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Microenvironments Engineered by Inkjet Bioprinting Spatially Direct Adult Stem Cells Toward Muscle- and Bone-Like Subpopulations

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ABSTRACT

In vivo, growth factors exist both as soluble and as solid-phase molecules, immobilized to cell surfaces and within the extracellular matrix. We used this rationale to develop more biologically relevant approaches to study stem cell behaviors. We engineered stem cell microenvironments using inkjet bioprinting technology to create spatially defined patterns of immobilized growth factors. Using this approach, we engineered cell fate toward the osteogenic lineage in register to printed patterns of bone morphogenetic protein (BMP) 2 contained within a population of primary muscle-derived stem cells (MDSCs) isolated from adult mice. This patterning approach was conducive to patterning the MDSCs into subpopulations of osteogenic or myogenic cells simultaneously on the same chip. When cells were cultured under myogenic conditions on BMP-2 patterns, cells on pattern differentiated toward the osteogenic lineage, whereas cells off pattern differentiated toward the myogenic lineage. Time-lapse microscopy was used to visualize the formation of multinucleated myotubes, and immunocytochemistry was used to demonstrate expression of myosin heavy chain (fast) in cells off BMP-2 pattern. This work provides proof-of-concept for engineering spatially controlled multilineage differentiation of stem cells using patterns of immobilized growth factors. This approach may be useful for understanding cell behaviors to immobilized biological patterns and could have potential applications for regenerative medicine. STEM CELLS *2008;26:127–134*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Spatial patterning of hormones, including growth factors, is known to be critical in directing all aspects of cell behavior throughout life, including embryogenesis [1, 2] and wound repair [3–5]. Simple, controllable methods to engineer the physical placement and concentration of immobilized exogenous growth factors in a physiologically relevant manner and to study cell behavior in register to such persistent patterns are important for biological research, as well as being a logical consideration for developing tissue regeneration therapies. Therefore, we have developed an inkjet-based bioprinting system and methodology to create concentration-modulated two-dimensional patterns of growth factors immobilized onto fibrin substrates using native binding affinities [6, 8].

There are several methods for creating protein-based arrays, including pen spotting [9], soft lithography [10, 11], photolithography [12, 13], and drop-on-demand inkjet printing [14, 15]. We chose to use our inkjet bioprinting method because it is programmable (no custom stamps are required), it is noncontact (there is no possibility of damaging the substrates), and it does not require any modifications to the proteins or substrates. In addition, since we use overprinting with a dilute bio-ink to modulate the surface concentrations of the immobilized growth factors, we have direct control over surface concentrations, limited only by the number of available binding sites on the printing substrate surface (e.g., fibrin). One limitation of drop-on-demand inkjet printing is the resolution compared with state-of-the-art protein array technologies [16]. Our system delivers drop volumes of approximately 14 pl (corresponding to spot sizes of ~75 μm in diameter); however, we have found that our current inkjet printing resolution is sufficient for studying the behavior of cell populations [6, 8].

Growth factors such as bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs) are essential for basic developmental mechanisms [17, 18]. BMPs are also potent osteoinductive factors and stimulate differentiation of osteoprogenitors via cooperative mechanisms with extracellular matrix (ECM) components. We and others have demonstrated synergistic activities among BMPs, other growth factors, and ECM proteins [19–24]. We have previously reported that inkjet-printed patterns of FGF-2 direct proliferation and migration of osteoblast-like cells dose-dependently [6, 8]. In the work reported here, we applied our inkjet printing strategy to investigate spatially controlled differentiation of a stem cell population. We hypothesized that BMPs immobilized within the ECM of stem cell microenvironments likely direct stem cell fate in a spatially defined manner. As such, inkjet printing is an ideal strategy to create and study stem cell response to solid-phase BMP-2 patterns. We used muscle-derived stem cells (MDSCs), which are

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a population of self-renewing [25] and multipotential cells [26] isolated from adult mice, as a model stem cell population to demonstrate spatially defined cell differentiation toward the osteogenic and myogenic lineages in register to inkjet-printed patterns of BMP-2.

MATERIALS AND METHODS

Inkjet Printing of BMP-2 Patterns on Fibrin Substrates

Fibrin-coated glass slides were prepared as previously described [6, 8]. Recombinant human BMP-2 (R&D Systems Inc., Minneapolis, http://www.rndsystems.com) bio-ink was labeled using cyanine 3 (Cy3) monofunctional reactive dye, synthesized in-house following established protocols [26]. Cy-3-labeled BMP-2 was purified from free dye over a heparin-Sepharose column and stored at −80°C to maintain viability. Prior to printing, the printhead was sterilized by first rinsing with 70% ethanol followed by three rinses with 0.2 μm of filtered deionized water prior to loading the ink in the jet. The bio-ink, consisting of 100 μg/ml Cy3-labeled BMP-2 in phosphate-buffered saline (PBS), pH 7.4, was loaded into the printhead, and patterns were printed onto fibrin-coated glass slides as previously described [6]. These relatively high growth factor bio-ink concentrations were used to aid visualization of the patterns. The printed patterns were 2 × 2 arrays of 750-μm squares with a spacing of 75 μm between the printed drops and 1.75 mm between the squares. Squares were printed as 2, 8, 14, and 20 overprints of the bio-ink to modulate the surface concentrations. The patterns were incubated at 37°C with 5% CO_2 and 95% humidity in serum-free Dulbecco's modified Eagle's medium (DMEM) with 1% penicillin/streptomycin and were imaged every 24 hours up to 144 hours by epifluorescence microscopy with a Cy3 filter set.

