Cancer development in esophageal epithelium

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Abstract

Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) are aggressive forms of cancer affecting the esophageal epithelium. These cancers are associated with a poor prognosis and a 5-year survival rate of approximately 25%. The epithelial cells lining the esophagus have built-in defense mechanisms against cancer, known as epithelial defense against cancer (EDAC). One mechanism involves clonal competition within the esophageal epithelium. Normal cells with *Notch1* mutations compete with other mutated cells, restricting the clonal expansion of mutant clones. However, ESCC and EAC tumors persist, suggesting that cancer cells find ways to evade these natural safeguard mechanisms. Recent research suggests that cell competition also plays a role in tumor initiation. Furthermore, there seems to be a critical role for the interaction with the tumor microenvironment in supporting tumor development. This review aims to explore the latest discoveries in esophageal cancer development, shedding light on the molecular mechanisms that enable tumor cells to evade the suppression mechanisms and establish cancerous growth. Understanding these mechanisms will contribute to uncovering new therapeutic interventions and improve outcomes for ESCC and EAC patients.

Laymen summary

Esophageal cancers can occur as two different diseases, esophageal squamous cell carcinoma (ESCC) or esophageal adenocarcinoma (EAC). Despite the increasing number of patients diagnosed with esophageal cancers, our understanding of this disease remains insufficient. Consequently, treatment options for these patients are limited, and the survival rates are very poor (5-year survival of ~25%). Cancer in the esophagus originates from the epithelial layers. The function of epithelial tissues is providing a barrier to protect underlying tissues from the harmful external milieu, exemplified in the gut, skin and esophagus. Epithelial tissues typically use various mechanisms to prevent cancer development. These are all grounded in the concept of cell competition. Cell competition is used to eliminate unfit cells in the tissue, while dividing the cells that are fit to maintain homeostasis in the tissue. This process occurs from embryonic stages through adulthood and is tightly regulated to make sure the organism functions optimally. Cells that have a mutation in a tumor promoting gene are potentially dangerous and need to be eliminated through cell competition. In the epithelium, a mechanism known as epithelial defense against cancer (EDAC) removes cells carrying these potentially harmful mutations. Furthermore, the esophageal epithelium has additional measures to prevent cancer formation. Normal esophageal epithelial cells can also undergo clonal competition and specific mutations in the *Notch1* gene result in the out-competition of the harmful cells. Despite these tumor-suppressive mechanisms in the esophagus, cancer occasionally emerges when mutated cells evade their cell competition fate. Recent studies suggest that escaping cell competition, as well as the interaction of mutated cells with the tumor microenvironment, contributes to aggressive tumor formation. In this review, we discuss the latest research on the precise mechanisms in the esophageal epithelial tissue during tumor development. In the future, this will contribute to a better understanding on esophageal cancers which potentially leads to the development of targeted therapies and improvement of the poor survival outcomes of ESCC and EAC patients.

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Content

Front page image: Pan,C. et al. (2021)

Introduction

Esophageal cancer

In the Netherlands, esophageal malignancies account for 2% of all cancer cases. Esophageal squamous cell carcinoma (ESCC) is the most predominant esophageal cancer type, yet the prevalence of esophageal adenocarcinoma (EAC) is on the rise in Western countries[1]. Despite ongoing research, the causes of these cancers remain not fully understood. However, risk factors include alcohol abuse, tobacco usage and diets deficient in fruits and vegetable intake. Due to difficulty in early diagnosing, the lack of precision medicine and the resistance to therapies, the prognosis for ESCC and EAC patients remains very poor resulting in a 5-year survival rate of merely 25% (KWF). Currently, targeted therapies are unavailable and standard treatment comprises (a combination of) partial resection of the esophagus, chemotherapy and radiotherapy.

Esophageal epithelial tissue

ESCC and EAC originate from epithelial cells in the esophagus. Biologically, the esophagus is an organ that passes through the mediastinum and diaphragm to connect to the stomach and primarily functions as a conduit for food passage[2]. Unlike other gastrointestinal organs, the esophagus, as far as it is known, lacks further absorbing or endocrine functions. The main function of the epithelium is to provide a continuous protective layer lining organs that are exposed to the external environment, such as the skin, gastrointestinal tract, respiratory passages and esophagus[3]. Epithelial tissues experiencing frequent exposure to environmental factors undergo frequent cell divisions to maintain the integrity and function of the tissue. This constant renewal of epithelial tissues is carried out by the mechanism of cell competition. Cell competition ensures tissue fitness and integrity in multicellular organisms[4]. Apart from monitoring tissue homeostasis, cell competition in epithelial layers is also important in a cancer context. For example, cells harboring oncogenic mutations can outcompete wild-type epithelial cells, a phenomenon termed ‘supercompetition’. Together, this suggests that epithelial cells possess the ability to compare relative fitness with neighboring cells and subsequently eliminate the least fit counterparts.

Tumor suppression in esophageal epithelium

Human esophageal epithelial tissue accumulates oncogenic mutations with age. Yet, the incidence of esophageal tumors does not correlate with the high number of acquired oncogenic mutations. This paradoxical discrepancy suggests the presence of a protective mechanism or positive selection preventing tumor formation in normal adult tissues. The average human esophageal epithelium comprises a mosaic of cells with different acquired mutations that colonize a large part of the epithelium. For example, mutations in *NOTCH1* are present in up to 80% of all esophageal cells[5]. Certain mutations appear to confer a general surveillance function, providing a competitive advantage to normal cells and facilitating the elimination of emerging pre-cancerous cells in order to maintain tissue integrity. Mechanisms where normal epithelial cells exert an anti-tumor strategies are defined as epithelial defense against cancer (EDAC). Despite these protective measures in esophageal epithelium, malignancies do occasionally arise, suggesting the involvement of mechanisms such as supercompetition in tumor initiation and development.

In this review, we discuss the latest insights regarding the mechanisms underlying cell competition in both cancer evasion and development within mammalian esophageal epithelial tissues. We elaborate on the mechanisms through which mutant cells escape their elimination fate and ultimately establish malignant tumors. In the future, a deeper understanding of esophageal pathogenesis will contribute to novel therapeutic strategies for ESCC and EAC patients aimed at mechanisms driving tumor development.

