Review

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Bringing balance by force: live cell extrusion controls epithelial cell numbers

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To function as an intact barrier, epithelia must maintain constant cell numbers despite high rates of turnover. If the rate of death exceeds proliferation, epithelial barrier function could become compromised; if it lags behind proliferation, cells could amass into tumors. Although the balance between cell death and division is critical for preventing pathology, most studies focus on each process in isolation. Loss of contact inhibition is a hallmark of cancer cells and has suggested that cell contacts are important for linking rates of cell division and death. However, epithelial cells continuously divide and die while maintaining contacts with each other, so other factors must control this balance. Recent studies have found that cell-crowding forces from cell proliferation can drive cells to die by extrusion from the epithelium. Factors that alter this response to cell crowding may lead to barrier function diseases or promote hyperplasia and cancer.

Epithelial tissue homeostasis and contact inhibition

Epithelia consist of tightly adherent cells that coat and protect our organs and body. Cells within epithelia have some of the highest rates of turnover in the body [1–3], where the number of dividing cells is tightly balanced by a similar number of dying cells. Given that most cancers arise in cell populations with high turnover rates, such as the blood and epithelia, it is likely that they arise from misregulation of cell number homeostasis. Because most solid tumors originate from epithelia, understanding what controls the link between cell division and cell death in epithelia is critical to our understanding of how tumors initiate. However, most studies have focused separately on either what controls cell death in response to apoptotic stimuli or what controls cell division in response to mitogens. Remarkably few studies investigate how these two processes are coordinated *in vivo* to maintain overall cell numbers.

The studies most relevant to cell number homeostasis began over 60 years ago with the discovery of 'contact inhibition'. Contact inhibition refers to two separate fundamental findings: contact inhibition of growth and contact inhibition of locomotion. The former is based on the fact that cells dramatically reduce their rate of mitosis when they contact each other and establish a monolayer [4,5]. Contact inhibition of locomotion refers to the fact that migrating cells will stop moving once they contact each other to form a monolayer [6]. By contrast, cancer cells are not contact inhibited in either their growth or motility and will instead pile upon one another and continue dividing [7–9]. The absence of contact inhibition forms the basis for testing whether cells are transformed by their ability to grow in soft agar. But what does contact inhibition mean *in vivo* for epithelial cells that are continuously migrating and dividing, despite the fact that they must maintain tight contacts with each other to preserve their function as a barrier? How do epithelial cells adherent to one another in an epithelium maintain constant cell numbers?

Although establishment of cell contacts may not be sufficient to control cell numbers in epithelia, the extent of cell contacts with each other and their substratum may, instead, control whether cells divide or die. Contact inhibition of growth and migration *in vivo* may depend on cells reaching a threshold number of cell–cell or cell–matrix contacts, because both types of inhibition are dependent on cell density rather than the formation of cell–cell contacts [10]. However, when cells reach still higher densities, crowding may lead to fewer engaged cell–cell and/or cell–matrix adhesions compared with neighboring cells, which can promote anoikis, or cell death by loss of adhesion-based cell-survival signaling [11]. In mathematical models, epithelial crowding can lead to increased mechanical tension on cells, which promotes cell loss [12,13] to regulate epithelial tissue homeostasis [14]. Thus, the mechanical strain and alterations of cell contacts in crowded epithelial regions may be at the heart of contact inhibition. Here, we discuss how cell contacts and density within epithelia may impact whether cells divide or die (Figure 1a).

Density-dependent control of cell proliferation

Exactly how cell contacts affect the decision for a cell to proliferate is not entirely clear; however, hints come from the discovery of the Hippo pathway. In *Drosophila* and mammals, mutations in the Hippo pathway lead to tissue overgrowth [15–22], suggesting that this pathway is critical for regulating proliferation and cell numbers. E-cadherin and alpha-catenin, essential proteins for cell–cell adhesion, control proliferation in response to changes in cell density by regulating the subcellular localization of the critical Hippo downstream effectors

