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S.I. : Biomaterials - Engineering Cell Behavior

Emerging Concepts and Tools in Cell Mechanomemory

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Abstract—Studying a cell's ability to sense and respond to mechanical cues has emerged as a field unto itself over the last several decades, and this research area is now populated by engineers and biologists alike. As just one example of this cell mechanosensing, fibroblasts on soft substrates have slower growth rates, smaller spread areas, lower traction forces, and slower migration speeds compared to cells on stiff substrates. This phenomenon is not unique to fibroblasts, as these behaviors, and others, on soft substrates has been shown across a variety of cell types, and reproduced in many different labs. Thus far, the field has focused on discerning the mechanisms of cell mechanosensing through ion channels, focal adhesions and integrin-binding sites to the ECM, and the cell cytoskeleton. A relatively new concept in the field is that of mechanical memory, which refers to persistent effects of mechanical stimuli long after they have been removed from said stimulus. Here, we review this literature, provide an overview of emerging substrate fabrication approaches likely to be helpful for the field, and suggest the adaption of genetic tools for studying mechanical memory.

Keywords—Biomaterials, Cancer, Mechanobiology.

INTRODUCTION

The mammalian cell's response to the rigidity of the extracellular matrix (ECM) is mediated predominantly by integrins, heterodimeric receptors directly link the ECM and the cytoskeleton, and activate intracellular biochemical-signaling.[26,35] Synthetic, protein-, and sugar-based biopolymer material networks have been employed to study the effects of mechanics on cell behavior, including polyacrylamide,[23,65,66] Matrigel™,[19] Type I Collagen,[28,93] poly(ethylene glycol),[67] and alginate.[78] It has been suggested that cells respond to mechanical cues *via* protein structural changes,[18,59] alterations to complexes of many proteins (focal adhesions),[4,76] or by regulating the polymerization and stabilization of several micron long cytoskeletal fibrillar polymers (actin, microtubules, and intermediate filaments).[11,50,85]

The stiffness of the ECM can cause significant phenotypic changes in cells.[16,20,34,48] Structurally, cells respond to ECM stiffness *via* conformational changes in the focal adhesion proteins vinculin and talin, which link to the actin cytoskeleton and reveal cryptic kinase domains to initiate downstream signaling.[31,98] These signaling cascades from focal adhesions lead to (1) alterations in the cell cytoskeleton through Rho/ROCK and myosin-regulated tension[94] that feedback to focal adhesion structures and changes in cell adhesion and motility,[68] and (2) changes in transcription factor activation and eventual gene expression.[80] The mechanisms responsible for mechanosensing include stretching of ion channels, or inside-out vs. outside-in sensing of substrate stiffness through integrins and focal adhesions.[50,56] These changes in cytoskeletal tension may directly control gene expression *via* altering force on the cell nucleus and modifying chromatin states.[89] Much is known about these short term phenotypic and longer term transcriptomic changes in cells, but less is understood about the long term changes in cell population dynamics that could be regulated by the stiffness of a cell's substrate or surroundings.

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WHAT IS MECHANO-MEMORY?

The effects of past mechanical cues on cells can persist long after the removal of those cues. Such behavior has been called "mechanical memory".[5,14] Early evidence of mechanical memory in the context of hydrogel stiffness came from experiments with primary rat lung fibroblasts which were cultured on a PDMS substrate of a specific stiffness for defined periods, followed by culture on PDMS substrate of a different stiffness.[5] On stiff but not soft substrates, primary fibroblasts typically differentiate into a myofibroblast phenotype, characterized by expression of α-smooth muscle actin and increased contractility. When these fibroblasts were cultured on stiff substrates for 3 weeks, which promoted myofibroblast differentiation, and then switched to soft substrates, the myofibroblast phenotype persisted up to the longest time point they measured (2 weeks). Conversely, culture on soft substrates for 3 weeks reduced the extent of myofibroblast differentiation when these cells were transferred to stiff substrates. These experiments showed that mechanical 'priming' or 'dosing' can induce long-term effects in cells which are irreversible on time scales of weeks after removal of the mechanical dose.

