Cell Competition in Carcinogenesis

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ABSTRACT

The majority of human cancers evolve over time through the stepwise accumulation of somatic mutations followed by clonal selection akin to Darwinian evolution. However, the in-depth mechanisms that govern clonal dynamics and selection remain elusive, particularly during the earliest stages of tissue transformation. Cell competition (CC), often referred to as 'survival of the fittest' at the cellular level, results in the elimination of less fit cells by their more fit neighbors supporting optimal organism health and function. Alternatively, CC may allow an uncontrolled expansion of super-fit cancer cells to outcompete their less fit neighbors thereby fueling tumorigenesis. Recent research discussed herein highlights the various non–cell-autonomous principles, including interclonal competition and cancer microenvironment competition supporting the ability of a tumor to progress from the initial stages to tissue colonization. In addition, we extend current insights from CC-mediated clonal interactions and selection in normal tissues to better comprehend those factors that contribute to cancer development.

Cell Competition: Key Principles and Findings

Accumulation of damage and genetic aberrations within a cell may render the cell suboptimal, or less fit, but still viable. Over time, retention of suboptimal cells likely results in tissue abnormalities, disease, or loss of function. Cell competition (CC) is a highly conserved biological mechanism among multicellular organisms that results in the removal of less fit, suboptimal cells by their more fit neighbors. CC is active during tissue development to ensure the contribution of robust cell populations and throughout life as a homeostatic surveillance mechanism to maintain tissue function and health. CC was first experimentally identified in *Drosophila* when it was found that within mosaic wing discs composed of wild-type (WT) cells and heterozygous *Minute* (*M*/+) mutant (MT) cells that have impaired protein translation and slower growth, WT cells grew at the expense of their slower-growing *M*/+ cellular neighbors, which were markedly eliminated and not detected in the adult wing (1). However, no CC occurred in a homotypic wing disc of *M*/+ cells alone, indicating that relative, not absolute, state of fitness dictates CC; CC did not affect total wing disc size. These observations show that homeostatic CC is restrained within tissue compartmentalization and thus acts to maintain tissue integrity. Additional studies revealed that the elimination of suboptimal *M*/+ cells proceeded through apoptosis and suppression of apoptosis obviated CC-induced expansion of WT cells (2). Altogether, these studies demonstrate that expansion of distinct cell populations depends not only on cell-autonomous traits, but the outcome of active selection preceded by communication of cell fitness. We now understand that multiple parameters dictate cell fitness, and differences in growth and proliferation rates alone between interacting cells do not always trigger CC (3). Instead, several complex mechanisms of CC have been identified: mechanical forces (4); cell surface ligand-receptor systems (5); direct communication of fitness status via fitness fingerprints (6); competence in capturing nutrients and signaling cues (an indirect mode of CC similar to trophic theory; ref. 2), and various signaling pathways. Through these mechanisms, CC has been shown to operate via direct cell-cell contacts (6), or longer-range up to several cell diameters apart (4); and by secretion of soluble signaling cues (2). Several CC mechanisms first realized in fruit flies have been identified in advanced multicellular systems whose complex tissue organizations demand multiple regulatory features wherein CC appears to function as a highly responsive, tunable parameter that integrates environmental cues with inherent fitness inequalities amongst heterogeneous populations.

Role of CC in cancer

Homeostatic CC optimizes the expansion of fit cells but is controlled within normal tissue size and patterning constraints. Thus, it is not surprising that critical genes identified in CC include *Myc*, which regulates growth and proliferation (7), and also genes within the Hippo signaling pathway, a master regulator of organ size (8, 9). Increased expression of the c-Myc proto-oncogene (7, 10), hyperactivation of

