SNAIL-induced epithelial-to-mesenchymal transition produces concerted biophysical changes from altered cytoskeletal gene expression

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ABSTRACT A growing body of evidence suggests that the developmental process of epithelial-to-mesenchymal transition (EMT) is co-opted by cancer cells to metastasize to distant sites. This transition is associated with morphologic elongation and loss of cell-cell adhesions, though little is known about how it alters cell biophysical properties critical for migration. Here, we use multiple-particle tracking (MPT) microrheology and traction force cytometry to probe how genetic induction of EMT in epithelial MCF7 breast cancer cells changes their intracellular stiffness and extracellular force exertion, respectively, relative to an empty vector control. This analysis demonstrated that EMT alone was sufficient to produce dramatic cytoskeletal softening coupled with increases in cell-exerted traction forces. Microarray analysis revealed that these changes corresponded with down-regulation of genes associated with actin cross-linking and up-regulation of genes associated with actomyosin contraction. Finally, we show that this loss of structural integrity to expedite migration could inhibit mesenchymal cell proliferation in a secondary tumor as it accumulates solid stress. This work demonstrates that not only does EMT enable escape from the primary tumor through loss of cell adhesions but it also induces a concerted series of biophysical changes enabling enhanced migration of cancer cells after detachment from the primary tumor.—McGrail, D. J., Mezencev, R., Kieu, Q. M. N., McDonald, J. F., and Dawson, M. R. SNAIL-induced epithelial-to-mesenchymal transition produces concerted biophysical changes from altered cytoskeletal gene expression. FASEBJ. 29, 000–000 (2014). www.fasebj.org

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The problem of cancer metastasis is undoubtedly one of the biggest hurdles facing cancer clinicians, as this deadly process is currently responsible for over 90% of cancer-related deaths. A large body of evidence suggests that EMT, whereby less motile regularly shaped polygonal epithelial cells transition into elongated invasive mesenchymal cells, is critical for cancer cells to leave the primary tumor to spread to distant sites (1). This increased invasiveness is largely attributed to loss of E-cadherin responsible for cell-cell adhesion and increasing expression of matrix-binding integrins (2). Furthermore, increased expression of matrix-degrading matrix metalloproteinases allows for degradation of the basement membrane and dissemination to other tissues (3).

In addition to these molecular changes, the emerging field of physical oncology has highlighted that cells modulate their mechanical properties to disseminate effectively (4). Moreover, during the morphologic rearrangement the epithelial cells lose their apical-basal polarity causing dissolution of cortical actin structure as the cells gain the more elongated mesenchymal morphology with front-rear polarity (5). Despite extensive study into EMT over the past three decades, how it affects these critical biophysical properties remains largely unknown. Environmental factors used to induce EMT, such as soluble factors, matrix stiffness, or adhesive ligands (6), alter other biophysical and biochemical properties confounding the analysis of the effects of EMT alone. These pathways use a variety of intermediate signaling molecules, but in all studied cases of EMT, they converge on the activation of SNAIL genes (7). SNAIL is a transcriptional repressor primarily thought to induce EMT from direct repression of E-cadherin (8).Thus, to directly examine EMT in isolation without confounding environmental cues, we utilized a robust genetically engineered model of EMT in epithelial MCF7 breast cancer cells. To do so, we chose a SNAIL variant modified to be stable and localized within the cell nucleus, which was recently shown to be a superior model of EMT compared with overexpression of wild-type SNAIL (9).

Here, we demonstrate that EMT directly produces biophysical changes resulting in a prometastatic phenotype...
and further elucidate the underlying alterations in gene expression by microarray transcriptomic analysis. SNAIL-transformed cells were significantly more compliant than an empty vector control, both softening their cytoplasm by an order of magnitude and relaxing their nucleus approximately 5-fold to allow for navigation of the extracellular environment. These mechanical changes coincided with structural changes including decreased polymerized actin and abnormal nuclear morphology, correlating with decreased expression of actin cross-linkers such as FLNA (Filamin A) and structural nuclear proteins such as LMNA (Lamin A). In addition to these changes in intracellular compliance and structure, EMT also increased extracellular force exertion and contractile gene expression to further expedite migration. We show these changes allow for increased motility regardless of extracellular mechanics, possibly helping cells adapt to their new environment as they disseminate. However, more migratory mechanically compliant SNAIL cells lacked the structural stability to form large spheroids analogous to secondary tumors. Taken together, this work not only demonstrates that biophysical changes associated with cancer progression are produced by EMT, but integrates this mechanical analysis with gene expression profiling for a more complete portrait of this key process in cancer metastasis.

