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RNA-seq reveals diverse effects of substrate stiffness on mesenchymal stem cells

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Abstract

Substrate stiffness has been recognized as an important regulator of cell fate and function, but an understanding of the full extent of processes affected by stiffness is lacking as its transcriptome-wide effects have not been mapped. This limited understanding has restricted the contexts in which engineers can employ stiffness as an engineering design parameter. To address these limitations, we performed RNA-seq on mesenchymal stem cells (MSCs) cultured in alginate hydrogels over a range of moduli to broadly map the transcriptome-wide changes associated with stiffness sensing. We found a large number of stiffness-sensitive genes, and that many genes respond to stiffness in nonlinear ways. Informed by these differential expression results, we explored a hypothesis related to current MSC clinical activity, and found that stiffness can regulate the expression of MSC immunomodulatory markers in response to cytokine stimulation. Overall, these results reveal previously unknown features of MSC stiffness response and demonstrate the value of coupling –omics approaches with biophysical experiments.

Keywords

mesenchymal stem cells; RNA-seq; systems biology; alginate; transcriptome; immune response

For almost two decades, engineers and biologists have studied the effects of adhesion substrate properties on cell phenotype^[1, 2]. Over that time, approaches to control and study these features have evolved, revealing myriad ways in which cells sense and respond to their

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Author Contributions

M.D., L.G., and D.J.M. conceived of and designed the study. M.D. performed the RNA-seq and MSC immunomodulation experiments and analyzed the data. M.D. and D.J.M. wrote the paper.

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Methods

Detailed methods are included in the supplementary information.

Data Accessibility

RNA-seq data can be found in the NCBI Short Read Archive under project [PRJNA419311](https://www.ncbi.nlm.nih.gov/sra/PRJNA419311).

culture substrates^[3]. For instance, Tekin et al found significant transcriptome-wide changes associated with 2D versus 3D tissue culture^[4]. Within 3D cell culture, material characteristics such as adhesion ligand composition and density^[5–7], time-dependent material properties^[5, 8], nanoscale topography^[9], and degradability^[10], among others, have all been shown to affect phenotype, stiffness has perhaps been the most widely explored^[11–13]. In both 2D and 3D cell culture, and recently *in vivo*, stiffness has been shown to affect cell morphology^[2], proliferation^[14], lineage commitment^[15], cell-cell interactions^[16], and tissue regeneration^[11] in experiments that span a wide variety of cell types and materials systems. Mechanistic studies have uncovered biophysical assemblies underlying stiffness sensing^[17] and a number of signal transduction modes that convert substrate sensing into changes in intracellular signaling pathways and gene expression^[18].

The majority of studies on the role of substrate stiffness to date have been largely phenomenological, and a relatively narrow range of gene expression changes have been reported. The lack of an unbiased and broad exploration into these gene expression changes likely limits the field's understanding of the number and extent of processes influenced by stiffness. These limitations have implications for biomaterial design in that they both restrict what processes have been engineered through stiffness, and specific stiffness conditions could unintentionally lead to broad and potentially off-target effects. This knowledge gap also hinders the ability to predict *in vivo* implications for stiffness in regulating physiology.

To address these limitations, we employed a hydrogel system with tunable stiffness along with RNA-seq to understand broad changes in the mouse MSC transcriptome as a function of stiffness. Alginate hydrogels were utilized due to the ability to easily and reliably control their mechanical properties, and their history of use in mechanotransduction studies^[12, 19, 20]. Cell adhesion to these hydrogels was enabled by covalently decorating the polymer with fibronectin mimicking RGD peptides. MSCs are widely used today in various basic and clinical studies, with over 600 ongoing clinical trials^[21], and here, a clonally-derived mouse MSC line was used to minimize the well described effects of cell-cell heterogeneity found in primary cultures^[22]. Additionally, this MSC line has been shown to be responsive to substrate stiffness in similar alginate hydrogels using alternative, functional outputs^[5, 12]. We uncovered wide-ranging changes in gene expression, and found that mMSCs respond to different stiffness ranges in varied ways. After generating and subsequently testing a notable hypothesis from our bioinformatics analysis of the RNA-seq data, we found that stiffness regulates the mMSC immune response under cytokine stimulation. Overall, our results expand on phenomenological explorations of substrate stiffness by providing a view into broad transcriptional changes in mMSCs induced by stiffness.

Results

The transcriptional response to stiffness was profiled in MSCs encapsulated in alginate hydrogels. Cells were cultured in hydrogels at three stiffnesses that span a range previously shown to influence MSC fate choice (Fig. 1a)^[12, 13]. MSCs maintained high viability and were distributed uniformly throughout the hydrogels (Fig. 1b-c, 2a). The zonal mechanism of alginate crosslinking has been previously demonstrated to allow for the maintenance of

hydrogel nanostructure even at different levels of crosslinking, minimizing differences in porosity and diffusion through the gels^[5]. After 40 hours, we performed RNA-seq in order to, in a global and unbiased way, address the early events involved in MSC stiffness sensing and to generate hypotheses about the effects of stiffness sensing. At this time point, cultures had not undergone any significant cell proliferation, as measured by cell number (Fig. 1d), and adipogenic differentiation in all conditions was insignificant (Fig. S1). In stiff gels, a slight increase in the expression of alkaline phosphatase, an early marker of osteogenesis, was noted, consistent with previous work exploring the effects of stiffness on mMSCs^[12] (Fig. S1).

