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NEW PARADIGMS IN THE ESTABLISHMENT AND MAINTENANCE OF GRADIENTS DURING DIRECTED CELL MIGRATION

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Abstract

Directional guidance of migrating cells is relatively well explored in the reductionist setting of cell culture experiments. Here spatial gradients of chemical cues as well as gradients of mechanical substrate characteristics prove sufficient to attract single cells as well as their collectives. How such gradients present and act in the context of an organism is far less clear. Here we review recent advances in understanding how guidance cues emerge and operate in the physiological context.

Directed cell migration is mainly governed by the ability of cells to sense an external gradient of chemotactic factors. This can occur through a temporal sampling mechanism, as observed during bacterial chemotaxis, where cells measure and compare chemical concentrations over time [1] or through a spatial sampling mechanism by measuring differences across space, as observed during eukaryotic chemotaxis [2]. Several computational models have been employed to describe spatial directional sensing. Chemoattractant-mediated pseudopod biasing, where chemoattractants bias internal pseudopod dynamics [3,4,5], has been used to explain sensing of shallow gradients. The local excitation and global inhibition (LEGI) concept, where small changes in external chemoattractant gradients give rise to highly polarized intracellular responses [6,7], has been useful to explain adaptation and sensitivity of chemotaxing cells to chemical cues. The “network theory”, combines various feed-forward and feed-back signaling loops to explain the robust persistence of cellular motion towards chemotactic gradients [8]. Most models assume that the chemotactic gradient encountered by cells is at steady state, static and spatially stable and, under the simplest circumstances, it is assumed that the gradient is created by diffusion of the chemoattractant from the source site towards the target cells (Fig. 1A) [9]. However, in a physiological milieu, gradients of chemoattractants might be: (i) non-

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linear, with rapid decay of concentration as a function of distance from the secreting source, (ii) sequestered by extracellular matrix, (iii) degraded, (iv) self-generated and amplified, and (v) modified by extracellular enzymes (Fig. 1). In a given environment, these conditions may give rise to dynamic and discontinuous gradients. More importantly, cells can encounter more than one chemoattractant with different diffusivities and must therefore adapt to multiple cues. In extreme cases the chemoattractant itself is a non-diffusible, short-range cue that remains associated with cells. The mechanism by which cells and their chemoattractants have evolved to reach their desired designation is extremely varied and little attention has been paid to these while modeling single cell or collective cell migration in eukaryotes. This review intends to present an overview of these mechanisms.

THE REACTION-DIFFUSION/SOURCE-SINK MODEL FOR CREATING STABLE MICROGRADIENTS

In 1952, Alan Turing, the father of modern computing, proposed that “a system of chemical substances, called morphogens, reacting together and diffusing through a tissue, is adequate to account for the main phenomena of morphogenesis” [10]. His idea has since been modified and supplanted with various models to explain spatial patterning of biological tissues [11]. The notion of spatio-temporal pattern formation through interacting moieties has been further applied to understand the formation and stability of a chemotactic gradient [12]. In a typical case of reaction-diffusion, a uniform steady state concentration of chemicals may be destabilized when the balance between an active mechanism (such as production of chemoattractant) and an inhibitory mechanism (such as removal of chemoattractant) is disrupted. In the event of a rapid removal of chemoattractant through a local sink, a homogenous steady state distribution rapidly evolves into a spatially heterogeneous gradient [13]. This would also allow a stable and steep gradient to be formed over relatively small spatial distances. The reaction–diffusion/source–sink process therefore enables chemical gradients hitherto not possible through simple diffusion (Fig. 1B).

