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Micropatterned hydrogels and cell alignment enhance the odontogenic potential of stem cells from apical papilla in-vitro

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Abstract

Introduction.—An understanding of the extracellular matrix characteristics which stimulate and guide stem cell differentiation in the dental pulp is fundamental for the development of enhanced dental regenerative therapies. Our objectives, in this study, were to determine whether stem cells from the apical papilla (SCAP) responded to substrate stiffness, whether hydrogels providing micropatterned topographical cues stimulate SCAP self-alignment, and whether the resulting alignment could influence their differentiation towards an odontogenic lineage in-vitro.

Methods.—Experiments utilized gelatin methacryloyl (GelMA) hydrogels of increasing concentrations (5, 10 and 15%). We determined their compressive modulus via unconfined compression and analyzed cell spreading via F-actin/DAPI immunostaining. GelMA hydrogels were micropatterned using photolithography, in order to generate microgrooves and ridges of 60 and 120 μm, onto which SCAP were seeded and analyzed for self-alignment via fluorescence microscopy. Lastly, we analyzed the odontogenic differentiation of SCAP using alkaline phosphatase protein expression (ANOVA/Tukey $\alpha = 0.05$).

Results.—SCAP appeared to proliferate better on stiffer hydrogels. Both 60 and 120 μm micropatterned hydrogels guided the self-alignment of SCAP with no significant difference between them. Similarly, both 60 and 120 μm micropattern aligned cells promoted higher odontogenic differentiation than non-patterned controls.

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Author contributions

M.H and A.A designed assays, conducted cell experiments, and co-wrote the manuscript. A.T designed, fabricated and characterized the micropatterned hydrogel substrates. P.M assisted with interpretation of results. L.E.B. conceived the idea, conceptualized and supervised the study, and co-wrote the manuscript.

Significance.—In summary, both substrate mechanics and geometry have a statistically significant influence on SCAP response, and may assist in the odontogenic differentiation of dental stem cells. These results may point toward the fabrication of cell-guiding scaffolds for regenerative endodontics, and may provide cues regarding the development of the pulp-dentin interface during tooth formation.

Keywords

GelMA; Micropatterning; Mechanotransduction; Odontogenic differentiation; Regenerative dentistry

1. Introduction

Pulp regeneration has emerged as an exciting alternative to current root canal treatment methods [1]. There is growing evidence of the potential efficacy of various pulp regeneration strategies [1–3], and multiple technologies continue to be developed on a regular basis to address the challenges of engineering the dental pulp both on the lab bench and in the clinical practice. These include regenerative methods based on evoked bleeding protocols [4], where, in short, host periapical stem cells are recruited to the root canal via formation of an blood clot intracanal; cell delivery approaches, where exogenous stem cells are delivered to the site of repair with the aid of polymeric scaffolds [3,5,6]; and the so-called cell homing methods, where bioactive materials stimulate the recruitment of host stem cells through either chemotactic growth factors or their bioactive constituents [2]. A key requirement for dental pulp regeneration methods to work, regardless of the treatment protocol, is that stem cells need to differentiate toward the desired lineage to perform the function that is expected for a given phenotype. For the most part, a critical outcome to evaluating the efficacy of pulp regeneration strategies in-vitro has been the differentiation of stem cells into a mineralizing phenotype, which is consistent with the function of odontoblasts in the pulp. Despite outstanding progress in determining the differentiation potential of various stem cells in the tooth, the influence of microstructural and physical cues on the odontogenic potential of stem cells remains poorly understood.

It has been well documented that both physical and structural properties of the extracellular matrix onto which cells adhere can determine stem cell fate decisions. In a pioneering study Engler et al., demonstrated that the stiffness of polymeric hydrogels alone can guide stem cell differentiation toward multiple lineages [7]. A wide range of studies on the mechanotransduction mechanisms regulating stem cell behavior as a function of substrate stiffness have followed this work, and recent findings point to the importance of various physical properties (i.e. degradation, swelling, microporosity, viscoelasticity, in addition to stiffness) in stimulating odontoblast-like cell spreading, viability and differentiation. In a similar effort to harness the ability to control substrate physical properties to manipulate cell behavior, a vast number of reports have been dedicated to exploring the influence of substrate geometry on stem cell behavior. These works utilize a wide variety of microfabrication methods ranging from lithography-inspired techniques to bioprinting based strategies [8] to fabricate microscale scaffold features where cells are seeded (or embedded within), in order to control cell function and tissue morphogenesis (see Refs. [9–11] for a

review). A set of early reports has demonstrated, for instance, that substrate geometry alone, can guide stem cells to differentiate either toward adipogenic or odontogenic phenotypes [12]. More recent reports have shown that micropatterned hydrogels can also stimulate endothelial cell morphogenesis toward blood vessel formation [13], neuronal cell alignment for improved migration [14], and even cardiomyocyte alignment for enhanced synchronized beating [15]. Despite this growing body of evidence, to date, the influence of micropatterned surfaces on the odontogenic potential of dental pulp-derived stem cells has remained rather elusive. In this study, we employ photolithography to fabricate micropatterned substrates in order to study the response of dental pulp-derived stem cells to these microscale features.

