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Aligned Forces: Origins and Mechanisms of Cancer Dissemination Guided by ECM Architecture

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Abstract

Organized extracellular matrix (ECM), in the form of aligned architectures, is a critical mediator of directed cancer cell migration by contact guidance, leading to metastasis in solid tumors. Current models suggest anisotropic force generation through the engagement of key adhesion and cytoskeletal complexes drive contact guided migration. Likewise, disrupting the balance between cell-cell and cell-ECM forces, driven by ECM engagement for cells at the tumor-stromal interface, initiate and drive local invasion. Furthermore, processes such as traction forces exerted by cancer and stromal cells, spontaneous reorientation of matrix-producing fibroblasts, and direct binding of ECM modifying proteins lead to the emergence of collagen alignment in tumors. Thus, as we obtain a deeper understanding of the origins of ECM alignment and the mechanisms by which it is maintained to direct invasion, we are poised to utilize the new paradigm of stroma-targeted therapies to disrupt this vital axis of disease progression in solid tumors.

INTRODUCTION:

In addition to transformed cells, solid tumors are comprised of a complex ensemble of cellular and acellular components, collectively known as the tumor stroma. The stroma is a critical part of the tumor microenvironment (TME) and often plays a vital role in

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tumor progression and metastasis. Stromal components may be cellular, including cancer associated fibroblasts (CAFs), endothelial cells, tumor associated macrophages (TAMs), and various other immune cells, or acellular such as the extracellular matrix (ECM) on which the cellular components develop, interact and thrive. Many solid tumors, including those of the breast, pancreas and lung, are characterized by a robust desmoplasia - a fibroinflammatory response in which a dense, fibrotic ECM drives tumor progression, metastasis, and resistance to therapy. Robust deposition of fibrous collagen (mostly type I Collagen) is a hallmark of the desmoplastic tumor ECM, which not only contributes to elevated stiffness, but also creates discrete structural patterns in the TME. These patterns, called Tumor-Associated Collagen Signatures (TACS), have been shown to be important in local invasion of cancer cells, promoting directed cell migration into and through the stroma, by a process known as contact guidance (1–3).

The process of contact guidance has been experimentally studied for almost half a century (1, 4) and until a few of decades ago had been primarily described in the context of engineering cell behavior *in vitro* (2, 5), until imaging studies revealed it to be an important mechanism for guiding tumor cell invasion *in vivo* (3). Since then, along with a burgeoning interest in the field of mechanobiology, we have witnessed a plethora of studies seeking to better elucidate the prognostic relevance of collagen and more broadly ECM alignment in solid tumors and the mechanisms by which they promote invasion and metastasis. Here, we review recent advances in our understanding of the origin, diversity, and contextual relevance of ECM (mostly collagen) architectures in solid tumors, the molecular mechanisms of enhanced directed migration and invasion by contact guidance, and ways of targeting these stromal architectures to mitigate tumor progression and metastasis.

TYPES OF ORGANIZED ECM ARCHITECTURES IN TUMORS:

Collagen alignment as a driver of cancer cell invasion and metastasis was first described in Wnt-1 and PyMT breast carcinoma models (3), consistent with the role of ECM architecture and composition in mammary gland development and function (6, 7). Among the collagen patterns described in the TME were TACS-2 and TACS-3, which represent straight collagen fibers aligned parallel and perpendicular, respectively, to either the ductal or ductal carcinoma in situ boundary, around carcinoma cell clusters within the tumor mass, or at the tumor boundary (3), thus defining the relevant architectures dominant in early stage and advanced carcinomas. In particular, TACS-3 aligned collagen regions throughout the tumor mass provide conduits for carcinoma cell invasion (8–11)(Fig. 1). Along these lines, a recent study has presented an additional series of TACS (12), however it appears that these designations are the same or perhaps subcategories of the original TACS1–3 architectures. In addition, a few studies identify alignment of fibronectin (13, 14), instead or in addition to collagen, in the periductal space as driving the directed migration of cancer and stromal cells, which can also be broadly understood in the realm of anisotropic cell-ECM interactions. Indeed, such TACS features have now been identified in pancreatic (8, 15–17), lung (18), renal (19), prostate (20, 21) and skin (22) cancer, among others. Particularly in breast cancer, studies have demonstrated that collagen architecture, either alone or in combination with related stromal properties, is a prognostic for patient outcomes (12, 23–25). Notably, in complex, heterogeneous tissues such as carcinomas,

