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ABSTRACT

Tissue loss due to oral diseases requires the healing and regeneration of tissues of multiple lineages. While stem cells are native to oral tissues, a current major limitation to regeneration is the ability to direct their lineage-specific differentiation. This work utilizes polymeric scaffold systems with spatiotemporally controlled morphogen cues to develop precise morphogen fields to direct mesenchymal stem cell differentiation. First, a simple three-layer scaffold design was developed that presented two spatially segregated, lineage-specific cues (Dentinogenic TGF- β 1 and Osteogenic BMP4). However, this system resulted in diffuse morphogen fields, as assessed by the *in vitro* imaging of cell-signaling pathways triggered by the morphogens. Mathematical modeling was then exploited, in combination with incorporation of specific inhibitors (neutralizing antibodies or a small molecule kinase inhibitor) into each morphogen in an opposing spatial pattern as the respective morphogen, to design a five-layer scaffold that was predicted to yield distinct, spatially segregated zones of morphogen signaling. To validate this system, undifferentiated MSCs were uniformly seeded in these scaffold systems, and distinct mineralized tissue differentiation were noted within these morphogen zones. Finally, to demonstrate temporal control over morphogen signaling, latent TGF- β 1 was incorporated into one region of a concentric scaffold design, and laser treatment was used to activate the morphogen on-demand and to induce dentin differentiation solely within that specific spatial zone. This study demonstrates a significant advance in scaffold design to generate precise morphogen fields that can be used to develop *in situ* models to explore tissue differentiation and may ultimately be useful in engineering multi-lineage tissues in clinical dentistry.

KEY WORDS: scaffolds, growth factors, directed differentiation, dentinogenesis, osteogenesis, TGF- β 1.

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Multi-lineage MSC Differentiation via Engineered Morphogen Fields

INTRODUCTION

Clinical dentistry has always been at the forefront of biomaterials utilization to improve patient care. However, a large emphasis in these approaches has been on restoring diseased or missing tissues rather than regenerating them. Given the increasing availability of multipotent stem cells, both resident in oral tissues and derived, the ability of biomaterials to program or promote directed differentiation for functional tissue engineering is becoming reality. To date, biomaterials have been designed to exploit a range of signals, including the incorporation of biologically active motifs, nano-architecture interfaces, and the controlled release of factors to modulate cellular responses to promote regeneration (Lutolf and Hubbell, 2005; Burdick and Vunjak-Novakovic, 2009; Discher *et al.*, 2009; Guilak *et al.*, 2009; Aida *et al.*, 2012).

Morphogens are potent biological signals that induce signal transduction pathways to control developmental processes (Wartlick *et al.*, 2009) and have been widely explored to promote tissue regeneration (Reddi, 1998). These factors have historically been delivered in solution form, but this has significant limitations due to the short half-lives of these molecules in the body. In contrast, biomaterials-based delivery of these potent signaling molecules can lead to robust responses (Lee *et al.*, 2000; Wikesjö *et al.*, 2004; Chiu *et al.*, 2013). For example, recombinant bone morphogenetic proteins in a collagen carrier are used clinically to promote healing of vertical alveolar bone defects and critical-sized bone defects, and in spinal fusions (McKay *et al.*, 2007). However, the spatial gradients of these factors, and their temporal presentation, are key factors that have not often been thoroughly addressed in their applications to date. A major limitation of current *in vivo* approaches to the use of growth factors is likely the lack of spatial restriction, since this may generate undesirable, and often detrimental, effects in non-target tissues (Shields *et al.*, 2006).

