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Large-scale biophysics: ion flows and regeneration

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Regeneration requires exquisite orchestration of growth and morphogenesis. A powerful but still largely mysterious system of biophysical signals functions during regeneration, embryonic development and neoplasm. Ion transporters generate pH and voltage gradients, as well as ion fluxes, regulating proliferation, differentiation and migration. Endogenous bioelectrical signals are implicated in the control of wound healing, limb development, left–right patterning and spinal cord regeneration. Recent advances in molecular biology and imaging technology have allowed unprecedented insight into the sources and downstream consequences of ion flows. In complement to the current focus on molecular genetics and stem cell biology, artificial modulation of bioelectrical signals in somatic tissues is a powerful modality that might result in profound advances in understanding and augmentation of regenerative capacity.

> *'Wonderful as are the laws and phenomena of electricity when made evident to us in inorganic or dead matter, their interest can bear scarcely any comparison with that which attaches to the same force when connected with the nervous system and with life' (Michael Faraday, 1839).*

Introduction

The repair of tissues lost to injury, senescence or disease is a key goal of biomedicine. Regeneration (see Glossary) of complex organs is fascinating because it is a fundamental property of living things – recognition of damage and self-repair – that still eludes our engineering efforts. The enormous implications of understanding the mechanisms of this ability were recognized very early and were addressed by some of the greatest minds in the field at the turn of the previous century [1]. However, most of the fundamental questions are still with us today. How does a system know it has been injured and when repairs should stop? What is the nature of the information that controls the geometry of new tissue? To what extent is regeneration attributable to the function of a persistent group of undifferentiated cells versus transformation of mature cells from a differentiated state? Do the processes of regeneration recapitulate primary embryonic development or are they an alternative pathway [2]? Is regeneration a special adaptation or is it an emergent, fundamental feature of living systems? What is the role of stem cells and could biophysical parameters, along with biochemistry, control their properties?

A driving hypothesis is that understanding the signals that normally guide cell behavior in the context of complex morphogenesis will reveal the answers to these profound questions and allow us to modulate tissue growth at will. Biomedically induced regeneration is pursued today mainly through manipulation of stem cells and chemical control factors (secreted proteins and transcription factor cascades). This approach also dominates the fields of development and cancer biology. However, data collected over the past 100 years indicate that ionic phenomena (produced by ion transporter proteins in plasma and organelle membranes) underlie a little-understood but important aspect of cellular control [3].

Bioelectrical signals control cell behavior

Channels and pumps produce ion currents that segregate charges and in turn generate voltage gradients across cell membranes (the resting membrane potential). Gap junctions are a passive conduit between cells that can establish multicellular compartments in which voltage, pH and specific ion concentrations are equalized. Bioelectrical signals encode information by modulation of specific ion currents, which set up electric fields across cell and epithelial membranes [4]. Early work demonstrated a role for bioelectrical signals in regeneration (Box 1). Indeed, a comparative analysis of membrane voltage properties of various kinds of cell reveals a relationship between

Glossary

Ion flux: movement of charges (across a cell membrane or through conductive media, such as the cytoplasm or extracellular space). Current is the velocity of this flux.
Voltage: difference of electric potential between two points (achieved by separation of charges across a distance).
Bioelectricity: steady-state signals passed between cells and tissues and carried by voltage gradients, ion flows and concentration domains (e.g. pH) over a time scale of hours (in contrast to the rapid action potentials studied by neurobiologists).
Regeneration: restoration of complex three-dimensional structures (organs, tissues or appendages) after injury. This process can be achieved by renewal of existing tissues, dedifferentiation or transdifferentiation of cells into other cell types, or proliferation and differentiation of a resident stem cell population.
Morphostasis: maintenance of structure and form during physiological turnover.
Blastema: a mass of undifferentiated cells, appearing after injury, capable of growth and regeneration to replace damaged organs or appendages.

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Box 1. Classical studies – a rich literature on bioelectricity

Classical studies by H.S. Burr, E.J. Lund and others demonstrated using direct measurements of voltage gradients that changes in these physical parameters are often correlated with morphogenetic events in growth and patterning across the plant and animal kingdoms [3,11]. The characterization of endogenous bioelectrical signals was revolutionized by the development of the non-invasive extracellular probe [44–46], which allowed study of ion currents in the context of large-scale polarity [47]. Functional experiments subsequently showed that these currents were not merely physiological correlates of housekeeping events, but rather provided specific instructive signals regulating cell behavior during embryonic development and regenerative repair [48,49]. Roles for endogenous currents were found in polarization of cellular asymmetry in the alga *Fucus* [49], in the patterning of chick and frog embryos during gastrulation, neurulation and organogenesis [12,50,51], in the directional transport of maternal components in the insect oocyte [52], in the determination of anterior–posterior axial identity of regenerates in the flatworm [53], in neural differentiation [54], and in the guidance of a wide range of migratory cell types from vertebrate species [55,56]. In several cases, spatially instructive signaling could be demonstrated [53,57].

The mechanistic understanding of bioelectrical signals holds the promise of allowing manipulation of these signals to achieve rational control over growth and morphogenesis. Indeed, several workers during the first 'golden age' of bioelectricity attempted to modulate regenerative response [48,58]. Currently, the hypothesis that regeneration is guided in part by electrically mediated signals is supported by three main lines of evidence [48,59]: (i) strong currents accompany regeneration events, and their magnitude, direction and spatial distribution is often different in the wounds of species that normally do not regenerate; (ii) inhibition of endogenous currents specifically abrogates regeneration; and most strikingly (iii) artificial induction of currents can induce a significant degree of regeneration in normally non-regenerating species. Thus, this relationship is not epiphenomenal – ion fluxes are a causal factor in determining crucial aspects of cell state trajectory [59]. The possibility of altering the differentiation status of committed, somatic cells is of profound importance to the field of regenerative medicine, in which most of the focus has been on embryonic stem cells.

depolarization and control of differentiation and proliferation [5]. Importantly, a degree of dedifferentiation can be induced by ionic modulation [6,7] and even mature neurons can be coaxed to re-enter the cell cycle by long-term depolarization, raising the possibility that a degree of stem-cell-like plasticity could be induced in terminally differentiated somatic cells by bioelectric signals [7–9].