collagen and ECM architecture, as it pertains to driving the cell biology of disease, is best understood in relation to cellular and tissue organization in the TME. The TACS nomenclature exemplifies this approach, wherein the organization of the ECM is classified in relation to the transformed epithelium as the basic organizational structure and can be utilized as a template to universally classify collagen patterns in malignant tissue.

MECHANISMS OF LOCAL INVASION GUIDED BY ANISOTROPIC ECM:

Dissecting ECM alignment with respect to cellular organization in the TME reveals at least two distinct niches in which contact guidance plays a key role in directing cancer cell invasion and dissemination:

(a) Dissemination of single cells distal to ductal structures or tumor nests:

These are single cells already delaminated from the core tumor nest or neoplastic epithelium and continue to disseminate through the stroma using aligned and organized collagen tracks (TACS-3; Fig. 1). Such cancer cells have often undergone an epithelial-to-mesenchymal transition (EMT), and have been observed in live breast and pancreas tumors (26, 27). In other cases, such cells have also been found to track along blood vessels, using the regular structure of the endothelium as tracks instead of aligned collagen (28, 29). Multiple schools of thought have emerged to explain how single cells sense and migrate directionally along these organized ECM tracks, largely based on *in vitro* systems to probe the mechanisms of this process under controlled conditions. A prevailing theory, one that was proposed almost half a century ago (4), is where the cellular cytoskeletal and migratory apparatus responds to the discrete pattern and curvature of available adhesion sites, in the form of aligned fibers, leading to directional orientation. Recent work indeed demonstrates that the primary mechanosensitive sensors of the cell, focal adhesions, are confined by discrete substratum architecture resembling aligned ECM fibers *in vivo*, thereby maturing in an anisotropic fashion, leading to the reorganization the actin cytoskeleton and directional migration (8, 30, 31) (Fig. 2A). The anisotropic maturation of adhesions leads to traction force anisotropy (8) and is likely the result of enhanced local ECM stiffness in the direction of fiber alignment (32) and can be largely divorced from the bulk stiffness of the 3D tissue (31). Indeed, limitations on the direction of leading-edge protrusions (8, 14, 33–36), likely driven by a constraint to actin nucleation and branching at the discrete adhesion sites (33), lead to guided spreading and migration along the alignment cues. Regulators of leading-edge protrusions such as integrins, Rac1, FAK (14, 33, 37) and cellular contractility like myosin phosphorylation (8, 33, 38) are critical factors for directional sensing (Fig. 2A, B). The latter appears to be an important determinant of contact guidance on stiffer 2D substrates (8, 33, 38) and in generating fiber alignment, either locally (31) or globally (39), but can be dispensable for sensing already aligned tracks in softer 3D collagen matrices (35, 39). In fact, under low traction conditions, either due to low substrate stiffness or cell-intrinsic properties that dictate a more amoeboid migration mode, integrin and myosin-mediated anisotropic forces play a less significant role (Fig. 2B); rather, actin nucleation and branching at the contact site through the activity of Arp2/3 and formins drive the attenuated directional sensing under such conditions (8, 38, 40). These aforementioned mechanisms hold true when the alignment cues are of micron to sub-micron dimensions,

i.e., of the order of individual focal adhesions, as is the case for cancer cells interacting with individual collagen fibers or fibrils in the stroma (3, 8, 28). However, cancer cells in the stroma may also interact with aligned bundles of collagen fibers (3, 28), which are comparable to adhesion areas tens of microns wide, of the order of a cell width. In the latter context, theoretical and experimental work suggest that maximization of entropy by either non-adhesive gap avoidance at the single cell level or relative positioning and morphological state at the population level may play a role in driving contact guidance (41, 42), although the molecular players involved in this decision-making is largely unexplored.

