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The modulation of canine mesenchymal stem cells by nano-topographic cues

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Abstract

Mesenchymal stem cells (MSCs) represent a promising cellular therapeutic for the treatment of a variety of disorders. On transplantation, MSCs interact with diverse extracellular matrices (ECMs) that vary dramatically in topographic feature type, size and surface order. In order to investigate the impact of these topographic cues, surfaces were fabricated with either isotropically ordered holes or anisotropically ordered ridges and grooves. To simulate the biologically relevant nano through micron size scale, a series of topographically patterned substrates possessing features of differing pitch (pitch=feature width+groove width) were created. Results document that the surface order and size of substratum topographic features dramatically modulate fundamental MSC behaviors. Topographically patterned (ridge+groove) surfaces were found to significantly impact MSC alignment, elongation, and aspect ratio. Novel findings also demonstrate that submicron surfaces patterned with holes resulted in increased MSC alignment to adjacent cells as well as increased migration rates. Overall, this study demonstrates that the presentation of substratum topographic cues dramatically influence MSC behaviors in a size and shape dependent manner. The response of MSCs to substratum topographic cues was similar to other cell types that have been studied previously with regards to cell shape on ridge and groove surfaces but differed with respect to proliferation and migration. This is the first study to compare the impact of anisotropically ordered ridge and groove topographic cues to isotropically order holed topographic cues on fundamental MSC behaviors across a range of biologically relevant size scales.

Keywords

Mesenchymal stem cells; Topography; Canine; Nuclear alignment; Migration

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Conflict of interest statement

The authors have no conflict of interest to declare.

Introduction

Mesenchymal stromal/stem cells (MSCs) have now been isolated from a variety of species including humans [1] and dogs [2–4]. From both species, MSCs are capable of multi-lineage differentiation into adipocytes, chondrocytes, fibrous tissues and osteocytes [1–3 5–7]. MSCs can be obtained from a variety of tissue sources including bone marrow, adipose (Ad), placental, and liver tissues [1,8,9]. While there is no specific marker for MSCs, they are considered to be CD44 and CD90 positive, and negative for CD34, CD45, CD80, CD86, and MHC-II [2,3,5,10,11]. MSCs derived from canine sources are being used in veterinary clinical trials and canines serve as a model for many human diseases [12,13].

The therapeutic potential of MSCs is being investigated for tissue regeneration and repair [14] as well as for immunomodulation [15,16]. MSCs are being investigated for use in heart disease [17,18], rheumatoid arthritis [10], bone regeneration [19], and inflammatory bowel diseases [20]. Ad-MSCs are advantageous in therapeutic treatments due to the high number of cells that can be obtained and the less invasive nature of obtaining adipose tissue as compared to other MSC sources [21]. Ad-MSC can be derived from allogeneic sources due to their low immunogenicity [15,22,23] which allows for readily available therapeutic supplies of cells without the need for harvesting MSCs from each patient. Both systemic and local delivery of Ad-MSCs can be employed separately or in combination in the treatment of disorders. In many applications, MSCs either engraft in and around the injection site or migrate to distant sites with subsequent engraftment [5].

After administration, MSCs interact with diverse extracellular matrices (ECMs) [24–27]. Intrinsic to all matrices are biophysical attributes (e.g. topography and compliance) that have been shown to dramatically modulate fundamental cell behaviors [24,28–34]. These matrices are complex 3-D structures with a variety of architectures including intertwining networks of fibers and perforated sheets with fibers and pores that vary in dimension from the nano to micron scale [24,25,33,35–37]. Biophysical cues have been shown to dramatically modulate the cytoskeleton and potential for differentiation of MSCs [38–41]. However, a detailed investigation of the impact of a variety of topographic cues on MSC behaviors including alignment, orientation, proliferation, and migration has not been previously reported. With these aims in mind, we report the changes in fundamental MSC behaviors in the presence of topographic features having differing size scales, feature types and surface order.