In their native microenvironment, odonotoblast cells are known to polarize, and remain longitudinally aligned relative to the preferential orientation of dentin tubules in the pulp-dentin interface. These cells also have notably elongated processes that dictate the mineralization front in the tooth. Therefore, the longitudinal alignment of odontoblast cells that ensures the pseudo-palisade morphology of the odontoblast layer in the pulp-dentin complex is expected to be critical for the physiology of the tooth. Moreover, the highly elongated morphology of odontoblasts and their processes may be a geometrical feature that is associated with a mineralizing phenotype. We used SCAPs, which have been recognized as a valuable source of stem cells for both endogenous repair as well as cell-based therapeutic approaches to regeneration of the dentin-pulp complex [16] to test two inter-related hypotheses. First, we designed and developed micropatterned hydrogels which were seeded with SCAP cells to test whether such topographical cues could stimulate cell alignment when cultured under odontogenic conditions. Secondly, we tested whether the resulting alignment could significantly influence the differentiation potential of SCAP towards a mineralizing (odontogenic) phenotype. We argue that determining the influence of cell alignment in the odontogenic differentiation potential of stem cells could both elucidate poorly understood mechanisms of tissue formation, and in the future, pave the way for regenerative technologies that stimulate the self-organizing potential of stem cells toward their preferred orientation for the desired function. This, in turn, could guide future strategies of scaffold fabrication for pulp regeneration.

2. Materials and methods

2.1. Gelatin methacryloyl (GelMA) synthesis

GelMA synthesis followed previously published protocols [17]. A 10% (w/v) type A gelatin from porcine skin (Sigma) was dissolved in Dulbecco's phosphate buffered saline (DPBS, Sigma) at 50 °C. The solution was stirred while 8% (v/v) methacrylic anhydride (Sigma) was added in a dropwise manner. The reaction was maintained for 2 h at 50 °C before diluting the solution 5×, with 40 °C DPBS. This solution was dialyzed against distilled water using 12–14 kDa dialysis tubing and maintained at 45 ± 5 °C for five days. The distilled water was changed twice per day. The solution was filtered prior to freezing at −80 °C and lyophilized for five days prior to use. Methacrylation efficiency of 87.8 ± 3.3% was confirmed by ^{1}H NMR [17].

2.2. Hydrogel preparation and mechanical characterization

GelMA macromer at concentrations of 5, 10, and 15% (w/v) was dissolved in DPBS with 0.1% (w/v) 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (Tokyo Chemical Industries) photo initiator at 80 °C immediately prior to use. 5, 10 and 15% (w/v) GelMA hydrogel samples of 8 mm diameter were photocrosslinked under UV light (320–390 nm,) (EXFO Acticure 4000) with a power of 850 mW for 30 s and at a distance of 8.5 cm and stored in DPBS for 4 h prior to testing. Elasticity was measured under unconfined compression at a loading rate of 0.01 mm/s on a universal mechanical testing machine (MTS Criterion model 42) and the elastic modulus of the hydrogel was determined as the slope of the linear portion, corresponding to 0%–10% strain, of the resultant stress-strain curve.

2.3. Photomask design and hydrogel micropatterning

Photomask designs were first created using computer aided design (CAD) software. These masks were designed to have equal alternating transparent and opaque sections for each individual size. Three mask types consisting of 60, and 120 μm alternating patterns, and an OHSU logo mask were fabricated (Output City). Hydrogel constructs were fabricated by dispensing 10 μl of GelMA hydrogel precursor onto a TMSPMA (3-(Trimethoxysilyl)propyl methacrylate) (Sigma) coated glass slide. The hydrogel precursor was then compressed to 100 μm thick disks following previous methods [5]. This hydrogel disk was then photocrosslinked under UV light with a power of 850 mW for 30 s and at a distance of 8.5 cm. Patterned hydrogel was formed by covering the sample with an individual photomask prior to photo crosslinking. The resulting hydrogel was rinsed with DPBS to remove any remaining hydrogel precursor, as illustrated in Fig. 2.