Thus, membrane potential can serve as a cell-autonomous control of cell differentiation and proliferation; the fluxes resulting from ion transport at the cell surface can also provide long-range signals to neighboring cells (by net current or through movement of specific ions). Particularly important is the establishment of long-range electric fields and currents by coordinated ion transport through epithelia [10,11]. Such fields can provide cues for migratory cells [12], and establish coordination of cell behavior across large cell fields (Figure 1, Figure 2). Because of space constraints, this review ignores several fascinating signaling modalities, including biomagnetism, ultraweak (mitogenetic) radiation [13] and electrostatic properties of cells [3]. We also largely avoid the considerable literature on chemical gradients of ions (e.g. chemical Ca^{2+} signaling and pH) as distinct from the generation of fields by their electrical properties (although of course there is a very tight relationship between the two). Instead, the focus will be on the biggest recent strides that have come in the understanding of the roles of endogenous bioelectrical events: long-term (steady state) voltage gradients and ion flows.

The resurgence of studies on ion fluxes in cell and developmental biology

Although the importance of bioelectrical events for regeneration has been suspected for over 100 years [14] and pursued using physiology, now is a particularly exciting time because of a convergence of multidisciplinary techniques and datasets, which promises to enable a radical advance in our understanding of the integration of biophysical and biochemical control mechanisms [15]. First is the development of highly sensitive ion-selective extracellular electrode probes [16] and fluorescent reporter dyes, which enable the noninvasive real-time monitoring of pH, membrane voltage and ion flow in any optically tractable system. For the first time, such four-dimensional datasets are allowing an unprecedented level of understanding of endogenous electrical properties of morphogenetic systems. Second, in recent years, ion transporters have been implicated in several aspects of cell biology relevant to regenerative ability, including control of cell number, location and differentiation (Box 2, Box 3).

The work of neurobiologists and kidney physiologists has resulted in the availability of a large number of expression constructs encoding ion transporters that can be used as a molecular tool for rationally altering the electrical activity of cells and tissues. Morpholino knockdown and construct misexpression are finer tools than the classical technique of applying current with electrodes and enable both specific loss-of-function for electrical signals as well as rescue experiments, allowing elegant demonstrations of necessity and sufficiency. Finally, several recent studies have made significant inroads towards merging genetic and biophysical analysis at a cellular level, providing the first clues into the mechanistic control of regeneration [17,18], as well as the molecular steps transducing membrane voltage changes into canonical second messenger pathways [19]. This review will discuss several recent studies that have synthesized physiological and molecular data, and highlight the most interesting themes emerging in this field that have the potential to alter significantly the landscape of regenerative and developmental biology.

Considerations for bioelectrical control of regeneration and complex morphogenesis

Coherent regenerative response requires integration of proliferation, cell movement and differentiation (Box 3) to restore large amounts of tissue while avoiding the runaway growth of cancer (Box 4). Not surprisingly, given the role of ion flux in proliferation control, 'channelopathies' have increasingly been implicated in neoplasm [20,21]. In programmed cell death, the flip side of induction of mitosis, it is now clear that ion transport (especially by K^+ channels) and membrane hyperpolarization are paramount [22,23]. Because cell death is becoming increasingly appreciated as a required component of regeneration [24], control of apoptosis might be one mechanism by which