1. Epithelial homeostasis in the esophagus

1.1 Histological characteristics esophagus

Histologically, the esophagus has a rather straightforward composition and is lined with multiple layers of squamous epithelial cells[6]. This stratified epithelium contains proliferating cells in the basal layer that differentiate upon movement to the upper layer. During embryogenesis, the esophagus develops from the endodermal foregut and surrounding mesenchyme. The esophageal epithelium (EE) provides a protective barrier against harmful content in the external environment, such as food and refluxed gastric content[1]. Comparable to the epidermis of the skin and the epithelium of the gastrointestinal tract, the EE undergoes constant renewal. Despite its apparent simplicity, the regulation of cellular turnover in the EE is complex and there are key differences between murine and human EE (figure 1). In mice, the EE renewal is driven by the proliferation of keratinocytes solely located in the basal layer[2]. Furthermore, the basal-luminal axis displays a proliferation-differentiation gradient. Towards the top of the epithelial tissue, murine EE cells keratinize and lose their nuclei. The keratinized layer is thought to provide a protective barrier against the external milieu. Conversely, the human EE displays a more complex architecture with multiple layers of keratinocytes folded along the papillae. Additionally, keratinocyte proliferation in human EE occurs in the 5-6 (supra)basal cell layers instead of exclusively at the basal layer. Similarly to murine EE, basal cells are committed to differentiation once exiting the cell cycle. After that, they migrate to the external surface and are eventually extruded from the tissue at the luminal side. Upon differentiation, keratinocytes change their shape to larger and flattened cells with a large cytoplasm. In humans, esophageal cells do not keratinize and retain their nucleus. Instead, the protection of human esophageal tissue relies on a high turnover of epithelial cells.

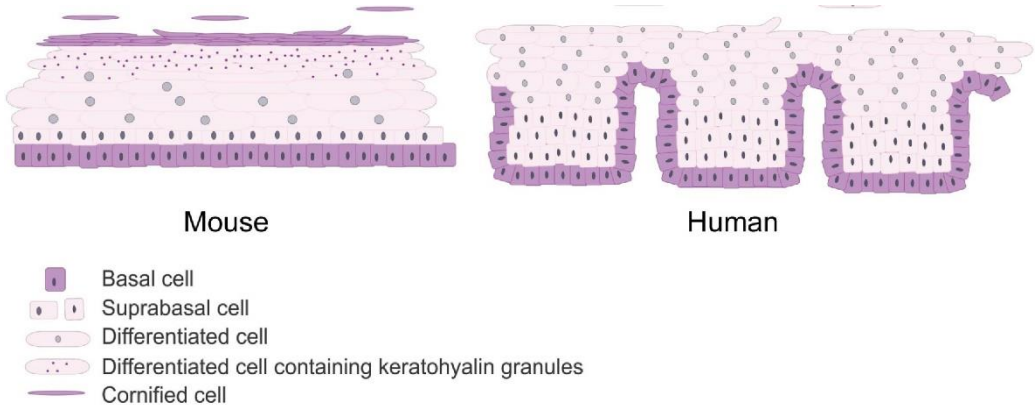

Figure 1: Schematic representation of the murine esophageal epithelium vs. the human esophageal epithelium. Adapted from Rosekrans, S. *et al.* (2015)

1.2 Esophageal single-progenitor model vs. stem-cell model

In the field of esophageal epithelial research, the organization of proliferating precursor cells is a topic marked by debate and varying interpretations. Currently, two different models are dominant in declaring how cells in the EE are renewed and maintained. This paragraph provides an overview of the methodologies and evidence underpinning these different perspectives.

Single-progenitor model

The first model, discovered by studying mice EE, describes a single-progenitor model. In this model, EE maintenance is executed by a homogenous population of progenitor cells that balances cell loss by cell division generating both dividing and non-dividing cells[7]. After cell divisions in the basal layer, the non-dividing progeny exits mitosis. Thereafter, the non-dividing cells will leave the basal layer, while the dividing cells remain in the basal layer. The esophageal

progenitor (EP) undergoes stochastic and unpredictable divisions, generating either two dividing cells, two proliferating cells, or both a dividing and proliferating cell[8]. However, the probability between dividing and non-dividing cells on average is balanced. This provides a homeostatic state in the esophageal tissue under normal circumstances. This single-progenitor model, lacking hierarchical stem cell/ transit-amplifying organization, is also applicable to the skin epidermis[9]. However, this model contrasts with crypt stem cells in the intestine, where the fate of a progenitor cell is dependent on the competition for limited niche space. The population of EPs was first identified in murine esophageal tissue by lineage tracing experiments[7]. Utilizing *in vivo* Histone2B-GFP labeling experiments, the researchers demonstrated that the presence of "label-retaining cells" (LRC) in the murine esophagus is infrequent and from non-epithelial origin. The LRCs in these experiments represent slow-cycling stem cells. Thus, the absence of LRCs indicates that esophageal epithelium is not maintained by a stem cell pool. Furthermore, employing a cre-lox-based genetic marking technique, researchers tracked the fate of single-cell derived clones in adult mice. Combined with a mathematical model, it was predicted that the basal layer dynamics align with the single-progenitor model, positing that the basal layer predominantly consists of esophageal progenitors dividing approximately twice per week. Additionally, transgenic assay experiments revealed a homogeneous cell-cycle period between the proliferating cells in the basal layer best explained by the single-progenitor model[8]. Taken together, lineage tracing and single-cell fate-tracking experiments prove the single-progenitor model to be important for esophageal homeostasis.

Stem-cell model

Another model explaining the composition and maintenance of the EE is explained by a slow-cycling stem cell pool that gives rise to more differentiated cells in the basal layer[10,11]. Based on cell surface markers, various studies have identified distinct subpopulations in the murine EE, indicative of a more hierarchical composition. Although lacking a defined stem cell niche, the EE possesses a small fraction of non-quiescent stem cells and a majority of faster dividing transit-amplifying cells, according to the stem-cell model. In human tissue, researchers identified the presence of LRCs exhibiting stem cell characteristics, including long lifespan, slow-cycling behavior and multipotency[12]. In this study, using human esophageal endoscopic mucosal resections, LRCs were more frequently found along the papillary basal layer than in the basal layer. Nevertheless, the interpretation of a proliferative pool is more complex in human esophageal tissue compared to mouse, due to the multiple layers of proliferating cells arranged along irregular papillary and glandular structure[13].