Methods

Cell isolation and culture

Canine MSCs were isolated and cultured as previously described. In brief, MSCs were isolated from Ad tissue and characterized by flow cytometry [42,43]. Cells were cultured in low-glucose DMEM (Mediatech, Manassas, VA), 10% fetal bovine serum (HyClone Inc, Logan, UT), and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). Cells were incubated at 37 °C, 5% CO₂ and passaged at 70% confluence. The 6 primary cultures were used prior to the 7th passage for all experiments.

Patterned substrate fabrication

Silicon master surfaces were prepared at the Center for Nano-technology (University of Wisconsin) and the Nm2 LLC (Cambridge, MA) as previously described [29,44]. In brief, anisotropically ordered patterned substrates possessing alternating ridges and grooves as well as isotropically ordered substrates patterned with holes were fabricated in arrays containing 6 different pitch sizes separated by planar control regions. Pitch (pitch=ridge width+groove width or hole diameter and lateral distance to the margin of the adjacent hole)

dimensions for patterned surfaces were 400 nm, 800 nm, 1200 nm, 1600 nm, 2000 nm, and 4000 nm. Feature depth was at least 300 nm in all cases. Polydimethylsiloxane (PDMS) stamps were fabricated from the silicon masters as previously described [29,44]. Norland Optical Adhesive -81 (Norland Optical Adhesives, Cranbury, NJ) was then spin-coated into tissue culture polystyrene dishes and stamped as previously described [31] to create the surfaces used in all experiments.

Immunofluorescence

Approximately 1×10^4 MSCs were seeded onto 35 mm dishes containing either ridge and groove surfaces or holed surfaces for 24 h. After incubation, MSCs were fixed with 4% paraformaldehyde for 20 min followed by 5 min digestion with 0.1% Triton X-100. MSCs were then labeled with phalloidin-568 (Invitrogen, Carlsbad, CA) to detect actin filaments and a nuclear label, DAPI (BioGenex, Fremont, CA). The fluorescently labeled cells were imaged with a Zeiss Axio Scope.A1 upright microscope using a $10 \times /0.3\text{NA}$, AxioCam HRc camera (Carl Zeiss Inc., North America)

Alignment, aspect ratio, and orientation

A total of 6 different MSC primary cultures were analyzed. Approximately 3×10^4 MSCs were seeded onto 35 mm dishes containing arrays of patterned surfaces for 24 h prior to imaging. These dishes contained all 6 different pitch sizes (holes or ridges and grooves) as well as intervening planar regions. Phase contrast images were then taken at 4 randomly determined locations per surface pitch. Images were obtained with a Zeiss Axiovert 200 M inverted microscope with a $10 \times /0.4\text{NA}$ objective and an AxioCam HRm camera (Carl Zeiss Inc., North America). For each canine MSC primary culture, a total of 5 dishes (20 fields) per surface type were imaged. The perimeters of the cells that were not in contact with any other cells were traced using Image J ver. 1.42q (NIH, Bethesda, MD). At least 297 cells per pitch were traced and analyzed for angle alignment to the direction of the ridges or the y -axis and the aspect ratio using the fitted ellipse model in the Image J program. To analyze the MSC populations that were both oriented and aligned, 95% confidence intervals were calculated using MSCs on planar controls. The lower bound of the alignment angle (43.72°) and the upper bound of the aspect ratio (4.88) on planar surfaces were used to determine the number of MSCs that were both aligned (angles $< 43.72^\circ$) and elongated (aspect ratio > 4.88) on holed or ridge and groove surfaces. The cell count of oriented and aligned MSCs was divided by the total cells per surface type and pitch to determine that percentage of cells that were both oriented and aligned.

Area

Total cell area was determined using the perimeter tracings described above along with the area calculation function of Image J ver. 1.42q (NIH, Bethesda, MD). Using measurements based on the 4000 nm pitch ridge and groove surfaces, the scale was set to 3.5 pixels per $4 \mu\text{m}$ for all images. At least 297 cells per surface pitch and type were analyzed from the 6 different primary MSC lines.

