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Review

Bioelectric mechanisms in regeneration: Unique aspects and future perspectives

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ABSTRACT

Regenerative biology has focused largely on chemical factors and transcriptional networks. However, endogenous ion flows serve as key epigenetic regulators of cell behavior. Bioelectric signaling involves feedback loops, long-range communication, polarity, and information transfer over multiple size scales. Understanding the roles of endogenous voltage gradients, ion flows, and electric fields will contribute to the basic understanding of numerous morphogenetic processes and the means by which they can robustly restore pattern after perturbation. By learning to modulate the bioelectrical signals that control cell proliferation, migration, and differentiation, we gain a powerful set of new techniques with which to manipulate growth and patterning in biomedical contexts. This chapter reviews the unique properties of bioelectric signaling, surveys molecular strategies and reagents for its investigation, and discusses the opportunities made available for regenerative medicine.

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Contents

1. Introduction

Bioelectrical signals are mediated by the steady-state electrical properties of cells and tissues. Despite much fascinating data on the role of endogenous bioelectric signals controlling limb and spinal cord regeneration [1–3], cell and embryonic polarity [4–6], growth control [7,8], and migration guidance of numerous cell types [9], the field as a whole is unfamiliar to several generations of modern cell and developmental biologists. However, some well-known processes, such as the fast, electrical polyspermy block [10,11], are in fact good examples of such signaling.

This chapter discusses the roles of ion-based physiological processes in guiding cell activity during regeneration, and more broadly, pattern formation. Functional experiments throughout the last decades showed that some bioelectric events were not merely physiological correlates of housekeeping processes, but rather provided specific instructive signals regulating cell behavior during embryonic development and regenerative repair [12,13]. Roles for endogenous currents and fields were found in numerous systems (Table 1), and in several cases, spatially instructive signaling was demonstrated [14–19]. Here, I discuss bioelectric controls of morphogenesis in the larger context of pattern formation, outlining controls of individual cell behavior and the unique properties of electrical processes that may underlie the orchestration of higher-order patterning. Specifically excluded in this review are action potentials in neurons, and electromagnetic radiations

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Nomenclature

Allometric scaling remodeling of tissue during changes of cell number or type that maintains correct proportions between organ dimensions (an example of control of non-local, large-scale structure)

Bioelectrical signals information transmitted via spatio-temporal properties of membrane voltage, ion flux, or electrical fields. These are produced by ion channels or pumps functioning in an individual cell or in cell sheets (e.g., epithelial cells arranged in parallel to maximize current) and sensed by the cell itself, neighboring cells, or distant cells. Some are instructive—they carry specific morphogenetic cues used to determine position, differentiation, or proliferation/apoptosis decisions by cells

Epigenetic morphogenesis induced by mechanisms other than changes in DNA sequence or transcription. Bioelectric signals are often epigenetic because these physiological processes can accomplish much patterning via post-translational and physical (e.g., electrophoresis) events not relying on transcription or translation. Ultimately, bioelectric events do induce changes in gene expression

Gavanotaxis ability of cells to utilize field lines and voltage gradients as migratory cues, moving towards the anode or cathode (depending on cell type)

Morphostasis maintenance, throughout life, of large-scale pattern despite death or injury of individual cells or cell groups

Second anatomy coding (in terms of positional, gene expression, or signaling factor gradients) of the components of any system. Roughly, this is the molecular identity by which the embryo or regenerating field spatially addresses (maps) its different parts

State space the set of all possible states of a dynamical system. When applied to cell properties, this is a multi-dimensional theoretical construct where each orthogonal dimension reflects a specific parameter such as voltage, pH, potassium content, etc. Current modeling efforts often make use of the X, Y, Z, t, g space where cells occupy a given point in this space corresponding to their three-dimensional position, gene expression, etc. We propose a physiological state space that instead groups cells by their bioelectrical properties

and biophotons generated by cells. The review concludes with a discussion of the molecular mechanisms transducing bioelectrical events into genetic cascades, and the opportunities provided for the field of regenerative medicine by state-of-the-art molecular tools for the study and manipulation of bioelectric cues.

Bioelectric signals are generated by specific ion channels and pumps within cell membranes. The segregation of charges achieved by ion fluxes through such transporter proteins gives rise to a transmembrane voltage potential (usually on the order of −50 mV, inside negative). Ion channels and pumps are localized to distinct regions of some cell types; in particular, the apical-basal organization of epithelial cells results in a parallel arrangement of battery cells which in turn gives rise to a transepithelial potential [9,20]. Thus, all cells – not just excitable neurons and muscle – generate and receive steady-state bioelectrical signals. These transmembrane potentials, electric fields through tissue and surrounding fluids, iso-electric and iso-pH cell groups established by gap junctions [21], and fluxes

Table 1
Physiological data on endogenous bioelectric signal roles in morphogenesis.

Role	Species/system	References
Cellular polarization (anatomical asymmetry of cell or epithelium)	Alga *Fucus*	[13]
Patterning in gastrulation, neurulation, and organogenesis	Chick, axolotl, frog	[108,169,208,248]
Directional transport of maternal components into the oocyte	Moth, *Drosophila*	[249]
Growth control and size determination	Segmented worms	[250]
Neural differentiation	*Xenopus* embryo	[251]
Polarity during regeneration	Planaria and annelids	[15,18,19,27,28]

of individual ions, all carry information to the source cell as well as to its neighbors, and in some cases, to distant locations.

Early discoveries of "animal electricity" can be traced to Luigi Galvani in the late 1700s, and as early as 1903, it was found that hydroids have a specific electrical polarity [22]. However, the majority of the literature in this rich field has come from several subsequent major waves. Lund, through the 1920s and 1930s, focused on currents and showed that polarity was predicted by, and in some cases controlled by, the bioelectric polarity of ion flows *in vivo* [23]. Burr (1930s and 1940s) focused on measuring and correlating voltage gradients with future developmental pattern in a wide range of species and organs [24,25]; the measurements suggested that the voltage gradients are quantitatively predictive of morphology, and suggested that the measured fields carried patterning information (an example of Slack's "second anatomy" [26]). Some of the best early functional results were obtained by Marsh and Beams [15,27,28] who were able to specifically control anterior–posterior polarity in planarian regeneration by supplying bioelectrical signals to fragments. Enormously influential for the field was the work of Jaffe and co-workers including Nuccitelli, Robinson, and Borgens [12,13,20,29–37], who demonstrated that electrical properties of individual cells, epithelia, neural structures, and entire limbs were instructive for growth, pattern, and anatomical polarity.

The rise of molecular genetics has drawn attention away from a huge literature of not only descriptive, but also solid, well-controlled functional work using physiological techniques. However, in the last decade, state-of-the-art work has begun to identify proteins responsible for the well-characterized bioelectric signals, the genetic networks that shape them, and the mechanisms that allow cells to transduce the information into growth control decisions. Molecular and cell biology are now being applied to this problem in the areas of wound healing, neural guidance, and cell orientation responses to physiological electric fields [38–42], as well as the role of specific ion transporter activity in tail regeneration, left–right patterning, control of adult stem cells and regenerative polarity, and the switch between embryonic stem cell and neoplastic phenotypes [43–49].