Overall, the presence of quiescent slow-cycling stem cells in rodent and human EE remains controversial and additional evidence of cell functions *in vivo* is required to confirm the presence or absence of an esophageal stem cell. Furthermore, understanding the role and turn-over capacities of progenitor- or stem- cells in the EE is crucial for elucidating the mechanisms underlying processes such as homeostasis.

1.3 Injury

The EE is regularly exposed to damage from various influences on the luminal side of the tissue. Assuming the single-progenitor model as the premise for esophageal tissue homeostasis, there are no slow-cycling stem cells to increase self-renewal and compensate for the cell damage upon injury. Therefore, it was proposed that the homogenous population of progenitor cells not only maintains homeostasis but also actively participates in tissue repair during injury[7]. During wound healing, local EPs exhibit

a temporary cell fate bias, producing more proliferating than non-dividing progeny[14]. Once the injury is resolved, the EP population reverts to its homeostatic behavior. This reversible transition to a regenerative state and increasing the likelihood of producing proliferating cells as progeny, provides a rapid and durable response to injury without the presence of a distinct stem cell pool. However, the adjustable fate of EPs raises the potential for mutant clonal expansion upon the acquisition of oncogenic mutations during aging.

2. Cell competition

To preserve the integrity and precise organization of multicellular tissues, such as esophageal epithelial sheets, cell populations conduct the mechanism of cell competition. For that, relative cellular fitness is monitored within a cell population after which specific clones expand or are lost to maintain homeostasis within the tissue. During cell competition, cells within the epithelial layer compete for survival and space. The 'loser' cell is eliminated, while the 'winner' cell can divide and occupy more of the available area. However, during the lifetime of an organism, most replaced cells are (almost) genetically and phenotypically wild-type. In this case, there is no distinction between fit and unfit cells and the stochastic nature of cellular processes will balance the number of cells in a tissue. Based on the context of the epithelial cells, there are different ways cell competition takes place, either actively or passively. This chapter will elaborate on the different types of cell competition and in what context this is conducted.

2.1 Passive cell competition

Neutral competition

Neutral competition is a form of passive competition and occurs in situations where cells have equal fitness[15]. During esophageal homeostasis, the precursor cells balance the proliferating and non-proliferating cells within the tissue to maintain an equal number of cells. Because these stem- or progenitor cells in the EE are as fit, the replacement of a cell is random, resulting in the stochastic expansion or contraction of clones in the epithelial tissue. This neutral drift at population level will result in a decrease in clonal diversity and an increase in clone size over time.

Biased drift

Another form of passive competition is biased drift. Fitter cells are now more likely to replace less fit cells than the other way around. This happens for example in the intestinal stem cell crypt, where stem cells in the crypt compete for niche factors. Stem cells deeper in the crypt are more likely to stay in the niche while other stem cells will move to the villi and unavoidably differentiate. Thus, biased drift describes the situation where a fitter cell is more likely to proliferate more and is less likely to be replaced by other cells. However, the stochastic nature of these events will not automatically result in expansion of the fittest clones, while some competition events can even result in the expansion of less fit clones.

2.2 Active cell competition

In contrast to passive cell competition, active cell competition involves the intentional elimination of less fit counterparts, followed by the repopulation of the fitter clone[15]. Moreover, clones with a fitness advantage will expand at the expense of the less fit clones. The active elimination of the 'loser' cells can take place in different manners as described in the following paragraph (figure 2).

Apoptosis induction

Firstly, the elimination of a particular cell in an epithelial sheet can occur by the induction of apoptosis. This was first described when studying the ribosomal gene mutations in *Drosophila melanogaster*[4]. In this classical example of *Drosophila* $Rp^{+/-}$, viable and functioning organisms were formed. It was discovered that the mutant cells in genetic mosaics underwent caspase-dependent apoptosis when in contact with wild-type cells in *Drosophila*[16].

Mechanical forces

Another situation where active cell competition plays a role is during tissue crowding. Whenever epithelial sheets have too many cells, mechanical forces maintain homeostasis by extruding live cells from the tissue[17]. In the crowded region, epithelial cells are actively extruded from the tissue in a cell-death-independent manner and undergo anoikis or cell death afterward. To initiate extrusion, the cell designating to die secretes sphingosine-1 phosphate (S1P), which serves as a signal for neighboring cells to form a ring of actin and myosin IIA, contracting the cell out of the epithelial layer.

Survival signals

Lastly, a cell competition mechanism independent of direct contact between cells operates through soluble signals. These signals are being transmitted between cells, without direct cell-cell interactions being present and stimulate the differentiation of specific counterparts. For example, in the intestinal crypts reside stem cells that compete for niche factors and space. The secretion of WNT antagonists by $APC^{-/-}$ intestinal stem cells results in the differentiation of wild-type stem cells[15,18]. Ultimately, the differentiating stem cell is eliminated from the stem cell niche and moves towards the villi upon differentiation.

All in all, the different forms of active cell competition contribute to the elimination of less fit counterparts in different contexts and tissue origins to maintain epithelial homeostasis.

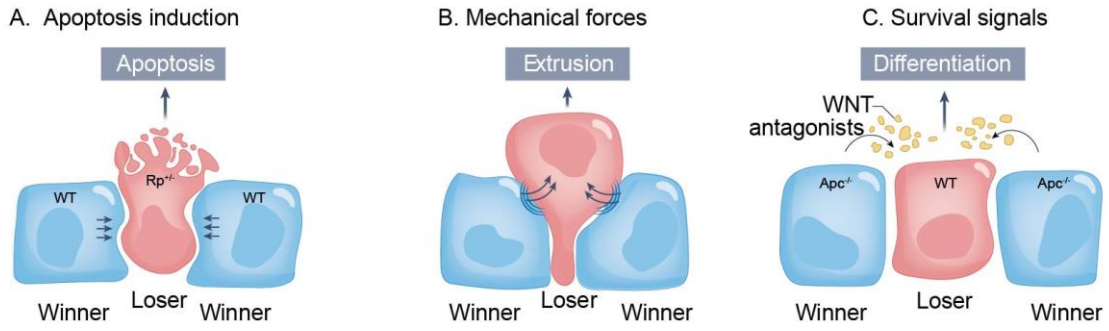

Figure 2: Mechanisms of cell competition in epithelial tissues. (A) Upon contact of $Rp^{+/-}$ cells with wild-type cells, the transformed cells undergo apoptosis. (B) Tissue crowding results in the extrusion of epithelial cells. (C) Contact-independent cell competition in the intestinal crypt. Adapted from van Neerven, S. & Vermeulen, L. (2023)

