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Micro-scale and meso-scale architectural cues cooperate and compete to direct aligned tissue formation

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ABSTRACT

Tissue and biomaterial microenvironments provide architectural cues that direct important cell behaviors including cell shape, alignment, migration, and resulting tissue formation. These architectural features may be presented to cells across multiple length scales, from nanometers to millimeters in size. In this study, we examined how architectural cues at two distinctly different length scales, "micro-scale" cues on the order of ~1–2 μm, and "meso-scale" cues several orders of magnitude larger (>100 μm), interact to direct aligned neo-tissue formation. Utilizing a micro-photopatterning (μPP) model system to precisely arrange cell-adhesive patterns, we examined the effects of substrate architecture at these length scales on human mesenchymal stem cell (hMSC) organization, gene expression, and fibrillar collagen deposition. Both micro- and meso-scale architectures directed cell alignment and resulting tissue organization, and when combined, meso cues could enhance or compete against micro-scale cues. As meso boundary aspect ratios were increased, meso-scale cues overrode micro-scale cues and controlled tissue alignment, with a characteristic critical width (~500 μm) similar to boundary dimensions that exist *in vivo* in highly aligned tissues. Meso-scale cues acted via both lateral confinement (in a cell-density-dependent manner) and by permitting end-to-end cell arrangements that yielded greater fibrillar collagen deposition. Despite large differences in fibrillar collagen content and organization between μPP architectural conditions, these changes did not correspond with changes in gene expression of key matrix or tendon-related genes. These findings highlight the complex interplay between geometric cues at multiple length scales and may have implications for tissue engineering strategies, where scaffold designs that incorporate cues at multiple length scales could improve neo-tissue organization and resulting functional outcomes.

1. Introduction

Cellular organization and alignment play key roles in determining tissue formation and function across a wide range of tissues, contributing to both biological and mechanical functions of a tissue. For example, during development of collagenous tissues such as tendon [1] cells become highly aligned and assemble a dense, extremely ordered collagen fiber extracellular matrix that is critical to the tissue's function of resisting tensile mechanical loads. An important factor driving cell organization and alignment behaviors during neo-tissue formation is the extracellular microenvironment within which cells reside. In addition to soluble mediators such as growth factors, physical signals such as tissue stiffness, topography and geometry, and extracellular matrix (ECM) ligands have been shown to affect and direct a wide variety of cell behaviors including migration, shape, aggregation, and differentiation (reviewed in Refs. [2,3]). During tissue development, these cues are often presented to cells in complex combinations with spatial and temporal gradients that may be distributed across different length scales. For tissue engineering applications, biomaterial scaffolds that present specific microenvironmental cues (e.g., nano- and micro-topography, cell-adhesive ligands, growth factor gradients) to cells have shown considerable promise for locally guiding cell organization, phenotype, and resulting tissue formation [4–11]. However, relatively little is known about how combinations of these cues interact to precisely control cell and tissue organization.

Tissue architecture is one important category of microenvironmental cue that, via contact guidance [12,13] or cell confinement (i.e., tissue geometrical constraint) [14,15] mechanisms, direct a variety of

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cell behaviors. These cues may occur across a wide range of length scales, from nanometers to millimeters in size. At the nano- and micro-scales, topographies such as fibers, grooves, and pits have been shown to direct cell behaviors including adhesion, shape, survival, focal adhesion and cytoskeletal architecture, gene expression, and stem cell differentiation [16–24]. These cues have been utilized for tissue engineering applications, where, for example, aligned nanofiber scaffolds promote aligned cell morphologies, matrix organization, and tendon-specific cell differentiation [25–28]. Additionally, tissue microenvironments may also include larger meso-scale (hundreds to thousands of microns) geometrical constraints that impose confinement boundary conditions on multicellular cultures, yielding emergent, spatially varying patterns of cell proliferation, growth, differentiation, mechanotransduction, and organization within developing neo-tissues [8,29–34].