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Hippo downstream signaling component *Yorkie* (*Yki*, *Drosophila* homolog of mammalian *Yap*; refs. 8, 9), results in supercompetitors, which are cells that proliferate at the expense of neighboring normal (or WT) cells that undergo apoptosis. In addition, deletions in the tumor suppressor gene, *APC* (11) is also associated with competitive advantages and elimination of their WT neighbors. Notably, the tumor suppressor gene, *p53*, plays a context-dependent role in CC wherein cells with relatively lower *p53* expression outcompete cells with higher *p53* in the irradiated hematopoietic stem cell niche (12); and in UV-exposed epidermis (13), or irradiated esophagus (14). MT gain-of-function (GOF) *p53* exerts a fitness advantage with clonal expansion at the expense of WT progenitors fated for terminal differentiation and elimination. These observations of activated oncogenes and inactivated tumor suppressor genes conferring this 'supercompetitor phenotype' led to speculation that disrupted CC may result in tumorigenesis. Supercompetitor elimination of WT cells is analogous to the phenomena of tumor outgrowth at the expense of normal tissue wherein cancer cells kill normal cells to clear space for their own expansion. Indeed, apoptosis of normal cells in peritumoral tissue has been detected in multiple cancers (6, 9, 11). In theory, if supercompetitor clonal expansion goes unchecked, this would result in "field cancerization," which can result in tissue dysplasia and tumors overtime owing to additional mutations. These studies provide intriguing data to suggest that many advantageous cancer-associated mutations are actively selected based on their acquired ability to exploit CC and overcome barriers within heterogeneous multicellular populations required for tumor formation and growth.

CC between Cancer and the Microenvironment: Friend or Foe?

The tumor microenvironment (TME), particularly the tumor-adjacent stroma, plays a crucial role in cancer initiation and progression. The TME can both impede and facilitate tumor development and growth. The tumor–stroma interface is a highly dynamic zone that directly mediates tumor outgrowth and local invasion. In this section, we review recent studies that have attempted to model the initial stages of cancer development and growth and mechanisms by which CC may dictate clonal selection and growth under selective pressures exerted by the tissue microenvironment.

Whether tumor-host CC facilitates the growth of precancerous lesions was experimentally tested using the *Drosophila* midgut as a model of intestinal adenomas harboring $APC^{-/-}$, resulting in hyperactive Wnt signaling. In this model, the tumor cells behaved as supercompetitors and induced apoptosis in the tumor-adjacent tissue and strikingly, inhibition of apoptosis significantly impeded lesion growth (11). One mechanism of tumor-host CC fueling oncogenesis is via direct fitness sensing through Flower transmembrane proteins, which were considered to be fitness fingerprints that allow cells to directly communicate the fitness status of their neighbors. Flower is highly conserved in multicellular organisms, and in humans, *Flower* is encoded by four isoforms; two isoforms are named Flower-Win and two are named Flower-Lose. Distinct membrane isoform expression marks cells as fit (Flower-Win) or as suboptimal (Flower-Lose) for neighboring cells to recognize. When Flower-Win cells are cocultured in direct contact with Flower-Lose cells, Flower-Win cells eliminate via apoptosis Flower-Lose cells and undergo compensatory proliferation. Strikingly, several types of human cancers express high levels of Flower-Win, in contrast to the adjacent stroma, which expresses high levels of Flower-Lose, concomitant to increased expression of several

proapoptotic genes. These results suggest that in human cancers, Flower-Win expressing cancer cells outcompete and eliminate Flower-Lose expressing stromal cells, resulting in tissue attrition, and enabling tumor outgrowth (**Fig. 1A**). In addition, *in vivo* mouse models showed that xenografts of Flower-Win expressing cancer cells against a Flower-Lose tissue background resulted in aggressive tumor growth and metastasis, which is in stark contrast to the negligible tumor growth observed from xenografts of Flower-Lose cells against a Flower-Win background. Importantly, inhibition of Flower-mediated competition between tumor and stroma substantially reduced tumor volume and incidence of metastasis and heightened sensitivity toward chemotherapy (6). These results indicate that tumor growth is not the outcome solely of the cancer genome but is mostly a function of fitness inequalities and the elicited CC between the tumor and the host microenvironment.

