

Dissecting the Molecular Mechanisms of Electrotactic Effects

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Significance: Steady electric fields (EFs) surround cells and tissues *in vivo* and may regulate cellular behavior during development, wound healing, or tissue regeneration. Application of exogenous EFs of similar magnitude as those found *in vivo* can direct migration, growth, and division in most cell types, ranging from bacteria to mammalian cells. These EF effects have therapeutic potential, for instance, in accelerating wound healing or improving nerve repair. EFs are thought to signal through the plasma membrane to locally activate or recruit components of the cytoskeleton and the polarity machinery. How EFs might function to steer polarity is, however, poorly understood at a molecular level.

Recent Advances: Here, we review recent work introducing genetically tractable systems, such as yeast and *Dictyostelium* cells, that begin to identify proteins and pathways involved in this response both at the level of ion transport at the membrane and at the level of cytoskeleton regulation.

Critical Issues: These studies highlight the complexity of these EF effects and bring important novel views on core polarity regulation.

Future Directions: Future work pursuing initial screening in model organisms should generate broad mechanistic understanding of electrotactic effects.

SCOPE AND SIGNIFICANCE

THIS REVIEW WILL PROVIDE an overview of the recent advances made in understanding the molecular mechanisms of galvanotactic effects, which is the process by which cells sense and utilize small electric fields (EFs) to orient polarity, migration, or division. These effects have long been known to influence many physiological processes, including development and wound healing, and the discovery of gene products regulating these effects promises to open new avenues for medical applications in these contexts.

TRANSLATIONAL RELEVANCE

The study and molecular understanding of EF effects on cell polarity will aid in understanding many medically relevant *in vivo* tissue behaviors. The most important one is wound healing, which is known to be influenced by endogenous EFs *in vivo*. Other *in vivo* relevance also includes nerve regeneration and metastasis.

CLINICAL RELEVANCE

The discovery of genes and proteins regulating electrotaxis will likely provide the driving knowledge to design chemical enhancers of



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Submitted for publication February 3, 2013.
Accepted in revised form April 7, 2013.

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Abbreviations and Acronyms

cAMP = cyclic adenosine monophosphate
cGMP = cyclic guanosine monophosphate
EF = electric field
GCA = guanylyl cyclase A
GTPase = guanosine triphosphate hydrolase enzyme
PI3K = phosphatidylinositol 3-kinases
PIP = phosphatidylinositol-phosphate
PIP2 = phosphatidylinositol bisphosphate
PMv = transmembrane potential value
PTEN = phosphatase and tensin homolog
sGC = soluble guanylyl cyclase
WT = wild-type

wound healing *in vivo*. Additionally, the control over cellular behavior provided by exogenous EFs may serve as a potent tool to drive repair- or target-specific cells to sites of infections.

BACKGROUND

Cell polarization describes the ability of a cell to use external and/or internal stimuli to decide in which direction to grow, migrate, or divide. It is a prerequisite for the development of a multicellular organism and is involved in numerous biological processes such as tissue repair, cancer metastasis, or cell–cell communication.¹ Cell polarity is usually regulated by internal polarity effectors that promote the assembly of actin and microtubule cytoskeleton, which trigger cell movement and shape changes.² Conserved polarity hubs include the one regulating the small guanosine triphosphate hydrolase enzyme (GTPase) cdc42p, or the one controlling the phosphorylation state of phosphatidylinositol lipids (phosphatidylinositol-phosphate [PIP]).^{3,4} In tissues, these internal polarity modules are usually biased and oriented by external cues, such as chemical gradients, mechanical signals, and electrical signals, which allow cells to organize spatially at the tissue level. Although the effects of extracellular cues on single-cell or tissue polarity have been described in many contexts, the molecular details of the cross-talk between external and internal cues remain unclear in most cases. Here, we review the molecular mechanisms underlying this cross-talk in the context of electrical signals.

Cells and tissues in our body are surrounded by organized electrical currents and ion flux, yet the role of such electrochemical signals in organizing cellular behavior remains poorly appreciated. Steady electrical currents and fields have been measured across epithelial layers and proposed to guide cellular behavior in wound healing, development, and metastasis.^{5–9} Even single cells may organize ion flux and electrical currents in large polarized single cells, such as developing eggs or pollen tubes; organized ionic currents around the cell have been measured and are implicated in helping to establish a global order to maintain polarized growth.^{10–13}

It has been observed for decades that the exogenous application of an EF on the same order of magnitude as those measured *in vivo* (ranging typically from 0.1 to 10 V/cm) can direct cell polarity, migration, and division in many different cell types ranging from bacteria and fungi to neurons and neutrophils.^{9,14–17} This near-universal

process is known as galvanotaxis when the cell migrates directionally in the EF, and galvanotropism when the cell reorients its growth axis with respect to the EF. EF effects may have important therapeutic and diagnostic values, for instance, in nerve repair, wound healing, or to control the orientation of cells within tissues. For instance, it has been widely appreciated that EFs may serve as prime directional cues to direct cell migration and division during wound healing, and that their manipulation affects wound closure *in vivo*.¹⁸ Although these effects have long been described and investigated, both molecular and biophysical mechanisms remain elusive. A deep understanding of these EF-sensing mechanisms should enable clinicians and engineers to develop new therapeutic methods for improving wound-healing treatment.