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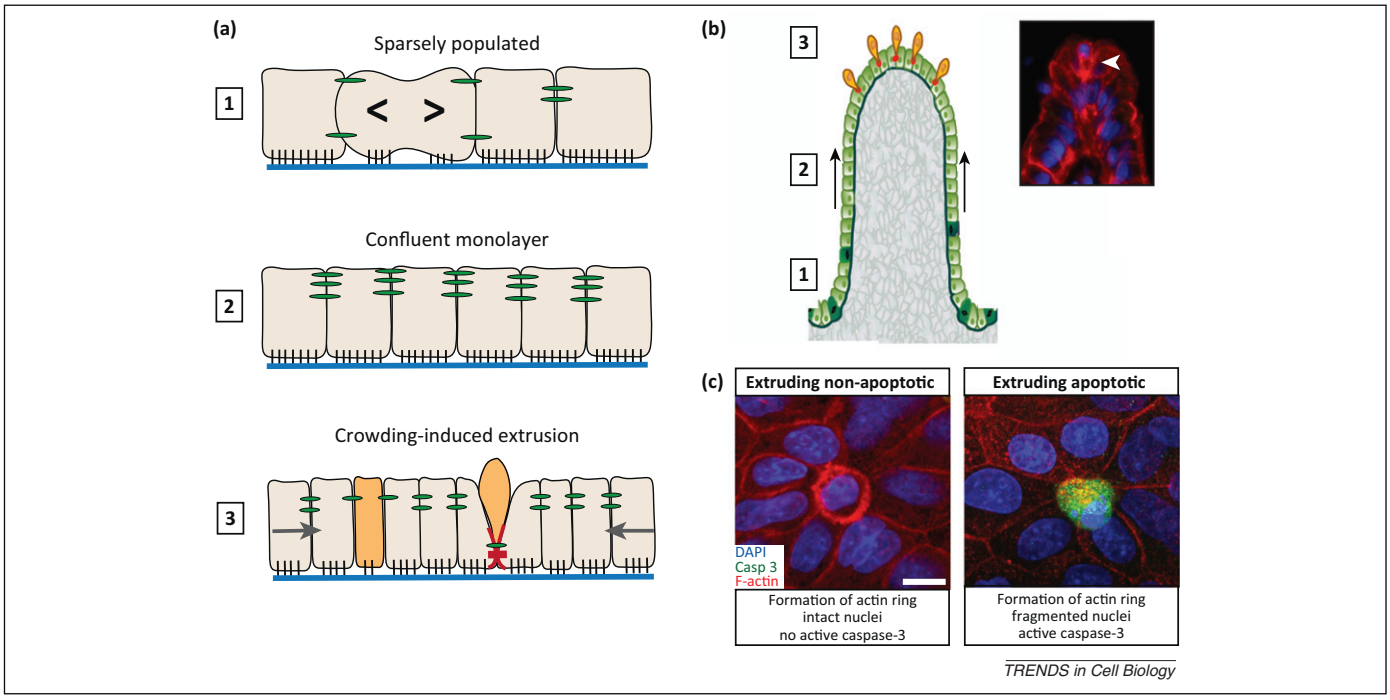

Figure 1. Crowding-induced live cell extrusion in epithelia. (**a**) Schematic outlining the life cycle of epithelial cells. (1) In sparsely populated cells, proliferation occurs where cell–extracellular matrix (ECM) contacts are high but cell–cell contacts are low. (2) Once cells reach a given density where cell contacts with other cells and the ECM are in equilibrium, proliferation decreases. (3) Increased cell crowding may reduce cell contacts with the ECM and other cells, causing extrusion of live cells to maintain constant numbers. (Green bars, cell–cell contacts; black lines, cell–ECM interactions; blue line, ECM). (**b**) Schematic of defined zones of cell division and cell loss in a colonic villus. Cells divide in the crypts where cells are less crowded (1), then migrate toward the villus tip (2), where they extrude (demarcated by arrowhead in inset) (3), probably due to pressure from converging cells. (**c**) Representative images and hallmarks of apoptotic versus non-apoptotic extruding MDCK cells.

yes-associated protein (YAP) and TAZ [23,24]. YAP is predominately cytoplasmic in confluent cells, but at lower cell densities accumulates in the nucleus where it acts as a transcriptional coactivator to promote proliferation [23–26]. Disrupting cell–cell contacts or cell–extracellular matrix (ECM) interactions is sufficient to shift YAP to the nucleus [23,27]. Thus, the Hippo pathway is emerging as the long sought-after pathway for controlling cell proliferation in response to cells contacts, yet its regulation does not depend merely on cells making contacts with one another but on the density of contacted cells. Mechanical forces generated from matrix stiffness can also activate YAP/TAZ independently of the Hippo pathway [28], suggesting that crowding forces independent of cell–cell contact could also signal density-dependent proliferation. We do not elaborate on contact-dependent regulation of cell proliferation here, because a recent review covers this topic in depth [29], but instead consider how cell density affects cell death.

