Three Dimensional Culture with Stiff Microstructures Increases Proliferation and Slows Osteogenic Differentiation of Human Mesenchymal Stem Cells[**]

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Abstract

A small quantity of stiff microstructures in a 3D matrix regulates human mesenchymal stem cells to increase proliferation and slow differentiation after 10 days in culture. Cell morphology, clustering and gene expression are significantly different although the stiff microstructures are only 0.07% of the total gel volume.

Keywords

cell lineage; gene expression; niche; physical microenvironment; regenerative medicine

Stem cells are regulated not only by soluble stimuli, but also the less well studied physical microenvironment.[1] The physical niche is a combination of structural, cellular and biochemical components that vary among tissues and contribute to control proliferation and differentiation.[2] Cells function differently when grown in a three-dimensional (3D) environment than on a flat two-dimensional (2D) surface.[3] Indeed, it has been demonstrated that cell growth, death, differentiation and motility may all be controlled by culturing cells on 2D extracellular matrix scaffolds of defined geometry[4–8] or mechanical rigidity.[9–11] Recently, we have shown that microstructures in a 3D system inhibits fibroblast proliferation.[12] Nonetheless, the understanding of how topographical features in 3D culture can influence cellular behavior is comparatively understudied. The design and

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development of novel platforms for regenerative therapies requires an understanding of how physical and mechanical cues affect cells in three dimensions. Here, a 3D matrix contains a small quantity of stiff microstructures to probe how the local microenvironment regulates human mesenchymal stem cell (hMSC) functions. Microstructures are shown to cause an increase in hMSC proliferation and slow osteogenic differentiation after 10 days of 3D culture.

Bone marrow derived mesenchymal stem cells (MSCs) are self-renewing cells that retain their ability to differentiate into mesenchymal tissue including bone, cartilage and adipose tissue[13–15] as well as skeletal muscle cells,[16] liver cells,[17] neural cells,[18] smooth muscle cells and fibroblasts.[19] These properties make MSCs an attractive cell source for regeneration of damaged tissue. In this report, the 3D microenvironment is manipulated by including rigid, randomly oriented microstructures in a 3D gel and assessing hMSC function. To this end, photolithography is used to fabricate microstructures (100 µm long × 15 µm^2 cross section) of SU-8 photoresist material for culture in a 3D Matrigel environment as described previously,[12] with modifications (Supporting Information, Figure S1). hMSCs with or without microstructures are uniformly distributed throughout this 1–2 mm deep matrix.

hMSCs spread out with finger-like projections between days 1 and 2 with this morphology persisting throughout the 10 day culture period. The cell body spreading and area of hMSCs are smaller in 3D matrix than when grown on 2D tissue culture plastic. hMSCs begin noticeable interaction with microstructures as early as day 2 which becomes more pronounced between days 5–6. The hMSCs are flattened, more polarized and less stellate-shaped with microstructures compared to without (Figure 1; Supporting Information, Figure S2). The decreased amount of finger-like projections suggests less active filopodial activity which likely coincides with decreased motility. Cells have decreased rates of motility on large expanses of hard substrates compared to softer substrates,[20,21] and apparently the small contact area presented by the microstructure surface acts in a similar manner.

In addition to the organization of hMSCs by 10 days in 3D culture with microstructures, an interesting finding relates to hMSC number. Increased Matrigel concentration is stiffer[12,22] and the corresponding bulk viscosity is higher (Supporting Information, Figure S3A) which causes lower hMSC number (Supporting Information, Figure S3B). To rule out the confounding covariance of protein and growth factor content that changes with Matrigel stiffness, 50% or 75% Matrigel was coated as a 2D surface layer on culture dishes. No differences were observed in hMSC proliferation (Supporting Information, Figure S3C). However, in 3D culture, proliferation increases due to microdomains of stiffness introduced by microstructures (Figure 2A) within the gel. Note that equal cell numbers result on the bottom surface of the dish with or without microstructures in the overarching gel (Figure 2A). Thus, microstructures appear to induce increased proliferation of hMSCs. This was further probed by analysis of cell cluster size at both days 5 and 10. Cluster size and distribution are both larger with microstructures than in the gel alone at days 5 and 10 (Figure 2B, 2C and 2D). Additionally, although robust analysis and quantification is difficult (see Experimental section), cells attached to microstructures synthesize DNA as detected by EdU incorporation (Figure S2G), and thus it is likely that the increase in hMSC number with microstructures is due to a combination of proliferation of hMSCs after attachment to the microstructure as well as individual cells migrating to and collecting around the microstructures. Certainly physical properties affect both migration and attachment in 2D culture where fibroblasts adhere preferentially (durotaxis) to stiffer surfaces[23] and proliferate faster on stiff substrates.[21] Recent work extends these findings to three dimensions, showing that human dermal fibroblasts, initially seeded evenly throughout a collagen matrix with a stiffness gradient, accumulate at the stiffer end after 3 and 6 days of