3. Cell competition as tumor suppressive mechanism

As we age, our cells acquire an accumulation of mutations. Especially mutations affecting cancer driver genes are known to promote disease formation. Nonetheless, in epithelial tissues, cell competition between normal and potentially harmful cells usually results in the elimination of the transformed cells, subsequently preventing oncogenic clonal expansion. In other words, cell competition is thought to be the driving factor for tumor suppressive mechanisms in the epithelial layer. In stratified epithelia, such as the esophagus, transformed cells face different fates as a consequence of cell competition: they are either extruded from the epithelial layer, undergo apoptosis or adapt supercompetition characteristics and evade elimination. Escaping the 'loser' cell fate will disrupt homeostasis and could initiate cancer development (described in Chapter 4). Despite significant advancements in this research field, the complex and dynamic interaction between healthy and mutant cells remains elusive. This chapter covers the tumor suppressive mechanisms in epithelial tissues responsible for eliminating potentially harmful cells.

3.1 Epithelial defense against cancer in epithelial tissues

The ability of normal epithelial cells to recognize and eliminate transformed cells is referred to as 'epithelial defense against cancer' (EDAC). This term was first described in the epithelial Madin-

Darby canine kidney (MDCK) cells and was described by the 'immunity' of normal epithelial cells against transformed cells[19]. Over the last decade, more evidence has been retrieved on the context when transformed cells are eliminated in the presence of normal neighboring cells and the applications of EDAC across different epithelial tissues becomes more profound.

Mechanical extrusion

In line with the concept of EDAC, oncogene-expressing cells are extruded from the epithelial layer via apical extrusion or basal delamination. This happens exclusively upon contact of transformed cells with surrounding normal cells. In the absence of normal cells, neither apoptosis nor extrusion occurs when mutated cells are present[20]. This indicates that the presence of normal cells determines the fate of transformed cells. A well-known example of EDAC involves mutant *RAS* cells. The extrusion of these transformed cells is dependent on both E-cadherin-based cell-cell adhesion and the actin-myosin skeleton. Firstly, individual $Kras^{G12D}$ mutant cells in the pancreas have an elevated expression of the membrane receptor protein EPHA2, which is detected by healthy neighboring cells[21,22]. Due to E-cadherin cell-cell interactions, downstream EPHA2 signaling is triggered and the contractility of the transformed cell increases. Secondly, to physically extrude the transformed cell from the epithelial layer, the actin-myosin skeleton is utilized. A significant aspect that is required for this is the rearrangement of the extracellular matrix (ECM) at the interface of wild-type and mutant cells[23]. In the context of *RasV12*-tranformed cells, non-autonomous changes occur at the interface affecting both the normal and the mutant cell. This is evident in the case of MCDK cells, where normal epithelial cells actively eliminate transformed cells by accumulating the cytoskeletal protein filamin and intermediate filament protein vimentin directly at the interface of the transformed cells[20]. In the presence of *RasV12*-transformed cells, filamin interacts with filamin-binding protein RhoGTPase Cdc42, which drives the filamin to move to the interface of normal and transformed cells to initiate extrusion of the latter[23,24]. In the transformed cells, myosin-II is activated when the transformed cell is surrounded by normal cells, which leads to the increased elasticity of the cell. Furthermore, the increased presence of filamin in the ECM prompts the remodeling of the actin cytoskeleton in adjacent normal cells at the interface[23,19]. This facilitates the physical extrusion of the transformed cell. Also, this sustains the hypothesis that normal cells execute anti-tumor activity without the involvement of immune cells. Taken together, filamin acts as a key mechanosensing mediator for the interplay between normal and transformed cells.

The molecular mechanisms underlying the interaction between normal and transformed cells prior to mechanical extrusion are not fully understood[20]. However, it is suggested that alterations in the mitochondrial metabolism at the interface may play a role during cell competition (figure 3). Evidence indicates that *RasV12*-transformed cells surrounded by normal cells exhibit increased levels of epithelial protein lost in neoplasm (EPLIN) due to upregulated filamin in the neighboring cells. EPLIN positively regulates pyruvate dehydrogenase kinase 4 (PDK4) and phosphorylation of pyruvate dehydrogenase (PDH) in the transformed cell, leading to a reduced membrane potential. As a compensatory mechanism for this mitochondrial dysfunction, transformed cells have an enhanced aerobic glycolysis, known as the Warburg effect. Inhibition of mitochondrial dysfunction was found to suppress the extrusion of the transformed cells, suggesting the relevance of these metabolic changes in transformed cells in the extrusion process. The conventional Warburg effect is often observed in advanced stage cancers and is linked to tumor-promoting phenotype. In epithelia, the EDAC-mediated metabolic Warburg shift plays a role in tumor-suppressive mechanisms. Additionally, studies in *Drosophila* suggest that mitochondrial dysfunction in *RasV12* mutant cells causes a senescence-associated secretory phenotype that triggers the proliferation of neighboring normal cells[25]. In conclusion, these findings shed light on the role of mitochondrial metabolism in the extrusion of transformed cells and further research is needed to elucidate the precise mechanisms.

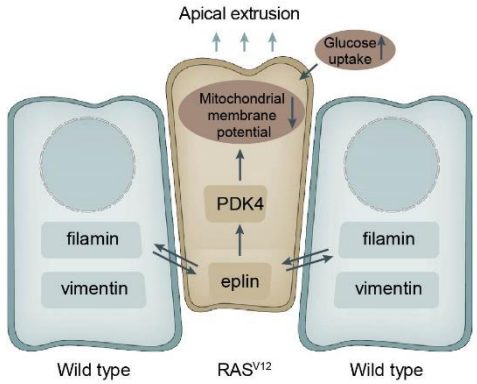

Figure 3: Mechanical extrusion during EDAC.
Upon contact of wild-type cells and RAS^{V12} cells, cytoskeletal proteins filamin and vimentin accumulate in the wild-type cell. In the transformed cell, eplin accumulates, which upregulates PDK4. Ultimately, this lowers the mitochondrial membrane potential and stimulates apical extrusion of RAS^{V12} cells. Apted from Baker, E. (2020).