Density-dependent control of cell death: epithelial cell extrusion

In addition to cell–cell contacts and density controlling whether a cell within an epithelium will proliferate, they also control whether it will die. Recent studies from a variety of epithelia, including developing *Drosophila* and zebrafish, human adult colon epithelia, and tissue culture epithelial monolayers, show that cells in crowded regions of epithelia extrude and later die [30,31]. Cells routinely were found to proliferate at defined regions within the epithelium where cells were least crowded. Cells then migrate away from sites of proliferation within the monolayer to converge at regions of high density, where they are pushed out, or extruded. This conveyor-belt model of cells within an epithelium is exemplified by intestinal epithelia (Figure 1b), but is also apparent in different epithelia *in vivo*, such as the edge of the fin epidermis in developing zebrafish [30]. The surprising finding from these studies was that cells rarely died by apoptosis *in situ*. Instead, live epithelial cells in crowded regions extrude [30,31] (Figure 1c) and later die by anoikis [11,32,33], or cell death due to detachment from the matrix. These studies support the idea that mechanical stresses and tension promote cell death by extrusion independent of the apoptotic pathway [34]. Although it is unclear what marks a particular cell for extrusion, loss of a threshold number of cell contacts with other cells and the underlying matrix may be a factor.

Cell extrusion was originally identified as a process that maintains the barrier function of an epithelium when cells within that layer die [35]. All epithelia observed have been found to extrude dying cells, including *Caenorhabditis elegans*, *Drosophila*, mouse, chick, zebrafish, and human epithelia [30,31,34–37]. Induction of apoptosis in cell culture monolayers and zebrafish has been critical for elucidating the mechanisms that control apoptotic cell extrusion in vertebrate epithelia. From these studies, we know that inducing apoptosis in epithelia through either the intrinsic or extrinsic pathway [38] results in extrusion of cells (Figure 2a). Additionally, these experiments defined a conserved mechanism for cell extrusion in vertebrate epithelia. To extrude, the cell destined to die produces and secretes sphingosine-1 phosphate (S1P) [39], to signal its live neighboring cells to form a ring of actin and myosin IIA around the dying cell [40]. Contraction of the multicellular actomyosin ring ejects the dying cell from the tissue and closes any gaps