Similarly, in several human cancers, cancer cells with increased expression of c-Myc and/or YAP, appear to outcompete and eliminate via apoptosis adjacent stromal cells with relatively less c-Myc/YAP at the tumor–stroma border (**Fig. 1B**; ref. 9). The ubiquity of cancer-stroma CC as mediated by the relative expression of c-Myc and YAP across a wide spectrum of human cancers suggests conservation in mechanisms exploited by oncogenic cells in epithelial cancers.

The beginning stages of tumor initiation remain the most elusive; we still have a nascent and largely theoretical view of the initial interactions between a transformed cell and its surrounding normal neighbors. Accumulating experimental evidence suggests that in some instances, normal epithelial cells can recognize and eliminate a transformed cell through CC. This process is termed epithelial defense against cancer (EDAC) and can be imputed as a tumor-suppressive mechanism. *In vitro* experiments using mammalian cell lines have shown that when oncoprotein RasV12-transformed cells are cocultured with WT epithelial cells, WT cells outcompete transformed cells, which are apically extruded from the epithelial monolayer (15). A recent study investigating the role of differential Hippo signaling between liver cancer cells and tumor-adjacent normal hepatocytes *in vivo* provides compelling evidence that the fitness and competitive advantages of normal cells can protect the epithelia from tumor expansion. Using a mouse model of Notch/Akt-activated driven liver cancer, researchers found that unlike hepatocytes in normal liver, peritumoral hepatocytes displayed accumulation of activated Yap (co-activator for the pro-cancerous transcription factor, TEAD). Deletion of *Yap/Taz* from peritumoral hepatocytes resulted in accelerated tumor growth (**Fig. 1C**), whereas Yap hyperactivation in peritumoral hepatocytes diminished tumor growth. Secondly, the reduction in tumor growth was a consequence of induction of apoptosis of the cancer cells surrounded by the peritumoral hepatocytes, suggesting that increased YAP in the surrounding normal cells exerts a tumor suppressive effect by outcompeting and eliminating cancer cells (**Fig. 2A**; ref. 8). Furthermore, in crypt organoid three-dimensional cultures generated from mouse intestinal epithelial cells, RasV12-transformed cells were extruded into the apical lumen when surrounded by WT cells. However, little extrusion occurred when the epithelia was nearly entirely RasV12-transformed (**Fig. 2B**; ref. 15).

