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Role of Membrane Potential in the Regulation of Cell Proliferation and Differentiation

Sarah Sundelacruz · Michael Levin · David L. Kaplan

Abstract Biophysical signaling, an integral regulator of long-term cell behavior in both excitable and non-excitable cell types, offers enormous potential for modulation of important cell functions. Of particular interest to current regenerative medicine efforts, we review several examples that support the functional role of transmembrane potential (V_{mem}) in the regulation of proliferation and differentiation. Interestingly, distinct V_{mem} controls are found in many cancer cell and precursor cell systems, which are known for their proliferative and differentiation capacities, respectively. Collectively, the data demonstrate that bioelectric properties can serve as markers for cell characterization and can control cell mitotic activity, cell cycle progression, and differentiation. The ability to control cell functions by modulating bioelectric properties such as V_{mem} would be an invaluable tool for directing stem cell behavior toward therapeutic goals. Biophysical properties of stem cells have only recently begun to be studied and are thus in need of further characterization. Understanding the molecular and mechanistic basis of biophysical regulation will point the way toward novel ways to rationally direct cell functions, allowing us to capitalize upon the potential of biophysical signaling for regenerative medicine and tissue engineering.

Keywords Biophysical signaling · Electrophysiology · Membrane potential · Proliferation · Differentiation · Stem cells

Introduction

It has long been known that in addition to the chemical determinants exchanged by cells during growth and development, bioelectrical signals represent a rich and interesting system for intracellular communication and cellular control [1–3]. These signals function also in the process of regeneration [4–6], a cornerstone aspect of modern biomedicine. This field is enjoying a resurgence [7, 8], as the powerful techniques of molecular physiology are being merged with developmental biology and biophysics to reveal novel mechanisms by which bioelectricity controls morphogenesis and can be harnessed to control it [9, 10]. In parallel with the growing importance of stem cell biology in cancer, in addition to the fields of embryogenesis and regeneration, a variety of channelopathies have drawn attention to the role of specific ion transport in neoplasm [11–13]. Here, we review exciting data implicating bioelectrical signals in the control of stem cell behavior, focusing on transmembrane voltage as a cell-autonomous signal (as distinct from exogenous electric fields).

Transmembrane potential (V_{mem}) refers to the voltage difference across a cell's bilayer membrane that is established by the balance of intracellular and extracellular ionic concentrations. Such a balance is maintained via passive and active ion transport through various ion channels and transporters located within the membrane. According to traditional membrane potential theory, the resting membrane potential of a cell is achieved when the electrochemical forces driving ion movement are equalized and ionic

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S. Sundelacruz · D. L. Kaplan (✉)
Department of Biomedical Engineering, Tufts University,
4 Colby St.,
Medford, MA 02155, USA
e-mail: david.kaplan@tufts.edu

M. Levin
Department of Biology, Tufts University,
200 Boston Ave.,
Boston, MA 02155, USA

equilibrium is maintained. Although maintenance of ionic homeostasis is a critical feature of cell viability and metabolism [14, 15], surprising specificity has been uncovered in the relationship between changes in V_{mem} levels and alteration of cell function. Furthermore, increasing evidence has pointed toward not only a correlation, but a functional relationship between V_{mem} and cell functions such as proliferation and differentiation. This relationship can be seen in many cell types, several of which will be reviewed here. That this biophysical relationship is conserved in a wide range of cell types (precursor and mature cells; proliferative and quiescent cells; normal and cancerous cells) suggests that V_{mem} regulation is a fundamental control mechanism. Better characterization of V_{mem}-regulating and V_{mem}-regulated pathways will uncover novel ways to control cell behavior. Such knowledge may significantly advance regenerative medicine applications, including stem cell-related tissue engineering efforts, where the potential of bioelectrical regulation is largely unexplored.

Membrane Potential Measurements

Several techniques are currently used to measure V_{mem}. They fall in two main categories: electrophysiological recordings and dye imaging.

Traditionally, electrophysiological recordings are obtained either by intracellular “sharp” microelectrode recording or by patch clamping. To obtain intracellular recordings, a glass microelectrode impales a cell to make direct contact with the cytoplasm, while another electrode is immersed in the bath solution surrounding the cell [16, 17]. The potential difference between the bath electrode and the penetrating electrode is the V_{mem}. Sharp microelectrode tips are small in diameter, on the order of tens of nanometers, to minimize damage to the membrane during insertion [16, 18].

In patch clamping, a patch electrode is brought in contact with the cell membrane but does not penetrate the membrane. Instead, the electrode is positioned against the membrane, allowing the glass to form a tight seal (gigaseal) with the membrane. There are several modes of patch clamping; however, only current clamping in the whole-cell configuration allows for direct membrane potential measurements [18]. In the whole-cell configuration, the patch of membrane sealed by the electrode is ruptured by a suction pulse or a large current pulse. Like intracellular recordings, the patch electrode is electrically connected to the cytoplasm of the cell, and when no current is injected, the endogenous V_{mem} can be recorded relative to a reference electrode in the bath solution. The patch electrode has a larger tip than a sharp microelectrode, has less resistance to allow for current injection, and is typically filled with a cytoplasm-like solution to measure endogenous membrane potential [18].