In this article, we review recent advances in the dissection of the molecular mechanisms underlying EF effects on cell polarity, with a particular emphasis on the introduction of genetically tractable organisms and quantitative approaches, which begin to bring understanding of these effects.

DISCUSSION OF FINDINGS AND RELEVANT LITERATURE

Cathode, anode, or perpendicular: which way to polarize in an EF?

Whereas most cell types respond to EFs by reorienting their internal polarity to guide migration, growth, or division, a puzzling result obtained over the years is that different cell types respond by orienting to different directions (Fig. 1A). Most migrating cells, including epithelial cells, fibroblasts, or neutrophils, respond to EFs by migrating to the cathode of the EF (negative electrode).¹⁸ In contrast, breast cancer cells and some endothelial cells migrate to the opposite direction, which is toward the anode of the field.^{19–22} Some cells also display additional atypical shape changes that accompany the directional migration phenotype. Mouse fibroblasts depict, for instance, a striking shape elongation perpendicular to the EF and start migrating to the cathode of the EF.²³

EFs may also orient cellular growth in a non-motile walled cell that displays polarized growth, such as rod-shape bacteria, filamentous fungi, and the rod-shape fission yeast.^{16,24,25} In this situation, the cells reorient their growth axis by bending or branching with respect to the EF direction (Fig. 1C). There again, different cell types appear to reorient differently. Most bacteria grow and bend toward the anode, while some fungi such as *Candida*

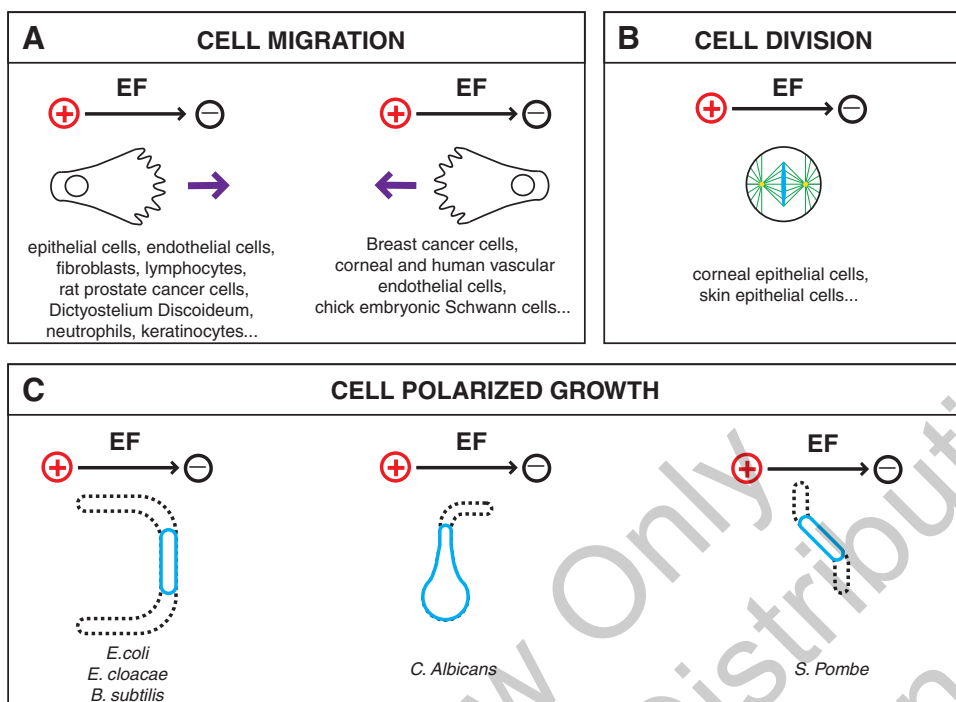


Figure 1. Polarity reorientation of different cell types to exogenous EF. **(A)** Different cell types that show directional migration to the cathode or anode of the EF. **(B)** Cells that depict a perpendicular orientation of the metaphase plate with respect to the EF during division. **(C)** Different cell types that orient their growth axis toward the anode (*left*), the cathode (*center*), or perpendicular to the EF (*right*). EF, electric field. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

albicans elongate its hyphal tip toward the cathode. Other mycelia fungi and the fission yeast *Schizosaccharomyces pombe*, reorient their polarity and grow perpendicular to the EF.^{25,26}

These different orientations, as well as the multiple effects caused by the EF on certain cells, highlight the complexity of these responses and reveal the putative existence of dominant modes that may have a prevalence to steer cells to the cathode versus anode versus perpendicular.