The microenvironment acts as a selective pressure that heavily dictates the relative fitness status of interacting cells and thus, the outcomes of CC-mediated clonal selection. Indeed, inflammation has been found to attenuate CC-mediated elimination of transformed cells. For example, obesity-induced chronic inflammation and metabolic alterations suppressed apical extrusion of RasV12-transformed cells by WT cells in mouse pancreatic and small intestine tissue. However, aspirin treatment rescued CC-driven extrusion of
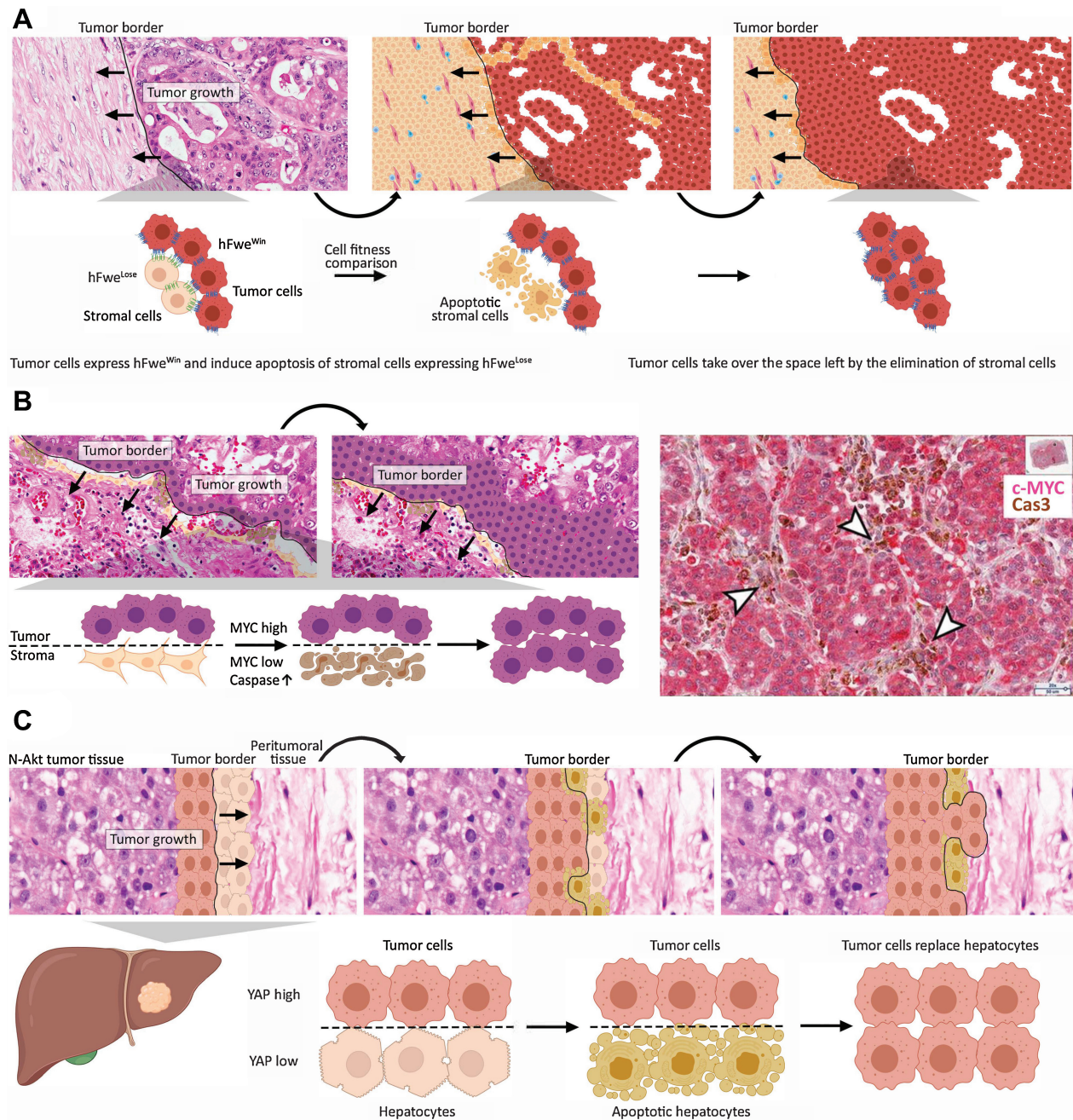
Figure 1.
A, hFwe-mediated tumor-host CC regulates tumor outgrowth along the tumor–stroma border. Left, hematoxylin and eosin staining of human colon cancer tissue demarcates tumor and stroma. hFwe^{Win}-expressing tumor cells compete with hFwe^{Lose}-expressing stromal cells within the microenvironment for space. Following cell fitness sensing and comparison, hFwe^{Win} cells outcompete and eliminate hFwe^{Lose}-stromal cells via apoptosis, creating space within which tumor cells can take over, resulting in tumor outgrowth at the expense of normal tissue. **B,** c-Myc–mediated CC drives cancer growth. Left, hematoxylin and eosin staining of human lung cancer tissue demarcates tumor and stroma. Myc-high tumor cells outcompete Myc-low stromal cells, which undergo apoptosis. Right, staining of human lung cancer sample shows high c-Myc protein in tumor cells and high caspase-3 (Cas3) in stroma cells, indicative of Myc-mediated competition-induced apoptosis. **C,** YAP–mediated CC mediates tumor outgrowth in liver cancer. Left, hematoxylin and eosin staining of human liver cancer tissue demarcates tumor and stroma. Differential YAP expression between tumor cells (YAP-high) and peritumoral normal hepatocytes (YAP-low) along the tumor–stroma border results in YAP-induced CC, resulting in expansion of YAP-high tumor cells and apoptosis of YAP-low peritumoral hepatocytes. (Created with BioRender.com.)
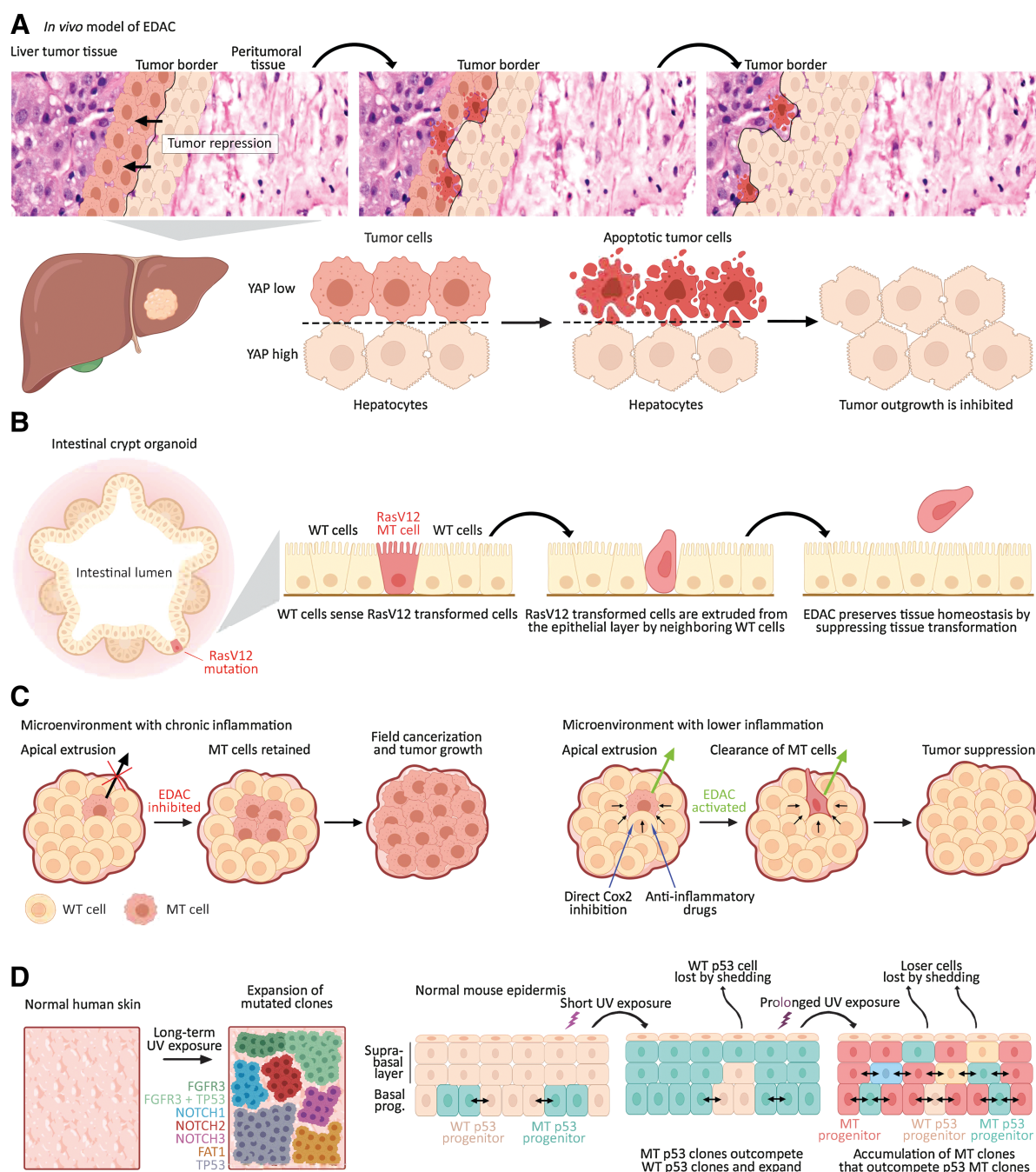
Figure 2.
A, *In vivo* model of EDAC. Top, hematoxylin and eosin demarcates tumor and stroma of human liver cancer overlaid by an animated depiction of YAP-mediated CC between tumor and stromal cells. Bottom, within liver tissue, YAP-high peritumoral hepatocytes outcompete YAP-low liver tumor cells, which undergo apoptosis. This results in proliferation of YAP-high hepatocytes and inhibition of tumor outgrowth. **B,** *In vitro* intestinal organoid model of EDAC. Within the crypt domain of intestinal organoids, RasV12-transformed cells (red cell) surrounded by neighboring WT cells are sensed by WT cells and undergo apical extrusion. This results in the elimination of MT cells and prevention of tumor development. **C,** Inflammation suppresses CC and EDAC. Left, a tissue microenvironment burdened with chronic inflammation inhibits homeostatic CC between WT and MT cells, resulting in suppression of EDAC and thereby retention of MT cells. This leads to field cancerization and potentially tumor growth. Right, targeting inflammation by COX-2 inhibition or anti-inflammatory drugs restores CC and activates EDAC; WT cells eliminate MT cells, and tumor growth is suppressed. **D,** MT clone dynamics within normal human skin and mouse epidermis. Left, DNA sequencing of aged normal human skin reveals a high degree of genetic mutations resulting in a patchwork of MT clones, most likely due to lifetime of UV exposure. Most frequent mutations were found in *FGFR3*, *NOTCH1–3*, *FAT1*, and *TP53*. Also detected was a subclone containing *FGFR3+TP53* mutations (size of clones relative to each other not drawn to scale). Right, mouse epidermis exposed to UV. UV exposure is highly mutagenic and generates a patchwork of MT clones. Short-term UV exposure results in GOF p53-MT epidermal progenitors that outcompete p53-WT progenitors and rapidly expand. Long-term UV exposure results in the out competition of p53-MT clones by MT clones with presumed increased fitness and competitive strength. (Created with BioRender.com.)