p53-induced elimination during cell competition

EDAC not only operates through mechanical cell competition but also involves the active induction of apoptosis in mutant cells. Research conducted on MDCK *scribble* knock-down cells elucidated the apoptosis-driven elimination process in epithelial cells[26]. In this type of cell competition, the tumor suppressor protein (p53) plays an important role. This protein is central to crowding hypersensitivity within epithelial tissues. Tp53 exhibited higher abundance in the *scribble* knock-down cells compared to the wild-type cells before cell competition ensued (figure 4). Furthermore, upon compaction of the cells, p53 levels are further elevated by activation of Rho-associated kinase (ROCK) and stress kinase p38. Elevated p53 levels correlate with a 'loser' status in terms of cell competition, subsequently leading to the induction of apoptosis in the transformed cells. Taken together, this form of apoptotic-mechanical cell competition describes the mechanism where crowding-induced compaction results in a lethal upregulation of p53 in transformed cells.

Given the high mutation rate of *Tp53* across most cancers, loss-of-function mutations could potentially cause evasion of crowding-induced apoptosis. Surprisingly, in epithelial layers, *Tp53* mutations often lead to the elimination of the emerging mutant cell through cell competition with neighboring normal cells[27]. Hereby, the transformed cells undergo necroptosis, which is a form of programmed necrosis. Overall, alteration of p53 activity triggers cell competition, and loser cells are determined in a cell-context manner.

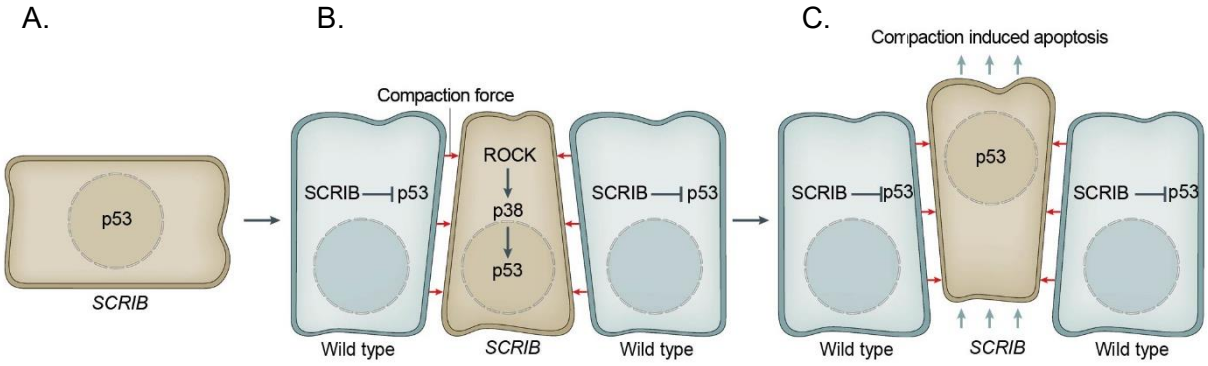

Figure 4. Elimination of *SCRIB* knock-down cells in epithelial tissues.
(A) *SCRIB* knock-down cells exhibit high levels of p53. (B) Upon compaction with wild-type cells, p53 levels in *SCRIB* knock-down cells further elevate by activation of Rho-associated-kinase (ROCK and stress kinase p38. (C) *SCRIB* knock-down cell undergoes compaction induced apoptosis. Adapted from Baker, E. (2020).

3.2 Spatial clonal competition in the esophageal epithelial tissue

Besides EDAC as an interplay between wild-type and transformed cells, the esophageal tissue employs additional measures to prevent tumor initiation. The tumor suppressive mechanisms of the esophagus are described by spatial clonal competition (figure 5). With aging, the progenitor cells in the EE acquire mutations, some providing a growth advantage, allowing mutant clones to colonize the tissue. However, as not all mutations initiate malignant transformation, this leads to a patchwork of histologically normal-looking clones in the EE. To unravel the genomic landscape of the EE, genome sequencing techniques were employed. These analyses revealed mutations in *NOTCH1* in 25 to 42% of healthy cells in middle-aged humans[5]. The frequency of the *NOTCH1* mutations correlates with age, showing a 30 to 80% prevalence in healthy EE of elderly humans. Interestingly, when comparing the healthy EE to ESSC cells, the mutations in *NOTCH1* were relatively low in the cancer tissues (~10%)[28,29].

Initially, *NOTCH1* was believed to be the driver of ESCCs. However, the prevalent presence of *NOTCH1* mutations in the normal esophagus suggests that cellular fitness does not equal oncogenic potential and that *NOTCH1* mutant cells provide tumor-suppressive features. *NOTCH1* encodes for a protein that plays an important role during cell fate determination, cell development and cell renewal[30]. It also plays a multifaced role in different cancers, acting as an oncogene in several leukemias, while functioning as a tumor suppressor gene in squamous cell carcinomas of the skin and esophagus[31]. Moreover, in the epidermis of the skin, deletions of *Notch1* in mice were correlated to impaired differentiation and the promotion of tumor formation[32,33]. Different studies provided evidence that suggests the tumor-suppressive role of *NOTCH1* mutant cells. In the first place, ESCC tumors are more likely to develop from clones without *NOTCH1* mutations, than clones with this mutation. Moreover, mutations in *NOTCH1* occurring in clones with pre-existing oncogenic mutations inhibit further expansion of that particular clone[34]. Furthermore, heterozygous *Notch1* knock-out cells in mice confer a competitive advantage over mutant progenitors. However, when two clones with equal fitness collide, neither of the clones prevail[35]. Subsequently, as the heterozygous clone grows, the likelihood of losing the remaining *Notch1* allele increases, enhancing the cellular fitness of these clones. Eventually, this leads to the colonization of *Notch1* mutant cells in the EE and a histologically normal-looking phenotype of the esophagus[28]. Concluding these findings, while *NOTCH1* exhibits divergent roles across different cancers, its influence in the esophagus comprises of a tumor-suppressive mechanism by out-competing other mutant clones and maintaining the integrity of the esophageal tissues.

