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3D collagen architecture induces a conserved migratory and transcriptional response linked to vasculogenic mimicry.

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The topographical organization of collagen within the tumor microenvironment has been implicated in modulating cancer cell migration and independently predicts progression to metastasis. Here, we show that collagen matrices with small pores and short fibers, but not Matrigel, trigger a conserved transcriptional response and subsequent motility switch in cancer cells resulting in the formation of multicellular network structures. The response is not mediated by hypoxia, matrix stiffness, or bulk matrix density, but rather by matrix architecture-induced β1-integrin upregulation. The transcriptional module associated with network formation is enriched for migration and vasculogenesis-associated genes that predict survival in patient data across nine distinct tumor types. Evidence of this gene module at the protein level is found in patient tumor slices displaying a vasculogenic mimicry (VM) phenotype. Our findings link a collagen-induced migration program to VM and suggest that this process may be broadly relevant to metastatic progression in solid human cancers.
an initial step in cancer metastasis is the migration of tumor cells through the extracellular matrix (ECM) and into the lymphatic or vascular systems. Several features of the tumor ECM have been associated with progression to metastasis. In particular, regions of dense collagen are co-localized with aggressive tumor cell phenotypes in numerous solid tumors, including breast, ovarian, pancreatic and brain cancers. However, sparse and aligned collagen fibers at the edges of tumors have also been reported to correlate with aggressive disease. It remains unclear whether and how collagen architectures have a role in driving metastatic migration programs or if they simply correlate with progression of the tumor.

Intravital microscopy studies have shown that distinct collagen architectures are associated with specific cell motility behaviors. Cancer cells migrating through densely packed collagen within the tumor use invadopodia and matrix metalloproteinase (MMP) activity to move, whereas cells in regions with less dense collagen and long, aligned fibers migrate rapidly using larger pseudopodial protrusions or MMP-independent ameboid blebbing. Likewise, we previously showed in vitro that cell migration speed, invasion distance, and cellular protrusion dynamics are modulated by collagen fiber alignment, but that this relationship breaks down at high collagen densities (>2.5 mg ml\(^{-1}\)). These findings suggest that distinct motility regimes exist in low-density and high-density collagen, which may have implications for metastatic progression.

Here, we explore the relationships between collagen density, collagen architecture, cell migration behavior, gene expression, and metastatic potential. To do this, we develop a 3D in vitro model system designed to probe the physical basis of cancer cell migration responses to collagen matrix organization. Using this system, we find that confining collagen matrix architectures with short fibers and small pores induce a conserved migration behavior in cancer cells leading to network formation and the upregulation of a conserved transcriptional module, both of which are mediated by integrin-β1 upregulation. We show evidence that this in vitro behavior is consistent with phenotypic and molecular features of clinical VM. Moreover, we show that the associated transcriptional response is conserved among cancer types in vitro and is predictive of patient survival in multiple clinical datasets for various tumor types. Our integrative study suggests that a collagen-induced migration phenotype and gene

**Fig. 1** High-density 3D collagen microenvironment promotes a switch to persistent cell migration in cancer cells. a Mean squared displacement (MSD) and persistent time of MDA-MB-231 cells before and after cell division in high-density collagen. The persistent time was calculated from the MSDs using the persistent random walk model (see “Methods”). MSDs are shown for 12 representative cell trajectories. b Mean MSD and persistent time of MDA-MB-231 cells before and after cell division in low-density collagen. The persistent time was calculated from the MSDs using the persistent random walk model (see “Methods”). MSDs are shown for 12 representative cell trajectories. c Single-cell mean velocity measured at 2 min intervals before and after cell division. d Single-cell net invasion distance before and after cell division for cells in high-density and low-density collagen. e Representative image of MDA-MB-231 cells cultured in a 6 mg ml\(^{-1}\) (left) and in a 2.5 mg ml\(^{-1}\) (right) collagen I matrix after 7 days of culture. Cells are stained with Alexa-488 Phalloidin (F-Actin) and DAPI (nuclei). Scale bar 250 μm. f Quantification of mean structure length in low-density and high-density collagen, from images acquired in three independent experiments. g PAS stain of MDA-MB-231 cells cultured for 7 days in a 3D collagen gel of high-density (left) and low-density (right). Scale bar 100 μm. h Immunofluorescence staining of MDA-MB-231 cells for collagen IV after 7 days of culture in 6 vs. 2.5 mg ml\(^{-1}\). Representative images of n = 2 biological replicates. Bar graph shows mean and s.e.m. of quantification of stained area performed in 15 different fields of view. Scale bar 100 μm. i MDA-MB-231 cells cultured on top of growth factor-reduced matrigel after 24 h (left) and after 72 hours (right). Scale bar 250 μm. j MDA-MB-231 cells cultured inside growth factor-reduced matrigel in 3D culture for 7 days. Scale bar 100 μm. Box plots show quartiles of the dataset with whiskers extending to first and third quartiles. n = 3 biological replicates for all experiments unless otherwise noted. Statistical significance was determined by Mann-Whitney U test and is indicated as *, **, *** for p ≤ 0.05, p ≤ 0.01, p ≤ 0.001, respectively.
expression program are linked to a metastatic clinical tumor cell phenotype and potentiates future work to identify mechanistic strategies capable of limiting metastasis in several cancers.