While both electrophysiological techniques have been widely and successfully used to record membrane potential, there are several inherent limitations of the electrophysiological recording setup. Most systems are designed to record from only single cells at a time and are therefore laborious and low-throughput [17, 19, 20]. Single-cell recordings are also unable to provide information about the spatial dynamics of V_{mem} change in a cell population and are unable to reflect the degree to which electrical changes in one cell affects neighboring cells [19]. As with spatial resolution in a multicellular system, spatial resolution across the surface of a single cell also cannot be resolved with these techniques [20]. Electrophysiological methods also generally do not provide information about long-term temporal changes in V_{mem}, since recordings are typically conducted over only minutes or hours. Some of these limitations have begun to be addressed with microchip-based patch clamping. For example, several chip-based devices use a planar patch clamp approach, where microchips are fabricated with apertures that serve as inverted patch electrode tips, allowing parallel processing of many cell recordings simultaneously [21].

Another approach to membrane potential measurements is the use of voltage-sensitive fluorescent dyes. Several of these dyes are thought to operate by an electrochromic effect, where the dye spectra are altered due to the coupling of molecular electronic states with the electric field present in the membrane, or an electrophoretic effect, where distribution of the dye across the membrane is voltage-sensitive [20, 22, 23]. These dyes typically respond to membrane potential with sensitivities of 10% per 100 mV [20, 22]. In addition to changes in fluorescence, second harmonic generation signals from some dyes also exhibit voltage sensitivities of up to 43% [22]. Advantages of optical detection of V_{mem} changes include ease of use, simultaneous monitoring of many cells over many different regions, and the ability to resolve spatial differences over the surface of a single cell [17, 19, 20]. Voltage-sensitive dyes also facilitate V_{mem} measurements in small cells or structures (such as the thin dendritic processes of neurons) that are traditionally difficult to impale or patch with electrodes [17]. One major disadvantage to optical methods, however, is the difficulty of dye calibration, and thus the difficulty of obtaining absolute values for membrane potential [17]. Most data are reported as percentage changes in fluorescence over a basal fluorescence value and are sometimes converted into an estimated membrane potential value based on reported dye sensitivities [17]. Ratiometric imaging using fluorescence resonance energy transfer (FRET) between a mobile voltage-sensing dye and a membrane-bound fluorophore can improve voltage sensitivity, reduce experimental error, and provide information about the magnitude of the voltage change [19].

Proliferation

It has long been observed that V_{mem} levels are tightly correlated with cell proliferation-related events such as mitosis, DNA synthesis, and overall cell cycle progression. Resting potentials of various cell types fall within a wide range (generally −10 mV to −90 mV), and cells' positions along such a V_{mem} scale generally correspond to their proliferative potential [24]. Somatic cells that have a high degree of polarization (a hyperpolarized V_{mem}) tend to be quiescent and do not typically undergo mitosis. Conversely, developing cells and cancerous cells tend to have a smaller degree of polarization (a depolarized V_{mem}) and are mitotically active [24, 25]. In addition, cells transferred to *in vitro* culture from an *in vivo* environment tend to undergo spontaneous proliferation, which is accompanied by V_{mem} depolarization [25]. Similarly, proliferation induced by malignant transformation of somatic cells is also accompanied by depolarization [25].

Cone (1971) theorized that this correlation is indicative of a functional relationship between V_{mem} and mitotic level: transmembrane potential in non-proliferative cells could act as an inhibitory signal for mitosis (or the preparative events associated with mitosis), which, upon stimulation, could be reversibly altered to a level that is permissive for proliferation [25]. In cell cycle progression, a plausible scenario is that a highly polarized V_{mem} level blocks quiescent somatic cells residing in the G1 phase of the cell cycle from entering the S phase of DNA synthesis, thus inhibiting mitosis [25]. From the observation that most non-proliferative cells have relatively hyperpolarized (more negative) V_{mem}, while proliferative and cancerous cells have relatively depolarized (less negative) V_{mem}, Binggeli and Weinstein (1986) further hypothesized that there may be a boundary V_{mem} level that serves as a threshold or trigger for DNA synthesis [24].

Several studies have confirmed that V_{mem} modulation can stimulate or inhibit proliferation in a predictable way. Cone and Tongier (1973) investigated the effects of different V_{mem} levels on mitotic activity of Chinese hamster ovary cells [26]. V_{mem} levels were varied by changing the ionic composition of the medium to simulate a range of V_{mem} normally seen *in vivo* (−10 mV to −90 mV). Complete mitotic arrest was achieved by hyperpolarizing the V_{mem} to −75 mV but could be reversed by returning to a normal V_{mem} of −10 mV [26].

Since these initial studies, ionic regulation of cellular behavior has been increasingly studied and has been found to play a critical role in proliferation. It has become clear that the relationship between V_{mem} and proliferation is not a simple one. Since V_{mem} is a parameter that reflects the cumulative activity of many ion channels and currents, V_{mem}-induced cell behavior could be the result of one or many ion-related events. In dissecting control pathways, it is important to determine whether downstream events are controlled by the pure voltage, or by the flow (or concentration) of individual ions [27].

Cell proliferation is a multi-step event regulated by a system of checkpoints at different phases of the cell cycle. Such complexity has been addressed in more recent work on the role of V_{mem} in proliferation, resulting in a better understanding of the major ion channels and currents involved, as well as stage-specific regulation of the cell cycle. Many of these studies have implicated K^+ currents as protagonists of proliferation and cell cycle progression [28, 29]. Correlations between K^+ channel inhibition and inhibition of proliferation have been shown in a variety of cell types, including lymphocytes, peripheral blood mononuclear cells (PBMCs), lymphoma, brown fat, melanoma, breast cancer, Schwann cells, astrocytes, oligodendrocytes, neuroblastoma, lung cancer, bladder cancer, and melanoma (reviewed in [28, 30]). Several model systems will be reviewed below. In most systems, K^+ flux changes resulting in depolarization favor proliferation, although there are cases where depolarization inhibits proliferation.

