NATURAL LEAF REPLICAS TO STUDY CELL CONTACT GUIDANCE
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ABSTRACT
We describe a method to fabricate cell culture substrates with multi-scale branching topographies that mimic reticulated venation. By replicating the structure of natural leaves in silicone sheets and using these as templates for cell culture substrates, we performed heuristic studies of cell responses to multi-scale topographical features. Cultured human multipotent stromal/stem cells (hMSC) and GFP-transfected retinal pigment epithelial (ARPE) cell morphologies were guided by the underlying substrate by forming branched networks. Cell nuclei were aligned on branches and elongated by factors of 1.8 (MSC) or 1.7 (ARPE) with respect to off-branch nuclei.

KEYWORDS: Biomimicry, Leaf, Vension, Vasculature, Contact Guidance

INTRODUCTION
Topographic features that guide cell behavior in living tissues are presented at multiple length-scales and with various degrees of spatial order. The extra-cellular matrix, surrounding cells, and tissue sub-structure boundaries range in size from nanometers to centimeters and are often striated or branched. Emerging biomimetic cell culture substrates include multi-scale abrasions [1] or wrinkles [2] to reproduce the anisotropy seen in native tissues. In this study, our use of plant leaf replicas includes physiologically relevant structures that are not achieved using existing methods. Plant and mammalian vasculatures are structurally similar because fluid transport through progressive branch points is optimized in both systems in accordance with Murray’s law [3]. Our approach recapitulates key vascular topographic features in vitro and circumvents challenging micro/nanofabrication.

EXPERIMENTAL METHODS
We replicated natural leafs using PDMS and metallic epoxies. These simple and robust methods are schematized in Figure 1. We cut PDMS leafs into circular shapes (diameter = 1 cm) using biopsy punches and placed them in 24-well plates for cell culture. We cultured human multipotent stromal/stem cells (hMSCs), D1 murine MSCs, human umbilical vein endothelial cells (HUVECS), and GFP-transfected retinal pigment epithelial (ARPE) cells on leaf replicas for up to 48 days. Leaf topography and cell morphologies were examined using confocal fluorescence microscopy, atomic force microscopy, and scanning electron microscopy. We used texture segmentation algorithms, based on elevation and slope analysis, to classify substrate features and define “on”- or “off”-branch regions. To study engineered vasculatures, an initial HUVEC layer was cultured on PDMS leafs, followed by fibrinogen coating and addition of a final top layer of HUVECs.

RESULTS AND DISCUSSION
Variously textured substrate regions gave rise to distinct cell and nuclear morphologies. Each of the four cell types formed branched networks of elongated cells that were aligned with the substrate’s branch axes (ARPE nuclei alignment is shown in Figure 2). Topography-dependent cell area and nuclear shape parameters are summarized in Table 1. Clustered stellate MSCs observed in the relatively flat off-branch regions showed increased osteoinduction compared to spindle-shaped MSCs observed within branch regions (Figure 3). As shown in Figure 4, HUVEC/fibrinogen/HUVEC cultures resulted in enclosed lumens and HUVEC top layer orientation depended on lumen diameter.

CONCLUSION
The methods described here enable contact guidance studies on biomimetic substrates with physiologically relevant multi-scale features. Positive and negative molds produce “hill” or “valley” branch structures and metallic replica molding ensures substrate reproducibility. These methods complement simple feature screening and help bridge the gap between in vitro models and in vivo complexity. Ongoing work is focused on hydrogel leafs, MSC/EC co-culture, vascularization, and analysis of topography-dependent EC phenotype.
Figure 1. Cell culture on silicone leafs. a) Schematic of silicone and metallic leaf replica fabrication steps. b) silicone leaf topography shown in panels left to right: CCD image, confocal microscopy brightfield image, elevation data and topographic feature detection/segmentation. c) Circular silicone leaf inserts for cell culture in well-plates (top panels) and cultured human multipotent stromal/stem cells (hMSC, bottom panel); F-actin (phalloidin, green) cytoskeleton and cell nuclei (Hoechst, blue) shape and alignment are topography dependent, scale bar is 100 µm.

Figure 2. Retinal pigment epithelial cell-nuclei orientation on (a) or off-branch (b). Nuclei appear blue (Hoechst), red lines indicate nuclear orientation and cell bodies appear green (green fluorescent protein, GFP). Polar plots show cell number (radial plot axes) as a function of the angle between nuclei and branch axes (circumferential plot axis, in degrees); branch axis is indicated by white arrows. Scale bars are 50 µm.

Table 1. Experimentally measured topography-dependent cell and nuclear shape parameters

<table>
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<tr>
<th>Shape parameter</th>
<th>Cell-type (on- or off- branch areas)</th>
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<tbody>
<tr>
<td></td>
<td>MSC-on</td>
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<tr>
<td>Cell Area (µm²)</td>
<td>1368 ± 330</td>
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<tr>
<td>nuclear elongation</td>
<td>2.37 ± 0.50</td>
</tr>
<tr>
<td>nuclear circularity</td>
<td>0.64 ± 0.12</td>
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Elongation is the ratio of maximum over minimum Feret diameters; Circularity = 4π × (Area/Perimeter²); all values are mean ± SD (N=20).
Figure 3. Osteocalcin immunofluorescent staining of hMSCs. a) hMSCs cultured on relatively flat regions had flat “stellate” morphologies and clustered together; b) Osteocalcin staining was observed within stellate hMSC clusters. Scale bars are 25 µm.

Figure 4. HUVEC lumens with scale-dependent cell orientation. a) Parallel alignment of the top layer of a ~50 µm diameter lumen, scale bar is 50 µm; b) perpendicular alignment of the top layer of a ~100 µm diameter lumen, scale bar is 25 µm; c) arbitrary alignment of individual cells bridging the top of a ~25 µm diameter lumen, scale bar is 15 µm.

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REFERENCES


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