Bioengineering Paradigms for Cell Migration in Confined Microenvironments

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Abstract

Cell migration is a fundamental process underlying diverse (patho)physiological phenomena. The classical understanding of the molecular mechanisms of cell migration has been based on *in vitro* studies on two-dimensional substrates. More recently, mounting evidence from intravital studies has shown that during metastasis, tumor cells must navigate complex microenvironments *in vivo*, including narrow, pre-existing microtracks created by anatomical structures. It is becoming apparent that unraveling the mechanisms of confined cell migration in this context requires a multi-disciplinary approach through integration of *in vivo* and *in vitro* studies, along with sophisticated bioengineering techniques and mathematical modeling. Here, we highlight such an approach that has led to discovery of a new model for cell migration in confined microenvironments (i.e., the Osmotic Engine Model).

Introduction

Cell migration plays a key role in both cell physiology, including embryonic development, wound healing, and the immune response, and in development of pathological conditions. For example, in cancer metastasis, cells migrate away from the primary tumor, through the surrounding microenvironment, and to the microvessels, where they can invade into the blood and/or lymphatic circulation for metastasis to distal sites [1–3]. After traveling in the

circulation, the tumor cells extravasate from a blood vessel and migrate to the site where a secondary tumor will form. Recent *in vivo* intravital microscopy studies suggest that the metastatic cascade involves migration of tumor cells through extremely complex microenvironments [4–8], and it is becoming increasingly evident that physical forces are at play during multiple steps of metastasis [3,9]. To achieve migration through such microenvironments, cells are required to either degrade matrix to create their own migration tracks [10] or find preexisting tracks [11,12] through which to migrate. Interestingly, recent intravital microscopy studies reveal that cells preferentially migrate along very narrow pre-existing tracks *in vivo* [4,8]. These tracks vary from <3 μm to ~30 μm in width and are 100–600 μm in length [13]. The microtrack width modestly increases during perimuscular invasion [7], which may be attributed to limited matrix metalloproteinase (MMP)-dependent proteolysis or outward pushing exerted by invading cells. It is noteworthy that no significant changes in track width are detected during migration through collagen networks, fat tissue, or perineural space [7]. Hence, invading tumor cells not only preferentially follow pre-existing tissue tracks, but also adapt their shape to the space available without significant tissue remodeling or degradation. This may partly explain why MMP inhibitors have largely failed clinically in cancer patients [14].

Cell migration through confined spaces plays important roles in both physiological and pathological cell migration events [8,15–17]. During the past decades, *in vitro* cell migration studies have been mainly performed on unconfined two-dimensional (2D) surfaces such as glass or plastic; while we have learned an extensive amount about how cells migrate from these 2D assays [18–21], they fail to recapitulate the *in vivo* microenvironment. A number of assays have been developed to provide additional information, such as how cells respond to biochemical [22,23], adhesive [24], topographical [25], mechanical [26–32], and dimensional [33–36] cues; however, each of these assays faces its own limitations (Fig. 1). Only relatively recently have microfabrication techniques been used to simulate microtracks *in vitro*. The fundamental question now is whether cells utilize the same machinery and mechanisms for confined versus unconfined migration, and how the biochemical and mechanical properties of the microenvironment affect these mechanisms. Answering this question will most likely require a multi-disciplinary approach through integration of *in vivo* and *in vitro* studies, along with mathematical modeling.

Engineering the cellular microenvironment

Given the physiological relevance of cell migration through confined spaces *in vivo* [4,7], it is necessary to create appropriate *in vitro* systems that enable understanding of cell migration in this context. Reconstituted three-dimensional (3D) collagen gels have been extensively used to study the mechanisms of random 3D migration *in vitro* [13,37–39]. However, these 3D assays fail to recapitulate the longitudinal tracks and the dynamic range of collagen-free pore sizes encountered by cells *in vivo* [7,13]. To circumvent the limitations presented by traditional 2D and reconstituted gel migration assays, engineering techniques such as microfabrication have recently allowed researchers to evaluate the effects of physical confinement on cell migration [40–50] (Fig. 1). For instance, the microfabrication technology has been applied to create *in vitro* models of cellular intravasation [41], which represents a form of migration in a confined space, as cells must squeeze between