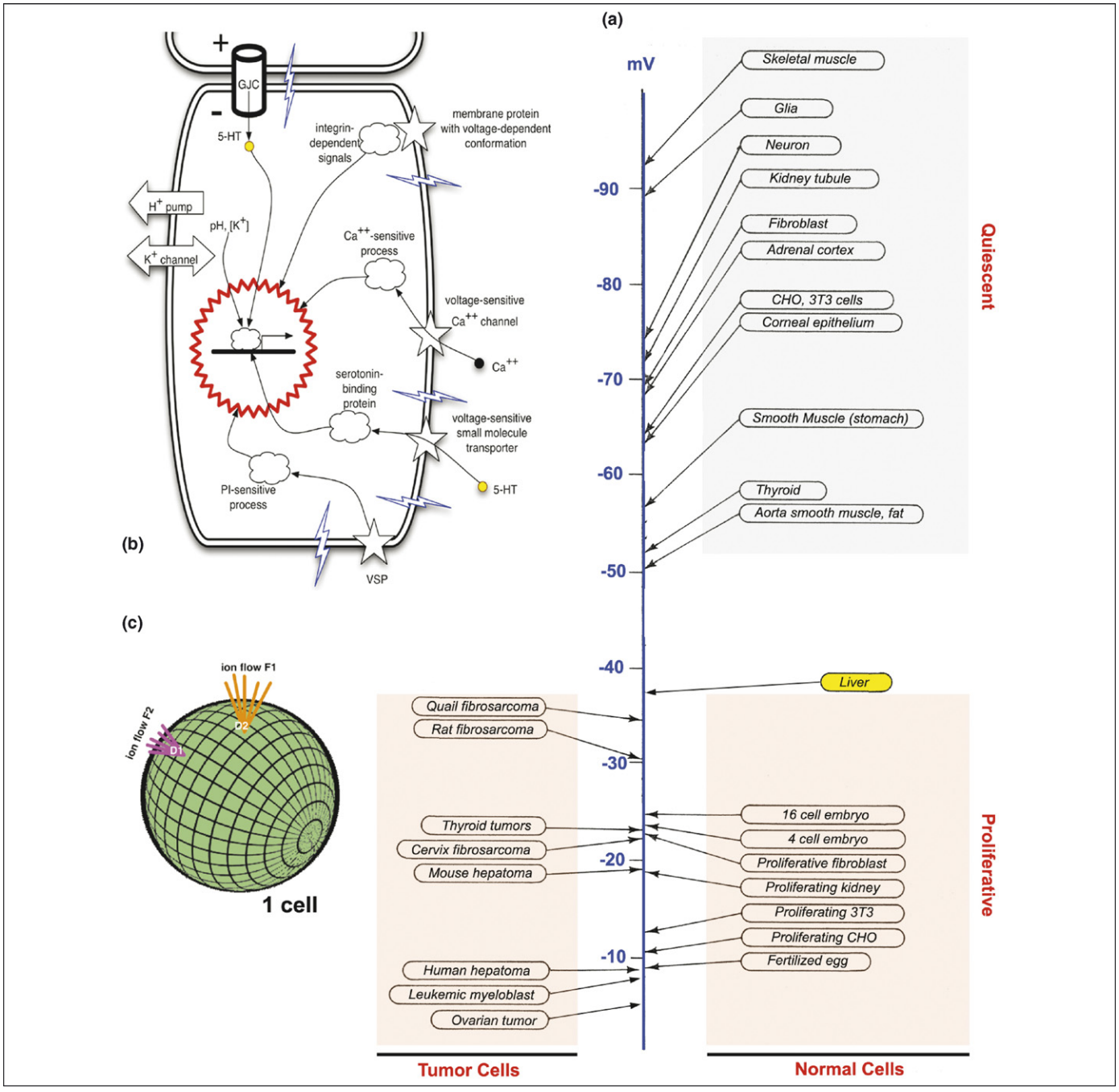

Figure 1. Cell-autonomous bioelectrical states. **(a)** Membrane voltage is correlated with proliferative potential and differentiation state. This sample of data (from Ref. [5]) illustrates the observation that tumor and embryonic (proliferative) cells tend to be highly depolarized; by contrast, terminally differentiated quiescent cells tend to be strongly polarized. The data indicate that this relationship is functional and not merely an epiphenomenon [3]. The position of the liver (highlighted in yellow) near the proliferative component of the scale is consistent with the relationship between membrane voltage and cellular plasticity, in light of its unique (high) regeneration potential. The properties of stem cells along this scale remain to be investigated. **(b)** Illustration of different possible mechanisms by which membrane voltage levels and intracellular pH and K^+ concentration are transduced to transcriptional responses. Some can impinge directly on transcriptional machinery, whereas others (schematized by connections to the nucleus as a whole) need to be transduced by canonical mechanisms. Clockwise from the top, these mechanisms include: entry of small molecule signals through voltage-gated gap junctions, voltage-dependent conformation changes in integrin-associated proteins, Ca^{2+} entry through voltage-gated Ca^{2+} channels, entry of small signaling molecules, such as neurotransmitters, through voltage-powered transporters, voltage-sensitive phosphatase (VSP) activity, and changes in cytoplasmic content of H^+, K^+ and other ions. 5-HT, serotonin, an example of a small molecule signal that could traverse gap junctional (GJC) paths under an electrophoretic force. Blue lightning-bolts represent membrane voltage potentials. **(c)** Membrane voltage can be thought of as a single number at first approximation, but varies along the cell surface as schematized here: each microdomain can support a different set of ion transporters because of complex localization. Thus, like an epithelium [10], the cell membrane can encode an enormous amount of information as a two-dimensional field of voltage potential values (expressing the effects of many nonisotropic domains of transporter function over the surface of a cell). Abbreviations: D1, domain 1; D2, domain 2; F1, flux from domain 1; F2, flux from domain 2.

ion fluxes shape regenerative response. Ion currents and transmembrane voltage potential thus control cell number – a key parameter in regenerative events.

Ion flows are also involved in differentiation control, which is an important aspect of both primary development and regeneration of complex structures. Recent findings have implicated the calcineurin pathway as linking Kir2.1-mediated hyperpolarization with differentiation in human myoblasts [25]. One recent example in which bioelectrical controls of large-scale morphogenesis have been