Here we describe a new approach for biomaterials-based morphogen presentation that was inspired by processes of embryonic development, where a uniform mass of undifferentiated cells develops into morphologically distinct, multi-lineage tissues in an organism under the direction of tightly regulated morphogen signaling (Arany and Mooney, 2011). In particular, developmental processes often depend on the activity of not a single, positive, signal, but instead often utilize two cues, one positive and one inhibitory, that are released from distinct spatial locations. As the two diffuse toward each other, each partially cancels the activity of the other, resulting in sharp boundaries

between spatial regions where the cue promotes a process, vs. adjacent regions where the process is inhibited (Jacobson and Sater, 1988; Davidson, 1993; Gilbert *et al.*, 1996; Lander, 2007). In contrast, diffusion of a single cue from a particular spatial location results in a much shallower gradient that provides less spatial information to direct a process (Ben-Zvi *et al.*, 2011; Shilo *et al.*, 2013). We have previously demonstrated that spatially or temporally segregated and biomaterials-based release of multiple cues can provide tight control over spatial regions and maturation of angiogenesis (Richardson *et al.*, 2001; Yuen *et al.*, 2012). In this study, we extend this concept to spatially and temporally regulate stem cell fate, and further demonstrate that the incorporation of two distinct morphogens, and their inhibitors, into these systems allows one to promote distinct stem cell fates in adjacent regions. Mathematical modeling was used to design these complex poly(lactide-co-glycolide) (PLG) scaffolds for this purpose. The utility of this approach to direct the differentiation of mesenchymal stem cells (MSCs) to two distinct mineralized tissue fates – bone and dentin – is demonstrated.

MATERIALS & METHODS

Microsphere Fabrication

Poly (lactide-co-glycolide) (PLG) (8515DLG7E, Lakeshore Biomaterials, Birmingham, AL, USA) microspheres were prepared by a solvent evaporation method using a double emulsion technique as described previously (Cohen *et al.*, 1991). Briefly, a 100- μ L quantity of proteins dissolved in molecular-grade water was pipetted into 1 mL of 5% PLG in ethyl acetate (Sigma-Aldrich Corp., St. Louis, MO, USA) and immediately sonicated (Sonics and Materials Inc., Newtown, CT, USA) for 1 min. A second emulsion composed of 1% polyvinyl acetate (PVA) (Sigma-Aldrich) and 7% ethyl acetate was then added and vortexed for 15 sec. This solution was added to a 1% PVA solution with continuous stirring for 3 hrs at room temperature. The solutions were filtered through a 0.2- μ m filter (Nalgene, Thermo Fisher Scientific, Rockville, MD, USA), collected by centrifugation at 2,000 rpm for 10 min (Eppendorf, Hamburg, Germany), freeze-dried for 16 hrs (Labconco, Kansas City, MO, USA), and stored at -20°C . The microspheres fabricated with this technique were between 2 and 50 microns (Appendix Fig. 1). Growth factors (Latent TGF- β 1, TGF- β 1, or BMP4), neutralizing antibodies (Pan-Anti-TGF- β or Anti-BMP4) (all from R&D Systems, Minneapolis, MN, USA), and TGF- β receptor I (Alk5) inhibitor, SB431542 (Calbiochem, EMD Millipore Chemicals, Billerica, MA, USA) were used (Appendix Table 1). For the MSC differentiation experiments, the mineralization supplements (100 mg/mL ascorbic acid, 10 mM β -glycerophosphate, and 10 mM dexamethasone) (all from Sigma-Aldrich) were also co-encapsulated with individual factors.

Scaffold Fabrication

Macroporous PLG scaffolds were fabricated with a gas-foaming-salt-leaching technique as described previously (Harris *et al.*, 1998) (Fig. 1A). Briefly, salt (NaCl, Sigma-Aldrich Corp.) was ground and sieved to particle size between 250 and 425 μ m and

mixed with PLG microspheres in a mass ratio of 5% PLG to 95% NaCl in a custom mold between two metal discs and compressed with a metal post for 1 min at 1,500 psi in a hydraulic press (Carver, Wabash, IN, USA). The resulting discs were allowed to equilibrate within a high-pressure (800 psi) CO_2 environment in a non-stirred pressure vessel (Parr Instruments, Moline, IL, USA) for 16 hrs, followed by a rapid, controlled reduction in pressure (Fischbach *et al.*, 2007). This causes the polymer to expand and fuse (foaming process) into an interconnected scaffold structure. Foamed scaffolds were stored at -20°C in a desiccated container, and, prior to use, salt was leached by immersion in water, yielding scaffolds that were 90% porous.