Activation of Proliferation

To understand endogenous regulation of proliferation, it is particularly useful to study systems in which cells endogenously switch from quiescent to proliferative phenotypes, or *vice versa*, or systems in which proliferative activity can be switched on by well-characterized stimuli (e.g., in response to injury or in response to mitogen exposure). Particularly impressive is the initiation of mitosis in normally post-mitotic cells, such as in the CNS; although the molecular details remain to be worked out, 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 [31–33].

Astrocyte cells display such behavior and have consequently been well studied. In several models of astrocyte injury (scarring of confluent spinal cord astrocytes; cortical freeze-lesions in rat brain), only astrocytes with relatively depolarized resting V_{mem} and lacking functional inward rectifier K^+ (Kir) channels displayed active proliferation in response to injury [34, 35]. In astrocytes from developing rat spinal cord, hyperpolarization of the resting membrane potential (approximately −50 mV to −80 mV) was accompanied by decreased cell proliferation and expression of Kir channels [36, 37]. Conversely, depolarization of quiescent astrocytes with ouabain or extracellular K^+ increased proliferation and DNA synthesis [28]. The correlation between ionic activity and proliferation was examined in further detail by studying the effects of cell cycle arrest on ion channel currents and the effects of exogenous current inhibition on cell cycle progression. In proliferating astrocytes, cell arrest in G_1/G_0 induced premature up-regulation of an inwardly rectifying K^+ current (IK_{IR}), resulting in a relatively hyperpolarized phenotype, while arrest in S phase induced downregulation of IK_{IR} with a concomitant increase in a delayed outwardly rectifying current (IK_{DR}), resulting in a relatively depolarized phenotype [28]. Pharmacological inhibition of IK_{DR} in normally proliferating astrocytes resulted in G_0/G_1 arrest, while inhibition of IK_{IR} in quiescent astrocytes resulted in increased proliferation and DNA synthesis [28]. These data imply that there is a G_1/S transition checkpoint where increased IK_{DR} and decreased IK_{IR} currents and the corresponding changes in V_{mem} are prerequisites for cell cycle progression.

Vascular smooth muscle cells (VSMCs) retain much plasticity even in the adult, and can undergo significant changes in phenotype (phenotypic switching, or modulation) in response to environmental stimuli. During vascular development and in response to vascular injury, VSMC modulation is characterized by a loss of contractile phenotype accompanied by an increase in proliferative and migratory ability [38]. One feature of modulation is a significant change in ion transport mechanisms between contractile and proliferative phenotypes [39]. Contractile VSMCs express an abundance of large-conductance calcium-activated K^+ channels (BK_{Ca}, also called MaxiK, $K_{Ca}1.1$), which modulate Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels ($Ca_V1.2$), which are also highly expressed. However, during VSMC modulation, these ion channels are downregulated [40, 41], while an intermediate-conductance calcium-activated K^+ channel (IK_{Ca}, also called $K_{Ca}3.1$) is activated [42]. BK_{Ca} is activated by depolarization, while IK_{Ca} is not depolarization-dependent and is thus able to open at more hyperpolarized V_{mem}, driving Ca^{2+} entry through voltage-independent, and possibly lipid-sensing, channels [39]. Proliferation-inducing switch from BK_{Ca} to IK_{Ca} may therefore hint at different voltage sensitivities driving Ca^{2+} transport during the two cell states.

Activation of quiescent human T lymphocytes and PBMCs by mitogens also involves ionic regulation of cell cycle progression. Upon activation by the mitogen phytohemagluttinin, lymphocytes undergo a transition from G_0 to G_1 and express the cytokine interleukin-2, which further stimulates a transition from G_1 to S [30]. T lymphocyte and PBMC proliferation can be blocked with peptide toxins with high affinity to K^+ channels [43–45], suggesting that mitogen-stimulated proliferation is mediated by K^+ channel activity. Similarly, activation of murine B lymphocytes and murine noncytolytic T lymphocytes can be blocked by K^+ channel inhibitors, which inhibit their progression through the G_1 phase of the cell cycle [46, 47]. Molecular studies targeting K^+ channels have implicated particularly the voltage-gated K^+ channel Kv1.3 in G_1 progression and lymphocyte activation. Ca^{2+} signaling, which is required for activation, is modulated by the activity of Kv1.3 and IK_{Ca} channels, which together regulate resting V_{mem} levels and thereby modulate Ca^{2+} entry [48]. The relative abundance of these channels changes during lymphocyte activation, and may account for the changes in Ca^{2+} signaling [49–51]. In quiescent cells, Kv1.3 expression is greater than IK_{Ca} expression and is therefore thought to control V_{mem}. However, upon mitogen stimulation, IK_{Ca} is upregulated and may play a greater role in modulating V_{mem} for further regulation of Ca^{2+} signaling [50, 52]. Thus, differential K^+ channel expression may be responsible for modulating V_{mem}, which regulates the Ca^{2+} signaling necessary for a downstream immune response [50].

Proliferation of Cancer Cells

Cancer cells, which show an abnormally high propensity to proliferate, are useful models in which to study ionic regulation, or mis-regulation, of proliferation and cell cycle progression [53, 54].