Results

High-density collagen promotes fast and persistent migration. To first investigate the role of 3D collagen density in modulating the migration phenotype of breast cancer cells, we embedded MDA-MB-231 cells in collagen I matrices at densities mimicking normal breast tissue, 2.5 mg ml$^{-1}$ collagen$^{10, 11}$, and cancerous breast tissue, 6 mg ml$^{-1}$ collagen$^{10, 11}$. We observed that cells migrating in dense collagen initially appeared to be trapped and were unable to invade. However, after one division cycle, most cells switched to a highly invasive motility behavior, significantly increasing their persistence, velocity, and total invasion distance (Fig. 1a–d, left panels). This behavior was not observed in cells embedded in the low-density matrix, where cell migration was the same before and after division (Fig. 1a–d, right panels). Interestingly, cells that were in contact with the coverslip and not fully embedded in the high-density condition did not undergo the same migration transition upon division (Supplementary Fig. 1a, b). The motility responses we observed in 2.5 and 6 mg ml$^{-1}$ collagen matrices were not unique to MDA-MB-231 breast cancer cells. Similar migration patterns were observed for HT-1080 fibrosarcoma cells embedded in the same collagen matrix conditions (Supplementary Fig. 1c), suggesting that these responses may be conserved among distinct cancer types. To further examine whether the observed migration behavior was cell-type dependent, we tested the response of normal mesenchymal human foreskin fibroblasts (HFF-1) to low-density and high-density collagen conditions. Over an observation period of 48 h, HFF cells migrated consistently with very low persistence. Cells invaded less than three cell lengths in low-density collagen. In high density, HFFs elongated to reach cell lengths up to 300 μm but did not invade significantly (Supplementary Fig. 1d).

Density-induced migration results in cell network structures. It was unexpected that both MDA-MB-231 and HT-1080 cancer cells migrated faster and further in high-density collagen conditions. Intuitively, cell migration would be expected to slow in dense conditions where more matrix must be remodeled to enable cell movement. Moreover, this behavior was common to both cancer cell types but not displayed by normal fibroblasts, which represent residents of the stroma and also undergo mesenchymal migration in collagen. This motivated us to investigate the long-term implications of the rapid migration phenotype induced in cancer cells under high-density conditions. After 1 week of culture in high-density collagen, breast cancer cells undergoing rapid, and persistent migration formed interconnected network structures that resembled the early stages of endothelial tubulogenesis (Fig. 1e, left). The average length of cell networks after 1 week was 437 μm (Fig. 1f). Interestingly, these network structures do not appear to be caused by cells aligning along collagen fibers (Supplementary Fig. 1e). In contrast, cells cultured in low-density collagen for 1 week migrated slowly with low persistence, and remained as single cells (Fig. 1e, right). HT-1080 cells also formed network structures in high-density collagen and remained as single cells in low-density collagen (Supplementary Fig. 1f). HFFs remained as single cells in both high-density and low-density conditions (Supplementary Fig. 1g). The transition of cancer cells from single-cell migration to network formation suggested a potential transdifferentiation event, and the cell networks were reminiscent of a cancer phenotype known as vasculogenic mimicry (VM). VM is thought to arise from tumor cells that acquire the ability to form networks in the tumor ECM lined with glycogen-rich molecules and basement membrane proteins that can be perfused with blood. However, the tumor cells lining these networks do not express endothelial surface markers such as CD31$^{12, 13}$. Periodic acid schiff (PAS) staining of the networks formed in our high-density collagen condition confirmed the presence of glycogen-rich molecules (Fig. 1g) and immunofluorescence confirmed the presence of basement membrane protein COL4A1 (Fig. 1h), as in VM.

Previous pioneering studies have shown that several aggressive melanoma cell lines, which produce VM in vivo also intrinsically form VM network structures when cultured on top of Matrigel or collagen I in a 2D in vitro context$^{15, 14}$. Recently, other aggressive tumor cell types have been shown to intrinsically form VM-like network structures on top of Matrigel or in 2.5D culture in Matrigel$^{15–19}$. Here, it is important to note that variations exist in the consistency of commercial ECM products as well as the terminology used to describe 3D culture. We define 3D culture strictly as a condition where cells are fully embedded, in contrast with ECM on all sides, and located a sufficient distance away from the coverslip bottom and sides of the culture dish to avoid their influence. We define 2.5D culture as a pseudo 3D culture where cells are embedded in the ECM but in contact with coverslip. Our previous studies have demonstrated the importance of these distinctions, as cell behavior and protein localization are differentially regulated in each context$^{20–22}$. Therefore, we sought to understand whether the network phenotype induced by a 3D collagen I environment was distinct from that induced by a 2D Matrigel environment. First, we asked whether our cells formed network structures on top of Matrigel. Few cells aligned within the first 24hrs of culture, and nearly all cells aggregated after 72 h (Fig. 1i). Next, we embedded MDA-MB-231 cells inside of Matrigel, in 3D culture. In this context, cells did not form network structures but instead formed rough-edged, disorganized spheroids (Fig. 1j). Thus, high-density collagen uniquely induced the network-forming phenotype in a more physiologically relevant 3D context.