2.4. Cell culture

To determine the ability of dental stem cells to differentiate and align on patterned and non-patterned GelMA hydrogels, we utilized SCAP derived from human third molars following previously published protocols [18]. Cells (P4–P6) were cultured in [H9251]-mem with 2 mM L-glutamine containing 10% (v/v) fetal bovine serum (FBS) with 1% (v/v) penicillin-streptomycin. All cells were cultured in a humidified, 37 °C, 5% CO_2 incubator, with media changes every two days and SCAP were passaged once per week.

2.5. Cell morphology and differentiation analysis

To study the cell morphology and differentiation on GelMA hydrogels, SCAPs (1×10^4 cells/cm^2) were first seeded onto non-patterned constructs of 5, 10, and 15% (w/v) GelMA and cultured for up to 7 days. Cell response to hydrogel concentration was assessed with Live/Dead stain (Molecular Probes), 10% GelMA hydrogel was selected for cell response and fabrication accuracy. Cell morphology was analyzed using ActinGreen/NucBlue assay kit (Molecular Probes) for samples fixed in 4% (v/v) paraformaldehyde (Electron Microscopy Sciences) for 30 min, permeabilized with 0.1% (w/v) triton X-100 solution for 20 min and blocked using 1% (w/v) bovine serum albumin (BSA) for 1 h. Samples were then submerged in Actin Green solution at room temperature for 45 min. The samples were rinsed then stained with NucBlue solution at 37 °C for 10 min. Cell proliferation was analyzed qualitatively. The angle of orientation of at least 100 cells per sample (n = 3) was

quantified using ImageJ (Fiji) and the frequency of alignment with micropatterns of each dimension was assessed using a bin size of 10°.

Cell differentiation was induced during experiments using osteogenic medium containing 50 μM ascorbic acid and 5 mM β-glycerophosphate. Cell differentiation was analyzed by staining samples with Alkaline Phosphatase (ALP) staining kit (Stemgent). ALP expression (n = 3) was quantified in three sample locations by measuring the expression intensity using ImageJ.

2.6. Statistics

Statistical analysis was performed using GraphPad prism 6. The values represent averages ± standard deviations. One-way ANOVA was used to analyze the differences between GelMA concentration, micropattern size and cellular alignment followed by Tukey post-hoc tests (α = 0.05).

3. Results

3.1. Effect of substrate stiffness on cell behavior

The mechanical properties of the substrate have been known to significantly influence cell behavior and function [7,19]. Here, we sought to characterize the elasticity of hydrogels with increasing polymer concentrations from 5% to 10% and 15% (w/v), resulting in increasing extents of crosslinking, in order to study cell response as a function of stiffness. As is evidenced by the steeper stress-strain curves (Fig. 1a) obtained from unconfined compression, the elastic moduli (Fig. 1b) of these hydrogels, increased significantly with increase in polymer concentration from 1.7 kPa for 5% GelMA to 7 kPa and 16.4 kPa for 10% and 15% GelMA hydrogels respectively (Fig. 1a and b).

We studied the response of SCAPs to the matrix mechanical properties by characterizing the ability of cells to cover the hydrogel upon spreading and proliferation, as an early indicator of cell response after 7 days of culture on hydrogel substrates of varying stiffnesses (Fig. 1c–e). We observed a proportionate increase in the rate of proliferation on stiffer hydrogels in comparison with softer hydrogel substrates, indicating a preference for higher mechanical properties for cell survival and function. However, there was no significant difference in cell density between cells cultured on 10% and 15% GelMA hydrogels suggesting that the cell response to matrix mechanics plateaued beyond 7 kPa. Previous work has linked cell spreading and morphology in response to matrix properties to lineage commitment, where stiffer substrates induced greater cell spreading leading to osteogenic differentiation while softer substrates impeded cell spreading and promotes adipogenic differentiation [7,20,21]. Since the goal of our study was to promote odontogenic differentiation of these cells, which we have previously shown to be associated with higher stiffness [5], we chose to use 10% GelMA hydrogels for subsequent studies.

3.2. Fabrication of micropatterns on GelMA hydrogel substrates

We hypothesized that microscale surface topography could influence cell function, where micropatterning substrates to form microscale parallel geometries, reminiscent of

organization of odontoblasts in a pseudo-palisade structure in the pulp dentin complex, could encourage self-alignment and odontogenic differentiation of SCAPs. In order to fabricate these micropatterned substrates, we employed a photo-lithography derived technique, where a photomask consisting of alternating, parallel light-blocking and transmitting sections was placed over the hydrogel precursors before photo-crosslinking to obtain substrates with parallel geometries (Fig. 2). Using this fabrication method, we were able to successfully micro-fabricate intricate micro-architectures (Fig. 3a) as well as parallel geometries of 60 and 120 μm width (Fig. 3b and c) on 100 μm thick GelMA hydrogels.