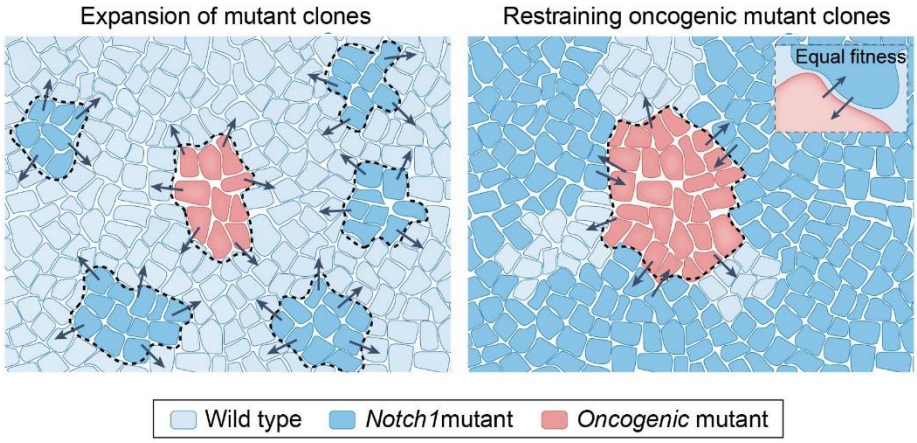

Figure 5 Clonal competition in esophageal epithelium.
Upon aging, epithelial cells in the esophagus acquire mutations, providing a growth advantage on cells in the tissue. While some mutations are oncogenic, *Notch1* mutations provide a tumor-suppressive mechanism to restrain oncogenic mutant clones in the EE. Adapted from van Neerven, S. & Vermeulen, L. (2023)

4. Esophageal epithelial cancer development

ESCC develops through different steps during carcinogenesis. However, the initial stage of tumor initiation remains largely unknown. Disruption of homeostasis of normal epithelial tissue and the emergence of (pre-) cancerous cells as 'winner' cells during cell competitions can lead to the formation of tumors in the EE. It is established that ESCC originates from the esophageal basal progenitors rather than cells in the differentiated layers of the esophagus in mice[36]. In human ESCC, there is also speculation that the tumors originate from progenitor cells. The mechanism by which mutant clones overcome the tumor suppressive mechanisms (explained in Chapter 3) and expand within the EE remains poorly understood, but it is hypothesized that cell competition plays a crucial role. For example, the failure of EDAC could facilitate the outgrowth of mutant clones, initiating carcinogenesis. Moreover, the transition from intraepithelial malignancy to invasive carcinoma is believed to result from cell competition within tumor clones. This chapter provides an overview of the latest research on the mechanisms through which mutant cells in the EE evade elimination and initiate cancer.

4.1 Evading EDAC

Field cancerization in the esophageal epithelium

The formation of esophageal tumors initiates with the development of intraepithelial neoplasia, a precancerous stage characterized by a bias towards producing proliferating cells over differentiating cells. To reach this stadium, an important aspect in developing neoplasia is field cancerization. This refers to the process of widespread mutant clonal genetic changes in histologically normal regions of the epithelium.

A mutation that regularly clonally expands in the EE is *TP53*. Heterozygous mutation of *TP53* is observed in 5-10% of phenotypically normal EE cells in middle-aged humans, increasing with age to 15-30% in individuals older than 70 years[5]. Despite the high number of mutations, cancer incidence related to *TP53* mutations remains low. Still, lineage tracing experiments revealed that *TP53* mutant clones can be important for tumor initiation through the mechanism of field cancerization. Heterozygous *Tp53* mutant mice revealed that mutant clones have a proliferative advantage over wild-type clones due to a bias in cell fate, without changing the total number of cell divisions in the tissue[37]. Thus, *Tp53* mutant clones are sufficient to drive clonal expansion. In this stage of field cancerization, the EE remains phenotypically normal and the epithelial structure is not disrupted. However, in a normal esophagus, heterozygous *Tp53* mutant cells only colonize up to 30% of the EE. This can be explained by the presence of *Notch1* mutant clones in adjacent healthy EE that are potentially responsible for out-competing *Tp53* mutant cells. In Notch1 inhibited mouse EE, it was demonstrated that differentiation of adjacent wild-type cells was promoted, while mutant cells could replace these cells and dominate the epithelium[34]. Thus, Notch seems to be important for competition with mutant cells in the EE. This could also explain the rather slow accumulation of heterozygous *TP53* mutants in the normal esophagus, which is tolerated in humans over decades. Taken together, heterozygous *Tp53* mutant clones are sufficient to drive mutant field cancerization, but there is competition with Notch1 mutant clones.

Subsequent to the clonal expansion of *TP53* mutant clones in the EE over the span of decades, a second event resulting in the biallelic loss of function of *Tp53* initiates tumor development. In mice, esophageal cancer arises from patches of *Tp53* mutant clones that lose the remaining *Tp53* allele (loss of heterozygosity) (figure 6). These tumors exhibited an unstable epithelium characterized by polyaneuploid cells and copy number alterations. Therefore, heterozygous *Tp53* mutations do not initiate tumor formation by themselves. However, the rare clones with *Tp53* mutations and a loss of heterozygosity of the second allele result in extensive chromosomal instability, which significantly increases susceptibility to tumor formation. Furthermore, the bias towards producing proliferating cells is smaller in dysplastic tissue and increases upon tumor development, contributing to the further colonization in the EE tissue[14].

As touched upon in Chapter 3, *Tp53* mutant cells are eliminated through necroptosis when in contact with normal neighboring cells. However, evidence shows that *Tp53* mutant cells can escape this elimination in the context of adjacent *RasV12*-transformed epithelial cells[27]. This contributes to the observation that there is a complex sequential cascade of oncogenic events in esophageal cancer development that are all dependent on the mechanisms of cell competition.

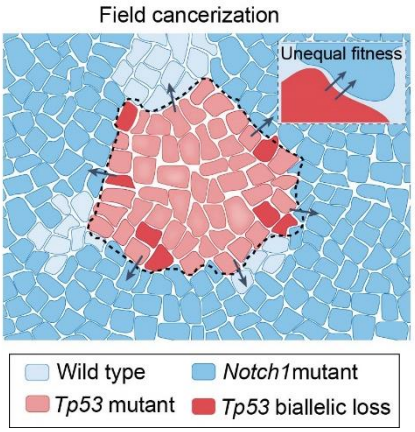

Figure 6. Field cancerization in the esophageal epithelium.
Within the clones of *Tp53* mutant cells, some cells can lose the remaining *Tp53* allele through loss-of-heterozygosity. Now, the oncogenic clones confers a growth advantage in the *Notch1* clone population and establish field cancerization. Adapted from van Neerven, S. & Vermeulen, L. (2023).