MATERIALS AND METHODS

Cell culture and phenotype verification

Human breast carcinoma MCF7 stably transformed with either SNAIL-6SA (mutated for internuclear stability) (9) or empty vector control, both encoding for neomycin resistance, were cultured in RPMI 1640 (Mediatech, Herndon, VA, USA) containing 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA) and 400 μg/ml G418 (Sigma-Aldrich, Carlsbad, CA). Cell aspect ratio was determined by manually tracing cells in ImageJ (U.S. National Institutes of Health). Gene expression analysis using RT-PCR was performed using primers as previously described (10, 11). All experiments were performed on glass coated with 10μg/ml collagen I or compliant substrates coated with collagen I synthesized as previously described (12) with either 10% acrylamide and 0.3% bisacrylamide (hard, 34.88 kPa) or either 10% acrylamide and 0.03% bisacrylamide (soft, 2.83 kPa).

Cell function analysis

Proliferation was quantified using 5-bromo-2-deoxyuridine (BrdU) staining as previously described (13) by labeling with 50 μM BrdU (Sigma-Aldrich) for 90 min before staining. For motility quantification, cells were labeled with NucBlue (Invitrogen, Carlsbad, CA) per manufacturer’s instructions and then imaged every 5 min on a Nikon Eclipse Ti (Nikon, Tokyo, Japan) inverted epifluorescent microscope equipped with an environmental chamber (InVivoScientific, St. Louis, MO, USA) for 6-12 h. Nuclei were tracked in MATLAB and traces used to determine mean velocity, defined as the average velocity over each 30 min time interval, directional velocity, defined as the net change in position over total tracking time, and straightness, defined as the total path length over the net displacement. Spheroids were formed by creating single-cell suspensions in either 0.5 or 1.0% low-gelling agarose and allowing them to grow for 4 wk before labeling live cells with green fluorescent Calcein AM to quantify spheroid area. Only spheroids isolated from other spheroids by at least 2 radii were imaged. Solid stress was calculated as described elsewhere (14) using a linear poroelastic model assuming no initial stress at time 0 and that stress goes to 0 at an infinite distance. Spheroids with elongated morphologies were discarded as symmetric assumptions were no longer valid; the remaining population had equivalent average area (t test, P = 0.52) and variance (F test for equality of variances, P = 0.41).

Microarray analysis

After achieving about 80% confluence, cells from triplicate cultures of both cell types were harvested by trypsinization; total RNA was isolated, processed to fragmented biotin-labeled cDNA, hybridized on Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA) and scanned as described in detail in the Gene Expression Omnibus repository (GEO, http://www.ncbi.nlm.nih.gov/geo/) data set under the accession number GSE58252. Additionally, data sets GSE37820 and GSE17802 were used for analysis of ESR1 knockdown and VM knockdown, respectively. Data were processed with the Expression Console software Build 1.2.1.20 (Affymetrix) using the Affymetrix default analysis setting for PLIER algorithm. Probe sets that displayed absent detection calls (MAS5.0 algorithm) across all chips were removed and PLIER16 values were used to identify genes differentially expressed between SNAIL and empty vector control cells using the Significance Analysis of Microarrays, version 4.01 program (Stanford University Labs, Stanford, CA, USA) (15). Genes were reported as differentially expressed between SNAIL and control classes at false discovery rate (FDR) = 2.12% and absolute fold change ≥ 1.5. Probe sets corresponding to differentially expressed genes were employed for enrichment analysis using the MetaCore suite 6.18 build 65505 (Thomson Reuters, New York, NY, USA). In brief, significantly perturbed pathways and process networks were identified by mapping differentially expressed genes onto manually curated GeneGO canonical pathway maps and process networks (16). Additionally, functional enrichment for up-regulated and down-regulated genes was determined using David 6.7 functional annotation tool (Laboratory of Immunopathogenesis and Bioinformatic, SAI-C-Frederick, Inc., Frederick, MD, USA) (17, 18). To test for the statistical significance of enrichment, P values were calculated based on hypergeometric distribution and corrected for multiplicity using the FDR procedure. Complementary to the differential expression and pathway enrichment analysis, the Gene Set Enrichment Analysis (GSEA) (19) was performed on PLIER16-processed data without any prefiltering of probe sets for SNAIL and control cells, using categorical phenotype labels and signal-to-noise metrics to identify gene sets significantly enriched in specific phenotypes. Gene set permutation type and Gene Ontology (GO) Biologic Processes gene sets (825 gene sets; Molecular Signatures Database, v4.0, Broad Institute, Cambridge, MA, USA) were the parameters used in this GSEA. Gene sets were considered to be significantly enriched in a given phenotype, if their q values were ≤ FDR threshold, for which the expected number of false positive gene sets was ≤ 1. The analysis of gene expression data presented in this report is focused on solely on cell mechanics and details relevant to other functional contexts will be reported elsewhere.