RasV12-expressing cells (16). Further investigations into the role of inflammation in CC subversion found normal cells surrounding RasV12-transformed cells *in vitro* display upregulated cyclooxygenase (COX)-2, a key mediator of inflammation, and prostaglandin E$_2$, a downstream mediator of COX-2. Induction of pancreatitis, a state of chronic pancreatic inflammation, in mice with RasV12-transformed pancreatic epithelia drastically suppressed extrusion of RasV12-transformed cells. Importantly, and similar to obesity-driven suppression of CC, reduction of inflammation by COX-2 inhibition promoted apical extrusion of RasV12 cells by WT cells (17). These results indicate that inflammation can directly impede the anticancer properties of CC, resulting in higher retention of transformed cells (**Fig. 2C**). Another aspect of the TME that selects for MT cells is the immune system. Tumors induce an immune inflammatory response that contribute to selection of aggressive clonal populations. During early tumor development, cytotoxic immune cells such as natural killer cells and macrophages recognize and eliminate immunogenic cancer cells. The remaining neoplastic tissue that is less immunogenic evades the immune system and progresses towards a clinically detectable tumor. Over time, cytokines from tumor and stromal cells elicit alterations in macrophage activation and function (18–20). These tumor associated macrophages that originally selected against MT cells now accelerate tumor growth by promoting angiogenesis (21), immune suppression, and metastasis (18, 22).