Sustained effects of mechanical stimuli were demonstrated in the context of Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding domain (TAZ) signaling in human mesenchymal stem cells (hMSCs).[97] Upon activation, YAP/TAZ localizes to the nucleus and triggers gene expression. This localization is mechanosensitive because YAP/TAZ is present in the nucleus of hMSCs on stiff substrates ($E \sim 40$ kPa) but not on soft substrates ($E \sim 1$ kPa).[22] Culture of hMSCs on stiff tissue culture plastic (Young's modulus ~ 3 GPa) caused YAP/TAZ translocation to the nucleus.[97] Subsequent transfer of these cells to soft substrates (Young's modulus of 2 kPa) caused YAP/TAZ deactivation only when hMSCs were cultured for short times on the stiff gels (~ 1 day). Longer cultures over several days on the stiff gels resulted in irreversible activation of YAP/TAZ, such that the nuclear localization of these proteins did not decrease even after culture on soft substrates for 3 days. Thus, YAP/TAZ signaling pathways are not only mechanosensitive, but their effects may persist depending on the time of 'mechanical dosing'. In addition to activation, YAP/TAZ localization caused osteogenic differentiation as measured by RUNX2 expression, again, in a mechanical dose dependent manner.[97] Differentiated human mammary MCF10A epithelial cells also possess the capacity for mechanical memory.[61] Continuous MCF10A culture on a collagen-coated polyacrylamide substrate with spatially variable stiffness showed that cells grown on the stiff portion migrated faster and retained nuclear YAP on the soft portion than cells initially on the soft substrate.

Because changes to YAP/TAZ signaling pathways in hMSCs persisted for 3 days of culture on soft substrates, it is possible that these changes are heritable across cell generations due to epigenetic alterations.[99] Anseth and coworkers investigated the effect of mechanical dosing on histone modifications. Histone acetylation in hMSCs was found to be higher on stiff substrates than on soft substrates,[44] and consistently, chromatin was more decondensed in these cells on stiff substrates. Furthermore, the levels of histone acetyl transferases (HATs) were higher, while those of histone deacetylases (HDACs) were lower on stiff substrates. Importantly, histone acetylation in cells cultured on stiff substrates followed by substrate softening was reversible only for short culture times (1 day) on the stiff substrates. For longer culture times (10 days), the acetylation was irreversible, such that it stayed high despite softening the gel for as long as 10 days post softening (the longest time point they measured). Collectively, these results suggest that epigenetic modifications may be a mechanism to store mechanical memory.

Alternatively, microRNA miR-21 has suggested as a key mediator of mechanical memory".[54] This was demonstrated with primary rat bone marrow-derived mesenchymal stem cells cultured on PDMS substrates. Priming of MSCs on soft substrates prevented the expression of alpha smooth muscle actin when subsequently cultured on stiff substrates. Conversely, stiff-primed cells retained alpha smooth muscle actin levels when transferred to soft substrates. Interestingly, knockdown of miR-21 at the end of the stiff priming period re-sensitized cells to the soft substrates. The authors suggested that while YAP/TAZ may act as a memory storage pathway on the shorter time scales, miR-21 may provide long-term storage of mechanical memory.

In summary, mechanical memory is the persistent effects of mechanical stimuli on cells, long after the mechanical stimulus has been removed (Fig. 1). Whether the word 'memory' is appropriate for such effects is not clear, given that "memory" implies retrieval of stored information. The experiments described above certainly support the notion that mechanical stimuli can cause permanent or irreversible effects on cell differentiation, activation, and growth rates, but it stands to reason that such irreversible effects do not necessarily imply a corresponding memory pathway. We suggest that to truly prove the presence of a memory encoded in signaling pathways, the information needs to be temporarily forgotten and then

