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## **The Role of pH and the V-ATPase in Electric Field Guided Cell Migration**

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## SUMMARY

Recent studies revealed a mechanistic link between proton transporters, intracellular pH and downstream signaling cascades related to cell and tissue polarity (Simons *et al*, 2009). In this context, we were able to demonstrate that a subunit of the proton pump V-ATPase contributes to planar cell polarity signaling in *Drosophila melanogaster* (Hermle *et al*, 2010).

Here, we set out to establish cell-based assays for studying the connection between pH and directional cell responses. Our main focus is the directional cell migration induced by electric fields (EFs), also referred to as electrotaxis.

Direct current electric fields (dcEFs) have been implicated in cell migration as well as during wound healing, development and metastasis (Robinson & Messerli, 2003), but little is known on the sensing mechanism as well as the immediate effect on the EF-exposed cells. Recently, the proton pump pma1, a yeast homologue of the V-ATPase, was identified as a mediator of electrically guided cell growth, pointing towards a possible link between dcEF effects and local intracellular pH gradient shifts (Minc & Chang, 2010).

We exposed keratinocytes and differentiated HL-60 cells to electric fields of physiological strength and observed cell polarization and directional migration towards the cathode. In an attempt to modulate the electrotactic response, we pharmacologically inhibited the V-ATPase, which resulted in impaired directionality of cells in the electric field. Furthermore, we observed a complete loss of the EF response in cells exposed to low extracellular pH. Wound healing assay outcomes were not affected neither by V-ATPase inhibition, nor in altered pH environments but showed decreased wound closure when inhibiting proton transporters of the Na<sup>+</sup>/H<sup>+</sup> exchanger type.

In summary, our studies introduced the V-ATPase and extracellular pH as crucial factors in EF-guided cell migration, while showing that directed cell migration in the wound healing model functions independently of these parameters. These results may contribute to the understanding of directional migration and the role of bioelectrochemistry in cell polarity, metastasis and development.

## ZUSAMMENFASSUNG

Neue Forschungsergebnisse deuten auf eine mechanistische Verknüpfung zwischen Protonentransportern, intrazellulärem pH, sowie Zell- und Gewebepolarität betreffenden Signalwegen hin (Simons *et al*, 2009). Wir konnten zeigen, dass eine Untereinheit des Protonentransporters V-ATPase im Planaren-Zellpolaritäts-Signalweg PCP in *Drosophila* involviert ist (Hermle *et al*, 2010). Da dieser Signalweg auch für gerichtete Zellmigration eine Rolle spielen kann, haben wir infolgedessen die genannten Faktoren mithilfe zweier Migrationsmessmethoden, nämlich *in vitro*-Wundheilung und Zellmigration in elektrischen Feldern unter Gleichstrom, Electrotaxis genannt, untersucht. Solchen elektrischen Feldern wird eine Funktion in Zellwanderung, Wundheilung, sowie Entwicklungsbiologie und Metastasierung zugesprochen (Robinson & Messerli, 2003). Einige bekannte Signalproteine für Zellmigration konnten bereits als Regulatoren von Electrotaxis ermittelt werden, wobei die zelluläre Perzeption der elektrischen Felder, sowie der unmittelbare Einfluss auf exponierte Zellen weitestgehend unverstanden bleiben. Ein der V-ATPase homologer Protonentransporter namens pma1 konnte in Hefezellen als relevanter Vermittler elektrisch gerichteten Wachstums identifiziert werden, was auf einen möglichen Zusammenhang zwischen elektrischen Signalen und lokaler Dynamik intrazellulärer pH-Gradienten hinweisen könnte (Minc & Chang, 2010). Wir konnten kathodengerichtete Wanderung von Keratinozyten und HL-60-Zellen induzieren und diese elektrisch induzierte Migration durch Inhibition der V-ATPase signifikant reduzieren. Ebenso konnten wir durch Exposition der Zellen gegenüber niedrigen pH-Werten Electrotaxis sogar gänzlich eliminieren, ohne dabei die migratorischen Fähigkeiten der Zellen per se zu beeinträchtigen. Die auf Wundheilung basierenden *in vitro*-Untersuchungen zeigten keine reduzierte Migration durch Inhibition der V-ATPase oder niedrigen extrazellulären pH, wohl aber durch die Inhibition von Protonentransportern aus der Reihe der Natrium-Protonen-Austauscher NHE.

Unsere Ergebnisse sollen beitragen zu einem umfassenderen Verständnis von gerichteter Zellmigration und der Rolle von bioelektrochemischen Vorgängen in Zellpolarität, Metastasierung und Entwicklungsbiologie.

# 1. INTRODUCTION

## 1.1 Electric fields in cell migration, wound healing and development

The hypothesis of electrical influence on biology in the form of electric fields emerged more than 150 years ago (du Bois-Reymond, 1843) (Geddes & Hoff, 1971). Extracellular steady, direct current (DC) electric fields have been shown to play different roles in various organisms from plants to hominids in physiological contexts like regeneration as well as in pathomechanisms underlying disease (Robinson & Messerli, 2003). The extensive interest in bioelectricity and possible medical applications in the early days led to unscientific explorations and quackery which, in turn, caused bioelectricity to fall into disgrace scientifically. One prominent example that epitomizes this reputational loss is Mary Shelley's "Frankenstein or The Modern Prometheus", a book that, to date, is a reference for criticism of unscrupulous science in the mainstream debate (McCaig *et al*, 2009). External application of electricity in experimental setups subsequently became rather unpopular with the result that the scientific focus shifted towards the measurement of dynamic intra- and extracellular electric signals. This led to diagnostic and experimental breakthroughs like the electrocardiogram, electroencephalography or the voltage- and patch-clamping techniques (Borgens *et al*, 1979a; McCaig *et al*, 2009; Turney, 1998). Nonetheless, in the last decades a number of scientifically robust studies have linked major cell biological processes like cell division, cell differentiation and cell migration to extracellular steady voltage gradients. Besides, those gradients were shown to be persistent for hours, sometimes for days. The origins of physiological electric fields were shown to be ion pumps or leaky cell layers generating ionic gradients that, in turn, can trigger ionic current flow, thereby generating voltage gradients (McCaig *et al*, 2005, 2009; Levin, 2007). One well studied model is electric field-induced cell migration in the context of wound healing, termed electrotaxis. Wound healing is a complex process, consisting of three, partially overlapping phases: inflammation, tissue formation, as well as tissue remodeling. Reepithelialization of the wound starts shortly after the injury takes place (Singer & Clark, 1999). The widely accepted cues orchestrating wound closure are believed to be chemoattractants, e.g. certain growth factors,

wound void and intercellular coordination of locomotion (Reid *et al*, 2005; Rørth, 2009). In contrast to the aforementioned cues, disruption of epithelial cell layers was shown to *immediately* result in the generation of endogenous electric fields, which have been linked to wound closure. Furthermore, exogenously applied electric fields in experimental monolayer scratch assays were shown to override other wound healing cues. EFs could enhance *in vitro* wound closure when applied in a physiological manner, with the cathode being at the wound site. Most notably, when the field vector was applied in the opposite direction, the wound scratch was shown to widen (Zhao *et al*, 2006). In the context of skin EF phenomena, the term "skin battery" evolved, highlighting the potential generation of electric fields in the context of skin wounding. Across the intact human epidermis, between stratum corneum and the dermis, transepithelial potentials (TEP) between -15 and -50 mV were measured, with relatively higher TEPs being found at the hands and feet (Foulds & Barker, 1983). TEPs are generated due to the asymmetry of epithelial ion transport across tightly sealed epithelial cells. In the case of experimental disruption, TEP differences were shown to generate a DC outward current of the magnitude of 4  $\mu\text{A}/\text{cm}^2$  in rat cornea and human skin after wounding, which subsequently increased to 10  $\mu\text{A}/\text{cm}^2$  and then decreased again to between 4 and 8  $\mu\text{A}/\text{cm}^2$ . A voltage gradient of up to 200mV/mm was shown between intact skin 1mm away from the wound edge and the wound itself (Barker *et al*, 1982; Reid *et al*, 2005; Zhao *et al*, 2006), see Fig 1.1. In various *in vitro* experiments, single cells were shown to migrate towards either the cathode or the anode of externally applied electric fields. Electrotactic behavior was reported in keratinocytes, fibroblasts, neutrophils, Dictyostelium discoideum, embryonic stem cells, neurons and fish keratocytes, among others. Field strengths of the exogenously applied EFs range between e.g. 10mV/mm and 600mV/mm or more, depending on organisms and cell type and sometimes higher voltage correlates with stronger electrotactic response (Song *et al*, 2007; Pu *et al*, 2007; Cooper & Schliwa, 1986; Zhao *et al*, 2006). Transepithelial potentials e.g. in human kidney tubules or in the skin were shown to be of much smaller magnitude (Barratt, 1976; Foulds & Barker, 1983). It needs to be clarified though, how exogenous

application of significantly higher magnitude EFs can be justified. Rat prostate glands shall serve as an example of physiological field strength calculation. The value of the transluminal potential of a rat prostate duct reportedly lies at around -10mV (Szatkowski *et al*, 2000), making the lumen an "endogenous cathode". Taking into account the relatively thin 20 $\mu$ m wall of the duct lumen, though, a relatively high possible field strength of 500mV/mm can be achieved.

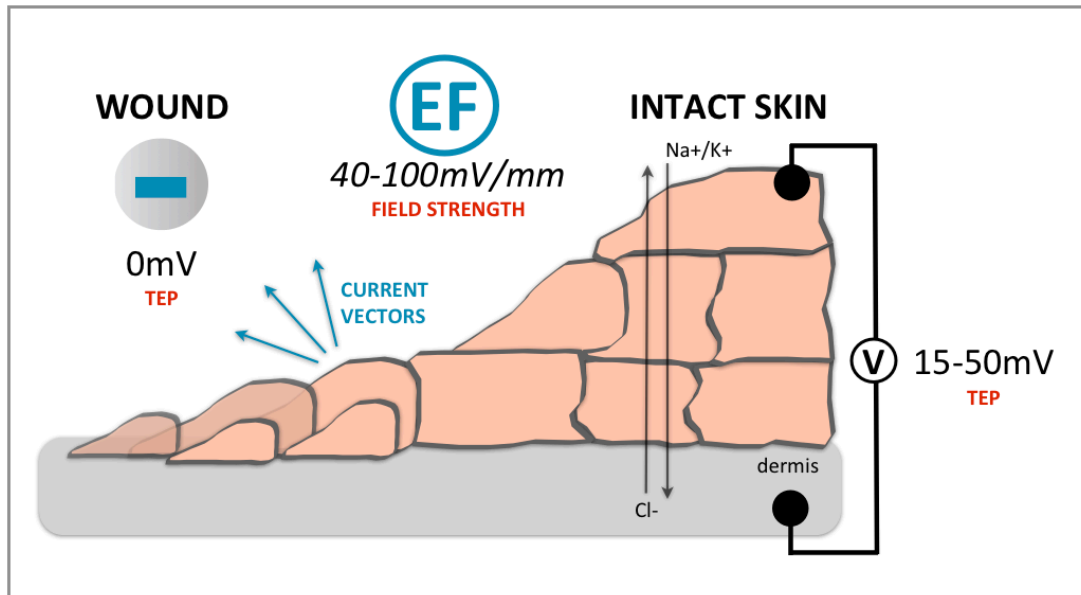


Fig. 1.1: Electric fields (EF) are generated in the context of skin wounding (drawing based on Zhao *et al.*, 2006)

The same holds true for breast ducts, albeit ductal lumina show a TEP of +30mV, meaning it represents an anode (Faupel *et al*, 1997). Its 50 $\mu$ m wall allows for a voltage gradient of 600mV/mm. In many cases though, only a fraction of the calculated physiological field strength is needed to induce electrotaxis (McCaig *et al*, 2009). Moreover, these two tissues helped to understand the role of electrical cues in the pathophysiology of cancer as well as in physiological EF directedness. First of all, both a metastatic rat prostate cancer cell line and breast cancer cells were shown to migrate directionally in electric fields. Secondly, breast cancer cells migrated anodally, in line with the anodal characteristics of breast duct TEPs, whereas prostate cancer cells migrated cathodally, which fits the cathodal TEPs of prostate ducts. Thirdly, in prostate cancer, highly metastatic cell lines exhibited strong electrotactical response, while weakly metastatic ones did not show electrotaxis, linking

metastatic potential to electrical cues (Pu *et al*, 2007; Djamgoz *et al*, 2001; Mycielska & Djamgoz, 2004). Thus, electrotaxis might be seen as a valid model system of directed migration studies, specifically, given the evidence of its physiological and pathophysiological relevance, as summarized above. Recent investigations in the field helped to determine possible sensing and signal transduction mechanisms involved in electrical guidance. In *Xenopus* spinal neurons, the small GTPases RhoA, Rac1 and Cdc42 were reported to influence growth cone guidance towards the cathode, supposedly mediated via high Rac and Cdc42 as well as low RhoA activity at the cathode and opposite activity at the anode (Rajnicek *et al*, 2006). In mouse keratinocytes and neutrophils, electric fields were shown to activate known chemotaxis signaling actors Akt, Src, ERK and p38 mitogen-activated kinase (MAPK), while phosphorylated Src even showed polarized localization to the EF-induced leading edge (Zhao *et al*, 2006). PI3K, known to localize to the leading edge and activate some of the mentioned actors in the migration direction in chemotaxis (Merlot & Firtel, 2003), was shown to be required for electrotactic response in multiple cell types. Besides, PI3K phosphorylation product PIP<sub>3</sub> localized to the cathode side in cathodally migrating cells, even independently of actin polymerization, indicating actin-independent EF-induced PI3K polarization to the leading edge. In contrast, loss of PI3K signaling counterpart PTEN enhanced electrotactic response, which led to the conclusion that the phosphatase is a negative regulator of electrotaxis (Zhao *et al*, 2006). As these discoveries show important parallels in downstream signaling of chemotaxis and electrotaxis, major questions arise concerning the direct sensing of electric cues and more upstream signaling mechanisms involved. How do EFs affect biophysical properties of cells? Are there interactions between EFs and molecular players responsible for chemotactic sensing? A number of hypotheses aimed at elucidating EF sensing have evolved. First of all, *in vitro* application of voltage gradients is known to produce electroosmotic medium flow that could possibly alter cell motility via directed shear stress (McLaughlin & Poo, 1981; Zhao *et al*, 2004). Additionally, EFs might cause secondary generation of pH or chemical gradients, implicating EF effects could originate from secondary chemotaxis

(Robinson, 1985). Both possible experimental flaws could be overcome with the application of a medium cross-flow. Extensive measurements of pH, temperature and calcium levels showed the respective values to be relatively stable, at least over a 4h timeframe. Cross-flow experiments did not show alterations of EF effects (Stump & Robinson, 1983; Cooper & Schliwa, 1986; Song *et al*, 2007). Still, the evidence does not rule out that electro-osmotic flow and possible secondary gradients might even play a role in the physiological setting. Another model suggests electric fields might cause electrophoretically triggered polarized relocalization of membrane receptors (Poo & Robinson, 1977; Jaffe, 1977; Poo, 1981). This model is in line with a number of studies that claim asymmetrical membrane distribution of certain receptors to be significant for electrotaxis or electrical growth cone guidance. In the latter, neurotrophin and neuronal nicotinic acetylcholine receptors were shown to play a role, whereas EGF (epidermal growth factor) and VEGF (vascular endothelial growth factor) are thought to transduce electric signals in EF guided epithelial and endothelial cell migration (Zhao *et al*, 2002; Erskine & McCaig, 1995; Zhao *et al*, 1999, 2004; Pullar *et al*, 2006a). The idea of EF-induced ECM alterations, e.g. collagen-reorientation, was calculated to be rather improbable, because voltage gradients required for such processes would by far exceed physiological and common experimentally applied field strengths (McCaig *et al*, 2005; Jaffe & Nuccitelli, 1977). Nonetheless, the extracellular matrix has been shown to affect EF-directed migration, as laminin and fibronectin were reported to enhance electrotactic directedness as well as overall motility under EF application. Interestingly, these coatings increased expression of the EGF receptor, too. Upregulation of gene expression might also represent a possible factor in EF signal transduction, although it is probably not involved in short-term EF dynamics. Indeed, EGF receptor expression was demonstrated to be upregulated under application of electric fields (Zhao *et al*, 1999). Again in the context of EGF signaling, truncation of  $\beta 4$  integrins and EGF withdrawal blinded cells with respect to electric cues, linking integrin signaling to EF-induced migration. Focal adhesion based convergence of both EGF and  $\beta 4$  integrin signals through Rac was discussed as an explanation of these results (Pullar *et*