While numerous studies have thus indicated the molecular pathways involved in contact guidance, many of these rely heavily on *in vitro* results, largely on 2D substrates, owing to the obvious challenges of controlled molecular perturbations *in vivo*. A further confounding factor is the fact that elevated stiffness and increased straightness and alignment of fibers often go hand-in-hand and similar molecular pathways are implicated for sensing of both of these ECM properties. Nevertheless, it is clear from this body of work that a force anisotropy at the single cell level through cell-ECM interactions favors polarization and movement in the direction of ECM alignment. An intriguing question is how this force anisotropy manifests when tightly connected cell clusters (often, reasonably well-organized as in the case of well-differentiated or early-stage carcinomas) encounter ECM alignment in the neighboring stroma. Indeed, this is another distinct niche in which contact guidance and anisotropic cell-ECM interactions are critical to understanding tumor cell invasion.

(b) Contact guidance of collectives and disruption of organized tissue structure:

Contact guidance in collective cell clusters have been implicated in important homeostatic processes like mammary gland development (6, 7, 43), and indeed it has important parallels in determining the architecture of tumor nests, comprising of neoplastic epithelium in case of carcinomas. For example, TACS-3 (perpendicular alignment of collagen to the epithelial boundary or throughout the tumor in later stages) leads to a collective migration front in the direction of alignment, with cells “peeling off” the organized epithelial structure (3, 9, 11, 17)(Fig. 1, 2C–D). Such epithelial disruption has been extensively observed *in vitro* using mammary acini, spheroids and tumor organoids, when these well-formed epithelial structures or cancer cell clusters are exposed to surrounding fibrillar ECM (44–47). Indeed, such structural disorganization is often observed in tumors, eventually leading to delamination of cancer cells into the stroma *in vivo* (3, 17, 48–50). While some studies propose a more quorum approach to understanding collective contact guidance, including an alternate, non-tensional sensing in cell sheets independent of cell-cell junctions (51), a sizeable body of work implicates molecules associated with cell-cell and cell-ECM adhesions including α5-integrin, DDR2, P-cadherin and Dia-1 as critical mediators of collective guidance (45, 46, 52, 53)(Fig. 2C). Further, cytoskeletal proteins such as cytokeratin-14 appear to be important in determining which cells are most susceptible to “break away” and become the leader cell in the invasive front (54, 55). The existence of heterotypic adhesions between fibroblasts and epithelial cells at the tumor-stromal interface (56) also imply that along with anisotropic cell-ECM interactions from proximal aligned ECM, cell-cell interactions (hetero and homotypic) are also critical mediators of this process. Indeed, the transition from a jammed (contained) to unjammed (not constrained)

state of the epithelium, driven by local density of cells is mediated largely through cell-cell adhesion proteins (9, 57) (Fig. 2D).

From this perspective, the contact guidance of collectives can certainly be viewed as a balance between cell-cell and cell-ECM forces (Fig. 1). Importantly, the disruptions in organized tissue structure created by the anisotropies in force distribution at the tumor-stromal interface may affect otherwise well-differentiated/early-stage cancers and contribute to early and extensive metastasis.

ORIGINS OF ECM ALIGNMENT IN THE TME:

Although it is well established that ECM architecture plays an important role in directing invasion and metastasis, the origins of collagen and ECM alignment in tumors are not fully established. Several studies implicitly or explicitly point to the role of cancer-associated fibroblasts (CAFs) in the deposition and reorganization of stromal collagen fibers mediated by DDR2 (52) or PTEN expression (58). This is also supported by *in vitro* studies where FAP-expressing CAFs, but not normal fibroblasts, generate aligned stromal collagen through Cav-1 (10) and TGF-beta (59)-dependent processes, and that CAFs from desmoplastic tumors can be utilized to generate aligned collagen matrices *in vitro* (60); Fig. 3A. Indeed, collagen organization by fibroblasts into aligned networks is likely a feed forward process as fibroblasts themselves are known to be highly responsive to ECM alignment and the orientation of matrix-remodeling fibroblasts predicts the orientation of the derived ECM (61), with a recent study demonstrating that cell-cell collisions among fibroblasts drive this behavior (62)(Fig. 3B). In addition, ECM-linking and cross-linking proteins such as HAPLN1(63) and LOXL2 (18) are critical regulators of CAF-mediated remodeling leading to aligned ECM. Other stromal cell types such as inflammatory TAMs (64) and adipocytes (65) have also been shown to regulate ECM remodeling in the TME through the expression of various matrix-remodeling enzymes. In reality, all these stromal cells likely work in concert or in a redundant fashion to align and modify the stromal ECM surrounding tumor nests, a niche that is often co-localized by these stromal cells.