Proliferation

Seeding and phase contrast imaging of each patterned and planar surface were performed as described above. The same locations were imaged 1 day and 5 days after seeding. The total number of cells in each of 4 images per pitch was summed with the proliferation rate determined by dividing the total number of cells on day 5 by the total number of cells on day 1. A total of 30 surfaces (120, $10 \times$ fields) per pitch from the 6 primary cultures were assayed. Box plots were then used to remove outliers. At least 15 replicates (60, $10 \times$ fields) per pitch size and shape were included in all statistical calculations. To control for seeding

density artifacts across the various pitch sizes and shapes, data were normalized to the respective planar controls for each topographic pattern.

Migration and tortuosity

The Zeiss migration tracking module was used to track individual cells at 10 min intervals for 12 h. Cells that did not remain in the field of view, contacted another cell, or divided during the course of recording were excluded from analysis. Box plots were then used to remove outliers. At least 28 cells per pitch from the 6 primary cultures were tracked. The total distance traveled was divided by the total elapsed time to determine the net migration rate. Tortuosity was calculated by dividing the total distance travelled by each cell by the length of a straight line connecting beginning and end points. Migration direction was determined using the x and y -axis coordinates provided for each cell at each time point. These coordinates were then plotted in a dot plot format for each surface shape and size. At least 28 cells were included in each dot plot.

Statistics

A Sigma Plot 11 software package (Systat Software, Chicago, IL) was used to analyze all data. Analysis of variance (ANOVA) was performed to compare more than two groups. The Tukey post-hoc test or Kruskal–Wallis ANOVA on ranks with Dunn’s post-hoc modification was used depending on the results of Chi-square normality tests. To determine the level of statistical significance between two groups after ANOVA analysis, un-paired two-tail Student’s t -tests and Mann–Whitney rank sum tests were employed depending on the results of Chi-square normality tests. All levels of significance were determined when $*/\# = p < 0.05$, $**/\#\# = p < 0.01$, or $***/\#\#\# = p < 0.001$.

Results

Alignment, cell shape and aspect ratio

Substratum topography modulated the architecture of the actin cytoskeleton in addition to the overall cell shape. On ridge and groove patterned surfaces, the actin fibers aligned parallel to the direction of the ridges compared to both planar and holed surfaces. Surfaces patterned with holes had a more meshwork-like actin architecture as did MSCs on planar surfaces. On hole patterned surfaces the overall cell shape was more rounded with fewer processes as compared to both planar and ridge and groove patterned surfaces (Fig. 1).

MSCs on ridge and groove patterned surfaces were significantly aligned in the direction of the long axis of the ridges on all pitch sizes compared to planar and hole patterned surfaces of identical pitch size. Alignment to the ridge and groove patterned surfaces increased with pitch size. MSCs on 400 nm ridge and groove patterned surfaces had an average orientation angle of 25° to the direction of the ridges as compared to 8° on 4000 nm ridge and groove patterned surfaces (Fig. 2A). MSCs on planar as well as hole patterned surfaces of all pitch sizes, except 400 nm, had an orientation of approximately 45° suggesting random orientation.

In contrast, MSCs on ridge and groove patterned surfaces with pitch sizes of 800 nm or greater were significantly more elongated compared to cells on planar surfaces. In addition, MSCs on ridge and groove patterned surfaces were significantly more elongated on all pitch sizes than cells on the identical pitch, hole patterned surfaces (Fig. 2B). The 95% confidence interval for the orientation angle on planar surfaces was 43.72° – 47.80° and 4.39 – 4.88 for the aspect ratio. Therefore, to be considered both oriented and aligned, MSCs had to have an orientation angle $< 43.72^\circ$ and an aspect ratio > 4.88 . The percentage of MSCs that were both

oriented and aligned was significantly greater on all ridge and groove patterned surfaces compared to both planar and holed surfaces of identical pitch (Fig. 2C).