Cells and tissues utilize various processes to create sinks for chemoattractants. Degradation of the chemoattractant is one of the most common mechanisms utilized (Fig 1D). For example, *Dictyostelium* cells secrete phosphodiesterases that specifically breakdown external cAMP cues during chemotaxis [14,15] and regulate migration in streams [16]. As opposed to the termination of signal through phosphodiesterases, gelatinase B, a major secreted matrix metalloproteinase (MMP-9) from neutrophils, truncates the amino terminus of IL-8 to increase the potency of chemoattractants [17,18]. MMP-9 also mediates the proteolytic degradation of bone marrow stromal cell-derived factor (SDF-1) during G-CSF-mediated hematopoietic stem/progenitor cells mobilization (Fig. 2A) [19]. In addition, chemoattractant endocytosis has been shown to be involved in the generation of transient gradients. Fgf8 morphogen gradients in zebrafish embryos have been reported to be regulated by a sink function associated with receptor-mediated endocytosis [20]. Similarly, CCR2 is thought to act as a scavenger for the chemokine CCL2 during monocyte chemotaxis, although it remains to be seen whether the CCL2 gradient is formed as a cause or a consequence of CCL2 endocytosis [21].

The best-studied source-sink system is the chemokine SDF-1, which is sequestered by its scavenger receptor CXCR7. Binding of SDF-1 to CXCR4b was shown to trigger downstream signaling cascades in zebrafish primordial germ cells that give rise to cell polarization and directed migration towards the attractant source [22,23]. In a series of elegant experiments, the Raz lab subsequently demonstrated that CXCR7 is key for the homing of germ cells. By internalizing and thereby clearing SDF-1 from the tissue, somatically expressed CXCR7 created an SDF-1 gradient that allowed directional migration of germ cells [24,25]. Several studies have since demonstrated similar mechanisms in different developmental systems. For example, zebrafish progenitor neurons, which surround the migrating trigeminal sensory neurons, express CXCR7b. Here again, CXCR7b internalizes SDF-1 and reduces its availability for CXCR4, the neuronal chemotaxis receptor [26,27]. An analogous mechanism has been described for the chemokine CCL21, which guides dendritic cells and lymphocytes into mammalian secondary lymphoid organs. Here, the scavenger receptor CCRL1 prevents the accumulation of dendritic cells in the subcapsular sinus, a lymph node compartment that is usually traversed by these cells. CCRL1 does so by eliminating excess CCL21 from the subcapsular sinus, thereby maintaining a gradient of CCL21, which guides dendritic cells further into the center of the lymph node [28].

SDF-1 also participates in a reaction–diffusion/source–sink system that is markedly different from those described previously. The posterior lateral line primordium in zebrafish is composed of a group of cells that migrate along a pre-patterned path of uniformly distributed SDF-1. Prior observations have shown that these cells maintain their directionality even in the absence of a SDF-1 gradient and that interplay between CXCR4 and CXCR7 was involved [29]. Recent reports [30,31] explain this apparent aberration through the presence of CXCR7 expressing cells at the rear of the migrating primordium. CXCR7 sequesters and internalizes SDF-1, hence creating a microgradient of SDF-1 across the primordium. This is a case where a moving mass of cell can shape a chemotactic gradient, even when an external sink is absent, and create a self-generated gradient (Fig. 2A). Other studies have demonstrated the existence of self-generating gradients not only in a collection of migrating cells but in single cells as well. For example, normal and cancer epithelial cells are known to migrate persistently in microscopic mazes filled with uniform concentrations of chemoattractants through a combination of EGF uptake, restricted transport of EGF and response to EGF microgradients [32].

THE RELAY OF CHEMOTACTIC SIGNALS AS A MEANS TO AMPLIFY GRADIENTS

In physiological settings, cells are exposed to a complex environment that contains cues from diverse sources. For example, neutrophils are exposed to and can sense a wide array of attractants in inflamed tissues. Primary attractants originate from damaged/dying cells or bacteria (via formyl peptide release) while secondary attractants are released from endothelial cells, neighboring immune cells or neutrophils themselves, acting in an autocrine and paracrine fashion [33,34]. It has been proposed that secondary attractants are secreted in sequential waves allowing the recruitment of neutrophils far away from primary attractants