culture.[24] However, the stiffness of the matrix is related to the collagen concentration, with its many cell anchorage motifs that complicates analysis of the purely physical variable. Here, we show that hMSCs proliferate and preferentially collect around randomly distributed microstructures of high stiffness within a Matrigel matrix of uniform stiffness and chemical composition.

Microarray experiments reveal many transcript level differences between hMSCs cultured in 3D gel alone and in 3D gel with microstructures. Raw p-values reveal 1659 significant differentially expressed transcripts that can be grouped by hierarchal clustering into enriched functional groups including "cell adhesion", "developmental process", "actin cytoskeletal organization and biogenesis", and "cell proliferation" (Table 1). Similar effects were found for cells grown in monolayer compared to cells grown in spheroids[25] where differences in stress fiber formation and cortical actin organization coincide with changes to structural genes related to extracellular matrix, cytoskeletal and adhesion molecules. Further filtering by correcting raw p-values by the Benjamini-Hochberg (BH) procedure identified 119 significant differentials between hMSCs in 3D gel alone or with microstructures (Figure 3A). In 2D culture, hMSCs transition from a dividing, proliferative state at day 2 to a more developed state at day 7.[26] Additionally, hMSCs cultured on flat polystyrene for 2 or 7 days[26] have large differences compared to cells in 3D Matrigel with or without microstructures (Figure 3A). Although there are differences between experiments in two dimensions[26] compared to those here, namely 2D culture on tissue culture plastic versus 3D culture in Matrigel as well as timing of experiments, a qualitative inspection of the heat-map suggests that the transcript levels of hMSCs with stiff microstructures at 10 days (Day 10, 3D GEL + MICROSTRUCTURES) are more similar to those of the likely less differentiated hMSCs on flat polystyrene at 2 (Day 2, 2D) or 7 days (Day 7, 2D) than hMSCs in gel alone (Day 10, 3D GEL).

Microarray data was confirmed by quantitative polymerase chain reaction (qPCR) for several genes. Initial analysis was performed on BH-corrected genes related to migration and the actin cytoskeleton. Importantly, there is significant agreement for 3 out of 5 of these genes ($p < 0.05$, Figure 3B), with a trend for agreement with the other two. Note that agreement is determined by comparison of qPCR expression levels to average expression levels of all microarray probe sets for each gene. Quantitative PCR was also performed on differentially expressed genes as identified by raw p-values ($p < 0.05$) which clustered into several functional groups, namely, "skeletal development" (bone morphogenetic protein 6, BMP6 and collagen 1, COL1a1), "contractile fiber" (ankyrin repeat domain 1 - cardiac muscle, ANKRD1) and "regulation of neurogenesis" (neuroplastin, NPTN) (Table 1 and Supporting Information, Table S1). BMP6 and COL1a1 were significantly down-regulated in hMSCs with microstructures, whereas there was no statistical significance for ANKRD1 or NPTN expression (Figure 3C). The qPCR results indicating decreased expression of bone related genes were further probed by analyzing microarray transcript levels for other genes related to bone development, including Runt-related transcription factor 2, Periostin and Osteocalcin (For complete list see Supporting Information, Table S2). Although lacking statistical significance 22 of 32 genes including 60 of 88 probe sets showed trends for decreased expression of the bone development program with microstructures. Altogether the data suggest that after ten days of culture, microstructures in 3D gel decrease or slow hMSC bone differentiation with gene expression profiles approaching the early expression levels seen in hMSCs in 2D culture at two days. This decreased differentiation likely corresponds (Figure 3D) to the increased proliferation of hMSCs with microstructures.