3.3. Effect of micropatterning on self-alignment of SCAPs

We studied the effect of microscale geometry of substrates on cell morphology and orientation by seeding SCAPs onto unpatterned, 60 μm and 120 μm wide parallel micropatterned GelMA hydrogel substrates (Fig. 4). After 3 days in culture, cells on the micropatterned substrates were preferentially aligned (±10°) along the length of the patterned geometries in the case of both 60 μm and 120 μm wide patterns, while the cells on the unpatterned substrates were more heterogeneously oriented, demonstrating an influence of microscale geometries on cell morphology and behavior. While the narrower pattern appeared to guide better alignment, the fraction of cells aligned in each pattern was not significantly different. Interestingly, self-aligned cells on the micropatterned substrates demonstrated highly elongated cell bodies indicative of the onset of extension into odontoblast-like processes, supporting our hypothesis that substrate geometry driven morphological changes may instruct cell differentiation.

3.4. Effect of micropatterning on odontogenic differentiation of SCAPs

To determine if self-alignment and elongation induced by the microscale parallel substrate geometry indeed induced better odontogenic differentiation even in the absence of dexamethasone, we measured ALP activity in samples of SCAPs cultured on unpatterned 60 μm and 120 μm wide parallel micropatterned GelMA hydrogel substrates (Fig. 5). Our results showed a significantly higher expression of ALP in cells cultured on micropatterned GelMA hydrogels over those cultured on unpatterned substrates. Again, there was no significant difference in ALP expression between cells on 60 μm and 120 μm wide patterned substrates.

4. Discussion

We hypothesized that the mechanical and topographical cues provided by the micropatterned hydrogels would elicit odontogenic response from SCAPs through alignment and morphological changes engendered by integrin mediated cytoskeletal remodeling. Indeed, our results demonstrate that SCAPs are mechanoresponsive and display spread morphologies on stiffer substrates. Moreover, the repeating micropatterns on the hydrogels guided self-alignment of the cells with elongated processes, resembling the highly elongated features of odontoblasts at the dentin-pulp interface. Most intriguingly, self-alignment induced by the micropatterning promoted odontogenic differentiation resulting in a mineralizing phenotype, as evidenced by the higher expression of alkaline phosphatase.

Therefore, substrate mechanics and geometry may inform cell response and potentially assist in the regeneration of dentine-pulp tissues.

The influence of extracellular matrix (ECM) properties in stem cell fate specification has been well established. Previous studies have shown that cell lineage specification can be directed by mimicking the elasticity of the desired tissue [7]. Regulatory cues originating from integrin-mediated 'sensing' of matrix mechanical forces are understood to be conveyed to the cell nucleus through cytoskeletal remodeling resulting in changes to cell morphology [22,23]. Cell morphology, in turn, has been associated with a range of regulatory cell functions including viability, proliferation and lineage specification through the RhoA/ROCK signaling pathway, and has largely been acknowledged as an early indicator of cell response to matrix properties [24,25]. In our study, the functionalization of natural ECM derived gelatin with photocrosslinkable methacrylate groups enabled precise modulation of matrix mechanical properties while preserving cell-matrix interactions through RGD peptide sequences, thereby allowing characterization of cell response to matrix mechanics over a range of stiffnesses [26]. Odontoblasts, however, occupy the pulp-dentin interface spanning soft and hard tissue types, making it difficult to predict the matrix properties that would elicit desired functional response. Nonetheless, mature odontoblasts adopt elongated morphologies with processes extending into the dentinal tubules, which is a hallmark of stem cell response to stiffer matrices. In keeping with our existing understanding of cell response to matrix mechanics, our results show that SCAPs assume elongated morphologies and proliferate better on GelMA hydrogel substrates with stiffness above 7 kPa (Fig. 1), which matches similar trends observed in preceding studies performed using odontoblast precursor cells [5].