For the biological studies, we used our overprinting strategy with a 10 μg/ml unlabeled BMP-2 bio-ink to create four squares (2, 8, 14, and 20 overprints) of differing BMP-2 surface concentrations. We quantified the amount of BMP-2 deposited on the fibrin surface following the methods we have previously described [6]. The amount of BMP-2 deposited was as follows: 2 overprints, 64 ± 18 pg/mm^2; 8 overprints, 256 ± 72 pg/mm^2; 14 overprints, 448 ± 126 pg/mm^2; and 20 overprints, 640 ± 180 pg/mm^2. After 48 hours in serum-free media, 22.4% ± 4% of the deposited BMP-2 was retained. For all experiments, 2, 8, 14, and 20 overprints were printed to create 2 × 2 arrays.

Cell Culture

MDSCs were isolated as previously described [26] and cultured in DMEM (high-glucose), 10% fetal bovine serum (FBS), 10% horse serum, 1% chick embryo extract, 200 mM glutamine, and 1% penicillin/streptomycin including prophylactic for myoplasma (Invivogen, San Diego, http://www.invivogen.com). C2C12 were obtained from American Type Culture Collection (Manassas, VA, http://www.atcc.org) and cultured according to the manufacturer's instructions (DMEM [high-glucose], 10% FBS, 1% penicillin/streptomycin) (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). Where noted, Noggin (100 ng/ml as conditioned media) or 50 ng/ml FGF-2 (Peprotech, Rocky Hill, NJ, http://www.peprotech.com) was added to the cultured media. Doses of FGF-2 and Noggin were determined on the basis of our prior studies [28–30]. For myogenic conditions, serum levels were adjusted to 1% FBS and 1% horse serum.

Quantitative Real-Time Polymerase Chain Reaction

BMP-2 (125, 250, or 500 ng in 10 μl H_2O) was hand-pipetted onto collagen-coated dishes (12 wells) and allowed to air-dry in a sterile hood. Plates with blotted BMP-2 were then incubated overnight in complete basal medium to release unbound BMP-2. Cells were then seeded to ~90% in basal medium on the BMP-2 blots. BMP-2 was added to the culture media in parallel wells (125, 250, and 500 ng/ml) as positive control. For negative control, cells were cultured in basal medium alone (no BMP-2). Cells were cultured for 24 hours prior to extraction of total RNA (RNeasy Mini Kit; Qiagen, Hilden, Germany, http://www1.qiagen.com). Quantitative polymerase chain reaction (qPCR) analysis for *Alp* and *Osx* was performed as previously described [21, 31]. Target gene expression was normalized to *18S* internal control.

Alkaline Phosphatase Activity

Cells were seeded to ~90% on BMP-2 patterns. BMP-2 (50 ng/ml) was delivered to the media in parallel wells as the positive control. Basal medium was the negative control. Cells were cultured for 72 hours and then rinsed in PBS and fixed for 2 minutes in 3.7% formaldehyde. Alkaline phosphatase (ALP) activity (SIGMA*FAST*) was detected according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com).

Immunocytochemistry for Myosin Heavy Chain (Fast)

Cells cultured on BMP-2 patterns were fixed in 3.7% formaldehyde and stained immunocytochemically for myosin heavy chain (fast) (MHC-f) as described previously [26] to detect the formation of myotubes. Cells were counterstained with 4,6-diamidino-2-phenylindole (1:1,000).

Time-Lapse Image Acquisition

Time-lapse experiments were performed as previously described [6]. Briefly, the microscope stage incubator was mounted on a Zeiss Axiovert 135TV microscope using a 5×, 0.15 numerical aperture EL plan-NEOFLUAR phase I objective and phase optics (Carl Zeiss, Thornwood, NY, http://www.zeiss.com) with an automated stage (Ludl Electronic Products Ltd., Hawthorne, NY, http://www.ludl.com). A ground-glass diffuser was used to smooth out the bright-field illumination. Time-lapse imaging of the patterns was automated using QED InVitro Version 3.2.0 (Media Cybernetics, Silver Spring, MD, http://www.mediacy.com). The patterns (located using the marks scored on the slide after printing) were imaged every 10 minutes using a QImaging RETIGA EXi CCD camera (QImaging, Burnaby, BC, Canada, http://www.qimaging.com). A total of four fields, representing the four different surface concentrations, were imaged.

Statistical Analysis

All experiments were repeated a minimum of three times. One representative experiment is shown. For qPCR assays, gene expression is displayed as one representative experiment of three independent experiments and represented as mean ± SEM. Intra- and interday variation did not exceed 5%. For all treatment groups and target genes analyzed, the coefficient of variation among polymerase chain reaction assay replicates did not exceed 3%. One-way analysis of variance followed by Fisher's least significant difference post hoc test using SYSTAT 9 software (Systat Software Inc., Richmond, CA, http://www.systat.com) was performed to determine significance among treatment groups. A p value $<.05$ was considered statistically significant.