*al*, 2006a). Similarly,  $\beta$ -adrenergic receptors were identified as actors in EF motility. While pharmacological activation of the receptors decreased electrotaxis and corneal wound healing, their inhibition caused an accelerated response. Based on these observations, decreased autocrine catecholamine binding was suggested to positively affect electrotactic behavior (Pullar *et al*, 2006b). In *Dictyostelium discoideum*, inhibition of both guanylyl cyclase and PI3K impaired cathodal migration, whereas simultaneous inhibition of both actors led to a reversal of migration polarity in the form of anodal attraction. This observation highlighted the possible significance of pathway interactions for signal transduction in electric field guided directional migration (Sato *et al*, 2009). Another prominent hypothesis related to EF sensing suggests that exposure of exogenously applied electric fields might alter cellular membrane potential in a polarized way, thus leading to depolarization and hyperpolarization at the two cell poles with consecutively polarized signaling. Downstream of the membrane potential alteration, calcium influx could be triggered either by hyperpolarization at the back of the cell due to increased driving force for cation influx, or via depolarization at the cell front facing the cathode and subsequent activation of voltage-gated calcium channels (VGCCs) (Robinson, 1985; Jaffe & Nuccitelli, 1977). As some cells show perpendicular alignment to electric fields, this longitudinal shape was suggested to decrease the voltage drop along the cells, thus reducing the impact of the hypothesized membrane perturbation by the EF (Cooper & Keller, 1984). It remains unclear though, if membrane potential changes induced by electric fields of physiological strength are potent enough to induce secondary signaling effects. A 60  $\mu\text{m}$  long keratinocyte exposed to a 100mV/mm field strength is exposed to a 6mV voltage gradient along its cell length. This implicates a 3mV depolarization at the cathode-facing side and a 3mV hyperpolarization at the anode-facing side. Assuming a resting membrane potential of approximately -70mV, those values would represent a 4% change in local membrane potential (Robinson, 1985; Nishimura *et al*, 1996). However, depending on the field strengths applied or potential secondary amplification mechanisms, membrane potential changes might individually differ in significance. In electrically



polarized yeast cells, for instance, relatively high field strengths were calculated to result in remarkable transmembrane potential changes. These local potential differences were hypothesized to lead to localized changes in proton-pump mediated proton flux which, in turn, might alter intracellular pH and thus, pH-dependent cytoskeletal rearrangements (Minc & Chang, 2010; Campetelli *et al*, 2012). Besides, voltage-gated proton transporters in various species and cell types have been discovered (Decoursey & Cherny, 1994; Morgan *et al*, 2009; Touret & Grinstein, 2002) and NHE1, one of the major regulators of intracellular pH, was reported to play a role in electrotaxis, as pharmacological inhibition thereof led to impaired EF guidance (Zhao *et al*, 2006). Another candidate for the coupling of EF sensing and cell migration machinery is the voltage-sensing phosphatase Ci-VSP. This PTEN-like phosphatase converts  $\text{PIP}_3$  to  $\text{PIP}_2$  in response to membrane voltage changes. Ci-VSP is the first known protein being activated by a S1-S4 voltage sensor without being an ion channel itself (Murata *et al*, 2005). With important processes like collective and single cell migration as well as the cell cycle being affected by extracellular electric fields, it seems possible that bioelectricity might have a more global impact, too, e.g. on development and regeneration (Song *et al*, 2002; McCaig *et al*, 2005). Voltage gradients exist across the wall of *Xenopus* neural tubes during neurogenesis (Hotary & Robinson, 1994) and in the same model organism, limb bud outgrowth was shown to be preceded by an endogenous  $7\mu\text{A}/\text{cm}^2$  outward current on the flank skin of embryos (Robinson, 1983). Similar currents were reported to exist in axolotl embryos, where they often reversed polarity becoming inward currents, following bud outgrowth (Borgens *et al*, 1983). Intriguingly, these currents have not only been shown in mouse and chick as well, but experimental reversal thereof resulted in pathological limb morphologies, suggesting they might play a significant role in vertebrate limb development (Altizer *et al*, 2001). Moreover, 5-10 days after newt limb amputation,  $10\text{-}100\mu\text{A}/\text{cm}^2$  outward currents were reported to leave the stumps, while inward sodium-dependent currents of  $1\text{-}3\mu\text{A}/\text{cm}^2$  were shown to enter the intact neighbouring skin (Borgens *et al*, 1977). Finally, limb regeneration was shown to be enhanced by the application of electric fields in *Xenopus* and rats

(Becker, 1972; Borgens *et al*, 1979b). Investigations on tadpole tail regeneration have demonstrated V-ATPase-induced membrane potential changes and proton flux to be both necessary and sufficient to drive tail regeneration, without affecting wound healing or development per se (Adams *et al*, 2007). In the context of global developmental polarity, V-ATPase- as well as  $H^+/K^+$ -ATPase-dependent ion flux and subsequent membrane voltage and pH gradients were shown to play a vital and upstream role in left-right patterning (Levin *et al*, 2002; Adams *et al*, 2006). In summary, electric fields have been shown to act as guidance cues in various species and cell types. Underlying sensing mechanisms remain poorly understood, albeit motility-related downstream signaling cascades have been elucidated in the past decade and, in many cases, resemble those known from chemotaxis (McCaig *et al*, 2009; Zhao *et al*, 2006). Many observations regarding bioelectrical phenomena have extended the understanding of fundamental cell biological and developmental processes. Further insight into the functional interplay between electric fields, receptors, ion transporters, membrane potential and pH gradients may provide better understanding of development and regeneration (Levin, 2007; Robinson & Messerli, 2003).

## **1.2 Planar Polarity signaling and its role in directional migration**

It is intriguing that some of the bioelectrically influenced processes such as left-right asymmetry or wound healing bear resemblance with processes regulated by the planar cell polarity pathway (Bayly & Axelrod, 2011). This pathway polarizes epithelia and other tissues in the plane and provides orientation for single cell or collective cell behaviour. On the molecular level, it is a branch of the so-called Wnt signaling pathway, also referred to as non-canonical Wnt signaling (McNeill, 2010). The so-called „PCP core module“ consists of the proteins Flamingo (Fmi, aka starry night), Frizzled (Fz), Dishevelled (Dsh) and Diego (Diversin and Inversin in vertebrates) localizing subcellularly at the distal side of cell-cell junctions and the proximally accumulating proteins Flamingo, Prickle (Pk) and Van Gogh (Vangl, also known as Strabismus) (Simons & Mlodzik, 2008). Linking directed cell migration to planar cell polarity, convergent

extension (CE), the developmental mechanism of lengthening and narrowing of tissues needs to be mentioned. Depending on the model organism, it can be dominated by cell rearrangement, cell shape change or directed cell migration. In *Xenopus*, for instance, it is entirely based on cell rearrangements involving mediolateral cell intercalation, whereas fish embryos seem to have included directional migration to their morphogenetic repertoire (Wallingford *et al*, 2002; Solnica-Krezel *et al*, 1996). It was shown in fundulus that all cells of the germ ring migrate in clusters towards the embryonic shield where extension takes place subsequently (Trinkaus *et al*, 1992). PCP signaling was mechanistically linked to convergent extension when mutants of *Xenopus dishevelled* showed defects that resembled direct mesodermal or neural CE inhibition (Wallingford & Harland, 2001). In this context another landmark work showed *Xwnt11* associated non-canonical Wnt signaling to regulate gastrulation via *Dishevelled* (Tada & Smith, 2000). Another prominent example of planar polarity signaling being involved in collective developmental cell migration is the population of neural crest (NC) cells. Neural crest cells are located between the neural plate and the epidermis. After being induced by Wnt-, FGF- or retinoic acid-signaling, they undergo EMT (epithelial-mesenchymal transition) in order to leave the neural tube and find a new destination somewhere in the embryo, where they finally differentiate into various cell types. This coordinated collective migration makes NC cells a model for directed migration studies *in vivo* (Carmona-Fontaine *et al*, 2008; Steventon *et al*, 2005; Thiery & Sleeman, 2006; Polyak & Weinberg, 2009; Yang & Weinberg, 2008)

### **1.3 Directed cell migration**

Apart from these *in vivo* models, directional cell migration is a widely studied process in cell culture. Numerous studies have contributed to a rather detailed understanding of the steps underlying migration. The so-called "cell motility cycle" consists of five steps. Polarized signaling within the cell, polarized protrusion formation, adhesion via integrins and contraction, followed by localized detachment of the cell (Lauffenburger & Horwitz, 1996; Ridley *et al*, 2003). Input into this cycle may originate from within the cell or from external

cues. Motogenic cytokines like EGF, FGF or PDGF can trigger intrinsic motility without promoting directionality (Stoker & Gherardi, 1991). Directed migration requires persistent asymmetry of a migrating cell and therefore, an asymmetrically distributed environmental cue is seen as a precondition for directionality (Petrie *et al*, 2009). The aforementioned motogenic cytokines can also act as cues, as soon as their distribution becomes asymmetrical or in combination with another cue (Arriemerlou & Meyer, 2005; Bourne & Weiner, 2002). Depending on the nature of the cue, different types of directional migration have been described and suffixed with -taxis. Soluble cues can lead to chemotaxis, which has been studied extensively. If the chemical factor, independently of asymmetrical or symmetrical distribution, only contributes to stimulation of motility without affecting directionality, the term chemokinesis applies (Petrie *et al*, 2009). One clinically relevant example of chemotaxis is ATP-mediated directional migration of dendritic cells and eosinophils in allergic lung inflammation, as e.g. in asthma. These cells were shown to migrate towards ATP and sensing was demonstrated to be mediated by a purinergic receptor named P2YR (Müller *et al*, 2010). Substrate adhesion differences can cause haptotaxis, pointing at the importance of the extracellular matrix, whereas other mechanical cues have been described to mediate durotaxis. In general, velocity of the migrating cells as well as their directional persistence, meaning the straightness of their migratory path, can be readouts of directionality (Petrie *et al*, 2009). The role of the extracellular matrix (ECM) in directional migration has been demonstrated in various experiments. Fibroblasts were shown to migrate in the direction of more rigid, higher tension surfaces in polyacrylamide gels (Lo *et al*, 2000). The ECM surrounding the cell was reported to drive cell motility and morphology, which was called contact guidance (Harrison, 1910; Letourneau, 1975). One example of this phenomenon is *Xenopus* gastrulation, where ECM fibrils affect mesodermal cell migration (Nakatsuji & Johnson, 1984). From a signaling point of view, the aforementioned polarity regulators Cdc42, Rac1, RhoA, the PAR proteins, calcium as well as PI3K, PTEN and PIP<sub>2/3</sub> need to be highlighted. The more stable the front-rear axis is, the more persistent the migratory directionality can be. Thus, major regulators of cell

polarity may be vital for migration directionality (Etienne-Manneville, 2008). Cdc42, for instance, is known to be activated itself via the ECM and integrin signals. The small GTPase, in turn, activates atypical protein kinase C (aPKC) which subsequently leads to formation of the Par (partitioning defective) complex including PAR3 and PAR6. This signaling complex is an important regulator of microtubule stabilization, centrosome reorientation and membrane trafficking. Thus, it is contributing to persistent migration by establishing and stabilizing the polarization of the cell (Etienne-Manneville & Hall, 2001). Apart from its role in front-back axis establishment, the PAR complex also mediates crosstalk between the three Rho-GTPases Cdc42, Rac1 and RhoA. Crosstalk differs spatially, resulting in leading edge formation when taking place in the front of the cell and to trailing edge regulation when happening at the rear of the cell (Sander *et al*, 1999; Pertz *et al*, 2006). Another regulator of directedness is calcium. Transient and local intracellular elevations of  $\text{Ca}^{2+}$  have been associated e.g. with chemotaxis, while in moving growth cones,  $\text{Ca}^{2+}$  reportedly activates Rac1 and Cdc42 and inhibits RhoA (Gomez & Zheng, 2006; Jin *et al*, 2005). TRPM7 channels (transient receptor potential cation channel, subfamily M, member 7) were shown to induce intracellular  $\text{Ca}^{2+}$  bursts at the leading edge of migrating fibroblasts and inhibition of this ion channel resulted in impaired chemotaxis to PDGF (Wei *et al*, 2009). When it comes to describing the steadiness of a directional cell response, the term persistence is used. The term means a stable directional response of a migrating cell. In order to gain persistence, stability of the leading edge is often considered an important prerequisite (Petrie *et al*, 2009; Bourne & Weiner, 2002). A key determinant of leading edge formation is the small GTPase Rac1. Importantly, it is not only the presence of Rac1, that determines the migratory capabilities and morphology of the cell, but its activity and localization. When Rac is found abundantly in the cell, without polarized localization and at high activities, lamellae are formed at multiple spots. This promotes random migration, whereas intermediate Rac activity leads to more polarized and concentrated lamellae and thus, directional and e.g. chemotactic migration (Petrie *et al*, 2009; Pankov *et al*, 2005). PI3K (phosphoinositide3-kinase) was shown to target Rac1 at the leading edges of

migrating cells and inhibition of the kinase led to impaired motility and chemotaxis in a range of cells (Kölsch *et al*, 2008; Hirsch *et al*, 2000). Above all, localized PI3K at the leading edge drives the production and accumulation of PIP<sub>3</sub> at the front of the cell. PIP<sub>3</sub> is of paramount importance for motility and directionality, as it is later amplified via feedback loops at the front of the cell, while being degraded at the rear and sides (Haugh *et al*, 2000; Niggli, 2000). In line with these observations, the phosphatase and tumor suppressor PTEN (phosphatase and tensin homologue), which represents a signaling counterpart to PI3K, dephosphorylating PIP<sub>3</sub> to PIP<sub>2</sub>, was also reported to affect cell migration (Tamura *et al*, 1998). Further studies revealed that PTEN down-regulates Cdc42 and Rac1, explaining increased cell motility in PTEN<sup>-/-</sup> cells, which led to the conclusion that PTEN was a negative regulator of motility per se (Liliental *et al*, 2000). From a directionality point of view, though, taking into account the importance of balanced leading edge signaling via e.g. Rac, PTEN might also have been considered to act as a mediator of directedness. This relationship was subsequently elucidated, showing PTEN acted as a mediator of chemoattraction in Dictyostelium discoideum. Cells lacking PTEN showed reduced persistence when chemotacting and PTEN was shown to localize to the membrane at the cells' trailing edges, requiring the binding of PIP<sub>2</sub> for chemotaxis (Iijima & Devreotes, 2002). Thus, PI3K and PIP<sub>3</sub> signaling at the leading edge and PTEN-mediated PIP<sub>2</sub> at the sides and the rear were considered to be important regulators of directional migration (Petrie *et al*, 2009). Nevertheless, chemotaxis was shown to function independently of PIP<sub>3</sub> in a sextuple Dictyostelium mutant lacking all PI3K isoforms and PTEN, again widening the field of possible indispensable mediators of directionality (Hoeller & Kay, 2007). Similarly, in fibroblast chemotaxis, Cdc42 and Rac were shown to play a role in cell motility and persistence, without being required for the directional response to the chemoattractant (Monypenny *et al*, 2009). Cofilin, a protein mediating actin depolymerization and cytoskeletal rearrangement, has also been implicated in directional migration by regulating integrin signals, as inhibition thereof increases random migration (Danen *et al*, 2005). NHE (sodium-proton exchanger)-mediated deprotonation of cofilin inhibits its binding

to PIP<sub>2</sub>, which serves as a negative regulator of cofilin. This link between NHE, cofilin and phospholipids might explain, how leading edge activation of cofilin enables cytoskeletal dynamics at the front of the cell, finally promoting directed cell migration (Frantz *et al*, 2008; van Rheenen *et al*, 2007).