[35]. The arachidonic acid-derived leukotriene B₄ (LTB₄) has been shown to be a central attractant in the initial phases of inflammation [36–38] and inhibition of LTB₄ production has been shown to decrease the recruitment of leukocytes and inflammation in a variety of pathological conditions, including spinal cord injury, asthma and arthritis [39–42]. Interestingly, while it was presumed that LTB₄ is released only once neutrophils reach the inflammation site, it was recently demonstrated, *in vitro* and *in vivo*, that LTB₄ is actually actively secreted by neutrophils as they migrate toward inflammation sites, therefore acting as a signal relay between neutrophils (Fig. 2B) [43,44]. A similar situation occurs in *Dictyostelium*, where the relay of cAMP signals between cells is required for cells to align in a head-to-tail fashion during chemotaxis [45]. This relay of signals dramatically increases the recruitment range of cells and maintains directionality over long distances [46]. Remarkably, it has been shown that the enzyme that synthesizes cAMP, the adenylyl cyclase ACA, is localized within multivesicular bodies that coalesce at the back of migrating cells and are released as exosomes during chemotaxis [47]. It therefore appears that signal relay between chemotaxing cells is an evolutionary conserved property that serves to increase recruitment range to primary chemotactic sites. The release of secondary chemoattractants amplifies the chemotactic landscape of primary chemoattractants by relaying signals to neighboring cells that are too far to sense the primary signal.

MECHANICAL GRADIENTS, MECHANOTAXIS AND HAPTOTAXIS

The discovery that fibroblasts tend to move from softer to stiffer regions of a matrix-coated substrate put forward the notion that physical gradients can act as cues to direct cell migration [48]. Since then, several types of physical cues have been reported, collectively called mechanotaxis (Fig. 2C). For example, durotaxis, guidance by the stiffness of the substratum, plithotaxis, guidance of a collection of cells by intracellular tension, and cohesotaxis, collective cell migration guided through intracellular force gradient, have all been described [49]. These behaviors have largely been observed by studying cell migration on a given substratum (i.e. collagen) and, as such, cannot be decoupled from other physical attributes such as adhesion, osmotic swelling and chemical composition of the substratum. Moreover, a direct *in vivo* demonstration of mechanotaxis is still lacking.

A widely accepted principle of directional guidance is haptotaxis, a concept that was first proposed by Carter (Fig. 2C) [50]. Here cells migrate along a concentration gradient of adhesive ligands, usually extracellular matrix (ECM) proteins, that are recognized and bound by integrin receptors. Like mechanotaxis, haptotaxis can be robustly demonstrated *in vitro*, where fibroblasts migrate towards increasing concentrations of immobilized integrin ligands [51]. Yet, a clear demonstration of haptotaxis *in vivo* has not been reported. An alternative variant of haptotaxis is the migration of cells in response to immobilized guidance cues. Neuronal growth cones have been shown to navigate along immobilized gradients of netrin-1 [52] and ephrin [53] *in vitro*, while fibroblasts are able to interpret surface anchored gradients of platelet-derived growth factor [54]. As gradients are notoriously difficult to detect and manipulate in organisms, it again remains to be determined to what extent the concept translates *in vivo*. However, the fact that most chemokines and chemotactic growth factors harbor binding sites for glycans, especially heparan sulfates [55], argues for an important functional role of immobilization. Direct

evidence for a role of chemokine-glycan interaction in the directed migration of leukocytes was provided in two recent studies. First, it was demonstrated that the chemokine CCL21 forms interstitial gradients in murine skin, which guide dendritic cells towards lymphatic vessels. A stretch of charged residues serves to anchor CCL21 to interstitial heparan sulfates, thus forming a spatiotemporally stable gradient [56]. Second, Sarris and colleagues [57] established that in zebrafish the chemokine CXCL8 induces the directional migration of neutrophils and that the gradients established by CXCL8 depend on its heparin sulfate binding capacity. How glycan interactions shape, stabilize and functionally regulate chemotactic gradients is largely unknown and extensive literature on morphogen gradients offers many potential scenarios [58]. Furthermore, it remains to be shown if haptotaxis indeed holds true at the molecular level. Although it is plausible that the guidance cue remains bound to the immobilizing substrate while it is sensed by the receptor, it is also possible that glycans rather attract dense “clouds” of chemokines, which are then sensed in their soluble state. As chemokine affinities towards heparan sulfates vary across orders of magnitude it is likely that individual chemokines show very distinct behaviors.