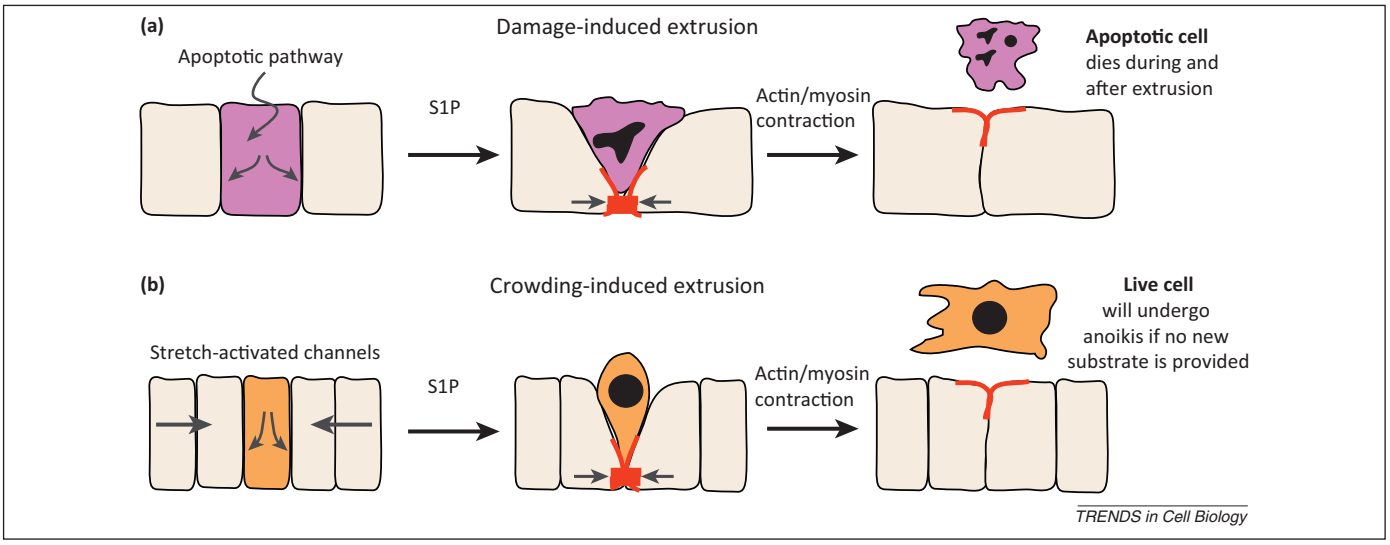

Figure 2. Schematic of damage-induced and crowding-induced extrusion in epithelia. (**a**) During damage-induced extrusion, apoptotic stimuli trigger a cell to simultaneously die and extrude to preserve epithelial barrier function (pink cell). (**b**) During live cell extrusion, localized epithelial cell crowding triggers extrusion via stretch-activated channels (orange cell). Although different upstream signals initiate each pathway, in both cases the extruding cell secretes sphingosine-1 phosphate (S1P) to its surrounding live neighbors to promote the formation and contraction of an actin/myosin ring that ejects the cell from the epithelium.

that may have resulted from its exit. It is unclear whether this mechanism and signaling pathway are conserved throughout all species, because cells can extrude apically or basally, depending on the organism. In *Drosophila*, cells extrude predominantly basally, in a process typically referred to as cell delamination [31,34,37], whereas vertebrates extrude epithelial cells predominantly apically. Whether a cell is shed apically or basally could depend on different signaling pathways or molecular alterations in a conserved pathway [40,41]. Mutant or transformed cells may also use extrusion to exit an epithelium. Expression of oncogenic K-Ras, Src, or mutant DPP/BMP cells leads to preferential elimination of those cells when mixed with wild type neighboring cells in cell culture or the epidermis of developing *Drosophila* and zebrafish [42–46], although it is currently unclear whether these delaminations use the same mechanism as apoptotic and homeostatic extrusions. Regardless of whether a common mechanism is responsible for extrusion in all organisms, extrusion appears to serve the same purpose throughout all species: it ejects cells while preventing any breaches of barrier function, a function essential to all epithelia.

How does the apoptotic extrusion pathway relate to the live cell extrusion that occurs in response to cell crowding during normal homeostasis in the body? Live cell extrusion, like apoptotic extrusion, requires S1P signaling to activate ROCK-mediated actomyosin contraction [30]. Yet, blocking apoptosis by Bcl-2 overexpression, which blocks apoptotic cell extrusion [38], does not inhibit live cell extrusion. Likewise, blocking cell death in developing *Drosophila* by overexpressing p35 does not affect extrusion [31,42]. Instead, live cell extrusion from crowding strain during homeostasis requires stretch-activated signaling, presumably upstream of S1P signaling. Inhibiting stretch-activated signaling or knocking down Piezo1, a recently identified stretch-activated channel (SAC) that transmits calcium currents [47,48], prevents live cell extrusion *in vivo*. Importantly, SAC inhibition also leads to accumulation of epithelial cell masses at sites where extrusion would have occurred, suggesting that extrusion is the chief mechanism for controlling epithelial cell death *in vivo* [30]. Thus, although the S1P pathway controls both apoptotic and homeostatic cell extrusion, SACs control live cell extrusion during homeostasis (Figure 2b). This raises the question of how cell-crowding strain may activate SACs to induce extrusion of live cells.