In order to understand the link between pH regulators and directional motility, the next chapter will serve as an introduction of the aforementioned NHEs in the context of directed cell migration.

## **1.4 Na<sup>+</sup>/H<sup>+</sup> exchangers and their role in cell migration**

The role of NHE1 in cell migration has been studied extensively and various cell types, such as fibroblasts, MDCK, leukocytes, melanoma cells or neutrophils, were shown to depend on NHE1 function for migration (Denker & Barber, 2002; Klein *et al*, 2000; Ritter *et al*, 1998; Rosengren *et al*, 1994; Stock *et al*, 2005).

One particularly important study showed that NHE1 acts as a cytoskeletal anchor, binding directly to actin binding ERM proteins (ezrin, radixin, moesin) and promoting cortical actin filament attachment to fibroblast leading edge lamellopodia (Denker & Barber, 2002). In this context, NHE1 is thought to act as a scaffold for certain signaling complexes that, in turn, regulate the cytoskeletal architecture and maintain the exchanger's own localization at leading edge lamellipodia via ERM activation (Baumgartner *et al*, 2004). Another possible explanation of the mechanistic role of NHE1 in cell migration is based on its supposedly central function, which is sodium/proton exchange. Cell migration has been linked to intracellular pH in several studies and as NHE1 plays a central role in pH homeostasis within the cell, substantial investigations on this coherence have established a network of interacting partners (Stock & Schwab, 2006). It was proposed, that transient intracellular alkalinization is necessary for actin polymerization, which is a process that is considered necessary for generation of lamellipodia and for rear retraction and thus, for cell migration per se (Mitchison & Cramer, 1996; Small *et al*, 1995; Wang, 1985; Condeelis *et al*, 1988; Merkel *et al*, 2000). This direct connection, shown in sea urchin eggs and echinoderm sperm (Begg & Rebhun, 1979; Tilney *et al*, 1978), was refined and eventually adapted to mammalian cells. In these cells, cofilin, the

aforementioned actin-binding protein, is activated and recruited to the leading edge, as soon as cytoplasmic alkalinization takes place (Arber *et al*, 1998; Mouneimne *et al*, 2004; Bernstein *et al*, 2000; Bowman *et al*, 2000). This might be considered a substantial mechanistic link between intracellular pH, cytoskeletal dynamics and cellular locomotion. Nevertheless, investigation on the role of NHE1 in neutrophil migration and in directed cell migration in the form of chemotaxis have come to the conclusion, that sodium/proton exchange and intracellular alkalinization rather have a permissive role in the context of cell motility, whereas chemotaxis in neutrophils did not require NHE activity at all (Hayashi *et al*, 2008).

### **1.5 The V-ATPase and its role in cell migration and metastasis**

Another important pH regulator is the V-ATPase, or vacuolar H<sup>+</sup>-ATPase. This ATP-driven proton pump is evolutionarily conserved, being composed of two multi-component subcomplexes consisting of 14 subunits. The membrane-bound V<sub>0</sub> subcomplex mediates electrogenic proton translocation, whereas ATP hydrolysis takes place in the peripheral V1 part (Wilkins & Forgac, 2001). The manifold functions of the V-ATPase in different locations within the cell and in different tissues might be based on its complex molecular composition. Thus, the V-ATPase is an example of an ATP-driven, energy consuming transport mechanism providing active proton extrusion. Historically, these proton pumps were known above all for their role in pH regulation of intracellular organelles, such as lysosomes or endosomes (Forgac, 2007; Nishi *et al*, 2000). In some cells the V-ATPase is also integrated into the plasma membrane on the cell surface. The functions of the proton pump in this cell-specific context range from renal acidification, osteoclast-mediated bone resorption and cytosolic pH homeostasis to sperm maturation (Wagner *et al*, 2004; Toyomura *et al*, 2003; Pietrement *et al*, 2006). Physiological regulation of the V-ATPase is possible either by affecting its activity or by interfering with its localization and overall expression pattern. Activity of the V-ATPase can be influenced through conformational alterations or changes in the coupling of ATP



hydrolysis and proton transport. In different organisms, different metabolic situations demand for modulation of V-ATPase activity (Forgacs, 2007).

In the context of experimental regulation of the V-ATPase the specific inhibitors Bafilomycin A1, a macrolide, and its derivative Concanamycin A need to be mentioned. They both show potent inhibition at nanomolar concentrations and are supposed to function via binding to the c subunit of  $V_0$  (Bowman & Bowman, 2002, 2005; Bowman *et al*, 2006). In the context of cell migration, V-ATPase was shown to play a role in metastatic cell lines, where highly metastatic cells showed plasma membrane localization and higher activity of the proton pump. V-ATPase inhibition in these cells led to impaired migratory capacity (Sennoune *et al*, 2004a).

## **1.6 Aim of the study**

With this study we aimed to elucidate the role of the V-ATPase and pH in directional electric field-guided cell migration. The main task was to establish an electrotaxis model for keratinocytes, which we could subsequently use for the screening of different ion channel inhibitors and pH values. Based on our findings regarding the function of the V-ATPase in planar polarity, we were particularly interested to study the role of this proton transporter in electrotaxis. In addition, we asked whether the manipulation of intra- and extracellular pH values could have more general effects on a cell's capability to migrate directionally. As electrotaxis has been implicated in wound healing, we also performed *in vitro* scratch assay experiments. By doing so, we aimed not only at learning more about the validity and mechanisms of electrotaxis per se, but also about the role of the V-ATPase and pH in different experimental contexts of directional migration. Finally, we compared the V-ATPase and the well-studied NHE1 in our directed cell migration assays.

## **2. MATERIALS & METHODS**

### **2.1 Fly genetics**

The UAS/GAL4 system was used for *Drosophila* RNAi and overexpression. Yellow-white (yw) or *inx2* RNAi strains were used for control experiments and all RNAi crosses were grown at 25°C or 29°C. For CG8444 (*Dmel/VhaM8.9*) knockdown experiments two nonoverlapping transgenic UAS-RNAi lines (5830 or 105281) came from the Vienna *Drosophila* RNAi Center. Ap-GAL4 was used as a driver in the *notum*, while *dpp*-GAL4 served as a wing expression driver. Both were obtained from the Bloomington Stock Center. CG8444 RNAi was coexpressed with the *notum* driver (*ap*)-GAL4 and two distinct, non-overlapping RNAi lines were used to rule out knockdown-related artefacts. Both RNAi lines caused the same phenotype (Hermle *et al*, 2010).

### **2.2 Cell culture**

HPV E6E7-transduced immortalized human foreskin keratinocytes (Hawley-Nelson *et al*, 1989) were a gift from Prof. Cristina Has of the Department of Dermatology, University Hospital Freiburg. Cells were maintained at 37° C in a humidified atmosphere with 5% CO<sub>2</sub> in Keratinocyte Serum Free Medium (KSFM, Gibco) supplemented with 50 µg/ml penicillin/streptomycin, 0.3ng/ml recombinant epidermal growth factor and 30µg/ml bovine pituitary extract, adapted from (Andl *et al*, 2003; Pullar *et al*, 2006a). Cells were grown in 6-well plates and split 1:3 to 1:4 in a three-day splitting routine. During splitting procedure cells were trypsinized for 5-8 minutes with 1ml trypsin/EDTA solution and once detached, 2ml of 10% fetal calf serum (FCS) containing PBS was added to halt trypsin digest. Thereupon, cells were spinned down at 650 RPM for 5 minutes, supernatant was discarded and the remaining cell pellet was resuspended in 1ml KSFM medium. HL-60 cells were a gift from Prof. Ralph Wäsch of the Department of Hematology/Oncology of the University Hospital Freiburg. These cells were kept at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> in RPMI-1640 medium, supplemented with 20% FCS (Gibco) and 50µg/ml gentamicin as previously described (Collins *et al*, 1977; Collins, 1987). HL-60

were differentiated into neutrophil-like cells by adding DMSO, as detailed elsewhere (Collins *et al*, 1978).

### **2.3 Transfections**

HL-60 cells were transfected before DMSO-differentiation with Fz-myc and Dsh-GFP or other plasmids using Amaxa Nucleofection technology from Lonza, which is an electroporation-based transfection technique. 1µg of each plasmid was diluted in 100µl nucleofection solution containing 18µl of supplements. Cells were transfected at a density of  $4 \times 10^6$ . The program Y-001 was applied and subsequently, 500µl of RPMI medium with 20% FCS were added to the cells for a 30 minute recovery incubation. The cells were added to 1.5ml of prepared recovery medium in a 6-well-plate. Expression was visible as early as 5 hours later. Our approach was based on an optimized protocol of the Weiner lab (Millius & Weiner, 2009). Keratinocytes were transfected using Fugene transfection reagent with a reagent/DNA ratio of 3:1. 300.000 cells were seeded into one well of a 6-well-plate and incubated over night until cell density of 50-70% was reached. At first, the transfection reagent was added to 150µl KSFM medium followed by vortexing and a 5 minute incubation at room temperature. DNA was added (e.g. 1.5µg each of Fz-myc and Dsh-GFP plasmids) and the mix was incubated for another 5-20 minutes at room temperature. Cells were washed with 2ml PBS/well and 2ml KSFM were added to each well. Subsequently, the prepared plasmid/transfection reagent complex was added, followed by an 8 hour incubation. Then the medium was changed to 3ml of fresh KSFM and cells were incubated for another 24-48 hours until transfection was evaluated (adapted from Fugene protocols and Yamano *et al*, 2010).

### **2.4 Production of Wnt5a in L cells**

L cells (available from ATCC; control L Cells # CRL-2648, Wnt5a producing L cells # CRL-2814; deposited by R. Nusse) were maintained and amplified in G418 containing Dulbecco's modified Eagle medium (DMEM) at 37°C and 5% CO<sub>2</sub>. Control, Wnt3a and Wnt5a conditioned media were collected from L cells, which are fibroblasts, following a medium production protocol by ATCC. Cells were split 1:10 in 10ml DMEM without G418 and distributed in petri dishes

where they were grown to confluency in 4 days. The first batch of conditioned medium was taken off and stored at 4°C while 10ml fresh DMEM without G418 was added to the petri dishes. After another 3 days the second batch of conditioned medium was harvested and the two batches were mixed and sterile filtered. The soluble Wnt purification method using L cells was described by (Willert *et al*, 2003).

## **2.5 Electric field application to cells in vitro**

Cells were grown to slight confluency in 6-well plates after a 3-day growth period strictly following the above-mentioned splitting scheme. On the day before the experiment uncoated, hydrophobic Ibidi  $\mu$ l-slides were coated with rat tail collagen I (Invitrogen) in a 25 $\mu$ g ml<sup>-1</sup> dilution in 0.02 M acetic acid for one hour, following the manufacturer's protocol. After three PBS washing steps, slides were left to dry before seeding 15 000 cells per slide for overnight attachment and incubation. Shortly before the experiment, the normal KSFM was changed to KSFM containing 10mM HEPES titrated to desired pH levels from 7.0 to 9.0. 12-15 cm parts were cut off a plastic tube and bent in order to obtain v-shaped bridges with a short arm (4 cm) and a long arm (8cm). Those tubes were dried and cleaned with ethanol before being filled with 2% agar solution. The agar was diluted in sterile-filtered Steinberg's solution as described before and boiled at 95°C for 1 minute (Song *et al*, 2007).

Tubes could be filled after a 2 minute cooling-down of the gel. After a 10 minute cooling-down of the freshly filled tubes, the microscopy slide, the agar bridges and a custom made stage with two holders containing 50ml beakers of Steinberg's solution were brought to the microscope. The microscope incubator was set to 37°C one hour prior to the experiment. Agar bridges were put with the short end into the beakers containing Steinberg's solution and with the long ends into the reservoirs of the ibidi  $\mu$ l-slides, which were filled with the 30V DC power supply via alligator clips and dipped into the Steinberg solution beakers (see Results, Fig 3.3) (Ozkucur *et al*, 2009). Platinum wires of 1cm length (WPI) were clipped to alligator clips and connected to a voltmeter for live field strength measurement throughout the experiment. Once regions of interest (ROIs) were

chosen the power supply was turned on and voltage set to 5V on the voltmeter before starting image acquisition. Initial voltage was applied and augmented slowly. During the course of the experiment voltage had to be kept very stable at values of 5V +/- 5% at the voltmeter display for 100mV/mm experiments or 3V respectively for 60mV/mm experiments. A second voltmeter was daisy-chained and used for current monitoring. During the course of the experiment, current should not exceed 600  $\mu$ A. Inhibitor experiments in this assay were conducted with V-ATPase inhibitor Bafilomycin A<sub>1</sub> (300nM, 1:500, Invitrogen). Dosing was based on cell viability testing at different concentrations with our cells and (He *et al*, 2003). Besides, Concanamycin A (1 $\mu$ M, 1:150, Invitrogen)(Luanpitpong *et al*, 2012) as well as NHE<sub>1</sub>-inhibitor EIPA (50  $\mu$ M, 1:670)(Lagana *et al*, 2000) were used in cell migration assays.

## **2.6 Live imaging of cell migration**

All live cell imaging experiments were conducted at a Zeiss Observer workstation with Axiovision 4.8 software and at 37°C. Directionality and speed measurement videos were made either with a 5x magnification using phase contrast I or with 20x or 40x objectives using either differential interference contrast (DIC) II or III. In the standard video setup for quantification (5x) three regions of interest (ROIs) with similar cell density across the slide, usually at the outmost left, right and the middle part of the slide, were chosen and filmed for 60 minutes using 1 minute time frames. The multi-position acquisition tool in the Axiovision software was used to record videos from the different locations on the slides and in case of different ROIs being in different focal planes, the Zeiss Definite Focus hardware was used to maintain stable focus over longer periods of time in multiple positions. For insert based gap closure assays a custom multi-position acquisition setup was created using phase contrast at 5x magnification and 42ms exposure time. One ROI for each well was chosen usually in the middle of the scratch due to better contrast conditions in the middle of each well. In total, at least 8 ROIs were filmed over 12 hours using 5 minute time frames for the insert based gap closure assays. For these assays cells were incubated at 37°C and 5% CO<sub>2</sub>. Live imaging with fluorescently

labelled cells containing GFP or RFP tags or cells being stained with LysoSensor or DiBac<sub>4</sub>(3) was conducted by adding desired RFP or GFP channels to the phase contrast/DIC channels using a HXP lamp for multi-channel acquisition.

## 2.7 Insert-based wound healing assays

Uncoated hydrophobic ibidi 8-well slides were coated with rat tail collagen I (Invitrogen) in a 25 µg ml<sup>-1</sup> dilution in 0.02 M acetic acid for one hour. After three washing steps with PBS, complete removal of residual liquids and extensive drying, ibidi 2-well-inserts were attached to each well in the ibidi slides. Afterwards 35.000 keratinocytes were seeded into each reservoir in 50 µl medium and were incubated for 24 hours. After removal of the inserts, cells were washed with PBS and imaged in KSFM medium at 37°C and 5%CO<sub>2</sub> incubation for 12 hours on the Zeiss Observer workstation using automated multi-position acquisition at 50x magnification (Fig. 2.1).

