Osmotic cues from the environment mediate rapid detection of epithelial breaches by leukocytes in larval zebrafish tail fins. Using intravital luminescence and fluorescence microscopy, we now show that osmolarity differences between the interstitial fluid and the external environment trigger ATP release at tail fin wounds to initiate rapid wound closure through long-range activation of basal epithelial cell motility. Extracellular nucleotide breakdown, at least in part mediated by ecto-nucleoside triphosphate diphosphohydrolase 3 (Entpd3), restricts the range and duration of osmotically induced cell migration after injury. Thus, in zebrafish larvae, wound repair is driven by an autoregulatory circuit that generates pro-migratory tissue signals as a function of environmental exposure of the inside of the tissue.

Introduction

Rapid epithelial wound closure is essential for metazoan life, as it restricts exposure of the inside of an organism to the noxious outside environment. Wound closure mechanisms operate efficiently in animals that occupy different habitats (e.g., land/river/sea), and whose epithelia are exposed to vastly different physicochemical environments (air, fresh/salt water, etc.). Wound closure shows striking similarities to morphogenetic processes, such as dorsal closure in Drosophila melanogaster (Redd et al., 2004). These developmental events are thought to be regulated by organism-intrinsic cues; i.e., the external environment does not instruct them. Given its robust functionality throughout phyla, and analogy to developmental mechanisms, it is intuitive (and in fact common) to regard wound repair as an intrinsically guided, postdevelopmental program. Yet, it remains unclear whether the conserved function of wound repair derives from an insulation against extrinsic influences, or an adaption to them. The question arises whether epithelial wound detection and repair are obligatory tissue-intrinsic processes, or if they also integrate information from the environment.

Zebrafish is a powerful system to study wound responses and their possible environmental adaption in the intact animal (Redd et al., 2004; Huttenlocher and Poznansky, 2008; Richardson et al., 2013). The tail fin fold of 2–4-d-old zebrafish larvae is a double-layered epithelium consisting of a basal epithelial layer that is attached to a basal lamina, and a suprabasal layer in which cells are connected by adherens and tight junctions (Fig. 1 a; Sonawane et al., 2009). This stratified skin fold protects the inside of the zebrafish (∼270–300 mOsm, i.e., common vertebrate extracellular tonicity) from its natural hypotonic freshwater environment (∼10 mOsm), analogous to the stratified linings of mouth and esophagus, which protect mammalian tissues from hypotonic saliva (∼30 mOsm). The thinness and transparency of zebrafish tail fins facilitates interrogation of tissue damage detection mechanisms through pharmacologic/genetic perturbations and intravital microscopy. Using the zebrafish tail fin wounding assay, we previously demonstrated that a drop in interstitial osmotic pressure initiates eicosanoid-mediated leukocyte recruitment (Enyedi et al., 2013). In the present study, we asked whether osmotic signaling is an environmental master regulator of wound responses by examining its potential involvement in epithelial repair.

Results

Environmental hypotonicity triggers rapid wound closure in zebrafish larvae

To test for a role of external tonicity, we imaged wound closure in larval zebrafish tail fins after UV laser wounding of fish
Figure 1. A transepithelial osmotic pressure gradient is required for rapid wound closure and barrier reconstitution of zebrafish tail fin wounds. (a) Simplified scheme of larval zebrafish tail fin epithelium ~3 dpf. Putative cell–cell contacts are indicated. (b, left) Representative time-lapse montage of zebrafish larvae immersed in hypotonic (Hypo) or isotonic (IsoNaCl, IsoSucrose) solutions at the indicated times after UV laser puncture injury. The actin cytoskeleton is labeled with GFP-Utr-CH. Bars, 50 µm. (b, right) Quantification of wound area as a function of time after injury. (c, left) Time-lapse montage of suprabasal AKT-PH-GFP (suprabasal) at 0′, 40′ (shift), and 90′. (c, right) Quantification of wound area as a function of time after injury. (d) Hypo + H2O2 and Iso + H2O2 oxidized area as a function of time after H2O2 addition.
immersed in either normal, hypotonic bathing medium or bathing medium that had been adjusted to the ionic composition and/or tonicity of vertebrate interstitial fluid (with the addition of NaCl or sucrose). The actin cytoskeleton and plasma membranes were labeled using GFP-utrophin-calponin homology domain (GFP-Utr-CH; Burkel et al., 2007), and AKT-pleckstrin homology domain (AKT-PH)–GFP (Kwon et al., 2007), respectively. Injection of mRNA into one-cell stage embryos led to ubiquitous labeling. In contrast, injection at the 4–8-cell stage gave rise to mosaic labeling of predominantly basal epithelial cells (Fig. S1 a). Basal epithelial cell labeling was also performed by injection of DNA constructs containing a fluorescent protein under the control of a basal cell–specific ΔNp63 promoter (Reischauer et al., 2009). Suprabasal labeling was achieved by expression via a keratin promoter (Gong et al., 2002). Several pulses of a micropoint laser (435 nm) were used to produce wounds on both sides of the epithelial fold. Importantly, these full-thickness wounds are unlikely to close by contraction of underlying structures, because those are ablated by the laser blast. In hypotonic fish bathing solution (standard E3 medium), closure of ~5,000-µm² puncture wounds was completed within ~20 min, i.e., ~5× faster than closure of similar sized lesions in Drosophila larvae (Geiger et al., 2011). Iso-tonicity (IsoNaCl or IsoSucrose) inhibited wound closure, with NaCl showing a more pronounced inhibition (Fig. 1 b and Video 1). Isotonic inhibition of wound closure was reversible (Fig. 1 c and Video 2). We also tested whether isotonicity blocks restoration of barrier function. To this end, we amputated the tail fin tips of transgenic zebrafish larvae ubiquitously expressing a genetically encoded, reversible fluorescent H2O2 reporter (HyPer; Belousov et al., 2006) in isotonic medium. After the endogenous, injury-induced HyPer signal (Niethammer et al., 2009) had subsided, the Tg(actb2:HyPer) transgenic fish were mounted in isotonic agarose and overlaid with isotonic or hypotonic solution supplemented with H2O2. Intact tail fin skin is impermeable to both H2O and H2O2, H2O2 in the bathing solution probes wound permeability by eliciting a HyPer signal upon entering the fish. This signal was sustained upon isotonic, but not hypotonic, bath exposure, which is consistent with delayed barrier recovery in isotonic solution (Fig. 1 d and Video 3). Similar to the initial inflammatory response (Enyedi et al., 2013), rapid repair of zebrafish tail fin wounds depends on the osmotic difference between the freshwater environment and the interstitial fluid of the fish.