RESULTS

Inkjetted BMP-2 Remains Immobilized to a Fibrin Substrate

Printed square patterns of Cy3-labeled BMP-2, each measuring 750 by 750 μm, were created on fibrin-coated glass slides. These patterns persisted for up to 144 hours under standard cell culturing conditions (Fig. 1A–1D). Increasing surface concentrations of BMP-2 were created by repeatedly overprinting onto the same pattern (Fig. 1A–1D). There was no apparent loss of fluorescent signal for up to 144 hours of incubation. Square patterns of Cy3-labeled BMP-2 were immobilized to fibrin in four different surface concentrations (2, 8, 14, and 20 overprints) on a single glass slide using an overprinting strategy (Fig. 2). For all subsequent experiments, unlabeled BMP-2 was inkjetted on fibrin slides to create the immobilized patterns.

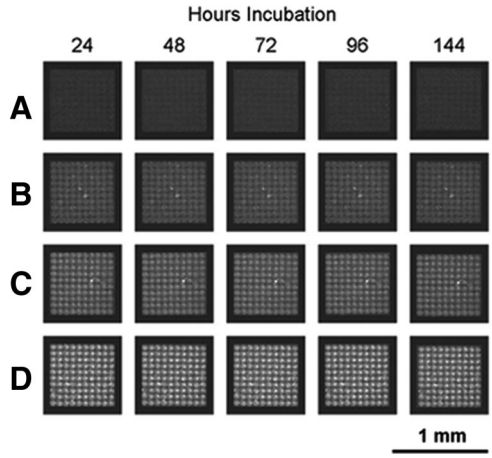

Figure 1. Cyanine 3-labeled bone morphogenetic protein 2 two-dimensional patterns created using inkjet printing persist on fibrin films. Surface concentrations were varied using an overprinting strategy and imaged every 24 hours under standard cell culture conditions. **(A):** Two overprints. **(B):** Eight overprints. **(C):** Fourteen overprints. **(D):** Twenty overprints.

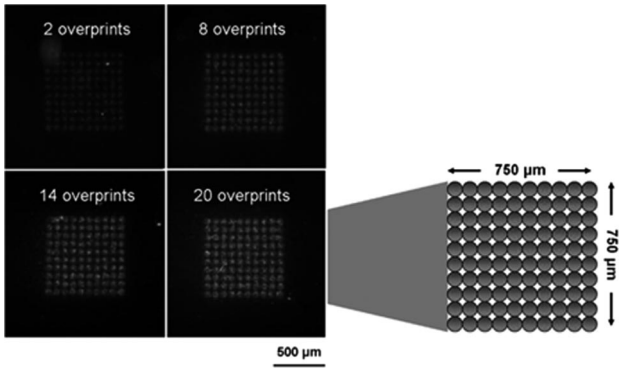

Figure 2. Cyanine 3-labeled bone morphogenetic protein 2 patterns inkjet printed on fibrin films at various surface concentrations using an overprinting strategy.

Solid-Phase BMP-2 Is Biologically Active

Prior to printing BMP-2, *Alp* and *Osx* gene expression was quantified in cells cultured on solid-phase blots of BMP-2 dried on collagen-coated tissue culture wells (Fig. 3A, 3B, respectively). Both C2C12 cells and MDSCs expressed basal levels of *Alp* gene expression that were dose-dependently upregulated in response to a 24-hour exposure of increasing concentration of BMP-2 dried and immobilized to collagen-coated tissue culture wells. The lowest dose of solid-phase BMP-2 (125 ng/10 μl deposited) was not sufficient to induce a significant increase in *Alp* gene expression relative to negative control for either MDSC ($p = .9575$) or C2C12 ($p = .4557$). However, 250 ng/10 μl deposited stimulated significant upregulation of *Alp* in MDSC of 33.17-fold ($p = .0255$), but 500 ng/10 μl deposited was not significantly different from 250 ng/10 μl deposited, with an upregulation of 26.51-fold ($p = .0511$) relative to control. Both 250 and 500 ng/10 μl deposited were significant compared with negative control ($p < .0001$). Similarly, *Alp* upregulation was quantified in C2C12 as 3.18-fold ($p = .0377$) for 250 ng of solid-phase BMP-2 and 17.8-fold ($p < .0001$) for 500 ng/10 μl deposited relative to control. Neither MDSC nor C2C12 expressed basal *Osx* in this experiment. However, the highest dose of solid-phase BMP-2 (500 ng/10 μl deposited) was sufficient to induce detectable *Osx* gene expression in both MDSC and C2C12.