The biophysics of galvanotactic effects

To dig into the understanding of galvanotactic effects, one needs to start asking questions on the biophysical effects that EFs may cause to cells. One well-accepted view is that EFs signal at or through the plasma membrane of cells, which serves as an electrical insulator. In other words, the cell response to EFs is not caused by the movement or direct rearrangement of certain proteins or organelles inside the cytoplasm. Rather, this response may involve a complex signal transduction, which eventually leads to the reorganization of the cytoskeleton and polarity machinery with respect to the EF direction (Fig. 2).

Several biophysical mechanisms for EF effects have been proposed throughout the years and are

supported by experimental evidence (Fig. 3). A prevalent model is that the EF causes local inhomogeneity in transmembrane potential values (PMV) around the cell: the cathode-facing side would be depolarized (reduced PMV) and the anode-facing side would be hyperpolarized (increased PMV), while the parts of the cell facing the

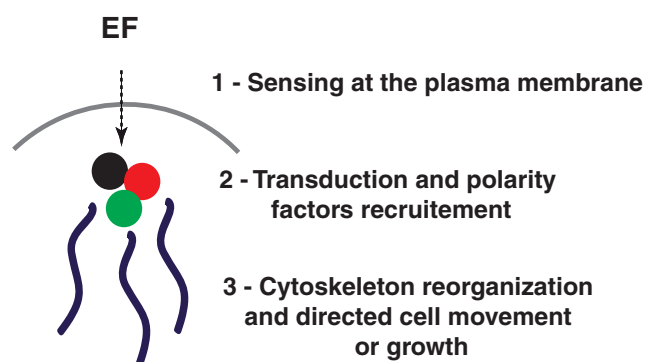


Figure 2. Schematic representation of how EFs may signal to reorganize cell polarity and the cytoskeleton. The EF signal is transduced at or through the plasma membrane, which acts as an electrical insulator. This initial effect may trigger a complex signaling cascade, which eventually leads to the reorganization of the cytoskeleton and polarity machinery with respect to the EF direction. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