While the ability of ECM to modulate cell behavior through elasticity mediated mechanotransduction has been greatly explored, there is also evidence to suggest that matrix geometry at both micro and nano scales significantly influence cell shape and response [27–29]. Several microfabrication techniques have been developed to preferentially orient cells according to specific geometric patterns [30,31], which typically involve soft lithography techniques such as micro contact printing [32,33] or photolithography [34] to control spatial distribution of adhesion proteins, which can create sub cellular structures down to 1 μm in size. Other micropatterning approaches involve the use of electric potential [35], magnetic fields [36], temperature [37] or light [38] to fabricate micropatterns through distribution of cell adhesion constraints. Photolithography techniques eliminate the need for additional etching steps, chemical processing, or sophisticated tools for the generation of patterned substrates, and can create feature sizes down to 1–2 μm. In this study, photolithography with GelMA hydrogel allowed creation of micropatterned substrates with feature sizes of 60 and 120 μm containing uniformly distributed cell adhesion molecules. However, our attempts to reproduce these features in 20 μm size resulted in very low-resolution patterns, which we attribute to bleeding of light around the edges of the photomask (data not shown). Despite the dimensions of our micropatterns being 20–40 fold those of the dentinal tubules, the SCAPs nevertheless responded to the geometry of the substrate, and aligned themselves along the lengths of the patterned grooves.

There is accumulating evidence that the microenvironmental architecture and topography control cell behavior and fate through mechanotransductive signaling in native tissues [39]. While it is clear that substrate topography plays a significant role in adult stem cell morphology and lineage specification [40,41], possibly through clustering of adhesion proteins resulting in reorganization of the cytoskeleton [21,42]. Through micropatterning the spatial arrangement of adhesion proteins, it is possible to guide other important cell functions such as cell motility, cell division or signaling processes through morphogenesis and recent studies have endeavored to utilize cell response to geometric patterns to elicit specific cell functions for regenerative applications [10,43], immune response and wound healing, [44] or to study cancer progression [45]. This concept of interlinking of form (micropatterns) and function (differentiation) has been utilized in recent studies to elicit desired cell function through morphogenesis by microfabrication of specific geometric architectural cues present in native tissues. For example, micropattern guided vasculogenesis has been used to enhance lumen formation by endothelial cells by introducing cells to vessel like architectures [46] and axonal direction and growth was guided by substrates patterned with narrow grooves [47,48]. Similarly, bioinspired fabrication to emulate osteon like structures by creating microchannels within hydroxyapatite substrates encouraged organization and differentiation of odontoblasts [49]. Nevertheless, studies investigating the interface of geometrical features and dental stem cell differentiation have remained elusive. We contend that the architectural similarity between our micropatterned grooves and dentinal tubules stimulated odontogenic differentiation in stem cells. These findings further emphasize the role of microfabrication techniques in designing tissue engineering scaffolds in order to accurately mimic the physiological microarchitectures of native tissues for adequate cell response. Lastly, the use of scaffolds with a planar foot print, such as ours, in the regeneration of 3D tissues, such as the dental pulp, has obvious limitations, and therefore these studies were not intended to progress to in-vivo analyses of tissue regeneration, but rather to provide fundamental science information for the development of future scaffold systems.

5. Conclusion

In summary, we demonstrate that SCAPs had enhanced survival and proliferation on stiffer hydrogels, and that micropatterned hydrogels guided self-alignment of cells with elongated morphologies. More importantly, such self-alignment induced by the micropatterning promoted odontogenic differentiation of SCAP indicating that substrate mechanics and geometry may inform cell response and potentially guide odontogenic phenotype of dental pulp stem cells. These results represent important considerations in scaffold design for applications in regenerative dentistry.

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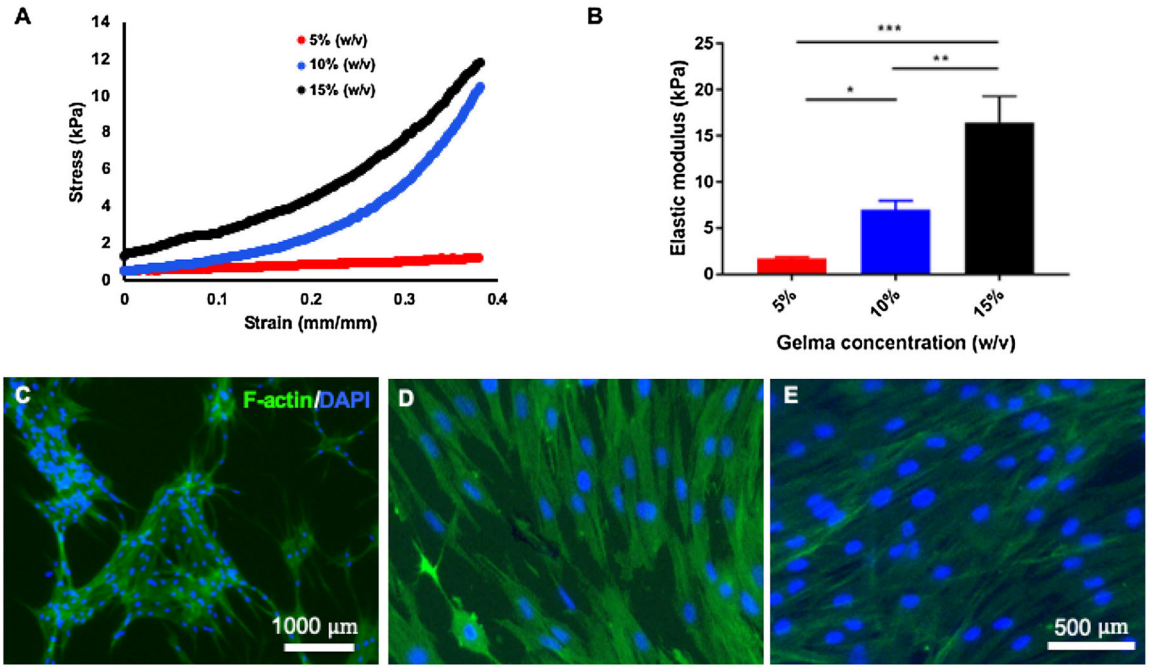