For example, MCF-7 human breast cancer cell proliferation has been shown to require a characteristic V_{mem} hyperpolarization during the G_0/G_1 phase transition [55, 56]. Hyperpolarization occurs via an ATP-sensitive, hyperpolarizing K^+ current, comprised of several K^+ currents including human ether à go-go (hEAG) and IK_{Ca} currents [57–61]. Inhibition of hEAG and IK_{Ca} channels induces membrane depolarization and a decrease in intracellular Ca^{2+}, resulting in early G_1 phase arrest [62]. K^+ channel inhibition also results in accumulation of cyclin-dependent kinase inhibitor p21, which is known to block the G_1/S transition [62]. A current model for cell cycle regulation by hyperpolarizing K^+ channels is that hEAG is activated during early G1, when V_{mem} is depolarized to about −20 mV. hEAG expression is then further upregulated during late G_1, causing V_{mem} hyperpolarization and increasing the driving force for Ca^{2+} entry. Ca^{2+} entry triggers activation of hIK_{Ca} channels, resulting in further hyperpolarization that drives the G_1/S transition [62].

A glioma cell model has also been used to demonstrate the functional importance of the inwardly-rectifying Kir4.1 channel in glial cell proliferation. Kir4.1 is not expressed in immature, proliferating glial cells [36, 63, 64], but is widely expressed in glial-differentiated astrocytes [28, 65], and its expression is associated with a hyperpolarized phenotype and an exit from the cell cycle [36, 37]. Functional Kir4.1 channels are also absent in glial-derived tumor cells, and the resulting depolarized phenotype has been suggested to contribute to uncontrolled glioma tumor growth [66]. When Kir4.1 channels were selectively overexpressed in astrocyte-derived gliomas, glioma cells exhibited a differentiated astrocyte-like phenotype, including membrane hyperpolarization and cell growth inhibition by a transition from the G_2/M to the G_0/G_1 phase of the cell cycle [66]. This study demonstrated that Kir4.1 expression was sufficient to induce cell maturation characterized by changes in V_{mem} (hyperpolarization) and proliferative capacity.

Proliferation of Precursor and Stem Cells and Proliferation in Regenerating Systems

V_{mem}-associated changes have also been shown to regulate proliferation in precursor cells, stem cells, and regenerating systems. In neural precursor cells (NPCs) isolated from neurospheres derived from adult mice, IK_{IR} and IK_{DR} channels were responsible for establishing a hyperpolarized resting V_{mem} of approximately −80 mV [67]. Depolarization by extracellular Ba^{2+} or K^+ accelerated mitosis in NPCs, resulting in an increase in cell number and neurosphere size. It is hypothesized that V_{mem} depolarization via modulated Kir channel activity is responsible for NPC proliferation and cell cycle progression [67]. Interestingly, the effect was maximal at 100 μM but declined at 1 mM. This biphasic effect reveals the presence of an optimal membrane potential range—a window effect which will have to be taken into account when designing modulation techniques for biomedical applications.

In human (hESCs) and mouse (mESCs) embryonic stem cells, IK_{DR} currents are present and are permissive for proliferation, as application of K^+ channel blockers inhibited DNA synthesis [68]. In *Xenopus* embryos, the K^+ channel KCNQ1 (also called Kv7.1) contributes significantly to the membrane potential [69]. When its regulatory subunit KCNE1 (also called minK, Isk) was misexpressed in the embryo, KCNQ1 currents were suppressed, resulting in V_{mem} depolarization and ectopic induction of the neural crest regulator genes *Sox10* and *Slug*. Consistent with these two genes' known roles in neoplastic progression [70–73], reduction of KCNQ1-dependent currents induced overproliferation of melanocytes and conferred upon them a highly invasive, migratory phenotype resembling metastasis [69]. Thus, a functional role was identified for the channel KCNQ1, whose V_{mem}-controlling activity regulates the mitotic and invasive activity of the melanocyte neural crest lineage through known signaling pathways. A functional role was also found for the vacuolar ATPase H^+ pump (V-ATPase) in *Xenopus* tadpole tail regeneration, which requires the H^+ pumping activity of endogenously expressed V-ATPases [74]. Loss of V-ATPase function, and the resulting depolarization in the tail bud region, decreased the number of proliferating cells in the bud and abolished regeneration. Conversely, expression of a heterologous H^+ pump repolarized the bud and induced a significant degree of regeneration in normally non-regenerative conditions [74]. These studies demonstrate the importance of V_{mem} modulation by specific molecular species in regulating cell growth during embryogenesis and regeneration.

Differentiation

Regulation of proliferation and cell cycle progression is closely associated with differentiation, since cells must coordinate their exit from the cell cycle with the initiation of their differentiation programs [75]. Thus, since V_{mem} regulates proliferation in many cell types, V_{mem}-related signals may also act as triggers for differentiation. A thorough understanding of these signals would be an invaluable resource for characterizing and controlling cell development and maturation in many systems. Two key questions must be answered in order to extract therapeutically relevant information about V_{mem} in differentiation: (1) what are the electrophysiological differences between the differentiated and undifferentiated states, and (2) are these differences instructive for differentiation? Following the identification of functionally significant changes in bioelectric state during differentiation, we may be able to manipulate the parameters so as to control differentiation outcomes for therapeutic applications.