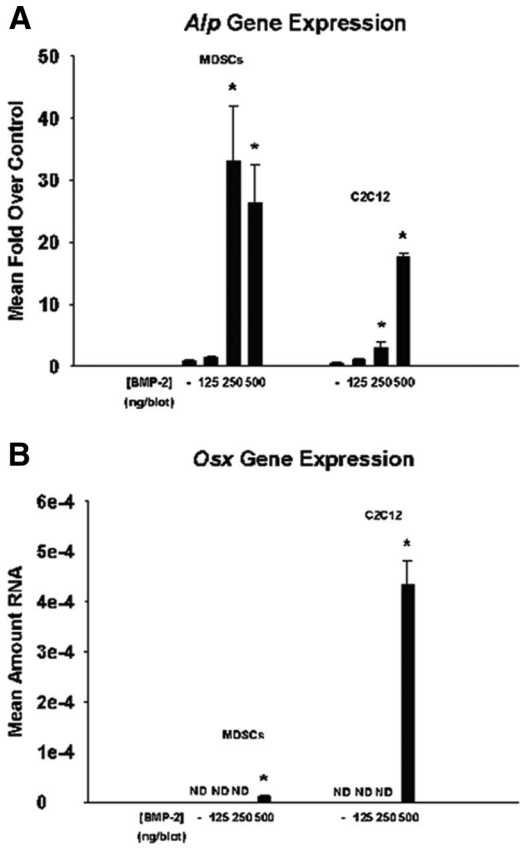

Figure 3. Quantitative real-time polymerase chain reaction analysis of *Alp* **(A)** and *Osx* **(B)** gene expression in MDSC and C2C12 cells exposed to solid-phase BMP-2. Columns represent mean ± SEM ($n = 3$) fold over control (−BMP-2) for *Alp* and mean amount RNA expression (arbitrary fluorescent units) for *Osx*. ∗, Significantly different from control; $p < .05$. Abbreviations: BMP, bone morphogenetic protein; MDSC, muscle-derived stem cell; ND, not detected.

Spatial Control of Osteogenic Differentiation

MDSC and C2C12 have the capacity to differentiate toward the osteogenic lineage, evidenced here by induction of ALP activity by liquid-phase BMP-2 for 72 hours (Fig. 4A, 4B) and as previously reported [28, 32–35]. MDSCs and C2C12 cultured on patterns of printed BMP-2 also differentiate toward the osteogenic lineage, as evidenced by increased ALP activity (Fig. 4A, 4B, respectively) in register to the printed BMP-2 patterns. A patterned differentiative response was visible by eye within tissue culture wells, as shown by the scanned images of the culture wells (Fig. 4A, 4B, middle). An increased magnification of the tissue culture wells shows the dose-dependent pattern response to cells on BMP-2 patterns for both MDSC and C2C12 (Figure 4A, 4B, far right). MDSC and C2C12 osteogenic differentiation was dose-dependent with respect to the surface concentration of printed BMP-2, with the highest surface concentration inducing a near-perfect square region of differentiating cells that corresponds to the BMP-2 array (Fig. 4C, 4D, respectively), whereas cells seeded outside the spatially defined BMP-2 pattern did not exhibit appreciable ALP activity and thus presumably remain undifferentiated. These data provide proof-of-concept for engineering spatially controlled engineered cell fate toward the osteogenic lineage.

FGF-2 and Noggin Inhibit Cell Differentiative Response to Printed BMP-2 Patterns

We next asked whether BMP signaling antagonists such as FGF-2 or Noggin present in the liquid-phase would influence cell response

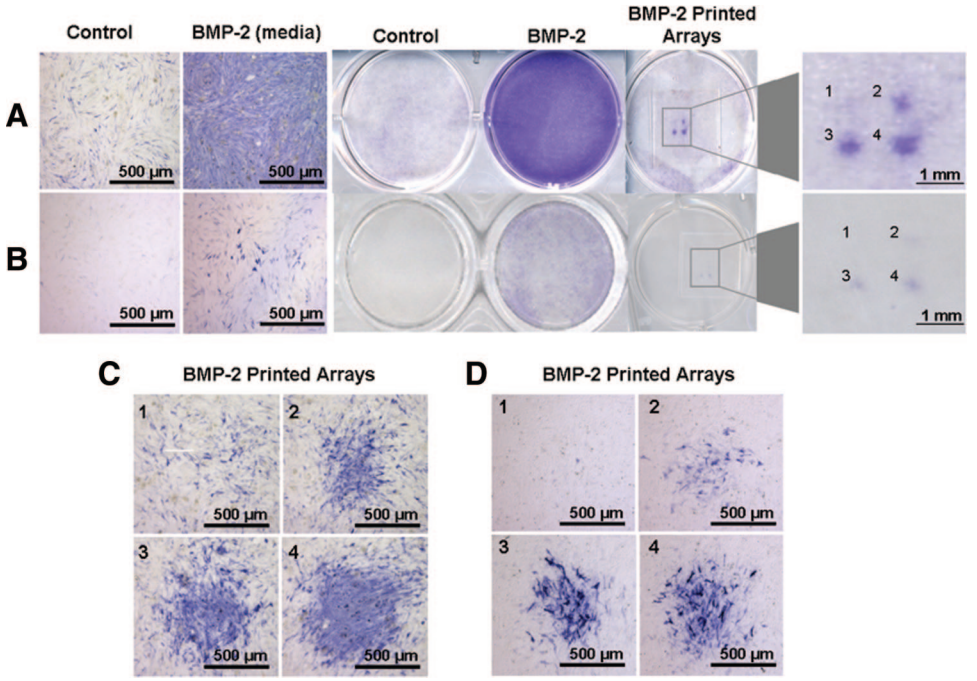

Figure 4. Spatial control over osteogenic lineage progression for muscle-derived stem **(A, C)** and C2C12 **(B, D)** cells. Overprints of BMP-2 were as follows: (1) 2 overprints; (2) 8 overprints, (3) 14 overprints; and (4) 20 overprints. **(C, D):** Osteogenic differentiation in register to printed patterns. Abbreviation: BMP, bone morphogenetic protein.