Multilayered Design

Multiple PLG layers were fabricated by modifying the protocol above as follows (Fig. 1B): Each layer was sequentially generated by light compacting of the salt-polymer mix (mass ratio of 7.4% PLG to 92.6% NaCl) with a hammer and metal post within the mold. After all layers were assembled, the layered scaffold was compression-molded at 2,500 psi for 1 min in a hydraulic press and routinely processed further.

Core and Shell Design

Two separate custom molds, a 4-mm mold for the core and an 8-mm mold for the shell, were used (Fig. 1C). The 4-mm core scaffold was made as described above for a single-layer scaffold, except that the core was compacted at 500 psi for 30 sec in the hydraulic press. The core scaffold was then transferred to the center of an 8-mm mold, and the PLG-NaCl shell mixture was added to fill the remaining space in the mold; this core-shell scaffold was compressed at 1,000 psi for 1 min, and the scaffolds were then processed as above.

Release and Uptake-Degradation Studies

The cumulative profile was assessed for the release of factors from microspheres (small molecules) or single layers of microsphere-foamed scaffolds (TGF- β 1) suspended in PBS with 0.05% sodium lauryl sulfate (Sigma) at 37°C in an incubator on a rotator (Labnet, Edison, NJ, USA). At given time intervals, supernatant was collected to assess release kinetics with either ELISAs or liquid chromatography-mass spectrometry (LC-MS). TGF- β 1 ELISA (Promega, Madison, WI, USA) was used as *per* the manufacturer's instructions. Briefly, samples were incubated in microplate wells coated with the monoclonal capture antibody, followed by the addition of secondary antibody and detection with a colorimetric substrate. Absorbance was read on a microplate reader (Synergy HT, Bio-Tek Instruments, Winooski, VT, USA). LC-MS for release of the inhibitor (SB431542) was performed as described previously (Arany *et al.*, 2014). To monitor cell uptake and degradation of morphogens, we added factors to low (0.2%) serum cell-culture dishes and collected aliquots of conditioned media at various time-points for assessment.

Tissue Culture

TGF- β reporter (p3TP luc, Mv1Lu cells), BMP4 reporter (BRE-luc, C2C12 cells), kind gifts from Dan Rifkin, New York

Figure 1. Fabrication of PLG scaffolds. **(A)** Procedural steps of conventional, single-phase PLG scaffold involve a single-stage compression of porogen and polymer mix, followed by gas foaming and salt leaching, to generate a macroporous scaffold. **(B)** Steps of layered PLG scaffold fabrication involve sequential, manual (light pressure) compression of several porogen and polymer mixes, followed by gas foaming and salt leaching, to generate multilayered, porous scaffolds. **(C)** The concentric, core-shell PLG scaffold was fabricated in a two-step process with two molds, 4 and 8 mm. The first, smaller (4 mm, core) scaffold was generated routinely and placed in the center of a larger (8 mm) mold surrounded by the porogen-polymer mix and compressed. Routine gas-foaming and leaching was performed to yield the core-shell, porous scaffolds. In (B) and (C), zone (layer or disc) thickness was controlled by varying the quantities of the porogen-polymer mix. Distinct biological factors (morphogen, neutralizing antibodies, or inhibitors) within the polymer (PLG microspheres) were used for individual morphogen zones, as outlined in Figs. 3A and 4A.

University, Langone Medical Center (Abe *et al.*, 1994; Zilberberg *et al.*, 2007), and D1 (mouse mesenchymal stem cell) (ATCC, Manassas, VA, USA) (Diduch *et al.*, 1993) were cultured in complete media composed of 10% FBS, DMEM, Glutamax, penicillin (100 U/mL), and streptomycin (100 µg/mL) (all from Gibco, Life Technologies, Thermo Fisher Scientific, Grand Island, NY, USA) in a 37°C incubator with 5% CO₂ and 90% relative humidity.