DIFFUSION-INDEPENDENT MECHANISMS OF GRADIENT FORMATION

Cell migration during organogenesis in response to morphogens is unique in terms of the delivery and dispersal of the attractants. Organogenesis involves positioning and differentiation of primordial cells based on the graded distribution of morphogens. Paradoxically, in spite of their ability to distribute at tissue spaces far from their site of production, some of the best-studied morphogens are lipid modified or bind tightly to membrane associated proteins. For example, hedgehog (Hh), which acts as an attractive cue during germ cell migration [59], is covalently attached to cholesterol and palmitate [60]. Various mechanisms have been suggested to explain dispersal of these modified morphogens. Modified, membrane bound morphogens such as Hh, Wnt3a and Dpp (decapentaplegic) have been shown to be packaged in exosome-like membrane vesicles called argosomes [61], transported along filopodia-like protrusions called cytonemes [62], and transcytosed across cells [63] (Fig. 1C). These processes alleviate the need for diffusion-based dispersal of chemoattractants. More importantly, similar principles of chemoattractant dispersal have been suggested beyond organogenesis. For example the transport of Wnt-PCP (planar cell polarity) components from fibroblasts to breast cancer cells occurs through exosomal packaging of the signaling components [64] and, as discussed above, the packaging of cAMP in exosomes is essential for collective migration of *Dictyostelium* cells [47]. Chemokines have also been shown to be transcytosed from tissue to vascular lumen promoting chemokine-mediated leukocyte transmigration through the Duffy antigen receptor for chemokines (DARC) [65]. Although not directly demonstrated, vesicular packaging seems to have other attractive advantages for chemoattractant dispersal. It could allow the chemoattractant to be protected from various degradative elements present in the extracellular milieu. In addition, integral proteins bound to the surface of vesicles could play a role in chemoattractant targeting and lipids on the surface could act as tethers to specific tissue elements.

GRADIENT FORMATION BY SMALL, HIGHLY DIFFUSIBLE MOLECULES

Small, highly diffusible chemotactic molecules present an inherent problem because of their rapid diffusivity and inability to persist long enough and reach far enough to convey spatial information to target cells. Yet, there exists a growing body of literature that provides evidence to the contrary. Gradients of hydrogen peroxide (H_2O_2), important for the recruitment of leukocytes, have been shown to extend ~100–200 μm into injured zebrafish tail fin epithelium [66]. Gradient of hydrogen (H^+) ions emanating from human colon adenocarcinoma xenograft to blood vessels result in a decrease of pH by 0.7 units over a space of 350 μm [67] and have been implicated in acid-mediated tumor invasion [68]. More recently osmotic shock and water permeation in confined channels were found to be involved in tumor cell metastasis [69]. Reaction-diffusion of chemoattractants by delimiting product concentration is one of the most widely used mechanism to maintain gradient of highly diffusible molecules, as is the case for the degradation of H_2O_2 by extracellular dismutase [70]. Gradients may also be maintained through the spatially graded production of chemoattractants by neighboring cells. For example, the NADPH oxidase, DUOX, expressed in wounded epithelial cells in zebrafish, produces extracellular H_2O_2 in a graded manner and is responsible for the generation of wound margin H_2O_2 (Fig. 2D) [66]. This H_2O_2 gradient is detected in leukocytes through the Src-Family kinase Lyn, which changes its redox state in response to changes in H_2O_2 concentrations and activates the Erk cascade [71]. A more thought provoking concept is the possibility of a cell to self-generate pH gradients. For example, single melanoma cells can generate an extracellular H^+ gradient at their surface that increases from the rear end to the leading edge of the lamellipodium along the direction of movement by preferentially expressing the Na^+/H^+ exchanger, NHE1, at the edge of the lamellipodium [72]. Small molecule diffusion also enables a cell to detect injury even in the absence of cell death. For example, changes in osmotic gradients between wound and extracellular environments in zebrafish have been shown to be sufficient to recruit leukocyte through the release of leukotrienes as a result cell swelling [73].