Electrophysiological Changes During Cell Development and Differentiation

Currently, most work in this area has focused on comparing the electrophysiological profiles of differentiated cells and undifferentiated cells. In addition to providing clues about potential control points for differentiation, these profiles can be used to better characterize the maturation of stem and progenitor cells, as well as to identify and distinguish between subpopulations that may not show other differentiating phenotypes. The majority of work has been done in neural and muscular systems, as the acquisition of electrophysiological features contributes to the excitability of the mature cell. During early stages of development, maturing neural crest (NC) cells express human ether à go-go related gene encoded K^+ currents (I_{HERG}) and IK_{DR} currents, while during later stages, NC cells exhibit V_{mem} hyperpolarization and expression of IK_{DR}, IK_{IR}, and Na^+

(I_{Na}) currents [76–79]. Based upon these data, it was suggested that the ordered expression of ion channels defines NC cell developmental stages [78]. Similarly, the NC-derived SY5Y neuroblastoma cell line exhibits specific electrophysiological profiles (relative ratios of I_{HERG}, I_{KDR}, I_{Na}) depending on differentiated state and specific subtype, N- or S-type [80]. N-type cells display an immature, nonexcitable neural phenotype and are characterized by I_{HERG} and IK_{DR} currents and a depolarized V_{mem}. Upon stimulation, they differentiate into excitable neural cells and express IK_{IR} [80]. S-type cells display negligible I_{HERG}, IK_{DR}, and I_{Na} currents, but upon differentiation along a smooth muscle pathway or a neural abortive pathway, display characteristic (and different) levels of these currents [80]. Characteristic changes in Na^+ and K^+ channel expression and ionic currents have also been found to accompany neural differentiation of other stem-like cell types, such as neural stem-like cells from human umbilical cord blood [81], immortalized human neural stem cells [82], and mESCs [83]. These studies suggest that electrophysiological profiles can be coupled with traditional immunocytochemical techniques to describe the maturation state of cells and to distinguish between different cell populations originating from common precursors.

Similarly, the electrophysiology of myocyte differentiation has also been characterized. Differentiation of mouse embryonic stem cells [84] and of embryonic carcinoma P19 cells [85] into cardiomyocytes correlates with upregulation of cardiac-related ion channels in specific temporal patterns. P19 cells express L-type Ca^{2+} and transient outward channels early during differentiation, and Na^+ and delayed and inward rectifier channels later during differentiation [85]. Skeletal myoblasts also exhibit different current and ion channel profiles in their proliferating and differentiating states. Murine C_2C_{12} myoblasts undergoing active proliferation express an ATP-induced K^+ current, a swelling-activated Cl^- current, and an IK_{Ca} current [86–88]. Upon initiation of differentiation, these currents are replaced with a tetrodotoxin-sensitive Na^+ current, an IK_{DR} current, an IK_{IR} current, and an L-type Ca^{2+} current [87–90]. In muscle satellite cell-derived human myoblasts, voltage-gated Na^+ and Ca^{2+}-activated K^+ channels are expressed during proliferation [91], while hEAG, IK_{DR}, IK_{IR}, T-type Ca^{2+}, and L-type Ca^{2+} channels are expressed in differentiated fusion-competent myoblasts [92–96].

Functional Role of V_{mem} Signaling During Differentiation

Beyond their role as markers of the maturation process, electophysiological changes play functional and instructive roles in the differentiation process. In such a role, they could provide more than just a passive readout of developmental stage or of linage commitment, but could actively contribute to transcriptional and other activity leading to expression of a differentiated phenotype. Observations made over 30 years ago showed that neural differentiation depended on the function of specific ion transporters such as the Na,K-ATPase [97, 98]. By uncovering the molecular and mechanistic basis of the underlying signaling pathways, we may find novel ways to direct stem cell behavior by modulating V_{mem} and related ion channel expression.

Several recent studies have demonstrated that endogenous V_{mem} modulation does indeed have an instructive role during cell differentiation and maturation. For example, V_{mem} hyperpolarization not only precedes human myoblast differentiation, but is also required for differentiation, as myocyte fusion and transcription factor activity are blocked when hyperpolarization is blocked [96, 99]. It is the earliest detectable event in the differentiation process, and is therefore thought to be a trigger for myoblast differentiation [99]. Hyperpolarization, and thus differentiation, is thought to be initiated by tyrosine dephosphorylation of the Kir2.1 channel and results in Ca^{2+} influx through T channels, calcineurin (CaN) activation, and expression of two myocyte transcription factors, myogenin and myocyte enhancing factor 2 [93, 95, 99–101]. Similarly, expression of the chloride channel ClC-3 and its corresponding Cl^- current is required for fibroblast-to-myofibroblast differentiation [102], and expression and function of two inward K^+ rectifier channels is essential for the differentiation of human hematopoietic progenitor cells [103, 104]. Taken together, these results support a relationship between ion channel modulation and the intracellular signaling pathways involved in the differentiation process. That the endogenous hyperpolarization happens upstream of known conventional biochemical signaling events also hints at the possibility of using a single control point (e.g., V_{mem}, or Kir2.1 channel phosphorylation) to modulate the differentiation-related signaling pathways that diverge from that point.