Sensing the strain: cell density changes promote live cell extrusion

How do epithelia sense crowding and activate stretch-activated signals in response to strain? Interestingly, extrusion *in vivo* always occurs in regions of the epithelium that are 1.8 times more crowded than other areas [30]. Importantly, this is a scaling effect, because the crowding response is relative to the size of other cells within the epithelium rather than an absolute cell size. This suggests that cells, like people, have a sense of personal space that is defined by the tissue, and when they become too crowded some must leave for the group to return to comfortable densities. Interestingly, mathematical simulations have predicted a similar threshold in tension to promote 'pressure-induced apoptosis' in response to cell growth [13] (Figure 3a). Pressures near extrusion-induced apoptosis have been measured by membrane recoil following laser ablation and suggest that the amount of local mechanical tension influences the frequency of extrusion [31,34,37]. Crowding-driven extrusion was also demonstrated experimentally where cells are crowded in a stretching device used in reverse. Over a 6-hour period following crowding, epithelia equilibrate to homeostatic cell densities by activating cellular extrusion [30]. Increasing the amount of cellular crowding, both in mathematical models [31] and in experiments [30], increases the amount of live cell extrusion (Figure 3b,c). As the cell density increases by more than a factor of 1.4–1.6, the number of live cells ejected from the tissue increases dramatically (Figure 3c). *In vivo*, extrusion may initiate at similar crowding densities, but the lag in extrusion activation may make it appear to occur

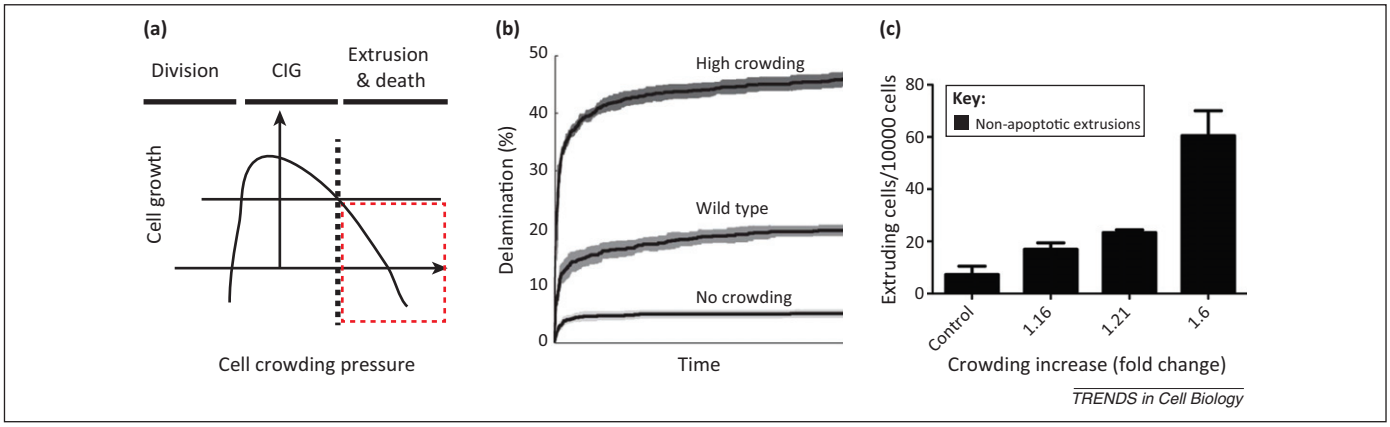

Figure 3. Increased cell density induces live cell epithelial extrusion. (**a**) Mathematical simulations showing that increasing pressures due to cell growth can prevent proliferation and eventually cause cell loss by apoptosis (CIG, contact inhibition of growth). Adapted, with permission, from [13]. (**b**). Increasing the amount of crowding in simulations correlates with increasing amounts of extrusion, based on models in *Drosophila* [31]. (**c**) Experimental data showing that when cell densities reach a critical threshold, live cell extrusion dramatically increases. Adapted, with permission, from [30].

at the 1.8-times density measured. The *in vitro* crowding studies establish a critical crowding concentration where cells activate extrusion that may be used to predict situations where cells will be extruded.