Traction force cytometry

Substrates (34.88 kPa) were synthesized as above except embedded with 200 nm red fluorescent particles (Invitrogen). Cells were first labeled green fluorescent with Calcein AM (AnaSpec, Fremont, CA, USA) and NucBlue (Invitrogen) per manufacturer’s instructions. An initial image of the cells and the stressed gel was captured, and then the cells were lysed with 0.5% SDS before capturing a final unstressed gel image. An average of 100 cells per experiment was captured, values represent average of 3–5 experiments. Cell-induced displacements from particle...
images, a freely available MATLAB particle-tracking algorithm (MatPIV, http://www.math.uio.no/~jks/matpiv/) was used with minor modifications and used to determine traction forces as described previously (20).

**Intracellular mechanics**

MPT microrheology was used to quantify intracellular rheology based on the thermal motion of nanoparticles (reviewed elsewhere) (21). Cells were injected as described previously (22) on collagen-coated 35 mm dishes and then immediately passed to collagen-coated 35 mm glass bottom dishes (MatTek, Ashland, MA, USA). Cells were allowed to adhere overnight and imaged the following day in the live-cell chamber described above with a Nikon CFI Apochromat TIRF 100× oil-immersion lens and QuantEM CCD (Princeton Instruments, Trenton, NJ, USA) at 30 frames per second for 20 s. Particle displacements were tracked in MATLAB based on previous established algorithms (23) by first using a bandpass filter to isolate particles and the subpixel accuracy centroid was determined based on the intensity-weighted centroid. After building tracks using a Hungarian linker algorithm the time-dependent 2-dimensional particle mean squared displacements (MSDs) were calculated as $<\Delta^2(D_t)> = <(x(t+\Delta t) - x(t))^2 + (y(t+\Delta t) - y(t))^2>$, which can then be used to determine rheological properties including diffusion coefficient, creep compliance, as well as the viscous ($G'(\omega)$) and elastic ($G''(\omega)$) moduli (24). The ratio of viscous to elastic moduli is represented by the phase angle ($\delta$, where $\delta = \arctan(G'(\omega)/G''(\omega)$), which ranges from 0° for an elastic solid to 90° for a viscous liquid (25). To quantify nuclear mechanics, we tracked Hoechst-labeled chromatin (26) using techniques as described above for MPT. The relaxation time was determined as when the logarithmic slope of the MSD was equal to 0.5, indicating a transition from a viscoelastic solid to a viscoelastic liquid.

**Cytoskeletal staining and image analysis**

Cells were stained for F-actin with rhodamine phallodin (Invitrogen) as described previously (22). Images were captured at ×20 magnification on an inverted Nikon Microscope with a CoolSNAP camera (Photometrics, Tucson, AZ, USA) and quantified in MATLAB. For quantification, image histograms were first adjusted to fill the entire range before applying a median filter and then segmenting by Otsu’s method. F-actin intensity was defined by taking the average intensity of the segmented region (cells) minus the average intensity of the unsegmented region (background) of the original, unmodified image. Confocal images were captured on a Zeiss LSM 700 confocal microscope equipped with a ×63 lens and rendered in ZEN 2012 software (Carl Zeiss, Jena, Germany).

**Statistics**

All studies were performed in triplicate or more. The data are reported as mean ± SEM. Statistical analysis was carried out using a Student’s t test for comparison considering $P < 0.05$ to be significant.

**RESULTS**

**SNAIL transformation induces a mesenchymal phenotype and increased malignant character**

After transformation with SNAIL-6SA, MCF7 breast cancer cells exhibited a more mesenchymal phenotype than their empty vector transformed controls. This was confirmed both by cell morphology, where SNAIL cells showed a 2-fold increase in aspect ratio (Fig. 1A, B), as well as gene expression that revealed classic changes associated with EMT such as down-regulation in E-cadherin (CDH1) as well as up-regulation of N-cadherin (CDH2) and vimentin (VM) (Fig. 1C). To understand the functional consequences of this transformation, we next evaluated the migratory and proliferative capacity of the transformed cell lines. We tracked the migration of over 500 cells per condition (Fig. 1D) and found mesenchymal SNAIL-transformed cells showed a large ($P < 1^{-15}$) increase in motility relative to their control counterparts with an over 2-fold increase in cell speed (Fig. 1E). Moreover, SNAIL cells utilized a different migratory phenotype characterized by “bursty” migration as opposed to persistent migration over time. We quantified this difference by the coefficient of variation (27), which increases as the velocity of a cell becomes less consistent, to find a significant increase following SNAIL transformation (Fig. 1F). Finally, we evaluated proliferation in both 2 and 3 dimensions. When grown on a 2-dimensional surface, the proliferation was significantly increased in SNAIL-transformed cells (Fig. 1G). Proliferation was also increased in 3 dimensions, as determined with an anchorage-independent spheroid formation assay in agarose (Fig. 1H). This increase in proliferation and migration indicative of a more aggressive phenotype are conserved across multiple substrate rigidities by repeating the proliferation (Fig. 1I) and motility (Fig. 1J) experiments on soft (2.83 kPa) and hard (34.88 kPa) substrates.