Hyperpolarization also plays a role in the development and maturation of mammalian cerebellar granule cells. Developing granule cells hyperpolarize from −25 mV to −55 mV, and it is hypothesized that these V_{mem} changes alter Ca^{2+} signaling via CaN and Ca^{2+}-calmodulin-dependent protein kinase to control stage-specific gene expression [105, 106]. V_{mem}- and CaN-mediated changes in granule cell gene expression were found after treatment with depolarization agents and/or a CaN inhibitor FK506 [107]. Interestingly, ~80% of developmentally-relevant genes corresponded to depolarization-regulated genes, and the correlation was such that developmentally-upregulated genes were downregulated with depolarization, while developmentally-downregulated genes were upregulated with depolarization [107]. Furthermore, there was a large overlap and inverse relation seen between depolarization- and FK506-regulated genes [107]. These data suggest that endogenous regulation of V_{mem} level controls genes associated with maturation of granule cells, and that this regulation may be mediated by CaN.

More recently, we have shown a similar connection between V_{mem} and differentiation propensity in bone marrow-derived human mesenchymal stem cells (hMSCs). Similar to what was found for human myoblast and cerebellar granule cell differentiation, and also in line with Binggeli and Weinstein's hypothesis [24] about V_{mem} levels in developing vs. quiescent cells, hMSCs undergo hyperpolarization during both osteogenic (OS) and adipogenic (AD) differentiation [108]. More importantly, hyperpolarization was found to be necessary for differentiation. When normal V_{mem} progression was disrupted by depolarization with high K^+ or ouabain, OS and AD differentiation markers decreased significantly, suggesting suppression or delay of differentiation under depolarized V_{mem} conditions [108]. Conversely, during OS differentiation, treatment with hyperpolarizing agents pinacidil or diazoxide induced upregulation of bone-related gene expression [108]. These depolarizing and hyperpolarizing experiments demonstrate that hMSCs are sensitive to bidirectional changes in V_{mem} and provide compelling evidence for an instructive role of V_{mem} in differentiating hMSCs. The discovery that V_{mem} can regulate long-term cell behavior in a non-excitable cell type is exciting because it highlights the fact that ion flows are important for a broad range of cell functions, of which excitability is only a small part. Rational modulation of ion currents and ion channel expression may therefore be potential control mechanisms for a variety of cell signaling pathways. Possibilities include maintenance of a renewable stem cell population *in vitro* and acceleration or augmentation of stem cell differentiation for therapeutic purposes or for tissue engineering.

It should also be noted that bioelectric signals may function in de-differentiation, a process of considerable importance for understanding regeneration of some structures [109, 110]. This work [111–115] is largely pre-molecular, and it remains to be seen what ion transporters and mechanisms can be capitalized upon in order to de-differentiate somatic cells for biomedical applications.

Electrophysiological Characterization and Profiling of Stem Cells

Realizing the potential of bioelectric control for stem cell therapies and for general understanding of stem cell biology requires thorough characterization of ion channel and current expression during proliferation and differentiation of stem cells [83, 116–120]. Alongside transmembrane voltage gradient, cells' dielectric properties reveal surface charge, membrane conductivity, nucleic acid content, cell size, and presence and conductivity of internal membrane-bound vesicles; this can be used to distinguish stem cells and their differentiated progeny [121].

A number of studies have begun to profile ion current and channel expression in undifferentiated stem cells [80, 122]. For example, hMSCs derived from bone marrow express IK_{Ca}, IK_{DR}, transient outward K^+ currents, and slow-activating currents [123, 124]. They display high expression levels for several channel subunits, including Kv4.2, Kv4.3, MaxiK, α1c subunit of L-type Ca^{2+} channels, and hyperpolarization-activated cyclic nucleotide-gated ion channel isoform 2 [123]. Similar to bone marrow-derived stem cells, undifferentiated human adipose tissue-derived stem cells express IK_{DR} and IK_{Ca} currents [125]. Human and mouse ESCs exhibit IK_{DR} currents at different levels of homogeneity, and it is hypothesized that differential ion channel gene expression is responsible for current expression in the two cell types [68]. Rat embryonic neural stem cells can be characterized by a signature ion channel profile, including several Na^+, Ca^{2+}, and K^+ channels [126]. Bioelectrical components (e.g., Kir current) are differentially present in mesenchymal stem cells, and can perhaps be used to identify distinct sub-populations [127]. Human vascular endothelial cells consist of 3 discrete sub-populations based on their ion channel properties [128].

We believe that obtaining a full profile of stem cell bioelectric state will be a key step in understanding the major players in ionic regulation and will help identify potential targets for manipulation. Such characterization may also supplement current immunohistochemical techniques for identifying stem cell sub-populations, and the development of sensitive fluorescent dyes that report V_{mem}, pH, and individual ion content will allow cells in unique physiological states to be segregated non-invasively via FACS. Profiling of stem cell bioelectric state may prove to be a novel technique to identify stem cells that are difficult to phenotype by traditional methods (such as hMSCs, whose set of identifying markers is still unclear), or a technique to distinguish between a heterogenous stem cell population (again, such as an hMSC population, which is thought to be composed of cells with varying differentiation propensities) [129, 130].

Migration

The function of stem cells *in vivo* is often determined by their position within tissues and organs; this is crucial because microenvironment controls stem cell behaviors [131–133], and because targeting stem cells towards areas of injury is a key goal of regenerative medicine. Moreover, stem cells are now an increasingly promising vector for cancer therapies, because some types, such as neural stem cells, appear to preferentially target aggressive tumors such as gliomas [134, 135]. While the cues that guide stem cells' homing in mammals have mainly been studied from the perspective of chemical gradients and ECM molecules [136–140], there is a model in which physiological signals have begun to be investigated.