How do cells sense crowding at a molecular level? One might expect cells experiencing compression in crowded zones of the epithelium to become thinner and taller. However, crowding instead causes the total cell volume to decrease [30]. Because SACs are likely to play a role in translating the crowding strain force into activation of proteins controlling extrusion, changes in cell volume could ultimately affect the expression or activity of SACs [49,50]. A key requirement for mechanosensitivity is that membrane stress reaches the channel so it can change shapes between open and closed states [51–54]. Therefore, cell density could directly impact SACs in the membrane. Either aqueous influx or resistance due to the state of the cytoskeleton could counteract increased pressure on a cell [55–57]. Interestingly, we have found an early role for potassium channels in promoting apoptotic cell extrusion, because addition of 4-aminopyridine, a potassium channel inhibitor, blocks cell death and extrusion following UV-C exposure [35]. Although a role for potassium channels has not yet been established for crowding-activated live cell extrusion, these channels might also enable cells to condense and activate SACs. Further, both actin/myosin contraction and destabilized microtubules [40] have been noted early in the extruding cell, both of which could compact the cell and activate SACs. Likewise, rearrangements in the actin and microtubule cytoskeleton occur in both the extruding cell and its neighbors during cell delamination in the *Drosophila* amnioserosa [34]. Alternatively, cell condensation could affect the number of cell–cell or cell–matrix contacts, which could target particular cells in crowded regions for extrusion. Although the Hippo pathway plays a role in controlling contact inhibition of proliferation, mediators of this pathway, YAP and TAZ, could also act to control extrusion in response to crowding, because cytoplasmic phosphorylated YAP increases when cells are plated in a small, restricted pattern of matrix [28]. Recent mathematical simulations also show that the activity of the *Drosophila* YAP homolog yorkie can be influenced by mechanical stresses during development [58]. Further, activation of the S1P/$S1P_2$/Rho pathway that controls extrusion also activates YAP [59]. Although YAP and TAZ regulate proliferation through transcriptional activation in the nucleus, during crowding they could act in the cytoplasm to regulate extrusion.

Cell crowding and epithelial pathologies

Because extrusion appears to be critical for regulating overall cell numbers during epithelial homeostasis, alterations of any step in pathway controlling cell extrusion could lead to epithelial pathologies that either disrupt barrier function or lead to hyperplasia and cancer (Figure 4). Here, we discuss how defects in the ability to sense and respond to cellular crowding may promote common epithelial diseases.

Carcinomas

An inability to sense engaged cell contacts and crowding could prevent extrusion of cells, leading to the accumulation of defective cells that then develop into a carcinoma (Figure 4a). In support of this, colon adenomas (polyps) comprise densely crowded epithelia that lack extruding cells [30]. The idea that preventing the ability to sense crowding could lead to hyperplasia is supported by the fact that blocking the SAC Piezo1 results in epithelial cell mass formation in zebrafish epidermis. Additionally, changes in the intrinsic density of cells or their surrounding matrix could also affect the ability of cells to sense crowding. Tumors are denser than the surrounding normal tissue [60,61], a property that is evident by the ability to palpate tumors. Increased tumor density is due to a stiffer matrix underlying the tumor cells and denser cells that comprise the primary tumor, as measured by atomic force microscopy (AFM) of tumor tissue sections [62]. Stiffer tumor tissues would be less responsive to crowding pressures than would normal tissue so that their cells may not be triggered to extrude and instead accumulate in masses. Alternatively, AFM measurements have also determined that metastatic tumor cells isolated from pleural effusions are softer than neighboring benign or primary tumor cells [63]. A comparatively softer cell might have a higher