Differential expression analysis revealed 1,637 differentially expressed (DE) genes between the 3kPa and 30kPa conditions, which included the majority, although not all of the differentially expressed genes from the intermediate stiffness comparisons (Fig. 2b). Enrichment analysis for processes run on the DE genes from each comparison showed significant enrichment for processes such as bone remodeling, cell-matrix adhesion, proteolysis, and IL-1 and androgen receptor signaling (Fig. 2c).

DE genes were next classified by the ranges of stiffness to which they responded and the direction of that response (Fig. 3). Binning genes by the stiffness response profiles and ranking by significance revealed a large number of genes that were up or downregulated across each stiffness range, and an even larger number of genes that were up or downregulated only across the extreme stiffness values, consistent with the Venn Diagram (Fig. 2). Intriguingly, a number of genes displayed non-monotonic profiles, either displaying a biphasic response to stiffness, or a response to only the lower or higher stiffness regime. Notable in the Up-Up profile were known mechanosensitive genes *Sdc3*, *Wnt4*, and *Col1a1* along with the aforementioned *Il1rl1*, while the Down-Up profile featured genes known to be tangentially related to mechanics, such as *Mmp9* and *Fgfr3*, and the epigenetic modifier *Chd4*. A large number of genes were only responsive to the extreme stiffness range, such as the actin binding protein *Twf2* and proliferation related *Triaf1*, which were downregulated, and the kinase *Src* and the Rho-A responsive *Dgkq*, which were upregulated with the large increase in stiffness.

We next used the set of DE genes to infer transcription factors likely to display altered activity as a function of stiffness. After surveying the DE genes across the different stiffness pairwise comparisons and performing a transcription factor enrichment analysis on each of those, we noted the presence of the p65 subunit of NFkB, also known as RelA (Fig. 4a) in the 3kPa vs. 18kPa comparison. While the influence of substrate properties on MSC differentiation has been widely studied, an understanding of their influence on immunosuppression is lacking, but has significant relevance given the clinical use of MSC therapy for immune modulation^[23]. The relevance of stiffness to immunomodulatory MSC function is additionally supported by the presence of immune-related processes enriched in our gene ontology analysis (Fig. 2c). We chose to focus on NF-kB, as it is responsive to substrate stiffness, acts as a signaling hub for immunosuppression^[24, 25], and its enrichment in our inferred stiffness-responsive transcription factor list suggests that substrate stiffness could affect other NF-kB-related processes in MSCs. Stiffness was then compared by using 3kPa and 18kPa hydrogels and the MSCs were cultured for three days in media with or

without the known NF- κ B activators TNF α and IFN γ , and with or without an NF- κ B inhibitor peptide (Fig. 4b). Three days was chosen since previous studies on MSC immunostimulation had cultured cells on this timescale^[26, 27]. No differences in proliferation, as indicated by total cell number, nor differentiation down the osteogenic or adipogenic pathways were noted with cytokine exposure over this time frame (Fig. S2). Analysis for activated NF κ B-p65 showed an increase with stiffness and further increases with cytokine stimulation (Fig. 4c). NF κ B-p65 was inhibited with the blocking peptide in all cases, although in the stiff condition with cytokines, the blockade was not complete. The downstream effects of the stiffness-induced enrichment were then explored by examining expression of MSC immunomodulatory markers IDO1, OPN, SDF-1, and COX-2. Stiffness was found to dominate IDO1 expression relative to cytokine stimulation (Fig. 4d). Cytokine stimulation upregulated COX-2 additively with stiffness, an effect that was only NF κ B-p65 dependent in the soft condition (Fig. 4e). Cytokine stimulation inhibited OPN in soft conditions but stimulated it in stiff conditions; only the effects in the stiff conditions were NF κ B dependent (Fig. 4f). SDF-1 was only diminished by cytokine stimulation in the stiff condition, which was also NF κ B dependent (Fig. 4g).

Discussion

This work indicates that the transcriptome-wide response of MSCs to substrate stiffness is significant and varied. Substrate mechanical properties modulate MSC immunomodulatory behavior in response to pro-inflammatory cytokines, showing that a molecular signal can be contextualized by the material microenvironment of the cell. These results suggest a model in which persistent inputs from the adhesion substrate can bias certain network modules and transcription factor activity, setting a “material background” or boundary condition for the cell with which other cellular processes interact.

The RNA-seq analysis of the effects of substrate stiffness on MSCs encapsulated in 3-dimensional hydrogels generated a large number of differentially expressed genes. DE genes binned by their expression across the three different moduli displayed a variety of profiles. The presence of biphasic profiles is consistent with reported “optimal” moduli for MSC differentiation^[12], but also suggests that similar biphasic responses exist for other processes. The response of certain genes to only low or high stiffness ranges is consistent with the quantitative nature of stiffness sensing and motivates the notion that stiffness could “switch” certain other processes on or off. Moreover, the composition of genes within these profiles was both consistent with previous phenomological studies and provided new insights. For instance, genes already associated with bone formation were upregulated with stiffness, consistent with their physiologic context; however, genes previously unassociated with substrate mechanics were found to display rich stiffness-dependent expression, such as Cox6a and Cox6b being oppositely affected by stiffness.