**Increased traction forces during SNAIL-induced EMT**

Because of the major role of actomyosin contraction in cell migration, we used traction force cytometry to quantify alterations in cell-exerted traction forces after EMT. Traction force maps of control (Fig. 2A) and SNAIL (Fig. 2B) cells show altered force arrangement. Control cells have forces distributed throughout the cell body, whereas SNAIL forces are more localized to the tips of the cell. In addition to this altered intracellular distribution, SNAIL cells also exerted significantly ($P < 0.01$) higher forces (Fig. 2C). Because of the increased “bursting” migration in the SNAIL cells, we hypothesized that this would translate to capturing some cells “postburst” and others “preburst” resulting in a larger variance within the population compared with the control cells that moved more consistently. Supporting this idea, we found that the coefficient of variance for traction stress within each individual experiment was increased almost 2-fold in the SNAIL cells (Fig. 2D). Microarray analysis of gene expression data revealed that this force increase coincided with up-regulation of genes that promote actomyosin contraction such as MYLK (myosin light chain kinase) and MYH10 (myosin IIb) as well as down-regulation of genes that reduce contractility such as PPP1R12B (myosin phosphatase) (Fig. 3D).

**EMT softens the cytoplasm and alters actin structure**

To investigate how EMT altered intracellular mechanics, we utilized MPT microrheology to determine rheological information from the thermal motion of ballistically
injected fluorescent nanoparticles (21). Larger MSDs of particles in SNAIL cells indicate less resistance to particle motion within their cytoplasm. At all evaluated time scales, SNAIL cells displayed a higher MSD (Fig. 3A). This result translated to a 5-fold higher effective diffusion coefficient (Fig. 3B) and an order of magnitude increase in cytoplasmic compliance (Fig. 3C). Calculation of viscoelastic parameters further revealed that control cells have an appreciable elastic moduli of 29 ± 5 dynes/cm², but this value is almost negligible for SNAIL cells at 0.6 ± 0.4 dynes/cm² (Fig. 3D) with similar trends in viscosity (Fig. 3E). Moreover, though control cells maintained some elastic character over all frequencies, SNAIL cells became entirely viscous (phase angle of 90°) at frequencies higher than 1 Hz (Fig. 3F).

We hypothesized that these changes in cytoplasmic mechanics were largely due to decreases in actin polymerization. To test this hypothesis, we stained cells for F-actin, which showed that control cells had organized actin filaments and that tension from these cortical actin stress fibers helps create a more uniform, flat cell shape (Fig. 4A). Overexpression of SNAIL dissolved these stress fibers (Fig. 4B) and produced more rounded, 3-dimensional cells (Fig. 4B). SNAIL cells also displayed enhanced lamellipodial ruffling at either pole (blue arrows in Fig. 4B), as well as additional filopodial structures extending from the cell body (red arrow in Fig. 4B). We additionally characterized intermediate filaments cytokeratin and vimentin, which are known to be down- and up-regulated during the EMT (6), respectively, and are known to play a role in cell mechanical properties (28–30). Though control cells display robust keratin networks, these are completely lost following EMT in SNAIL cells (Fig. 4C). Conversely, ectopic SNAIL expression increased the expression of vimentin (Fig. 4D). To quantify these changes, we took the integrated fluorescence density of each filament, revealing that SNAIL cells have a 3-fold decrease, 10-fold decrease, and a 4-fold increase in F-actin, cytokeratin, and vimentin, respectively (all P < 0.001, Fig. 4E). This was further confirmed by gene expression data showing the up-regulation of vimentin as well as down-regulation of several keratin isoforms (Fig. 4F).