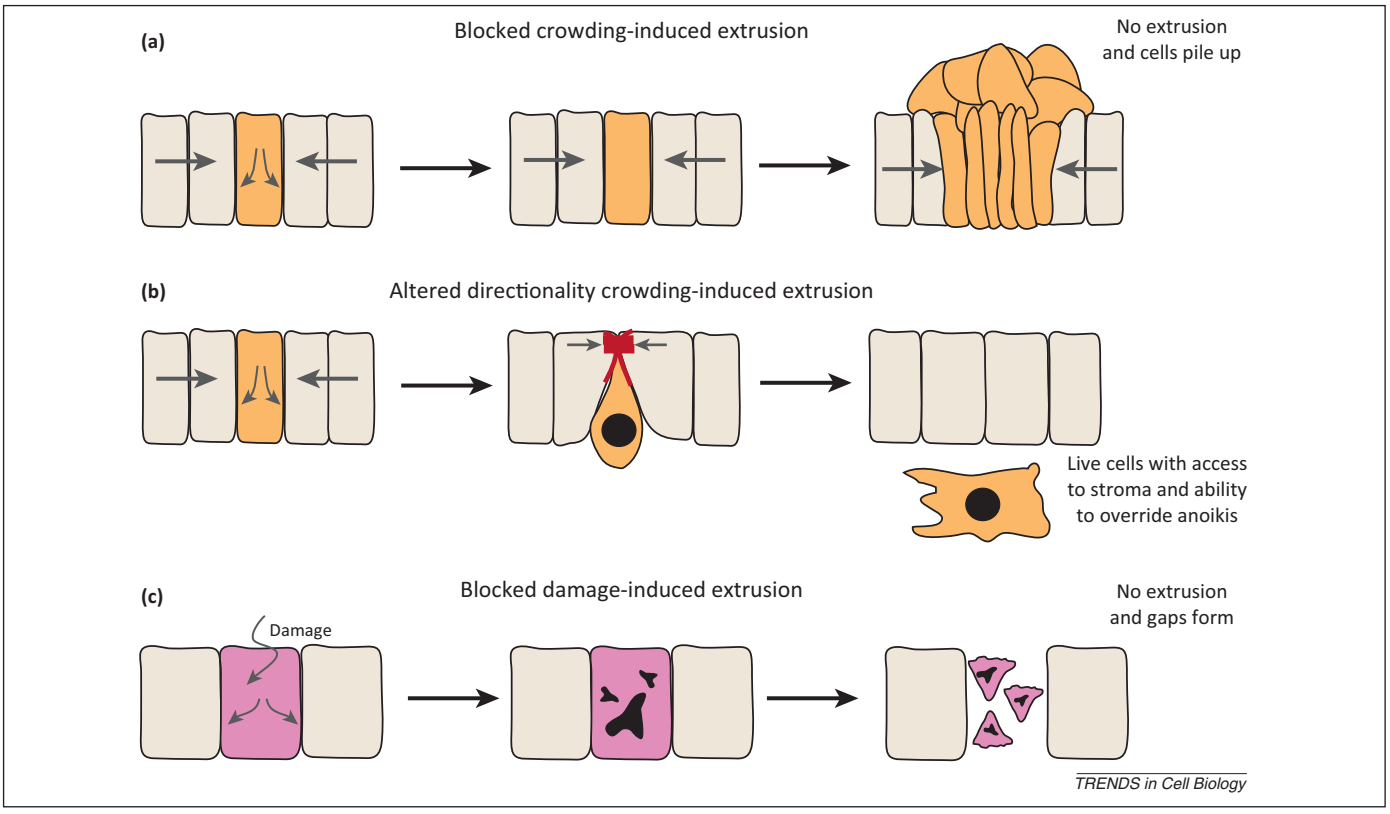

Figure 4. Cellular crowding and epithelial pathologies. Schematics depicting scenarios of altered extrusion events in epithelia. (**a**) Blocking crowding-induced live cell extrusion leads to cells amassing in the tissue. Based on *in vivo* evidence in [30]. (**b**) Epithelial cells that extrude basally and have aberrantly high survival signaling could invade the underlying tissue and access other parts of the body [40,41]. (**c**) Preventing extrusion in cases where apoptosis is induced could lead to gaps in the monolayer and disrupt barrier function [38–40].

probability of getting extruded from the tumor or epithelium from which it originates, facilitating its invasion and migration through the body. In support of this, increased physical pressure on tumors can promote the shedding of cells [64] and mechanical stimulation enhances cancer cell invasion [65,66]. Moreover, tumor cells can be more contractile than normal cells [67] and may be primed to extrude at a lower threshold than normal. Changes in density of tumor cells could even work together to promote invasion. Primary tumors could induce enough pressure to promote extrusion and metastasis of softer tumor cells.