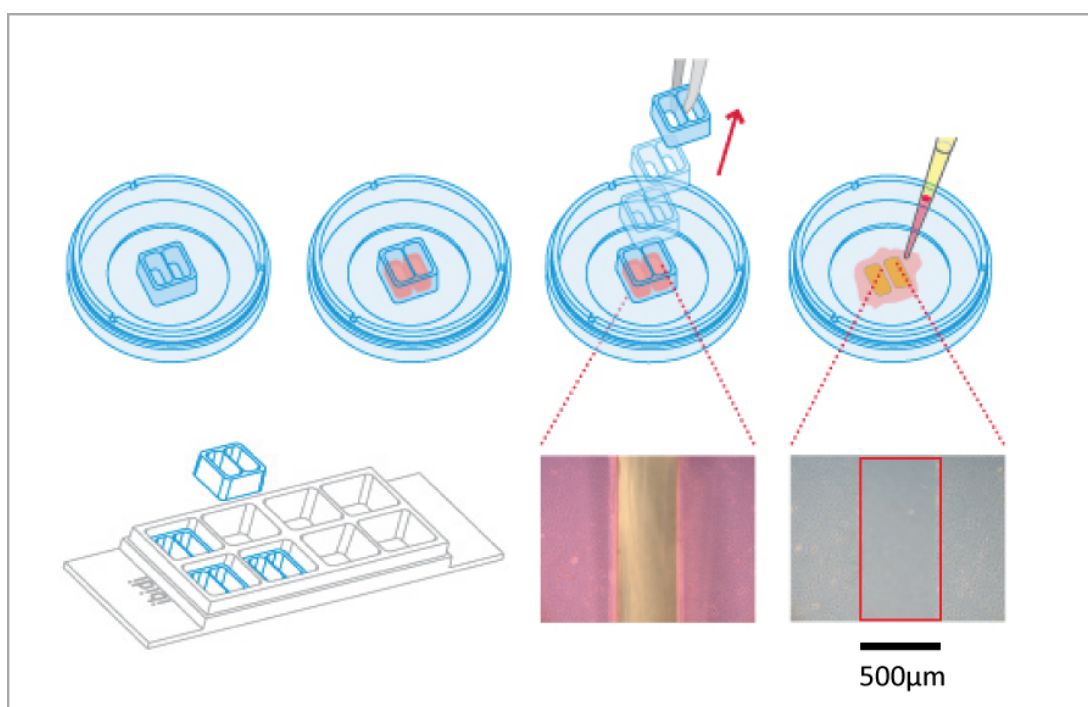


Fig. 2.1: Insert based gap closure in 8-well slides was used as a wound healing assay (figure adapted from ibidi gmbh drawings with permission from the company)

Inhibitor experiments in this assay were conducted with 300nM concentration of the V-ATPase inhibitor Bafilomycin A<sub>1</sub> (Invitrogen), 30µM and 50µM application of NHE-inhibitor EIPA, EGF small-molecule inhibitor AG1478 at 200nM (He et

*al*, 2001) and myosin inhibitor Blebbistatin at 50 $\mu$ M (Kolega, 2006). pH of the media was measured directly prior to all experiments with a pH-meter and random testing of post-experiment pH with litmus paper showed approximately stable pH and prevailing pH differences in case of application of varied pH.

## **2.8 Immunostainings and mounting procedure**

Human keratinocytes and HL-60 were seeded into uncoated, collagen-coated or insert-equipped wells of slides from ibidi (8-well-slides) or Labtek (16-well-slides) for high magnification confocal microscopy using Zeiss LSM 510 microscope workstations running Zen software. Cells were fixed in 100 $\mu$ l of 4% paraformaldehyde (PFA, kept at 4°C) per well for 15 minutes. After cautious removal of PFA and PBS washing, cells were exposed to blocking solution containing 0.2% BSA and 0.1% Triton-X 100 and fixed cells were incubated therein for 30 minutes to 1 hour prior to incubation with primary antibodies. Primary antibodies we used were chicken anti-V<sub>E</sub> subunit (1:200, abcam), goat anti-PRR (1:200, abcam), mouse anti-LAMP1 (1:200, abcam), goat anti-Arp2 (1:50) and rat anti-Fz3 (1:100) among others. The following secondary antibodies were used: Alexa 488 anti-mouse/chicken/goat/rabbit/rat, Alexa 555 anti-mouse/goat/rabbit, Alexa 350 anti-mouse and Alexa 633 (Cy-5) anti-goat. Besides, the dyes Rhodamine Phalloidin (invitrogen) and DAPI were used in 1:200 and 1:10.000 concentrations. Incubation for primary antibodies as well as secondary antibodies and dyes took 1 hour respectively at room temperature. Antibodies were diluted in blocking solution and secondary antibody incubation took place in a light-protected environment (in a drawer). In between antibody incubations, the wells were washed at least twice with PBS and antibodies were vortexed and spinned down prior to usage. Mounting of the slide took place after three PBS washing steps. The reservoir parts of the 16-well-slides were removed and ProLong Gold mounting medium was added prior to the addition of a cover glass. Cells in ibidi 8-well-slides were mounted using ibidi mounting medium and adapted cover glasses.

## 2.9 Lysosomal pH measurements

Cells were incubated in 1  $\mu$ M LysoSensor (Invitrogen), diluted in KSFM medium at 37°C and 5% CO<sub>2</sub> for 15 minutes (Matarrese *et al*, 2010). After replacing dye containing medium with standard medium, cells were either imaged under the same incubation conditions at the live imaging setup consisting of a Zeiss Observer workstation or fixed in 100 $\mu$ l of 4% paraformaldehyde per well for 15 minutes and mounted in Ibbidi Mounting Medium (Ibbidi) or ProGold (Invitrogen) for imaging at Zeiss LSM 510 confocal microscopes.

## 2.10 Quantification of electrotaxis and wound healing assays

Electrotaxis experiments were performed in triplicate, unless stated otherwise, with 3 videos per slide, hence resulting in nine to twelve videos per condition. Initially, the manual tracking tool built into Zeiss Axiovision software was used to track 20 cells per video. These cells were randomly chosen by persons not involved in our project or the imaging process. Initially detached, dead or non-motile cells were excluded from the quantifications. Directionality was assessed by measuring the angle between the axis of abscissae, which represented the axis of the applied electric field, and the cell displacement axis connecting start and end positions of the cell. Alternatively, the cell displacement parallel to the axis of abscissae can be divided by the square root of the sum of the squared cell displacement parallel to the axis of abscissae (x-axis) and the squared cell displacement parallel to the axis of ordinates (y-axis), which represents the division of the adjacent by the hypotenuse according to the theorem of Pythagoras. This means, cathodally migrating cells have directionalities of around +1 and anodally migrating ones usually show directionality of around -1, based on individual migration angles (Tai *et al*, 2009)(Fig. 2.2 A&B). One experimental condition yielded individual cell trackings with directionality, speed, track length, displacement and straightness (Fig. 2.2 C) information. The experimental conditions of 0mV/mm, 60mV/mm, 100mV/mm, 100mV/mm+Bafilomycin and 100mV/mm+ Concanamycin were processed this way, yielding a total of over 4000 manually tracked cells.



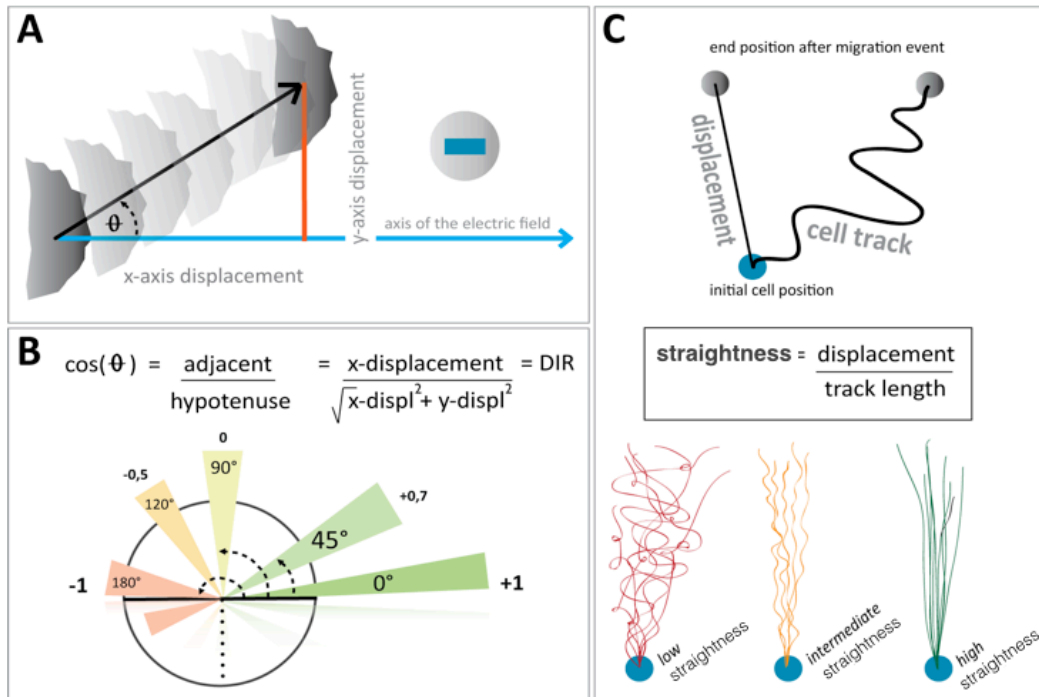


Fig. 2.2: Introduction to directionality and straightness values

A cooperation with the cell migration analysis start-up Wimasis in Munich was initiated and videos were transferred for automated tracking. Algorithms were optimized until tracking results were close to manual quantifications. After the establishment of automated cell tracking analysis all electrotaxis assays were sent to Wimasis for blinded quantification of directionality, overall speed, center of mass weight shift and acceleration. Subsequently, a cell tracking and quantification software was developed in our lab by Deniz Saltukoglu in order to address some issues with aforementioned automated and outsourced trackings yielding faster, more reliable and highly customizable trackings. All experiments were re-analysed using this software. Wound healing assays were performed with Ibidi inserts and resulting scratch closure videos were uploaded to Wimasis for automated wound edge tracking and closure statistics. A randomly chosen, debris-free part of each scratch video was cut out in a manner as to provide a video with a 4:3 ratio (400 by 360 pixels) and 20-30% cell colonization at the start of the analysis to reduce variance in closure analysis. Cell gap closure was evaluated with one image per hour. Image sequences sent to Wimasis usually ended with an image representing manually approved gap closure, if the experiment yielded such outcome.

### 3. RESULTS

#### 3.1 V-ATPase subunit CG8444 RNAi causes PCP phenotype in *Drosophila*



Fig. 3.1: CG8444 knockdown leads to PCP phenotype in *Drosophila*.  
(T.Hermle)

In 2009, a genome-wide RNAi screen in *Drosophila melanogaster* led to the discovery of a link between the proton pump NHE2 and Frizzled-mediated PCP signaling (Simons *et al*, 2009). Another genome-wide RNAi screen looking for Notch signaling phenotypes revealed that knockdown of the gene *CG8444* caused bristle polarity defects (Mummery-Widmer *et al*, 2009).

*CG8444* was also termed *Vha-M89*, *VhaPRR* or *dPRR*, as it shows a 26% sequence homology with the human prorenin receptor (PRR) gene *ATP6AP2* (Hermle *et al*, 2010; Buechling *et al*, 2010). The encoded protein PRR consists of a N-terminal signal peptide and two domains with distinct functionalities. While full length PRR supposedly binds prorenin and renin, promoting subsequent intracellular signaling, a 28kDa extracellular domain was shown to be soluble, being involved in (pro)renin binding. A c-terminal 8.9kDa domain, termed M8.9, was reported to be complexed with the V-ATPase, containing a transmembrane and a cytoplasmic domain. We could confirm the *CG8444* loss-of-function planar polarity phenotype, as seen in Fig. 3.1 (Hermle *et al*, 2010). In addition, *VhaPRR* functions in organellar acidification and lysosomal degradation (Hermle *et al*, 2013).

### 3.2 A cell-based approach to PCP in directional cell migration: The electrotactic response of neutrophil-like differentiated HL-60 cells is not enhanced by Wnt5a treatment

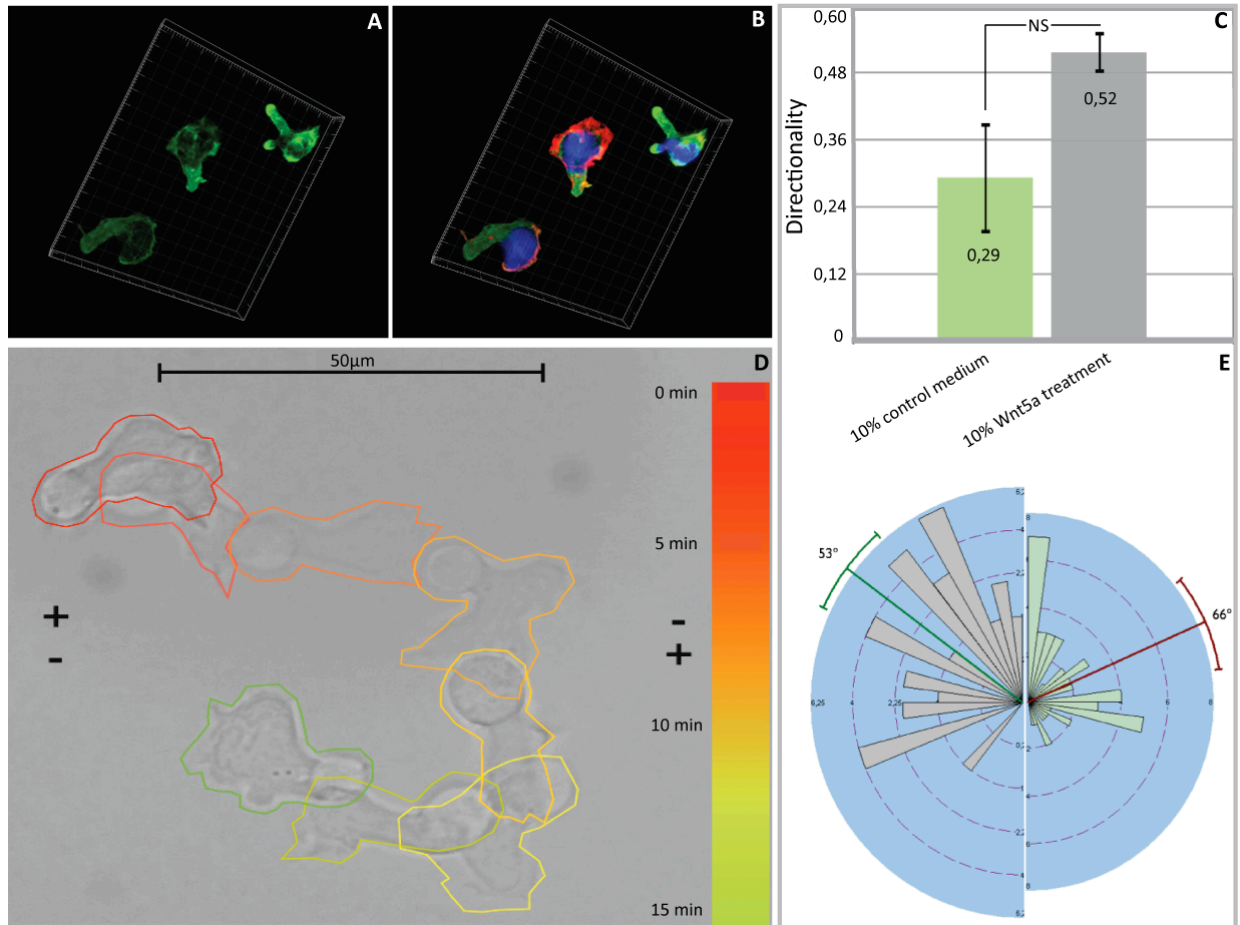


Fig. 3.2: EF migration and Wnt5a treatment of differentiated HL-60 cells, A&B: antibody immunostainings in confocal microscopy of migrating differentiated HL-60 cells: green = Fz3, red = phalloidin = actin, blue = DAPI, C&E: Wnt5a treatment does not show enhanced EF directionality in HL-60; D: example of a differentiated HL-60 cell reacting to a change in EF polarity

In order to further investigate the role of the V-ATPase in planar polarity signaling we decided to focus on directional cell migration, as this process is known to be linked to PCP (Petrie *et al*, 2009; Witze *et al*, 2008).