Although cells likely experience multi-scale combinations of architectural cues simultaneously *in vivo* and in biomaterial scaffolds, there has been limited work examining how architectural cues from different length scales interact to influence cell behaviors. Several recent studies have investigated the effects of combining aligned topographical cues at nano- and micro-scales on the alignment of single cells, with findings suggesting that cell alignment can be controlled by nano-cues [35,36] or micro-cues [37], and enhanced when cues at both length scales are aligned [38]. However, each of these studies has examined the response of single cells to just one combination of nano/micro-cues (one set of cue geometries arranged in parallel or opposition), and without exploration of downstream behaviors of complex multicellular systems such as tissue assembly or gene expression. Thus, there remains an incomplete understanding of how cues at different length scales, potentially acting through different mechanisms (contact guidance versus multicellular geometric confinement), combine or interact to direct cell and resulting tissue organization.

The objective of this study was to examine how microenvironment architectural cues at two distinctly different length scales, "micro-scale" cues on the order of ~1–2 μm, and "meso-scale" cues approximately two orders of magnitude larger (>100 μm), combine and interact to direct aligned neo-tissue formation. Using a microphotopatterning (μPP) system to mimic architectural features of native fibrillar matrices and electrospun fiber scaffolds, cell-adhesive cues at micro and meso-scales were precisely arranged in a variety of combinations, and the effects on human mesenchymal stem cell (hMSC) organization and aligned collagen fibril assembly were examined. Our findings identify a complex interplay between cues at different length scales and illustrate how these cues may cooperate or compete (depending upon their arrangement) to direct the formation and maintenance of aligned tissues.

2. Materials and methods

2.1. Microphotopatterning (μPP)

Cell-adhesive patterns were created within a non-fouling hydrogel layer [39]. Glass-bottomed cover dishes (MatTek Corp) were amino-silanated (1% (3-aminopropyl)trimethoxysilane, Sigma), activated with 0.5% glutaraldehyde, and spin-coated with polyvinyl alcohol (Sigma, 5.6% w/v in 0.2 N HCl) to create a thin (~150 nm thick) hydrogel layer that resists protein adsorption and cell adhesion (stable for >1 month in culture). Cell-adhesive regions within the gel layer were created via photoablation using a two-photon microscope (Olympus FV1000, 25X 1.05NA objective, Ex: 725 nm), and functionalized with fibronectin (20 μg/mL in PBS with 0.1% pluronic F127, Sigma, followed by blocking with 1% heat-denatured BSA, Life Technologies) to promote cell adhesion. For this study, three "micro-scale" μPP cell-adhesive architectures were investigated: *aligned* 2.03 ± 0.05 μm parallel lines spaced 5 μm on center; feature size measured by staining non-ablated regions with Hoechst 33342 (Sigma) and imaging (Zeiss LSM 510, 63X 1.2NA), measurements in FIJI (NIH), $n = 156$), *grid* (lines of same dimensions added in orthogonal direction), and *unpatterned* (fully-ablated). Additionally, the "meso-scale" pattern boundary dimensions were also varied as described below.

2.2. Cell culture

Human mesenchymal stem cells (hMSCs) isolated from bone marrow aspirates (cells pooled from 3 de-identified donors, surgical waste approved as exempt from review by Duke University Institutional Review Board) were expanded in monolayer (passage 5) and seeded onto μPP substrates (1000 cells/cm^2 for single cell experiments, 18,000 cells/cm^2 for all other experiments) with unattached cells removed via media wash. Cells were cultured (5% CO_2, 37 °C) on patterns in culture media (Advanced DMEM, Life Technologies) with 10% FBS, 200 μM L-ascorbic acid 2-phosphate, 2 mM L-glutamine, and 1% penicillin-streptomycin) for either 2 h (single cell experiments) or 3–12 days (all other experiments), then fixed (4% formaldehyde, Electron Microscopy Sciences) for analysis.