Examination of differentially expressed genes revealed a host of processes influenced by stiffness. The enrichment of processes such as bone remodeling^[28, 29], cell-matrix adhesion^[17], and proteolysis^[10] are consistent with previous mechanotransduction studies; the enrichment of IL-1 and androgen receptor signaling suggests the mechanical regulation of diverse processes that have been less studied in mechanosensing. While consistent with

previous studies concerning the role of substrate stiffness in regulating metabolism and the cell cycle^[18], the identification of modular signaling hubs such as androgen receptor as well as promiscuous transcription factors such as E2F1 suggest that stiffness-sensing could not only be involved in crosstalk with a number of other cellular processes, but also could be an important epigenetic regulator. Stiffness-mediated epigenetic changes, such as histone status and the lamin A/lamin B ratio have been reported^[30, 31], so these results suggest that the activity of broad-acting transcription factors could act as another layer of stiffness-mediated epigenetic regulation.

Additionally, the apparent effects of substrate stiffness on metabolic factors and processes are notable. With recent work emphasizing the importance of metabolic cues in determining stem cell fate choice^[32, 33] such as shifting from glycolysis to oxidative phosphorylation, the enrichment of metabolic processes with substrate stiffness presents a potential route through which substrate-mediated fate choice could act. Activation of early response genes represents one avenue to induce these metabolic changes, as their activation and subsequent MAPK activity (also seen in these results) has been noted in cells exposed to mechanical stretch^[34].

The inferred enrichment of NFkB in stiffness sensing combined with previous work on MSC immunomodulation led to the hypothesis that the MSC response to pro-inflammatory cytokines could be altered by substrate stiffness. We found that the expression of several immunomodulatory markers was stiffness-dependent between 3kPa and 18kPa, and in several cases, mediated by a stiffness-dependence in NFkB-p65 activity. These results indicate that stiffness can act as a switch for the processing of an independent molecular input signal, and that at least in certain cases, this stiffness switch is transduced through NFkB-p65. A precedent for these results is found in the context-dependent regulation of transcriptional modules in immune cells^[35]. Further, since tumors tend to display altered mechanical environments, altered stiffness in that microenvironment could potentially regulate the immunological role of MSCs in a tumor.

These results show that substrate stiffness regulates a diverse array of processes in mesenchymal stem cells and that genes respond to stiffness with a variety of different profiles. As this work expands on the molecular details of previous descriptions of stiffness sensing, we anticipate that similar approaches employing next generation sequencing will expand upon the molecular details of other biophysical properties, such as substrate viscoelasticity and composition, as well as their respective interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:
Alginate hydrogel and mMSC encapsulation characterization.

- a)** Young's Moduli of alginate hydrogels used for mMSC culture. Mean + S.D.
- b)** Viability staining of mMSCs embedded in alginate hydrogels for the three gel moduli after 2 days in culture. Live cells stain green and dead cells stain red. Scale bar - 100 μ m.
- c)** Quantification of fraction of viable cells in hydrogels after 2 days in culture, computed from five representative fields of view for each gel. Triangles represent individual gels. Cells were segmented and counted using ImageJ. No statistically significant difference was found with ANOVA.
- d)** Percentage of original mMSC cell numbers after 2 days in culture in alginate hydrogels. Triangles represent individual gels. No statistically significant difference was found with ANOVA.

Figure 2:
RNA-seq analysis of MSC stiffness-sensing

- Schematic of MSCs encapsulated in alginate hydrogels with representative phalloidin stain showing actual encapsulated cell distribution.
- Venn Diagram of DE genes across different stiffness comparisons. Differential expression was defined as at least a two-fold change and $FDR < 0.05$.
- Enrichment analysis of DE genes for each stiffness comparison.

Fig. 3. Classification of DE genes by stiffness response profile. The bar chart depicts the number of DE genes corresponding to each stiffness response profile. The most significantly DE genes as defined by FDR are listed, where the lowest FDR across any of the stiffness ranges is used for the ranking. Gray color-coding corresponds to a stiffness range with no statistically significant differential expression, while green and red color-coding correspond to positive and negative, statistically significant differential expression, respectively.

- c-g)** Percentage of MSCs positive for pNFkB-p65 (c), IDO1 (d), Cox-2 (e), OPN (f), SDF-1 (g), respectively, after stimulation for 3 days in soft or stiff hydrogels. Cyt+ refers to culture with IFN γ and TNF α , while cyt– refers to culture without these factors. Inh+ refers to culture with NFkB-p65 inhibitor and inh– refers to without NFkB-p65 inhibitor. Data represent mean $\pm$ S.D. Results are representative of at least two independent experiments. (One-way ANOVA, Tukey post-hoc test, for visual clarity given the large number of comparisons, summary statistics are given in Table S2).