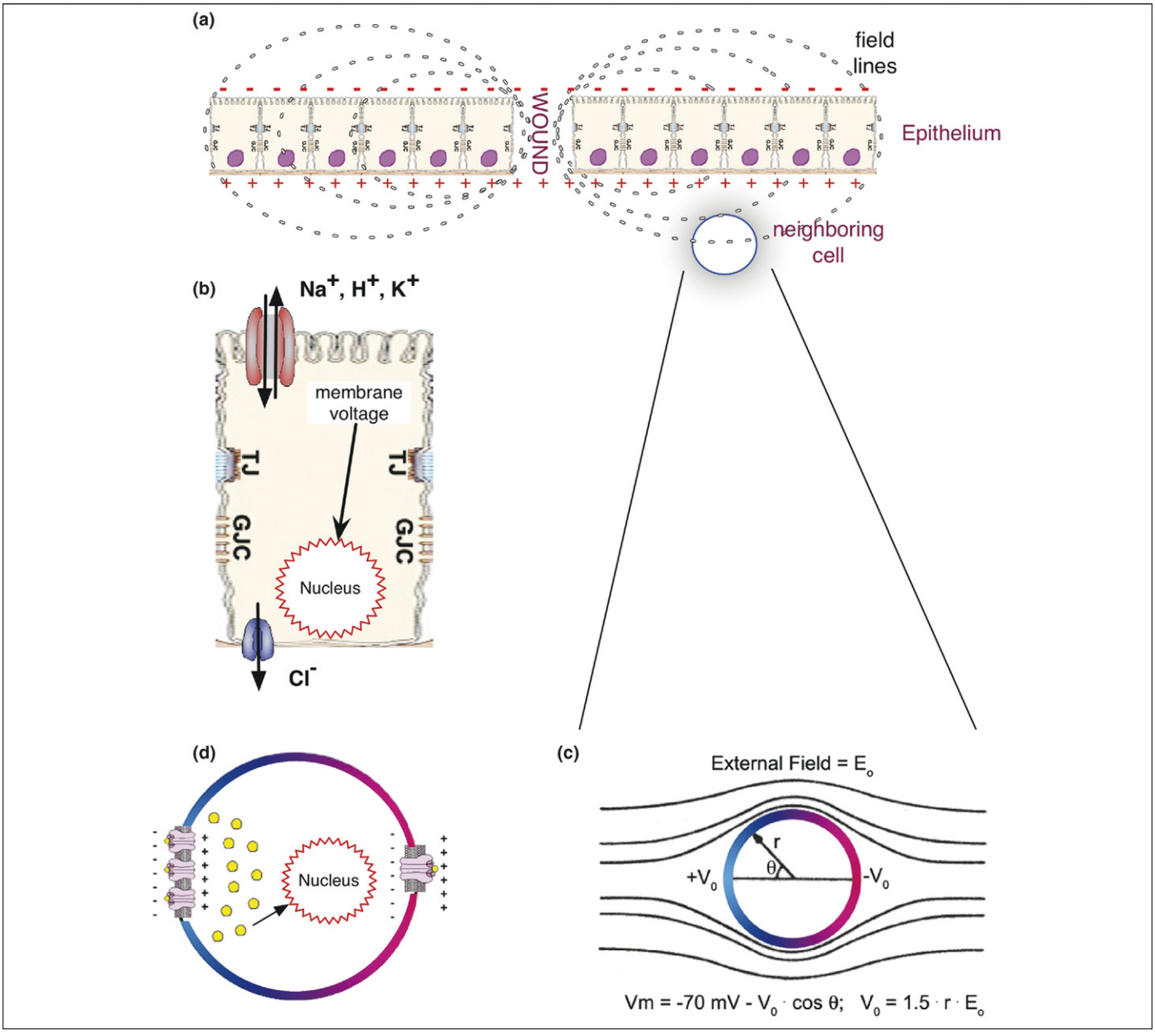

Figure 2. A schematic of long-range bioelectrical events that link subcellular ion transporter localization to long-range signaling. A sheet of cells connected by tight junctions **(a)** generates a transepithelial potential because each cell is physiologically polarized: by localizing particular ion transporters at its apical versus basal end **(b)**, the sheet achieves separation of bulk ions in the same way as occurs across a cell membrane. Any wound made in the sheet allows the ions to short-circuit, generating strong currents; thus, not only are individual cells affected by their own membrane voltage levels (Figure 1), but can also signal to neighboring cells through the induced electric field and ion flow. **(c)** Cells finding themselves in the presence of a surrounding field E_O will experience spatially inhomogeneous depolarization or hyperpolarization along the direction of the field. Membrane voltage at a point in the circumference at angle θ (angular position around the periphery) will be equal to the baseline membrane voltage of the cell in the absence of the field (e.g. −70 mV) minus $V_0 \bullet$ cosine (θ), where V_0 is the voltage change due to the field E_O for a cell of radius r. The equation shows how the initial voltage of a cell (−70 mV) is altered around the periphery of the cell. On the left side, it will be driven more positive by an amount equal to V_0. On the right side, it will be driven further negative by an amount equal to V_0. In between, it will be affected by smaller amounts, equal to $V_0 \bullet \cos(\theta)$. This in turn can provide directional signals by differentially gating small molecule transporters on opposite sides of the cell with respect to the field direction. **(d)** Yellow circles show a small signaling molecule, such as serotonin, entering on one side of the cell via the membrane-voltage-gated transporter. These events can provide migration cues as well as alter proliferation and differentiation states in a manner coordinated with the axial patterning of the organism (as the epithelial field can convey large-scale orientation information with respect to the whole organism or an appendage). The outline of the epithelial cell in (b) is taken from a diagram by Jim Swan at webanatomy.net.

characterized in significant molecular detail is in patterning of the left–right axis in vertebrate embryos [26]. It is now known that H^+ fluxes and membrane voltage gradients are oriented with respect to the prospective left–right axis of the embryo at very early stages of development [27]. Imposed across fields of cells connected by gap junctions, this asymmetric battery appears to exert an electrophoretic force that produces a net gradient of intracellular serotonin through long-range paths of gap-junction-coupled cells (demonstrating how bioelectric events on the scale of a single cell can pattern a major body axis). This system has recently been mathematically modeled [28], as a step towards the synthesis of genetic and physiological data into quantitative, predictive models of the events linking ion transporter protein localization in single cells to large-scale morphogenetic gradients.

Box 2. Bioelectrical controls of regeneration – limbs and nervous system

Amputated amphibian limbs maintain a current of injury – a direct-current signal that is very different in regenerating and non-regenerating animals. In the latter, the current decreases slowly as the limb heals, whereas the former exhibits first a positive polarity (similar to the non-regenerative organism), and then a sharp switch to negative polarity, the peak voltage of which occurs at the time of maximum cell proliferation. For example, in salamander and newt limbs, which have superb regenerative ability, several hours after amputation the density of stump current reaches 10–100 mA/cm^2 and the electric field is about 50 mV/mm [60]. Currents leave the end of the stump, and re-enter the skin around the limb. The relevant currents can be measured for weeks or months – much longer than the time needed for the damaged cells to either recover or die, refuting the simple model that the fields reflect passive ion leaks from damaged cells. The studies that simply correlated changes in voltage and currents were followed by functional experiments. Interfering with the required regeneration gradients via electrical isolation, shunting, ion channel blockers or exogenous reversal of the gradient inhibited regeneration in several systems [51,61–63], demonstrating that these biophysical events were causal factors regulating regeneration.