Although many modern workers are unaware of this rich field, the connection between molecular-genetic pathways and bioelectric signaling is being forged by the data itself. A variety of relevant channelopathies has now been discovered by unbiased approaches [50,51], though ion transporters are usually de-prioritized for analysis when they show up on comparative microarray experiments because it is not yet second nature for cell and molecular biologists to think in terms of bioelectrical signaling. It is hoped that by highlighting the techniques and tools now available, and illustrating strategies for integrating bioelectrical signals with mainstream pathways, workers in multiple sub-fields will consider that modulation of ion flows, currents, and voltages may be at the root of their favorite patterning or mis-patterning problem when ion channels and pumps are identified in genetic screens or subtraction analy-

ses. A superb example of such a convergence is the recent elegant study implicating sodium/hydrogen exchange in planar polarity in *Drosophila* [52], a relationship that was predicted by bioelectric signals during left–right patterning of embryonic epithelia [53].

Because recent reviews address the role of ionic phenomena and specific ion transporter proteins in wound healing [41,54–56], neoplastic growth [51,57], and cell cycle [58–60], this review has a different goal. Here I will consider the unique properties of bioelectrical signals, as well as the novel techniques being used in this field and the major directions that promise significant advances for regenerative biology and biomedicine [61], both of which require the development of techniques for the rational modulation of three-dimensional structure at multiple scales.

2. Context: bioelectric signals as a component of the morphogenetic field

One way to view regeneration of complex structures is as an example of morphostasis – the maintenance of "target morphology" by an organism. This is the shape, defined on multiple scales of size and levels of organization, which a biological system acquires during development, and maintains against cellular turnover (aging), stresses of life (remodeling and wound healing), and major injury requiring regeneration. This is a perspective, focused on information processing in cells and tissues, which emphasizes mechanisms common to the patterning events that occur during embryonic development and regeneration, or fail to occur during neoplastic growth.

The target morphology can be analyzed via mathematical tools – formalized descriptors allowing comparisons of form and of shape transformation, as well as analyses of complexity [26,62–65]. Its presence is revealed not only through highly stereotypical outcome of embryonic self-assembly, but also in the morphological remodeling over time, observed in both vertebrate and invertebrate systems where deviations from normal shape are slowly corrected. Examples of patterning driven by non-local morphogenetic information include allometric scaling during whole-body remodeling in planaria [66] and the long-term transformation of a tail into a limb when a tail blastema is grafted at the flank in amphibia. Although the origin of blastema cells is local to the site of injury [67] and the initial pattern formation is determined by the original position of the blastema within the donor, the host's morphogenetic fields exert their influence remotely, and slowly transform the ectopic tail into a limb—the structure appropriate to the large-scale global context in which the blastema is placed [68–71].

The mediator of pattern formation and remodeling can be viewed as a "morphogenetic field" [72–74] – the sum total of local and long-range patterning signals that impinge upon cells and bear instructive information that orchestrates cell behavior into the maintenance and formation of complex three-dimensional structures (Fig. 1). While this is currently studied with respects to gradients of chemical messengers [75–77], bioelectric signals are also ideal mediators of distributed, non-local field properties in large-scale patterning.

The morphogenetic field, while a classical concept [78–81], has recently been reinvigorated through the discovery and molecular characterization of several long-range patterning systems that use the same genetic components to carry patterning signals in embryonic development and regeneration [82,83]; it is this same information that may be ignored by cells during neoplasm [84–86]. This view is a different perspective on regeneration because, rather than focusing on individual molecules and on the special features of regeneration in adults (e.g., scarring), the goal is to understand, and learn to rationally modulate, large-scale patterning processes. This is broadly relevant to many other biomedical areas that can be formulated in terms of establishment, maintenance, and deviation of morphology (e.g., aging, birth defects, and cancer). Examples of underlying mechanisms that establish long-range order are planar cell polarity [86–89] and neural signaling. The latter in particular is known to be crucial for regenerative ability [90–92], and involved in the maintenance and organization of multicellular structures in the organism such as tongue buds [93], which become disorganized when their innervation is perturbed. This chapter describes one fascinating and molecularly tractable component of the morphogenetic field: endogenous bioelectric signals.

3. Cellular-level processes: what can bioelectric signals do?

Coherent regenerative response requires integration of proliferation, cell movement, and differentiation into needed cell types to restore large amounts of organized tissue. Large-scale morphogenesis is the ordered orchestration of lower-level cell behavior, and it is helpful to consider briefly the cell functions that are controlled by endogenous bioelectrical signals.

Cell movement and positioning is an important component of regeneration [94]; movement of progenitor cells towards wounds is observed in planaria [95], zebrafish brain [96], and in mammalian stem cell homing [97]. One of the earliest-observed effects of electric fields on cells was change of orientation (parallel or perpendicular to field lines), growth (extension of processes), or migration (towards the anode or cathode) [98,99]. Modern protocols avoid artifacts due to polarization of substratum molecules and release of electrode products into medium [100]. Despite some controversy [101] over which cell types respond to physiological-strength electric fields (usually on the order of 50 mV/mm, and as high as 500 mV/mm within the neural tube [102,103]), it is clear that a large variety of embryonic and somatic cells exhibit galvanotaxis in electric fields of the magnitude often found *in vivo* [104–107]. In embryos, it has been suggested that patterns of voltage gradients form coordinates guiding cell movement during complex morphogenetic processes [108]. Electric guidance also occurs in several types of tumor cells [109]; recently, voltage-gated sodium channels have been strongly implicated in this phenomenon [110,111] suggesting that endogenous bioelectric states may be a factor in metastatic invasion. It is also now known that bioelectric events are important not only for the generation of guidance signals, but for cell-autonomous responses to fields during migration [112] where channels such as $K_{Ca}3.1$ (KCNN4) provide instructive signals for the direction of cell movement [113].

In addition to cell positioning, regeneration requires the presence of numerous distinct cell types. Early links between ion flow and differentiation were observed by Barth and co-workers, who showed that ventral ectoderm explants could be differentiated into a variety of different cell types by careful modulation of extracellular medium ion content [114,115]. Bioelectric signals apply not only to embryonic cell differentiation, but also to stem cells, which have unique profiles with respect to ion channel expression and physiological state [116–123]. Moreover, it has been recently shown by functional experiments that membrane voltage controls human mesenchymal stem cell (MSC) differentiation *in vitro* [43]. Much remains to be learned about this process, but it is known for example that Kir2.1 (KCNJ2) channel-mediated hyperpolarization controls differentiation in human myoblasts via a calcineurin pathway [124]. Importantly, a degree of *de*-differentiation can be induced by ionic modulation [125,126], and even mature neurons can be coaxed to re-enter the cell cycle by long-term depolarization. This raises the possibility that a degree of stem cell-like plasticity could be induced in terminally differentiated somatic cells by bioelectric signals [7,126,127].