A recent investigation into the stem cell dynamics within mosaic mouse intestinal crypts attempted to quantify the competitive advantage of distinct MT clonal populations (oncogenic *Kras* MT, $Apc^{-/-}$, *p53*-MT) and their potential in tumor initiation. MT *Kras* and $Apc^{-/-}$ stem cells outcompeted WT cells, but WT cells overtime replaced MT cells and prevented field cancerization effects (23). These findings are reminiscent of clonal dynamics observed from lineage tracing of *p53*-MT clones in normal epidermis and esophagus, in which over time, the tissue was able to re-achieve homeostasis (13, 14). The ability of epithelial tissues to return to homeostasis following initial MT clonal expansion is puzzling, and future investigations into how CC regulates tissue plasticity long-term are crucial. Interestingly, in the intestinal crypt, *p53*-MT had a selective advantage and outcompeted WT stem cells in colitis-affected intestines, in which the surrounding cells exhibited reduced fitness due to colitis-induced chronic inflammation (23). Collectively, the results of these studies suggest that an inflammatory microenvironment impacts the fitness status of normal, non-transformed cells, which likely play crucial roles in determining outcomes of CC in early stages of tumor development. Total stem cell number within the crypt itself may also modulate CC outcomes: increased total stem cell population enhanced CC-removal of MT stem cells by WT cells (24).