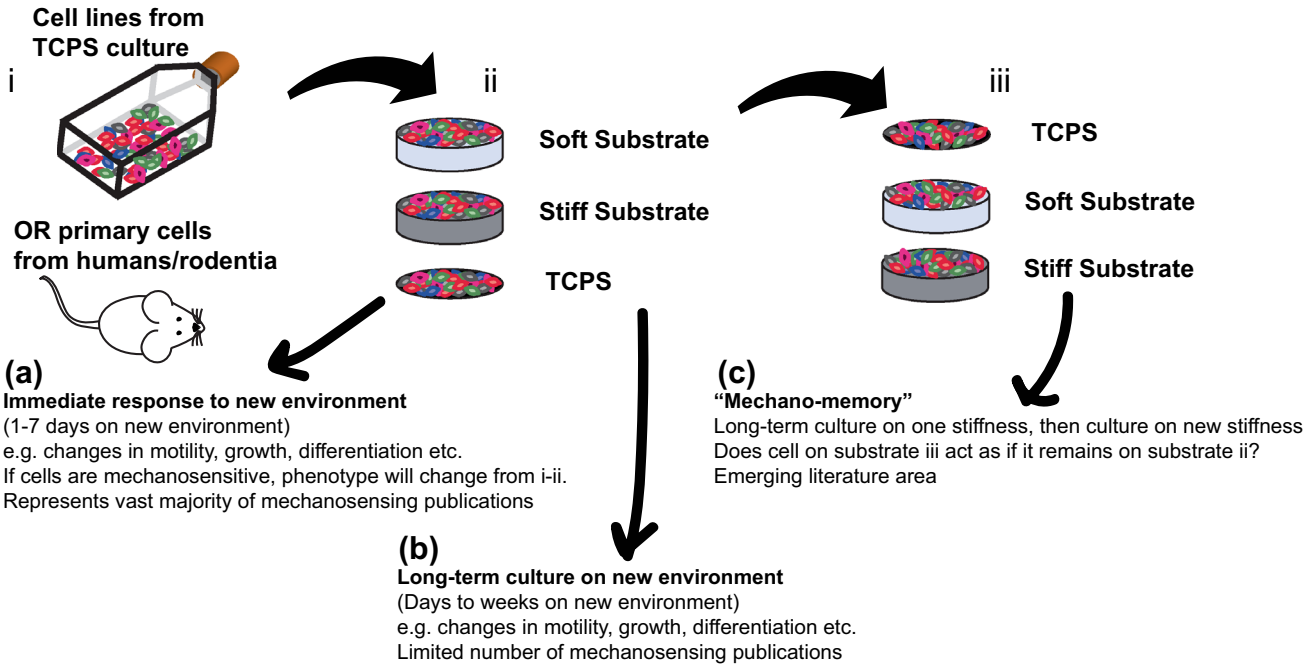

FIGURE 1. Schematic of a possible mechano-memory experiment. (a) Traditional mechanosensing experiments involve cell lines from standard culture on plastic, or primary cells plated on a substrate with some stiffness (ii) for a short period of time. Their ability to mechanosense is determined by different phenotypic responses to different stiffnesses. (b) More recently, cells have been cultured on gels of defined stiffnesses for much longer periods of times (days, weeks, or even months) on a substrate of stiffness ii to drive longer phenotypic changes. (c) To determine ''mechano-memory'' cells are cultured on a substrate of stiffness ii and switched to a substrate of stiffness iii. If the cells are mechanosensitive, then the phenotype as well as biochemical pathways will be different between i, ii, and iii. If the phenotype measured in ii persists on iii, then the experiment leads to the conclusion of persistent mechanical effects long after removal of the ii stimulus. This has been termed ''mechanical memory''.

remembered in the appropriate context. Studies performed so far do not appear to meet such a threshold. Perhaps the term 'persistent mechanical activation' is more appropriate for these effects.

IMPLICATIONS FOR PERSISTENT MECHANICAL ACTIVATION OF CANCER CELLS

These studies in other cell types raise the possibility that sustained exposure to changes in the ECM *in vivo* may impact cell functions in as yet unknown ways. This concept of a mechanical memory or a persistent mechanically activated state, though not yet addressed in the literature, has particularly important implications in cancer (Fig. 2a). During cancer initiation and progression, the tumor microenvironment stiffens *via* deposition and crosslinking of ECM proteins (Fig. 2b).[1,17,40,53,82] This ECM stiffening alters the mechanical forces experienced by the resident cancer cells.[40] As one example, the reported moduli of breast tumors vary considerably, but can range from 100 s of Pa to nearly 100 kPa.[12,32,33,37,49,53,63,64,71,90] Further, cells that have metastasized can reside at tissue sites mechanically distinct from their original environments from days to months to years, likely continually adapting to this new mechanical environment over time (Fig. 2c).