Planaria, flatworms with impressive regenerative abilities [141–143], possess a resident adult stem cell population. These neoblasts appear to migrate to wounds and recreate the necessary tissues [144, 145]. Importantly, this requires the stem cells to be informed as to where the damage occurred, what cell types are missing, what morphogenetic structures must be recreated by a combination of coordinated proliferation and differentiation, and when the target morphology is complete (regenerative stem cell activity can cease); the process involves molecules like PTEN [146], which are powerful regulators of stem cell activity in mammals. Recent data show that gap junctions, direct channels between adjacent cells that allow transfer of ions and small signaling molecules, are centrally involved in this process in planaria [147, 148] and many other systems [149]; gap junctional communication is an ideal way for stem cells to rapidly communicate with their niche. Gap junctions are both gated by V_{mem} [150, 151] and establish iso-potential cell fields (define physiological compartments) [152, 153]. Moreover, recent molecular data implicated KCNQ1 potassium channels [154] and NaV sodium channels [12, 155, 156] in the regulation of migration and invasiveness of several stem-like cell types. These findings implicate the cell-autonomous property V_{mem} in migration control, and complement the long-known ability of stem cells to migrate in physiological-strength electric gradients in their environment [157–159]. Thus, an investigation of voltage in the guidance of stem cell position during regeneration and morphostasis is a crucial area for future work.

Mechanisms: How is V_{mem} Transduced into Cellular Behaviors?

Since V_{mem} signals may be control points for guiding stem cell behavior in biomedical settings, it is necessary to identify the V_{mem}-sensing pathways that link bioelectric signals to cell behavior such as proliferation and differentiation. In order to mechanistically dissect bioelectrical signals, it is important to distinguish which aspect of ion flow bears the instructive signals for cell behavior: membrane potential change, long-range electric field, or flow of individual ions. In many cases, this can be very difficult to untangle. However, it has been accomplished in some developmental studies by using mutants of ion transporters that allow separation of individual biophysical events [160]. It is now possible to use molecular-genetic reagents in gain- and loss-of-function approaches to specifically modulate different aspects of ion flux [154], controlling corneal healing [10], inducing tail regeneration [9] at non-regenerative stages, and drastically altering the positioning and proliferation of neural crest cells [154]. 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 [161]. 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.

Table 1 Mechanisms for transduction of electrical signals

What happens	Mechanisms of action	References
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)	Membrane voltage potential	[195, 196]
Activation of integrin or other signals by conformational changes in membrane proteins	Membrane voltage potential	[185, 186, 197]
Depolarization-induced translocation of NRF-2 transcription factor	Membrane voltage potential	[198]
Alteration of PTEN enzyme activity	Membrane voltage potential	[10, 193, 199]
Redistribution of charged receptors along the cell surface	Electric field	[200–205]
Directional electrophoresis of morphogens through cytoplasmic spaces	Electric field	[206, 207]
Electroosmosis	Electric field	[208]
Direct changes of specific transcriptional elements, 2nd messenger systems like NF-kB, differentiation, and cell cycle	Ion-specific effects (K^+ and Cl^- fluxes and pH)	[102, 209–216]
Fixed charges around cell surface control neoplasm	Zeta potentials	[217–221]

The question of which aspect of ion flow is relevant in any instance of cell behavior is intimately tied to transduction mechanism: how does the cell (or a neighboring cell) know the membrane voltage has changed? Mechanisms that transduce electrical signal into second-messenger cascades [162] include those outlined in Table 1. While many questions remain, the molecular details of at least some such transduction pathways have recently been revealed (Fig. 1). One major candidate for V_{mem}-sensing mechanisms is Ca^{2+} signaling [163, 164]. Calcium signaling is crucially important to many cell behaviors, including proliferation [165–167], differentiation [168, 169], and galvanotaxis [170]. It also functions as a patterning signal in large-scale morphogenesis [171–175]. It is not possible to do justice here to the enormous literature on calcium signaling, and this has been well-reviewed elsewhere [176–179]. Most of these signaling events take place through specialized receptors such as calmodulin or calcineurin [180], since calcium signals largely by virtue of its unique chemical properties—it is not a true electrical signal. However, one area where Ca^{2+} signaling is integral to bioelectrical cues is in the transduction of membrane voltage to downstream cellular effector mechanisms. This often occurs through voltage-gated calcium channels [181–184], 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+}. Other V_{mem}-transducing mechanisms may include integrin-linked signaling involving hERG1 channels [185–189]; voltage-sensitive phosphatases operating through the phosphoinositide kinase pathway [190–193]; voltage-dependent changes in the