One cannot conclude that the microstructures *per se* induce these changes, since cell clustering occurred, introducing factors of cell-to-cell mechanical interaction or paracrine factor differences. Thus, the gene expression differences could be due to the clustering

resulting from the inclusion of microstructures. Indeed, higher cell density affects the differentiation potential of MSC.[13,27] Further, nanotopography causes lower cell density which correlates to higher osteogenic gene expression.[28]

In this report, we modulate the uniformity of a soft 3D gel with stiff, randomly oriented microstructures and assess the function of stem cells. hMSC morphology, clustering and gene expression are significantly different although the volume percentage of the stiff microstructures is very low, at only 0.07% of the total gel volume and the bulk properties are unchanged.[12] Thus, microstructures have large effects on stem cells and perhaps injection of small quantities of microstructures *in vivo* could alter proliferation of endogenous or injected stem cells and act to collect such cells preferentially at a desired location and improve engraftment. Optimization of the material, topography and stiffness of the microstructures coupled with growth factor incorporation and release may lead to improved properties of stem cells for regenerative therapy.

Experimental Section

Cell Culture

Institutional approval was received to obtain and use human mesenchymal stem cells (hMSCs) from the Tulane Center for Gene Therapy through a grant from NCRR of the NIH, Grant # P40RR017447. Passage 2 (P2) hMSCs were confirmed to possess "stem" characteristics exhibiting clonogenicity and multi-lineage differentiation potential (osteogenic and adipogenic) after chemical induction as described previously[26] (data not shown). Microarray analyses indicate that gene expression is consistent between hMSCs from different donors, isolated and expanded as described,[26] however, experiments were performed on P2 hMSCs obtained from 2 separate donors. Additionally, some experiments were repeated with P2 hMSCs isolated from whole marrow (AllCells, Berkely, CA) as described previously.[29]

P2 hMSCs were cultured with or without stiff (4.0 GPa) SU-8 microstructures (100 µm long × 15 µm^2 cross section) in a 3D gel environment[12] with modifications. Briefly, hMSCs were detached from tissue culture plates with TrypLE Express (Invitrogen, Carlsbad, CA). Cells were resuspended in DMEM and counted. Growth Factor Reduced Matrigel (GEL, BD Bioscience, San Jose, CA), cells and microstructures or DMEM (control) were mixed carefully on ice in a microcentrifuge tube. This mixture was incubated at 37 °C for 1.5 minutes to initiate gel setting. Mixtures were again mixed carefully and unless indicated otherwise, drops (80 µL) with 25,000 cells with or without 2500 microstructures (~0.07% total volume) were plated onto tissue culture dishes and incubated at 37 °C for 20–30 minutes to complete gel setting (Supporting Information, Figure S1). Finally complete culture media (CCM, consisting of αMEM, 16.5% FBS, 2 m$_M$ L-glutamine and penicillin/streptomycin) was added to each dish. Media was changed once at day 5 of the 10 day experiment.

Microstructure fabrication

SU-8 negative photoresist (Microchem, Newton, MA) was used to create microstructures designed to be 100 µm long with a 15 × 15 µm cross section. Microstructures were micro-fabricated on 3 inch silicon wafers. Each wafer was cleaned in piranha solution (3:1 H_2SO_4:H_2O_2) for 20 minutes, and rinsed with deionized water three times. Wafers were then rinsed with acetone, methanol and isopropanol and then baked at 200 °C for 2 minutes. A thin sacrificial layer of Omnicoat (Microchem) was spun onto each wafer and baked for 1 minute. SU-8 2010 was then spun onto each wafer to achieve a desired thickness of 15 µm. The SU-8 coated silicon wafer was then prebaked at 95°C for 3 minutes. The wafer was exposed using a Karl Suss MJB3 mask aligner to a 365 light source through the microrod

patterned photomask at 5 mW cm^{-2} for 30 sec. The coated wafer was then postbaked (95 °C for 4 minutes), and developed in SU-8 developer until uncross-linked photoresist was dissolved. The height of the microstructures was confirmed using an Ambios Technology XP-2 profilometer. Microstructures were removed by dissolving the sacrificial Omnicoat layer in Microposit developer (Micro Dev, Marlborough, MA). Collected microstructures were centrifuged and rinsed in 70% ethanol three times. Microstructures were then centrifuged and rinsed with sterile 1× phosphate-buffered saline (PBS) three times. Microstructures were finally resuspended in cell culture media.