This variability in stiffnesses that cancer cells can experience could have dramatic effects on cancer cell phenotypes. For example, we and others have shown that cellular response to chemotherapy and/or other targeted drugs is sensitive to the stiffness of the surrounding ECM (Fig. 2d).[15,30,51,53,57,60,62,64,81,91,101,102] Second, ECM stiffness plays a critical role in regulating cancer cell growth[21,79,83] and motility.[24,87] However, these studies are all reports of mechanosensing in the traditional sense, where cells are cultured on tissue culture polystyrene (TCPS) and then exposed to a certain substrate stiffness for a limited experimental window. We found one study that points toward persistent mechanical activation: cancer cells were adapted to a soft biomaterial for 3 passages on polyacrylamide substrates.[88] They found that MDA-MB-231 cells improved their attachment and increased their cell spread area on soft substrates increasingly as they were passaged on soft substrates (Fig. 2e). These studies suggest that sustained exposure to the mechanics of the ECM can have an impact on cancer

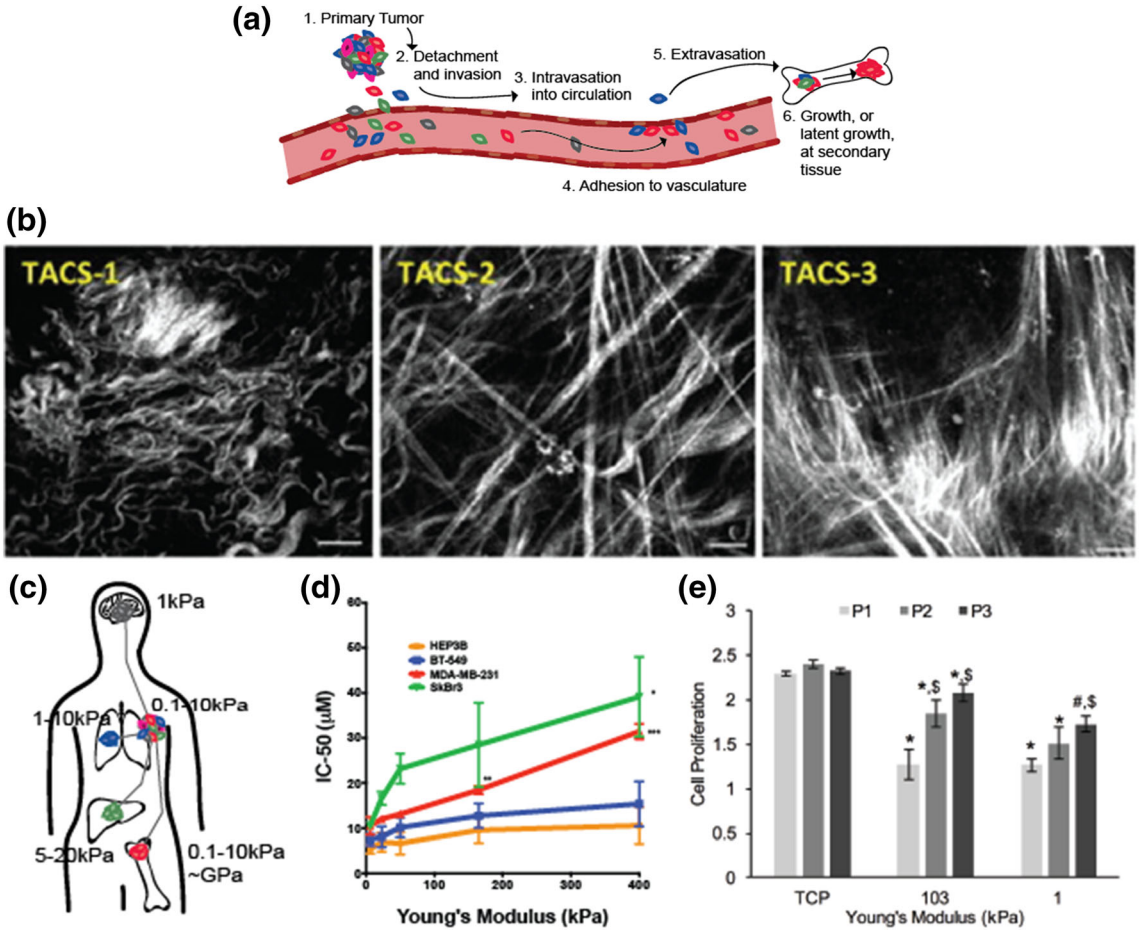

FIGURE 2. Stiffness and mechano-memory in cancer. (a) During metastasis, cancer cells see a variety of different stiffness environments that could impact their phenotype. Further, their residence time at these different locations will vary. (b) The stiffness of the primary tumor site is known to increase as the tumor grows, due to increased density of fibrillar collagens (Tumor-associated collagen signatures, TACS). Figure reproduced with permission from Ref. 72 (c) In breast cancer, the stiffness of the tissues to which cells most commonly metastasize ranges several orders of magnitude. (d) The stiffness of the culture substrate (x-axis) impacts cancer cell (colored lines) response to therapy (y-axis) in a cell-line dependent manner. Figure adapted with permission from Ref. 62 (e) The Zustiak lab has reported that passaging MDA-MB-231 cells continually on soft substrates causes adaptation to those substrates. Figure reproduced with permission from Ref. 88.

cell phenotype, but it is not yet clear if this is a phenotypic, genetic, or epigenetic response.