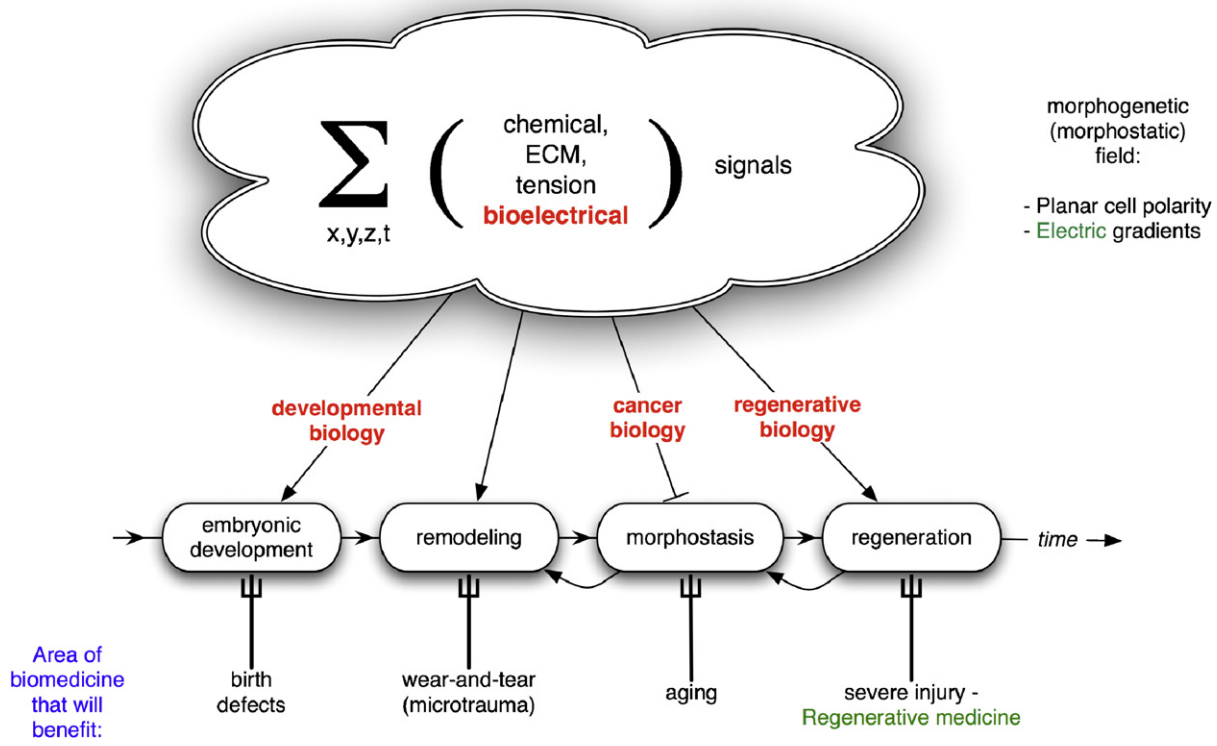

Fig. 1. Morphogenetic fields and biomedicine. The morphogenetic field can be defined as the sum, integrated over 3 spatial, and 1 temporal dimensions, of all non-local signals impinging on cells and cell groups in an organism. Functionally, these signals carry information about the current and desired pattern of the organism. This allows the initial development of complex form from a single fertilized egg cell, as well as the subsequent maintenance of form in adulthood against trauma and individual cell loss. Errors in various aspects of the establishment and interpretation of this field result in birth defects, cancer, aging, and failure to regenerate after injury. Thus, almost every area of biomedicine is impacted by our knowledge of how cells interact with this set of complex signals. Bioelectrical aspects of the morphogenetic field are crucial, although planar polarity systems and chemical gradients also form components of this information field. ECM = extracellular matrix.

Bioelectric signals also appear to control mitosis rate, which is closely linked to differentiation, as plastic cells tend to proliferate more than most terminally-differentiated somatic cells. Indeed, a comparative analysis of membrane voltage properties of various kinds of cells reveals a striking relationship between depolarization and control of differentiation and proliferation [128]. Numerous studies have implicated K^+ currents as protagonists of proliferation and cell cycle progression [129,130], reviewed in [60,129]. Cell proliferation appears to be controlled mostly by membrane potential [131,132], although the effect is not always cell-autonomous: depolarized cells can induce distant neural crest derivatives to overproliferate [49].

A considerable literature now exists on the role of specific ion transporters, including the sodium–hydrogen exchanger and a variety of K^+ and Cl^- channels, in cell cycle progression, although many questions remain about mechanistic details [129,133–136]. In the zebrafish eye, the V-ATPase is required for retinoblast proliferation [137]. Thus, because of its many patterning roles spanning from the elongation of the tadpole tail [48] to that of pollen tubes [138], as well as in neural stem cells in the regenerating fish brain [139], H^+ efflux is a widely relevant transporter for efforts to augment regenerative growth.

The converse of growth through mitosis, that is—cell elimination through programmed cell death, is known to be a part of regeneration in a variety of systems utilizing stem cells [140], tissue renewal [141], and transdifferentiation [142]. Apoptosis is regulated by hyperpolarization via a set of K^+ and other channels [59,143–145]; for example, inhibition of K^+ channels can promote apoptosis [146–148] while activation of K^+ channels can inhibit it [149,150]. Surprisingly, programmed cell death has recently been shown to be *required* for regeneration [151], suggesting that tight control over programmed cell death (by bioelectric means as well as genetic) may need to be an important aspect of regenerative interventions.

Thus, the data point to transmembrane potential as a broadly conserved aspect of orchestrating the proliferation, reduction, differentiation, and movement of cells. This is of particular relevance for bioengineers and those seeking to transition findings in regenerative biology into therapeutics: bioelectric events are a powerful, largely untapped set of cellular control knobs. Gaining the ability to modulate cell number, position, and identity provides the opportunity to manage the alteration or generation of any desired shape.

4. Higher-level integration: the roles of bioelectric signals in morphogenesis

Use of ion-based signals in higher order patterning necessitates coupling groups of cells with respect to electrical signals. This often occurs through gap junctions [152,153], which not only augment cells' ability to sense extracellular electric fields [154], but also are a common mechanism for organizing cells into functional domains, for example when delimiting regions of neurogenic precursors in the spinal cord [155].

The simplest examples of the roles of ionic signals in multicellular systems involve healing epithelial layers, where the fields resulting from disruption of the integrity of the polarized layer

provide guidance cues for growth of migratory cells that repair the wound; much molecular data is now available about the alveolar epithelium [156] and the cornea in particular, where not only electric fields [41,55,157,158] but also cell-autonomous changes in transmembrane potential [159,160] are involved. Other tissues where bioelectric cues contribute to repair include the spinal cord [161–163], and indeed this modality is now in human clinical trials with paralyzed patients [164].

A more complex example of morphogenetic control by bioelectric cues is revealed by the role of currents during appendage regeneration. Excellent reviews of the early work of bioelectric effects on regeneration (augmentation of innervation, control of polarity, and alteration of differentiation) are given in [12,165,166].

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, while 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 salamanders and newt limbs, which have superb regenerative ability, several hours after amputation the density of stump current density reaches 10–100 mA/cm^2 and the electric field is on the order of 50 mV/mm [167]. Currents leave the end of the stump, and re-enter the skin around the limb. The relevant currents can be measured for weeks—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 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 [6,36,168,169], demonstrating that these biophysical events were necessary factors regulating regeneration.