Luciferase Reporter Assays

PLG scaffolds were prepared as described above, and luciferase reporter cells (3×10^6 cells/mL) were seeded as described in the Appendix. In some studies, laser treatments were performed 4 hrs after cell-seeding. Following overnight incubation, scaffolds were rinsed briefly in PBS, coelenterazine (25 µg/mL) (Nanolight

Technology, Pinetop, AZ, USA) was added for the evaluation of luciferase activity with a bioluminescence imaging system (Xenogen IVIS-200, Caliper Life Science, Hanover, MD, USA), and images were analysed on Living Image software (Ver 4.1, Caliper Life Science). For 2D reporter studies, cells underwent lysis in passive lysis buffer, and luciferin substrate (both Promega) was added for the evaluation of luciferase activity in a microplate reader (Synergy HT, Bio-Tek Instruments).

MSC Differentiation Assays

To differentiate MSCs in 2D, we plated cells (6×10^4 cells/mL) in 24-well tissue culture dishes (Becton Dickinson, Franklin Lakes, NJ, USA) in complete media. After 24 hrs, the media were replaced with mineralizing media containing growth factors (TGF-β1, 2 ng/mL; or BMP4, 10 ng/mL) in alpha Minimal Essential Medium (αMEM) with Glutamax and non-essential amino acids (NEAA), 10% FBS, 1% penicillin-streptomycin (all from Gibco, Invitrogen, Carlsbad, CA, USA) with mineralization supplements. Media were replaced every 3 days and analysed for matrix markers at 14 days. For 3D differentiation studies, PLG scaffolds were sterilised with 70% ethanol, washed with sterile PBS, and serum-coated overnight. Scaffolds were dried by fluid-wicking, and D1 (10×10^6 cells/mL) were seeded as described in the Appendix. Scaffolds were then allowed to float freely in mineralizing media, replaced every 3 days, on a rotator at 70 rpm (VWR, Arlington Heights, PA, USA) for 14 days.

Laser Treatments

A single treatment with a near-infrared laser, 810 nm, GaAlAs diode laser with a fiber optic delivery system (400 µm fiber) (Newport, Irvine, CA, USA) was used in continuous mode. The laser dose was checked with a power meter before each experiment (Newport). Power density (irradiance, W/cm²) was adjusted by varying distance, while treatment time was kept constant at 5 min to achieve a total energy density of 3 J/cm².

Immunoblotting

Cell-seeded scaffolds were lysed in RIPA buffer (Sigma-Aldrich) with Complete Mini protease inhibitor (Roche, Madison, WI, USA), followed by repeated sonication (Sonics,

Newtown, CT, USA) in lysis buffer on ice. Lysates were centrifuged at 14,000 rpm at 4°C for 20 min, and total protein was estimated with a Bradford assay (BCA, Thermo Scientific Inc.). Samples with equal total proteins were separated in precast Tris-glycine gels and transferred onto nitrocellulose membranes (both Invitrogen, Life Technologies, Thermo-Fisher Scientific). Blots were incubated with various primary antibodies (Appendix Table 2) at 4°C overnight, washed with TBST, and incubated with species-specific secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and chemiluminescence was detected by films (Kodak MR, Sigma-Aldrich).

RESULTS

Fabrication of Simple, Layered Scaffold Systems

Porous polymeric scaffolds were generated (Figs. 2A, 2B) and subsequently seeded with cells (Figs. 2C, 2D). These scaffold systems assisted in the development of morphogen fields that could be assessed with growth-factor-specific luciferase reporters as well as facilitated directed differentiation studies of stem cells, MSCs in this study, into specific mineralized tissue lineages. The luciferase reporters for TGF- β (p3TP-luc) and BMP (BRE-luc) responded to both growth-factor treatments and cell densities in a dose-dependent manner in both 2D (Appendix Figs. 2A-2D) and 3D cultures (Appendix Figs. 3A-3C) with minimal cross-reactivity. In the first study, morphogen gradients were developed by simple placement of two distinct morphogens, TGF- β 1 and BMP4, into opposing outer layers of a three-layer scaffold with an empty intervening layer to separate the two morphogen zones (Fig. 2E). This scaffold design demonstrated diffuse, poorly delineated morphogen fields that did not appear to be conducive for the precise induction of MSC differentiation (Figs. 2F, 2G).