Environmental hypotonicity triggers migration of basal epithelial cells
Wound margin contraction through an actin cable (Martin and Lewis, 1992; Bement et al., 1993) or epithelial cell migration are the most plausible mechanisms for rapid wound closure in our model (see Discussion). To experimentally define the processes underlying isotonic inhibition of closure, we imaged the morphological responses of epithelial cells by expressing fluorescent markers for actin, myosin, and plasma membrane in different epithelial layers. Time-lapse videos revealed rapid formation of lamellipodia in the basal, but not the suprabasal, layer after injury in hypotonic solution (Fig. 2, a and b; Fig. 3 a; and Video 4). Notably, migrating cells that had arrived at the wound margin abruptly ceased migration, and underwent a drastic morphological change that involved cell rounding, apparent shrinkage, and extension of phosphatidylinositol (3,4,5)-trisphosphate (PIP3)-positive fingerlike protrusions (Fig. 2 a and Video 4). By terminating cell migration at the cell margin, and making room for subsequent rows of cells (through compaction of wound margin cells), this process may contribute to the orderly and sequential advancement of migrating basal cells toward the wound. Propagation of lamellipodia formation distal to the wound was strongly inhibited by isotonicity (IsoNaCl or IsoSucrose), with NaCl having a slightly more pronounced effect (Fig. 3 a and Fig. S1 b). Suppression of lamellipodia formation by isotonicity or cytochalasin D was paralleled by inhibition of basal cell translocation (Fig. 3 b) and wound closure (Fig. S2 a). Both the morphological transition of basal cells (see above) to a rounded shape at the wound margin and the propagated wave of basal cell crawling were absent in isotonic wounds, while fingerlike protrusions positive for PIP3 could be observed at the basal cell margin.

We could detect recruitment of actin and myosin II at the wound margin by imaging ubiquitously expressed GFP-Utr-CH (Fig. 1 b) and mKate2-labeled myosin regulatory light chain (MRLC-mKate2; Fig. 3 c), or suprabasally expressed GFP-Utr-CH (Fig. S1 c). Together, these data indicate the formation of a “purse string” (Martin and Lewis, 1992; Bement et al., 1993) in the suprabasal layer. We cannot formally exclude purse string formation in the basal epithelial sheet, but our experiments do not bear evidence for it. Unlike at the rounded suprabasal margin (Fig. 2 a, white arrow; and Video 4), fingerlike protrusions appear to predominate at the basal wound margin as mentioned above (Fig. 2 a, yellow arrow; and Video 4). Actin and myosin recruitment to the wound margin seemed little affected by tonicity (Figs. 1 b and c and 3 c). Likewise, wound margin rounding, which is indicative of a functional purse string, was visible in isotonicity (Fig. 1, b and c; and Videos 1 and 2). Thus, spatially separated wound closure mechanisms (i.e., suprabasal purse string contraction and basal cell migration) mediate rapid wound closure in zebrafish larvae.

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motility in response to hypotonic exposure, we decided to directly measure wound-induced epithelial sheet motility by PIV analysis of the suprabasal layer (Fig. 4a).

tissue, and their spatial coordination, as well as their mutual interactions into a single endpoint measurement. To obtain more specific information about the mechanisms that initiate cell

Figure 2. Epithelial cell layers within the larval zebrafish epidermis exhibit distinct morphological wound closure mechanisms. (a) Time-lapse images of a representative 2.5–3-dpf zebrafish larva at the indicated times after UV laser-induced injury. (a, top) Transgenic Tg(krt4:AKT-PH-GFP) expression of AKT-PH-GFP driven in the suprabasal cell layer (green) is observed simultaneously with mosaic AKT-PH-mKate2 in underlying basal epidermal cells (4–8-cell-stage mRNA injection; red) after puncture wounding in hypotonic E3 medium. Basal cells form lamellipodia and translocate collectively toward the wound, whereas the suprabasal cells translocate and elongate without visible lamellipodia. Note that basal cells at the margin can protrude across the wound opening (yellow arrow), whereas suprabasal cells at the margin align to form a smooth wound edge indicative of contractile “purse string” closure (white arrow). Basal and suprabasal cells maintain a largely consistent proximity; representative center of mass tracks for basal (yellow) and suprabasal (white) cells are shown (10’, top right panel). Bars, 50 µm. (a, bottom) Enlargement of a region in the top panel; basal cell (yellow x) and neighboring suprabasal cell (white x) correspond to upper tracks in top right panel. Bars, 25 µm. All images are from a partial z projection to capture an individual epidermal bilayer. See Video 4. (b) Representative images of a 2.5–3-dpf zebrafish larva mosaically expressing GFP under the control of a basal cell–specific ΔNp63 promoter, immersed in hypotonic bathing solutions shown at indicated times after UV laser cut injury. Broken white line, position of wound. Broken yellow lines, outlines of representative lamellipodial protrusions. Bars, 25 µm.
Some residual motion remained after isotonic injury (Fig. 4 b, red curve), which was further suppressed by preincubation with the Rho kinase inhibitor Y27632 (Fig. 4 b, green curve), a compound known to abrogate purse string formation (Abreu-Blanco et al., 2011). Interestingly, Y27632 prolonged sheet motion after hypotonic but not isotonic injury (Fig. 4 b, compare blue and gray curves), and appeared to interfere with coordination of tissue movements, at least to some degree. Specifically, closure movements appeared less regular, and not as concentrically balanced around the wound as in the control (Video 5). However, it remains unclear whether this effect is caused by the abrogation of the