A similar concept to how differences between cells could selectively remove some cell types at the expense of others is exemplified genetically in the phenomenon of cell competition, seen in *Drosophila* and cell culture epithelia [68,69]. In *Drosophila*, mosaic patches of cells with compromised growth [70] or altered polarity [71] will be eliminated by surrounding wild type tissue through cell competition. Conversely, patches of mutant cells that have enhanced growth rates compared with surrounding wild type cells can act as 'supercompetitors' to eliminate their neighbors [72,73]. During cell competition, winning cells could promote extrusion of less fit cells by causing increased pressure through faster growth and proliferation. Mathematical models also support this idea, because patches of mutant clones with enhanced growth cause 'pressure-induced apoptosis' of surrounding neighbors [13], a process that may be due to crowding-induced live cell extrusion. In addition to altering the ability of a cell to sense crowding, mutations in the extrusion pathway could alter the ability of a cell to extrude in response to increased pressures. For instance, several regulators of the extrusion pathway, such as sphingosine kinase-1, a precursor of S1P, S1P receptor 2, and RhoA and C, are misregulated in numerous tumors [74–79]. Future work will determine how alterations in the extrusion pathway could cause cells to accumulate or promote invasion.

Although extrusion of live cells normally promotes their death by anoikis, it is important to note that most aggressive, metastatic tumors upregulate survival signaling to override anoikis [33,80,81], a property that allows them to colonize elsewhere in the body. When cells can no longer die by anoikis, the direction a cell extrudes could have a dramatic impact on its later fate. Typically, cells extrude apically into the lumen [30,35,40,41], which is a dead space, so even if cells continue to survive after extrusion, they would still be essentially eliminated by extrusion. In this way, extrusion could act to suppress tumor formation. In fact, cells expressing oncogenic K-Ras or Src or mutant DPP/BMP are preferentially eliminated from epithelia in cell culture and from the epidermis of developing *Drosophila* and zebrafish [42–46]. However, if cancer cells with upregulated survival signaling extrude basally underneath the epithelium [40,41], they could potentially invade the stroma and gain access to other sites in the body (Figure 4b). Interestingly, loss or mutation of the tumor suppressor adenomatous polyposis coli (APC) shifts cells to extrude predominantly basally [41], suggesting an additional way that wild type APC may act to suppress tumor formation. Therefore, aberrations in any step of sensing crowding,

activating extrusion, or ensuring that extrusion occurs in the correct apical direction could contribute to the formation or progression of tumors.

Barrier function diseases

Epithelia provide a barrier to the outside world and disruption of this barrier results in various diseases. Although hyperimmune response is often thought to be the basis of diseases such as asthma, coeliac disease, and irritable bowel syndrome (IBS), an alternative hypothesis is that the primary cause of these diseases is poor epithelial barrier function. In support of this idea, many of these diseases are associated with mutations in cell–cell adhesion genes [82–86]. Epithelia in these situations are thought to have poor barrier function simply due to poor adherens and tight junctions. However, alterations in cell adhesion could also affect the ability of a cell to extrude effectively. Additionally, mutations in the extrusion pathway could disrupt the ability of an epithelium to extrude in response to pathogens or insults that trigger cell death. For instance, perturbation of Rho activity downstream of the apoptotic pathway can block cell extrusion but still allow death to occur [35,39,40], which could produce gaps in the epithelial layer (Figure 4c). Loss of epithelial barrier function in colitis or asthma would then lead to inflammation and further exacerbation of the disease.

Another possibility is that epithelial barrier lesions could arise from excessive extrusion. For example, bronchoconstriction of airway smooth muscle during asthma may lead to excessive overcrowding of the attached bronchial epithelia. Excess epithelial crowding could cause hyperextrusion, which could lead to the epithelial denuding seen in asthma [87–90]. The resulting poor barrier could then lead to the increased inflammation and infection commonly seen in people with asthma following an attack of bronchoconstriction [91–93]. Further, excess extrusion caused by pathogens such as *Vibrio parahaemolyticus* may contribute to the disintegration and inflammation seen in intestinal villi [94]. Future studies will define what roles, if any, extrusion plays in the pathobiology of epithelial barrier diseases.

Concluding remarks

Despite its importance to human physiology and disease, how epithelial tissues maintain overall cell numbers has been a mystery. Previous studies implicated a role for cell contacts controlling proliferation of cells. Recent findings now add a role for epithelial cell densities in controlling cell division and death, suggesting that crowding forces and relative levels of cell contacts may be critical for regulating overall numbers. This emerging view of an old problem is also revealing new molecules that might translate changes in density, force, and contacts into whether a cell will live or die. These findings should, hopefully, also bring new models for how epithelial diseases initiate and new targets to treat or prevent these diseases.

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