Fig. 1 –.

(a) Stress-strain curves and (b) elastic modulii of 5%, 10% and 15% (w/v) GelMA hydrogels show an increase in mechanical properties of the hydrogels with increased polymer concentration. Representative images of SCAPs cultured on (c) 5%, (d) 10% and (e) 15% GelMA hydrogels for 7 days and immunostained for F-actin (green) and DAPI (blue) shows increased substrate coverage due to apparent enhanced proliferation on stiffer hydrogels as indicated by increased number of cell nuclei. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

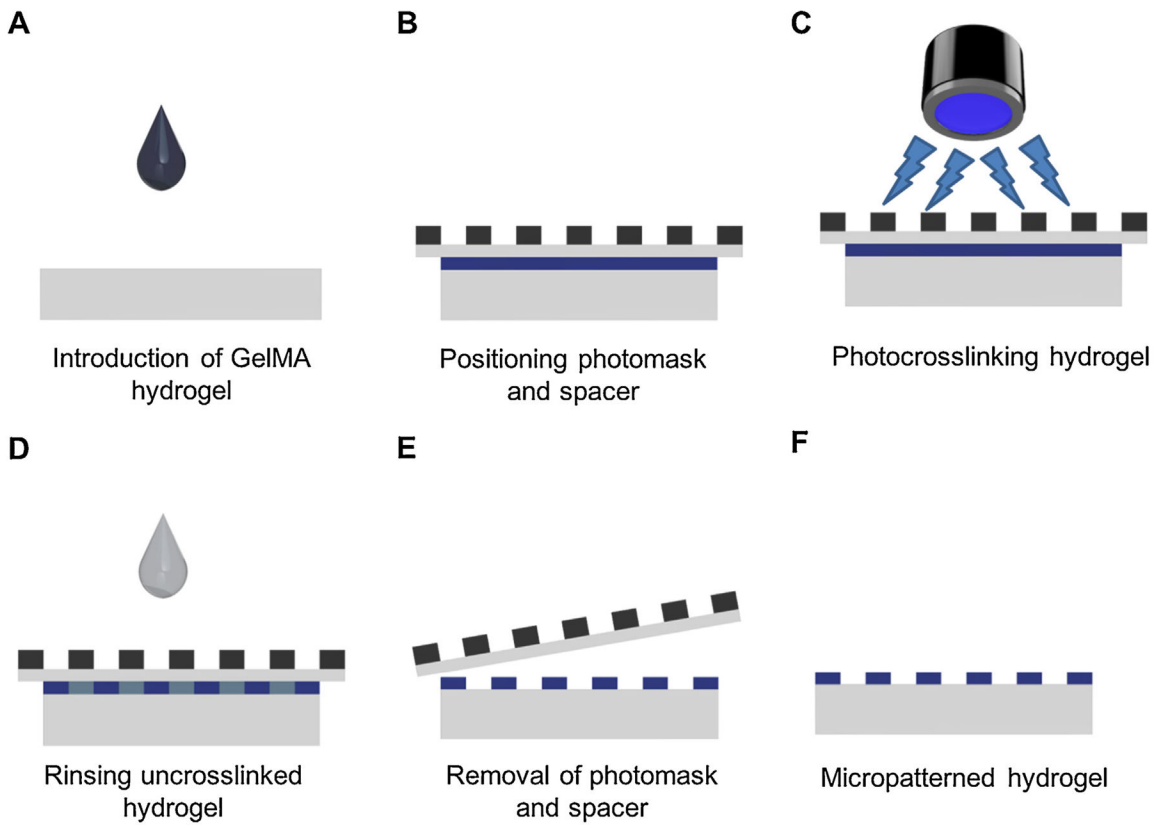

Fig. 2 –.