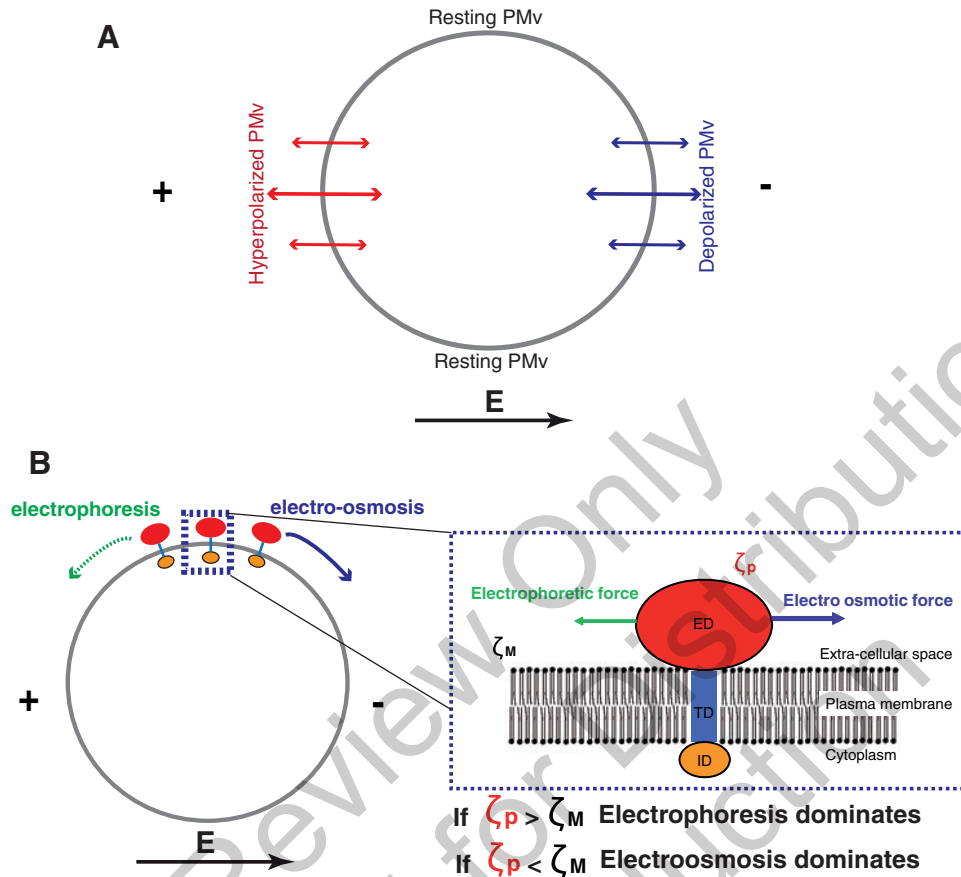


Figure 3. Biophysics of galvanotactic effects. **(A)** EF can cause local inhomogeneity in PMv around the cell, leading to depolarization (reduced PMv) at the cathode-facing side and hyperpolarization (increased PMv) of the anode-facing side. **(B)** EF can cause movements of membrane proteins along the plasma membrane through electrophoresis or electro-osmosis of membrane proteins with a charged extracellular domain. This effect involves competitive forces on the extracellular domain of membrane proteins, and the dominance of steering electrophoretic versus electro-osmotic forces may depend on the surface charge of the domain. PMv, transmembrane potential value. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

perpendicular axis would stay at their resting PMv (Fig. 3). These changes in PMv may yield local imbalances in ion fluxes, or turn on or off voltage-gated channels, or have other yet uncharacterized effects that would initiate a signaling cascade recruiting polarity component. Quantitatively, the extra-transmembrane potential caused by the EF scales with the intensity of the EF multiplied by the typical size of the cell, and thus, if this effect is dominant in EF experiments, larger cells are expected to respond at smaller EFs, which is most likely true from inspecting values in the literature.⁶ These effects on PMv have been directly highlighted using membrane potential dyes²⁷ and genetically encoded proteins.²⁸ Best supports for the role of PMv in EF responses come from experiments in which PMv is altered, from changing specific ion concentrations (H^+ or K^+) in or out the cell, or genetically inhibiting membrane potential regulators.^{14,25,29}

A second important view is that the EF may cause movements of membrane proteins, along the plasma membrane. These movements may result from the electrophoresis or electro-osmosis of membrane proteins, which have charged extracellular domains protruding the plasma membrane. If the Zeta potential (effective surface charge) of the extracellular domain of the protein is more negative than the local Zeta potential of the surrounding membrane, then the prediction is that the protein should move toward the anode; in the opposite case, the protein will be moved by electro-osmosis toward the cathode. Several models coupled with experimental data depicting movements of different membrane proteins, provide support for this view,^{30–32} although very little functional data linking protein movement and polarity re-orientation have been reported so far. It is plausible that in any given cell type, some extracellular domains of some proteins may display enough

surface charge to yield movements, but the question is whether these movements really drive polarity downstream. Modeling considerations provide arguments for how this effect would depend on cell size, protein charge, and diffusion constant in the membrane.³¹ Trafficking and recycling of these membrane proteins is also likely to bias these modeling predictions, and should be taken into account in future extensions of these models.

How might cells sense and transduce EF?

The hidden side of galvanotactic effects is found in the molecular machinery transducing an EF into a defined internal cell polarity. Until recently, there has not been a complete picture in a single cell type that provides a pathway linking biophysical effects of EFs at the membrane down to cytoskeletal organization. In Table 1, we summarize some of the most important proteins or types of proteins that have been suggested to sense and transduce EF effects and be involved in reorganizing polarity in response.^{9,14,21,25,33–38}

Connections between EF effects and downstream cytoskeletal regulators, including the small GTPase cdc42p, the Rho/Rac pathways, integrin signaling, and phosphatidylinositol (PIP) signaling, have been suggested in different cellular systems.^{9,25,33–35} A pioneering work, performed in the context of mammalian wound healing, showed that neutrophils and keratinocytes wound-directed migration depended on phosphatidylinositide 3-kinases (PI3K) and on the phosphatase tensin homolog (PTEN) which, respectively, positively and negatively regulate phosphatidylinositol bisphosphate (PIP2) homeostasis.^{9,39} Wound-healing relies, in part, on endogenous EFs in the wound, and can be inhibited or accelerated by exogenous application of EFs pointing toward or away from the wound, respectively.⁹ In this electrotactic assay, exogenous EFs induce the activation of signaling kinases, including ERK, p38, Src, and Akt. In mouse models lacking the catalytic γ -subunit of

PI3K, neutrophils and keratinocytes displayed reduced activation of these kinases, reduced electrotactic migration, and defective wound closure. Conversely, PTEN deletion enhanced EF-induced Akt and Src phosphorylation and directional migration, and accelerated wound healing. Thus, PIP signaling regulates electrotactic migration of cells in the wounded tissue and supports proper healing.¹⁸ It is interesting to note that PIP signaling also regulates chemotaxis in neutrophils.⁴⁰ The downstream machinery required for directional migration is thus likely to be similar regardless of the nature of the spatial cue in this situation.