Another mechanism of ECM organization is that cancer cells themselves, in addition to or instead of stromal cells, can align the ECM around them. Recent work in this realm suggests that cancer cells secrete a collagen-remodeling protein WISP1 that promotes the formation of aligned stromal collagen (66)(Fig. 3C). In addition to this biochemical mechanism, biophysical mechanisms play a critical role in aligning ECM in the tumor stroma. Cancer cells, particularly those that have undergone EMT have long been known to generate large amounts of force and maintain homeostasis in the stiff tumor microenvironment (39, 67). In vitro studies in many contexts demonstrate that collections of cancer cells, either in the form of spheroids, organoids, plugs or acini can remodel surrounding ECM to generate aligned collagen patterns using integrin-actomyosin contractility-dependent pathways, while also subsequently migrating along those highways (37, 39, 68)(Fig. 3A). Furthermore, theoretical and experimental modeling of the mechanotransduction indicates that pioneer symmetry breaking processes (69, 70) can lead to cascading force anisotropy at the epithelial-stromal interface and eventual dissemination along aligned ECM. Thus, it seems likely that multiple mechanisms may be sufficient to produce TACS-3 perpendicularly aligned collagen

architectures, but also that these mechanisms may be at play simultaneously following an initiating alignment event, such as contractile reorganization, and then feed forward by promoting each other. Indeed, since newly aligned matrices produce mechanical and structural cues that incite signaling feedback that is conducive to each of the described mechanisms of ECM organization (i.e., anisotropic traction forces, directed migration, activation of the cellular secretome), they likely promote or compensate for one another, to ultimately produce robustly aligned ECM in the tumor stroma.

TARGETING ECM ALIGNMENT AND CONTACT GUIDED INVASION:

Given that ECM alignment plays such a key role in directing local invasion and metastasis, an increasing number of studies in the nascent field of stroma targeted therapies (STT) explore the possibility of mitigating these effects by targeting either the ECM architecture itself or the cellular recognition and response to the anisotropic ECM. While historically STT studies have focused on increased drug delivery to drug-free sanctuaries in solid tumors, e.g. (71, 72), these approaches are also being evaluated to disrupt disease progression, and more recently improve anti-tumor immunity. Specifically, targeting ECM-remodeling fibroblasts in desmoplastic tumors such as pancreatic cancer by FAK inhibition (48), Halofuginone (72) or Losartan (73) show a reduction in collagen (as well as other ECMs), which at sufficient levels of ablation could reduce or eliminate alignment cues and thus produce favorable outcomes for invasion and metastasis. This is particularly applicable to early disease, prior to recurrent disease or to stop continual disease spread during a cytotoxic treatment. Indeed, targeting Rho GTPase-mediated contractility with Fasudil alters the ECM in a manner conducive to less aggressive disease while simultaneously increasing efficacy of standard-of-care chemotherapy (74) Apart from these compounds, specific targeting of LOXL2 (18, 64, 75) in pancreatic, lung, and breast cancers led to significant ECM remodeling, decrease in the level and alignment of collagen and decrease in invasion and metastasis. Indeed, similar effects were observed by specifically targeting molecules that are involved in the active sensing and guidance of cells on these aligned ECM patterns such as focal adhesion and actin remodeling proteins (14, 48). Thus, it seems clear that STT to re-engineer tumor microenvironments to stop or slow disease progression, while also increasing drug delivery and anti-tumor immunity, is likely to be part of rational strategies, and perhaps personalized strategies, to combat solid malignancies.