Common sites of breast cancer metastasis include the bone, lung, liver, and brain, which are mechanically distinct tissues.[21] As cancer cells disseminate, they can reside at these distant tissue sites, which have moduli far distinct from breast tissue, for decades. One example of this phenomenon is cancer dormancy. Even after apparently successful therapy, disseminated tumor cells can remain dormant for many years, often in the bone marrow, before outgrowth. The presence of these disseminated, quiescent tumor cells in the marrow is a marker of poor prognosis.[9,46] These dormant cells are also notoriously difficult to treat, and cannot be killed by the traditional chemotherapies typically given to patients with metastatic disease. Breast tumors are highly heterogeneous and drug treatment is known to enhance mutagenesis and clonal selection.[10] Therefore, it is quite possible that the stiffness of these distant tissue sites could be priming cancer cells for fast growth, invasion, and drug resistant qualities. This is thus far an unexplored area in need of the phenotypic, genomic, and mechanistic studies underway for fibroblasts and stem cells described earlier.

BIOMATERIALS DEVELOPMENT TO INVESTIGATE PERSISTENT MECHANICAL ACTIVATION OF CELLS

Many biomaterial systems have been developed to capture the elastic modulus of real tissue. Bioengineers, and increasing numbers of cell biologists, have used these to study how the rigidity of the microenviron-

ment affects cell behaviors. Biomaterials made from synthetic polymer networks are attractive for this application because they have more control over mechanical properties compared to naturally-derived protein and polysaccharide biopolymers.[45] Polymer biomaterials such as polyacrylamide (PAA) and poly(ethylene glycol) (PEG) are excellent model systems in which to understand the biophysical aspects of cell-material relationships.[42,43,67–70] PAA was the first popularized material used to parse the role of substrate modulus on cell behavior[65]; however, its main limitation is that it cannot be used as a 3D cell culture environment. PEG, in comparison, is also not cell-degradable on its own, but can be engineered to contain hydrolytically[58,100] or enzymatically degradable sites[73] for 3D cell culture. PEG is inherently resistant to protein adsorption, but can be coupled with short peptides or full-length proteins[55,95] to target specific receptor-ligand interactions in cells.[29,69]