Tissue velocity was measured as a function of time after injury (global PIV; Fig. 4 b) and/or distance from the wound (spatial PIV; Fig. 4 c). Averaging the motion profiles of multiple animals permitted quantitative comparison of dynamic “tissue-motion phenotypes” after experimental perturbations. Spatial PIV revealed that tissue motion spreads ~150–200 µm away from the wound after hypotonic injury (Fig. 4 c), which is consistent with the length scale of basal cell translocation (Fig. 3 b). Highlighting statistical significant differences by subtracting hypotonic and isotonic velocity maps (Fig. 4 c) showed that isotonicity reduced amplitude and spatiotemporal spreading of motion through the tissue, in line with our morphological observations. Some residual motion remained after isotonic injury (Fig. 4 b, red curve), which was further suppressed by preincubation with the Rho kinase inhibitor Y27632 (Fig. 4 b, green curve), a compound known to abrogate purse string formation (Abreu-Blanco et al., 2011). Interestingly, Y27632 prolonged sheet motion after hypotonic but not isotonic injury (Fig. 4 b, compare blue and gray curves), and appeared to interfere with coordination of tissue movements, at least to some degree. Specifically, closure movements appeared less regular, and not as concentrically balanced around the wound as in the control (Video 5). However, it remains unclear whether this effect is caused by the abrogation of the

Figure 3. A drop in interstitial osmotic pressure after injury stimulates basal cell migration. (a) Representative images of 2.5–3-dpf zebrafish larvae mosaically expressing AKT-PH-GFP in basal cells (4–8-cell-stage mRNA injection), immersed in hypotonic or isotonic bathing solutions at the indicated times after UV laser cut injury. Broken white lines, wound margin. Yellow x, representative cell developing a lamellipodium (yellow dotted line) in response to a wound-induced exposure to hypotonicity. (b) Quantification of basal cell migration in zebrafish larvae mosaically labeled by one-cell stage actb2:AktPH-GFP DNA injections. Cell migration was tracked for ~19 min after wounding in hypotonic medium (Hypo), isotonic medium (Iso), and hypotonic medium supplemented with 100 µM cytochalasin D (Hypo CytoD) to probe actin-dependent motility, DMSO control (Hypo DMSO), isotonic medium supplemented with 100 µM cytochalasin D (Iso CytoD), or Iso DMSO control (Iso DMSO). Mean basal cell displacement as a function of initial distance to the wound is shown for n = 123 cells/8 tail fins (Hypo), 114 cells/8 tail fins (Iso), 62 cells/5 tail fins (Hypo DMSO), 86 cells/6 tail fins (Hypo CytoD), 79 cells/7 tail fins (Iso DMSO), and 122 cells/8 tail fins (Iso CytoD). Error bars indicate SEM. (c) Representative images of myosin II recruitment 2 min after UV-puncture injury in hypotonic (left) or isotonic (right) medium. Myosin recruitment is visualized by mKate2-labeled myosin regulatory light chain (one-cell stage mRNA injection). Bars: (a, main panels) 50 µm; (a, insets) 10 µm; (c) 50 µm.
Figure 4. Quantitative analysis of wound-induced epithelial sheet movement by PIV. (a) Representative example of PIV analysis (PIVlab, MATLAB) of epithelial sheet movement after UV puncture injury of a Tg(krt4:AKT-PH-GFP) larva exhibiting plasma membrane labeling in the suprabasal layer. Green arrows, velocity vectors derived by comparing particle movements between subsequent frames. Red area, extra-tissue area excluded from analysis. Bar, 50 µm. (b) Global PIV analysis of UV-puncture wounded Tg(krt4:AKT-PH-GFP) larvae immersed in solutions of indicated composition (Y = Y27632, Rho-kinase inhibitor, 100 µM). The graph displays the mean of all velocity vector magnitudes within the unmasked field of view as a function of time after injury. (c) Spatial PIV analysis representing the Iso and Hypo datasets from b as spatially resolved 3D plots. (i and ii) Averaged spatial PIV plots of the indicated number of Tg(krt4:AKT-PH-GFP) larvae after UV-laser puncture injury in hypotonic or isotonic medium. Tissue velocities are color-coded (blue to green to yellow to red), and represented as a function of time after injury (x axis) and distance from injury site (y axis). (iii) Differential plot derived by subtraction of the indicated velocimetry plots, and t test filtering of statistically significant differences between groups (unpaired t test, P < 0.05 = significant difference). Statistically significant velocity differences are color-coded (turquoise to pink). Pink, positive values. Turquoise, negative values.
purse string, or decreased tissue tension in general. The fact that lamellipodial cell migration occurs only in the basal layer, but that motion is detected in the suprabasal layer even when the purse string is inhibited, suggests that the layers are mechanically coupled (e.g., desmosomes; Fig. 1 a; Sonawane et al., 2009) and that movement is largely promoted by the basal cells (Fig. S2 b). Supportive of this idea, basal and superficial cells maintain relative positions during movement (Fig. 2 a and Video 4). We therefore conclude that the superficial layer is dragged toward the wound primarily by osmotically induced basal cell migration, and potentially assisted (e.g., through spatial coordination) by an intrinsically triggered purse string contraction of the suprabasal wound margin.

Environmental hypotonicity stimulates wound-induced ATP release

Next, we investigated the mediators of basal cell migration after hypotonic injury. We previously showed that osmotically induced arachidonic acid release stimulates leukocyte migration to tail fin wounds (Enyedi et al., 2013). Arachidonic acid produced sporadic increases in plasma membrane activity (“wobbling”) of the basal cells, but unlike hypotonic solution, it did not induce wound-polarized lamellipodia or directional cell migration after isotonic injury (Video 6). This suggests that the mechanisms of rapid epithelial defense and repair diverge downstream of the shared osmotic trigger.

Many cells respond to osmotic swelling with nucleotide secretion (Hoffmann et al., 2009). Nucleoside triphosphates (NTPs), such as ATP, are present in the cytoplasm of all cells at high concentrations ([ATP] ~5 mM; Beis and Newsholme, 1975). Vesicular NTP concentrations can be an order of magnitude higher ([ATP] ~90 mM; Johnson, 1988). NTPs may enter the interstitial space via cell lysis, exocytosis, or nucleotide conducting pores. In mammalian cell culture wound healing models, ATP has been found to mediate cell migration via P2Y2 receptor signaling (Yin et al., 2007; Block and Klarlund, 2008; Boucher et al., 2010). It is unclear if similar mechanisms are operant in vivo.