Another set of crucial experiments demonstrated sufficiency of the electrical signals in inducing or augmenting regeneration [170,171]. Guided by measurements of field density, voltage gradient, and direction in endogenous regenerating systems, several labs showed that application of exogenous fields (with physiological parameters) can induce limb regeneration in species which normally do not regenerate, including amphibia [172–175], aves [176], and possibly even mammals [177,178], although the rodent data have not been widely reproduced. 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 [174] 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.

Recently, molecular details have been uncovered about the guidance of regenerative events in vertebrate appendages. The tail of *Xenopus* tadpoles contains spinal cord, muscle, vasculature, and epidermal components. A combination of pharmacological, and molecular-genetic analyses using dominant-negative and constitutively active ion transporters implicated strong H^+ pumping from the wound as an instructive factor in regeneration [48], controlling the appearance of proliferative cells and required for the correct pattern of innervation. Thus, tadpoles normally rely on the V-ATPase hydrogen pump to drive regeneration during early stages. More importantly, during later stages when tadpoles cannot regenerate, the entire regenerative cascade can be reproduced by artificially driving H^+ efflux by misexpression of the heterologous (yeast) pump PMA-1 [179].

5. How are changes in membrane voltage transduced to canonical pathways?

Bioelectric signals are found both upstream and downstream of biochemical and genetic elements (Fig. 2). Ion flows are produced by channels and pumps (which are regulated by transcriptional, translational, and gating mechanisms). Conversely, they control the expression of other genes and the function of physiological mechanisms at the cell surface and in the cytoplasm. Biophysical processes can often achieve considerable patterning in the absence of changes in transcription or even translation, due to the rich regulation of ion transporter activity and the redistribution of macromolecules by electric fields. For example, the stimulation of the sodium-hydrogen exchanger in tumor cell lines results from an increased affinity of the internal H^+ regulatory site without changes in expression [180]; likewise, the electrophoretic mechanisms underlying early left–right patterning in frog embryos occur during the first few cleavages, when the zygotic genome is not transcribed [47,181]. Nevertheless, eventually these processes feed into subsequent pathways that alter gene expression. A commonly occurring theme of this type is the determination of a cell's bioelectrical polarity by its anatomical (apical-basal) polarity, which in turn is controlled by the electric fields produced by specifically localized ion transporters [4,182–184].

Specialized sensory cells can distinguish signals as weak as 5 nV/cm [185,186]; moreover, these mechanisms can exhibit window effects [187], where a stronger applied signal does not necessarily induce the same effects as a more physiological one. The most common mechanism linking membrane voltage change and downstream events is calcium influx (voltage-sensitive Ca^{2+} channels) [188], though in some instances of K^+-dependent signaling, Ca^{2+} fluxes were not affected by K^+ channel activity, showing that effects on cell behavior can sometimes bypass modulation of intracellular Ca^{2+} [189]. Additional mechanisms that transduce electrical signal into second-messenger cascades [190] include: modulation of the activity of voltage-sensitive small-molecule transporters (e.g., the serotonin transporter, which converts membrane voltage into the influx of specific chemical signals); redistribution of charged receptors along the cell surface; directional electrophoresis of morphogens through cytoplasmic spaces; and activation of Integrin or other signals by conformational changes in membrane proteins [191–193]. These elements can be capitalized upon, for the design of bioelectrical intervention in regenerative processes.

Several more exotic possibilities may be fertile areas for future work. First, it is now clear that the nuclear membrane possesses its own complement of ion transporters, the activity of which expands the relevance of bioelectricity past cell surface events [194] and opens the possibility of specific gene regulation by the membrane potential across nearby nuclear envelope regions. 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 CREB (cAMP response element-binding protein) [195]. Third, depolarization has recently been shown to lead to subcellular translocation of NRF-2 transcription factor, providing a mechanistic link between membrane voltage and transcriptional targets [196].

An exciting recent discovery involves VSP—a phosphoinositide phosphatase that converts $PI(3,4,5)P_3$ to $PI(4,5)P_2$ in a manner regulated by a voltage sensor domain [197]. Local levels of $PI(4,5)P_2$ control the cytoskeleton and nuclear effectors. The identification of a protein able to transduce membrane voltage into all of the potential downstream pathways controlled by this powerful second-messenger system [198] provides a plethora of testable