Imaging of hMSCs in 3D

A Nikon TMS inverted phase contrast microscope was used to observe 3D hMSC cultures daily. Images were recorded with a SPOT RT camera (Diagnostic Instruments Inc., Sterling Heights, MI). In order to observe live cells in a 3D Matrigel environment, hMSCs were incubated with membrane-permeant Calcein-AM (1 μ$_M$ in PBS) and Hoechst (5 μg mL^{-1} in PBS) for 20 minutes. After careful washing with PBS, confocal images were obtained with a Zeiss LSM 510 META microscope.

RNA isolation and reverse transcription

Total RNA was isolated from hMSCs within 3D gel alone or 3D gel with microstructures. Ten days after plating, the droplets of cells with gel or gel and microstructures were gently removed leaving behind the cells adherent to the bottom of the dish. Trizol and the PureLink Micro to Midi total RNA Purification system (Invitrogen) were used to isolate RNA per manufacturer's protocols. RNA concentration was quantified using the Qubit Quantitation Platform (Invitrogen). RNA was reverse-transcribed for 50 minutes at 37 °C and 15 minutes at 70 °C (inactivation) using M-MLV Reverse Transcriptase (Invitrogen) and a thermal cycler (BioRad iCycler, Hercules, CA).

Microarray analysis

For microarray analysis, total RNA was pooled from four independently prepared cultures of hMSCs in gel with or without microstructures. Quality of RNA was assessed by a spectrophotometer (A260/A280 between 1.8 and 2.0) and by a denaturing agarose gel. RNA was labeled, hybridized onto 3 microarray chips per condition (Human GeneChip U133 Plus v 2.0, Affymetrix, Santa Clara, CA) and scanned by the Genomics Core Facility at the University of Illinois at Chicago. All hybridizations passed standard quality criteria. Raw data were normalized by quantiles and summarized by robust multiarray average. Data was analyzed in 'S-Plus' 6.2 statistical package with the 'S+ArrayAnalyzer' v2.0.1 (Insightful, Palo Alto, CA). For pair wise comparisons, statistically significant, differentially expressed transcripts were identified by raw local-pooled- error (LPE) test p-values. For global functional clustering analysis a list of these genes was imported into the DAVID Functional Annotation Clustering tool.[30] Raw LPE test p-values were then corrected for False Discovery Rate by Benjamini-Hochberg (BH) procedure.

Affymetrix *.CEL* files containing data for hMSCs cultured for two or seven days on flat polystyrene[26] were downloaded from Gene Expression Omnibus, processed identically, and used for comparison to the genes that were significantly different (BH-adjusted p-value < 0.05) between the gel alone and gel with microstructures groups. Heatmaps were completed using GenePattern software (Cambridge, MA, http://www.broad.mit.edu/cancer/software/genepattern/).

Quantitative PCR

Initial quantitative polymerase chain reaction (qPCR) confirmation was done on significantly (raw LPE test p-values corrected for False Discovery Rate by BH procedure) and differentially regulated genes (FN, MMP13, MMP1, ACTR2, PHACTR2, Figure 3B) related to observed functional differences (i.e. morphology, migration and actin cytoskeleton) between hMSCs with or without microstructures, as determined by microarray analysis. Next, genes related to differentiation of hMSCs (COL1a1, BMP6, ANKRD1, NPTN, Figure 3C) were chosen if they were significantly (raw LPE test p-values) and differentially regulated as well as classified into functional groups related to differentiation (i.e. skeletal development, regulation of neurogenesis and contractile fiber, Table 1). Primer sequences for all genes are available upon request.

For qPCR experiments, total RNA was isolated and reverse transcribed, as described above, but without pooling from independently prepared cultures of hMSCs with or without microstructures. Experiments were performed with the SYBR Green PCR Master Mix and a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Amplification was achieved by the following protocol: 1 cycle of 50 °C for 2 minutes; 1 cycle of 95 °C for 10 minutes; 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Control preparations without cDNA template were run in parallel for each analysis to identify potential amplification of contaminating genomic DNA. To ensure specificity of polymerase chain reaction (PCR), melt-curve analyses were performed at the end of all PCRs. The relative amount of target cDNA was determined from the appropriate standard curve and divided by the amount of GAPDH cDNA present in each sample for normalization. Each sample was analyzed in triplicate, and results were expressed relative to control condition (hMSCs without microstructures, GEL) as means ± standard error.