To test for osmotically induced ATP release, we wounded larvae in isotonic medium supplemented with firefly luciferase and luciferin. This experimental setup generates luminescence when ATP is released from the fish into the bathing medium. Detection of ATP by firefly luciferase is highly specific (Moyer and Henderson, 1983). The membrane-impermeable DNA-specific dye SYTOX Orange, which enters cells upon plasma membrane damage, was added to highlight the wound margin and monitor cell lysis. Over an isotonic incubation period of 10 min (unpublished data), or 20 min after tail fin tip amputation, there was no detectable ATP secretion at the wound margin (Fig. 5 a, left; and Fig. 5 b, kymograph between ~20’ and 0’). After overlaying the isotonic mounting agarose with a bolus of hypotonic medium, the time required for toxicity equilibration largely depends on the agarose/medium volume ratio. In this experimental setup, we typically observed the first physiological responses, including wound margin compaction and leukocyte recruitment, at ~10–20 min after hypotonic shifting. Dilution of isotonic bathing medium triggered luminescence flashes at the wound margin (Fig. 5 a, right, green; and Fig. 5 b, kymograph, ~30’–80’) after a 16 ± 3 min (mean ± SEM) lag period. Notably, control experiments revealed that light emission by luciferase itself is quenched ~2x by isotonic solutions (Fig. S3 a).

However, given the typical amplitudes of ATP flashes and the detection sensitivity of our assay, the complete absence of flashing activity during isotonic incubation cannot be explained by luminescence quenching (Fig. S3 b). Consistent with previous findings (Enyedi et al., 2013), hypotonicity did not considerably increase cell lysis (SYTOX Orange staining; red in Fig. 5 a; and quantified in Fig. 5 c and Video 7). We conclude that a drop in interstitial osmotic pressure triggers nonlytic ATP secretion from wounded tail fins.

Extracellular nucleotide hydrolysis regulates the range and duration of epithelial cell recruitment

Owing to rapid extracellular hydrolysis by nucleotidases (e.g., ecto-NTP diphosphohydrolases [ENTPDs]; Zimmermann et al., 2012), extracellular ATP half-life in intact tissues is extremely short (ranging from seconds to milliseconds), starkly contrasting its approximately minute-scale half-life in cell culture (Orriss et al., 2009, 2013). Thus, increasing ATP half-life through ENTPD inhibition presents a sensitive method to probe the physiological role of NTPs as regulators of the wound response in vivo. We identified entpd3 as the most abundant entpd isoform in the basal epidermis by FACS sorting of basal epidermal cells expressing GFP under the Δnp63 promoter (Reischauer et al., 2009), and semiquantitative RT-PCR (Fig. S4, a and b). Analogously, mammalian ENTPD3 is expressed in wet epithelia of the digestive tract (e.g., stratified linings of the esophagus; Lavoie et al., 2011). For genetic interference, we designed a translation-blocking morpholino (entpd3 MO1) and a splice-blocking morpholino (entpd3 MO2) against zebrafish entpd3. entpd3 MO1 produced no major morphological defects besides cardiac edema (Fig. S4 c). entpd3 MO2 produced a truncated entpd3 mRNA, which exhibited no gross morphological defects (Fig. S4 c). Both morpholinos had no obvious morphological effects on the tail fin epithelium (Fig. S4 c). To chemically interfere with extracellular nucleotide hydrolysis, we used adenosine 5’-[γ-thio] triphosphate (ATPγS; a slow-hydrolyzing ATP analogue that, owing to its structural analogy to ATP, is expected to competitively inhibit all ATP hydrolyzing enzymes in the extracellular space) and the ENTPD subgroup-selective antagonist polyoxometalate (POM)/compound 7 (Müller et al., 2006).

Entpd3 knockdown by both morpholinos consistently increased the range and duration of wound-induced epithelial sheet motion (Fig. 6, a and b; and Fig. S4 d). This motion phenotype could be partially rescued by co-injecting entpd3 MO1 with morpholino-resistant entpd3 mRNA (Fig. 6 b, blue vs. red curve), arguing against an off-target effect. Enhanced tissue motion was not observed upon injection of an entpd3 MO1 five-nucleotide mismatch control morpholino (MO1 5 mm), excluding nonspecific morpholino effects (Fig. 6 c). Wound-induced epithelial sheet motion was blunted by the addition of apyrase to the fish bathing medium (Fig. 6 d). The genetic
Importantly, these dramatic epithelial migration phenotypes were not induced by pharmacological inhibitor treatment per se, but only in conjunction with hypotonicity (Fig. 6, f and g). This is consistent with the idea that hypotonic, but not isotonic, injury leads to the release of a pro-migratory signal that is NTPase sensitive. Interestingly, although inhibition of interstitial NTP breakdown generally increased sheet motion, aberrant motion typically did not lead to faster wound closure. Rather, it tended to antagonize the normal spatially and highly coordinated, concentric closure movements. Not unexpectedly, efficient wound closure likely requires both epithelial sheet motility (in this case driven by lamellipodia formation in the basal epithelial layer) and proper spatial coordination of collective cell migration.

Results were corroborated by the pharmacologic inhibition data. As expected, ATPγS produced a dramatic increase in wound-induced tissue motion, which is consistent with its ability to compete with extracellular ATP hydrolysis in general (i.e., not only with ENTPD-mediated hydrolysis; Fig. 6, f and g). POM produced a similar, though less pronounced, motion phenotype (i.e., prolonged epithelial migration), which is consistent with its more restricted target selectivity (as compared with ATPγS; Fig. 6 g). As expected, POM and ATPγS (Video 8) promoted lamellipodia formation in cells far from the wound margin in hypotonicity. The migratory response that ATPγS elicited in hypotonic solution was often so massive that it globally distorted the tissue structure, producing sample drifts (which were corrected by computational image registration when possible; Video 8). Importantly, these dramatic epithelial migration phenotypes were not induced by pharmacological inhibitor treatment per se, but only in conjunction with hypotonicity (Fig. 6, f and g). This is consistent with the idea that hypotonic, but not isotonic, injury leads to the release of a pro-migratory signal that is NTPase sensitive. Interestingly, although inhibition of interstitial NTP breakdown generally increased sheet motion, aberrant motion typically did not lead to faster wound closure. Rather, it tended to antagonize the normal spatially and highly coordinated, concentric closure movements. Not unexpectedly, efficient wound closure likely requires both epithelial sheet motility (in this case driven by lamellipodia formation in the basal epithelial layer) and proper spatial coordination of collective cell migration.
Figure 6. Epithelial movements in response to hypotonic injury are regulated by extracellular NTP hydrolysis. (a, i and ii) Spatial PIV analysis of the indicated number of Tg(krt4:AKT-PH-GFP) larvae subjected to UV laser cut injury in hypotonic medium, with or without translation morpholino-mediated
ATP reconstitutes cell migration in the absence of environmental hypotonicity