To determine the molecular mechanism for this loss of mechanical structure, we utilized several approaches to...
analyze microarray gene expression data. The GSEA analysis, which does not depend on an arbitrary selection of differentially expressed genes revealed that the “Actin Polymerization and/or Depolymerization” (M2403, Supplemental Fig. 1A) and “Rho Protein Signal Transduction” (M7069, Supplemental Fig. 1B) gene sets were significantly enriched in the control phenotype ($q$ values 0.223 and 0.196, respectively). Because Rho GTPase is known to regulate actin cytoskeleton remodeling during cell morphogenesis and motility (31), our data suggest that phenotypic differences between mesenchymal-like SNAIL and epithelial-like control cells probably include Rho-mediated reorganization of actin cytoskeleton. Consistent with this finding, pathway enrichment analysis by MetaCore suite identified alterations in several actin cytoskeleton-related gene signaling networks, including “Regulation of Cytoskeletal Rearrangement” ($P = 5.06 \times 10^{-7}$, FDR $q = 2.70 \times 10^{-5}$, Supplemental Fig. 2A) and “Cytoskeleton Actin Filaments” ($P = 9.55 \times 10^{-18}$, FDR $q = 1.14 \times 10^{-7}$, Supplemental Fig. 2B). Moreover, analysis by David

Figure 2. SNAIL-induced EMT increases cell-exerted traction forces. Traction heat maps in units of Pascals ranging from 0 (dark blue) to 850 (dark red) overlaid with matrix displacements (black arrows). A) Control (CTRL) cells forces are located predominately in the cell interior (inset heat map is rescaled to a max value of 300 Pa for visualization). B) SNAIL cells exert forces closer to the cell periphery. C) Peak traction stresses in SNAIL cells were 3-fold higher than those exerted by control cells. D) Coefficient of variation (Coeff. Var.), defined here as the standard deviation of the cells within each experiment over the average of all the cells within the experiment, is increased as seen with motility results in Fig. 1. E) Microarray analysis shows differential regulation of contractile markers between SNAIL and control cells. FC, fold change. Scale bars = 10 µm.

Figure 3. Analysis of intracellular microrheology reveals greatly softened cytoplasmic space by SNAIL transformation through loss of polymerized actin. A) MSDs of 200 nm particles injected into the cytoplasmic space are increased by nearly half a decade across all time lags. B) Intracellular diffusion coefficient at $\tau = 1$ s is decreased over 5-fold in SNAIL cells. C) Creep compliance of cytoplasm is also decreased by an order of magnitude at $\tau = 1$ s. D) Elastic moduli at a frequency of 1 Hz is significantly decreased in SNAIL cells. E) Shear viscosity shows similar decrease in SNAIL cells. F) Evaluation of the phase angle, $\delta$, which quantifies the relative ratio of viscous to elastic character shows that at frequencies above 0.3 Hz SNAIL cells show significantly lower elastic character. CTRL, control. **$P < 0.01$, ***$P < 0.001$. 
6.7 functional annotation tool identified KEGG pathway “Regulation of Actin Cytoskeleton” (hsa04810, Supplemental Fig. 3A) as significantly enriched ($P = 4.7 \times 10^{-13}$, FDR $q = 0.00092$) for genes down-regulated in SNAIL cells.

To verify that these changes were primarily caused directly through SNAIL-induced EMT and not through downstream modulation of non-EMT genes, we compared microarray results from other related perturbation experiments with MCF7 cells. Because the loss of estrogen receptor-α (ERα) is associated both with breast cancer progression and SNAIL expression (32), we analyzed changes in MCF7 cells induced by siRNA-mediated knockdown of the expression of $ESR1$ gene that encodes ERα. This analysis revealed minimal changes in the expression of EMT-associated genes and no enrichment of the actin-related processes seen in the SNAIL cells. Furthermore, analysis of vimentin knockdown only revealed one conserved actin-related gene, the cross-linker cingulin, indicating it may have some effect on the actin cytoskeleton in isolation.

For brevity, a subset of actin cytoskeleton-related genes is displayed in Table 1. There was no clear trend in actin severing proteins, but several actin cross-linking genes such as filamins A and B were down-regulated along with a series of actin stabilizing proteins. This implies that the loss of actin structure was largely mediated via decreased expression of actin cross-linking, not an increased rate of disassembly. We further tested this hypothesis by treating cells with membrane permeable jasplakinolide, which acts to polymerize and stabilize actin filaments, and found that this treatment was only able to partially recover cell mechanical properties (Supplemental Fig. 3B, C). This incomplete recovery may be due to the lack of filamins to cross-link the actin fibers in SNAIL cells, or because some of the mechanical stability originates from keratin intermediate filaments, which were lost following SNAIL transformation. Though actin severing proteins showed no direct relation to actin cytoskeletal architecture, the large down-regulation of gelsolin is notable because reduced expression in breast cancer is associated with poor patient prognosis (43, 44). This indicates decreased gelsolin may be a conserved feature of breast cancer progression. Moreover, several genes associated with filopodia formation such as $CDC42$, $ACTR3$, and $PARVB$ were up-regulated, suggesting