endothelial cell-cell junctions in order to enter a blood vessel. Microfabrication techniques have also been employed to generate surfaces, wells, or molds with adhesive areas of varying size and shape in order to evaluate the effects of spatial confinement on cellular differentiation [51], proliferation [52], angiogenesis [53], and protein expression [52]. Recently, perfusable engineered vascular channels have been developed [54] by 3D printing of rigid filament networks of carbohydrate glass, which served as a template for the casting of either a synthetic or natural extracellular matrix containing cells around the lattice. Upon dissolving the carbohydrate glass away, endothelial cells are introduced into the vascular architecture and perfused with media to simulate blood flow and the physiological endothelial cell function. This microfabrication approach could also be used to create microtracks to investigate cell migration in confined microchannels.

We and others have developed PDMS-based microfluidic devices where physical cues (e.g., microchannel cross-sectional area and topography) and biochemical cues (e.g., chemoattractant gradient and surface protein presentation) can be simultaneously varied within the same device [45–47,55–57]. Furthermore, we have used this device to investigate the molecular mechanisms and signaling pathways involved in cell migration in unconfined versus confined spaces [47,55]. While this PDMS-based device is likely relevant in the context of stiffer *in vivo* microtracks such as those which might be found along muscle and nerve fibers [7], a limitation is its narrow range of tunable stiffness (Fig. 1). To address this limitation, a polyacrylamide gel-based device has been fabricated consisting of 3-wall microchannels of varying width (10–40 μm) and stiffness (0.4 kPa to 120 kPa) [42,43]. However, this device cannot replicate a truly confined microenvironment, as it is comprised of 3- rather than 4- wall microchannels of 10 μm or larger in width, nor does it incorporate a chemotactic gradient. To circumvent these limitations, we are currently developing a new model of our chemotaxis-based device in which the stiffness of the narrow (3 μm wide) microchannel walls can also be manipulated systematically; this will allow for modeling of softer microtracks, such as those that would occur between bundles of collagen fibers within the ECM [4].

Using the microchannel device, two classes of cell responses have been discovered – (1) those that utilize a Rho/Rac crosstalk mechanism (e.g., in normal fibroblasts, fibroblast-like cells, primary murine T-cells, and α4-expressing A375 melanoma cells), where Rho-mediated cell contractility is necessary for migration in confined spaces [55], and (2) those that do not require actomyosin during migration in confined spaces (e.g, metastatic cells such as murine S180 sarcoma cells, human MDA-MB-231 breast tumor and CH2879 chondrosarcoma cells) [45,47] (Fig. 2).

By incorporating a bed of micropillars onto the bottom wall of the microchannels within the microfluidic device, cellular traction forces have been measured during migration in confined and unconfined spaces [46]. This assay has revealed that traction forces exerted by cells in confined microchannels are lower than those in unconfined (2D) channels. These observations are in line with studies demonstrating that cells exert lower traction forces on 1D micropatterned lines in comparison with 2D substrates [36]. As expected, treatment of human osteosarcoma (HOS) cells by blebbistatin, which suppresses myosin II-mediated contractility, or calyculin A, which increases cell contractility, decreases or increases cell

traction forces, respectively, in wide channels (i.e., unconfined spaces) [46]. Remarkably, neither blebbistatin nor calyculin A has any effect on cell traction forces in narrow channels (i.e., confined spaces). Thus, myosin-mediated cell contractility appears to play reduced role in HOS cell confined migration, as in other metastatic cell lines [45,47]. In agreement with these observations, tumor cells have recently been shown to exert less frictional forces along channel walls in comparison with normal cells [58].