Cell area and proliferation

In addition to measuring orientation and alignment, the shape and size of substratum biophysical cues was found to impact MSC size and proliferation. MSCs on ridge and groove patterned surfaces of all pitch sizes had a significantly smaller area compared to cells on planar surfaces (Fig. 3A). Similarly, the areas of MSCs on surfaces patterned with holes were significantly smaller compared to planar surfaces on all pitch sizes except 1600 nm (Fig. 3A). When comparing ridge and groove patterned surfaces to hole patterned surfaces, MSCs on 400 nm ridge and groove patterned surfaces had significantly larger areas than MSCs on 400 nm hole patterned surfaces. Also, MSCs on 1200 and 1600 nm ridge and groove patterned surfaces had significantly larger areas than MSCs on the same respective pitch, hole patterned surfaces (Fig. 3A). In contrast to area variations, proliferation rates on patterned surfaces did not vary significantly from planar surfaces. However, proliferation rates were significantly greater on 800, 1600, and 2000 nm ridge and groove surfaces compared to hole patterned surfaces of the same pitch dimensions (Fig. 3B).

Migration rate and direction

Overall, the rate of MSC migration on ridge and groove patterned surfaces increased as pitch increased. In contrast, MSCs on hole patterned surfaces with pitch sizes ranging from 400 to 1200 nm trended towards faster migration rates as compared to planar and larger pitch, holed surfaces. Interestingly, MSCs on 400 nm hole patterned surfaces migrated with the fastest rate (Fig. 4A). Their migration rate was significantly faster than planar as well as 4000 nm hole patterned surfaces though not significantly different from 400 nm ridge and groove surfaces.

In addition to measuring the overall rate of migration, the directional vectors of the migration paths were used to determine tortuosity. On ridge and groove patterned surfaces, MSCs tended to follow a more tortuous path on smaller pitch sizes. Similarly, MSCs on 800 nm hole patterned surfaces moved in a significantly more tortuous path than cells on 4000 nm hole patterned surfaces (Fig. 4B). Dot plots of the migration paths of all cells tracked on planar as well as patterned surfaces of 2000 nm pitches demonstrated the influence of substratum topography on MSC migration (Fig. 5). Overall, MSCs on ridge and groove patterned surfaces traveled greater distances with less tortuosity compared to hole patterned and planar surfaces.

Discussion

After MSCs are injected for the treatment of diverse disorders, they ultimately interact with the ECMs intrinsic elements of the local microenvironment [35–37, 45]. The surface chemistry [46], relative compliance [38,40], and substratum topography [38,39] of the ECM have all been shown to profoundly impact the behavior of MSCs. The present study demonstrates that the size scale, shape and surface order of substratum topographic features have an impact on a variety of fundamental MSC behaviors.

The alignment of the actin cytoskeleton to substratum ridges and grooves as well as the assumption of a more rounded cell shape in response to surfaces patterned with holes are consistent with previous findings in other cell types (Fig. 1). Alignment of the actin cytoskeleton has been previously demonstrated with human bone marrow derived MSCs [39]. More detailed analyses with a number of other cell types including endothelial cells [28,31] and corneal epithelial cells [29,30,32] demonstrate the area and shape response to

biologically relevant topographic features. The similarity of the current results to those observed in other cell types demonstrates a conserved response to substratum topographic cues.

The shape and orientation of a cell relative to the long axis of underlying topographic features of the cell is a consequence of the 3-dimensional organization of the actin cytoskeleton. The vast majority of MSCs on ridge and groove patterned surfaces exhibited increased aspect ratios and a higher degree of alignment. This was consistent with the actin staining pattern and similar to previous reports investigating vascular endothelial cells [31]. In contrast to endothelial cells, previous investigations with corneal epithelium demonstrate that some cell types sense topographic cues as small as 60 nm in width [47]. In our studies, the 400 nm pitch patterned surfaces induced poorly defined alignment of the actin cytoskeleton compared to larger sized pitch patterned surfaces. Thus, the 400 nm pitch of these anisotropic surfaces may start to define the lower limit for MSC biophysical cueing on this topography.