Another set of crucial experiments demonstrated sufficiency of the electrical signals in inducing or augmenting regeneration [64,65]. Guided by measurements of field density, voltage gradient and direction in endogenous regenerating systems, several laboratories showed that application of exogenous fields (with physiological parameters) can induce limb regeneration in species that normally do not regenerate, including Amphibia [66–69], Aves [70] and possibly even mammals [71,72], although the rodent data have not been reproduced in other laboratories. For example, when 0.1 mA DC current was artificially pulled out of the stumps of amputated adult *Xenopus* and *Rana* forelimbs, treated animals (but not controls) formed broad bifurcated structures [68] containing nerve trunks within the cartilage core and mature epidermal papillae. Cathodal current initiated partial regeneration (including extension of severed ulna, and production of muscle, ligament and isolated partially segmented cartilage). Implantation of sham electrodes (carrying no current) produced no deviations from the normal response.

Nerve supply is a key factor in regeneration [73]. The observation that neurites are galvanotactic [74] and that applied fields induce a striking hyper-innervation of electrically treated limbs led several investigators to hypothesize that blastema currents induce regeneration by attracting migratory neuronal cells to the regeneration site [75]. Exogenous currents can induce regeneration of both peripheral and central nervous tissue, and have shown promise in treating spinal cord injuries in human patients [76].

Bioelectrical control of cell migration during regeneration

Recent work has explored the mechanisms of cell orientation and growth guidance in physiological electric fields [11]. One study focused on molecular characterization of the signaling pathways that allow endogenous electric fields to guide cell migration in the mammalian corneal epithelium *in vivo*. Corneal wounds short-circuit the transepithelial potential, leading to 4 μA/cm^2 currents directed into the wound center [18]. Studies using an *in vitro* assay showed that fields of physiological strength could act as directional cues for neutrophils, fibroblasts and epithelial cells. Moreover, chemical and pharmacological manipulation of ion flux *in situ* can accelerate or retard corneal wound healing in a manner consistent with the modulation of transepithelial potential [18].

Crucially, they were able to use this elegant system to probe the mechanisms by which electric cues are transduced into cellular responses in this system. Exposure of both neutrophils and keratinocytes to electric fields in serum-free medium induced rapid, specific and long-term phosphorylation of protein kinases including mitogen-activated protein kinase (MAPK), ERK, Src and Akt. As occurs in the canonical chemotactic cue responses, phosphorylated Src and phosphatidylinositol-3,4,5-triphosphate [PtdIns(3,4,5)P_3; PIP$_3$] were shown to be polarized in the direction of

Box 3. Does ion transport function in the mapping of three-dimensional pattern on to two-dimensional skin?

In normal development of the vertebrate limb and in regeneration in *Hydra*, the ectoderm possesses 'organizing' properties and is required for outgrowth. Indeed, ion currents are spatially predictive for initiation of limb development [77]. Classical surgical recombination and irradiation experiments showed that in species that regenerate limbs, it is the skin that appears to possess the ability to initiate regeneration [78]. Thus, an attractive hypothesis [2,10] is that this crucial ability is mediated, at least in part, by the capacity to produce ion currents into the outside milieu that affect underlying mesenchyme cells. A passive 'leak' mechanism results (see Figure 2 in the main text) when breakdown of tight junctions leads to strong fields generated by the ubiquitous transepithelial potential [2]. This might be especially significant in the MRL mouse strain, in which basement membrane breakdown appears to be a key component of the augmented healing response [79]. By contrast, the epithelium can also provide an active bioelectric component by upregulating ion transporters that provide specific ion flows overlying a blastema or growth bud after it covers the wound. Both processes could result in depolarization-induced dedifferentiation of mature cell types to provide stem-like cells that are the basis for new tissue growth, and galvanotactic induction of innervation (which is known to be a crucial factor for regenerative potential).

Box 4. Cancer – regeneration's bioelectric twin?

Regeneration has a fascinating inverse relationship with cancer [80–82]: regenerating species rarely exhibit tumors, and if limb regeneration is initiated by cutting in the middle of a tumor, the tumor cells can be transformed into normal limb tissue [83]. The inverse relationship between ability to regenerate and susceptibility to cancer throughout taxa is unexpected, because simple theories of 'growth potential' would predict that animals with more permissive growth programs would also be more amenable to cancer. Likewise, it is surprising that many normal cells (in tissues with strong regenerative potential), such as epithelial cells within the small bowel crypt, have very rapid proliferation rates but show an extremely low rate of malignant transformation [84]. Interestingly, both of these cases involve tight control over ion flux (strong physiological polarization). As predicted by the proposal that ionic mechanisms form a fundamental system of growth control, bioelectric events have been strongly implicated in neoplasm.

Manipulation of membrane H^+ flux can confer a neoplastic phenotype [85]; voltage-gated sodium channels potentiate breast cancer metastasis [86], and channels of the ERG family have been implicated in a variety of cancers [20]. In the case of KCNK9, it is known that the oncogenic potential depends on K^+ transport function *per se*, not on another role of the protein [87]. Ion channels and pH levels (both intracellular and extracellular) have been implicated in cell cycle control [88]. The sodium–hydrogen exchanger NHE1 can control the $G_2 \rightarrow M$ transition by control of intracellular pH [89], and depolarization appears to interface to the cell cycle through cyclin D1 degradation [90]. Taken together, these data underscore the fundamental underlying unity of pattern control mechanisms in regeneration, development and cancer and indicate that advances made in fundamental control mechanisms of shape and cell number will have profound impact on several important fields.

migration. Interestingly, they also reported that a mutant strain of *Dictyostelium* lacking the Gβ protein subunit was unable to demonstrate chemotaxis but did show a robust electrotactic response. Thus, although electrical stimuli can be detected by cells independently of chemokine sensory pathways, it appears that they both feed into the same second messenger cascade.