One innovation we have brought to the field of stiffness-tunable 2D hydrogels is based on combining PEG with the zwitterionic monomer phosphorylcholine (PC).[29] PCs and other zwitterions have been exploited for their hydrophilicity and their mimicry of cell membrane phospholipids. These features make polymers including PCs ideal for drug delivery,[38] but their use in a biomaterial hydrogel has been limited.[47,84,96] PCs are extremely resistant to nonspecific protein adhesion, with performance better than poly-HEMAs, acrylamides, and pyrrolidones,[36] and this makes them particularly attractive for the long-term culture time points required to study persistent mechanical activation of cells. Hydrogels made from combining PEG and PC can be polymerized with as little as 0.5 wt% PEG crosslinker, resulting in a Young's modulus range over four orders of magnitude, which is also a key design criterium for studies attempting to differentiate cells based on the rigidity of the substrate.

3D hydrogels developed by us and others[25,39,81] are less frequently used for long-term cell cultures. A potentially cumbersome challenge here is how, technically, to repeatedly release and re-seed cells from a 3D environment as one does during cell passaging on 2D substrata. The prime candidates for 3D hydrogels would be Matrigel, type 1 Collagen, and Fibrin. Since these hydrogels are protein-based, cells can be released by proteolytic degradation (MMPs, collagenases, trypsin, etc.). 3D synthetic hydrogel environments could be adapted for this purpose if they were to include enzymatically degradable crosslinks.[29,62] In both cases, however, enzymatic digestion of gels would be expensive for continuous passaging. Finally, a lingering challenge with any 3D gel system is the limited range of moduli these gels can achieve (typically between 10 s of Pa to 10 kPa). This is significantly lower than that achievable by 2D hydrogel systems. Additional chemistries need to be developed to achieve these higher moduli and still be appropriate for cell culture in order to study persistent mechanical activation of cells in 3D.

GENETIC TOOLS TO STUDY PERSISTENT MECHANICAL ACTIVATION OF CELLS

Epigenetic Memory in Transcriptomic Cell States

Gene regulatory networks determine the coordinated dynamics of gene and protein expression programs, giving rise to distinct cell states. Networks are defined by the nodes, or the molecular players including proteins or genetic elements, and the molecular interactions, or wiring diagrams, that that govern their expression and activity. A cell state is reinforced and stabilized by the feedback of these interconnected pathways. It is these self-stabilizing patterns of gene activation across the genome that account for "epigenetic memory", rendering a cell state change irreversible (or difficult to reverse), as in development and differentiation. Thus, even in the absence of the initiating stimulus that triggered a cell state change, the pattern of gene expression persists.

Transcriptomic changes may be assessed through qPCR analysis of panels of selected genes or by RNA-seq. Importantly, advances in single cell RNA-Seq (scRNA-Seq) have now made possible the analysis of gene expression states in individual cells, with thousands of individual cell transcriptomes simultaneously measured. Recent studies that identify irreversible or partial irreversible gene expression changes induced by mechanical perturbations have relied on measuring only a small number of gene expression changes. As discussed earlier, one adipogenic marker (PPARg) and two osteogenic markers (alkaline phosphase and OCN) were assessed as markers of mechanical memory in mesenchymal stem cells,[97] and expression of actomyosin was measured in epithelial cells primed on stiff vs. soft ECM.[61] It therefore remains to be seen, whether other dimensions of the gene regulatory network sustain heritable changes in gene expression upon removal of a mechanical signal.

Measurements of Epigenetic Memory in Chromatin

Chromatin organization and epigenetic regulators also play key roles in the determination of cell state. To assess changes in binding of histones (such as H3K27me) and other regulatory factors, chromatin immunoprecipitation (ChIP) is performed. In this as-

say, proteins are covalently crosslinked to genomic DNA, providing a snapshot of histone or other protein–DNA interactions at a particular time point or in response to mechanical or biochemical signals. Following the isolation and fragmentation of chromatin, the protein–DNA complexes are isolated by binding to an antibody specific to the histone or factor of interest. The covalent crosslinks are then reversed, freeing the DNA for purification and analysis by qPCR or high-throughput sequencing (ChIP-seq). Another technique, Formaldehyde-Assisted Isolation of Regulatory Elements (FAIRE) does not rely on the availability of a specific antibody. Instead, DNA is bound to chromatin proteins by formaldehyde and sheared *via* sonication. Tightly packed chromatin regions will have abundant DNA/protein crosslinks, while DNA regions with no or few nucleosomes will have little or no crosslinked DNA/protein complexes. Quantification of this free DNA compared to a reference of total DNA sample allows the identification of the chromatin free regions. The FAIRE method can be used for the characterization of individual genomic regions or for the identification of genome-wide chromatin accessibility when coupled to deep sequencing.[27]