Tumor cells also display an altered actin cytoskeleton, with fewer stress fibers [59,60], and increased deformability [58,61,62]. Similar to observations in 3D collagen gels [38], focal adhesions are also suppressed in tumor cells within narrow microchannels [45]. Furthermore, physical confinement induces F-actin remodeling, such that stress fibers are drastically diminished in physically confined spaces [45]. Moreover, actin appears to be concentrated on the leading and trailing edges of cells migrating in narrow channels [45]. In line with our observations, HL60 neutrophil-like cells chemotactically migrating in confining microchannels (5×5 μm^2) form a "slab" of actin that fills the entire cross-section of the channel at the cell's leading edge, rather than assembling thin ~200 nm-thick actin-rich lamellipodia at the leading edge, as occurs on 2D surfaces [56]. Our findings, along with experimental observations [45] and a mathematical model [63] showing that tumor cells are able to migrate even in the absence of integrin-mediated adhesion, may help explain the reduced magnitude of traction forces measured in confined relative to unconfined migration [46]. The marked decrease in the formation of stress fibers and focal adhesions may also explain why inhibition of cell contractility via blebbistatin has no effect on tumor cell migration in confined spaces [45–47]. In contrast, normal fibroblast-like cells intrinsically displaying a higher level of stress fibers and focal adhesions demonstrate decreased confined migration upon blebbistatin treatment [55]. Intriguingly, tumor cells are still able to undergo confined migration in the presence of latrunculin-A, which disrupts actin polymerization, even though the same treatment completely abrogates migration in wide channels, as it does on a 2D surface. It is thus becoming increasingly apparent that the cellular mechanisms utilized during tumor cell migration in confined spaces can be fundamentally different from migration in unconfined spaces (i.e. 2D planar surfaces).

New Model for Cell Migration

Recently, we discovered a new model for confined cell migration that is driven by water permeation through the cell membrane [47]. This mechanism, termed the "Osmotic Engine Model" of cell migration, requires the coordinated activity of ion channels and aquaporins and is based on water flux into the cell at the leading edge and water flux out of the cell at the trailing edge (Fig. 3A). Ion pumps and aquaporins have previously been implicated in 2D cell migration [64–66], however their function has been both underappreciated and not well understood. Their role as cellular migration machinery has mostly been associated with the cytoskeleton. For example, the Sodium Hydrogen Exchanger-1 (NHE-1) physically interacts with the actin cytoskeleton, and in turn, the actin cytoskeleton regulates the activity of ion channels [67–69]. Indeed, inhibition of NHE-1 decreases 2D migration speed in several cell types [47,70,71]. Furthermore, Aquaporin 5 (AQP5) is overexpressed in lung and breast tumor cells [72,73] and acts to facilitate actin polymerization [64] while stabilizing microtubules [74], thus supporting 2D cell migration. In addition, aquaporins

crosstalk to cell-matrix adhesion molecules (i.e., integrins) during cell migration, especially in renal cells and cancer cells that abundantly express aquaporins [75–77].

Our work provides a new context in which ion channels and aquaporins (e.g., NHE-1 and AQP5) are not only required but can also drive migration through confined spaces when the function of actin polymerization is disrupted [47]. By integrating theory and experiments, we derived an analytical expression for cell migration velocity in confined spaces. The mathematical model takes into account the kinetics of water, kinetics and diffusion of ions, flow of the cell cytoplasm, and mechanics of the cell cortex (i.e., the friction between the cell and channel wall and between the cell cortex and cytoplasm) [78,79]. Importantly, the model predicts that a nonzero cell velocity can be achieved even without actin polymerization and myosin II activity in confined spaces, which aligns with experimental observations, and distinguishes our model from previous mathematical frameworks [18,63,80–88]. For instance, although prior work has modeled the cell as a soft, fluid-infiltrated sponge surrounded by a water permeable barrier capable of taking in water across the cell membrane, it couples hydraulics and cytoskeleton-dependent cellular mechanics [89]. Both theoretical and experimental data from our Osmotic Engine Model reveal that confined cell migration depends on osmotic and hydrostatic pressure differences across the cell membrane at both the cell and trailing edges. For instance, application of a hypotonic shock at the cell leading edge or of a hypertonic shock at the trailing edge causes a rapid reversal in the direction of cell migration (Fig. 3B–C). In both cases, cells repolarize to migrate towards the higher osmolarity regions.