Figure 4. Cytoskeletal architecture is altered by EMT. To visualize corresponding changes in cytoskeletal structure, cells were stained for F-actin and visualized via confocal microscopy. Three-dimensional confocal reconstruction color-coded by Z-distance for (A) control (CTL) cells and (B) SNAIL cells shows not only cortical actin structure present in control cells (black arrows) but also increased ruffling (blue arrows) and actin protrusions (red arrow) in SNAIL cells. C) To visualize intermediate filaments, cells were stained for cytokeratins and vimentin, (D) both shown in green, and then counterstained for F-actin (red) and nuclei (blue). E) Quantification of fluorescence intensities show that SNAIL cells have decreased levels of F-actin and cytokeratin but increased level of vimentin (all values reported normalized to the average of both cell types). F) Gene expression analysis shows up-regulation of vimentin and down-regulation of several cytokeratins consistent with protein expression findings. FC, fold change. ***$P < 0.001$. All scale bars = 10 μm.
TABLE 1. Microarray analysis of gene expression data

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Microarray analysis of gene expression data reveals significant dysregulation of genes associated with actin cytoskeletal structure.

these regulate the protrusion formation in SNAIL cells. Interestingly, Snail cells also overexpress the WASF3 gene encoding WAVE3 member of WASP/WAVE protein family known to regulate actin cytoskeleton remodeling through Arp2/3 and enhance invasive and metastatic potential of breast cancer cells in vitro and in vivo (33).

SNAIL relaxes nuclear stiffness and produces defects in nuclear morphology

Although relaxing cytoskeletal rigidity may help cells navigate through tight pores in the extracellular matrix, the nuclear deformation is often the rate-limiting step for effective invasion (34). Thus, we sought to probe whether SNAIL-induced EMT would likewise soften nuclear mechanics by performing particle tracking in the nucleus but instead of using injected nanoparticles using Hoechst-labeled chromatin as tracer particles (26). Consistent with previous studies, we found at short time scales the nuclei of both cells behaved as elastic solids, corresponding to a MSD slope close to 0 (Fig. 5A). However, at later time scales nuclei began to transition to more viscous behavior indicated by an increasing MSD slope approaching 1 for a perfectly viscous material. The time for this transition, or relaxation time, was 5-fold faster in SNAIL cells (Fig. 5B). This relaxation produced a significantly softer nucleus after SNAIL transformation, quantified by an effective diffusion coefficient nearly an order of magnitude higher than control cells (Fig. 5C).

Based on this loss of mechanical stability, we sought to determine whether this produced alterations in bulk nuclear structure. Although there were some visually elongated nuclei in both the control cells (Fig. 5D, red arrow) as well SNAIL cells, the latter additionally displayed frequent aberrations in nuclear morphology including elongation, but also excessive creasing and folding within the membrane (Fig. 5E, blue arrows). Morphologic quantification based on nuclear shape factor (35), defined as 4π·area/perimeter² ranging from 0 for a line to 1 for a perfect circle, demonstrated that control nuclei were significantly more circular (Fig. 5F). Analysis of microarray gene expression data by David 6.7 functional annotation tool revealed enrichment of the nuclear lumen (GO: 0031981; P = 3.5 × 10⁻¹⁶, FDR q = 3.5 × 10⁻¹⁵), nucleoplasm (GO:0005654; P=3.6 × 10⁻¹¹, FDR q =5.5 × 10⁻¹⁰) and nu-clear pore (GO:0005643; P = 1.0 × 10⁻³, FDR q = 1.5 × 10⁻²) cellular component gene ontologies for genes up-regulated in SNAIL cells. This result provides an evidence for molecular differences between SNAIL and control cells in their nuclear compartments and implies the role of nuclear compartments in phenotypic differences between these two cell types. Notably, several genes coding for nuclear structural proteins were differentially expressed including lamin A/C, Nesprrin 2, and several Sun proteins (Fig. 5G). Down-regulation of lamin A was recently shown to decrease nuclear stiffness and enhance cancer cell migration (36).

Epithelial phenotype supports higher levels of solid stress during tumor growth

To expand against agarose gels, cells must have structural stability to maintain the induced solid stress, so we sought to test if increasing this solid stress would differentially block proliferation between control and SNAIL cells by doubling the percentage of agarose used during spheroid growth. Control cell spheroids showed a slight but insignificant decrease in spheroid area in 1.0% gels, whereas SNAIL cell spheroids in 1.0% agarose were 5-fold smaller than in 0.5% agarose (P < 0.001, Fig. 6A). This indicated that the induced solid-stress affected mesenchymal SNAIL cells (86 ± 6.2% growth inhibition) significantly more than the epithelial controls (18 ± 1.4% growth inhibition) (Fig. 6B). Quantification of the solid stress exerted by the spheroids demonstrated that SNAIL cells were able to withstand a peak solid stress of ~35 mm Hg regardless of gel concentration, whereas control cells were able to withstand at least 80 mm Hg in stiffer gels (Fig. 6C).