to the BMP-2 printed patterns. We found that Noggin delivered to the media inhibited BMP-2 pattern response in both MDSCs and C2C12 cells (Fig. 5A). FGF-2 partially inhibited patterned cell differentiation in MDSCs, whereas inhibition was apparently complete in C2C12, where no appreciable ALP activity was detected in the presence of FGF-2 (Fig. 5A). Noggin present in the culture media likely binds to the printed immobilized BMP-2 and thus inhibited BMP-2 signaling on patterns. Noggin-BMP-2 binding likely occurs prior to cell attachment and BMP receptor activation, since Noggin was administered to the media at the time of cell seeding. In contrast, FGF-2 has been shown to elicit cross-signaling mechanisms with BMP, as was previously reported by others [17, 18]. Therefore, the simultaneous signaling of FGF-2 and BMP-2 likely results in an inhibition of cell differentiation on BMP-2 printed patterns.

We sought to further elucidate the mechanism of the inhibition of BMP-2-directed cell differentiation in the presence of Noggin or FGF-2. We reasoned that although Noggin and FGF-2 may have different mechanisms of action (i.e., extracellular prevention of BMP receptor binding by Noggin and intracellular cross-signaling by FGF-2), the net result of blocking BMP-2 signaling might be similar. We therefore quantified the gene expression of the osteoblast gatekeeper transcription factor *Osx,* which is an essential factor for osteogenesis [36]. We have previously shown *Osx* to be strongly upregulated in human adult mesenchymal stem cells, as well as mouse osteoblasts, fibroblasts, pluripotential cells [19, 21, 30, 37], and, in the present study, MDSCs (Fig. 3B). We found that costimulation of MDSC or C2C12 cells with BMP-2 and either Noggin or FGF-2 resulted in a significant downregulation of *Osx* gene expression (Fig. 5B, 5C). Noggin reduced *Osx* levels to baseline in the presence of BMP-2 for MDSC ($p = .3178$) and below baseline for C2C12 (not detectable) compared with control. The addition of FGF-2 in the presence of BMP-2 resulted in a significant downregulation of *Osx* gene expression compared with BMP-2 stimulation alone for both MDSC ($p = .0021$) and C2C12 ($p < .0001$), which was at the level of baseline *Osx* expression for MDSC ($p = .2678$) and C2C12 ($p = .7788$). BMP-2 might continue to signal through its receptors, but the addition of FGF-2 likely contributes to some intracellular cross-signaling, which influences the BMP signaling cascade downstream, leading to *Osx* expression.

MDSCs Can Be Patterned Toward the Osteogenic and Myogenic Lineages Simultaneously on the Same Printed Array

We took advantage of the inherent myogenic potential of the MDSCs [26] to spatially control the differentiation of two lineages simultaneously in the same culture well using our inkjet printing approach. MDSCs were cultured on 2 × 2 patterns of four different surface concentrations of BMP-2 (as described above), under myogenic culture medium conditions. MDSCs exhibited ALP activity in register to the printed BMP-2 patterns (Fig. 6A) as before in normal serum (Fig. 4). All patterns showed dose-dependent ALP response and no myotubes on pattern. The 14-overprint BMP-2 pattern is shown as a representative image. Outside of the BMP-2 pattern, cells began to fuse, forming multinucleated myotubes, as shown by time-lapse microscopy (Fig. 7; supplemental online video) and immunocytochemical staining for MHC-f (Fig. 6B, 6C). Cells formed multinucleated myotubes outside of the BMP-2 pattern within 72 hours of culture, indicating differentiation toward the myogenic lineage. Cells were cultured in 20% serum as a negative control for myogenic differentiation. No myotubes formed off BMP-2 pattern under 20% serum conditions (Fig. 6G, 6H). C2C12 responded similarly to form distinct subpopulations of ALP+ cells on BMP-2 pattern and MHC-f+ multinucleated myotubes off pattern (data not shown). This demonstrates proof-of-concept for spatial patterning of multiple lineages from a single population of stem cells simultaneously within the same culture well.