To mimic transient ATP concentration peaks that cells in a tissue may experience immediately after nonlytic ATP release by exocytosis or channel conductance, we supplemented isotonic bathing solution with cytoplasmic levels of ATP. This strongly stimulated basal cell migration in isotonicity as indicated by induction of lamellipodial protrusions toward the wound, and velocimetry analysis (Fig. 7, a and b; and Video 9). As expected, the response was abrogated by pre-incubating the ATP solution with apyrase before adding it to the larvae (Video 9). This excludes the possibility that contamination of the ATP solution stimulated motility. UTP, like ATP, stimulated basal cell motility (Fig. S5 a). The finding that physiologically relevant extracellular concentrations of ATP or UTP can promote epithelial cell motility after isotonic wounding is consistent with secreted NTPs providing a wound-relevant chemokinetic signal. This also shows that isotonicity blocks a migratory stimulus for epithelial cells, but not their general ability to migrate. Although UTP potently induced basal cell migration, our data do not determine whether it is actually released, as luciferase is highly specific for ATP, but not UTP (Moyer and Henderson, 1983). UTP and ATP can be rapidly interconverted by ecto-nucleoside diphosphokinases in the extracellular space of tissues (Lazarowski et al., 1997).

The NTP sensing mechanism underlying basal epithelial responses in our system remains unclear. Our current data indicate that it differs from previously reported purinergic mechanisms involved in wound repair in vitro. Suramin, a nonspecific P2 receptor inhibitor commonly used to block P2Y2, had little effect on rapid wound closure, even at high concentrations (Fig. S5 b). The agonist profile of basal epithelial migration in our system does not match known P2 receptor profiles (Table S1 and Videos 9 and 10). Furthermore, tail fin wound closure occurs much faster than P2Y2-dependent monolayer healing in cell culture. Phylogenetic variations of P2Y specificity or formation of receptor heterodimers may account for this noncanonical behavior. Alternatively, unknown nucleotide receptors may exist. The unexpected discovery of novel ATP receptors in plants, which do not bear sequence similarity to traditional P2 receptors (Choi et al., 2014), underlines this possibility. Our ongoing efforts are directed toward identifying the molecular specifics of NTP release and sensing in our model.

Discussion

Purse string contraction of the wound margin and basal cell sheet mechanisms are the two most plausible wound closure mechanisms that are relevant to our model. Alternative wound closure phenomena, such as cell or matrix contraction, are unlikely to contribute because of the rapid nature of the wound closure and full thickness loss of cells and matrix in the wound region. Although we observe fingerlike protrusions at the wound margin, the almost perfectly circular shape of the wound during closure and its remarkably rapid kinetics argue against a slower zippering mechanism.

Pharmacological Rho kinase inhibition reveals that our laser puncture wounds largely close in the absence of a purine string, albeit not as well coordinated as in the control samples and with a higher incidence of “jagged” and deformed wound margins. Hence, the contractile actin cable cannot be the major mediator of osmotically stimulated wound closure. Likewise, our data do not bear evidence that purinergic formation or contraction depends on osmotic cues. The actin cable still forms and rounds up at the wound margin after isotonic injury (Fig. 1 b), which indicates intrinsically stimulated local force generation at the margin even in the absence of the hypotonic trigger. In contrast, we find that lamellipodia formation, migration of basal epithelial cells, and epithelial sheet movement are strongly inhibited by environmental isotonicity. Collectively, this argues that rapid wound closure is mainly mediated by hypotonicity-induced basal cell migration in our model, although basal cell-specific inhibition of lamellipodial migration would be required to test the contribution of the basal cells to wound closure more directly. To our knowledge, this is the first study delineating different wound closure mechanisms in a complex, bilayered epidermis. Intriguingly, contractile- and actin polymerization-mediated modes of wound closure appear to be spatially separated between different tissue layers, and show different environmental sensitivity. In motile cells, Rho-driven myosin contractility and Rac-driven actin polymerization are believed to antagonize each other, requiring spatial front-back separation to allow coordinated movements. Spatial separation of these mechanisms within multicellular structures may facilitate coordinated tissue movements during wound closure.

Consistent with preceding cell culture studies (Praterios and Leipziger, 2009), we find that environmental hypotonicity induces nucleotide release at the wound site, most likely through...
nonlytic cell swelling. Notably, our luciferase/luciferin bathing technique is limited by diffusion of luciferase (dimer mol wt $\sim$120 kDa) and luciferin from the bathing medium through the open wound into the tissue. While highlighting the fraction of ATP directly released into the bathing solution, our current measurements may underestimate the actual ATP release pattern due to the limited availability of luminescence-generating enzyme/substrate inside the tissue. Future studies, e.g., with transgenically expressed, membrane-bound luciferase, may allow measurement of ATP release deeper within the tissue.

The mechanisms of swelling-induced ATP release in cells are incompletely understood even in simplified cell culture systems. Besides osmotic cell lysis, hemi-channel conduction (e.g., via connexins or pannexins) and vesicular NTP release appear to be important. Our current results do not argue for cell lysis being a mediator of wound-induced ATP release. Whether any of the other known nonlytic mechanisms accounts for wound-induced ATP release in zebrafish tail fins remains to be investigated.