DISCUSSION

EMT is hypothesized to be a key step in cancer metastasis, predominately through loss of cell-cell adhesions leading to escape of cells from the primary tumor (1). However, recent studies have shown that biophysical changes may be equally important for cancer metastasis (37). To bridge the gap between these observed molecular and biophysical changes, we utilized a genetically induced model of EMT in epithelial MCF7 breast cancer cells transformed to a mesenchymal phenotype with a stable SNAIL mutant (9). As previously observed when comparing invasive and noninvasive cells (38), we found that the more motile
SNAIL cells exerted larger traction stresses on their underlying matrix (Fig. 2). Although this result was somewhat surprising considering the decreased level of polymerized actin, we find it to be consistent with results reported by other investigators. For instance, more aggressive cancer cells are known to be soft with decreased actin polymerization (39–41) and exert larger traction forces (37). Analysis of relevant gene expression revealed key changes, consistent with those previously observed in other models of EMT, such as a concomitant up-regulation of MYH10 (myosin IIb) and down-regulation of MYH14 (myosin IIc) (42). We further observed up-regulation of genes that would increase contractility including myosin light chain kinase and ARHGEF3 as well as decreased expression of contractility inhibitors such as myosin phosphatase and ARHGAP1 (Fig. 2E).

In addition to the extracellular forces exerted by cells, the intracellular mechanics of cancer cells have also been shown to correlate with metastatic potential with softer cells displaying a higher degree of invasiveness (40, 41). To determine if EMT could be responsible for this loss of cytoskeletal stiffness, we probed intracellular mechanical properties using MPT microrheology. This

Figure 5. Nuclear structure is compromised by SNAIL-induced EMT. A) MSDs of Hoechst-labeled chromatin show that while at low scales nuclei behave primarily as an elastic solid regardless of cell line; at later time MSDs begin to increase, indicative of more viscous diffusion. B) Quantification of the time until this relaxation from elastic to viscous character shows that it happens over 3-fold faster in SNAIL cells. C) The nuclei of SNAIL cells show a nearly order of magnitude increase in effective diffusion coefficient at 10 s. Loss of nuclear integrity was verified by examining 3-dimensional reconstructions of confocal micrographs where control (CTRL) cells (D) show some elongated nuclei (red arrow), but SNAIL nuclei (E) show several abnormal structural deformations (blue arrows). F) This structural abnormality was quantified by nuclear shape factor, or 4πarea/perimeter2 ranging from 0 for a line to 1 for a perfect circle(147,868),(276,953) showing a significant decrease in SNAIL cell nuclei. G) Gene expression analysis shows down-regulation of several genes associated with nuclear structure in SNAIL cells. *P < 0.05, **P < 0.01, ***P < 0.001. Scale bars = 10 μm.

Figure 6. Mesenchymal SNAIL cells do not support solid stress. A) Spheroid cross-sectional area after 4 weeks growth in either 0.5% or 1.0% (w/v) agarose gels shows no significant difference between the 2 concentrations for control (CTRL) cells, but a dramatic decrease for SNAIL cells. B) Four-fold higher growth inhibition from increasing solid stress by altering gel concentration in SNAIL cells. C) Calculation of radial stress during spheroid growth shows that control cells are able to support at least 80 mm Hg, whereas SNAIL cells plateau at about 50% of this value. ns, not significant. ***P < 0.001.
analysis revealed that across all time lags particles within SNAIL cells had higher mean squared displacements indicative of a softer cytoplasm (Fig. 3A). Quantification of creep compliance demonstrated that SNAIL cytoplasm is deformed nearly an order of magnitude easier than their control counterparts (Fig. 3C). Moreover, increased effective diffusion coefficient within SNAIL cells may allow for more rapid transport of chemical signals or actin retrograde flow (Fig. 3B). Control cells had elastic moduli and shear viscosity mirroring other epithelial cancer cells (24), but both parameters were nearly totally abrogated following EMT (Fig. 3D, E).

Consistent with this loss of mechanical structure, SNAIL cells also displayed decreased polymerized actin (Fig. 4E). Though SNAIL cells lost this internal actin structure, they did display increased filopodium-like protrusions (Fig. 4B). The formation of these protrusions has been shown to be governed largely through β-parvin (PARVB), which we found to be up-regulated as a result of EMT (Table 1). This effect was most likely further enhanced by increased expression of several actin nucleating/branching proteins such as ARP3 (Table 1). On the basis of transcriptional analysis, the loss of actin structure was largely mediated via decreased expression of actin cross-linking proteins, not increases in actin severing proteins (Table 1), supported by the stabilization of actin filaments only being able to partially recover the mechanical stability of control cells (Supplemental Fig. 3B, C). This may open the possibility for new therapeutic approaches to reduce metastatic potential of cancer cells.