A conserved transcriptional response precedes migration. We hypothesized that the persistent migration phenotype of cancer cells leading to network formation in high-density collagen conditions (collagen-induced network phenotype, CINP) could be the result of a transdifferentiation event wherein a unique cell motility gene module was upregulated. To test this, we conducted RNA sequencing of MDA-MB-231, HT-1080, and HFF cells cultured in low-density and high-density collagen matrices after 24 h (Fig. 2a), the time point just before most cancer cells in the high-density collagen matrix underwent at least one cycle of cell division and began to invade with increased persistence. As the majority of cancer cells cultured under high-density conditions participated in network formation, we expected their bulk transcriptional profile to be dominated by this phenotype$^{23}$. Then we asked whether common stem cell and differentiation markers were upregulated in association with the network-forming phenotype. Indeed, several known stem cell markers were upregulated (Fig. 2b), and three were common to both cancer cell types: JAG1, ITGB1, and FGFR1. This suggested that both cancer cell types harbored stem-like qualities, which could facilitate significant transcriptional reprogramming.

Analyzing more broadly, we then asked which genes were differentially regulated (TPM fold change $\geq 1.5$) in high-density collagen compared to low-density collagen in each cell type and whether these genes represented unique or conserved transcriptional response modules. As expected, cell type accounted for the most variance in gene expression (Fig. 2c). However, after a...
z-score transformation of the gene expression of each cell type, the collagen matrix condition accounted for the bulk of the remaining variance in gene expression (Fig. 2d). This suggested the presence of gene expression programs linked to collagen matrix conditions.

Using a Venn diagram approach to identify conserved expression modules, we discovered a set of 70 genes that were upregulated by both cancer cell types but not normal cells in response to high-density collagen (Fig. 2e; Supplementary Fig. 2a). Gene ontology (GO) enrichment analysis revealed that these 70 common-to-cancer genes were significantly enriched for annotations in blood vessel development and regulation of migration (Fig. 2f, g). Importantly, changes in the threshold for differential expression did not significantly alter the primary gene ontology categories identified (Supplementary Fig. 2d; Supplementary Table 1). Key genes involved in Notch signaling, i.e., BBP1 and LENG, were among the 70. Importantly, LAMC2, JAG1, and THBS1 genes identified in this common-to-cancer gene set have been previously associated with a VM phenotype intrinsically displayed by metastatic melanoma, which was assessed by targeted microarray analysis for angiogenesis, ECM, and cell adhesion genes. Upregulated surface markers were not endothelial in nature, and did not represent any specific tissue or cell type (Fig. 2g).

Further exploration of our dataset with respect to individual cancer cell types revealed that, beyond the conserved transcriptional response, high-density collagen also triggered the expression of genes related to vasculogenesis in a cell type-dependent manner. For example, breast cancer cell networks upregulated VEGFA fold change = 1.65 and MMP14 fold change = 1.72, but fibrosarcoma cell networks did not. Some of these genes have been previously associated with the VM network phenotype of melanoma cells (Supplementary Fig. 2c).

Next, we assessed the 35 genes that were upregulated in response to high-density collagen by all three cell types (Fig. 2e). These genes were enriched primarily for annotations in regulation of cell differentiation (Fig. 2h). However, it is important to take into account the inherent flaws associated with GO enrichment analysis. For example, some categories showing enrichment in the 35 genes common-to-all cell lines contain very few genes and may not represent real enrichment. However, this limitation is not observed in the top enriched categories in the 70 genes common-to-cancer cells, where most category contains at least 10 genes (Fig. 2f). The genes associated with each enrichment category are given in Supplementary Tables 2 and 3.

Interestingly, SERPINE1, a secreted protease inhibitor involved in coagulation and inflammation regulation, was identified in the common-to-all gene module (Supplementary Fig. 2b). Several Serpine protein family members have previously been implicated...
as drivers of metastasis correlating with VM18 and with brain metastases of lung and breast cancers26.

Integrin-β1 upregulation is required for CINP. We next sought to identify the matrix feature triggering transdifferentiation. The physical parameters of stiffness, pore size, and fiber organization differ between the low density 2.5 mg ml⁻¹ and high density 6 mg ml⁻¹ collagen matrices10. Chemical cues may also change. For example, adhesive ligand density and binding site presentation to integrins and other matrix receptors may differ27, 28 as well as accumulation or release of autocrine and paracrine signals sequestered by the ECM 29, 30. Each of these features could potentially impact cancer cell motility behavior and gene expression.