Evasion of mechanical extrusion

Recently, it was discovered that an important parameter to facilitate the success of EDAC is extracellular matrix (ECM) stiffening[23]. Cancer-associated stiffening is a known phenomenon across different cancers and plays a critical role in cancer progression and eventually the formation of metastases[38]. Fibrosis, obesity and aging are all pathological conditions that contribute to tissue stiffening[39,40]. In epithelial tissues, ECM stiffening alters the cellular localization of force-sensitive cytoskeleton proteins[23]. Therefore, on a stiff matrix, mimicking fibrotic tissue, the dynamics of filamin distribution change the filamin and cannot locate to the interface of the normal and transformed cell. Molecularly, the elevation in stiffness results in lower differential activation of Cdc42 at the cell-cell interface. This protein is important for the interfacial localization of filamin. Instead, upon stiffening of the tissue, perinuclear cytoskeleton proteins (FAM10B) are responsible for interacting with filamin and localizing them at the perinuclear region. There seems to be a form of competition between filamin-Cdc42 and filamin-FAM10B interaction, which is responsible for the success rate of EDAC. In a soft ECM environment filamin-Cdc42 interactions are stronger, and in a stiff ECM environment the filamin-perinuclear cytoskeleton interactions are stronger. The efficacy of EDAC is inhibited by the stiffening of the ECM and the extrusion of transformed cells fails at the initial stage of oncogenesis. Moreover, it was also investigated that this ECM stiffening phenotype can be rescued by either disrupting the interaction of filamin with FAM10B or by decreasing nuclear mechanotransduction.

4.2 Tumor maintenance and progression

Tumor heterogeneity in established tumor

Tumors in the EE display a complex development that includes various molecular alterations. Consequently, ESCC and EAC tumors display high tumor heterogeneity, which could indicate that the colonizing mutations present in a specific clone are possibly responsible for the varying cancer risks and outcomes in patients[5]. In recent years, studies incorporating single-cell multiomics on ESCC and EAC tumor samples have revealed the diverse spectrum of intra- and inter-tumoral heterogeneity and aberrant gene signatures[41,42]. This revealed distinct cellular transcriptome

profiles for ESCC and EAC, where ESCC tumors resemble other squamous cell carcinomas, and EAC tumors resemble gastric cancers. In the ESCC transcriptome landscape, it was found that *TP53* mutations correlate with a proliferative and immunosuppressive characteristic[41]. Additionally, NOTCH1 alterations were found in a subpopulation of ESCC tumor cells. It is hypothesized that these alterations are 'passengers', carried over from normal tissue, with the requirement for wild-type NOTCH1 in carcinogenesis bypassed by the other genome changes[28]. Moreover, cancer cell transcriptomics associated different somatic mutations to the patient's survival outcome. Among these markers are genes important for innate immunity and homeostasis and integrity of the mucosa. Furthermore, a variety of new potential biomarkers have been identified that could lead to better diagnostic and potential precision medicine options in the future. Taken together, the unique pattern of gene expression for each esophageal cancer population contributes to the difficult cancer treatment process of ESCC and EAC patients.

Tumor microenvironmental changes promote esophageal cancer development

Besides genomic alterations within the tumor populations, inter-tumoral feedback with infiltrating immune and stromal cells is also linked to ESCC progression[41,42, 43]. This favorable microenvironment is needed for tumor development and maintenance. Firstly, studies have suggested that cancer development is dependent and enabled by the contribution of stromal cells in the tumor microenvironment (TME). For example, there is an important role for cancer cells that manipulate normal fibroblasts into cancer-associated fibroblasts (CAF)[44]. Investigating scRNA-sequencing and spatial transcriptomics data of ESCC tumor and normal esophageal cells revealed a reduced ANXA1-FPR2 signaling between the (pre)cancerous epithelial cells and fibroblasts[45]. In a normal situation, ANXA1 is produced by wild-type EE cells and acts as a ligand for FPR2 on fibroblasts to control the homeostasis of the fibroblasts. In malignant cells ANXA1 is downregulated due to transcription factor KLF4 silencing. Furthermore, the infiltration of CAFs in the TME provides aberrant ECM and a vascular niche for progressive and invasive tumor cells to reside in[41]. The interaction with stromal cells in the TME is supported by studies that suggest that somatic mutations in specific driver genes (such as *TP53*) may drive expression profiles that promote interaction between the cancer cells and TME in the EE. Taken together, this proposes that there is an important role for CAFs for a precancerous clone to progress to ESCC.

Secondly, there is also a crucial role in the cellular interaction between TME and immune cells. For example, different subsets of macrophages were enriched in ESCC samples[43]. The infiltration of these so-called tumor-associated macrophages has shown to promote tumor immune evasion and eventually tumor progression in ESCC. Furthermore, distinct subsets of T-cells had exhausted signatures compared to normal tissue, indicative of an immunosuppressive TME phenotype. In summary, the esophageal cancer TME is a comprehensive network of communication between tumor cells and infiltrating stromal and immune cells, further highlighting the complexity of ESCC and EAC tumors.

Nrf2-dependent cancer progression and relapse

When tumors are established, cell competition within the tumor population occurs to form an advanced malignant tumor. Although little research has been conducted on mechanisms to form an invasive esophageal tumor, it has been suggested that cellular stress plays an important role. The EE is frequently exposed to oxidative and electrophilic insults (originating from food, tobacco, alcohol etc.)[2]. In normal esophageal tissues, this stress results in the accumulation of transcription factor NRF2 in the nucleus, which is under the control of KEAP-1[46]. Here, NRF2 regulates the expression of cytoprotective genes which encodes proteins that protect the cell against reactive oxygen species (ROS). In patients with advanced esophageal cancer, constitutively active NRF2 is reported and is associated with a malignant and resistance potential of the cancer[47]. Work in mouse models demonstrated that Nrf2-deficient cells are susceptible to chemical carcinogens

and are selectively eliminated through cell competition[48,49]. Taken together, these data suggest that NRF2 gain-of-function tumor cells are considered as 'winner' cells in terms of cell competition and that treatment with chemotherapeutics positively selects for these cells. This not only contributes to clonal expansion, but also the relapse of these clones in the patients treated with chemotherapeutics.