DISCUSSION

Our bioprinting technology enables engineering of cell fates using spatially controlled deposition of hormonal stimuli that

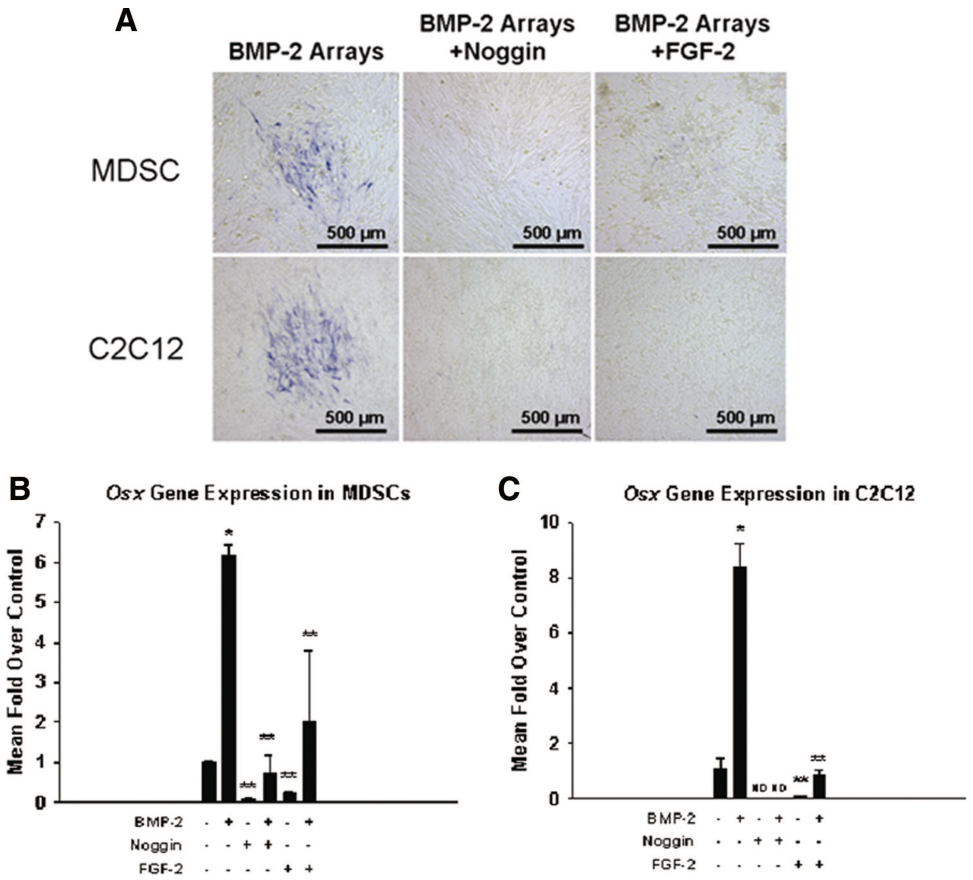

Figure 5. Inhibition of BMP-2 signaling by Noggin and FGF-2. **(A):** Media-delivered Noggin and FGF-2. **(B, C):** *Osx* gene expression MDSC and C2C12, respectively. Columns represent mean ± SEM, fold over control (−BMP-2/−Noggin-FGF-2). ∗, Significantly different from control; $p < .05$. ∗∗, Significantly different from BMP-2-treated; $p < .05$. Abbreviations: BMP, bone morphogenetic protein; FGF, fibroblast growth factor; MDSC, muscle-derived stem cell; ND, not detected.

direct cell responses. We have previously demonstrated this technology for spatial control of cell proliferation using printed immobilized patterns of FGF-2 [6, 8]. Whereas others have reported spatial control of osteogenic [38] and neural precursor cell differentiation [39] using immobilized growth factors, we now report on the use of this approach to engineer a microenvironment that spatially regulates stem cell differentiation into subpopulations of multiple lineages (e.g., osteogenic and myogenic) in a spatially controlled manner. We engineered the fate of MDSCs by culturing them on patterns of BMP-2 to induce osteogenic lineage progression dose-dependently and spatially restricted to the printed BMP-2 pattern. MDSCs located outside of the BMP-2 pattern but directly adjacent to the BMP pattern differentiated toward the myogenic lineage. No myotubes were found localized to the printed BMP-2 pattern. BMP-2 is known to prevent myotube fusion [40], potentially through cross-talk with the Wnt signaling pathway [41], and to promote osteogenic differentiation of MDSCs [42] and C2C12 [33]. This is the first demonstration, to our knowledge, of spatially controlled multilineage differentiation from a single population of stem cells within the same culture vessel. We are currently expanding this enabling methodology to other cell types and tissues to better understand tissue-specific repair and regeneration. Furthermore, this approach may have clinical relevance for addressing the need for replacement tissues.

Previous approaches to differentiating stem cells have been conducted in separate culture vessels. However, given that the human body is one complete vessel that was derived from a single population of primitive stem cells, we sought to create a more physiologically relevant test bed for understanding the biological patterns of immobilized growth factors that help determine stem cell fates. In this approach, we used our established inkjet printing technology to create engineered microenvironments to direct MDSCs toward osteogenic and myogenic lineages. Our *biological rationale* has been to model native growth factor-ECM interactions to study cell behaviors such as proliferation [7], migration, and, in the present study, differentiation.