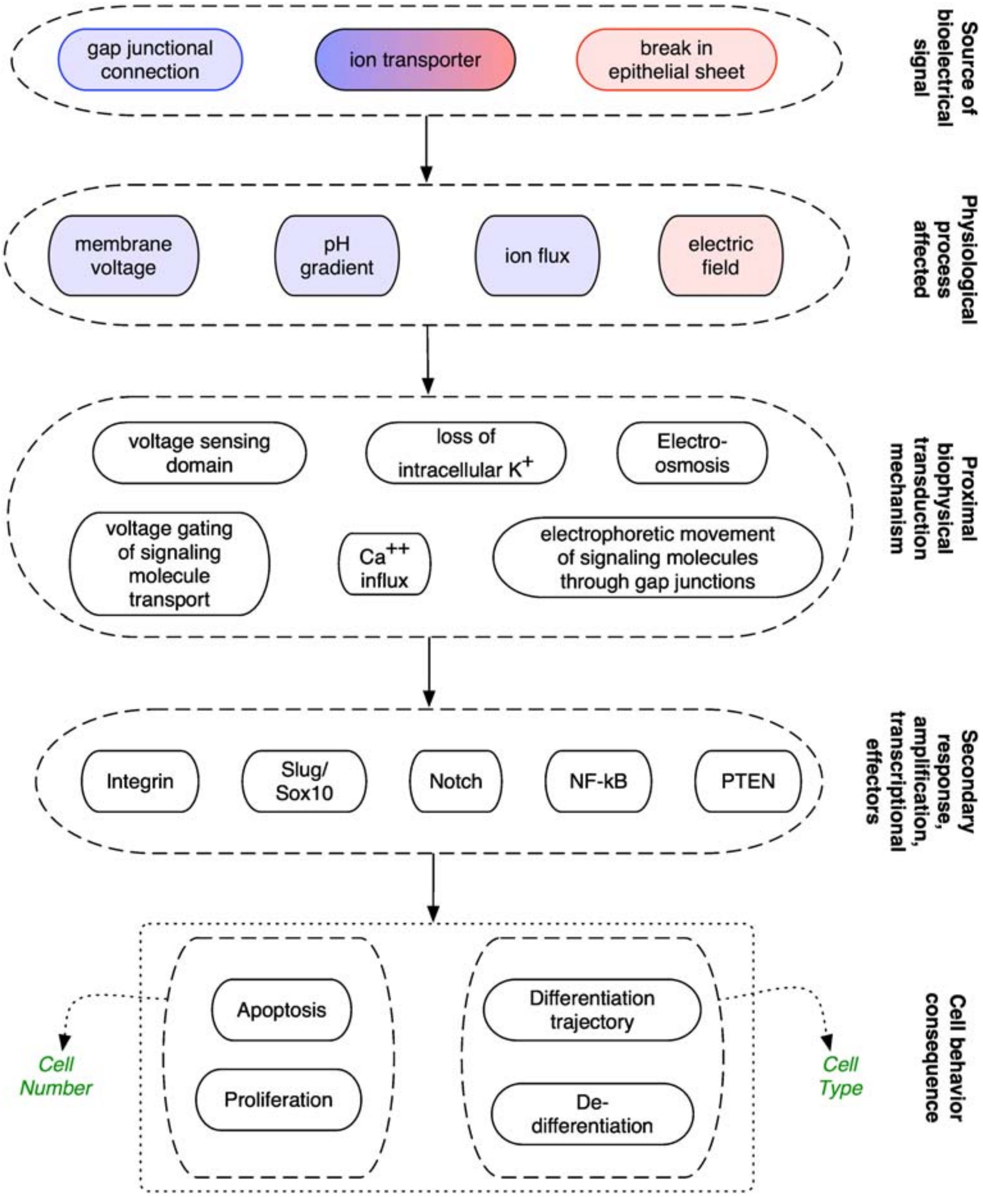

Fig. 1 Integration of bioelectric events with canonical biochemical and genetic pathways occurs through a number of sequential phases. Such signals can be initiated at the cell membrane of individual cells (function of ion transporters), can arrive through gap junctional connections to their neighbors, or be imposed through breaks in an epithelium that carries a transepithelial potential. Physically, such signals are carried by changes in transmembrane potential, pH gradients, flows of specific ions, or long-range electric fields. A number of mechanisms serve as biophysical receptors for these signals, including voltage-sensing domains within proteins, changes of intracellular ion content, electro-osmosis, changes in the gating of transporters for signaling molecules, calcium influx, and electrophoresis of morphogens through gap junctional paths between cells. A number of early response genes have been identified immediately downstream, including integrins, Slug/Sox10, Notch, NF-kB, and PTEN. Because these transcriptional cascades can control all aspects of cell behavior, including proliferation, differentiation, and migration, transduction into these secondary pathways allow bioelectrical signals to control cell number and type during complex morphogenetic events such as tissue regeneration

function of intracellular transporters of signaling molecules such as serotonin [162]; and others.

A key issue for future work concerns specificity. How much information can be encoded in a number such as V_{mem} (do cells interpret it as a binary depolarized vs. hyperpolarized switch, or a larger number of discrete levels)? Do cells have a single V_{mem} value, or more likely, is the cell membrane a manifold containing a huge number of local microdomains expressing different transporters and thus presenting a very rich amount of information to neighboring cells as well as intracellular processes within the same cell? Do individual ion channels provide different signals to cells even when their effect on V_{mem} is similar? What are the time-dependent kinetics of V_{mem} changes in non-excitable cells (slow changes in transmembrane potential)? Such mechanistic understanding will be critical for pinpointing the most effective and specific molecular targets for pharmacological and molecular-genetic interventions [130].

Conclusions

Ionic regulation is a rich yet largely untapped toolbox for rational manipulation of cell behavior [194]. From existing studies, it is clear that ion flows contribute to much more than cell excitability, playing a functional role in proliferation, cell cycle progression, and cell maturation and differentiation. To capitalize upon the potential of bioelectric regulation for regenerative medicine, the fields of electrophysiology and stem cell biology must converge to uncover the molecular and mechanistic basis of ion channel and current contributions to cell behavior, with a view to using V_{mem} and other biophysical properties as (1) a profiling tool with which to characterize cell populations as they undergo changes in behavior and (2) as a control point with which to modulate their behavior. With the availability of pharmacological agents and molecular genetics tools to regulate ion channel activity and expression, we have many tools at our disposal for probing biophysical parameters and learning how to exploit them to direct cell functions for therapeutic applications.

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