The amoebae *Dictyostelium discoideum* has long served as a genetic model to dissect molecular mechanisms of directional cell migration and chemotaxis.^{41,42} When exposed to homogeneous concentrations of cyclic adenosine monophosphate and small EFs, these cells depict striking galvanotaxis, orienting their migration to the cathode of the EF within minutes.³⁷ This EF response is independent of chemotactic receptors.⁴³ Downstream signaling modules regulating directional cell migration during chemotaxis include PIP and cyclic guanosine monophosphate (cGMP) signaling. These effectors promote actin polymerization at the leading edge for directed migration.^{44,45} In a recent work, Sato *et al.* tested the role of these signaling modules in galvanotaxis.³⁵ Intracellular cGMP is produced mainly by two enzymes, soluble guanylyl cyclase (sGC) and guanylyl cyclase A (GCA). Mutants lacking the sGC and GCA (*gca*⁻/*sgc*⁻) and mutants lacking the cGMP-binding protein C (*gbpC*⁻), which display reduced levels of cGMP, exhibited attenuated cathodal electrotactic migration. Similar phenotypes were obtained when PIP signaling was repressed through PI3-kinase inhibition (Fig. 4). Strikingly, when both PIP2 synthesis and cGMP pathways were knocked down, cells migrated to the opposite direction, to the anode of the EF. These results suggest the existence of parallel pathways participating in regulating electrotaxis and point to the existence of a third pathway promoting anodal migration.³⁵ These studies support the role of PIP signaling for electrotaxis in another cell type, and provide detailed genetic characterization of the molecular mechanisms involved. Cross-talk between EFs and polarity in these systems have been proposed to be mediated by calcium transport and membrane potential,^{23,36,37,46} yet the details of this transduction remain to be studied.

Fungal cells and yeasts are model systems to dissect molecular mechanisms of cell polarity. These nonmigrating cells exhibit polarized growth,

Table 1. Examples of gene products and putative pathways identified in electric field responses in different cells

Cell Type	Sensing at the Membrane	Sensing in the Cytosol	References
<i>Xenopus</i> neuron growth cones	Unknown	Cdc42/Rho/Rac	34,36
Keratinocytes	Integrin	Rac/cAMP	21,33
<i>Dictyostelium discoideum</i>	NHE2/Ca2+	PI3K/PTEN/cGMP	9,35,37
<i>Candida albicans</i>	Cch1	Rsr1/cdc42	14,38
Fission yeast	Pma1	Cdc42/for3	25

cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; PI3K, phosphatidylinositide 3-kinases; PTEN, phosphatase and tensin homolog.