Human MSC number

WST-1 reagent (Roche Applied Sciences, Indianapolis, IN) was used to determine the relative number of hMSCs in gel alone or in gel with microstructures. At day 10 of culture, gel drops were removed by gentle washing with DMEM from culture dishes as described above and collected into a microcentrifuge tube. WST-1 reagent was added (10% volume of DMEM used to collect gel drops) and incubated for 2 hours. Tubes were then centrifuged and the supernatant removed for analysis. The absorbance of the supernatant was determined at 450 nm. hMSC number was determined in 3D gel alone or 3D gel with microstructures at 10 days. Relative cell number was determined by dividing the absorbance at 450 nm for the gel with microstructures group by the gel alone group.

Human MSC clustering

In order to determine the effect of microstructures on cell clustering, counts were made for hMSCs cultured in 3D gel alone and 3D gel with microstructures. The 25× objective of a Zeiss LSM 510 META microscope was used to observe Calcein and Hoechst labeled hMSCs. The field of view (FOV) was set in the XY-plane on the bottom of the dish and then manually moved up through the Z-plane (~150 µm) and cell clusters were tallied. The number of cells per cluster was determined by proximity of nuclei (within 100 µm to the microstructure) and overlap of green cytoplasmic staining. The FOV was then reset back to the XY-plane on the bottom of the dish and moved to the adjacent FOV, systematically covering most of the gel drop in a snake-like pattern. At day 5, for cells in the gel alone or gel with microstructures, 786 or 240 clusters were counted respectively for 4 drops over 4 separate culture weeks. At day 10, for cells in the gel alone or gel with microstructures, 431 or 295 clusters were counted respectively for 4 separate culture weeks. To determine whether cell clustering was partially due to hMSC migration and collection or whether some of the hMSCs were proliferating, the Click-iT EdU Imaging kit (Invitrogen) was used to