One member of the family of secreted Wnt proteins, Wnt5a, has been reported to play a central role in cell polarity and directional migration in several studies. Wnt5a was shown to activate PCP signaling via the receptor tyrosin kinase Ror2 through phosphorylation of Vangl2 (Gao *et al*, 2011). Besides, Wnt5a was reported to colocalize with Fz2 at the leading edge of migrating cells, enhancing

the binding of APC and Dishevelled that form a complex with Fz2 (Matsumoto *et al*, 2010). It could be shown *in vitro*, that Wnt5a acts as a chemoattractant requiring Ror2 for migrating palate cells during development (He *et al*, 2008). Two studies further elucidated the role of Wnt5a and Ror2 in cell migration. Firstly, Wnt5a-induced cell migration was shown to require Ror2-promoted filopodia formation, whereas Ror2 was demonstrated to be capable of mediating filopodia formation without requirement for Wnt5a, depending rather on the actin-binding protein filamin A. This protein, in turn, was reported to associate with the cytoplasmic proline-rich domain of Ror2 (Nishita *et al*, 2006). Secondly, a landmark study by Witze *et al* revealed how Wnt5a controls single cell polarity and directional migration in a melanoma cell line. Wnt5a was shown to trigger the formation of an intracellular structure, termed W-RAMP (Wnt-mediated receptor-actin-myosin polarity) containing actin, myosin IIB, Frizzled 3 and melanoma cell adhesion molecule. This very structure accumulated asymmetrically in cells exposed to a chemokine gradient and the formation and relocation process was reported to depend on endosome trafficking. Additionally, it was proven that Wnt5a regulates the process via the small GTPases RhoB and Rab4. Once being located asymmetrically within the cell, the structures caused local polarized membrane contractility and movement of the nucleus towards the membrane retraction sites (Witze *et al*, 2008). There seem to be multiple signal transduction mechanisms by which non-canonical Wnt signaling affects migration of a collective of cells or single cells, making it a candidate pathway that might contribute substantially to the initiation and persistence of directionality in cell migration (Petrie *et al*, 2009). We chose to use a cell culture based approach that would allow us to take advantage of different *in vitro* based directional cell migration assays: electrotaxis and insert-based wound healing assays. At first, we decided to use electric field guided cell migration as a tool to generate directional migration which should subsequently help us to study possible PCP-related directional migration properties. We chose differentiated HL-60 cells as they have been reported to respond to electric fields and due to their relatively high migration speed, being neutrophil-like cells (Zhao *et al*,

2006; Lämmermann *et al*, 2008). Immunostainings showed Fz3 being located at the uropod as well as at the pseudopod structures (Fig. 3.2 A & B).

Although overall cathodal electrotactic response was rather low, showing average directionality of only around 0.3 (Fig. 3.2C), some single cells with a robust response could be re-directed promptly via inversion of the electrical field orientation (Fig. 3.2D). Based on the findings of Witze *et al*, we tried to enhance the directional response of the electrotacting cells with the addition of 10% conditioned medium containing Wnt5a during EF exposure.

At least three independent experiments with 7-18 cells each were performed. Cells were exposed to electric fields in ibidi  $\mu$ 1 slides as described before (Ozkucur *et al*, 2009, Fig 3.3).

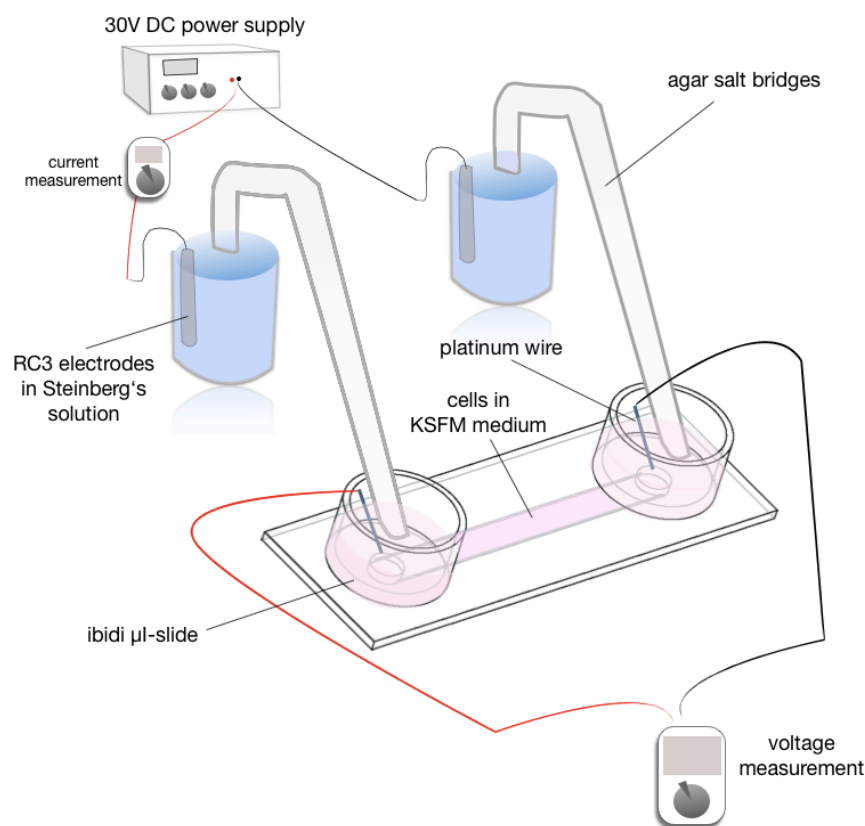


Fig.3.3: Experimental setup of *in-vitro* electric field application to cells (figure adapted from ibidi drawings with permission from the company)

Wnt5a treatment did not enhance directionality of HL-60 cells exposed to electric fields, as compared to addition of L-Cell control medium (Fig. 3.2 C and



E: error bars in C represent s.e.m., p-value=0.14; E shows respective distribution of angles used for quantification). EF directionality in the control medium experiment was heterogeneous. Further investigations using Wnt5a media would require testing Wnt5a activity in an independent readout and application in a more robust electrotaxis model.

### 3.3 E6E7 keratinocytes respond to electric fields as directional cues

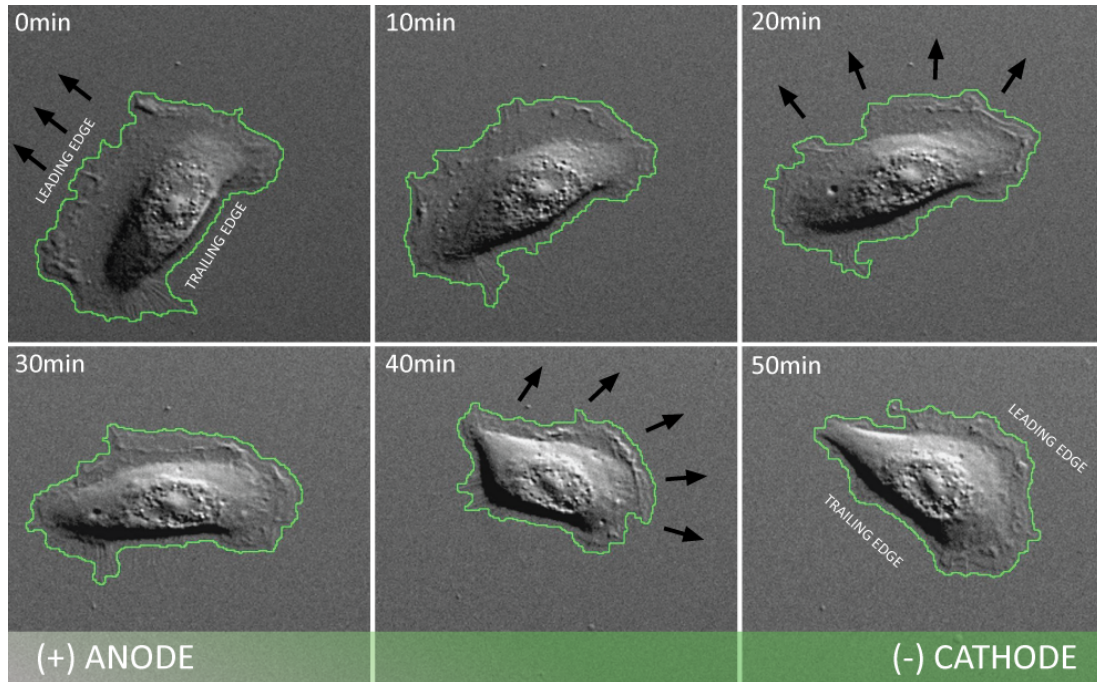


Fig. 3.4: Single keratinocyte response to an EF of 100mV/mm field strength; cell orientation indicated at 10min timepoints after beginning of EF exposure

Subsequently, we decided to shift our attention to keratinocyte electrotaxis, as these cells show a more homogenous electrotactic behaviour (Nishimura *et al*, 1996). Moreover, this cell type is physiologically known to be exposed to skin wounding and thus, to electric fields generated by the disruption of the epidermal layer. Primary as well as HPV E6E7-immortalized human keratinocytes have been shown to migrate cathodally in electric fields in a dose-dependent manner (Pullar *et al*, 2006a; Nishimura *et al*, 1996). We could reproduce dose-dependent electrotactic responses in E6E7-immortalized keratinocytes using an ibidi  $\mu$ 1 slide as an electrotactic chamber (see Fig. 3.5, based on manual quantifications, error bars represent s.e.m.). The time course of a cell totally re-orienting itself to the cathode after the application of an EF

can be seen in Fig. 3.4 (cell outline was generated automatically by Wimasys GmbH). Although electric field guided migration was seen at 60mV/mm field strength, results at 100mV/mm seemed more robust (Fig. 3.5, quantification based on manual trackings). Thus, we used this field strength for most EF migration studies. Interestingly, further augmentation of the field strength led to a longitudinal alignment of the cells perpendicular to the EF (see Fig. 3.5, right column). This cell behaviour was demonstrated before in different other cell lines and it was explained by the EF-induced membrane potential perturbations that might cause the cells to form protrusions only at their longitudinal tips, where membrane potential is stable, thereby minimizing the voltage drop across the cell (Cooper & Keller, 1984).

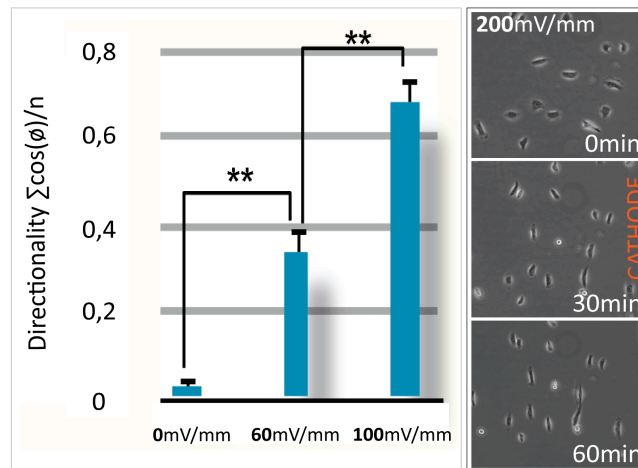


Fig. 3.5: Dose-dependent response to electric fields.  
Directionality towards the cathode, \*\* =  $p < 0.01$ .  
Side panel shows elongation at 200mV/mm.

In our cells, we did not observe desensitization over the course of 1 hour EF exposure experiments. Withdrawal of the field or reduction of field strength usually led to a partial or complete loss of directional migration towards the cathode, suggesting the impact of the electric field on cellular sensing and response mechanisms are of rather transitional nature. Different quantification methods were used to establish a robust keratinocyte electrotaxis readout. At first, manual tracking was performed with Axiovision software by Zeiss. Subsequently, we used online quantification solutions by Wimasys GmbH that were developed by correlating our manual trackings with automated trackings.

Finally, Deniz Saltukoglu developed a tracking software which we used for all quantifications.

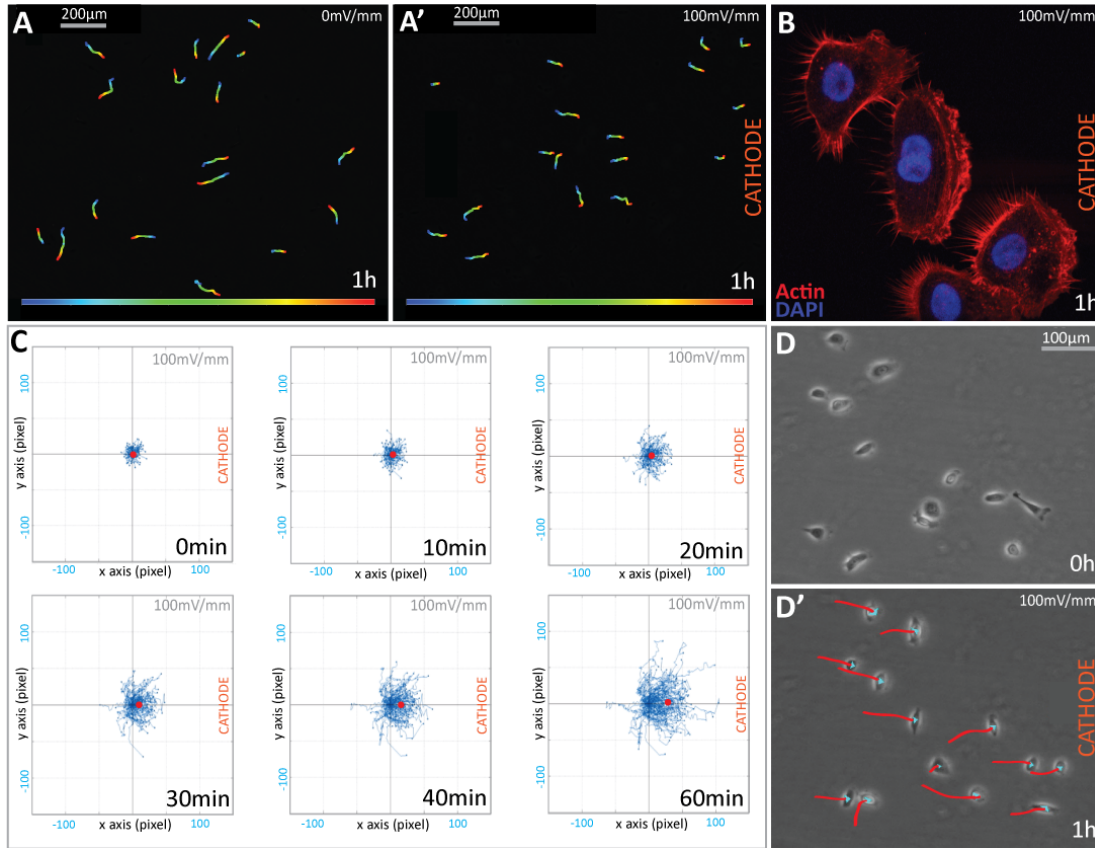


Fig. 3.6: Quantification of directionality and group cell response to 100mV/mm: A and A' show manual Axiovision-based cell trackings of randomly migrating and EF guided (A') cells with a visual representation of the experimental time course. B shows actin and nucleus staining of cathodally migrating keratinocytes after 1h of EF exposition. C shows automated cell trackings done by Wimasis, setting all cells to a common starting point in the middle of the plot, thereby facilitating visual directionality pattern recognition. Cathodal polarity of the cellular response becomes visually evident between 10 and 20 minute time frames. In D and D' a group of cells is shown in a phase contrast image before and after a one- hour EF treatment.

Immunostainings of cathodally migrating cells showed considerable actin accumulation at the leading edges after EF exposure (Fig. 3.6B). The red dots in the middle of the plots in Fig 3.6 C represent the center of mass weight, which is a parameter that averages all cell localizations into one single spot. Localization changes of this cellular center of mass are called center of mass



weight shifts and in the Wimasis quantifications this readout could be used to show directedness and straightness of a cell population over time. Having established keratinocyte electrotaxis in ibidi  $\mu 1$  slides, we defined other parameters we wanted to include in our data analysis. Obviously, it was vital to observe if pharmacological treatments of electrotacting cells might cause overall reduction of cell viability. Thus, we included cell velocity, displacement and track length information in our analysis. Furthermore, we used the ratio of displacement and track length to generate a parameter of migrational directedness and persistence, known as straightness. We were particularly interested in the question if undirectional cells showed reduced straightness, which might add to the understanding of non-directedness. Finally, we aimed at testing different pharmacological inhibitors in the newly established readout of keratinocyte electrotaxis.