To demonstrate conclusively that these signaling cascades were indeed important for electrotaxis, the group showed that electric guidance was significantly abrogated in cells from a phosphatidylinositol-3-OH kinase-γ (PI3Kγ) knockout mouse (in both monolayer wound healing and single-cell migration assays), as well as when PtdIns(3)K activity was pharmacologically blocked [18]. This is the first identification of a specific gene involved in electric-field-induced cell migration. The study then followed the PIP_3 mechanism further, capitalizing on a tissue-specific deletion of the *PTEN* gene in keratinocytes (the lipid phosphatase PTEN negatively regulates the PI3K and Akt pathway by reducing the available amount of PtdIns(3,4,5)P_3). Genetic abrogation of *pten* enhanced ERK and Akt phosphorylation, and potentiated field-induced keratinocyte migration. These results implicate function of the tumor suppressor PTEN in the response of cells to electrical signals. This is a very important result, because PTEN is an important regulator in several other regulatory contexts, and thus might implicate membrane voltage in novel areas of cancer and developmental biology [29].

This system also allowed the authors to isolate and focus upon the mechanisms sensing the fields (rather than those producing them). Although the magnitude and direction of corneal and skin wound currents were the same in wild-type and PI3Kγ knockout mice, a corneal explant system revealed that the mutant cells had significantly reduced ability to respond to fields that normally accelerate or retard healing [18]. These data are not only an exciting example of the molecular-cell-biological dissection of how complex cell behaviors in a biomedically important context are induced by external bioelectric cues, but they also provide the opportunity to use these knockout mice to examine what other aspects of healing might rely on bioelectrical signals.

Bioelectrical signals in the regeneration of complex tissues

A recent study has uncovered the involvement of H^+ flux in the regeneration of a complex vertebrate appendage: the tail of *Xenopus* larvae, which contains spinal cord, muscle and vasculature [17]. Although ion flows have been implicated in regeneration previously (Box 2), this is the first time that both the molecular source of the relevant currents and the downstream signaling cascades have been integrated with the physiological data. This study complements the type of work described above because it highlights cell-autonomous mechanisms (rather than the extracellular electric field guidance) and reveals an active pump mechanism upregulated specifically during regeneration, in contrast to 'passive' injury currents that result from breaks into ubiquitously polarized epithelia during limb regeneration [2].

An inverse pharmacological screen was performed [30], using a panel of channel and pump inhibitors to identify ion transporters involved in tail regeneration [17]. The screen implicated the V-ATPase H^+ pump, and subsequent molecular loss-of-function and rescue experiments showed that it is necessary for appendage regeneration but not for simple wound healing. Cell-surface V-ATPase is upregulated (at the mRNA and protein levels) within 6 h of amputation. Although it is still unclear what events lead to the upregulation of V-ATPase in wound epithelium, the very rapid response and V-ATPase-dependent expression of downstream gene expression indicate that this is an extremely early step in the regeneration process.

Membrane voltage dye analysis revealed two special zones: a hyperpolarization of the bud driven by H^+ efflux at the wound edge, and a depolarized region at a distance of ~300 μm from the regeneration bud. In the absence of this state (achieved by altering H^+ pumping and membrane voltage in several different ways), the mitotic increase that normally rebuilds the bud does not take place. This inhibition of mitosis was not detected outside the regeneration zone, showing that regeneration (but not morphostasis and normal growth) is uniquely dependent on this set of bioelectrical events. The axons approaching the regeneration bud were severely disorganized when H^+ pump activity was abrogated, indicating that this signal provides not only a permissive 'go' or 'no-go' signal for the regeneration program, but also contributes morphogenetic information. Most excitingly, it was shown that within a natural 'refractory' period, during which tails cannot normally regenerate at all [31], normal regeneration of the whole tail can be induced by artificial expression of a heterologous (yeast) plasma membrane H^+ pump, demonstrating that it is indeed the specific ion flux *per se* that is necessary and sufficient for regeneration. This finding is important because several ion channels and pumps have scaffolding roles unrelated to their ion transport [32]. Although the experimental induction of regeneration response remains to be fully optimized, these data indicate that artificial modulation of wound physiology by gene therapy, with a judicious choice of ion transporters, is a biomedically promising direction for augmenting and inducing regeneration in otherwise non-regenerating tissues.

The data suggested a model whereby the induction of strong H^+ pump expression and activity at the wound induces two distinct physiological events: an electric field that attracts innervation to the bud [33] and a depolarization of more rostral cells that perhaps upregulates proliferation [9] (and, consistent with previous data [5], can induce a degree of plasticity necessary for mature somatic cells to rebuild the tail). These events are upstream of the induction of mitosis, the expression of known regeneration cascade genes and the correct patterning of nerve and muscle components. The next steps must involve characterization of the roles of the rostral cell group (and identification of other ion transporters responsible for their rostral depolarization), as well as a precise quantitative specification of the pH and membrane voltage ranges that are permissive for regeneration, so that cocktails of expression constructs can be rationally designed for testing in mammalian systems.

How are changes in membrane voltage transduced to downstream pathways?

The linkage of membrane voltage changes to canonical genetic readouts is one of the major questions facing the field today. Voltage-gated ion channels are certainly affected by membrane potential changes, but this merely puts the problem off one step, because their activity still needs to be transduced into changes in gene expression. In some studies, effects seem to be dependent on precisely which transporter is involved, indicating that the type of ion and perhaps the dynamics of its movement also act as signals [34]. Because membrane voltage is only a single parameter (having limited information capacity), it is likely that the true richness of bioelectrical signaling can be fully appreciated only by considering the microdomains of transporter activity distributed across the entire two-dimensional surface of a cell: these inhomogeneities comprise a field of potential values that, because of their spatial distribution, can encode enormous amounts of developmental information (Figure 1c).

Several candidate mechanisms are now available for testing. The most obvious possibility is Ca^{2+} influx (voltage-sensitive Ca^{2+} channels) [35], although in some instances of K^+-dependent signaling, Ca^{2+} fluxes were not affected by K^+ channel activity, showing that proliferative effect is not always due to modulation of intracellular Ca^{2+} [36]. Other possible mechanisms [37] include: (i) modulation of the activity of voltage-sensitive small-molecule transporters, such as the voltage-sensitive serotonin transporter, which could thus convert membrane voltage into the influx of specific chemical signals; (ii) redistribution of membrane receptors; (iii) electrophoresis of morphogens through cytoplasmic spaces; and (iv) activation of β1 integrin signals by conformational changes in membrane proteins [31].