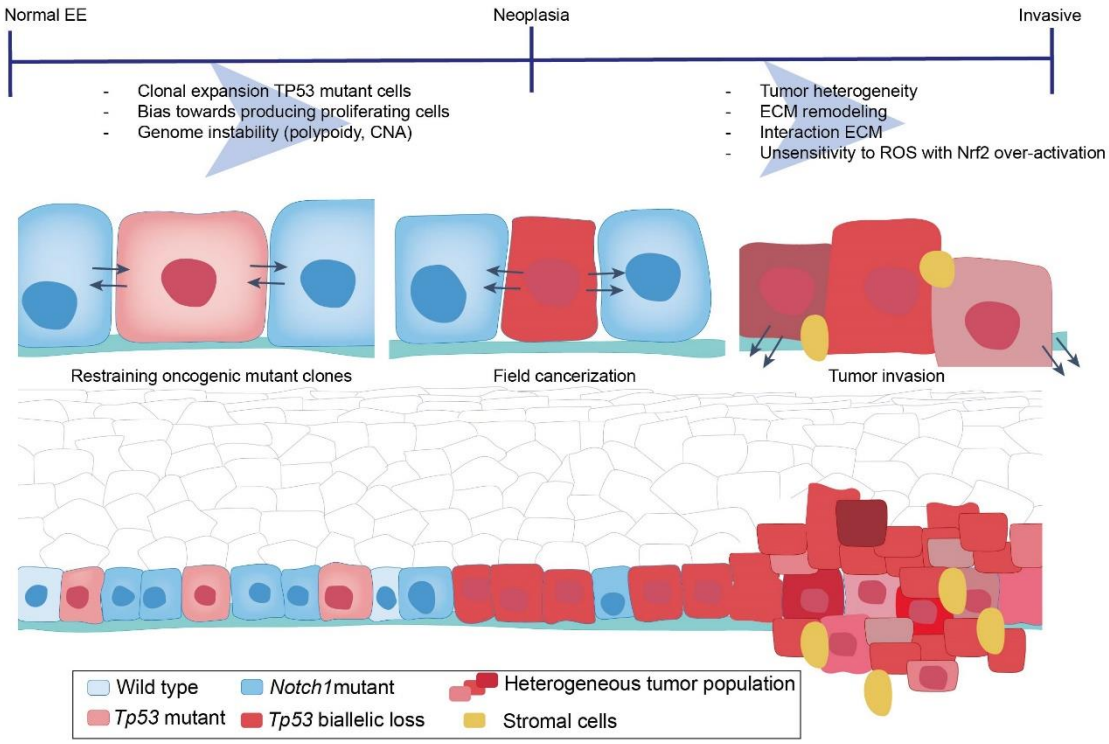

Figure 7. Summary of cancer development in the esophageal epithelium.
In normal EE, *Notch1* mutant clones restrain the clonal expansion of oncogenic (*Tp53)* mutant clones. If oncogenic cells within the clonal patches lose the remaining *Tp53* allele, field cancerization occurs and mutant clones dominate the tissue. Further cancer expansion and tumor invasive characteristics occur due to tumor heterogeneity and interaction with infiltrating stromal cells in the TME. Adapted from van Neerven, S. & Vermeulen, L. (2023)

Discussion

Deciphering the complexity of esophageal cancers is fundamental for establishing accurate diagnostics and discovering new precision medicine treatments. To achieve this, it is important to understand how cancer cells interact with other cells in the epithelial tissue, within the tumor population and with the TME. This includes comprehension of how cells recognize and respond to differences between (mutant) cells and whether there exist universal regulators of cell competition that play a general role in this process.

Genomic characterization of esophageal cancers has identified various potential cancer driver events in ESCC. However, not all events may contribute to tumorigenesis, as exemplified by NOTCH1 mutants in the EE. Under normal circumstances, diverse mechanisms of cell competition function to eliminate mutant cells, thereby preserving the integrity of the EE. Consequently, epithelial tissues are equipped with anti-tumor activity independent of the immune system. Nonetheless, while cell competition is initially perceived as a tumor-suppressive mechanism of the epithelial tissue, mutant cells can exploit it to achieve field cancerization and ultimately facilitate tumor initiation. In other words, the multifaced role of cell competition can both inhibit and stimulate cancer. Despite advancements in understanding the initial stages of carcinogenesis in esophageal cancers, much remains unknown about the molecular mechanisms

through which mutant cells escape the loser fate and initiate tumor formation. Moreover, clonal competition and clonal field cancerization mechanisms driving tumor evolution could have an impact on the response to conventional therapies. Multi-omics single-cell approaches indicate a prevalent role for inter-cellular communication of tumor cells with the TME in tumor growth and maintenance. In future research, human esophageal epithelial organoid models could be used to investigate the role of the microenvironment in the competitive selection for specific driver mutants. Besides a better understanding of the different stages of cancer development, this could also lead to the establishment of novel biomarkers for esophageal cancer.

The survival rates of ESCC and EAC are overall still very poor, with a 5-year survival rate of approximately 25%. Currently, ESCC and EAC follow the same treatment plan that is based on (a combination of) chemotherapy, surgery and radiotherapy. However, the use of targeted therapies in esophageal cancer patients is difficult, since the esophageal tumors are very heterogeneous. Also, current treatments often result in the development of resistance in a subpopulation of the tumor cells. For future prospects, targeting the basis that underlies cell competition within esophageal cancers could provide a better therapeutic intervention. For example, by promoting the attacking forces of normal cells by enhancing EDAC against transformed cells. Alternatively, by attenuating the defensive force of the transformed cells, making use of their imbalanced fate towards proliferation, tumor progression could be arrested. It must be noted, the profiling of tumor samples revealed that the molecular characteristics of ESCC and EAC display unique traits. This indicates the importance of treating ESCC and EAC as separate entities in future clinical trials for targeted therapy.

In summary, our current knowledge of cell competition in esophageal epithelium during homeostasis and cancer is rapidly expanding. However, fundamental questions remain on the (molecular) mechanisms underlying cell competition in different contexts. In the future, answering these questions will contribute to a better characterization of esophageal cancers and improve the current diagnostic and treatment options.

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