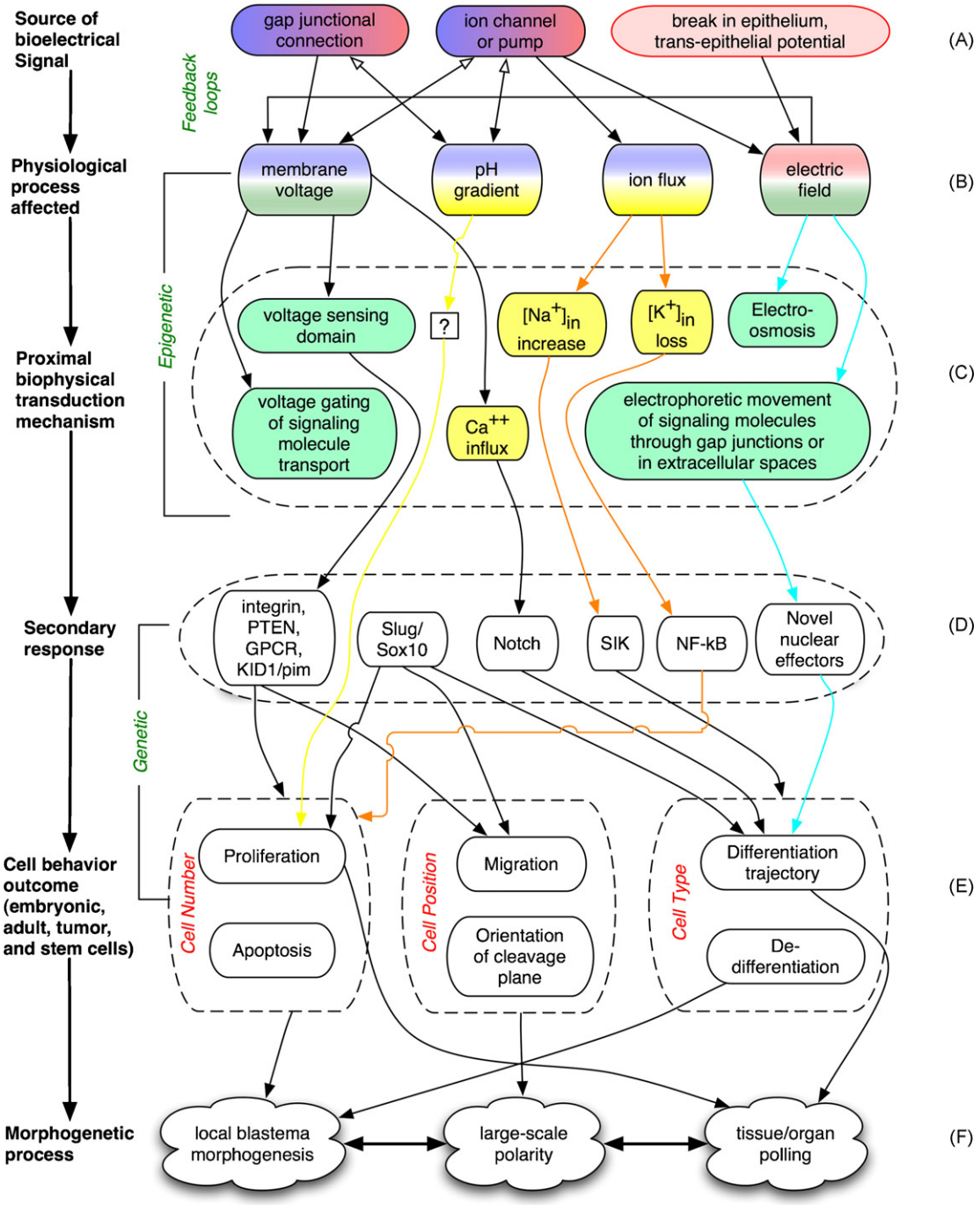

Fig. 2. Integration of bioelectric signals with canonical pathways. (A) Expression of ion channels or pumps, gap junctional connections, or epithelial damage all give rise to bioelectric signals. (B) These signals manifest as changes in transmembrane potential, pH gradients, specific ion flows, or electric fields. In the first two rows, pink shading indicates non-cell-autonomous signals while purple indicates cell-autonomous cues. Some nodes are both. (C) These processes are transduced via a variety of proximal epigenetic mechanisms including voltage-sensing domains on proteins, electro-osmosis, gating of morphogen transporters, and movement of specific ions like calcium. Green indicates a true electrical effect, while yellow indicates a biochemical effect due to ion identity. (D) These processes feed into several known genetic signaling pathways, including NF-kB, Notch, PTEN, Slug/Sox10, and Integrins. (E) Downstream of these signaling molecules are changes in cell cycle, apoptosis, position, orientation, and differentiation. (F) The ultimate result of orchestrated changes in cell behavior are morphogenetic processes including patterning of blastemas and embryonic fields, polarity decisions on several scales, and polling of remote tissues that enable wounds to decide what already exists and what must be recreated. The arrows indicate sample cases where the whole pathway has been traced for bioelectrical control of patterning.

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 [41], 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.

6. Unique features of bioelectrical signaling processes

Bioelectric networks are essentially recursive. For example, changes in membrane voltage gradients affect the function of voltage-sensitive ion channels, which in turn alters membrane potentials further. Likewise, gap junctions shape electrical properties of cell groups and are themselves sensitive to changes in

transmembrane potential and pH. This offers very rich opportunities for biological systems to use ion flows to implement both positive and negative feedback mechanisms. The former, such as that created by the hydrogen/potassium exchanger regulation via potassium-sensitive NF-kB [199], can be used to amplify small physiological signals, while the latter, such as that created by depolarization-induced activation of the hyperpolarizing V-ATPase pump [200], can be used to ensure robustness of patterning against perturbations. For example, consistent left–right patterning is driven by bioelectric cues in early embryogenesis [46,47,201–203], despite the very significant differences in actual content among normal embryos [204] because the physiological networks can buffer against such genetic and environmental variability.

Bioelectrical signals span several orders of magnitude in scale and levels of organization, controlling the distribution of subcellular components [205,206] and the structures of epithelia, appendages, and entire embryos [166,169,207,208]. While the penetration of endogenous electrical fields into distant tissue is a function of the complex resistivity and thus often hard to quantify in practice, bioelectric events can exert influence far beyond the local microenvironment. For example, in left–right patterning, a pump-driven battery in ventral cells appears to distribute small-molecule morphogens across the entire early frog embryo through long-range gap junction paths under an electrophoretic force [181,209]. Intriguingly, transplanted tumors can induce large-scale changes in voltage potentials detectable at considerable distances from the primary site [210].

Bioelectrical signals derive some of their behavior from the intrinsic properties governing electric fields, and are an epigenetic mechanism because physiological networks can regulate and generate order in the absence of changes in DNA, RNA, or protein expression. They are likely to be an evolutionarily ancient example of living systems capitalizing upon "order for free" [211], derived from basic physics which ensures that injury automatically provides cells with a vector cue indicating the position of the damage (Fig. 3). An interesting and important consequence of multi-scale control of bioelectrical signals is their ability to act as "master regulators": to activate coherent downstream morphogenetic cascades. It has already been shown in physiological experiments that localized interference with signals such as reversal of potential across the neural tube, or shunting specific currents at various anatomical sites, had broad and global effects on patterning [34,169,208].

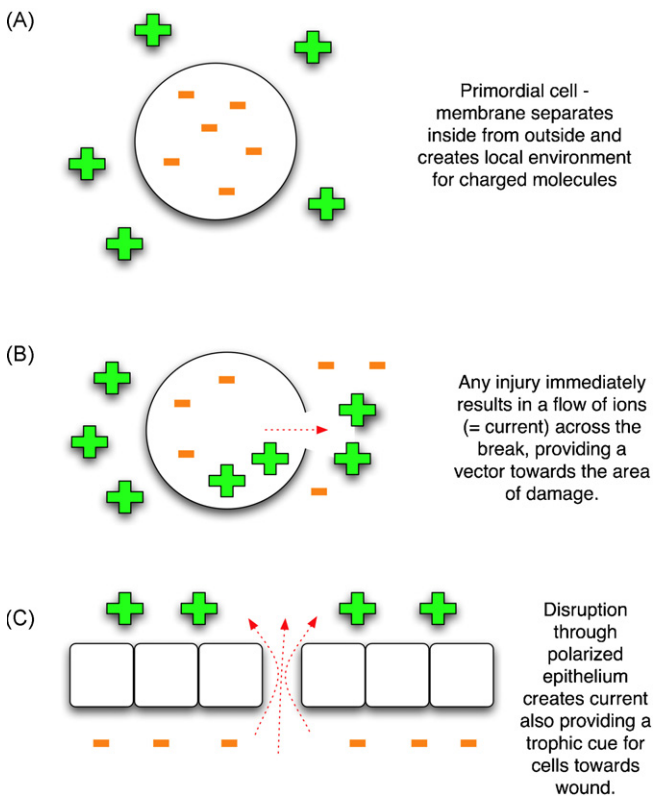

Fig. 3. Bioelectrical signals leverage the laws of physics into information for living systems. (A) In an early primordial cell, which has only a membrane separating inside from outside, a separation of charges will occur. When the membrane is damaged (B), the flow of ions that occurs (as the gradient tries to equalize across the break) provides a vector cue indicating the direction of damage to intracellular components. This occurs "for free"—it does not require the cell to have any specific machinery for this purpose. In more complex systems, an epithelium (C) maintains a transepithelial potential due to the segregation of charges by the component cells and their apical-basal polarization. When this is broken, nearby cells likewise experience electric fields which direct them towards the damage; this is especially useful for migratory cell types such as neoblasts and homing mesenchymal stem cells. Interestingly, this can be co-opted by normal developmental mechanisms; Borgens has proposed that a programmed tight-junctional breakdown in the flank results in "injury" currents that guide migratory cells to the right place during limb induction in embryonic development [166]. A final layer of complexity can be added to the passive fields that occur from breaks in epithelia by directing specific up-regulation of channels and pumps in wound cells (e.g., the V-ATPase in the regeneration bud of the amputated tadpole tail), thus shaping necessary fields further. These targeting cues, meant for the organism's own cells, could potentially be capitalized upon by galvanotactic fungi/bacteria and metastatic cells to identify areas of weak epithelialization that can be more easily attacked.