Schematic depiction of the fabrication of micropatterned hydrogels where (a) a volume of GelMA precursor is placed on a silanized substrate and then (b) covered with a photomask before being (c) exposed to UV light for photopolymerization. (d) Uncrosslinked hydrogel is then rinsed with PBS before (e) removal of the photomask (f) to retrieve the micropatterned hydrogel.

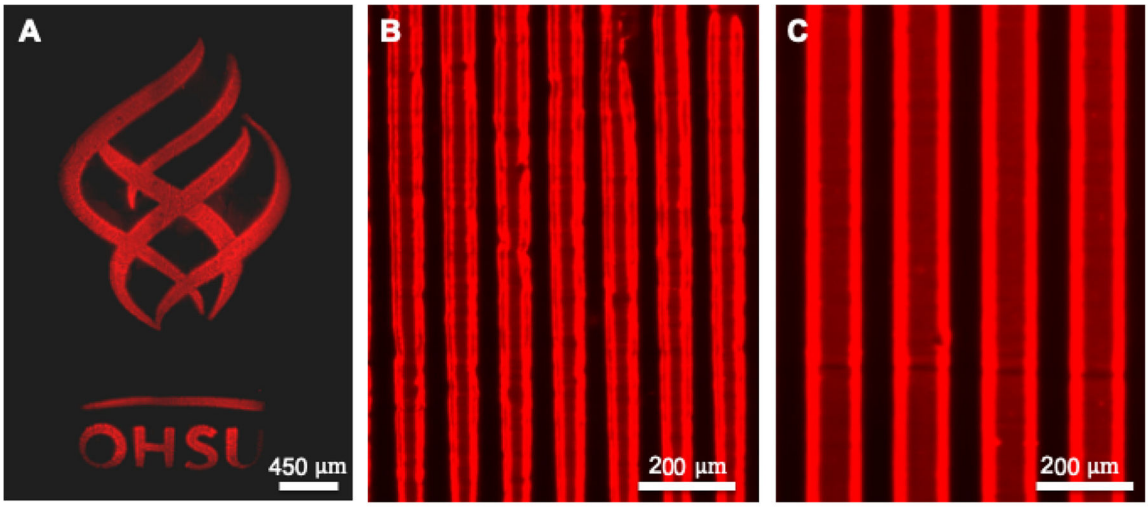

Fig. 3 –.

The photolithography based micropatterning method was successfully used to form the (a) OHSU logo, (b) 60 µm and (c) 120 µm wide grooves in rhodamine stained GelMA hydrogels.