Simulations to Engineer Scaffold Designs with Distinct Morphogen Fields

To further develop more relevant scaffold designs, we generated mathematical models to simulate the concentrations of free growth factors when antagonistic factors (neutralizing antibodies) were also incorporated and released from the scaffolds in

Figure 2. Fabrication of polymeric scaffolds. (A) SEM image of porous PLG scaffold. Scale bar = 2 mm. (B) SEM image of a single pore in the scaffold at high magnification. Scale bar = 100 μ m. (C) SEM image of PLG scaffolds seeded with Mv1Lu reporter cells (blue circles) at low magnification. Scale bar = 100 μ m. (D) SEM image of cells (pseudocolored blue) at higher magnification. Scale bar = 30 μ m. (E) Outline of three-layer scaffold design with morphogens (TGF- β 1 or BMP4) only (no neutralizing antibodies). (F) Luciferase imaging of TGF- β (p3TP-luc) reporter seeded in three-layered scaffolds at 24 hrs. (G) Luciferase imaging of BMP (BRE-luc) reporter seeded in three-layered scaffolds at 24 hrs. Scale bars (F, G) = 2 mm. Data are representative of at least two independent experiments.

opposing locations as their corresponding morphogen. Necessary inputs to the modeling are the release kinetics, cell uptake, and stability of the various agents from the scaffolds. The kinetics of TGF- β 1 release from microsphere-foamed PLG scaffolds as well as uptake and degradation of the factors in cell cultures were assessed with an ELISA (Figs. 3A, 3B). The release of the neutralizing antibodies, being prototypical immunoglobulins, was extrapolated from previous studies. Using these measurements, we first verified development of morphogen fields in simple, three-layer models (Figs. 3C, 3D; Appendix Video 1). We noted the development of diffused morphogen fields as observed in the previous luciferase reporter imaging study (Fig. 2F). Further modifications to simulation conditions by varying

Figure 3. Modeling to design scaffolds with distinct morphogen fields. **(A)** TGF-β1 release from PLG microspheres was assessed with an ELISA, $n = 4$; means and SDs are presented. **(B)** TGF-β1 levels in conditioned media following addition to MvLu1 cells was assessed with an ELISA to calculate uptake and degradation, $n = 2$; means and SDs are presented. **(C)** Outline of three-layer scaffold with morphogen (TGF-β1) alone in a single layer. **(D)** Simulation results demonstrating development and loss of a morphogen field in three-layer scaffold with release of one morphogen (individual frames indicate morphogen concentration at the noted time-point). **(E)** Outline of the five-layer scaffold with morphogen (TGF-β1) and neutralizing antibody. **(F)** Simulation results demonstrating morphogen field resulting from a scaffold design including morphogen and inhibitor, with five layers; again, individual frames document morphogen concentration at each noted time-point.

initial morphogen concentrations within layers, additions of neutralizing antibodies, varying layer sizes as well as addition of diffusion-restricting, outermost scaffold layers suggested that a five-layer design could theoretically yield distinct, discrete morphogen fields (Figs. 3E, 3F).