CONCLUSION AND OUTLOOK:

Over the past 15+ years our understanding of ECM architectures and directed migration in solid tumors has expanded profoundly. However, while considerable advances have been made in our understanding of directed migration from contact guidance, and in particular TACS-3 directed contact guidance in solid tumors, many aspects of the process remain elusive. For instance, the in-depth signaling mechanisms governing contact guidance have yet to be fully described, particularly during *in vivo* scenarios such as simultaneous exposure to complex combinations of different ECMs, the superposition of structural alignment cues and mechanical cues, and complex cell-cell interactions. Furthermore, the impact of superposed signals that can be additive or competing in tumor microenvironments, such as chemical gradients, mechanical gradient, and contact guidance

architectures in different directions has remained uncovered. However, as engineered tumor microenvironment platforms and murine models of human cancer continue to evolve, coordinately with advanced optical imaging technologies (such as increased spatial and temporal resolution, optogenetics advances that allow for local spatial control of key events, spatial transcriptomics) with quantitative analysis of signaling and cell dynamics (i.e. biophysical modeling and systems biology approaches) we are well poised to define the fundamental *in vivo* mechanisms governing this key process influencing tumor progression and develop rational therapeutic regimes to halt disease progression.

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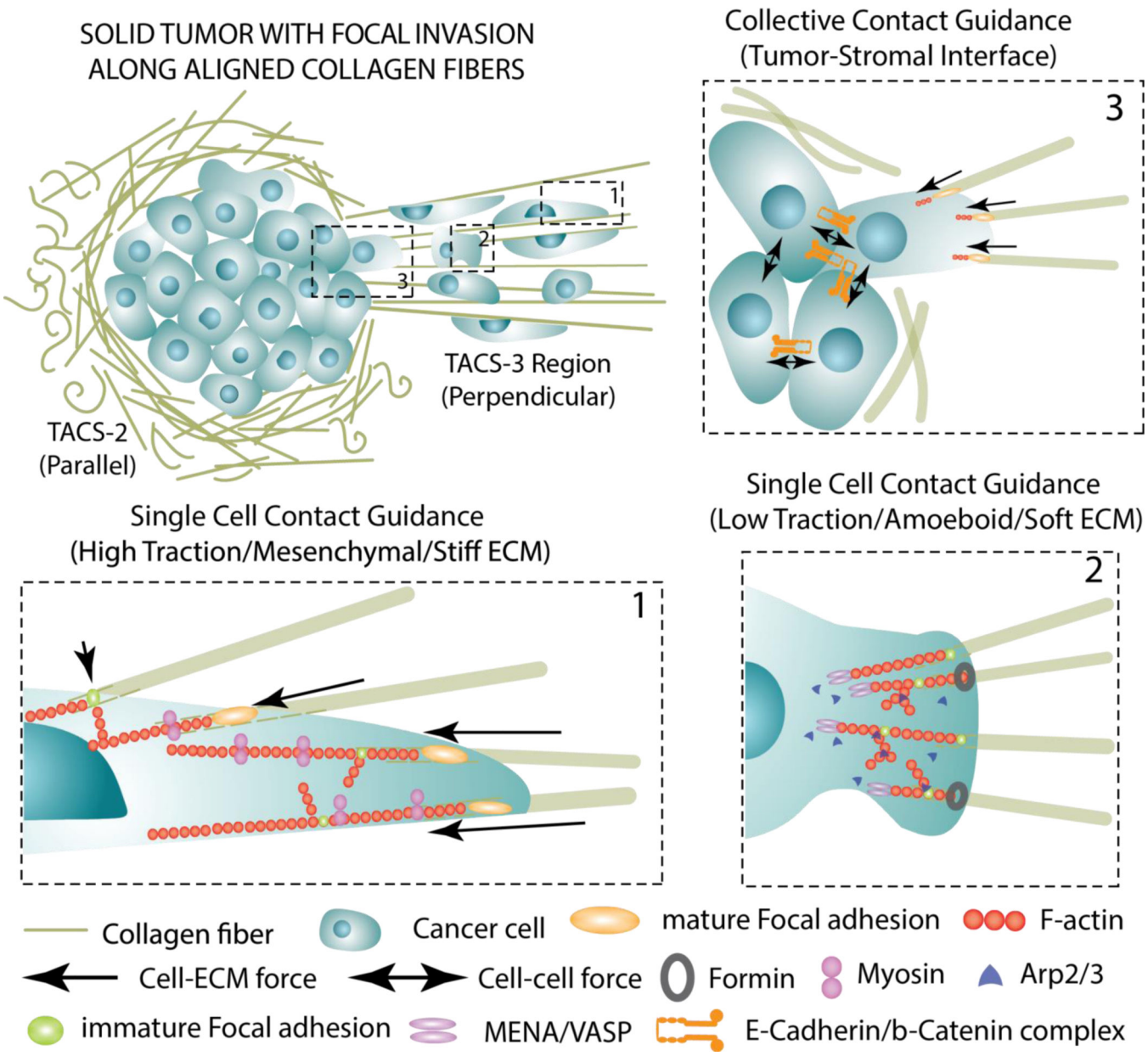