7. Implications for controlling regeneration

One of the key aspects of understanding signaling in morphogenesis is to ask what information is being carried by a given physiological process and what information capacity the signaling system has. For example, since membrane voltage is only a single parameter, it is likely that the true richness of bioelectrical signaling can only be fully appreciated by considering the microdomains of transporter activity distributed across the entire 2D surface of a cell or epithelium: these inhomogeneities comprise a field of potential values that, because of their spatial distribution, can encode enormous amounts of developmental information [212–214]. Although it was appreciated as early as 1983 [215] that individual cells can have more than a single transmembrane potential value, it is still largely unclear how adjacent domains maintain different voltage values and avoid equalizing short-circuits across the underlying cytoplasm. It is likely that we still have a very inadequate picture of all of the bioelectrical signals received and generated by cells *in vivo*.

With the advent of molecular tools, it is becoming easier to capitalize on this property for augmenting regeneration by providing specific signals. For example, in the case of tail regeneration, a single event – the continuous pumping of H^+ at the wound – induces the complete, normal regeneration of the tail. Its patterning and size are correct and its growth is appropriately halted when it catches up with the size of the tails of uncut controls. Two other illustrations are shown in Fig. 4. The ability of relatively simple bioelectrical signals to trigger orchestrated morphogenetic subroutines is a very desirable property for regenerative medicine applications: modulation of physical cues can leverage off the patterning capacity of the host's genetic programs without needing to micromanage the details of the regenerative process.

The implication of bioelectrical parameters in regulation suggests the idea of the physiological state space, proposed as a hypothesis for guiding future research in this field. Analyses have shown that generally, plastic, embryonic, stem, and tumor cells tend to be depolarized, whereas quiescent terminally differentiated somatic cells are hyperpolarized [128]. The use of membrane voltage to control cellular state is a powerful tool [49,216] but it is likely to be only a primitive approximation to the true richness of

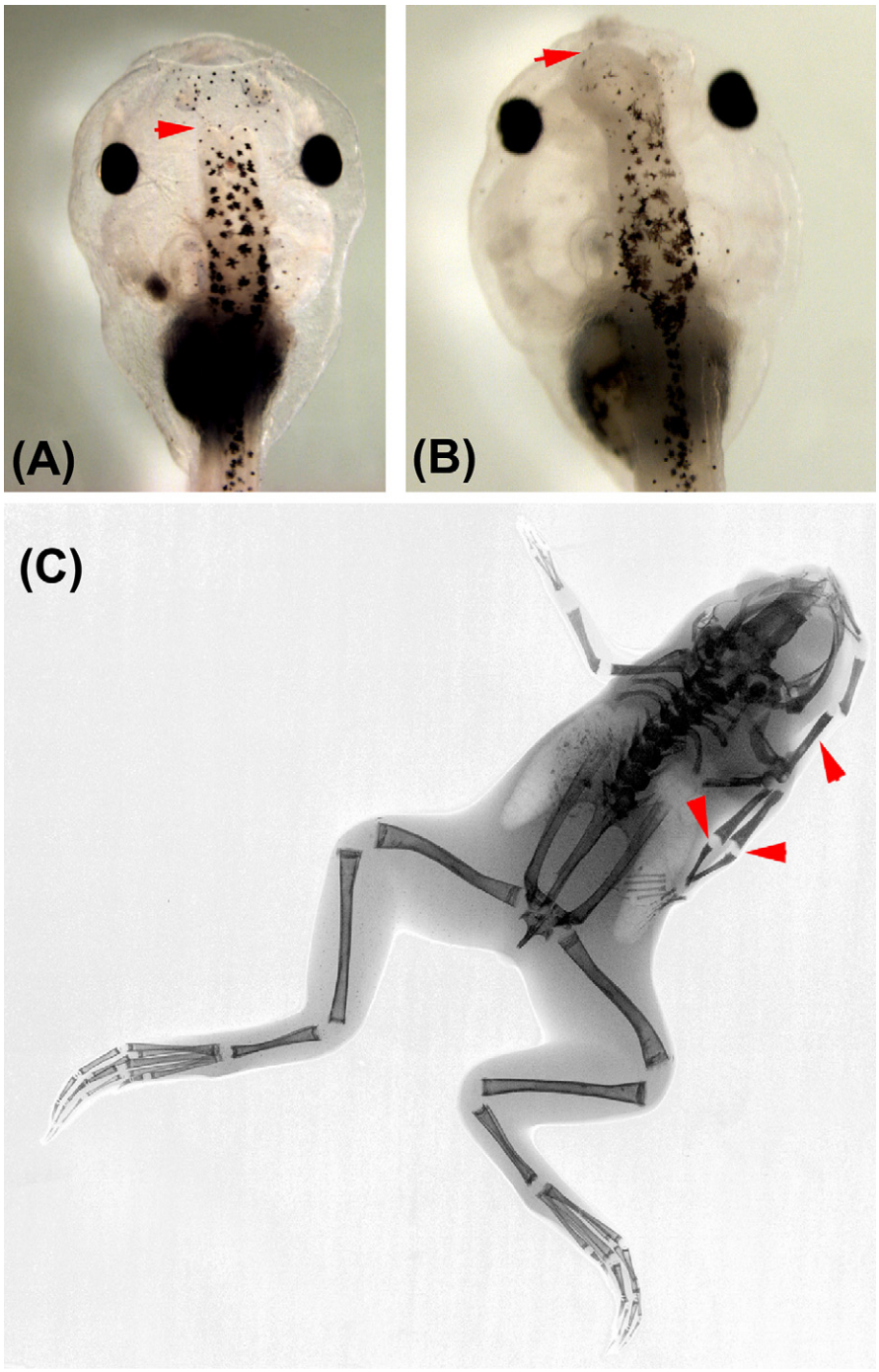

Fig. 4. Sample phenotypes arising from molecular-genetic modulation of bioelectrical cues in *Xenopus* laevis. Unpublished data from our lab showing that misexpression of ion channel constructs during embryogenesis can make coherent changes in pattern. Experiments performed with potassium channels by Sherry Aw result in the normal forebrain (A) being drastically increased (B); red arrow indicates anterior border of forebrain. Similarly, entire limbs can be induced (C), with X-ray imaging revealing the normal skeletal pattern in the ectopic limbs in this adult frog.

bioelectrical control. A more useful idea is that cells can be localized in a multi-dimensional physiological state space with a number of orthogonal dimensions indicating membrane voltage, intracellular pH, K^+ content, nuclear potential, Cl^- content, surface charge, etc.