Several other possibilities can now be examined. First, it is now clear that the nuclear membrane possesses its own complement of ion transporters, the activity of which expands this field past cell surface events [38]. Second, direct changes of specific transcriptional element activity by intracellular potassium ion concentration might mediate ion-specific events independent of membrane voltage *per se*; this mechanism can involve the DNA-binding activity of such important signaling molecules as p53, forkhead and cAMP-response-element-binding protein (CREB) [39]. Third, depolarization has recently been shown to lead to subcellular translocation of NRF-2 transcription factor, providing a mechanistic link between membrane voltage and transcription [40].

A recent discovery: voltage-gated phosphatase activity

Perhaps the most exciting recent discovery bearing on this issue is voltage-sensitive phosphatase (VSP) – a phosphoinositide phosphatase that converts PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2 in a manner regulated by a voltage sensor domain [19]. Local levels of PtdIns(4,5)P_2 affect other channels in feedback loops and control the cytoskeleton and nuclear effectors. The discovery of a protein that can transduce membrane voltage into all of the potential downstream pathways controlled by this powerful second messenger system [29] provides a plethora of testable hypotheses of how membrane depolarization functions in a variety of patterning systems involving migration, apoptosis and proliferation. Crucially, it was shown that wound healing control by endogenous electric fields is mediated by PTEN [18], adding weight to the possibility that PTEN could be a widely conserved and important means of integrating cell-autonomous ion flows into second messenger and transcriptional responses.

Thus, modulation of PTEN signaling by the voltage-dependent activity of VSP, of serotonin transport by the cell membrane serotonin transporter SERT (which is driven by transmembrane potential), and other mechanisms discussed above provide an extremely rich toolkit for manipulating morphogenesis during regeneration. All of these mechanisms necessarily feed into transcriptional cascades, and the *Notch* pathway is emerging as an important genetic 'sensor' for bioelectrical events [41]. A second major direction in this field is to determine which types of regenerating system depend on bioelectric events as functional, instructive signals. Examination of biophysical aspects of regeneration in species that use tissue renewal (deer antlers, *Xenopus* tail), adult stem cells (planaria), and dedifferentiation or transdifferentiation (axolotl limb) is likely to reveal that all of these types of regenerative responses involve ionic signals. In each of these cases, it will have to be determined (i) whether the bioelectric component contributes morphogenetic information or is a simpler initiator of regeneration programs; (ii) whether localized ion concentrations are key or whether long-range electric fields are primary; (iii) how changes in the expression and function of specific transporters are initiated by the earliest events resulting from injury; and (iv) whether cell position, proliferation and/or differentiation trajectories are being controlled by the bioelectrical events. Work in planaria, *Xenopus*, chicks and rodents is ongoing; particularly fruitful mammalian systems include the liver and the MRL 'superhealer' mouse (a strain with significantly increased regenerative abilities).

Bioelectrical signals are able to guide the morphogenesis of structures on several scales (from single cells to whole embryonic axes). Thus, the linking of different levels of morphogenetic organization by ion flux-dependent mechanisms is an important component of systems biology (Figure 3); such mechanisms allow the coordination of subcellular polarization mechanisms with long-range (potentially organism-wide) morphogenetic polarity – a profoundly powerful and flexible system of cellular control. The field is now poised for crucial advances in the biophysics of regeneration. The implication of ion flow controls in other regenerative organs and appendages can now be facilitated by reverse pharmacological (transporter inhibitor) screens [30] and by ever-growing microarray data, which implicate specific transporters. Datasets comparing gene expression in disease states and normal developmental processes often contain upregulated or downregulated ion transporter transcripts that are waiting to be functionally tested and characterized using the multidisciplinary tools of molecular biophysics [15]. Insight into their functional roles and into the ways in which cell behavior can be manipulated by this novel set of controls will be transformed by two new