Both of these tools have been utilized to uncover epigenomic changes in response to mechanical perturbations. For example, human epidermal progenitor cells exposed to biaxial cyclic mechanical strain undergo striking changes in gene expression with nearly 4,000 genes downregulated and no genes significantly upregulated. Polycomb repressive complex (PRC) is one key player in this process, catalyzing dimethylation and trimethylation of histone 3 on Lys27 (H3K27me3) through the methyltransferase activity of Ezh1/Ezh2. Genes regulated by H3K27me3 or by the PRC pathway were over-represented in the set of transcripts downregulated by cyclic strain.[52]

Another approach to quantify chromatin remodeling is the assay for transposase-accessible chromatin sequencing (ATAC-seq), which identifies regions of open chromatin across the genome. By taking advantage of a Tn5 transposase that preferentially cleaves DNA and inserts sequencing adapters in regions of open, accessible chromatin, ATAC-seq enables high-throughput comparison of accessible genomic regions across samples. Subsequent next-generation sequencing and mapping of the fragments identifies putative regulatory regions that exhibit signatures of active transcriptional state and chromatin accessibility. In recent work, Stowers, *et al*. utilized this technology to compare regions of chromatin accessibility in breast cancer cells cultured in soft and stiff 3D interpenetrating networks (IPNs) of reconstituted basement membrane (rBM) matrix and alginate. This culture system enables specification of the elastic modulus independent of matrix architecture and ligand density. Differential analysis of ATAC-seq peaks revealed more than 1600 significantly more accessible peaks for cells cultured in stiff matrices (~ 2000 Pa, corresponding to malignant tissue), with no regions found to be significantly more accessible in soft matrices (~ 100 Pa, mimicking normal mammary tissue).[86] New developments in this technology now permit measurements of DNA accessibility at the single cell level by single cell ATAC-seq.[13] This approach could be critical for quantifying the cell-to-cell variability in epigenetic regulation of persistent mechanical activation.

Tracking Histories of Individual Cells

To investigate the mechanisms of cellular alterations and adaptation to mechanical stimuli, it is necessary to distinguish between two broad categories of responses. Does the mechanical perturbation induce persistent, heritable changes in individual cells or is there selection (by differential survival or differential growth rates) for a subset of cells with particular pre-existing characteristics? Either of these scenarios could produce a lasting shift in the cell state of the overall population in response to a mechanical stimulus. To determine which of these general mechanisms is at work, it is critical to track individual cells in the population over time.

While fluorescent cell labels and time-lapse microscopic imaging have enabled decades of study, these approaches are limited in the number of labels that can be monitored simultaneously and the duration over which individual cells can be observed. In 3D culture systems, tracking individual cells over days and weeks adds another layer of technical challenges. To address these limitations, novel nucleic acid-based tools have been developed to label and quantify cells and their clonal descendants within heterogeneous populations.[92] DNA barcoding is uniquely capable of tagging and measuring large numbers of cells over time.[8,41] In this approach, each cell in a population is tagged with a unique random DNA sequence that is stably integrated into the genome and thus heritable by all daughter cells. The potential space of unique sequence tags is extremely large (for example, the theoretical diversity of a library of random 20-mer barcodes is more than 10^{88} distinct sequences), enabling the faithful labeling of large cell populations. After stable integration (typically by viral delivery), barcode abundance can be measured by targeted next-generation sequencing of the barcode region (Fig. 3).