### **3.4 V-ATPase inhibition decreases electrotactic response**

As a first molecular target, we decided to test the role of the V-ATPase in electrotaxis. We used Bafilomycin to inhibit the proton pump. To our surprise, V-ATPase inhibitor treatment in electrotaxis led to a significant reduction of cathodal directionality. Nonetheless, cathodal orientation of migrating cells still persisted, as seen in Fig. 3.7 A. To confirm the effectiveness of V-ATPase inhibition, we used Lysosensor assays, which showed reduced lysosomal and vesicular signal in the presence of Bafilomycin (3.7B). Bafilomycin treatment did not lead to an abrogation of the electric signal, as mean directionality towards the cathode was still measured at around 0.25. Compared to 0mv/mm experiments in Fig. 3.5 a directionality of 0.25 still appears to indicate a certain degree of EF polarity. Importantly, the effect of Bafilomycin-induced reduction of directionality was not due to overall reduction of cell viability, as average migration velocity was not affected. Furthermore, straightness also remained relatively stable under V-ATPase inhibition when observed over a 1 hour time frame (3.7A).

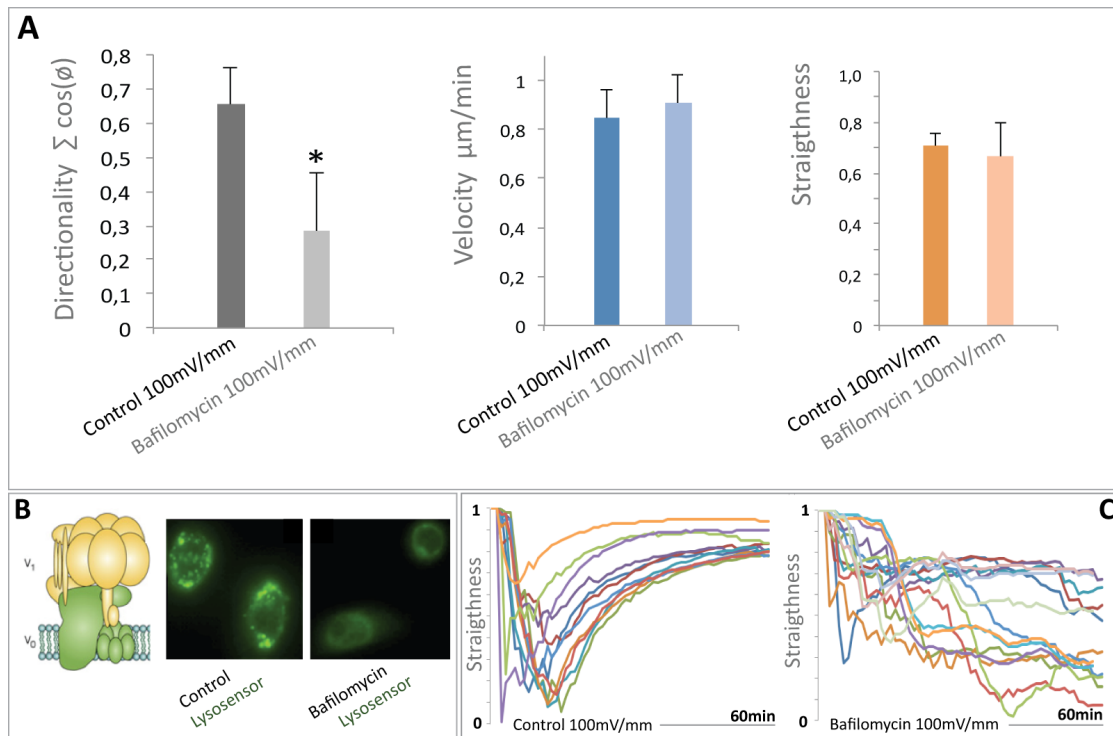


Fig. 3.7: Effect of V-ATPase inhibition on cell directionality, velocity and straightness, \* =  $p < 0.05$ ; A shows effects of V-ATPase inhibition on 100mV/mm EF. B shows V-ATPase structure and reduced Lysosensor signal under Bafilomycin treatment. In C straightness of center of mass weight shifts appears to be reduced under Bafilomycin treatment. (V-ATPase drawing in B is taken from Nishi & Forgac, 2002)

However, judging from straightness using center of mass weight shift data (3.7C), it can be concluded that Bafilomycin treated cells do not develop the same center of mass weight straightness over the course of an 1 hour EF exposure as untreated cells do. This might implicate that straightness analysis could benefit from higher resolution on the time axis.

### 3.5 Low extracellular pH abrogates electrotactic response

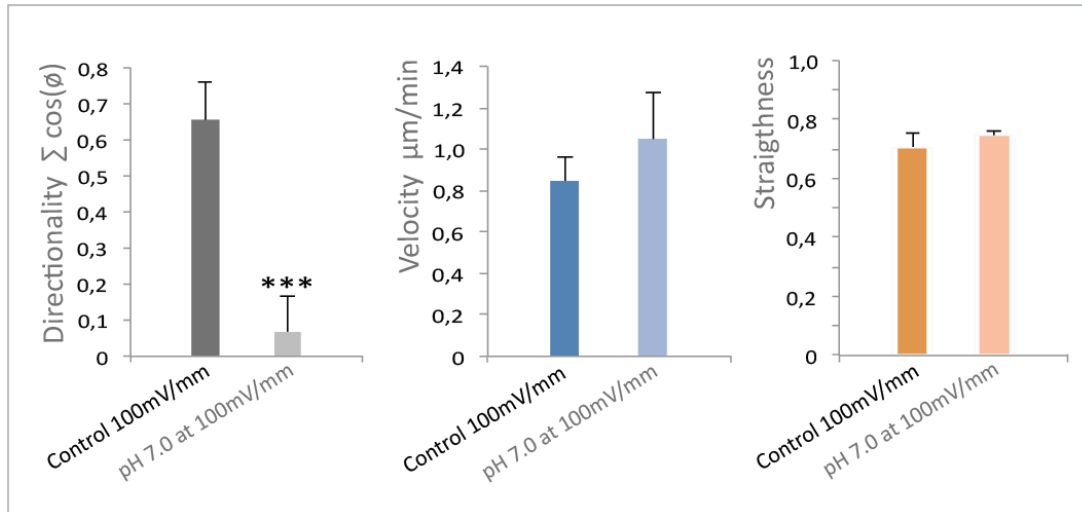


Fig. 3.8: Effect of low extracellular pH on cell directionality, velocity and straightness, \*\*\* =  $p < 0,001$ ; Abrogation of EF guided migration under pH of 7.0 without significant alterations of cell velocity or straightness.

Based on the insights gained from V-ATPase inhibition in electrotaxis experiments, we wondered if one could phenocopy Bafilomycin treatment by manipulation of medium pH. V-ATPase activity is altered by the rate of (dis)assembly of the  $V_0$  and  $V_1$  subunits and it was reported, that extracellular pH may affect this process. Also, the V-ATPase can establish a proton gradient across the plasma membrane in some cell types.

In this context, lowering of extracellular pH was shown to reduce vesicular V-ATPase activity in yeast (Diakov & Kane, 2010; Padilla-López & Pearce, 2006). Remarkably, exposure of electrotacting cells to an extracellular pH of 7.0, instead of 7.4 as in control experiments (at least three independent experiments per condition), not only reduced cathodal directionality, but fully abrogated the electrotactic response (Fig. 3.8). On the other hand, velocity and straightness were not affected significantly under this treatment. This suggests, cells were viable and still showed persistent migration, although without showing collective response to the bioelectrical cue.

### 3.6 Electrotactic response in elevated external pH environments

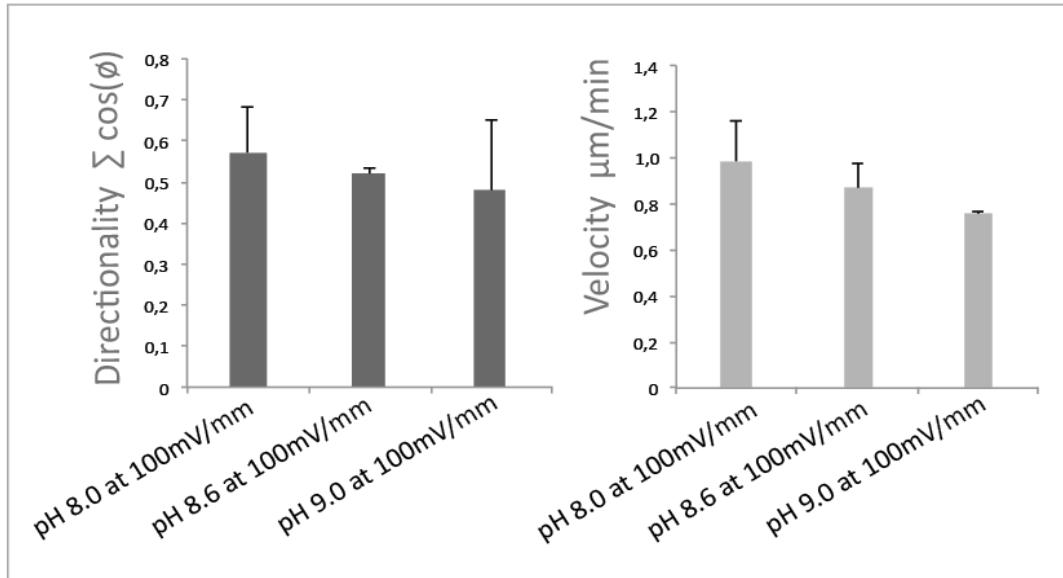


Fig. 3.9: Directionality and velocity of EF guided cells in high pH environments pH from 8.0 to 9.0 does alter neither EF guidance nor cell velocity.

Subsequently, we tested whether increasing extracellular pH might enhance the directional response to the electric field.

Experiments at different pH values ranging from 8.0 to 9.0 (three to four separate experiments per condition from two cell batches) did show neither significant alterations of directionality, nor of overall velocity (Fig. 3.9).

Thus, these experiments showed that *in vitro* electrotaxis was possible at extracellular pH values ranging from 7.4 to 9.0, while lowering pH to 7.0 led to a total abrogation of EF sensing. While pH change did not alter velocity significantly in either direction, higher pH values seemed to cause a tendency of slowing down cell motility.

### 3.7 V-ATPase inhibition does not affect migration in a wound healing assay

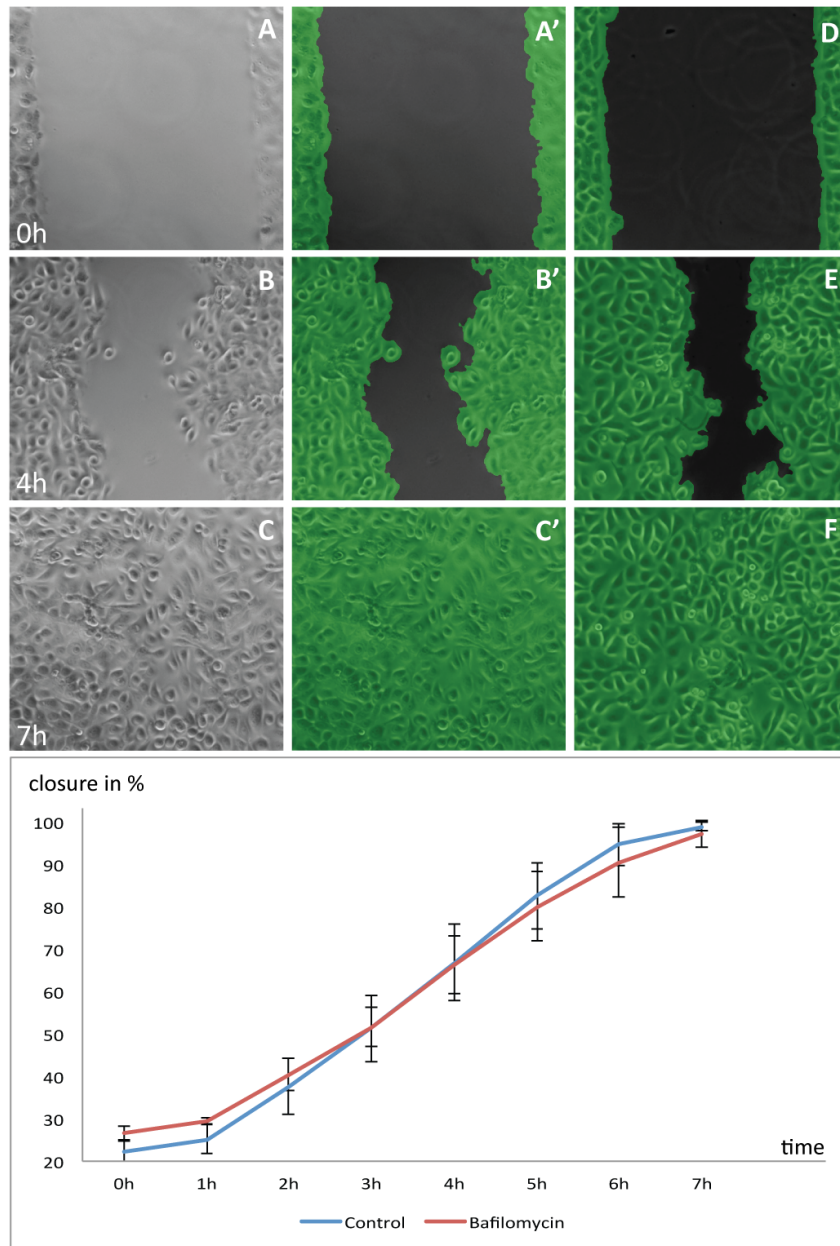


Fig. 3.10: V-ATPase inhibition does not impair directed gap closure  
A-C show unprocessed phase contrast images. A'-C' show processed counterparts of representative control experiments for indicated time points after external automated image analysis by Wimasis GmbH. In D-F, gap closure is shown in the presence of 300nM V-ATPase inhibitor Bafilomycin. Time dependent gap closure of the two conditions are plotted in the graph beneath.

To have an additional assay for directional migration, we studied 2-dimensional wound healing in so-called *in vitro* scratch assays (Liang *et al*, 2007).

We used ibidi® culture inserts to study the closure of a predefined gap without having to manually disrupt intact cell layers. E6E7 keratinocytes were used for the wound healing assay (WHA). Randomly chosen sections of insert-based scratches from at least three independent experiments were used for quantification. Inhibition of the V-ATPase did not show impaired gap closure (Fig 3.10). Previously, V-ATPase inhibition treatment in microvascular endothelial cells was shown to significantly inhibit conventional scratch assay closure (Rojas *et al*, 2006b).