As matrix stiffness has been implicated in driving epithelial-to-mesenchymal transitions (EMT) and aggressive phenotypes11, 32, 33, we first asked whether increased stiffness of the high-density collagen matrix was responsible for triggering transdifferentiation. To test this, we developed a collagen polymerization procedure (“Methods”) that increases the stiffness of the low-density matrix to match the stiffness of the high-density matrix (Fig. 3a). By lowering the polymerization temperature from 37 to 20 °C, polymerization slowed, allowing fibers to form more organized and reinforced fiber structures with larger pores (Supplementary Fig. 1I). Breast cancer cells cultured in this stiffened low-density condition did not undergo network formation (Fig. 3b), suggesting that 3D stiffness is not sufficient for triggering the transdifferentiation.

Next, we sought to determine whether the smaller pore size of the high-density matrices triggered transdifferentiation. One way in which smaller pore sizes could influence cell behavior is by restricting the diffusion of molecules to and from the cells34. More specifically, the imbalance between oxygen diffusion to cells and oxygen consumption by cells in 3D matrices has been shown to promote hypoxic conditions in some cases35. Since regions of VM have previously been associated with markers of hypoxia in vivo36, 37, we hypothesized that cells in high-density collagen created a more hypoxic condition than in low-density collagen and that low-oxygen levels could trigger network formation. To test this, we cultured MDA-MB-231 cells in low-density collagen under a hypoxic atmosphere of 1% oxygen for 1 week. To confirm that a hypoxic response was achieved, we assessed the level of HIF1A mRNA expression by RT-qPCR at day 7 and found a significant decrease in HIF1A expression (Fig. 3c). This is a common response to long-term hypoxia by various cancer cell
Fig. 4 Role of β1 integrin in the formation of cell network structures in high-density collagen. a Schematic of lentCRISPR V2 vector used for targeting ITGB1 gene and western blot validation of the protein depletion after 7 days of cell transduction. b Comparison of MDA-MB-231 cells WT and ITGB1 depleted in low-density 3D collagen. Left: micrographs showing a representative image of a WT cell undergoing mesenchymal migration and an ITGB1-depleted cell undergoing ameboid migration. Right: quantification of mesenchymal vs. ameboid migration within the cell populations. c Quantification of the effect of ITGB1 depletion on mean cell velocity when cells are cultured in 6 mg ml\(^{-1}\) collagen. d Cell persistence and e cell invasion distance. Comparison for c–e was performed using Mann–Whitney U test. f MDA-MB-231 WT, ITGB1-depleted, and control sgRNA cell phenotypes after 7 days of culture in low-density collagen (top row) and high-density collagen (middle row). Scale bar 250 μm. Bottom row shows high-magnification micrographs highlighting the difference between chain structures and spheroids. Scale bar 100 μm. g Quantification of proportional number of structures in each cell line when cultured in high-density collagen. h Fluorescence-activated cells sorting (FACS) was used to separate the parental WT MD-MB-231 cell line population into high-ITGB1 and low-ITGB1 expressing populations. i ITGB1 high and ITGB1 low cells after 7 days of culture in high-density 3D collagen (top row) and low-density (bottom row). Scale bar 200 μm. j RT-qPCR quantification of a small subset of genes identified in the 70-gene module in WT control- and ITGB1-silenced cells when cultured in low-density and high-density collagen. Data show mRNA levels relative to GAPDH and relative to low-density collagen level. Statistical significance evaluated between WT and gITGB1 groups. Statistical significance was determined by ANOVA test. Bar graphs represent mean ± s. d. and data in box and whiskers plots is presented using Tukey method. n = 3 biological replicates for all experiments unless otherwise noted. Significance is indicated as *, **, *** for p ≤ 0.05, p ≤ 0.01, p ≤ 0.001, respectively.

To further explore whether pore size reduction induced transdifferentiation of cancer cells, we sought to interrogate this parameter independently of collagen density. In our model, the high-density condition contains 2.4 times more collagen than the low-density condition. This increase in total collagen reduces pore size, but also presents more adhesive ligands to cells, which could increase integrin activation. To separate pore size from bulk density, we developed a collagen structure engineering technique that reduced the pore size and fiber length of the low-density matrix to approximate that of the high-density matrix. Under normal polymerization conditions, low-density collagen self-assembles into relatively long, structured fibers. When non-functionalized, inert polyethylene glycol (PEG) was mixed into collagen monomer solution prior to polymerization, molecular crowding-restricted fiber formation. This resulted in shorter, more interconnected fibers yielding smaller pores (Fig. 3f–i) without increasing stiffness (Fig. 3a). Breast cancer cells encapsulated in this pore size-reduced low-density matrix underwent network formation over the course of 1 week (Fig. 3j).