label nuclei of cells that were actively synthesizing DNA. Although, high background signal resulting because of the alkyne – azide reaction occurring outside of the nucleus and the difficulty in washing these reagents out of the 3D Matrigel makes robust collection of data and thus quantification of this effect difficult, some imaging is possible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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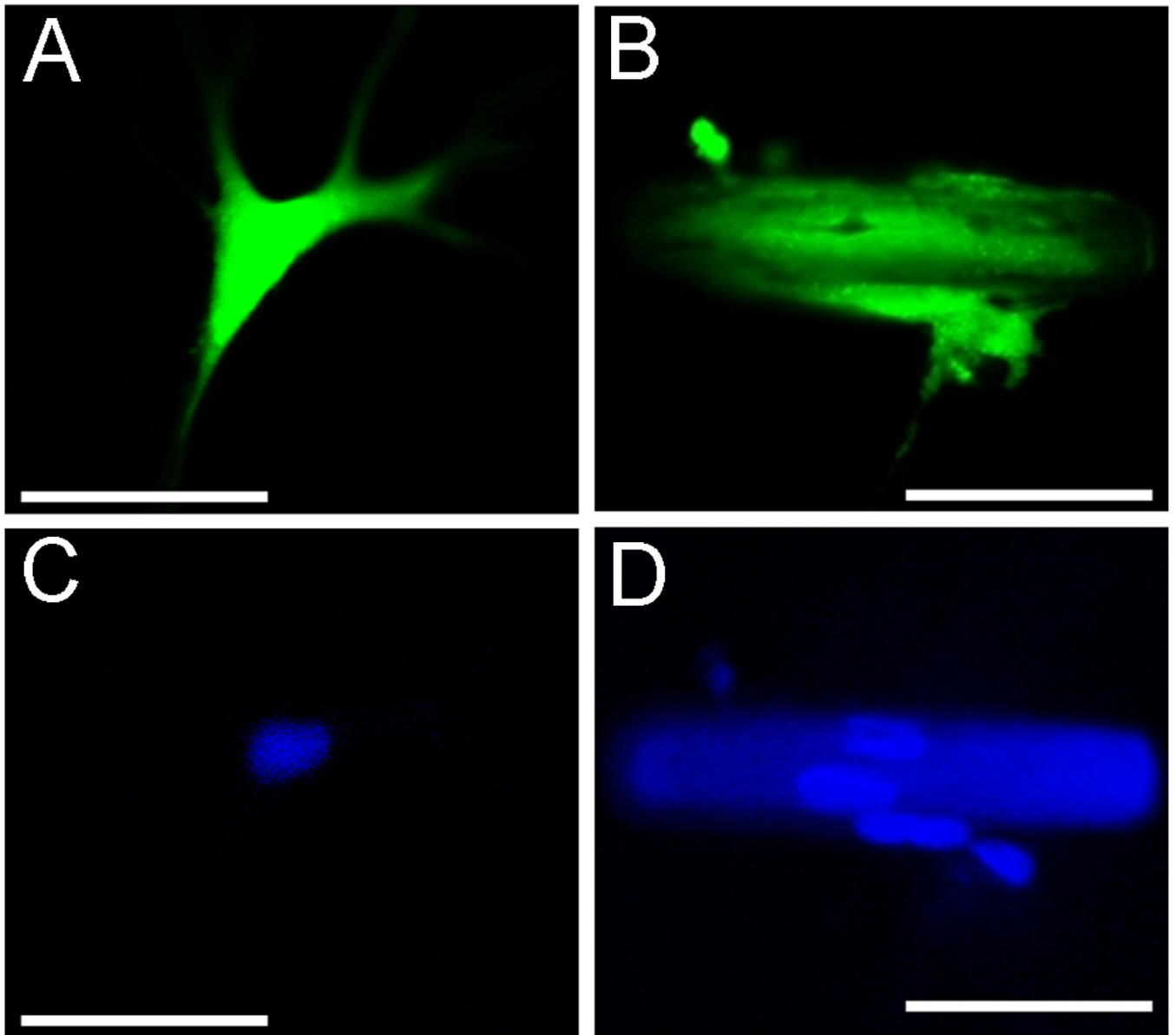30. Huang DW, Sherman BT, Lempicki RA. Nat. Protoc 2009;4:44–57. [PubMed: 19131956]