If extracellular NTPs are endogenous mediators of basal cell migration in our model, epithelial sheet motion should be inhibited by increasing extracellular NTP breakdown. Likewise, decreasing extracellular ATP hydrolysis should enhance epithelial sheet motility. Collectively, our genetic and pharmacologic perturbations of extracellular nucleotide metabolism, including morpholinoo-mediated knockdown of the most abundant ENTPDase of basal epithelial cells, (entpd3), confirm these predictions. ATP bathing triggers basal cell migration, but does not fully reconstitute hypotonic wound closure. A possible explanation for this could be that ATP bath application is unlikely to recapitulate the endogenous, spatiotemporal ATP release pattern, which may be crucial, e.g., for proper spatial coordination of collective movements. In addition, ubiquitous exposure of larvae to high ATP concentrations on the outside, apical surface of suprabasal cells may adversely affect the behavior of the suprabasal layer during closure. Finally, our data do not exclude that there are other unknown, hypotonically triggered processes, in addition to NTP release, that contribute to rapid wound closure.

In this study, we have identified an osmotic signaling circuit that initiates extracellular ATP release in response to a drop of interstitial osmotic pressure. This autoregulatory mechanism adjusts wound responses to wound size by coupling basal cell migration, but does not fully reconstitute hypotonic wound closure. A possible explanation for this could be that ATP bath application is unlikely to recapitulate the endogenous, spatiotemporal ATP release pattern, which may be crucial, e.g., for proper spatial coordination of collective movements. In addition, ubiquitous exposure of larvae to high ATP concentrations on the outside, apical surface of suprabasal cells may adversely affect the behavior of the suprabasal layer during closure. Finally, our data do not exclude that there are other unknown, hypotonically triggered processes, in addition to NTP release, that contribute to rapid wound closure.

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Figure 7. ATP reconstitutes basal cell migration and epithelial sheet movement in the absence of a transepithelial osmotic gradient. (a) Representative time-lapse images of zebrafish larvae exhibiting mosaic plasma membrane AKT-PH-mKate2 labeling of predominately basal cells (4–8-cell-stage mRNA injection). Larvae were subjected to UV-laser-cut wounding in isotonic mounting agarose. After 10 min of isotonic preincubation (red time indices), a bolus of isotonic solution ± 5 mM ATP was added to the imaging dish. Yellow x, representative morphological response after addition of isotonic solution ± 5 mM ATP. Note that formation of AKT-PH-mKate2–rich membrane protrusions [yellow broken line] after iso-iso/ATP, but not iso-iso shifting. The same representative iso-iso control and data set were used in Fig. S5 a. See also Video 9. Bars: (main panels) 50 µm; (inset) 10 µm. (b) Global PIV analysis of the indicated number of larvae exhibiting ubiquitous plasma membrane labeling (one-cell stage AKT-PH-mKate2 mRNA yolk injection). Larvae were subjected to UV-laser-cut wounding in isotonic mounting agarose. After 10 min of isotonic incubation, a bolus of isotonic medium ± 5 mM ATP was added to the sample. (c) Proposed circuitry scheme of tissue intrinsic and environmentally triggered branches of the wound response in zebrafish tail fins. Tissue-intrinsic mechanisms include purse-string contraction (not depicted). Environmentally dependent osmotic surveillance through secretion of nucleotides (epithelial cells) and eicosanoids (leukocytes) is depicted.
Epithelial cell tracking and morphometric analyses

Spinning disk confocal time-lapse stacks of wounded tail fins mosaically expressing PH-AKT-GFP in the basal cells were z-projected (maximum-intensity projection), and brightness and contrast enhanced in Fiji (an ImageJ package). The center of mass of basal cells in the imaging field was tracked using the MTrackJ plugin of Fiji. Linear cell displacement (D) was approximated as D = D(x) = \sqrt{D(x)^2 + D(y)^2}.

Distance between center of mass of a cell and the center of mass of the wound area either 0 min or 19 min after injury. We found this type of measurement to be more robust with regards to occasional sample drifts, as displacement is expressed in relation to wound center of mass. This calculation assumes that epithelial cells migrate roughly linearly on the shortest path to the wound, which is a good approximation for most epithelial cells on the fish. The displacement distance of these cells was plotted as a function of binned (12 µm), initial wound distance of these cells (D(x,y)).

Velocity analysis

Spinning disk confocal time-lapse stacks of wounded tail fins expressing PH-AKT-GFP (or PH-AKT-mKate2) in the suprabasal epithelial layer (Tg(krt4:AKT-PH-GFP) or Tg(krt4:AKT-PH-mKate2)) were z-projected (maximum-intensity projection), convoluted (3 x 3 x 1 matrix, Fiji “Convolve” command), background subtracted, and contrast enhanced in Fiji. Tissue velocities were measured by calculating particle motion between subsequent images of the time lapse using MATLAB (R2010b; MathWorks) with the open source PIV analysis software PIVlab v1.32 (developed by W. Thielicke and M. Stamhuis). In brief, the program split each frame into 63 x 63 interrogation windows for which the respective displacement vectors between subsequent frames were calculated. This yielded a 63 x 63 velocity vector matrix that was masked to eliminate regions of low image intensity. The masked PIVlab-generated velocity vectors were either 1 averaged within the program to generate global velocity (averaging of all velocity vectors magnitudes in the unmasked field of view) as a function of time after injury, or (2) matrices were further analyzed (spatially resolved) with self-written MATLAB functions to express particle velocity magnitude (|v|) as a function of binned distance (10 µm bins) of the respective PIV-interrogation window from the wound margin (Dmargin), and time after injury. For puncture wounds, the wound region was computationally determined in each image using an edge detection algorithm. The Euclidian distance (D) of the PIV-interrogation window from the center of mass of the detected wound area was determined. For puncture wounds (i.e., roughly round wounds), wound margin distance of each PIV-interrogation window was approximated as Dmargin = |D - r_b|, with r_b being the radius of a circle with the same area as the computationally determined wound region. For laser cut wounds, a polygonal region of interest was manually selected from the last frame of the timelapse stack to outline the wound area. Then, Dmargin was calculated as minimal distance of the PIV-interrogation window from any of the polygon’s ribs. Occasionally, fluorescent cell debris interfered with accurate edge detection. To remove bright spots, time-lapse stacks were post-processed using the freehand selection and flood filling tool (Fiji), and then further analyzed in MATLAB as usual. Tonicity shifting experiments typically caused unspecific bulk movements of the specimens due to liquid addition. To eliminate velocity components caused by unspecific bulk
movements, these time-lapse stacks were registered ("StackReg-> Rigid Body" command; Fiji) before PIVlab analysis.