To control for the possible influence of PEG itself, PEG was added into media on top of a normally polymerized low-density gel embedded with cells and allowed to diffuse into the interstitial

lines. Hypoxia was not sufficient to induce network formation in any portion of the cancer cell population in the low-density collagen matrix (Fig. 3d, left). For comparison, we also assessed the HIF1A mRNA expression of breast cancer cells cultured for 1 week in low-density collagen under 21% oxygen, in high-density collagen under 1% oxygen, and in high-density collagen under 21% oxygen (Fig. 3c). These results suggested that cells cultured in high-density collagen experienced increased hypoxia compared to cells cultured in low-density collagen under normal atmospheric conditions. Nevertheless, the hypoxic response achieved in low-density collagen under 1% oxygen exceeded that induced by high-density matrix alone. Cells in high-density matrix under 1% oxygen continued to predominately display a network phenotype (Fig. 3d, right), but the average network length (Fig. 3e) was significantly shorter than cells in high-density collagen under normoxic conditions (Supplementary Fig. 1b). Previous studies have reported that hypoxia is not sufficient to induce a VM phenotype in melanoma cells in vitro. It is possible that in vivo, additional stromal cell secreted factors or cell–cell interactions modulated by hypoxia may indirectly influence the VM process.
spaces among the fibers to reach the same final concentration as was used in the pore size-reduced low-density matrix (10 mg ml⁻¹ PEG). Cells maintained in this molecularly crowded condition over 1 week did not form networks, but instead remained as single cells. However, a noticeable slowing of cell migration occurred, which resulted in an anisotropic patterning of single cells throughout the matrix (Fig. 3k). These results suggested that the fiber architecture of high-density collagen induces network formation independently of the bulk increase in adhesive ligand density and confirms that bulk matrix stiffness is not involved.

The short, more isotropic arrangement of fibers associated with both the high-density collagen and low-density PEG crowded collagen conditions could act on cells through local cell–matrix interactions transduced by integrin signaling. Integrin-β1 (ITGB1) is a canonical receptor for collagen I, a central node in ECM signal transduction, and a critical mediator of breast cancer progression in mouse and in vitro models. Here, ITGB1 was upregulated by both cancer cell types in response to confining matrix conditions (Fig. 2b). Thus, we next asked whether the network-forming phenotype observed in confining matrix conditions was mediated by ITGB1. CRISPR-Cas9 technology was used to silence ITGB1 expression with single guide RNAs (sgRNAs), and constructs expressing sgRNAs targeting eGFP were used as controls (Fig. 4a). Silenced and control cells were embedded separately and sparsely in low-density and high-density collagen matrices. Cells were monitored by time-lapse microscopy for early migration behavior then imaged again after one week. In low-density collagen, ITGB1-silenced cells maintained a similar level of migration capability to wild type (WT) cells in low-density matrices, but used an ameboid blebbing migration phenotype instead of a mesenchymal migration phenotype (Fig. 4b). In high-density conditions, ITGB1-silenced cells migrated faster than WT cells, but were significantly less persistent and did not invade (Fig. 4c–e). Surprisingly, after 1 week ITGB1-silenced cells in high-density collagen-formed spheroid structures instead of cell networks, whereas control cells exhibited the same behavior as the WT in both collagen conditions (Fig. 4f). Retrospective analysis of WT MDA-MB-231 cells in high-density collagen revealed that a small fraction spontaneously formed spheroid structures (Fig. 4g). These findings suggest that either basal expression level or upregulation of ITGB1 dictates the network-forming phenotype. To distinguish between these two possibilities, we next sorted the parental WT population based on basal ITGB1 expression level and then embedded high and low expressing cells separately in confining high-density collagen matrices (Fig. 4h). We observed no appreciable differences in the percentage of networks versus spheroids formed by the sorted populations after one week. However, ITGB1 low cells proliferated less and displayed fewer total number of network or spheroid structures (Fig. 4i) even though the initial seeding density was the same (Supplementary Fig. 3a).

To further explore the link between the upregulated transcriptional module and the network-forming phenotype, we asked whether ITGB1-silenced spheroid-forming cells showed different gene expression patterns than WT network-forming cells. To assess this, we conducted qRT-PCR analysis of a subset of the 70-gene panel in the two cell phenotypes. Upregulation of several key genes were maintained in the spheroid-forming cells, whereas other genes were no longer upregulated (Fig. 4j). These results...
show that ITGB1 regulates some aspects of the transcriptional module associated with the network-forming phenotype.

Finally, we asked whether upregulated genes in our transcriptional module that have previously been implicated as drivers of VM in vitro were functionally active in our network-forming phenotype. LAMC2 (Ln-5, gamma 2 chain) was previously found to be upregulated in aggressive melanoma cells that intrinsically display the VM phenotype compared to less aggressive melanoma cells that do not display VM. Moreover, it was implicated as a driver of VM network formation, since the cleavage of this secreted matrix molecule by MMP-2 and MT1-MMP produces pro-migratory fragments. In 2D culture of aggressive melanoma cells on top of collagen I, the inhibition of LAMC2 cleavage blocked VM network formation. Using shRNA to knockout LAMC2, we found that LAMC2 KD MDA-MB-231 cells maintain their ability to form network structures in 3D high-density collagen complex. COL4A1 is another matrix molecule upregulated by cells undergoing the network phenotype and previously implicated in driving migration. COL4A1 KD in MDA-MB-231 cells also did not inhibit the ability of cells to form network structures in 3D high-density collagen complex.