In recent years, this approach has been leveraged to uncover evidence of pre-existing and induced responses to various biochemical stimuli, although to our knowledge it has not yet been applied to mechanical

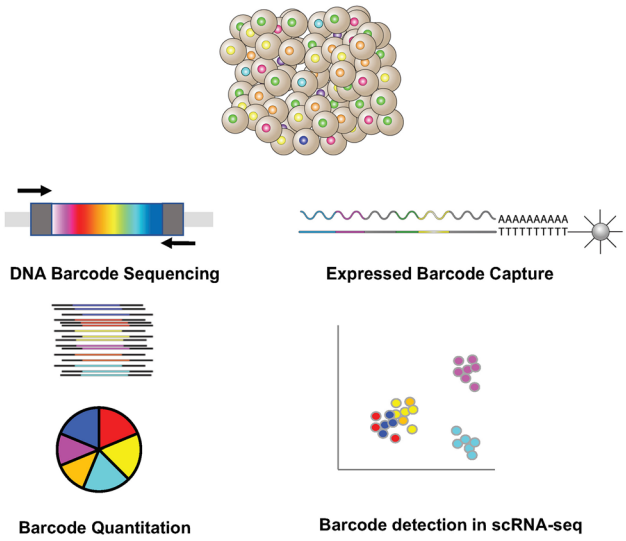

FIGURE 3. Cell barcoding approach to quantify effects of mechanics on cell populations. A population of cells is labeled with DNA barcodes and expanded. The abundance of each label can be measured by targeted sequencing of the barcode region of the genome (left). If the barcode is also an expressed sequence (right), it may be captured in workflows that analyze transcripts, such as by bead capture in single cell RNA-seq.

perturbations. For example, Bhang and colleagues utilized a high-diversity DNA barcode library to investigate the response of non–small cell lung cancer cells and chronic myeloid leukemia cells to targeted growth factor pathway inhibitors.[6] In all samples, a population of cells resistant to therapy emerged after treatment. To determine whether this shift in the cell population phenotype was caused by selection of a pre-existing subpopulation or induction of a resistance mechanism, investigators measured the abundance of barcoded cells in multiple parallel replicates. NGS revealed that the very same clones consistently escaped treatment across replicates, revealing the presence of a rare pre-existing drug-resistant sub-population prior to treatment. We propose that this same approach could be used to find clones that expand in different stiffness environments as well.

Variations on DNA barcoding systems have integrated these cell labels with other molecular and genomic assays. The use of RNA-based, expressed barcode tags enables the simultaneous read-out of the barcode label alongside the captured transcriptome in single-cell RNA sequencing (scRNA-Seq).[2,7,74,75,77] Expressed barcode systems with scRNA-Seq have been utilized to dissect the reprograming of mouse embryonic fibroblasts to induced endoderm progenitors (iEPs).[7] Barcode labeling and tracking the progenitor population revealed that cells from identical lineages follow similar reprograming trajectories within a replicate, but not across replicates. These data suggest that, rather than selection of a pre-existing stable cell state, multiple cells in the starting population are able to enter a temporarily privileged cell state, in which they are primed for IEP differentiation.[7] Similar processes may underlie persistent mechanical activation and can now be explored in the context of heterogeneity of cell responses to substrate stiffness.

Cell barcoding platforms offer powerful new tools to dissect the histories and trajectories of individual cells and relate these to population-level shifts in gene expression and behavior. They share one limitation - they are destructive measurements due to the requirement of sequencing the genome to quantify barcodes. To overcome this challenge, one of us developed a functionalized variant of DNA barcoding that uses stably integrated and expressed barcoded guide-RNAs, capable of isolating live cells carrying a particular barcode label of interest.[3] Borrowing from synthetic biology, this approach takes advantage of a transcriptional activator variant of dCas9 to activate a barcode-specific gene circuit and express a fluorescent reporter. This enables isolation of specific subpopulations of interest by fluorescent activated cell sorting for downstream molecular and cellular studies. This is a key technological advancement for studying persistent mechanical activation, so that we may harvest clones on soft vs. stiff environments and study important phenotypes relevant to cancer, such as their motility, growth rates, and drug responses. This could provide a much-needed link between genotype and phenotypes in cancer related to tumor ECM stiffness.

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