This normalization enabled averaging of spatiotemporal tissue motion profiles over multiple animals, and comparison between different experimental groups. For statistical comparison, averaged velocity maps of different experimental groups were subtracted as indicated, and the corresponding (i.e., with regards to distance from wound margin, and time after injury) velocity values of different experimental groups were tested for significant differences using an unpaired Student’s t-test (i.e., P < 0.05). All statistical insignificant velocity differences (P > 0.05) in the subtraction map were set to zero to highlight only statistically significant differences.

Means of global velocity vector magnitudes for time-lapse frames were extracted from PIVlab, averaged in Excel, and plotted using MATLAB. The differences in velocity ranges among different sets of experiments were tested for either different treatment methodologies or the type of wound (amputation, cut, or puncture) required to deliver selected compound into the tissue. For example, wounding regimes requiring isocitric to hypotonic shifting to deliver cell-impermeable compounds exhibit velocity ranges that differ from wounding regimes that deliver cell-permeable compounds where shifting was not used. Amputation wounds to deliver larger molecules (e.g., apyrase) also have different velocity profiles than cut or puncture wounds.

H2O2 barrier reconstitution assay
To measure epithelial barrier integrity, Tg(actb2:HyPer) larvae were used to visualize the penetration of exogenous H2O2, a molecule with similar physicochemical properties as H2O2, through the wound into the cytosol of the cells in the tail fin. The 2.5–3-dpf larvae were subjected to tail fin tip amputation in 1% Iso NaCl using a needle knife (Fine Science Tools), and were embedded in a small volume of 1% isocitric low-melting agarose (~300 µl) in a glass-bottom dish (MatTek Corporation) 1 h later. This waiting period was required for the endogenous, wound-induced H2O2 signal to diminish, allowing the consecutive measurement to assess the effect of exogenously added H2O2. At least a 10x agarose volume equivalent of standard-E3 (hypotonic) or IsoNaCl-E3 (3–4 ml) containing 1 mM H2O2 was added on top of the agarose pad when the acquisition started. Every minute, HyPer fluorescence was excited using LED light and 438/57 and 475/28 excitation filters (Lumencor). Emission was acquired using a 535/30 emission filter (Chroma Technology Corp.). Images were acquired at room temperature (~26°C) using NIS-Elements (Nikon) on a microscope (Eclipse Ti; Nikon), equipped with a 20× Plan-Apochromat NA 0.75 air objective lens, an Andor Clara charge-coupled device (CCD) camera, and a motorized stage. Hyper ratio images ([E500/E420] were created by dividing median brightness of the background and background subtracted YFP500 and YFP420 images using Fiji, as described previously (Niethammer et al., 2009; Enyedi and Niethammer, 2013). Oxidized tissue surface area was measured using Fiji by thresholding as described previously (Niethammer et al., 2009; Enyedi and Niethammer, 2013). Oxidation tissue surface area was measured using Fiji by thresholding for HyPer ratio values over 0.64.

Luminescence imaging
Zebrafish tail fin cats were performed on anesthetized 2.5–3-dpf larvae in a glass-bottom dish with a drop (~150 µl) of isotonic fish water (IsoNaCl-E3) supplemented with firefly luciferase (~1.7 mg/ml) and luciferin (100 µM), MgSO4 (10 mM), and SYTOX Orange (~7 µM) using a needle knife. For larval mounting, 100 µl of warm 1.5% low-melting agarose (Hypo-E3 or IsoNaCl-E3) was mixed into the drop containing the luminescence mix and the wounded larva. After agar solidification, the dish was transferred to an inverted microscope (Eclipse; Nikon) equipped with a 20× Plan-Apochromat NA 0.75 air objective lens, an Andor Clara CCD camera, an LED light source (Lumencor), and a motorized stage. During imaging, the dish was covered with an aluminum-foil covered plastic box to minimize environmental light exposure. Luminescence was acquired using a 525/30 emission filter (Chroma Technology Corp.) using 8 x 8 binning and a camera exposure time of 5 x. SYTOX orange fluorescence was excited using LED light and a 549/15 excitation filter (Lumencor), and its emission was acquired using a 632/60 emission filter (Chroma Technology Corp.). After imaging, isotonic wound responses for 10 or 20 min, a bolus (~3 ml) of hypotonic fish water (i.e., standard E3 + Tricaine) containing 10 mM MgSO4, 5 µM SYTOX Orange, and 150 µM sodium luciferin was added to the isotonic agarose drop to initiate the hypotonic wound response. Acquired image stacks were background subtracted, and contrast adjusted using the Fiji software (Schindelin et al., 2012). The luminescence kymograph was prepared by applying the Fiji “reslice” command on a line drawn along the wound margin (as visualized by SYTOX Orange staining). For presentation reasons, luminescence and fluorescence channels were color coded, and superimposed. All images were acquired at room temperature (~26°C) using the Fiji software (Schindelin et al., 2012).

response to hypotonicity, a circular region of interest (100 µm) was drawn around each individual ATP flash throughout the time-lapse (Fiji), to measure the intensity of luciferase luminescence and the local SYTOX orange fluorescence. The intensity measurements start one frame before, and end 1–2 frames after each luminescence peak (40 s per frame). Color-coded traces correspond to matching measurements of luminescence and fluorescence, respectively.

To test the effect of toxicity on light generation by luciferase, luminescence was measured in 5-µl drops of isotonic (145 mM NaCl) or hypotonic E3 (5 mM NaCl) supplemented with firefly luciferase (0.5 mg/ml), sodium luciferin (150 µM), and MgSO4 (10 mM), and indicated concentrations of ATP (10–100 µM). Luminescence was acquired using the same settings as above, but with a 4x Plan-Apochromat NA 0.2 air objective lens was used. Background of drop axes was acquired in a rectangular area outside the drop, and subtracted from the drop image. A rectangular region covering the center of the drop was measured to obtain the mean luminescence using Fiji. Three drops per ATP concentration were measured. Mean luminescence and standard deviation of triplets was plotted as a function ATP concentration, and toxicity (Fig. S3).