Although cytoplasmic deformation is required to navigate the extracellular environment, the nucleus is the largest organelle, and its deformation is often the rate-limiting step in migration (34). The nuclear relaxation following EMT (Fig. 5), may be important in allowing cells to navigate through small pores during migration, and in uncovering cryptic binding sites during transcription for increased gene expression (45). This nuclear softening has also been observed in stem cells (46) and supports the proposition that EMT creates stem cell-like cells (47).

Despite the body of evidence supporting the importance of EMT for cancer cells to migrate to distant sites, a continuing critique of this theory is the lack of mesenchymal signatures within secondary tumors (48). Recent studies have demonstrated that tumors in vivo are under mechanical solid stress and that this stress accumulates as tumors grow (49). Based on this, it reasons that softer cells would show impaired growth as this stress accumulates. Our results demonstrate that below a critical solid stress threshold, the mesenchymal SNAIL cells showed increased spheroid growth akin to the increased 2-dimensional BrdU incorporation (Fig. 1G, H). However, increasing the solid stress against the growing spheroids by doubling the agarose concentration effectively blocked SNAIL spheroid growth, whereas the area of control spheroids was unaffected (Fig. 6A). Calculation of the accumulated solid stress revealed SNAIL cells grew until reaching a maximum stress of approximately 35 mm Hg regardless of gel composition, whereas control cells were able to withstand at least 80 mm Hg of stress (Fig. 6C). One potential mechanism for this observation is that the reduced actin cytoskeletal structure from changes in cross-linking proteins does not allow the SNAIL-transformed cells to generate enough force to push the gel out allowing for further cell division. Alternatively, this could be a byproduct of decreased LMNA expression, which has been shown to not only expedite 3-dimensional migration, but also makes cells more prone to stress-induced apoptosis (36). This inability to survive in high-stress environments could potentially explain the inability to detect mesenchymal signatures in metastatic tumors, as stress acts as a selective pressure mitigating the presence of mesenchymal cells in favor of epithelial cells that can support the growing tumor.

In conclusion, this report demonstrates that the biophysical changes including increased traction forces and loss of cytoskeletal and nuclear structure associated with cancer metastasis are directly induced by epithelial to mesenchymal transition in absence of any extraneous environmental cues. Microarray gene expression analysis revealed concerted topographical alterations in gene expression networks associated with these phenomena. Finally, we show that these prometastatic biophysical changes may come at the cost of survival as secondary tumors progress in size, with accumulating solid stress only allowing for the survival of cells that return to a more mechanically stable epithelial phenotype. Further understanding of this complex interplay between changes in gene expression and cell biophysical properties with implications for cancer metastasis may help develop novel targeted therapeutics.

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SNAIL-induced epithelial-to-mesenchymal transition produces concerted biophysical changes from altered cytoskeletal gene expression


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Figure S1.
Heat maps of gene expression from GSEA analysis. (A) Actin Polymerization and/or Depolymerization gene set with mapped color-coded gene expression values. (B) Rho Protein Signal Transduction gene set with mapped color-coded gene expression values. Genes are sorted by correlation with SNAIL or control phenotype. Expression values range from low (blue) to high (red). S1-3: replicated Snail cells; N1-3: replicated control cells.
Figure S2.
Actin-related biological process networks (GeneGO) significantly enriched for genes differentially expressed between SNAIL and control cells. (A) “Regulation of Cytoskeleton Rearrangement” and (B) “Cytoskeleton Actin Filaments.” Red circles indicate up-regulation in SNAIL cells and blue circles indicate down-regulation in SNAIL cells. Directional links are activating (red), inhibitory (green) or unspecified (grey). For other details see the legend at http://pathwaymaps.com/pdf/MC_legend.pdf
Figure S3.
Changes in actin cytoskeleton and cell rheology. (A) Regulation of Actin Cytoskeleton – Homo sapiens (KEGG pathway hsa04810) with mapped color-coded genes identified as differentially expressed between SNAIL and control cells. Red: up-regulated in SNAIL cells; blue: down-regulated in SNAIL cells; pink: both up- and down-regulated genes were identified for given KEGG entry; green rectangles: genes not identified as differentially expressed. Changes in cell rheology following treatment with actin-stabilizing Jasplakinolide (Jas). (B) The elastic moduli of SNAIL cells is partially recovered following actin treatment, increasing an order of magnitude from untreated levels. (C) Shear viscosity is also partially recovered in SNAIL cells treated with Jasplakinolide increasing 2-fold.