Spatial patterning, in part, occurs via sequestration of growth factors in the interstitial space, which directly affects temporal and spatial control by immobilizing growth factors at specific locations within the ECM [43–46] or on the cell surface [47]. Solid-phase growth factors are enabled because many growth factors exhibit inherent binding properties to ECM molecules directly or through specific binding protein intermediaries [47]. For optimal growth factor activity, cell anchorage to the ECM is often required [48], and signaling through growth factor receptors is enhanced by growth factor-ECM interactions [49, 50]. Such observations are translated into immobilization strategies that permit local delivery of exogenous growth factors in physiological doses to induce cell proliferation and differentiation [51–54].

We chose fibrin as a printing substrate because of its inherent cell- and growth factor-binding capabilities. Furthermore, fibrin is an essential component of wound healing. The growth factor surface concentrations are modulated by overprinting each location within the pattern with a dilute growth factor bio-ink so that the deposited concentrations increase proportionately to the number of overprinted drops until the binding

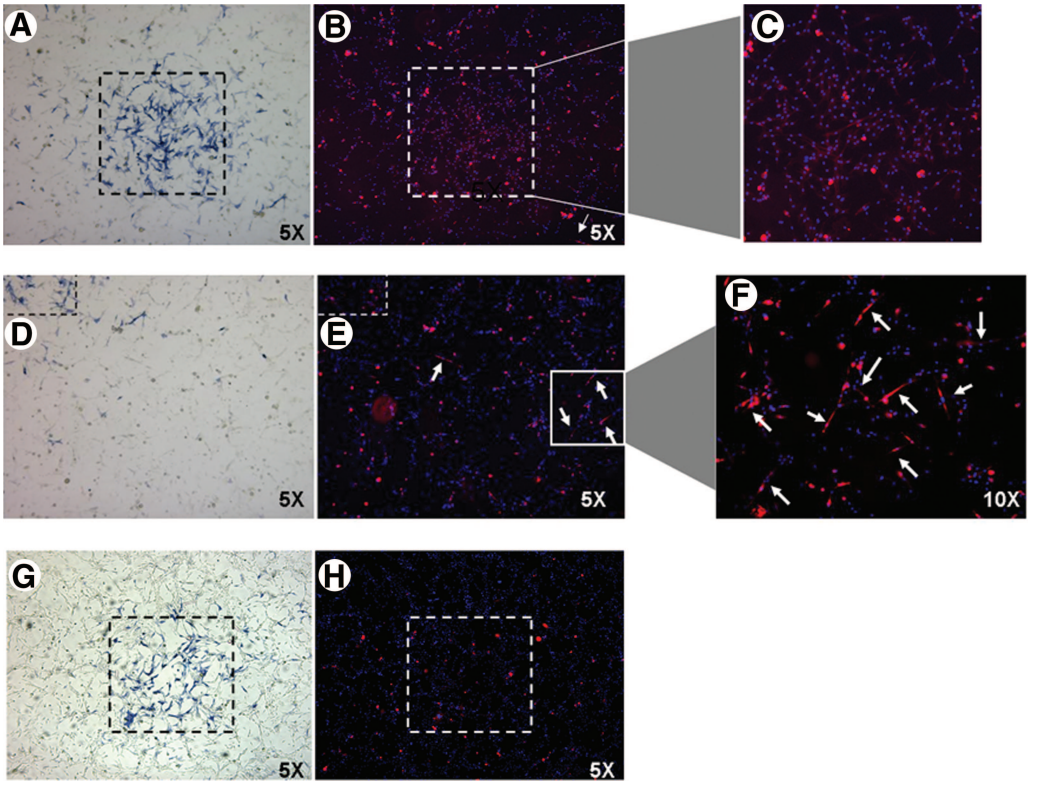

Figure 6. Patterning of multiple-cell types. **(A):** Fourteen-overprint bone morphogenetic protein (BMP) pattern under 2% serum. **(B, E):** Myotubes (arrows); red, myosin heavy chain (fast); blue, 4,6-diamidino-2-phenylindole. **(C):** Enlargement of square in **(B)**. **(D):** Lower righthand corner of **(A)**. **(F):** Enlarged view of box in **(E)**. Box, BMP-2 pattern. **(G, H):** Serum (20%) as negative control on **(G)** and off **(H)** pattern. Abbreviation: hr, hours.

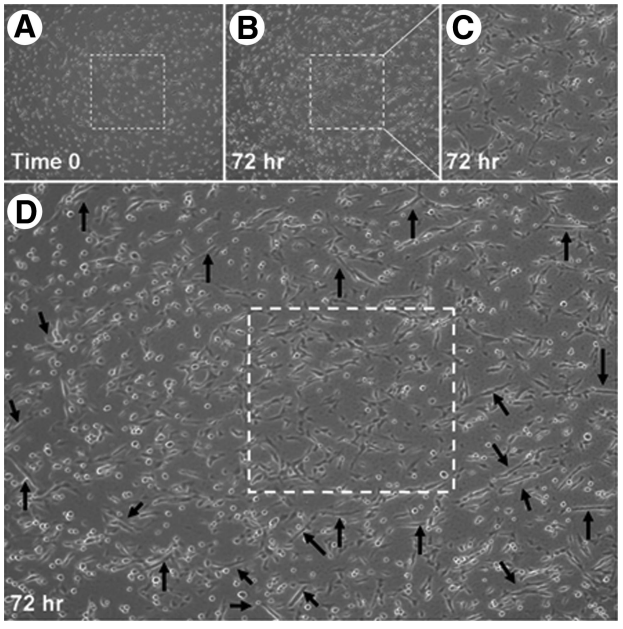

Figure 7. Time-lapse microscopy of muscle-derived stem cells on bone morphogenetic protein (BMP)-2 pattern. **(A):** Time 0. Dashed box indicates area of BMP-2-printed pattern. **(B):** Seventy-two hours. **(C):** Increased magnification of box in **(B)**. **(D):** Enlargement of **(B)**. Arrows indicate myotubes.