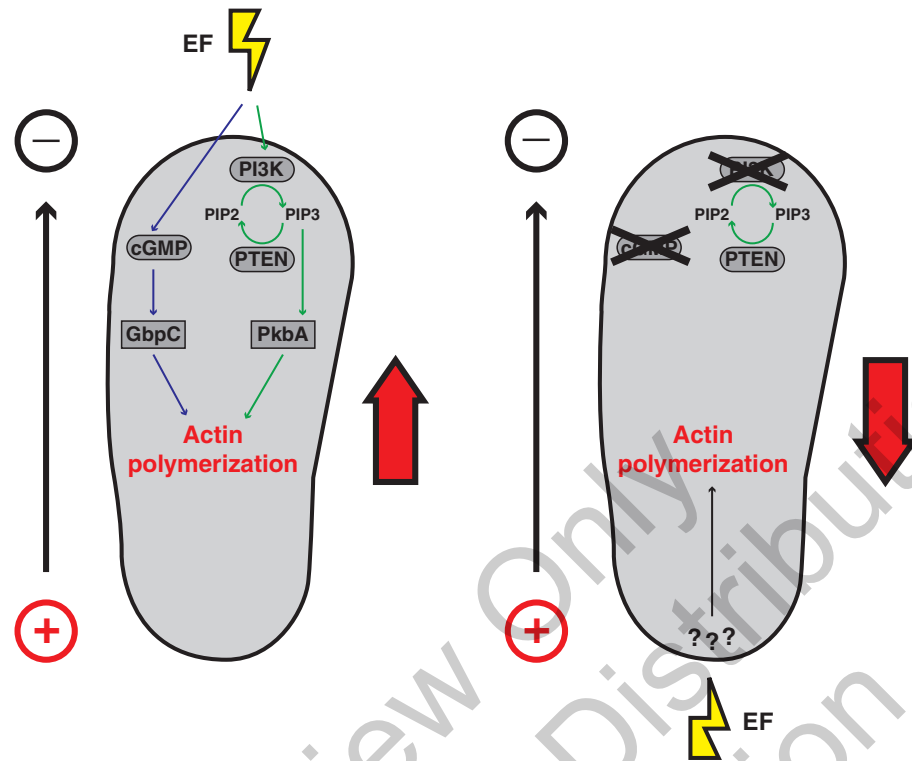


Figure 4. Molecular mechanisms regulating *Dictyostelium discoideum* galvanotaxis. WT cells migrate to the cathode of an applied EF. This polarized migration involves at least two different pathways: The PI3 kinase/PTEN pathway that lead to a polarized distribution of PIP (green arrows); and the cGMP pathway (purple arrows). Mutant cells deficient in the PI3K and cGMP pathway migrate to the anode, suggesting the existence of a third pathway for EF sensing and directional migration. cGMP, cyclic guanosine monophosphate; PI3K, phosphatidylinositol 3-kinases; PIP, phosphatidylinositol-phosphate; PTEN, phosphatase and tensin homolog; WT, wild-type. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

which involves similar regulatory modules and conserved effectors as many higher eukaryotes.⁴⁷ They usually possess a self-sustained internal polarity, which allows them to grow in a highly polarized manner even in the absence of external guiding cues. This polarity machinery may be biased and redirected by chemical gradients, for instance, during mating, or by mechanical signals in processes such as thigmotropism that may have relevance to infection in some hyphal species.^{14,38} Fungi and yeast also depict strong galvanotropism.^{25,26,48} The physiological relevance of the EF response in fungi is not well-established, but EFs are most likely present in the natural fungal habitat, such as on the surface of plants or in humid soils. Some fungi and molds have further been suggested to target wounds by following ion currents and EFs, and thus, the EF response could also have relevance in infection.⁴⁹ Ion transporters and membrane potential regulators are widely shared between fungi and higher organisms, and thus, fungal and yeast cells will likely serve as excellent prototype genetic systems to dissect molecular mechanisms of the EF response at different levels.

One such example can be highlighted from work on the pathogenic fungi *C. albicans*. This single-celled organism grows by budding and switches to highly polarized hyphal growth in certain conditions. EFs can direct both the site of bud emergence and the hyphal polarized growth toward the cathode.²⁴ Using forward genetic and chemical inhibitors, Brand *et al.* recently demonstrated that the galvanotactic response of *C. albicans* involved the conserved calcium transporter CaCch1p.¹⁴ This voltage-gated calcium channel shares high homology with mammalian homologues and with many other eukaryotes, and may serve to transduce membrane potential changes into calcium transport. Further work should reveal whether its function in EF sensing is conserved in other species. Other work from the same group further implicates the role of the Ras-like GTPase Rsr1 that serves as an internal landmark regulating Cdc42 activation in *C. albicans*.³⁸ This set of studies begins to identify important regulatory nodes at the membrane and in the cytoplasm, and further work should reveal how these different modules are connected to drive galvanotropism.

The fission yeast *S. pombe* serves as an excellent system to dissect the molecular mechanisms of eukaryotic polarized cell growth and cell form.⁵⁰ These cells depict a constant and quantitative rod-shape and grow exclusively at cell tips. Genetic libraries of individual knockout strains are available, and provide a very powerful tool to perform systematic genetic studies of basic biological processes. We recently introduced the use of this model to study EF effects on cell polarity.²⁵ The EF caused the fission yeast cells to reorient their growth axis by bending to a direction perpendicular to the EF, creating cells with a bent morphology.²⁵ Candidate genetic screens of mutants in major polarity regulators and in ion transporters suggested that this response depended on the conserved formin for3p and the small GTPase cdc42p,

which regulate actin cable polymerization for cell polarity.^{51,52} This screen identified a conserved plasma membrane ion pump, the proton ATPase pma1p, as a major regulator of EF effects. One interesting result was that mutants in these different genes still oriented to the EF, but to the wrong direction, toward the anode of the EF (Fig. 5). Coupling simulation of biophysical EF effects with detailed localization of these identified components suggested that the main mode orienting cell growth perpendicular to the EF involved membrane potential and local pH effects that may promote formin activation to nucleate actin cables. In turn, the anodal orientation in pma1 for3 or cdc42 mutants appeared to rely on the anodal electrophoresis of cell wall enzymes, beta-glucan synthases that possess highly charged extracellular domains. The role of