Cell sorting and semiquantitative RT-PCR
Basal cell sorting was performed by disaggregating ~200 Tg(lNp6:G4d, UAS-GFP) transgenic 2.5–3-dpf larvae into a single cell suspension as described previously (Bertrand et al., 2007). In brief, larvae were anesthetized using 0.2 mg/ml tricaine (Sigma-Aldrich) in E3, dissociated using Listerase TM (Roche; 13 U/ml, 15 min at 32°C), and disrupted mechanically with a pestle. Cell suspensions were passaged through a 40-µm nylon mesh and washed twice in FACS buffer (centrifugation at 250 g for 5 min). Cell sorting of GFP-positive cells was performed on a FACS (Aria III; BD) using 488-nm excitation and 530-500 nm emission wavelengths. mRNA was extracted with elago (di)25 Dyna Beads (Invitrogen), followed by cDNA synthesis with RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific), and PCR Phire Hot Start II DNA polymerase (Thermo Fisher Scientific) using the following gene primers (PCR cycle numbers referred to in Fig. S4 are indicated below):

entpd3_Fwd: 5′-ATAGTTCTTGGA-TGCGGCTC-3′, entpd1_rv: 5′-GGGGTTTCCGTGTTGCTG-3′ (33 PCR cycles).

entpd2a.1_fwd: 5′-ACATACAAA-GGGTCACAGGGCTT-3′, and entpd2a.2_rev: 5′-GTAGCTCTACGGCGCTTG-3′ (35 PCR cycles).

entpd2a.2 (ENSDARG0000003939), entpd2a.2_Fwd: 5′-CCATCAC-GTCTCAACGCGGATT-3′, and entpd2a.2_Rev: 5′-AAGAGACACCGCCGACAGG-3′ (33 PCR cycles).

entpd2b (ENSDARG00000044795), entpd2b_Fwd: 5′-GGAGACGAGATCCTTCTACACG-3′, and entpd2b_Rev: 5′-GGCTTGTAAGCTCTCTC-TAGTGT-3′ (41 PCR cycles).

entpd3.3 (ENSDARG00000035390), entpd3.3_Fwd: 5′-GGCTCTTCCGGA-TACGTCTGTA-3′, and entpd3.3_rv: 5′-CTGTTGCAAGCCGAGGAG-3′ (33 PCR cycles).

entpd8 (ENSDART00000011245), entpd8_Fwd: 5′-CTCTTCGAAACCGGACAGT-3′, and entpd8_Rev: 5′-ATCCCTGTGTAATCCGCTGC-3′ (35 PCR cycles).

tp63 (ENSDARG00000044356), tpc63_Fwd: 5′-AGCCAAAATCTCGCACAACCTGGAGCG-3′, and tpc63_Rev: 5′-AGGTGCCAGGGGCTTGCAT-3′ (33 cycles).

actb1 (ENSDART00000054987), actb1_Fwd: 5′-CCAGACATCAACAGG-AGTAGTGA-3′, and actb1_Rev: 5′-AGGAAGAAAGGCTGGCGAGAG-3′ (32 PCR cycles).

Pharmacological inhibitor treatments and morpholino injections
Cell-permeable pharmacological inhibitors were applied to 2.5–3-dpf zebrafish by preincubation for 60 min in standard E3 or isoionic E3iso, medium supplemented with the following compounds: Cytochalasin D (100 µM) or 1′-2763Z (100 µM). For UV laser wounding, larvae were mounted in 1% low-melting-point agarose (Hypo-E3 or IsoNaCl-E3) and overlaid with the respective inhibitor to containing the indicated concentration of antagonists and inhibitors. For inhibition experiments with POM (100 µM), ATP-5S (5 mM), or Suramin (1 mM), larvae were mounted, laser-wounded in 1% IsoNaCl E3 agarose supplemented with each compound, and incubated (10 min) with a drop of IsoNaCl-E3 placed at the interface between the dipping objective lens and mounted sample (containing the respective compound). The preincubation period was required for sufficient infusion of the water-soluble compound through the open wound (i.e., kept open with isoionic E3).
A 10x bolus of standard E3 or IsoNaClE3 medium supplemented with each respective compound was then added to the imaging dish. Apyrase (50 U/ml) inhibition was performed using the identical shifting regime described for POM, Suramin, or ATP-S, with the exception that tail fin amputation (needle knife; Fine Science Tools) was used to provide a larger opening for apyrase to infuse the tissue (osmolarity readings of the apyrase solutions were identical to the control solutions, ruling out salt contamination in the apyrase stock). For the in vivo analysis of ATP-S treatment, ATP-S was not administered in the agarose, but added to the interface drop and the shifting medium to prevent excessive drifting of the sample. Imaging was performed for the indicated time using a laser spinning disk confocal microscope (see “Confocal imaging and laser wounding”). Basal cell migration or suprabasal tissue movement were assessed for 20–30 min after shifting. Hydrophobic compounds were dissolved in DMSO, which was applied to samples at a maximal final concentration of 1%. 2.3 nl of 1 mM (~19 ng) translation-blocking morpholino (5′-GACTGGGACTCTTCTTATGACG-3′; MO1; Gene Tools, LLC), translation-blocking five-nucleotide mismatch control morpholino (5′-GAaTGGAaCTaCTATTaACG-3′; MO2; Gene Tools), targeting the exon3-intron3 of zebrashiftentpd3, were injected into the yolk of one-cell-stage embryos. For UV laser wounding, larvae were mounting in 1% low-melting-point agarose (HypoE3 or IsoNaClE3) and overlaid with the indicated medium, UV-cut-wounded, and imaged for the indicated time using a laser spinning disk confocal microscope (see “Confocal imaging and laser wounding”). To confirm knockdown efficiency of MO2, mRNA from 2.5–3-dpf old morphant larvae (n = ~20) were extracted using oligo (dT)25 Dyna Beads (Invitrogen). cDNA synthesis was performed with the ReverAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific), and PCR Phire Hot Start II DNA polymerase (Thermo Fisher Scientific), using the following primers: 5′-ATGACATAAGGAGTCCCAGTC-3′ and 5′-ATGATCAATAAGGAGTCCCAGTC-3′. PCR products were separated on a 1% low-melting-point agarose gel. The indicated samples were excised, and the mRNA revealed a 31-nt transcript following RT-PCR. The control samples were treated with 5 U/ml apyrase for 2–2.5 h at 28°C before adding to samples.

### References