Spatially Defined Morphogen Fields to Direct MSC Differentiation

Based on the simulation predictions, we generated five-layer scaffolds (Fig. 4A) with morphogens and neutralizing antibodies constituting distinct scaffold layers. Fabrication protocols were optimized to ensure a contiguous, interconnected porous scaffold, as visualized with μ CT imaging (Figs. 4B-4D, Appendix Video 2). These scaffolds demonstrated well-delineated TGF-β1 (dentin) and BMP4 (bone) morphogen fields as noted, with luciferase imaging of reporter cells seeded onto the scaffolds (Figs. 4E-4H). To begin MSC differentiation studies, we first confirmed the ability of MSCs to respond to the dentin cue, TGF-β1, by assessing Smad 2/3 phosphorylation in 2D cultures (Appendix Figs. 4A, 4B). This MSC line, D1, has been previously shown to be inducible to an osteogenic fate following

BMP treatments (Honda *et al.*, 2013). Treating MSCs in 2D culture with either growth factor individually or in combination generated a distinct transcription factor profile (Appendix Fig. 4C) and ECM induction (Appendix Fig. 4D). Strikingly, seeding naïve MSCs throughout the five-layer scaffolds led to dentin-like ECM (DMP1, DSP) markers in one zone, while bone-like ECM (OCN, BSP) markers distinctly segregated in the other zone of the scaffolds (Figs. 4I, 4J), suggesting that cells were induced to distinct fates in the different regions of the scaffolds. However, we noted that the ECM composition in both dentin and bone demonstrated significant redundancy for these markers (Butler *et al.*, 2003). Interestingly, osteopontin appeared to be up-regulated by both morphogens, indicating its multifaceted role in both bone and dentin matrix, as suggested previously (Sodek *et al.*, 2000).

Temporal Activation of Morphogen for MSC Dentin Differentiation

While the layered scaffold design allows one to pre-define cell fate in distinct regions of a scaffold, based on local availability of a freely available morphogen, many biological processes involve a temporally activated, dynamic cue. To determine if this can be mim-

icked in a synthetic system, we took advantage of the ability of a latent morphogen complex, latent TGF-β1, which could be activated on demand by an external cue. We have recently observed the ability of low-power laser-generated reactive oxygen species (ROS) to activate the latent TGF-β1 complex (Arany *et al.*, 2014). As a counter-cue in this scaffold design, we chose a small molecule kinase inhibitor against TGF-β receptor I (Alk5), SB431542 (Laping *et al.*, 2002). A concentric scaffold design (core and shell) was used again to create distinct spatial regions, although in this case with inner vs. outer regions (Figs. 5A-5C). Scaffolds were uniformly seeded with the TGF-β (p3TP-luc) reporter cells, and laser treatments were performed. The scaffolds containing latent TGF-β1 encapsulated within PLG microspheres in the core demonstrated a distinct central zone of luciferase activity, indicating regulated spatial and temporal activation of the morphogen (Fig. 5D), while a reverse orientation did not show these zones as distinctly (Appendix Fig. 5). MSC differentiation studies in these scaffolds demonstrated a clear increase in dentin matrix markers (DMP1, DSPP, and OPN) within the central zone with latent TGF-β1 following laser treatment, compared with the outer zone containing TGF-β inhibitor, SB431542 (Fig. 5E).

Figure 4. Directing multi-lineage tissue differentiation of MSCs in morphogen fields. (A) Outline of multilayer, cylindrical scaffold design with morphogen (TGF- β 1 or BMP4) and neutralizing antibody within other layers. (B) Image of layered scaffold fabricated with polymer-admixed dyes (Orcein red and Alcian Blue) during fabrication to highlight distinct zones. Scale bar = 2 mm. (C) Microcomputed tomography imaging to assess pore continuity following layered scaffold fabrication. A 3D reconstruction of images is available online (Appendix Video 2). (D) Pseudocolored SEM image depicting continuity between scaffold layers (blue dotted box). Scale bar = 400 μ m. (E) Scaffold seeded with TGF- β (p3TP-luc) reporter cells imaged for luciferase activity at 24 hrs. (F) Scaffold seeded with BMP (BRE-luc) reporter cells imaged for luciferase activity at 24 hrs. (G) Scaffold concurrently seeded with both reporters and imaged for luciferase activity at 24 hrs. (H) Fluorescent imaging to assess scaffold orientation from the incorporated fluorescent nanoparticle-attached microspheres. Scale bars (F-H) = 2 mm. (I) Outline of MSC-seeded scaffolds cultured for 14 days; sectioning plane (broken lines) used to collect cell lysates from distinct zones is shown. (J) Immunoblotting for dentin matrix and bone matrix markers was performed, and normalization with HSP70 is shown. Normalized band densitometry values are presented at the bottom of each lane.