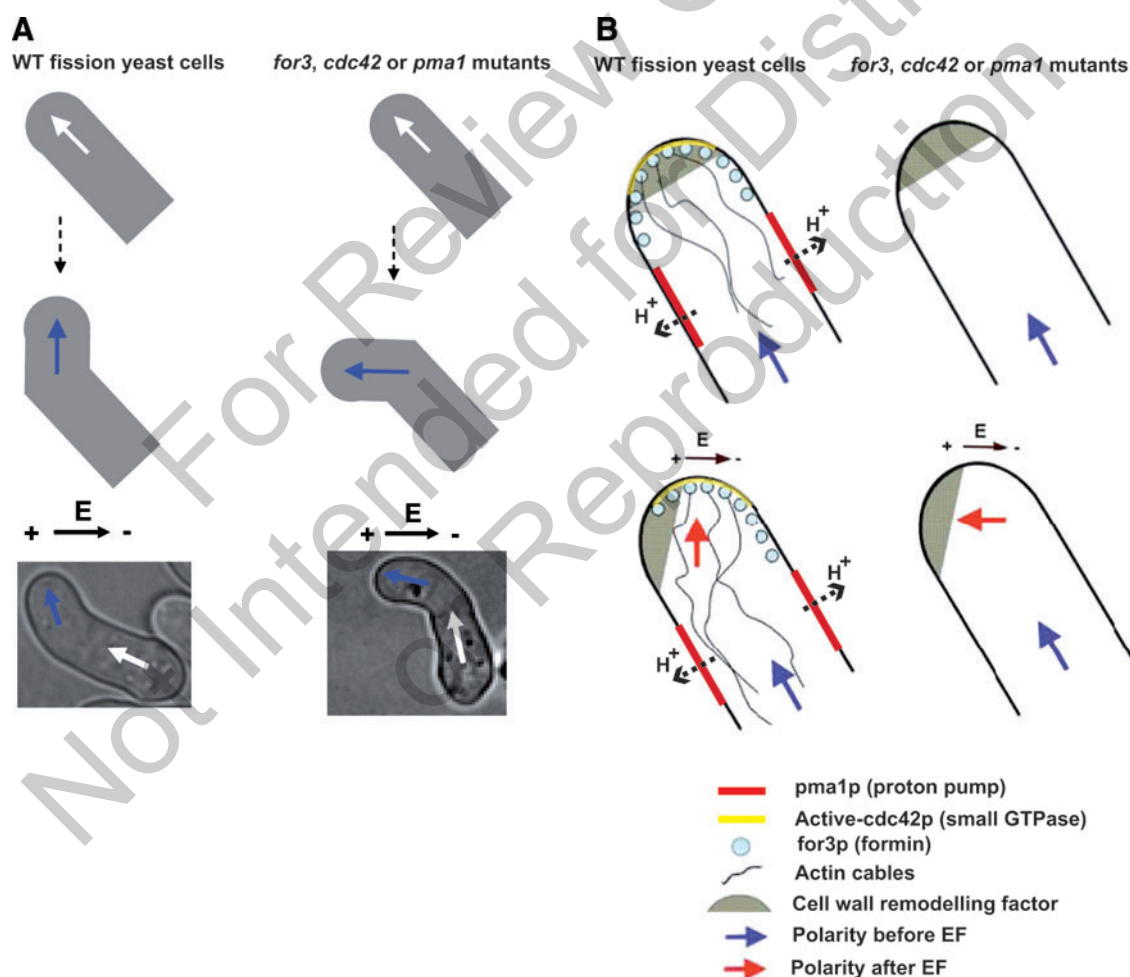


Figure 5. Molecular pathways regulating *Schizosaccharomyces pombe* galvanotropism. **(A)** In fission yeast cells that normally grow in perfect rod-shaped morphology; EF causes WT cells to reorient growth perpendicular to the EF direction. This reorientation depends on the proton ATPase pump pma1p, the small GTPase cdc42p, and the formin for3p. Pma1 functions as a pH regulator and is located on the side of the cell, which may establish putative transcellular proton currents and cortical pH gradients that transduce EF effects to for3p and cdc42p regulation. **(B)** The anodal orientation in pma1 for3 or cdc42 mutants may rely on the anodal electrophoresis of cell wall enzymes, which possess highly charged extracellular domains, and are important regulators of polarized cell growth in these cells. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

cdc42 and formin are consistent with findings in neuronal growth cone migration, which implicate function for the Cdc42/Rho/Rac pathway.³⁴

A very novel aspect of these studies is to promote the role of pH and/or membrane potential in mediating the cross talk between EFs and actin.²⁵ These layers of regulation may be important even in normal cells as *pma1* mutants depict strong morphogenesis and polarity defects. These studies thus bring important fresh views on general cell polarization mechanisms.

