Thompson FY, Brooks SK, Wiginton D, Whitsett JA. Temporal and spatial regulation of VEGF-A controls vascular patterning in the embryonic lung. Dev Biol 2003;264:443–55.
[17] Mrksich M, Whitesides GM. Using self-assembled monolayers to understand the interactions of man-made surfaces with proteins and cells. Annu Rev Biophys Biomol Struct 1996;25:55–78.
[18] Zhang S, Yan L, Altman M, Lassle M, Nugent H, Frankel F, Lauffenburger DA, Whitesides GM, Rich A. Biological surface engineering: a simple system for cell pattern formation. Biomaterials 1999;20:1213–20.
[19] Kane RS, Takayama S, Ostuni E, Ingber DE, Whitesides GM. Patterning proteins and cells using soft lithography. Biomaterials 1999;20:2363–76.
[20] Patel N, Bhandari R, Shakesheff KM, Cannizzaro SM, Davies MC, Langer R, Roberts CJ, Tendler SJ, Williams PM. Printing patterns of biospecifically adsorbed protein. J Biomater Sci Polym Ed 2000;11:319–31.
[21] Delamarche E, Geissler M, Wolf H, Michel B. Positive microcontact printing. J Am Chem Soc 2002;124:3834–5.
[22] Ito Y, Chen G, Imanishi Y. Micropatterned immobilization of epidermal growth factor to regulate cell function. Bioconjug Chem 1998;9:277–82.
[23] Chen G, Ito Y. Gradient micropattern immobilization of EGF to investigate the effect of artificial juxtacrine stimulation. Biomaterials 2001;22:2453–7.

[24] Watanabe K, Miyazaki T, Matsuda R. Growth factor array fabrication using a color inkjet printer. Zool Sci 2003;20:429–34.
[25] Lorthois S, Schmitz P, Angles-Cano E. Experimental study of fibrin/fibrin-specific molecular interactions using a sphere/plane adhesion model. J Colloid Interface Sci 2001;241:52–62.
[26] Vincent L, Soille P. Watersheds in digital spaces: an efficient algorithm based on immersion simulations. IEEE Trans Pattern Anal Mach Intell 1991;13:583–98.
[27] Russ JC. The Image Processing Handbook. Boca Raton, FL: CRC Press; 1999.
[28] Sahni A, Francis CW. Plasmic degradation modulates activity of fibrinogen-bound fibroblast growth factor-2. J Thromb Haemost 2003;1:1271–7.
[29] Bikfalvi A, Klein S, Pintucci G, Rifkin DB. Biological roles of fibroblast growth factor-2. Endocr Rev 1997;18:26–45.
[30] Martin I, Muraglia A, Campanile G, Cancedda R, Quarto R. Fibroblast growth factor-2 supports ex vivo expansion and maintenance of osteogenic precursors from human bone marrow. Endocrinology 1997;138:4456–62.
[31] Sahni A, Odrljin T, Francis CW. Binding of basic fibroblast growth factor to fibrinogen and fibrin. J Biol Chem 1998;273:7554–9.
[32] Clark RA. Fibrin and wound healing. Ann NY Acad Sci 2001; 936:355–67.
[33] van Griensven M, Zeichen J, Tschernig T, Seekamp A, Pape HC. A modified method to culture human osteoblasts from bone tissue specimens using fibrin glue. Exp Toxicol Pathol 2002;54:25–9.
[34] Campbell PG, Durham SK, Hayes JD, Suwanichkul A, Powell DR. Insulin-like growth factor-binding protein-3 binds fibrinogen and fibrin. J Biol Chem 1999;274:30215–21.
[35] Sakiyama SE, Schense JC, Hubbell JA. Incorporation of heparin-binding peptides into fibrin gels enhances neurite extension: an example of designer matrices in tissue engineering. FASEB J 1999;13:2214–24.
[36] Sakiyama-Elbert SE, Hubbell JA. Controlled release of nerve growth factor from a heparin-containing fibrin-based cell ingrowth matrix. J Control Rel 2000;69:149–58.
[37] Sakiyama-Elbert SE, Hubbell JA. Development of fibrin derivatives for controlled release of heparin-binding growth factors. J Control Rel 2000;65:389–402.
[38] Saltzman WM, Olbreicht WL. Building drug delivery into tissue engineering. Nat Rev: Drug Disc 2002;1:177–86.
[39] Schense JC, Hubbell JA. Cross-linking exogenous bifunctional peptides into fibrin gels with factor XIIIa. Bioconjug Chem 1999;10:75–81.
[40] Zisch AH, Schenk U, Schense JC, Sakiyama-Elbert SE, Hubbell JA. Covalently conjugated VEGF–fibrin matrices for endothelialization. J Control Rel 2001;72:101–13.
[41] Weiss LE, Amon C, Finger S, Miller E, Romero D, Verdinelli I, Walker L, Campbell PG. A Bayesian modeling approach to computer-aided experimental design of heterogeneous matrices for tissue engineering applications. Computer-Aided Des, 2005, in press, corrected proof, available online 1 April 2005.