### 3.8 Wound healing assay closure in different external pH environments

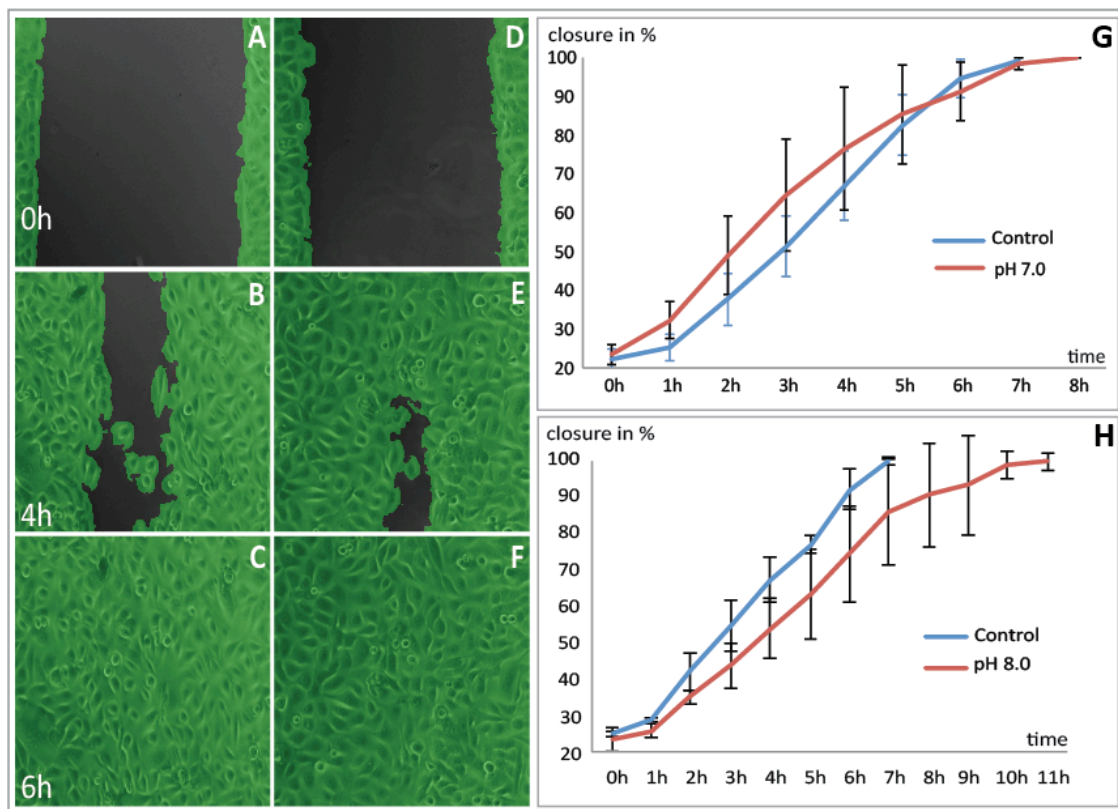


Fig. 3.11: pH 7.0 and 8.0 treatments do not affect wound healing assay outcomes. A-C show control and D-F show pH 7.0 insert gap closure images of one representative experiment. The graph (G) represents at least three independent experiments for each condition. Increasing the extracellular pH to 8.0 did not lead to a significant delay in gap closure either (H).

In order to test whether low pH treatment affects cell migration and directedness per se, we turned to the insert-based wound healing assay (WHA). However, application of KSFM medium at pH 7.0 did not lead to reduced gap closure. Overall cell viability did not appear to be reduced in the 12 hour timeframe and in some cases gap closure was even faster than in controls. Nonetheless, overall migration dynamics appeared to be reduced at pH 8, judged by the average amount of time until full closure. Thus, shifting extracellular pH in wound healing assays did not significantly alter overall gap closure outcomes, which is remarkable when seen alongside the impact of low extracellular pH on EF guided *single* cell migration.



### 3.9 NHE inhibitor EIPA and EGF inhibitor AG1478 slow wound closure

The eletrotactic response in the presence of V-ATPase inhibitor Bafilomycin was shown to be impaired in EF experiments. The wound healing assay experiments shown in 3.10, though, did not lead to a lack of wound closure in the presence of Bafilomycin. In order to compare the possible role of the sodium-proton exchanger NHE in wound closure to the V-ATPase, we used NHE inhibitor EIPA (ethyl-isopropyl amiloride) for a new set of wound healing assays. NHE inhibition was thought to help us determine the possible redundancy or predominance of one of the two pH regulators in the context of wound closure. We chose EGF inhibition to compare the Bafilomycin and EIPA effects to the inhibition of a rather downstream signaling target.

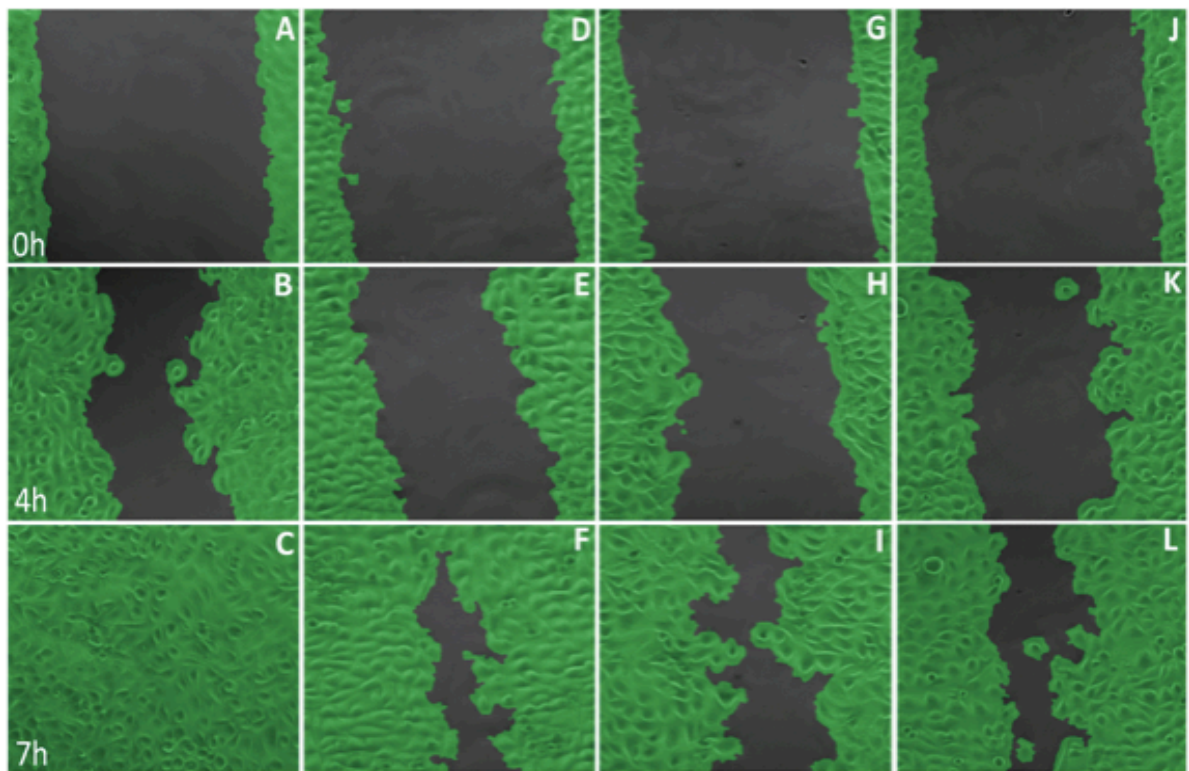


Fig. 3.12(A): NHE and EGF inhibition lead to impaired wound healing assay outcome. A-C show controls. Slowed wound closure at 30 $\mu$ M EIPA (D-F) and at 50 $\mu$ M EIPA (G-I), as well as under EGF inhibitor AG1478 (J-L).



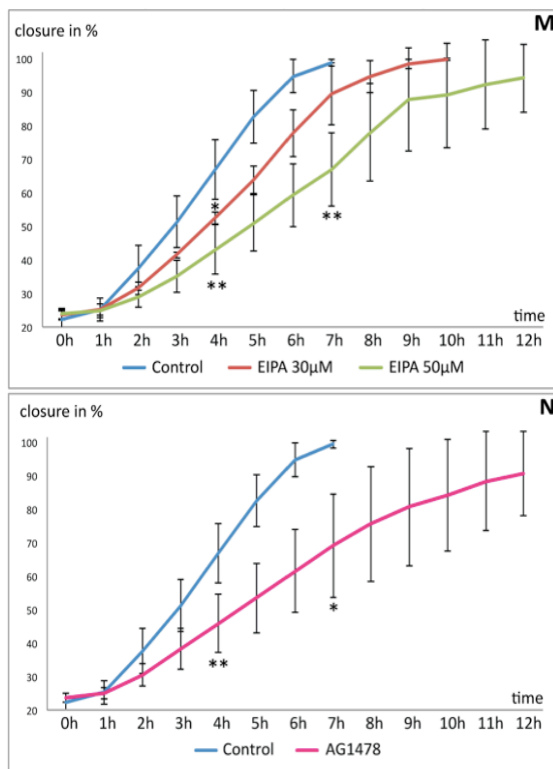


Fig. 3.12(B): NHE/EGF inhibition effects,  
 \* =  $p < 0,05$ , \*\* =  $p < 0,01$

Inhibition of NHE via application of the inhibitor EIPA (5-ethyl-isopropyl amiloride) significantly slowed gap closure in the insert-based wound healing assay in a dose-dependent manner (Fig. 3.12 D-I and M). Interestingly, EGF inhibition via AG1478 (Fig. 3.12 J,K,L and N), which was reported to inhibit keratinocyte migration before (Andl *et al*, 2003), led to a similar wound healing phenotype, when judged by gap closure dynamics.

We have not applied AG1478 in the electric field assays so far.

It remains to be determined experimentally, if and by what mechanisms, NHE interacts with the EGF pathway in the context of our wound healing assays. Comparing our Bafilomycin results with the inhibition by EIPA, it seems as if NHE has a far more important role in wound closure than the V-ATPase has.

## **4. DISCUSSION**

### **4.1 The V-ATPase & pH: Two possible players in electrotaxis**

In this work, we introduced electric field guided cell migration as a novel model system to study the influence of pH and ion transport on directional cell migration. Successfully establishing keratinocyte electrotaxis as a model system for directional migration was a critical endeavour. Our experiments led to the discovery of two factors that seem to affect electrotactic capabilities of the migrating cell. The V-ATPase seems to impair electrotactic guidance without affecting the sensing of the electric cue, while lowering the extracellular pH led to a complete loss of EF guidance. These results suggest a possible permissive role for the V-ATPase in electrotaxis, while extracellular pH above 7.0 seems to be an indispensable environmental necessity. Most importantly, both treatments do not reduce overall cell viability and in the more conventional wound healing assay, based on migratory closure of an insert-based void, pH 7.0 and Bafilomycin treatments do not affect scratch closure at all. In this assay though, NHE inhibition, which was previously shown to significantly impair electrotactic migration (Zhao *et al*, 2006), results in dose-dependent delay of gap closure. Some important questions arose based on these findings and, taken together, they might have the potential to advance the understanding of directional migration.

### **4.2 How do the V-ATPase and NHE affect transduction of the electric signal and directed migration per se?**

It remains unclear if the V-ATPase and the NHE share comparable mechanistic influence on downstream signaling or if their roles in cell migration differ substantially. In general, it seems as if the role of the V-ATPase was confined to intracellular vesicular processes or extracellular acidification in metastatic cells, when found at the plasma membrane (Forgac, 2007). NHE1 on the other hand is well known for its role in intracellular alkalinization and its localization to leading edge membranes. The correlation of NHE with pH-dependent cofilin activity is important to mention in this context. NHE1 clusters at the leading edge lead to local pH elevation, which in turn enables the actin-binding protein

cofilin to enhance local assembly of new actin networks (Stock & Schwab, 2006; Meima *et al*, 2007; Denker & Barber, 2002; Casey *et al*, 2010).

Thus, there seems to be a relative divergence of downstream effects of the two ion transporters, although both act as proton transporters. Two functions of the V-ATPase shall be highlighted in the context of this work, as they are tightly linked to the more global process of symmetry breaking and cell migration.

Firstly, the V-ATPase seems to be of fundamental importance for the establishment of left-right axis formation in *Xenopus*, zebrafish and chick, due to its contribution to asymmetrical proton flux in the early embryo (Adams *et al*, 2006). This finding seems to be in line with seeing the V-ATPase as a more upstream and possibly major regulatory actor in development. Secondly, the V-ATPase is also suspected to play a role in metastasis and invasion due to its prominent expression in tumorous cells, specifically in tumors or cell lines known for their metastatic potential (Martinez-Zaguilan *et al*, 1993; Sennoune *et al*, 2004c, 2004b). One could also see the V-ATPase as a permissive factor in adapting of tumors to challenging pH surroundings. Given the recent evidence of the localization of the proton pump at plasma membranes of highly metastatic breast cancer cells and its significantly higher activity in those, as compared to lowly metastatic cells, it is possible that upcoming insights will help to define the role of the V-ATPase in the context of cancer. It was shown that highly metastatic cell lines are more migratory and invasive than their lowly metastatic counterparts and their migratory qualities, e.g. in scratch assays, were impaired, as soon as V-ATPase inhibition was applied. Additionally, the lowly metastatic cell lines based their cytoplasmic pH regulation on NHEs and bicarbonate based proton transport, whereas V-ATPase dominated in their highly metastatic counterparts (Sennoune *et al.*, 2004). However, it remains to be elucidated by which means the V-ATPase affects cell migration per se. One possible explanation was again derived from a study using a direct comparison of two cell lines that, this time, differed mainly in their migratory capacity. The more migratory microvascular endothelial cells, involved in angiogenesis in acidic microenvironments, were compared with macrovascular endothelial cells. These less migratory and invasive cells were not affected by V-ATPase

inhibition and they did not show V-ATPase at the leading edges of cells facing a scratch-induced wound as their microvascular equivalents did. Besides, the more invasive cells were shown to have a higher cytoplasmic pH at their leading (pH 7.4) than at their lagging edge (7.2) (Rojas et al., 2006). Our wound healing data might also lead to the conclusion that NHE inhibition affects cell migration in a different way than V-ATPase inhibition does. In fact, it is possible that overall cell viability is reduced in the presence of NHE inhibitor EIPA. In some unquantified experiments, we saw shortened migration tracks and complete impairment of single cell migration. The data on NHE inhibition causing reduced electrotactic capability in fibroblasts by Zhao et al. does not include cell viability statistics (Zhao *et al*, 2006). Besides, it is not clear in how far these *in vitro* assays are valid enough to judge the role of the V-ATPase or NHEs *in vivo*, or in a pathological process as metastasis. Maybe overexpression of other membrane-targeted proton pumps might be used in an attempt to rescue V-ATPase or NHE inhibition and to help differentiate between the importance of ion transport and other possible e.g. structural or endocytosis-related functions of the two pumps. Additional experiments might contribute to a better understanding of NHE effects by quantifying the motility response in single cell migration. Judged by our knowledge of the role of the V-ATPase in cell migration, it seems plausible that endocytic or lysosomal processing of receptors involved in relaying directional signaling cues might halt reorientation of cells to the electrotactic cue. Independently of the actual sensing mechanism of the EF, downstream effectors of polarity and motility propagation might be the same as in chemotaxis. Reduced overall turnover of these effectors due to impaired V-ATPase function might also prevent the cell from polarizing towards the cue. This would explain suspected maintenance of electrical signal perception of the cells in the context of impaired directional response, which was the response of the cells to Bafilomycin treatment in our electrotaxis studies. A recent report on the role of the V-ATPase in tumor cell migration revealed that V-ATPase inhibition, both pharmacologically via archazolid and via RNAi silencing, impaired trafficking and spatial activation of

Rac1 as well as EGF receptors. Above all, tumor dissemination was abrogated in a breast cancer mouse model used in this study (Wiedmann *et al*, 2012). These results are in line with our observations of V-ATPase-induced reduction of cell directionality and this study is the first to show in detail how the V-ATPase affects metastasis not in the context of extracellular acidification, but solely based on its newly discovered role in directional cell migration. Maybe the differences in cell viability and morphology in V-ATPase and NHE inhibition experiments point to a more downstream, global motility-oriented function of NHE1, whereas the V-ATPase seems functionally closer to upstream sensing mechanisms of whatever guiding cue. In a recently published paper on the sensing mechanisms during electrotaxis neither Bafilomycin, nor Amiloride treatment altered the electrotactic response of fish keratocytes (Allen *et al*, 2013). Apart from the fact that this finding is based on a different model system (fish cells) it is possible that the much higher field strength of 500mV/mm used in these experiments overrides the impairment of electrotactic guidance we saw in our experiments. Similar to our studies, NHE inhibition also reduced cell speed significantly in these experiments while Bafilomycin treatment had no effect with regard to cell motility. Concerning mechanisms downstream of EF sensing- which based on the findings of Allen *et al* most probably seem to be based on electrophoresis- another recently published paper by Sun *et al*, delivers some substantial insights into the understanding of electrotaxis. In this work, keratocytes and fragments thereof were observed in the electric field. While keratocytes are known to migrate towards the cathode, keratocyte fragments were shown to migrate towards the anode. The authors also showed migration directionality of keratocytes being reversed to the anode by inhibiting PI3 kinase, while keratocyte cell fragments were not affected in their anodal orientation. On the other hand, though, inhibition of myosin with Blebbistatin, did not alter cell directionality but abolished directional EF sensing of the cell fragments (Sun *et al*, 2013). Taken together, the two publications might contribute to an understanding of EF sensing being based on transport of certain charged membrane components to the cell rear, while subsequent

signaling in the migrating cell is based on the polarization of PIP3, as well as actin and myosin networks.