locations saturate the fibrin-coated surface. Our printing approach differs from patterning of cell attachment molecules [55–59] because, in our approach, cells organize in response to growth factor patterns from initial homogenous cell dispersions that are independent of cell attachment. Whereas others have reported higher resolution for inkjet printing approaches, we find our jetted drop resolution of 14 pl sufficient to detect spatially defined differential cell response toward multiple lineages. Furthermore, our technology has the capacity to create cell-responsive growth factor gradients [6, 7]. Hence, concentration-dependent cell responses can also be addressed using our bioprinting technology.

The use of various substrates for printing may influence the cell response. We have used fibrin because BMP-2 contains a heparin-binding domain [60] that facilitates binding to fibrin. BMPs also bind to collagens [61, 62], and this event influences BMP activity during osteoblast differentiation [23, 63]. Noggin, chordin, and follistatin are examples of extracellular BMP antagonists [64]. These inhibitors function by directly binding BMPs, rendering them inaccessible to their receptors [65]. In the present study, when Noggin was added to the culture media of cells seeded on BMP-2 patterns, the patterned differentiative response was abrogated. This result provides further support for functional bioactivity of printed BMP-2 because Noggin is able to function as a pseudoreceptor for BMP-2 either by directly binding to BMP-2 or by localizing to the cell surface [66]. These data suggest that Noggin can bind to printed BMP-2. Furthermore, Noggin likely binds to printed BMP-2 before cells are exposed to BMP-2 on the fibrin film, and Noggin maintains its ability to prevent receptor binding throughout the culture period. We and others have demonstrated that other growth factors modulate BMP activity, such as FGF-2 [17], pregnancy-associated plasma protein A [31], and insulin-like growth factors [18, 20, 30, 37]. These growth factors, along with other ECM proteins, can activate parallel signaling pathways during osteogenic differentiation [21, 23, 24, 31] that complement BMP signaling and provide support for solid-phase presentation

of growth factors to cells that optimize the desired cell response (differentiation). Kalajzic et al. reported that stage-specific inhibition of osteoblast differentiation by Noggin and FGF-2 occurs in marrow stromal cells [67]. FGF-2 is thought to oppose BMP signaling by initiating parallel signaling pathways that influence Smads activated by BMPs [18]. Here, we show that FGF-2 inhibited BMP signaling and patterned differentiative response of stem cells on BMP-2 patterns. Our data indicate that such cross-signaling by FGF-2 and BMP-2 involves Osx. We suggest that concurrent signaling by multiple factors such as BMP-2 and FGF-2 converges on Osx to regulate overall osteogenic cell response. Thus, FGF-2 reduces the patterned osteogenic response through downregulation of *Osx*. The present study adds to our understanding of the Osx signaling network enabled by our inkjet bioprinting methodology. We are currently developing technologies that will incorporate solid-phase patterns of multiple hormonal stimuli to further elucidate osteogenic signaling within populations of stem cells. The inkjet printing approach will have applications in basic biology discovery and potentially for clinical translation to regenerative medicine applications.

For example, craniofacial surgeries often require repair of soft tissue, muscle, and bone. A single population of adult stem cells (such as MDSCs) that are multipotential may be particularly useful for treating defects that span multiple tissues in patients. To harness the therapeutic potential of adult stem cells, we must characterize the niche-driven signals that regulate stem cell lineage specification. The ECM, consisting of immobilized growth factors, is probably the most important player in stem cell biology, albeit the most complex one. Patterning of immobilized solid-phase growth factors is one novel approach that could enable the level of control required to regenerate complex tissues comprising multiple cell lineages that are spatially defined. Our approach is amenable to other growth factors [6, 7, 8], to ECM substrates such as collagen (data not shown), and to three-dimensional printing [68]. Our inkjet printing technology is a practical strategy to spatially control differentiation of stem cells and to address new biological questions regarding the influence of the microenvironment/ECM on stem cell behavior.

CONCLUSION

We have demonstrated controlled spatial differentiation of adult stem cells in direct register to printed patterns of BMP-2 immobilized to fibrin. We have shown that patterned growth factors can be used to derive subpopulations of multiple lineages (e.g., bone and muscle-like cells) in register to inkjet-printed patterns. These results provide proof-of-concept for engineering spatial control of stem cell fates. This approach may be useful to understand biological solid-phase patterns during tissue development, repair, and regeneration. Furthermore, such enabling bioprinting technologies may provide a strategy for engineering replacement tissues using a single source of autologous adult stem cells to regenerate multiple tissues simultaneously through precise patterning of immobilized growth factor bio-inks.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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