During cell entry into narrow microchannels, a highly polarized distribution of ion pumps (i.e., NHE-1) and aquaporins (i.e., AQP5) is detected along the longitudinal surface of the cell with an intense signal at the cell leading edge [47]. This polarized, spatial distribution of ion pumps and aquaporins is required for sustained migration through confined spaces when actin polymerization is disrupted, as also suggested by the theoretical framework. In contrast, the ion pumps and aquaporins are more randomly distributed on the surface of migrating cells on 2D substrates and act in coordination with the actin cytoskeleton to help drive cell protrusion at the leading edge. As such, interfering with actin polymerization is sufficient to abrogate 2D, but not confined, migration. Of note, knockdown or inhibition of ion pumps and aquaporins markedly suppresses both unconfined and confined migration [47,70,71]. It is also noteworthy that actin polymerization appears to be necessary to set up the polarization of ion pumps and aquaporins during cell entry into confined channels; however, once this polarization is established, actin polymerization is dispensable for confined cell migration [79]. Interestingly, actin is required for the re-polarization of aquaporins and ion channels in cells migrating inside narrow channels following the application of an osmotic shock. If actin polymerization is disrupted, the cells fail to repolarize aquaporins and ion channels to the post-shock leading edge, and are unable to sustain migration [79]. Collectively, the Osmotic Engine Model can predict cellular movement in confined spaces even in the case where actin polymerization is not the driving force, and it thus offers a new perspective into how we view cell migration.

In the classical model of cell migration, cell protrusion at the leading edge is driven by actin polymerization and is stabilized by integrin-dependent adhesion to the substrate, while de-

adhesion at the rear is facilitated by cellular contractile forces. Cells likely use this mechanism when migrating *in vivo* in situations where they are not laterally confined. However, cells may possess multiple power sources for motility, and it appears that tumor cells have evolved to be capable of using different mechanisms (i.e. actomyosin-based and water permeation-based) depending on the specific properties of the microenvironment. Of course, we cannot eliminate the possibility that normal cells also use the Osmotic Engine Model of migration, but the overexpression of aquaporins and ion pumps in numerous metastatic tumor cells [72,73,90] may cause the Osmotic Engine Model to be more evident in these tumor cells.

In confined spaces, water flux through the cell membrane is directed along a single axis longitudinally through the confined cell, allowing water permeation to be a major mechanism driving cell migration within the microchannel [79]. This mode of migration cannot be detected on 2D surfaces due to the lack of biological (e.g., ion channels and aquaporins) and geometrical (e.g., pill-shaped) polarization. Thus, in the 2D setting, actin polymerization is indispensable to guide the protrusions and drive migration. We speculate that Rho-associated kinase 1 (ROCK1), which phosphorylates myosin light chains to induce actomyosin contractility and is an upstream activator of NHE-1 [91], may serve as a linker between the actin-driven and water permeation-based mechanisms. NHE-1 is also involved in regulation of intracellular pH due to its role in exchanging Na^+ and H^+ ions; NHE-1 recruitment to cellular invadopodia is promoted by cortactin phosphorylation, thereby regulating both cellular pH and invasive capability [92]. Cellular pH could thus be incorporated along with osmolarity in future refinements of the Osmotic Engine Model. Prior modeling work suggests that the relative significance of hydraulics and cytoskeletal dynamics (i.e., actin-, microtubule-, or intermediate filament-based) on cellular morphology depends on the time-, length-, and force-scales involved [89]. In light of this model, we postulate that physical confinement alters cellular parameters such as the cytoskeletal mesh structure, membrane permeability, local contractility, and adhesion, all of which could heterogeneously alter cellular hydraulics and thus cellular migration in confined spaces.

A recent microfluidic study has also suggested that cells push water in confined spaces, where "barotaxis" can override chemotaxis in asymmetric hydraulic microenvironments [57]. Specifically, cells reaching an intersection "decide" to follow the path of least hydraulic resistance, which was manipulated experimentally by adjusting the length or width of the downstream channel far from a bifurcation (i.e., increasing channel length or reducing channel width increased hydraulic resistance). Interestingly, cell velocity is identical to the flow velocity of suspended 500-nm fluorescent polystyrene beads (and therefore also the bulk velocity), suggesting that these differentiated HL60 (dHL60) cells "push" fluid as they migrate forward. This mechanism may depend primarily on actin, given that actin-based motility is faster than the Osmotic Engine Model [47] and that dHL60 cells are fast-moving in comparison with tumor cells; however, the molecular constituents involved in this process have yet to be delineated. This phenomenon may occur in tandem with the Osmotic Engine Model, especially in slower-moving tumor cells, since cell water uptake may be limited by the number of aquaporins and ion channels on the cell surface. The question that remains to

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be unraveled is whether other conditions besides physical confinement encourage cells to favor the Osmotic Engine Model of migration.