All together, these genetic dissections in different organisms suggest downstream transduction of EF effects by small GTPase, lipid signaling, and actin regulation factors. The cross-talk between EFs and some of these polarity modules remains to be clearly defined, although some transporters and ions have been specifically identified in these different systems. Membrane potential, calcium, and pH regulation may play key roles in mediating EF effects into polarized reorganization of cytoskeletal regulators. Genetic studies in *Dictyostelium* and yeast begin to reveal why different cells may polarize to different directions, and suggest that directionality in response to the EF may be sensitive to the expression of a single protein, or a cellular component. These different directional responses found in different mutants support the existence of competing pathways steering the cell in different directions with one dominant mode. When the dominant mode is knocked down, the second pathway takes over to drive polarity in another direction.

Biological significance of galvanotactic effects

These EF effects are likely to reflect physiological events in wound healing, neuron organization, and development. As EFs are present around tissues, studying these effects in isolated cells *in vitro* will reveal important mechanisms of tissue organization and cell behavior *in vivo*. These effects offer one unique manner to control the orientation and shapes of many different cells, and have the potential to open new avenues in bioengineering and medicine.

Beyond their significance in basic biological processes, these galvanotactic experiments bring fundamental understanding in core polarity mechanisms.⁵³ The fact that most cell types can sense and orient to EFs suggest that galvanotactic effects involve an evolutionarily conserved layer of spatial organization. We speculate that EF effects

TAKE-HOME MESSAGES

- EFs may influence the spatial behavior of cells and tissues *in vivo*, during processes, such as wound healing, development, and cancer
- Exogenous EFs can direct cell migration, growth, and division in many different cell types, such as bacteria, neutrophils, and neurons.
- Biophysical and molecular mechanisms of EFs are poorly characterized, but may involve complex signal transductions at the plasma membrane.
- Recent work using genetic models such as *Dictyostelium* and yeast cells, begin to identify key molecular players at the level of membrane signaling and in the regulation of the cytoskeleton to direct migration and growth in response to EFs.

could reflect the natural electrochemical regulation of polarity and cytoskeletal elements. If this is the case, the EF effect may bias or exacerbate an existing electrical organization, leading to the polarized reorientation in the EF. A specific cytoskeletal regulator may, for instance, naturally bind to portions of the plasma membrane with specific charges,^{54,55} or be activated within a narrow pH window; the EF-induced perturbation on the membrane potential, membrane charge, or pH would cause the relocation or reactivation of this element to redirect polarity. It has long been a puzzle to understand how such small EFs, which perturb only 1–5% of the resting membrane potential, could orient polarity in such a striking manner.⁶ Positive feedback regulating polarization modules, the cytoskeletons and ion transport may begin to provide answers to these long-standing questions. There are many recent reports that highlight the role of membrane potential, pH gradients, and membrane inner leaflet charges as fundamental regulators of polarity processes in single cells, tissues, and whole organisms (for a recent review, see Campetelli *et al.*⁵³). Galvanotactic experiments will thus continue revealing important aspects of general polarization mechanisms, and may provide novel approaches to develop suitable therapeutic alternatives in the context of wound healing, development, and regeneration.

CONCLUSIONS AND FUTURE DIRECTIONS

In sum, the road to understanding the molecular mechanisms regulating galvanotactic effects is still long, before one can provide a system-level detailed understanding of such fascinating effects. Model organisms which allow reliable forward genetic studies, such as yeast or *Dictyostelium*, will help to rigorously identify and characterize gene products that may be involved in the electric response. It will then be possible to test these hits, either in mammalian cells using RNA silencing in cultured cell

lines, or in animal models, and to discern relevant signal transduction mechanisms directly relevant to human care. The identification of specific proteins also promises to pave the way for the synthesis of specific chemical inhibitors, which may be used to enhance the galvanotactic effect to improve healing or nerve repair and to develop accurate therapeutic methods for treating chronic wounds and spinal injury. Besides the genetic investigation of these sensing mechanisms, efforts at the biophysical level need to be made to generate a detailed understanding of the processes at play, and modeling together with detailed dynamic microscopy should help research move in this direction.

ACKNOWLEDGMENTS

N.M. acknowledges financial support from an Agence Nationale de la Recherche (ANR) 'retour post-doctorants' grant ANR-10PDOC-003-01 and a European FP7-People-CIG grant. D.B. is supported by an Institut Curie PhD fellowship.

AUTHOR DISCLOSURE AND GHOSTWRITING

The authors declare no competing financial interests. No ghostwriters were used.

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