Figure 1: Distinct niches and modes of contact-guided cancer cell invasion in the TME:
Schematic showing a solid tumor mass surrounded by TACS-2 (parallel arrangement of collagen fibers) along with a small window of TACS-3 (perpendicularly aligned collagen fibers), with focal invasion of cancer cells away from the primary cluster. *Magnified Region 1* shows a typical cancer cell, which has undergone EMT, aligned and migrating along the direction of fiber alignment. For such mesenchymal-like migration (involving leading edge protrusions, strong adhesion, elongated morphology), current understanding points to the role of constrained maturation of focal adhesions and resultant myosin-dependent anisotropic traction forces along aligned F-actin bundles as the primary driver of directed cell migration along aligned ECM; *Magnified Region 2* shows, in contrast, a low traction migration state (e.g., amoeboid migration involving blebs, low adhesion,

rounded morphology), where myosin-mediated force generation is dispensable for directed migration; instead competing pools of actin-modifying proteins play a critical role in mediating directed migration by contact guidance. In addition to single cells, anisotropic cell-ECM forces from aligned ECM at the tumor-stromal interface (*Magnified Region 3*) may facilitate the disruption of organized epithelial structures, leading to initiation of invasion. The nature (single cell or collective) and extent of the invasive front is largely dependent on the balance of cadherin-mediated cell-cell and integrin-mediated cell-ECM forces at the tumor-stromal interface.