Outlook

Cutting edge bioengineering techniques, such as microfabrication, *in vivo* and *in vitro* imaging, in combination with molecular biology, have led to new insights to the mechanisms by which normal and pathological cells migrate in heterogeneous microenvironments. These tools have allowed researchers to unravel the distinct effects of physical and biochemical cues, including confinement and dimensionality, matrix stiffness, topography, chemoattractant gradients, soluble factors, and matrix-bound adhesion proteins, on cell migration. Furthermore, we wish to emphasize the importance of mathematical modeling in combination with both *in vitro* and *in vivo* experimental analyses in order to help explain non-intuitive cellular behaviors. An integrated theoretical and multifaceted experimental approach can lead to discovery of new mechanisms for cell migration, just as the Osmotic Engine Model revealed novel roles for cellular machinery including ion pumps and aquaporins.

Open questions include how forces induced by the cellular microenvironment direct the distinct machinery cells use to move. For example, how does the force of physical confinement lead tumor cells to utilize an "Osmotic Engine" mode in addition to actin polymerization-based migration? We hypothesize that physical confinement induces biochemical signaling pathways within cells, similar to how cells transduce signals from biomechanical stimuli (e.g., matrix stiffness, fluid shear stress) [93–96]. Furthermore, the precise mechanisms by which physical confinement leads to polarization of ion pumps and aquaporins remain to be defined. Moving forward, it will also be critical to unravel the interplay between various mechanisms of migration (e.g., actomyosin-based and water permeation-based) and identify the specific microenvironments that promote one mechanism to become more dominant over the other; we predict it may depend on the type of cell (i.e., normal versus tumor), cell mechanics (e.g., traction forces, actin organization), and/or protein expression (e.g., Rho/Rac crosstalk, aquaporins, ion pumps), as well as the physical and biochemical properties of the microenvironment. An effective strategy may be to perform such analyses on the highly migratory population of tumor cells selectively isolated from a primary tumor that bear a gene signature predictive of cancer metastasis [97]. Engineering technology, combined with mathematical modeling, cutting-edge imaging and biological approaches, and *in vivo* studies will likely need to be integrated holistically in order to attack these questions.

Acknowledgments

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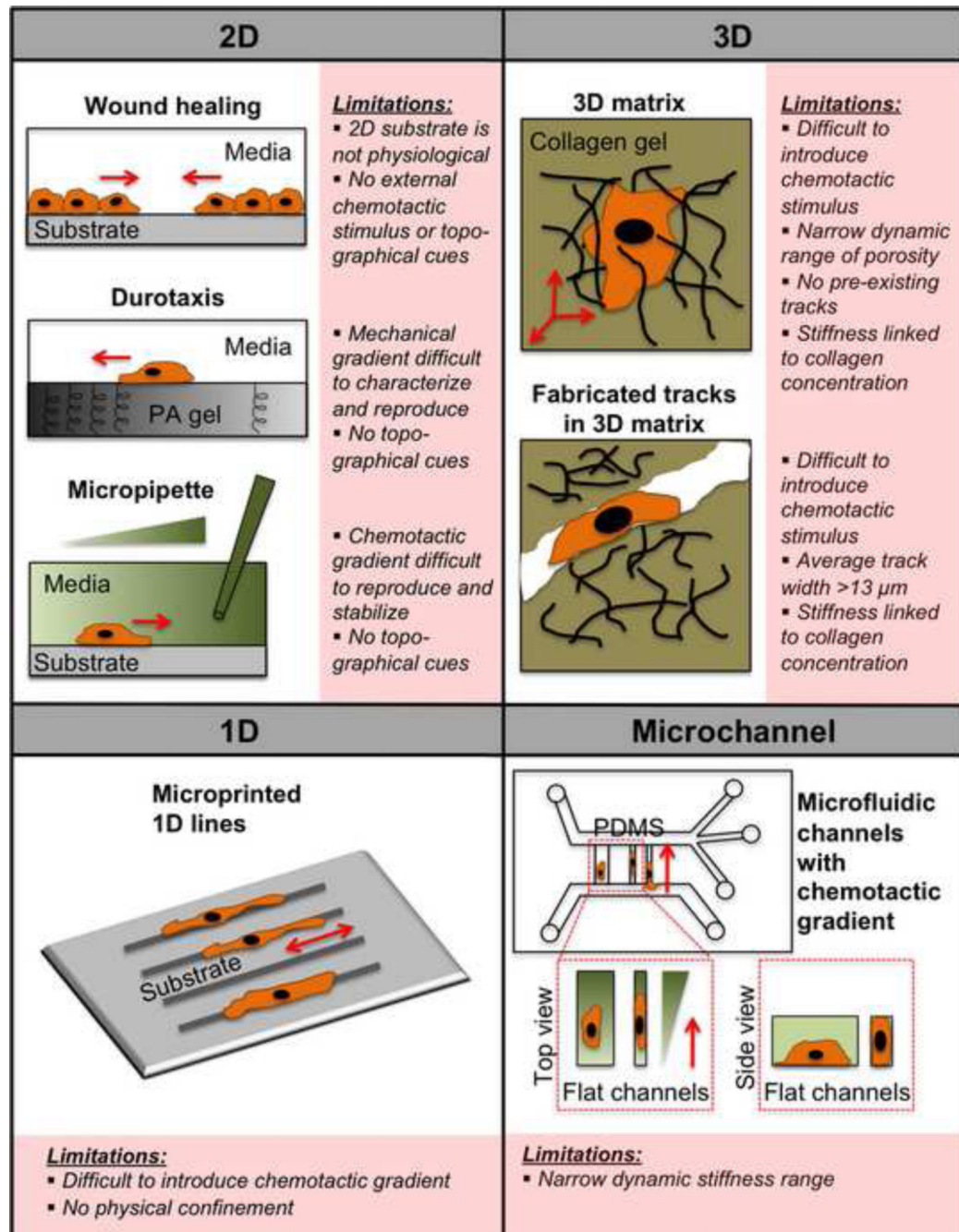
Figure 1.