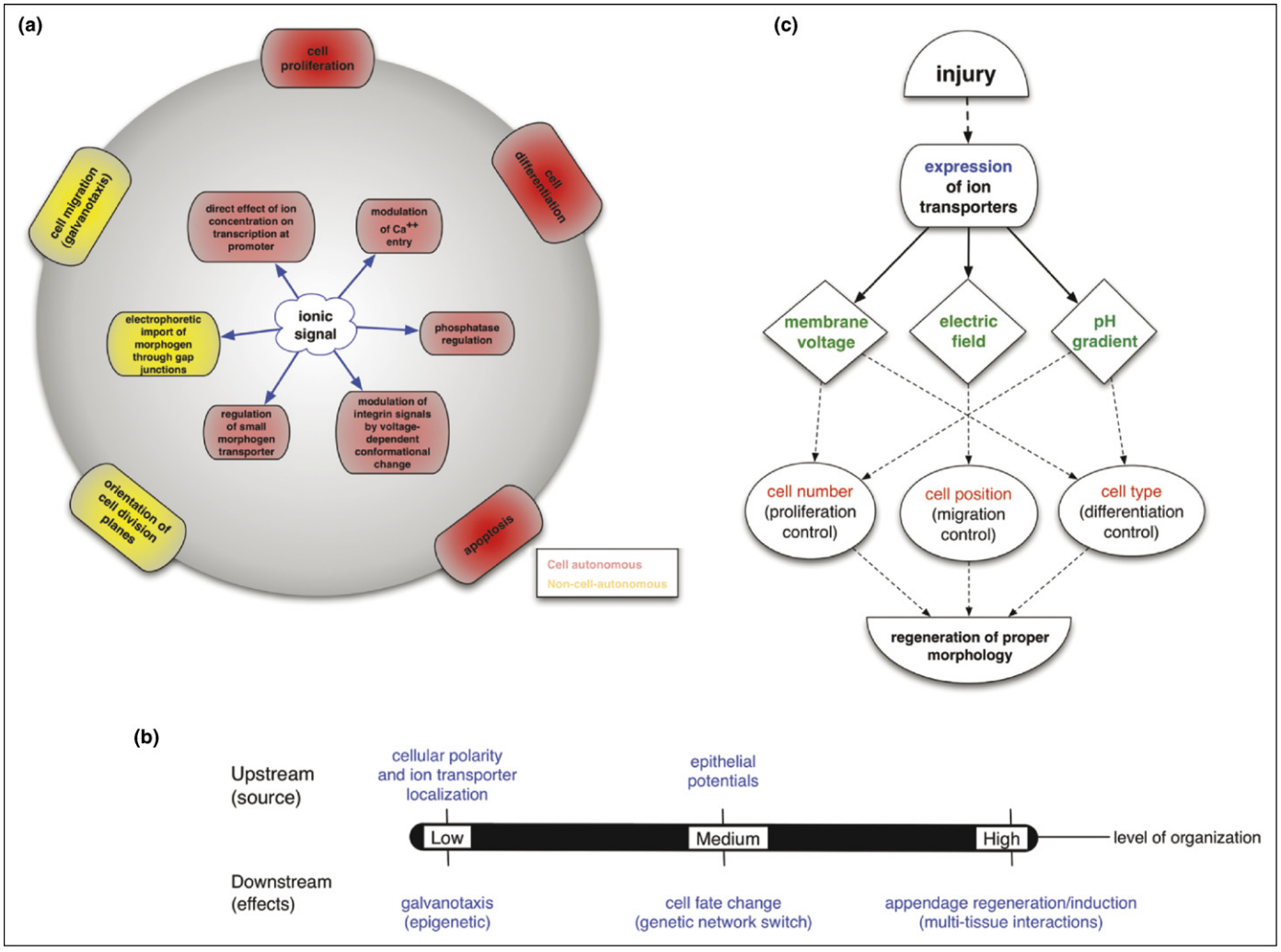

Figure 3. A functional schematic of subcellular and organism-wide bioelectrical control mechanisms. **(a)** This diagram illustrates the logical connection between two organizational levels affected by ion flux events (low-level, represented by the inner group of subcellular events, and higher-level, represented by the outer ring of coordinated cellular responses). A bioelectrical event gives rise to several specific subcellular events, including the import of Ca^{2+} and small molecule signals, changes in phosphatase activity and conformational changes in integrin signals (schematized in the inner portion of the diagram). Combinations of these changes, in the context of other events directed by canonical biochemical signals, in turn result in changes in cell number, position or fate. Events shown in red are cell autonomous; events shown in yellow are not cell autonomous. Events initiated immediately by ionic signals (blue arrows) are low level, occurring inside single cells. Events located on the outer ring encompass coherent cell behavior programs. **(b)** Ultimately, bioelectric events give rise to even higher-order morphogenetic events, such as outgrowth and remodeling, occurring on the scale of organs, appendages or whole organisms. The initiation and reception of ion-mediated signals thus integrates developmental programs across several levels of scale and organizational complexity, being performed by single cells, cell sheets and organs or appendages. **(c)** A logical schematic of one possible bioelectric cascade during regeneration, leading from injury to the expression of specific transporters in the wound. These set up specific pH and membrane voltage domains, as well as long-range electric fields – epigenetic signals that ultimately affect the number, position and types of cell in the blastema. These changes are coordinated with downstream genetic cascades to restore morphology of the tissue or organ being regenerated.

technologies made possible by developments in molecular genetics and fluorescence technology.

First, fluorescence-activated cell sorting (FACS) might be able to identify adult stem cells within a specific region of the phase space defined by independent pH, membrane voltage and $[K^+]$ detection using multiple fluorescent reporter dyes. Second, transgenic organisms expressing pH-dependent fluorescent reporters [42] will enable the monitoring of the physiome *in vivo* during a wide variety of endogenous and experimental conditions in a multitude of tissues and organs (for example enabling the testing of pH and depolarization-reporter dyes as a mechanism of identifying early-stage neoplastic cells). Even more exciting is the prospect of transgenic organisms constitutively expressing a light-gated ion channel or pump [43]. This is currently in development in our laboratory and will be an extremely powerful tool because the experimenter will be able to examine the consequences of activating a specific current in any region using light. In the context of embryonic development or regeneration, the ability to regulate ion flows precisely spatially and temporally will lead to a profound increase in our understanding of the functional roles of bioelectric events.

Summary

Physiological data have long implicated ion flows and voltage gradients in the guidance of regenerative processes in vertebrate and invertebrate systems. Gain-of-function applications have suggested that this powerful system of cellular control is a promising modality for augmenting limb and nerve regeneration. In the last few years, biophysical and genetic studies have shed light on molecular

mechanisms of cell motility guidance and left–right patterning by endogenous voltage gradients and several ion channels have been implicated in growth control relevant both to cancer progression and normal morphostasis. Most excitingly, recent studies have integrated cell biology, *in vivo* physiology and molecular genetics to reveal how endogenous bioelectric signals control corneal healing in the mouse [18] and regeneration of muscle and spinal cord in the tadpole tail [17]. Crucially, the discovery of a voltage-regulated phosphatase [19] suggests a tantalizing set of hypotheses about how changes in membrane voltage can be transduced to multiple downstream effector cascades. These findings, together with recent developments in fluorescent technology that allow an unprecedented degree of insight into, and control of, bioelectric events in real-time, position this extremely fertile area for rapid progress in novel directions. A multidisciplinary analysis of biophysical events in several tractable model species will reveal fundamental, conserved signaling mechanisms. The integration of genetic and physiological data into predictive, quantitative, computer models of complex morphogenesis will be of high significance not only for basic evolutionary, developmental and systems biology, but will also give rise to profound advances in regenerative technology and biomedicine.

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