**CINP transcriptional module predicts poor prognosis in human cancer.** Finally, we sought to determine whether the CINP triggered by our 3D system was clinically relevant. To test this, we first asked whether the 70 common-to-cancer genes associated with the CINP could predict cancer patient prognosis. We anticipated that if this gene signature was indicative of a more metastatic cancer cell migration phenotype, its expression would correlate with poor patient outcomes. Since late stage tumors are already characterized by migration of tumor cells to distant lymph nodes or organs, we hypothesized that a gene signature associated with metastatic migration would correlate with prognosis in early (stage I and II) but not late (stage III and IV) stage tumors. Using the cancer genome atlas (TCGA), we first analyzed data for breast cancer patients with respect to the expression of the 70-gene signature. An expression metagene was constructed using the loadings of the first principal component (CINP PC1) of a 195 Stage I patient by 70-gene matrix (Supplementary Fig. 3b, c). COLO1A1 is another matrix molecule upregulated by cells undergoing the network phenotype (Figs. 1h and 2g) and previously implicated in driving migration. COLO1A1 KD in MDA-MB-231 cells also did not inhibit the ability of cells to form network structures in 3D high-density collagen complex (Supplementary Fig. 3b, c).

**Table 1 CINP score potential to predict prognosis in stage I patients from METABRIC database broken down by molecular subtype**

| Metabolic molecular subtype | Patient count | Death observed | HR | Cox p |
|-----------------------------|---------------|----------------|----|-------|
| Luminal A                    | 126           | 33             | 1.2461 | 0.3194 |
| Luminal B                    | 202           | 34             | 1.5996 | 0.0162 |
| Triple negative              | 63            | 14             | 3.8537 | 0.0070 |
| HER2+                        | 39            | 13             | 0.7152 | 0.3405 |

Analysis of CINP score potential to predict prognosis in stage I patients from METABRIC database broken down by molecular subtype.

**Table 2 TCGA pan cancer analysis independent of stage**

| Cancer type | Patient count | Death observed | HR | Cox p |
|-------------|---------------|----------------|----|-------|
| LGG         | 508           | 92             | 1.8434 | 1.1–13 |
| ACC         | 79            | 25             | 3.1863 | 2.8–04 |
| CESC        | 304           | 60             | 1.6560 | 5.2–04 |
| MESO        | 85            | 28             | 1.6101 | 6.9–04 |
| PAAD        | 178           | 59             | 1.5948 | 2.2–03 |
| BLCA        | 409           | 111            | 1.3338 | 0.0053 |
| LUAD        | 521           | 124            | 1.2448 | 0.0169 |
| KICH        | 64            | 8              | 2.9277 | 0.0210 |

Table showing results from Kaplan-Meier and hazard ratio analysis across all cancer types in TCGA, where the CINP gene score is significant predictor of prognosis (p < 0.05).
genes from our CINP transcriptional module in the VM tumor tissue but little stain in healthy tissues.

Discussion
Our transcriptional, histopathologic, and phenotypic data suggest that the in vitro CINP and clinical VM share many commonalities. To our knowledge, this is the first time that collagen fiber architecture, characterized by short fibers and small pores, has been identified as an inducer of cancer transdifferentiation associated with a VM-like phenotype or more normal acinar phenotype, depending on the capacity of cells to upregulate ITGB1. More broadly, our findings show that collagen fiber architecture modulates the role ITGB1 plays in migration. In one architectural context, ITGB1 facilitates a switch from mesenchymal to ameboid migration and in another architectural context it mediates migration persistence and the shape of structures formed by collective morphogenesis.

Although ITGB1 was critical for directing the fate of cells during collagen-induced transdifferentiation, it was not necessary for initiating the transition from single cell to collective morphogenesis. Thus, it is not yet clear how cells sense the collagen architecture to initiate this process, but the response appears to be unique to stem-like cancer cells (MDA-MB-231 and HT-1080) as opposed to normal cells (HFF-1). Since, in our system, cells are embedded sparsely and undergo transcriptional reprogramming prior to cell division, the involvement of cell–cell interactions does not appear to have a role in transdifferentiation initiation. It is possible that cell interactions with the unique matrix architecture involve matrix sequestration of soluble factors and autocrine signaling. Indeed, TGFβ pathways were implicated by GO enrichment analysis (Fig. 2f). Alternatively, the initial confinement and rounded geometry of the cells enforced by the matrix may play a role. Several studies support a role for cellular geometry in numerous cellular processes including gene expression and differentiation17–51, some of which is mediated by RhoA and cytoskeletal tension. However, confinement in Matrigel did not trigger the same process, indicating a unique requirement for cell–cell interaction. Future work will address these questions.

ECM molecules, COL4A1 and LAMC2, were also upregulated by CINP cells and have previously been implicated in driving migration and VM network formation in 2D culture43, 44. In our 3D collagen system, knockdown of either gene was not sufficient to block the VM-like phenotype (Supplementary Fig. 3). This suggests that regulation of in vitro cell network formation in a more physiological 3D culture context is distinct from regulation in a 2D culture context, which has implications for understanding molecular mechanisms. Given the significantly different requirements for cell movement in 3D ECM, such as matrix degradation and remodeling, our study highlights the importance of both the type of matrix and the dimensional context for studying physiological migration strategies. This echoes previous studies, which have shown that cell motility proteins function distinctively in a more physiologically relevant 3D context52–22.