Advanced sequencing technologies have revealed that normal aged human tissue contains a remarkable degree of genomic mutations and other aberrations, despite the tissue maintaining structural and functional integrity with no clinically detectable cancer (25, 26). Revelations into the degree of mutations found in normal tissue postulate what mechanisms dictate expansion versus contraction of MT clones and their progression into malignancy. Two recent studies, which sought to trace MT clonal dynamics underlying CC in normal tissue, found that altering the exposure to specific stressors or altering fitness of WT cells greatly impacted CC outcomes (13, 14). Two model tissues were investigated—the epidermis and the esophagus—which are both squamous epithelial tissues, wherein tissue homeostasis is maintained by a pool of proliferating progenitor cells that terminally differentiate as they progress toward the tissue surface and are shed. Clonal lineage tracing in the normal murine esophagus found that p53-MT progenitors outcompeted their WT neighbors following low-dose ionizing radiation, but competition was obviated by antioxidant pretreatment. This indicates that alteration of the microenvironment affects CC outcomes (14) and that enhancement of relative fitness status of WT cells tilts CC in favor of tumor suppression. These concepts are supported from findings of a murine model of the mutagen-exposed esophagus that manifested a heavily mutated mosaic epithelium composed of *Notch* and *TP53* MT clones, wherein the growth of MT clones was directly impacted by competition with their neighbors, and the proliferative advantage of MT clones diminished when faced with cells with an equivalent fitness status (27). Mapping of MT clones in the normal human esophageal epithelium revealed a strong selection of clones mutated in various cancer genes, including *TP53* and most notably, *NOTCH1*, arguing these clones harbored a competitive advantage over their neighboring cells (25). In mouse esophageal epithelium, single-cell induction of Notch-inhibiting mutations in mice with sporadic p53 MT clones resulted in rare double-MT clones that grew larger than p53-MT clones (28), thus illustrating how cooperative mutations that advance competitive potential impacts clonal selection and perhaps eventual field effects.

In a similar study, it was shown that within the UV-exposed epidermis, progenitors accumulate GOF p53 mutations, which outcompete and proliferate at the expense of their WT neighbors. However, over time, the epidermis can achieve homeostasis and MT-progenitors no longer outcompete WT neighbors. Notably, long-term UV exposure decreases p53-MT clonal expansion, which is conceivably due to UV-induced accumulation of additional mutations, which generates additional aggressive MT clones that can outcompete p53-MT clones (13). This is similar to what has been observed in normal aged human skin subjected to a lifetime of UV-exposure, which is heavily burdened with many cancer-associated somatic mutations, including those in *TP53* (**Fig. 2D**). It is also possible that if increased density of MT clones persist, this may continue to promote WT cell loss by differentiation—this would help achieve homeostasis and fate balance between proliferation and differentiation, thus maintaining tissue integrity. However, if exposure to a specific mutagen or inflammation persists, this would tilt the fate of positively selected MT clones toward uncontrolled expansion, and overtime, accrual of additional mutations, and subsequent tumor formation.