### **4.3 How can extracellular pH affect migration in our experiments?**

Based on the idea of V-ATPase and NHE-dependent intracellular pH changes and the possible role of extracellular pH changes affecting *directional* cell migration by regulating extra- *and* intracellular signaling, we turned our attention to experiments that involved alteration of extracellular pH.

Initially, we wanted to elucidate in how far extracellular pH may affect intracellular pH. Interestingly, it was reported recently that some migratory neural progenitor cells show intracellular lowering of pH when exposed to extracellular acidification (Nordström *et al*, 2012). If this was the case in keratinocytes, this might point to a certain threshold of intracellular pH for the sensing or relay of electric signals, as extracellular alkalinization did not impair directedness. However, pH, be it extra- or intracellular does not seem to affect players involved in basic motility signaling, as migration per se and even directional wound closure were not impaired in our experiments.

In the melanoma cell line MV3 a study using proton-sensitive dyes revealed the generation of an extracellular proton gradient in polarized cells increasing from the trailing to the leading edge. This gradient, being localized at the glycocalyx or the outer membrane leaflet of the cells, was shown to depend on NHE1 and it was suggested it might contribute to cell adhesion and detachment processes at the front and the back respectively (Stock *et al*, 2007; Stüwe *et al*, 2007).

Interestingly, it was reported that extracellular alkalinization in amphibian heart cells leads to maximal and NHE-dependent p38-MAPK activation whereas acidification caused only moderate activation (Stathopoulou *et al*, 2006). p38-MAPK on the other hand, is a kinase that is known to be involved in relaying the electric signal (Zhao *et al*, 2006). Both models could explain impaired cell directionality in low pH based on either the manipulation of a potential extracellular proton gradient or the lower level of downstream motility signaling. With NHE1 being involved in both mechanisms, it might also explain our wound healing assay results. Another interesting possibility of extracellular pH

influence is related to the role of integrins in cell adhesion and migration. Melanoma cells, e.g., have been demonstrated to develop more lamellipodia and stronger adhesion to the substrate at low pH values (Stock *et al*, 2005). It is important to note that our cells did not show slower migration velocity in the presence of low pH, which could have been explained by possible stronger adhesion. Furthermore, binding of cell surface integrin receptors to ligands of the extracellular matrix has been shown to depend on pH. For instance, it was demonstrated that acidic extracellular pH leads to the activation of an integrin called  $\alpha_v\beta_3$  (Paradise *et al*, 2011). While integrin trafficking is known to be important in directional cell migration in general (Petrie *et al*, 2009), integrins have also been reported to play a role in electrotaxis (Pullar *et al*, 2006a). A recent publication by Allen *et al* demonstrated fish keratocyte electrotaxis could be abolished by lowering extracellular pH (from 6.2 and above to pH5.8)- which is in line with our results. The authors deduced that extracellular protonation of the migrating cell might be the cause of losing electrical guidance. As cells migrating towards the cathode have a positive membrane charge at the leading edge, it was assumed protonation abolished sensing of the electric field by "blinding" the negatively charged cell rear to the electric stimulus. In other words, the sensing mechanism seemed to demand for a specific alteration of the trailing edge cell membrane charge or composition. By experimenting with cell response time to the onset of an electric stimulus and changing aqueous viscosity, the authors showed that switching EF orientation capability of the cells was correlated to cell size. These findings strongly supported the idea of an electrophoretic redistribution of charged membrane components, probably to the rear of the cell, facing the anode. To confirm this hypothesis, it was shown that charged membrane components were redistributed to the anode by using a fluorescently labeled lectin (Allen *et al*, 2013). Due to this work, electrophoresis might now be considered the most important candidate for electric field sensing. Besides, Allen *et al* showed that extracellular pH plays an important, yet permissive, role in the electrophoretic sensing process due to its influence on the status of extracellular protonation.

#### 4.4 Is migrational directionality encoded in intracellular pH gradients?

Apart from events occurring on the external side of the plasma membrane, it remains unclear whether intracellular pH gradients might serve as a mediator of external cues, such as the electric signal. In two melanoma cell lines, B16V and MV3 significant rear-to-front gradients of intracellular pH could be demonstrated. These gradients were shown to be driven by NHE1, which is known to accumulate at the leading edge. However, other cell lines like NIH3T3 or MDCK-F1 did not show significant pH gradients (Martin *et al*, 2011). These findings can be linked to the hypothesis of cortical pH alterations being triggered by the aforementioned EF-induced membrane potential alterations. In a keratinocyte, membrane potential changes induced by an electric field of 100mV/mm field strength lead to a 3mV depolarization at the cathode-facing side and a 3mV hyperpolarization at the anode-facing side (Robinson, 1985; Nishimura *et al*, 1996). With the help of mathematical modeling of yeast cells exposed to electric fields of considerably higher magnitudes (5V/mm) it was demonstrated that transmembrane potential changes can affect proton flux and thus lead to EF-dependent intracellular cortical pH gradients (Minc & Chang, 2010; Campetelli *et al*, 2012). Such gradients, in turn, have been shown to exist in pollen, where they are believed to be associated with polarized tube growth (Feijó *et al*, 1999). Interestingly, pollen not only drives steady currents through itself but also shows cathodal or anodal growth patterns parallel to the electric field, depending on the location of its germination (Weisenseel *et al*, 1975; Malhó *et al*, 1992). Thus, based on the idea that cell polarity might rely on intracellular pH gradients (Denker & Barber, 2002; Casey *et al*, 2010), EF-induced cortical or intracellular pH gradient establishment represents a logical link between electric signals and cell polarity. Nonetheless, there are considerable objections to this hypothesis. First of all, in electric fields of low field strength, membrane potential changes are possibly not potent enough to alter ion flux dynamics across the membrane. This holds true for our experiments, too, as they were conducted at physiological field strengths. Secondly, intracellular pH gradients do not exist in all cells, meaning they cannot be a necessary prerequisite for cell polarity. Of course, knowledge of



intracellular pH in migrating cells is rather sparse. It is possible that pH gradients were simply not detected so far due to penurious interest in the topic, difficulty of experimental procedures and above all, lack of modern imaging techniques with higher resolution of pH measurements and temporospatial dynamics. Concerning membrane potential perturbations, it is important to keep in mind how small environmental changes can impact organisms in biology based on feedback loops and intracellular amplification. Yet, even if intracellular pH gradients were not necessary for cell polarization, they might still influence cells and, thus, could still explain the pH triggered actin-based polarization of cells towards the cathode.

However, membrane polarization related pH changes could not explain why some cells migrate towards the anode. All in all, the discussed hypothesis has the potential to explain a substantial part of EF induced polarity and cell migration. In the light of our results, extracellular pH alterations or inhibition of cortical V-ATPase-based proton flux might alter intracellular pH dynamics and thus lead to impaired gradient formation and EF response. Many objections could be addressed using elaborate intracellular pH measurements which are currently performed by members of the laboratory.

#### **4.5 pH and wound healing *in vivo***

From a clinician's perspective, wounds and wound healing are daily issues, being an underlying cause of many elderly patients' hospitalizations, specifically in the context of chronic wounds and associated complications due to infection and immobilization. In this paragraph I would like to point at some work related to *in vivo* wound healing in different pH environments. The skin surface pH usually lies at around 4-6 and is thought to contribute to the barrier function of the skin (Schmid-Wendtner & Korting, 2006). In wounds, internal layers of the body, usually comprising a pH of 7.4 get exposed. At later time points in the healing process of an acute wound, though, acidification by e.g. pus and lactic acid takes place, which is thought to be beneficial in the overall healing process. In chronic wounds higher pH values prevail. There is evidence for a link between the alkaline pH optimum of many proteases and the impaired

healing process in chronic wounds, in which catabolic activity dominates (Schneider *et al*, 2007; Schreml *et al*, 2010b). Nonetheless, it remains to be questioned if pH can be considered a homogeneously distributed factor in wounds. Some recent work used a new *in vivo* pH measurement technique to investigate pH distribution in wounds (Schreml *et al*, 2010a). With this technique a timely and locally differentiated distribution of pH could be detected within a healing wound. With the help of optical 2D pH sensors, an overall decrease in wound pH could be seen in the course of a healing skin-graft donor wound over a time course of 14 days. In comparison, a chronic venous ulcer showed a heterogeneous, rather alkaline pH, being surrounded by acidic skin at the wound edges (Schreml *et al*, 2010a). These results demonstrate the fundamental importance of pH in wound healing, possible differences between *in vivo* and *in vitro* approaches, as well as clinical implications in the field of wound healing disorders, which represent a common and growing public health issue.

#### **4.6 What might be the role of PCP signaling in the EF context?**

Taking into account our discovery of a V-ATPase subunit involved in planar polarity signaling, we tried to take a closer look at this pathway in electrotaxis using Wnt5a conditioned medium in HL-60 migration experiments. Although we could not enhance the electrotactic response, it is well established that planar polarity signaling has an important role in directional migration (Petrie *et al*, 2009). Besides, downstream effectors related to PCP were shown to be involved in EF-induced neural growth cone guidance (Rajnicek *et al*, 2006). All in all, it is possible that planar polarity signaling has a permissive role in the context of electrotactic guidance, being confined to downstream signaling and the generation of directed motility. However, sensing of the electric signal might not be related to the PCP pathway, except for the possibility of soluble Wnt gradients being generated secondarily to the application of electric fields.

## 4.7 Conclusions and outlook

Our findings point to an important role of extracellular pH in the context of directed cell migration. It seems probable that reduction of the electrotactic response due to V-ATPase inhibition is based on secondarily impaired intracellular trafficking and thus, altered receptor and downstream effector distribution, as demonstrated in the context of breast tumor metastasis by Wiedmann *et al.* Follow-up experiments to elucidate the role of the V-ATPase in this context should be targeted at the subcellular localization of the proton pump as well as the localization and distribution dynamics of other known, possibly PCP-related downstream actors, as e.g. RhoA. To see why electrotactic migration is reduced although wound healing assays did not show impaired scratch closure, these experiments should be performed in the presence and absence of electric fields using live cell imaging and *in vivo* wound healing assays. In order to differentiate between its vesicular role and plasma membrane related V-ATPase function, overexpression of membrane-targeted proton pumps might be used in an attempt to rescue V-ATPase inhibition.

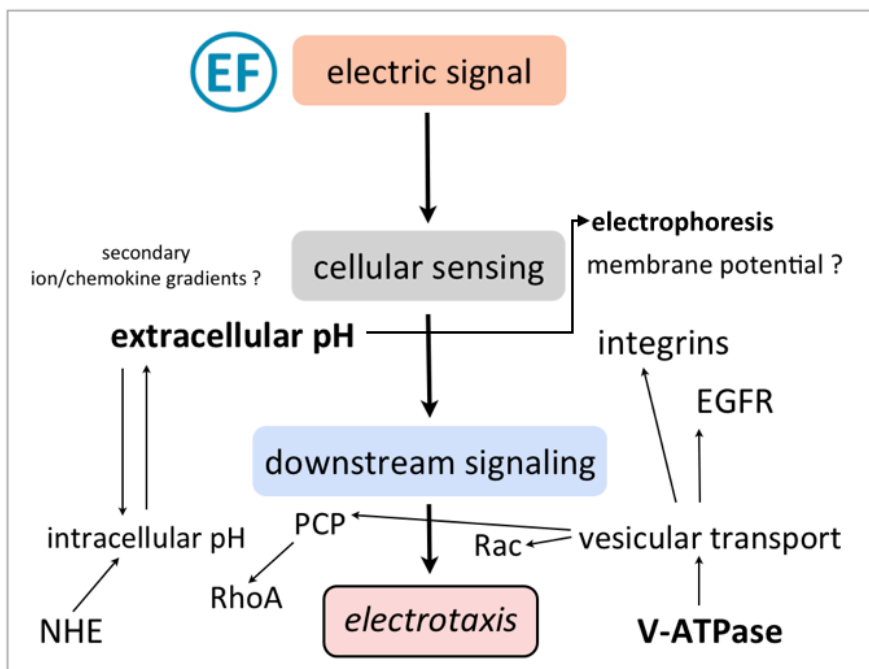


Fig. 4.1: Possible mechanisms and molecular actors behind electrotaxis

More importantly, the effect of low extracellular pH should be investigated in detail using high-resolution microscopy to detect temporospatial intracellular pH dynamics in cells undergoing random migration, chemotaxis and electrotaxis, as well as in wound healing assays. Live cell imaging of integrin dynamics in different pH environments and membrane potential measurements of cells exposed to the EF and low pH using voltage probes might also contribute to the understanding of underlying subcellular processes involved in electrotaxis. The experiments by Allen *et al* substantially enhanced the understanding of EF sensing by proposing electrophoresis of charged membrane elements to drive electrotactic orientation. Based on these findings, new assays identifying the membrane components that are being reoriented might contribute to enhance our understanding of electrotaxis. Ultimately, electric field guided cell migration might not only be a tool to study cell migration per se. Profound insight into mechanisms behind electrotaxis might contribute to the understanding of directed migration as a fundamental bioelectrochemical process in the physiological context of development as well as in the immensely relevant clinical context of metastasis.

## 5. LIST OF ABBREVIATIONS

AC/DC	alternate current/direct current
Akt	also known as Protein kinase B
APC	adenomatous polyposis coli protein
Arp2/3	Actin-Related Proteins 2 and 3
ATP6AP2	gene encoding PRR
Cdc42	cell division cycle 42 (rho family)
CG8444	drosophila gene with sequence homology to ATP6AP2, also known as VhaPRR, VhaM8/9 or dPRR
DAPI	4',6-Diamidin-2-phenylindol
DIC	differential interference contrast
DMSO	dimethyl sulfoxide
Dsh	Dishevelled
E6/E7 keratinocyte cell line	HPV-16 E6/E7 immortalized cell line
EF	electric field
EGF(R)	epidermal growth factor (receptor)
EIPA	5-(N-Ethyl-N-isopropyl)amiloride
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
Fz	Frizzled
GFP	green fluorescent protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HL-60 cell line	Human promyelocytic leukemia cells
JNK	janus kinase (MAPK family)
KSFM	keratinocyte serum free medium
MAPK	mitogen-activated protein kinase
MDCK cell line	Madin-Darby Canine Kidney cells
NHE	sodium-proton exchanger
NIH3T3 cell line	National Institute of Health
p53	(tumor) protein 53
PAR	partitioning defective
PBS	phosphate buffered saline
PCP	planar cell polarity
PCR	polymerase chain reaction
PFA	paraformaldehyde
PIP/2/3	phosphatidylinositol (bi/tri)phosphate
PRR	prorenin receptor
PI3-K	phosphoinositide 3 kinase
PLD	phospholipase D
PTEN	phosphatase and tensin homolog
Rab	"Ras-related in brain", G-protein , Ras superfamily
Rac1	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP

	binding protein Rac1)
Ras superfamily	class of small-GTPases involved in intracellular signal transduction
RFP	red fluorescent protein
RhoA	Ras homolog gene family, member A
RNAi	RNA interference, aka knockdown
ROCK	Rho-associated, coiled-coil containing protein kinase 1
ROI	region of interest
TRPM7	transient receptor potential cation channel, subfamily M, member 7
Tyrphostin AG1478	N-(3-Chlorophenyl)-6,7-dimethoxy-4-quinazolinamine
Wg	Wingless
Wnt	genes with sequence homology to <i>Drosophila</i> <i>Wingless</i> and mouse <i>Wnt1</i>
Wnt5a	soluble Frizzled receptor ligand
WRAMP	Wnt-mediated receptor-actin-myosin polarity
Zipper	myosin-II

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