One possibility is that cells can be grouped in distinct regions of this state space corresponding to stem cells, tumor cells, somatic cells, and other types of cells that are of interest to regenerative biology (Fig. 5). This hypothesis implies that in order to make rational changes in cell behavior, (1) data needs to be obtained on multiple cell types from different organs and disease conditions and (2) strategies need to be developed that use pharmacological reagents targeting natively expressed channels/pumps, and misexpression of well-characterized channel/pump constructs, to move cell states into desired regions (e.g., some cell may need to be depolarized by 30 mV and its internal pH acidified in order to induce proliferation). We are currently using quantitative modeling to expand the XYZTG (3D position, time, and gene expression) space [217] to include the systems biology of bioelectrical properties. The end result of the synthesis of experimental and modeling efforts should be the development of targeted channel/pump modulation strategies to achieve desired bioelectrical states of wound tissues for augmentation of regeneration.

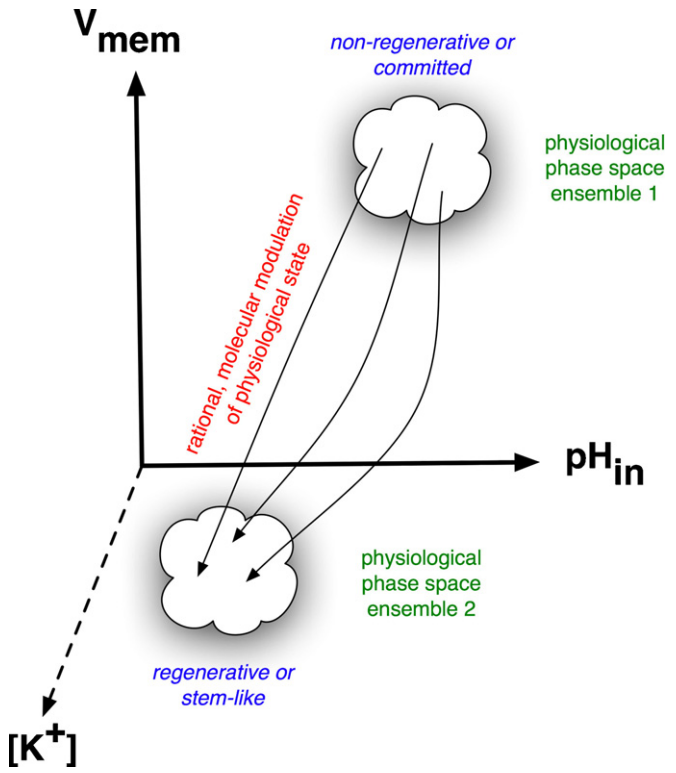

Fig. 5. Bioelectric state space. Cells live in a state space with a number of orthogonal axes corresponding to physiological properties. Here are shown only 3 (membrane voltage, V_{mem}, internal pH, and K^+ content). A more detailed dataset will contain additional semi-independent metrics such as the content of other ions, nuclear potential, surface charge, etc. One hypothesis is that cell types (e.g., stem cells, cancer cells, non-proliferative cells) will be seen to cluster in different regions of this space. If true, this will be not only a useful diagnostic framework but can also, when coupled with quantitative data and mathematical modeling, be used for rational modulation of cell behavior. Using well-characterized transporters in gene therapy, and pharmacological reagents targeting endogenous transporters in damaged tissue, bioelectrical properties can be specifically changed to move wound cells from a non-proliferative state towards a more plastic, regenerative condition.

One last key aspect of bioelectric signals (Fig. 6) is due to the fact that the same physiological state can be achieved by the function of many different sets of transporters; at the same time, regulatory (e.g., gating) events can result in the same ion transporter functioning very differently in different cells. This disconnect between molecular-genetic profile of cells and bioelectric state is very important: it cannot be assumed that cells expressing the same set of channels and pumps are in the same physiological state. Similarly, comparison of cell types based on microarray or differential expression analysis can be misleading with respect to bioelectric properties. Indeed, knockout of individual channel/pump genes can fail to reveal important aspects of ionic controls because many different transporters can compensate, masking phenotypes. This complexity has a benefit however. For example, in the tadpole, a yeast H^+ pump (which does not occur in vertebrates) was used to induce regeneration [48]. It appears that biomedical applications could potentially use any convenient channel or pump to achieve the desired change in cell physiology.

8. State-of-the-art tools for research in bioelectric signaling

A variety of new reagents and methodologies have been developed for molecular analysis of bioelectric signals in regenerative contexts [218]. Tools for the characterization of bioelectrical events now include highly sensitive ion-selective extracellular electrode probes [219,220], fluorescent reporter dyes, which enable the non-invasive real-time monitoring of pH, membrane voltage, and ion flow in any optically accessible tissue [221–224] (although much opportunity remains for the development of specific, bright, ratiometric dyes that localize exclusively to the desired subcellular locale), and nano-scale voltage reporters [225]. Especially exciting will be the use of multiple physiological dyes in FACS experiments to identify subpopulations of stem and other cell sets that differ in key bioelectric properties, as has been observed for HUVEC cells [226]. Importantly, such experiments on dissociated cells will clearly highlight properties that are cell-autonomous vs. those physiological conditions that can only be maintained as a group phenomenon.

To determine whether ion flow is a causal factor in a particular assay, and to inexpensively and rapidly implicate specific ion transporter proteins for further molecular validation, an inverse drug screen can be performed [227]. This is a chemical genetics approach that capitalizes on a tiered (least-specific → more specific) tree-based distribution of blocker compounds that enables an efficient binary-search for likely candidates. This is most often used to probe endogenous bioelectrical mechanisms and has resulted in the identification of channels and pumps as novel components of left–right patterning [228], anterior–posterior polarity [44], and stem cell regulation [43,45,49].

It is now possible to use molecular-genetic reagents in gain- and loss-of-function approaches to specifically modulate different