Overview of 2D, 3D, 1D, and microchannel cell migration assays and their limitations. In the **wound healing** assay, a monolayer of cells is scratched, or a physical barrier is removed, and the cells subsequently migrate towards each other to close the wound. In the **durotaxis** assay, a gradient of substrate stiffness is created by placing two drops of polymerizing polyacrylamide (PA) of different stiffnesses next to each other, and covering the solutions with a glass coverslip. Cells are then induced to migrate in response to the mechanical gradient of stiffness. In the **micropipette** assay, cells respond to a chemotactic gradient

created by a chemoattractant-filled micropipette. In a **3D matrix**, cells must enzymatically degrade the surrounding matrix in order to move, while in **fabricated tracks within a 3D matrix**, preexisting tracks are created in a collagen gel via microfabrication techniques. In the assay with **microprinted 1D lines**, cells adhere selectively to 1D protein lines of specific width. In the **microchannel** assay, cells are induced to migrate into confined or unconfined microchannels in response to a chemoattractant gradient. Some parts of the figure are adapted with permission from [9].
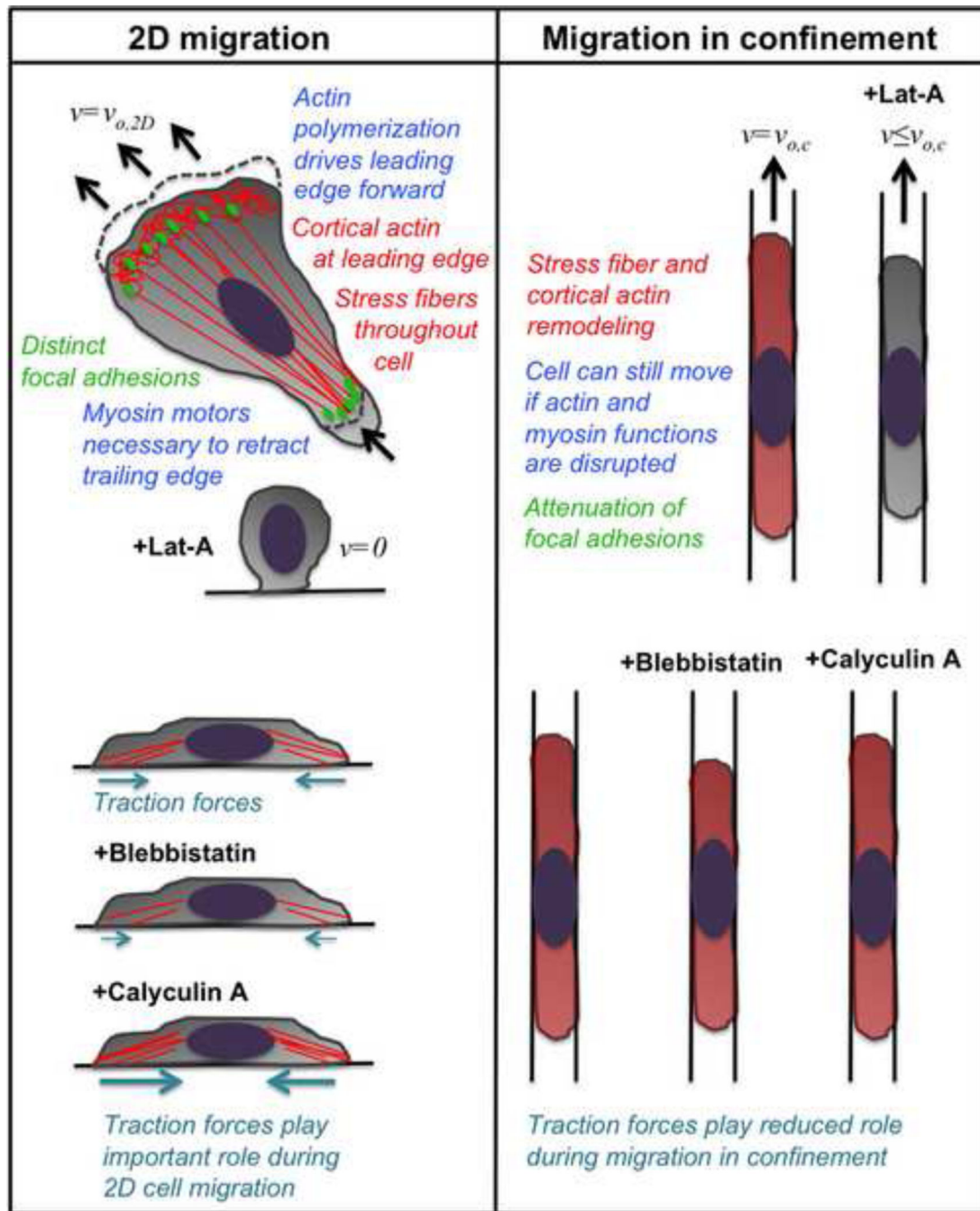
Figure 2.
Comparison of major differences between 2D migration and migration through confined spaces (i.e., microchannels). In **2D migration**, actin polymerization drives the leading edge forward, and both cortical actin and stress fibers are evident within the cell. Myosin motors are necessary to retract the cell's trailing edge. Distinct focal adhesions help anchor the cell and traction forces are generated through these focal adhesions. When the actin-disrupting drug latrunculin-A is added to cells in 2D, they lose attachment to the substrate, round up, and cell velocity goes to zero. Blebbistatin, which inhibits myosin II function and decreases