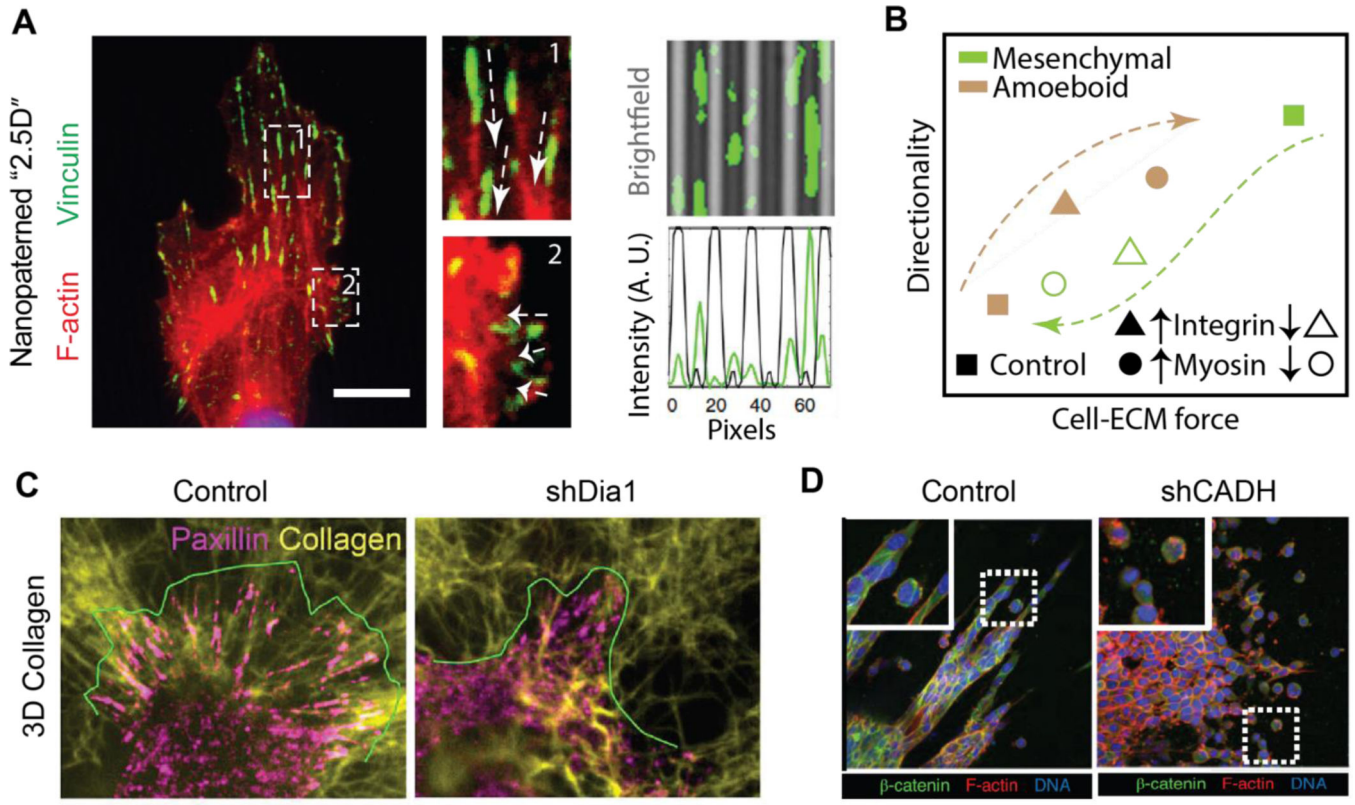

Figure 2: Mechanisms of single and collective dissemination on aligned ECM:

(A) *Left:* Typical lamellipodia of a MDA-MB-231 breast cancer cell on aligned patterned ECM (sub-micron dimension parallel ridges and grooves) showing large, aligned (region 1) and small, non-aligned focal adhesions (FA) connected to similarly aligned F-actin. In magnified images, arrows illustrate the magnitude and direction of myosin-mediated traction forces at those adhesion sites; *Right:* The anisotropic FA and F-actin distribution is derived from the constrained growth of FAs limited by the discontinuities (individual ridges and grooves) of the substrate (Modified from *Ray et al., Nat. Comm., 2017* with permission); **(B)** Baseline directional guidance is a function of cell migration mode, specifically the magnitude of myosin and integrin-mediated cell-ECM forces. While mesenchymal cells display higher directional guidance compared to amoeboid cells on the same stiff substrate at baseline (filled squares), the former can be attenuated by blocking integrin or myosin function (unfilled triangle and circle respectively); likewise an enhancement of directionality is achieved for amoeboid cells with integrin or myosin activation (filled triangle and circle respectively). *Figure adapted from data in Wang et a., Biomaterials 2017*; **(C)** FA confinement on individual collagen fibers is also observed in 3D collagen-based models of local invasion, the formin Dia1 is required for initiation of robust collective dissemination (*Figure reproduced from Fessenden et al., JCB, 2018* with permission); **(D)** Invasion along organized collagen patterns is a function of β-catenin and cadherin-mediated cell-cell interactions; while cadherin inhibition produces a higher percentage of disseminated single cells, the overall efficiency of invasion is not necessarily enhanced (*Figure reproduced from Ilina et al., Nature Cell Biology, 2020* with permission).