Figure 1. Microstructures affect hMSC morphology
A. At day 10, hMSCs in 3D Matrigel have a stellate appearance and are most often
dispersed as single cells (C). B. When cultured with stiff microstructures hMSC are more
polarized and tend to cluster around microstructures (D). (A,B) Green - Calcein AM staining
hMSC cytoplasm. (C,D) Blue - Hoechst stains nuclei and also SU-8 microstructures. Images
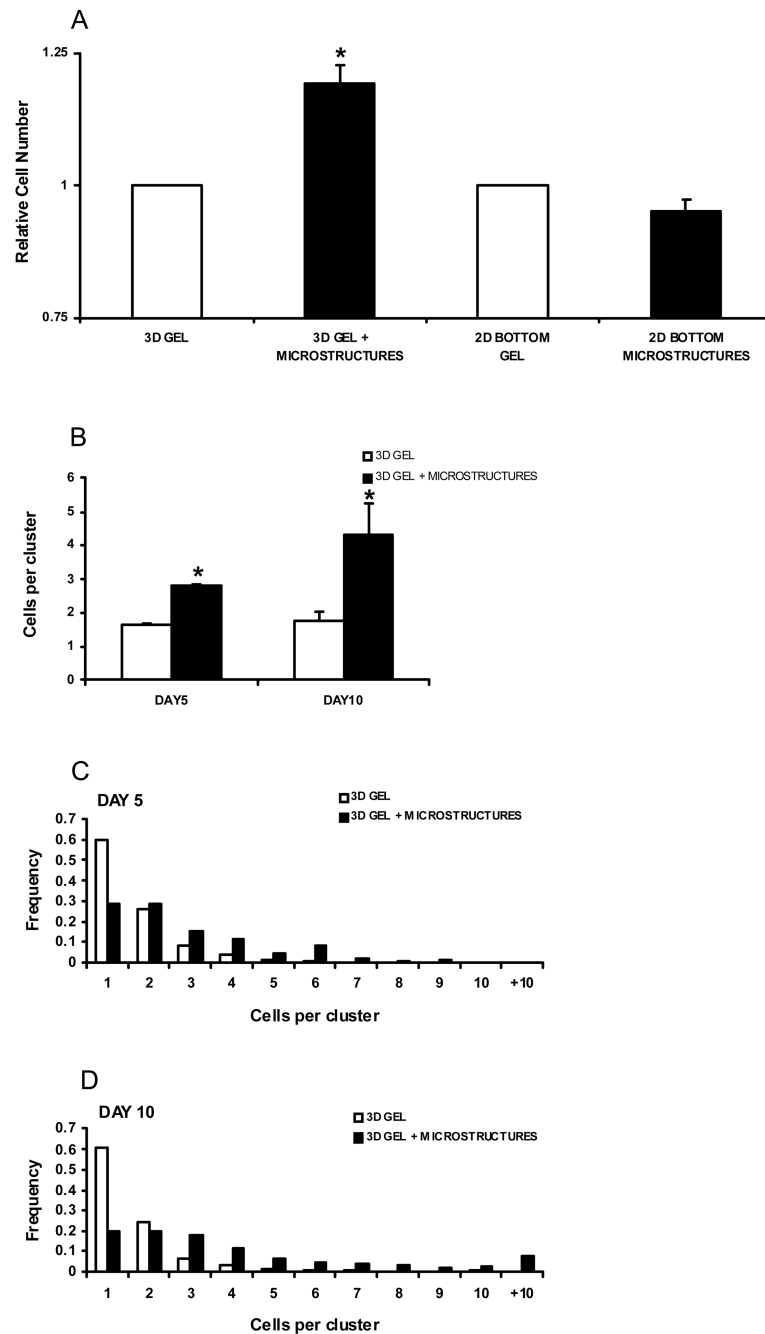representative of n=3 human donors. Scale bars = 50 µm.