CONCLUDING REMARKS

In vitro data have demonstrated that cells can migrate directionally in response to chemical or physical cues and modeling studies have provided important insight into the minimal requirements needed to mediate directed cell migration. But what cells actually do in complex living organisms is far less clear. In recent years, ample *in vivo* evidence for directional cell migration in response to various cues has emerged. However, in most cases, the evidence is still somewhat indirect and typical datasets include trajectories of migrating cells, which are perturbed upon elimination of the guidance cue. As the direct detection of the guidance cue itself, especially if it is in a soluble phase, is usually difficult, the molecular complexity hidden behind the observed process is often underestimated. In many cases a whole set of amplification loops might be nested into a seemingly simple attractant system. In addition, the spatial and temporal dynamics of gradient formation, propagation and interpretation remain largely unknown in physiological contexts. Here, the combination of novel imaging approaches, advanced *in vivo* manipulations that go beyond simple loss of function genetics, and reductionist systems to isolate and reconstruct processes will be

instrumental. Furthermore, the inclusion of more complex behaviors in modeling studies will help shed light into the ability of cells to decipher intricate *in vivo* environments.

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* of special interest

** of outstanding interest

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Figure 1. Modes of chemical gradient formation

A. Simple diffusion. In this mode, the spreading of particles occurs through free diffusion in the absence of cells or other hindrances. The mean squared displacement of particles (shown in green) is proportional to the diffusivity of the particles. **Inset.** At any given time, the concentration of the chemoattractant decays (broken line) logarithmically as a function of the distance from the source (green circle).

B. Hindered diffusion. Short acting gradients are formed when particles encounter geometric obstacle or are sequestered or immobilized through the presence of sinks (shown in red). **Inset.** A rapid decay in chemoattractant concentration as a function of the distance from the source is observed compared to simple diffusion. Although molecules have high local diffusivities, their effective global diffusivity is low.

C. Facilitated diffusion. The enhancement of chemoattractant diffusivity by a ‘positive regulator’. A positive regulator of diffusion (marked in blue) may be achieved via the transport of chemoattractants through cells (transcytosis), along cell extensions (cytonemes), by packaging into vesicles (exosomes) or any other form that prevents interactions that may otherwise hinder particle movement. **Inset.** The chemoattractant gradient shape is determined by the positive regulator and may not follow a logarithmic decay profile.

D. Complex diffusion. Complex chemoattractant gradient pattern may be obtained when two or more gradients interact with each other. For example, periodic gradient patterns (**Inset**) may be obtained in a situation where a diffusing chemoattractant (green) encounters a gradient of a degradative enzyme (red), the production of which depends upon the concentration of the chemoattractant itself.

Figure 2. Gradient formation in cellular environments

A. Cells acting as sinks. Receptors expressed either on the migrating cells or in surrounding tissues may sequester or internalize chemoattractants, resulting in the establishment of gradients. Preferential expression of these sequestering “sink” receptors at the back or the front of migrating cells may result in a self-generated gradient.

B. Signal relay by chemotaxing cells. Cells may produce a secondary chemoattractant in response to a freely diffusing primary chemoattractant. This secondary chemoattractant subsequently diffuses to recruit more cells, enhancing the sensitivity and robustness of the chemotaxis process.

C. Mechanical gradients. Cells demonstrate directed migration under various mechanical cues. Durotaxis is the directed migration of cells or group of cells from a softer to a stiffer substrate. Haptotaxis is the migration of cells towards immobilized gradients of chemoattractant trapped by extracellular substrates.

D. Gradients formed by small highly diffusible molecules. Gradients of small highly diffusible molecules may be created by a graded expression of a synthesizing enzyme. For example, the injured zebrafish epithelia produces H_2O_2 by a graded expression of NADPH oxidase, with higher expression in cells with higher degrees of injury.