MSCs on 400 nm pitch hole patterned surfaces were the only cells on these isotropic surfaces that had any significant degree of alignment to the neighboring cells (Fig. 2A). Evidence has shown that the differentiation ability of MSCs can be impacted by isotropic topography [7]. Previous reports have also shown that MSCs can align to nano-scale fibers layer on top of micron-scale holes [48]. However, there are no previous reports of cellular organization on isotropic cues for any other cell type. It is possible that the extremely short distance between features (200 nm) resulted in this organization. These results suggest that any impact of isotropic cues on cellular organization might be attributed to their close proximity.

Substratum topography also had a significant effect on the proliferation rate of MSCs (Fig. 3). This is comparable to reports investigating vascular endothelial cells and corneal epithelial cells [29,31]. The finding that the greatest rate of proliferation occurred on 1200 nm pitch patterned surfaces is similar to findings of corneal epithelial cells. However, while the size scale of topographic features has been documented to affect proliferation in a variety of cell shapes, the exact impact of a given feature type and size scale appear to vary to some degree between different cell types.

In addition to changes in proliferation, increased migration rates and contact guidance during migration have been previously documented on larger pitch ridge and groove patterned surfaces with vascular endothelial cells [31]. The MSCs migrated with less tortuosity on these features as well. A more tortuous path of migration was documented on surfaces patterned with holes compared to either flat or ridge and groove patterned surfaces. On hole patterned surfaces of 400 nm and 4000 nm pitch, less tortuosity was observed compared to the other pitch sizes.

The anisotropic surfaces appeared to direct MSCs to a higher degree than the isotropic cues. The data do point out, however, that the anisotropic substrates may be far less influential around the 400 nm pitch sizes. Conversely the isotropic surfaces at the 400 nm pitch size appear to influence MSCs while larger pitches do not. These studies have important implications for MSCs particularly in terms of proliferation and expansion *ex vivo* prior to injection. As these studies progress, it will be interesting to determine gene and protein expression patterns on these substrates.

Canines serve as a model for many human diseases that can be treated with MSCs including heart disease and osteogenic disorders [12,13]. To our knowledge, there are no reports that demonstrate a difference in the capabilities of canine MSCs as compared to human MSCs

once delivered locally or systemically. Results obtained in this study using canine MSCs should be applicable to both veterinary clinical and human patients.

Conclusion

Our data demonstrate modulation of an array of fundamental MSC behaviors by the presentation of substratum topographic cues. A greater effect was observed with the presentation of ridge and groove patterned surfaces compared to hole patterned surfaces. Anisotropic ridge and groove patterned surfaces of pitch sizes ranging from 800 to 2000 nm resulted in increased alignment, orientation, and migration. Prior work has also documented the modulation of differentiation of human MSCs by topographic cues [41]. Overall, it is clear that the impact of topographic cues is dependent on feature type, size scale, surface order and cell type. MSCs encounter a variety of ECMs in both their native environment and following transplantation. Our data show that the response of MSCs to each unique substratum will be at least partially regulated by the size and shape of the topographic cues the cells encounter. Since adipose derived MSCs are currently being used in clinical trials in animal models of human disease, these features can be utilized to direct MSC culture and expansion prior to injection. Additionally, the biophysical attributes of the ECM may play a critical role in determining the therapeutic efficacy of MSCs once engrafted.