A

B

C

D

Figure 2. Distribution of hMSCs in 3D gel with or without microstructures at day 10
A. Cell number is higher in 3D gel with microstructures ($19.4 \pm 3.3\%$, n=4 experiments, 3 human samples, * $p<0.05$) than in the 3D gel alone, while there is no difference in the number of cells on the bottom of the dish. B. Cluster size is higher with microstructures at both day 5 and day 10 compared to gel alone (n=4 experiments, 3 human samples, * $p<0.05$). C. Frequency distribution of cluster size with or without microstructures at day 5 (n=4 experiments, 3 human samples). D. Frequency distribution of cluster size with or without microstructures at day 10 (n=4 experiments, 3 human samples).
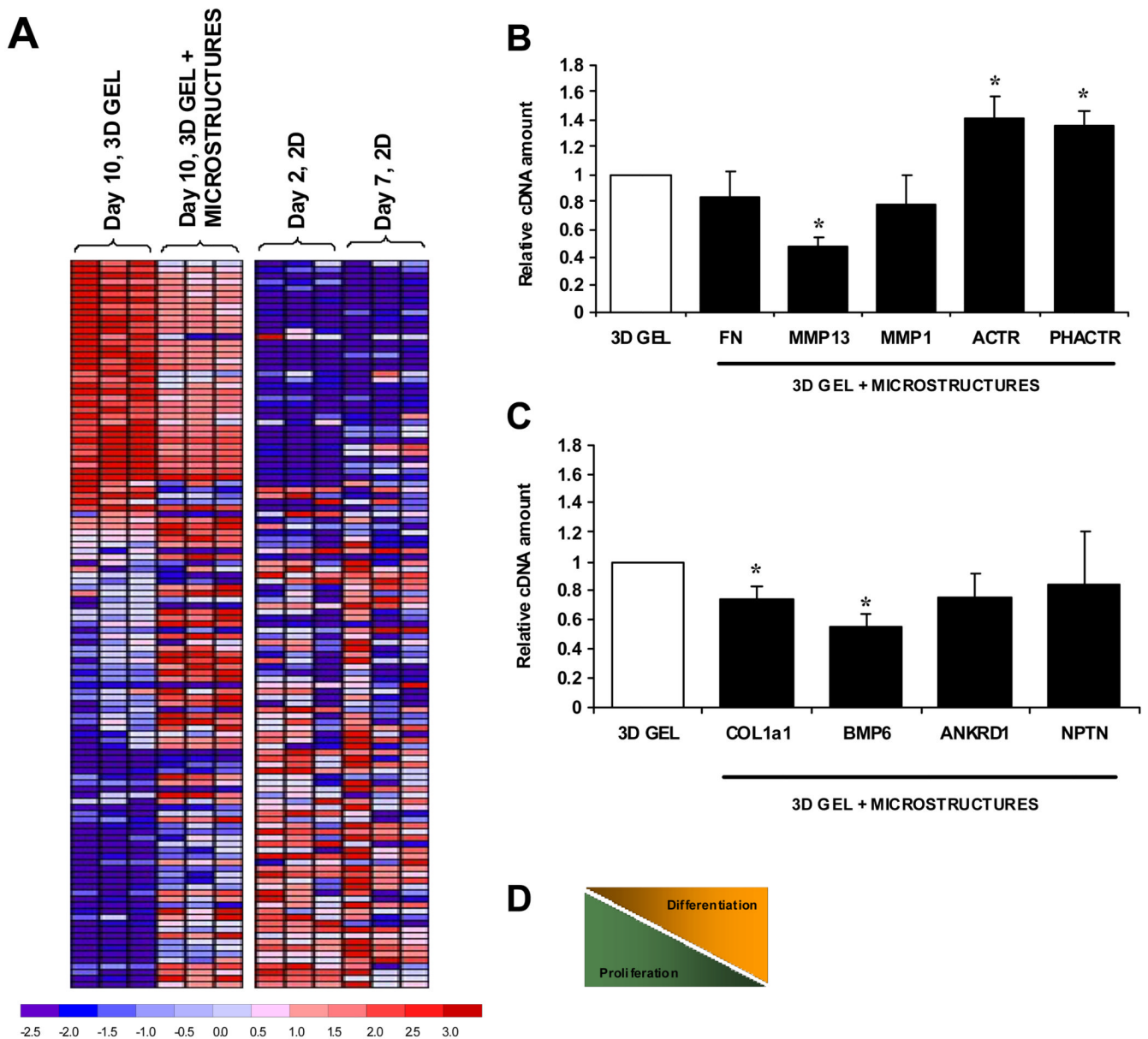
Figure 3. Microarray analysis of hMSC in 3D Matrigel with or without microstructures and confirmation by qPCR
After Benjamini-Hochberg correction, 119 significant differentials between Matrigel alone and Matrigel with microstructures after 10 days of culture were present. A. Heatmap illustrating the expression levels of hMSCs in 3D Matrigel culture with (Day 10, 3D gel + microstructures) or without microstructures (Day 10, 3D gel) versus in 2D flat culture for 2 days (Day 2, 2D) or 7 days (Day 7, 2D)[26] for the 119 significantly different genes (pooling of 4 independent cultures from 1 human sample, 3 microarray chips per experimental condition). B. qPCR confirmation. The direction of expression levels obtained by qPCR agree with those of the microarray with statistical significance for 3 of 5 genes selected and trends for agreement for the other two genes (n=3–4 experiments, 2 human samples, * p<0.05). C. qPCR of genes corresponding to functional groups "skeletal development" (COL1a1 and BMP6), "contractile fiber" (ANKRD1) and "regulation of

neurogenesis" (NPTN). Expression levels of hMSCs in 3D gel with microstructures (black bars) relative to 3D gel (open white bars). (n=3–4 experiments, 2 human samples, * p<0.05). D. Schematic illustrating the likely inverse relationship between proliferation and differentiation.

Table 1

Global gene expression ontology analysis [a].

GO TERM	Gene Count	p-value
Cell adhesion	98	< 0.0001
Developmental process	286	< 0.0001
Actin cytoskeletal organization and biogenesis	36	< 0.0001
Cell proliferation	84	< 0.0001
Skeletal development[b]	32	< 0.0001
Regulation of neurogenesis[b]	9	0.0072
Contractile fiber[b]	11	0.0170

[a] A list of genes differentially expressed (1659 genes, raw LPE p-value < 0.05) in hMSCs with versus without microstructures was input into the DAVID Bioinformatics Resources Functional Annotation Clustering Tool. Selected functional groups are displayed with the number of genes within each group and p-value for enrichment.

[b] See supporting information, Table S1 for lists of genes, corresponding fold changes and p-values.