Interestingly, SERPINE1, a secreted protease inhibitor involved in coagulation and inflammation regulation, was upregulated by cancer cells as well as normal fibroblasts in response to confining collagen architectures. A recent study of cancer cell heterogeneity using mouse mammary carcinoma 4T1 cells and validated in human MDA-MB-231 breast cancer cells showed that cells which intrinsically expressed SERPINE family members were most efficient at spreading hematogenously, a characteristic that also correlated with their capacity to undergo VM in vivo18. Together, with our findings, this suggests that both cell-intrinsic and ECM factors may contribute to the emergence of VM. Interestingly, our finding that fibroblasts and cancer cells both upregulate SERPINE1 expression in confining collagen conditions hints at a potential supporting role for stromal cells in SERPINE-mediated VM metastasis18.

The significant predictive value of our CINP gene signature in several tumor types may signify the physiological relevance of the ECM context and network-forming migration phenotype we created in vitro to a conserved mechanism of solid tumor metastasis. It is possible that gene expression analysis of additional cancer cell types induced into VM-like behavior by our 3D collagen system could help to further refine the conserved CINP gene module. This would facilitate prioritization of the genes for targeted functional studies to identify key regulators and potential therapeutic targets. In addition to regulators of the CINP, the conserved gene module also likely contains elements responsive to collagen but not directly involved.

Profiling additional cancer cell types and patient-derived tumor cells could also help to refine the gene module’s prognostic value in the nine tumor types already identified or define additional context-specific versions of the CINP. Validation of the prognostic value of this gene module could help patients avoid the long-term side effects of aggressive radiation and chemotherapy if the likelihood of metastasis is very low. A recent meta-analysis of histological VM in over 3000 patients with various solid tumor types found that the visual presence of this cancer phenotype is specifically associated with poor prognosis52. Molecular detection of VM markers could provide a more quantitative measure.

Methods
Cell culture. HT-1080 and HFF-1 were purchased from (ATCC, Manassas, VA) MDA-MB-231 cells were provided by Adam Engler (UCSD Bioengineering). All cell lines were cultured in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum (FBS, Corning, Corning, NY) and 0.1% gentamicin (Gibco Thermofisher, Waltham, MA), and maintained at 37 °C and 5% CO2 in a humidified environment during culture and imaging. The cells were passaged every 2–3 days. Cell culture under hypoxia was done on a humidified and temperature controlled environment at 1% O2. Cells were tested for mycoplasma contamination using the Mycoalert kit (Lonza, Basel, Switzerland) before performing experiments.

3D culture in collagen I matrix. Cells embedded in 3D collagen matrices were prepared by mixing cells suspended in culture medium and 10% reconstitution buffer, 1:1 (v/v), with soluble rat tail type I collagen in acetic acid (Corning, Corning, NY) to achieve the desired final concentration16, 20, 21. A total of 1 M NaOH was used to normalize pH in a volume proportional to collagen required at each tested concentration (pH 7, 10–20 µl 1 M NaOH), and the mixture was placed in 48-well culture plates and let polymerize at 37 °C. Final gel volumes were 200 µl.

Cell tracking and motility analysis. Cells were embedded in 3D collagen matrices in 48 well plates and left polymerize for 1 h in a standard tissue culture incubator and then 200 µl of complete growth medium were added on top of the gels. The gels were transferred to a microscope stage top incubator and cells were imaged at low magnification (<10) every 2 min for 48 h. Coordinates of the cell location at each time frame were determined by tracking single cells using image recognition software (Metamorph/Metavue, Molecular Devices, Sunnyvale, CA). Tracking data were processed using custom written python scripts based on previously published scripts53 to calculate cell speed, invasion distances, and mean-squared displacements (MSDs). For cell motility analysis before and after division the time-lapse videos were scanned to identify dividing cells within the imaging period and the division point was identified as the frame at which a clear separation could be identified between daughter cells. The dividing cell was tracked up to the division point and one of the daughter cells (randomly chosen) was tracked from that point until the 48 h time point. For collective cell invasion distance, the 48 h time-lapse video was processed to obtain the maximum intensity projection (MIP), which highlights the tracks taken by the cells/groups of cells. Individual tracks distinguished by the MIP were measured to obtain an equivalent invasion distance. All cell tracking data comes from three independent experiments performed on different days and with different cell passages.

Persistence random walk model implementation. To quantify the differences in the MSDs, we fitted the MSDs for each condition using a persistence random walk model (PRW model) as described in refs. 53, 54. Briefly, the MSDs were calculated as in Eq. 1. The Eq. 2 describing the PWR was fitted using python’s Imfit library for
Each MSD. The persistent time (parameter P) was then extracted to calculate the error in the position of the cell as described in ref. 34.