2.3. Cell imaging and analysis

Fixed cell constructs were permeabilized (0.1% Triton X-100, 2 min), fluorescently labeled for actin cytoskeleton (Alexa 488 or 633 phalloidin, Life Technologies) and cell nuclei (Hoechst 33342, Life Technologies), and imaged via confocal microscopy (Zeiss LSM 510, 40X 0.95NA). Image stacks were acquired over a depth of 4 μm above the pattern surface to isolate behaviors near the cell–pattern interface. Cell nuclear aspect ratios and nuclear alignment with pattern direction were determined using FIJI's (NIH) best-fit ellipse tool. For single cell experiments, Feret diameters (max, min caliper distances) for cells not in contact with other cells were measured from actin images (FIJI) and used to calculate cell elongation ($Feret_{max}$/$Feret_{min}$) and alignment ($Feret_{max}$ orientation relative to pattern). Orientation of cell sheets was determined from actin images using fast-fourier transform (FFT) analysis (FIJI Directionality tool), with pixel intensities summed at 2° increments over the power spectrum (−90°–+90°) to generate alignment histograms [40,41]. In cases where overall pattern dimensions were larger than a single image field, tiled images were stitched (FIJI Grid Stitching [42]) prior to FFT analysis. An image's degree of alignment was quantified by calculating the Alignment Index (AI) [40] using Equation (1), where θ_m is the mode of the FFT histogram and I is pixel intensity. In some cases (where specified), the AI was calculated about the μPP pattern direction instead of the histogram mode.

$$\mathrm{AI} = \frac{\int_{\theta_m-20^\circ}^{\theta_m+20^\circ} I\partial\theta}{(40^\circ/180^\circ)\times\int_{-90^\circ}^{90^\circ} I\partial\theta} \quad (1)$$

2.4. Fibrillar collagen imaging and analysis

Fibrillar collagen organization was assessed using both polarized light microscopy and second-harmonic generation (SHG) imaging. For polarized light analysis, samples were stained with picrosirius red (ScyTek) to enhance collagen birefringence and imaged using a polarized light microscope (Olympus E600) equipped with rotating polarizer/analyzer (images acquired at 10° increments; mean pixel intensity over entire μPP pattern area measured for each sample). To detect collagen alignment for a given pattern architecture, differences between mean pattern intensities with polarizer angle were tested (repeated measures ANOVA). To detect differences between pattern architectures, mean pattern intensities at a given polarizer angle were compared (t-test). Bonferroni corrections were made for multiple comparisons. SHG image stacks of samples (2 μm deep, starting just above pattern surface) were acquired using a two-photon microscope (Olympus FV1000, 25X objective, Ex/Em:880/440 nm). Directionality of SHG images was analyzed via FFT as described above, with image AI calculated about the histogram mode or pattern orientation.

2.5. Gene expression

Total RNA was extracted from cells grown on μPP patterns following 6 and 12 days of culture (RNeasy Micro kit, Qiagen), and RNA from each sample quantified (Nanodrop ND-1000, Nanodrop Technologies). Equal amounts of RNA were reverse transcribed (Superscript VILO cDNA Synthesis Kit, Life Technologies), and Real Time PCR performed (One Step Plus, Applied Biosystems; Express qPCR SuperMix, Life Technologies). Transcript levels for genes relating to tendon (collagen types I, III, tenomodulin, tenascin-C) were corrected for reaction efficiency, normalized to GAPDH (endogenous control), then expressed as fold-change relative to hMSCs prior to cell seeding (Day 0). Commercially available primers and probes (Applied Biosystems) were used to compare transcript levels: type I collagen (COL1A1, assay ID Hs00164004_m1), type III collagen (COL3A1, assay ID Hs00164103_m1), tenascin-C (TNC, assay ID Hs00233648_m1), scleraxis (SCX, assay ID Hs03054634_g1), GAPDH glyceraldehyde-3-phosphate dehydrogenase (GAPDH, endogenous control, assay ID Hs02758991_g1).

2.6. Statistical analyses

All data presented as mean ± standard error. Significance for analysis of variance (ANOVA) analyses detected via Tukey's HSD post hoc test unless otherwise noted.