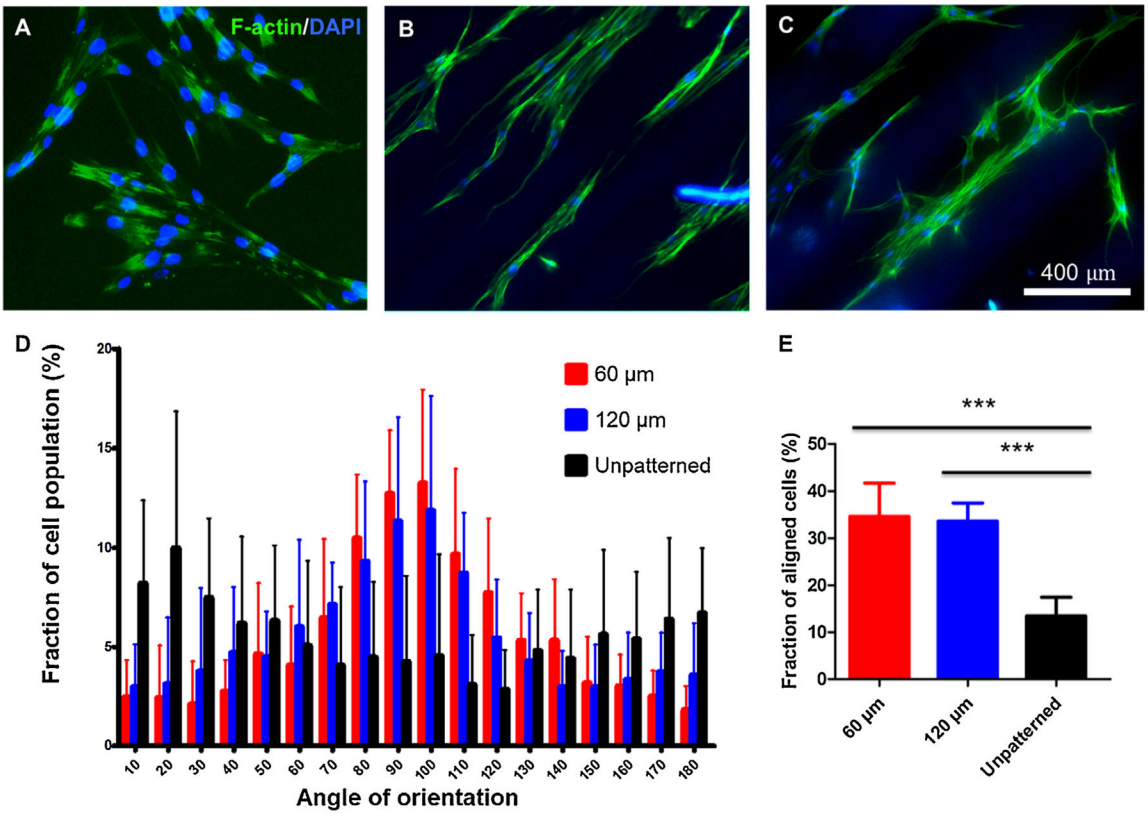

Fig. 4 –.

SCAPs cultured on (a) unpatterned, (b) 60 μm- and (c) 120 μm-patterned GelMA hydrogels showed a (d) tendency to self-align along the substrate geometry within 3 days with (e) no significant difference in aligned populations between 60 and 120 μm patterned groups.

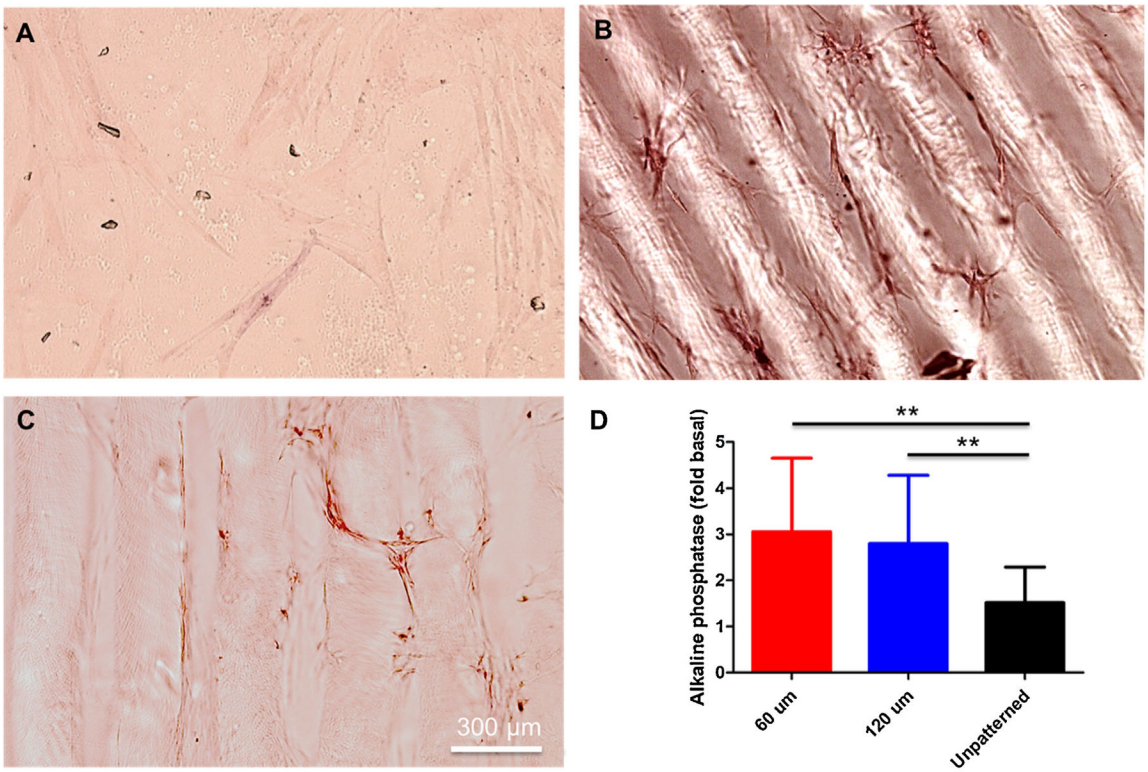

Fig. 5 –.

SCAPs cultured on (a) unpatterned, versus (b) 60 μm- and (c) 120 μm- patterned hydrogels showed (d) increased ALP expression in patterned substrates within 7 days in culture, indicating an increased tendency to an odontogenic phenotype in these hydrogels.