cell contractility, decreases cell traction forces in 2D; meanwhile, calyculin A, which inhibits protein phosphatases and increases cell contractility, increases cell traction forces in 2D. During **migration in confined microchannels**, the cell undergoes dramatic stress fiber and cortical actin remodeling, with both becoming more diffuse throughout the cell. Attenuation of focal adhesion size is also observed in microchannels. In contrast to 2D, the cell can still move in confined microchannels if actin and myosin functions are disrupted. Furthermore, neither blebbistatin nor calyculin A has any effect on the magnitude of cell traction forces in confinement, indicating that cell traction forces play a reduced role during migration through confined spaces. In confined spaces, the net direction of forces is towards the chemoattractant, though appreciable forces are also directed towards the side walls of the microchannels.
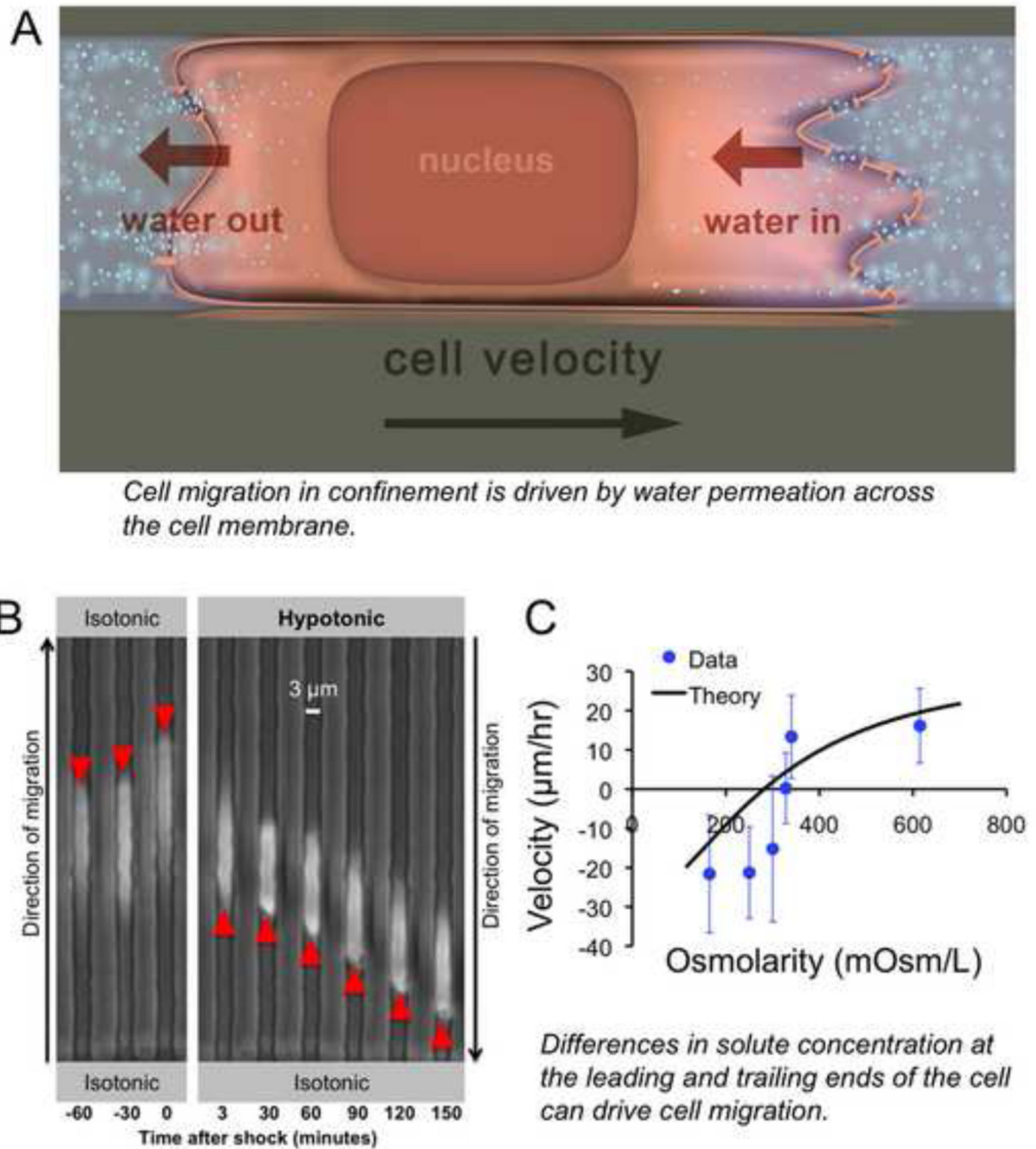
Figure 3.

Overview of the Osmotic Engine Model of cell migration. (A) Cell migration in confined spaces is driven by water permeation across the cell membrane. Water flows in at the cell's leading edge, which allows the front of the cell to extend forward, and water flows out at the cell's trailing edge, which allows the back of the cell to retract. This results in translocation of the cell body forward with little change in cell length (or volume). (B) The Osmotic Engine Model can be tested by applying osmotic shocks to the cell's leading (or trailing) edge. Here, a hypotonic shock is introduced within the microfluidic device at the cell's

leading edge, causing the cell to reverse direction and migrate away from the chemoattractant gradient. (C) The velocity of cell migration depends on the magnitude of osmolarity of the extracellular medium at the cell's leading edge. The reversal of cell migration direction in response to a hypotonic shock at the cell's leading edge is predicted by the theoretical framework of the Osmotic Engine Model. Thus, differences in solute concentration at the leading and trailing ends of the cell can drive cell migration. In the absence of osmotic shocks, the cell's ion channels and aquaporins must be polarized in order to sustain migration. Figures reproduced with permission from [79].