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Fig. 1. Substratum topography modulates the cytoskeleton of MSCs. Representative images of MSCs on planar (A), 1600 nm pitch holes (B), and ridges (C) demonstrate the impact of substratum topography on actin fiber orientation. Actin fibers (red) align in a parallel orientation with the ridge and groove surfaces (arrows) as compared to the more meshwork-like orientation observed on both planar and holed surfaces. MSCs on holed surfaces are also more rounded with fewer processes. Nuclei (blue) are labeled with DAPI. Scale bar=50 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Substratum topography modulates MSC alignment and aspect ratio. MSCs on topographically planar as well as holed surfaces of all pitch sizes, except at 400 nm, had an orientation of approximately 45° to the *y*-axis. MSCs on 400 nm holed surfaces had a significantly decreased average orientation angle. MSCs on ridge and groove surfaces were significantly aligned to the ridges on all pitch sizes compared to planar and holed surfaces of identical pitch size. Alignment to the ridge and groove surfaces increased with pitch size.

(A) MSCs on ridge and groove surfaces were significantly elongated compared to topographically planar surfaces on pitch sizes greater than 800 nm. MSCs on ridge and groove surfaces were significantly elongated compared to identical pitch holed surfaces on all pitch sizes. (B) The percentage of cells that were both oriented and aligned was significantly greater on all ridge and groove surfaces compared to planar as well as identical pitch, holed surfaces. and (C) All data are reported as mean±SEM. Significant differences compared to TCP are denoted with an “*” whereas significant differences compared to the different surface topographies of identical pitch are denoted with a “#”, * , # = $p < 0.05$, ** , ## = $p < 0.01$, *** , ### = $p < 0.001$.

Fig. 3.

Substratum topography modulates MSC area and proliferation. MSCs on holed surfaces were significantly smaller compared to planar surfaces on all pitches except the 1600 nm surface. MSCs on ridge and groove surfaces were significantly smaller compared to planar surfaces on all pitch sizes. MSCs on 400 nm ridge and groove surfaces were significantly larger than MSCs on 400 nm holed surfaces. MSCs on 1200 and 1600 nm ridge and groove surfaces are significantly larger than MSCs on holed surfaces of the same pitch sizes. (A) Proliferation rates were significantly greater on 800, 1600, and 2000 nm ridge and groove surfaces compared to holed surfaces of the same pitch sizes. and (B) All data are reported as mean±SEM. Significant differences compared to planar surfaces are denoted with an “*” whereas significant differences compared to the different surface topographies of identical pitch are denoted with a “#”, *, #= $p<0.05$, **, ##= $p<0.01$, ***, ###= $p<0.001$.

Fig. 4. Substratum topography modulates MSC migration rate and direction. MSCs on 400 nm holed surfaces moved at a significantly faster rate than MSCs on planar surfaces. MSCs on 400 nm holed surfaces also migrated at a faster rate than cells on 4000 nm holed surfaces. (A) Tortuosity was calculated by dividing the total distance travelled by each cell by the length of a straight line connecting beginning and end points of the migration path. MSCs on 800 nm holed surfaces moved in a significantly more tortuous path than cells on 4000 nm holed surfaces as well as 1600 and 2000 nm ridge and groove surfaces. MSCs on 800 nm ridge and groove surfaces moved in a significantly more tortuous path than cells on 2000 nm ridge and groove surfaces. MSCs on 2000 nm holed surfaces also moved in a more tortuous path than cells on 2000 nm ridge and groove surfaces. and (B) All data are reported as mean $\pm$ SEM. Significant differences compared to TCP are denoted with an “*” whereas significant differences compared to the different surface topographies of identical pitch are denoted with a “#”, *, #= $p<0.05$, **, ##= $p<0.01$, ***, ###= $p<0.001$.

Fig. 5. Substratum topography modulates the path of MSC migration. Dot plots of the MSCs' migration paths on planar (A), 2000 nm pitch holes (B), and ridges and (C) demonstrate the impact of substratum topography on migration. Migration on planar surfaces (A) is evenly distributed in all directions as compared to the more tortuous paths that more closely circle the point of origin on 2000 nm holed surfaces (B). MSCs on 2000 nm ridge and groove surfaces and (C), migrated further from the point of origin and in a parallel manner to the direction of the ridge and groove surfaces (arrows) compared to both planar and 2000 nm holed surfaces.