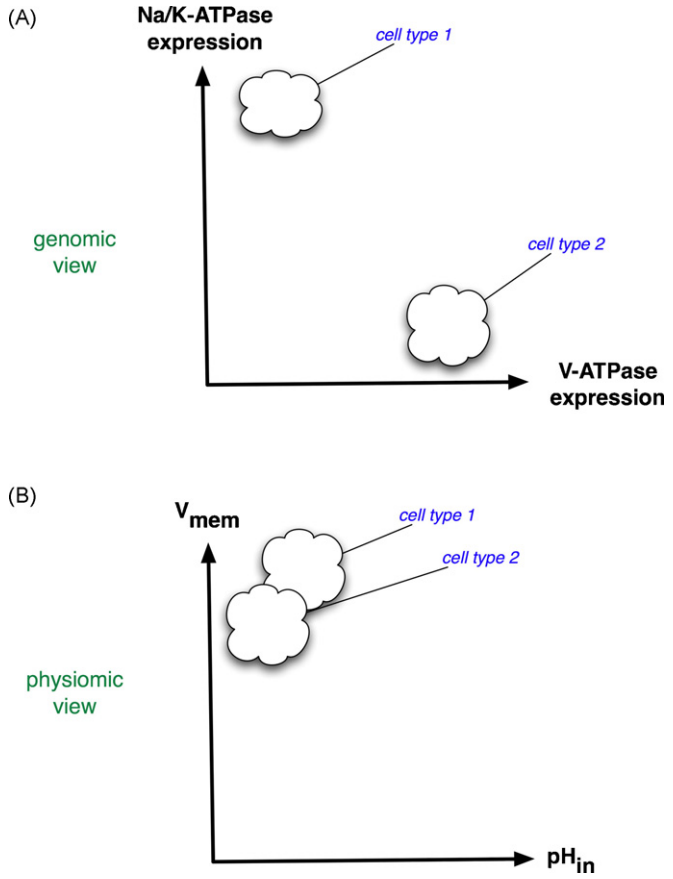

Fig. 6. Missing the physiological forest for the mRNA/protein expression trees. Analysis of gene or protein expression can often be very misleading with respect to physiological state. (A) Two hypothetical cell types have very different expression patterns. A microarray or differential analysis characterizes them as different, since one cell has low expression of Na,K-ATPase and high expression of V-ATPase, while the other cell is the opposite. However (B), analysis of pH and membrane potential may reveal that the cells actually have a similar proliferative potential because both pumps are hyperpolarizing (although genetically distinct), and the cells may in fact be similar from the point of view of bioelectrical controls.

aspects of ion flux [49], controlling corneal healing [41], inducing tail regeneration [48] at non-regenerative stages, and drastically altering the positioning and proliferation of neural crest cells [49]. 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 molecular tools for rationally altering the electrical activity of cells and tissues. Morpholino knock-down and mutant/constitutively active channel and pump construct misexpression are much finer-scale 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.

Indeed, analysis of the patterning phenotypes induced by such constructs can be used to dissect the mechanism of action, by distinguishing among different aspects of bioelectrical signals. For example, misexpression of electroneutral transporters can differentiate between the importance of voltage changes vs. that of flux of specific ions. Pore mutants can distinguish between ion conductance roles vs. possible functions of channels/pumps as scaffolds or binding partners (non-electrical signaling); for example, in the Na^+/H^+ exchanger, both ion-dependent and ion-independent functions control cell directionality and Golgi apparatus localization to wound edge [182]. Gating channel mutants and pumps with altered kinetics can, respectively, be used to reveal upstream signals controlling the bioelectric events, and the temporal properties of the signal. Heterologous transporters, combined with blockade of endogenous channels or pumps, can be used in elegant rescue experiments. Together, these tools can now be used to integrate bioelectrical signals with canonical downstream and upstream pathways, identifying transduction mechanisms leading from ion flow to patterning decisions.

9. Future prospects: what's next?

The field faces a number of major questions. One of the biggest issues is lack of sufficient quantitative data. Many measurements of pH, voltage, and ion content are needed on interesting cell types and model systems to flesh out the physiological state space concept, and compile enough data to develop predictive, quantitative physiological models that encompass the feedback loops and synthesize molecular-genetic and bioelectric data [209,229,230]. Issues of information content remain a rich area for discovery (what specific messages are encoded for cells by specific kinetics of individual ion fluxes, discrete ranges of transmembrane and transepithelial voltage, and distinct regions of different potential throughout the membrane of a single cell?). Oscillations in membrane voltage on a scale much slower than action potentials [231–237] are likely to carry important information and must be incorporated into pathway models. Voltage gradients across nuclear and organelle membranes [238] are only beginning to be measured, and their importance for cell function is not yet fully understood [194].

Importantly, even without all of the answers to these many fascinating issues, the existing data provide opportunities for modulation of regeneration. For example, it has been shown that Kv1.3 (KCNA3) and Kv3.1 (KCNC1) blockade increases neural progenitor cell proliferation [239]; likewise, induction of H^+ flux induces regeneration of a complex appendage [48] and blockade of gap junction-mediated signals results in the formation of a complete, properly patterned head in a planarian tail blastema [44]. These techniques can already be integrated into efforts to augment regeneration. The recent development of light-gated ion transporters has been particularly exciting; while these have so far been mainly used for neurobiological studies [240–242], they offer the potential of high-resolution spatio-temporal control of bioelectrical changes in cells during regeneration and development.

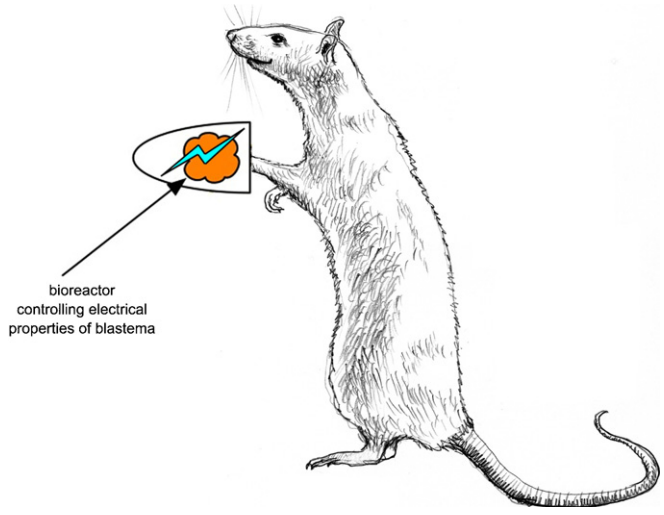

Fig. 7. A schematic of the regeneration sleeve: application to limb regeneration. Once sufficient quantitative data are available about the specific bioelectric states that promote regeneration, it will be necessary to develop sophisticated bioreactors, such as that pictured on the limb amputation wound in the rat model. These bioreactors will use microfluidics and light delivery to control, using pharmacological, genetic, and optical means the physiological properties of the wound. This is one vision of how information on bioelectrical controls of cell behavior can be transitioned into applications in regenerative biomedicine.

Three specific directions are being pursued in our group to provide additional opportunities for the field. One is the generation of mutant model species (e.g., *Xenopus*) expressing fluorescent proteins that report pH [243] or voltage [244], which will greatly augment the ability to study bioelectric properties of cells and tissues in a multitude of regenerative and disease states, or under molecular or pharmacological modulation. Another is the generation of mutants ubiquitously expressing light-gated ion transporters [245–247], which will allow unprecedented spatio-temporal control over bioelectric states in any tissue/organ of interest. Finally, in collaboration with bioengineers, we are working on the construction of regenerative sleeves (Fig. 7)—bioreactors to be applied to wounds (e.g., stump amputations) in which the physiological state of wound cells can be precisely controlled by pharmacological, optical, electrical, and genetic means to trigger regeneration and control patterning.

The widely conserved, multi-scale, instructive capacity of bioelectric events, coupled with their ability to induce complex downstream patterning cascades, make ion flow an extremely powerful control modality. Recent discoveries have shed light on the genetic response elements that are activated by ionic signals. The development of specific strategies for modulation of physiological state (whether through gene therapy with controllable transporters or by targeting endogenously expressed channels), in combination with efforts focused on biochemical factors, is sure to open exciting new vistas in regenerative medicine.

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