Collagen stiffness modification and measurement using shear rheology. To modify the stiffness of collagen matrices without increasing density of material, we kept 2.5 mg/m³ gels at 20°C for 30 min until they were fully polymerized. After the initial polymerization the gels were placed on a humidified tissue culture incubator at 37°C for at least 1 hour extra before adding cell culture media on top. To measure the effect of polymerization temperature on the gel stiffness, we recreated the polymerization conditions for rheology testing (hybrid rheometer (DHR-2) from TA Instruments, New Castle, DE) using a cone and plate geometry with a sample volume of 0.6 ml. Shear storage modulus G’ was measured as reported before16. Briefly, we first performed a strain sweep was from 0.1 to 100% strain at a frequency of 1 rad s⁻¹ to determine the elastic region. Then a frequency sweep was performed at a strain within the linear region (0.8%) between 0.1 and 100 rad s⁻¹. Three independent replicates were performed for each condition tested.

Collagen structure modification using polyethylene glycol. To modify the structure of the collagen fibers within the gels without changing the final collagen concentration, Polyethylene glycol (PEG, MW 8000, Sigma, St. Louis, MO) was solubilized in phosphate-buffered solution (PBS), filter sterilized. Solubilized PEG was then mixed into the cells, reconstitution buffer solution described above to produce a final PEG concentration of 10 mg/ml in the collagen gel. The gels were allowed to polymerize in the same conditions as collagen only gels. Collagen structure modification was verified using confocal reflection microscopy.

DNA isolation and purification. 3D collagen I gels were seeded in three independent experiments and harvested after 24 h of culture for DNA extraction and directly emergergent from a fold-change of 1.3 to a fold-change of 1.9. The probability of a gene given term, to assess the sensitivity of the enriched gene sets to the genes used in downstream analysis TPM was used as a measure of gene expression. A gene was considered detected if it had mean TPM > 5.

Western blotting. Cells were grown to >90% confluence in 100 mm dishes. After washing 2X with PBS cells were collected into 100 µl of lysis buffer with 1X Halt protease inhibitor cocktail (Pierce IP lysis Buffer, Thermofisher, Waltham, MA) by scraping the dish surface. Cell lysate was incubated on ice with constant shaking for 30 min and then centrifuged at 15,000 x g for 20 min for protein purification. Samples were loaded at 50 µg total protein concentration for SDS-PAGE. Membranes were probed with antibodies against ITGB1 (Catalog No: 44706 from Cell signaling Technology, Danver, CA). All cell motility data were analyzed for statistical significance using the Scipy Python package. Additional experimental data and statistics all cell motility data were analyzed for statistical significance using the Scipy Python package. Additional experimental data were analyzed using prism Graphpad (San Diego, CA). Significance (p) was indicated within the figures using the following scale: *p < 0.05, **p < 0.01, ***p < 0.001. Additional relevant information is detailed in the figure captions.

TCGA data reproprocessing and survival analysis. The TCGA raw data were downloaded from CGHub directly. The survival analysis was performed using an online survival tool, the Kaplan-Meier estimate (K-M) plotter.
RNA-seq fastq files were realigned and quantified using saffish v.0.7.6.2 with default parameters. Only primary tumors were considered in our analysis. In the analysis of breast invasive carcinoma, only the patients with reported histological staining for the three markers (Her2, ER, PR) could be associated with a molecular subtype. Patients for which any of the histological markers were not evaluated or were detected at an equivocal level were assigned to our analysis. In the analysis of breast invasive carcinoma, only the patients with genes to construct gene expression meta-markers as previously described. PCA-based score quantiles were mapped to CINP high and CINP low categories based on mean CINP gene expression levels. Because the CINP signature comprised only genes that were upregulated in the presence of the network phenotype, the overall mean expression of CINP genes was used to map PCA score to CINP signature activity level.

**Data availability**

Relevant scripts for the analysis of TCGA and METABRIC data are available at: https://github.com/brianyiktaktsi/Valentine_Mimicry.

**Human Protein Atlas data.** The online database Human Protein Atlas was used to identify breast cancer tumor slices displaying hallmarks of the VM phenotype and subsequently assess protein expression of the genes associated with our in vitro network-forming phenotype. The tumor of patient ID 1910 was found to display linear chains of cancer cells lining interconnected matrix networks and had been stained for numerous other proteins of interest. Histological images shown in Fig. 5d can be found at www.proteinatlas.org by searching for the gene name in the breast cancer database and selecting patient ID 1910.

**Code availability.** Relevant scripts for the analysis of TCGA and METABRIC data are available at: https://github.com/brianyiktaktsi/Valentine_Mimicry.

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### Author contributions

D.O.V. conducted all cell and collagen matrix experiments and analyses. T.G., A.H., D.O.V. and C.L.C. performed PEG-collagen matrix experiments and analyses. D.O.V. and B.T. analyzed RNA sequencing data. B.T. performed TCGA and METABRIC analyses. C.L.C. conducted COL4A1 and LAMC2 knockdown studies. S.I.F. conceived and supervised the project. S.I.F. and H.C. co-designed RNA-sequencing experiments and co-supervised sequencing data analysis. All authors wrote or edited the manuscript.

### Additional information

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**Competing interests:** The authors are inventors on a patent related to the prognostic gene signature reported herein.

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