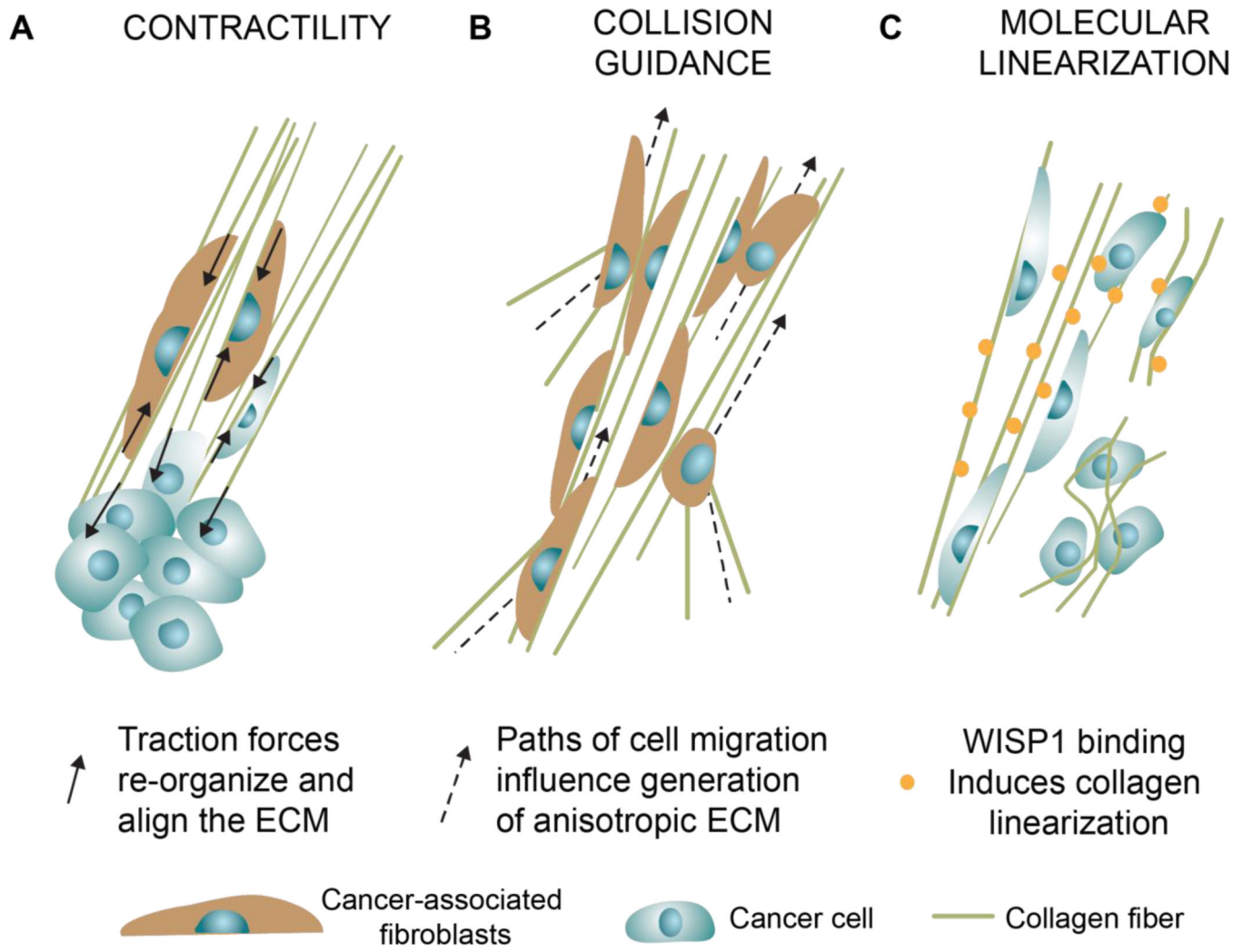

Figure 3: Origins of ECM alignment in the TME:

Schematics showing alignment of ECM in the TME by **(A)** traction forces from clusters of cancer cells or neighboring cancer-associated fibroblasts; **(B)** directional migration and alignment of matrix-producing and remodeling CAFs by collision guidance, driven by positional feedback from neighboring cells and **(C)** direct binding of matrix remodeling proteins such as WISP1 produced by proximal cancer cells.