DISCUSSION

In this study, we used multi-phase scaffold designs to develop morphogen fields capable of directing MSC differentiation along specific mineralized tissue fates. The use of growth factors and their neutralizing antagonists resulted in spatially discrete spatial zones. Further, the utilization of a laser-activated cue, latent TGF- β 1, allowed for temporal activation of the encapsulated latent factor. These multilayered zones were created without phase discontinuities, facilitating uniform cell seeding and preventing any physical barriers to the development of uniform morphogen fields.

The key role of TGF- β 1 in promoting dentin differentiation of MSCs was noted in this study. TGF- β has been shown to be

a central mediator of dentin pathophysiology (D'Souza *et al.*, 1998; Sloan and Smith, 1999; Oka *et al.*, 2007). We recently observed that laser treatment activates the latent TGF- β 1 complex and promotes a reparative, tertiary dentin response in dental stem cells (Arany *et al.*, 2014). Since tertiary dentin resembles bone-like, lamellar mineral deposition, a prime motivation for this study was to assess the nature of this osteodentin. We speculated that using a combination of osteo- (BMP4) and dentin (TGF- β) cues may generate this hybrid, mineralized tissue. This clearly does not appear to be the case, as evidenced by data from both 2D and 3D assays in this study. This suggests that tertiary dentin induction may need the concerted effects of multiple distinct cues, such as Wnt or FGF, besides TGF- β (Oka *et al.*, 2007; He *et al.*, 2008).

Figure 5. Temporal activation of morphogen fields. **(A)** Outline of core and shell scaffold design. The core was fabricated with Latent TGF- β 1 containing microspheres, while the shell contained the TGF- β RI inhibitor, SB431542. **(B)** Photograph of a top view of the core-shell scaffold. The core was fabricated with admixed gold nanorods to enhance contrast by SEM. Scale bar = 1 mm. **(C)** SEM image of the concentric zones. The interface is denoted (blue dotted box). Scale bars = 400 μ m. **(D)** Luciferase imaging of TGF- β (p3TP-luc) reporter seeded scaffolds with no treatments (Control) and following laser treatment (Laser) at 24 hrs. Scaffold images were photographed (top) and overlaid with luciferase imaging (bottom). Scale bar = 2 mm. **(E)** Immunoblotting was performed for dentin matrix markers from the two distinct concentric zones. MSCs were seeded in core-shell scaffolds; some were treated with laser irradiation. Cell-seeded scaffolds were cultured for 14 days, and normalization with actin is shown. Normalized band densitometry values are presented at the bottom of each lane.

We found that precisely designed and engineered polymeric scaffolds could be used to develop spatial and temporally restricted morphogen fields. The use of a morphogen cue alone generated a spatially diffused field, suggesting that directed morphodifferentiation may be difficult due to both tapering concentrations and interactions with other modifying cues (Wartlick *et al.*, 2009). Therefore, the use of a neutralizing or inhibitory agent not only generates precise, sharply delineated, instructive morphogen fields, but also ensures minimal residual, undesirable interactions (synergistic or antagonistic) with other cue fields. This study demonstrated the utility of using both a neutralizing antibody and a small-molecule inhibitor. However, the latter appears to have some limitations, since its small size leads to increased diffusivity and kinetics (turnover of inhibitor-bound receptors *vs.* stability of antibody-ligand complexes). Nonetheless, we have noted the utility of these small molecules in other contexts (Cao *et al.*, 2010), and their utilization in stem cell biology has potent future implications (Xu *et al.*, 2008). In summary, this study outlines the design and validation of precise morphogen niches within 3D scaffold systems able to provide instructive cues to direct MSC differentiation. These systems can have significant research as well as clinical translational applications for craniofacial tissue engineering.

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