CC in Clonal Expansion of Normal and Premalignant Human Epithelium

Epithelial homeostasis relies on appropriate stem cell behavior to maintain a population of differentiated daughter cells that form a functional epithelium. This process is tightly regulated but is susceptible to changes that can result in tumorigenesis. The risk of oncogenic progression within tissues such as the human colon is thought to be based on stem cells acquiring sufficient mutations in critical genes that become fixed within the epithelial unit, which clonally expands and forms a cancerized field effect from which tumors may arise. To achieve this, a MT stem cell must outcompete its WT neighbors, resulting in all future progeny being similarly mutated (29). Within epithelial units such as the intestinal crypt, there are several stem cells that each compete with their neighbors for space within the stem cell niche. Within the niche there is an optimum microenvironment that

maintains stem cell behavior and the longer a stem cell remains mitotically active at this location, the greater the likelihood that its stem cell progeny will outcompete its neighbors; a process often called niche succession (30). This ultimately results in the formation of a clone where all cells are derived from a single parental stem cell. Indeed, Corominas-Murtra and colleagues (31) were able to mathematically demonstrate that 'stemness' arises from the random neutral competition of cells within the niche using the mammary gland, kidney development, and the intestinal crypt as their models, in which competition for space is the limiting factor determining the number of stem cells within the niche. Competition in the normal epithelium is not solely restricted to neighboring stem cells; clonal units that comprise many stem cells may form within the epithelium, and the degree to which they expand is determined by selection or the lack thereof when competing for space in the presence of other clones (32).

In the absence of positive selection, clonal competition and expansion is thought to occur via a neutral process where clones stochastically appear and disappear over time. In humans, tissue stem cell behavior can be visualized through the use of lineage tracing methodologies to map clonal fate (33, 34). Naturally occurring mutations that result in biochemical defects is a common method to measure stem cell dynamics within human epithelial tissues. Using mutations within the mitochondrial genome that result in a biochemical deficiency of cytochrome *c* oxidase (CCO) it has been possible to both observe and measure stem cell dynamics within the normal human intestinal crypt, skin, pancreas, and breast (35, 36). CCO-deficiency can be easily identified histologically (**Fig. 3A**), and microdissection sequencing of multiple CCO-deficient cells can demonstrate they are clones, each sharing the same mitochondrial DNA mutation as their neighbors. As new MT stem cells arise, they compete with their WT neighbors, resulting in either losing or winning the right to seed the entire stem cell niche with their progeny. To determine the rate at which they do this, we can take advantage of the fact that daughter cells inherit their parent's stem cell genotype and in the intestinal crypt move in a conveyer belt manner (35, 37). This effectively means that cells located further up the crypt represent an older cell population than those at the crypt base. The proportion of CCO-deficient to CCO-normal cells at each physical level within the crypt represents the state of CC at that particular time point. Generating *en face* serial sections all the way through partially CCO-deficient crypts allowed the ability to trace the expansion and contraction of CCO-deficient clones over time, which is colloquially referred to as the 'wiggle' (**Fig. 3B**). Determining the change in the size of CCO-deficiency between each serial section in the reconstructed crypt allowed for calculation of the rate at which clonal expansion and competition between clones occur. In the normal adult human crypt, deviations in clone size relative to sequential sections were found to be balanced, and therefore CCO-deficiency represented a neutral marker of clonal expansion, suggesting that niche succession in morphologically normal crypts is based on stochastic stem cell divisions (37). Interestingly, neutral drift also occurs in partially CCO-deficient adenomatous crypts with the important distinction that if we view niche succession as a game, the game (the loss or replacement of stem cells) is played out at a faster pace yet the outcome of whether an adenomatous crypt becomes entirely CCO-deficient or CCO-normal, remains stochastic. Therefore, neutral stem cell drift occurs even in *APC*-mutated crypts if all stem cells possess the same genotype.

Crypts themselves compete for space within the colon and are able to divide by a process known as crypt fission, resulting in clonal expansion, and can also fuse with neighbor crypts, resulting in potential clonal contraction (38, 39). In the normal human colon, a lifetime of clonal expansion can result in a significant patch size of related crypts. Novelli and colleagues (38) have shown that random x-inactivation of a germline inherited MT allele for the *G6PD* gene early in development can result in the formation of large patches of clonally related crypts late in adult life. However, if MT crypts with no selective advantage arise later in life, such as CCO-deficiency, the patch size becomes very small (33, 40). Not only does this show that crypt fission is rare in the normal adult colon, but it is difficult for field cancerization to develop to any significant extent if there is no selective advantage over WT crypts. In addition, clonal expansion is countered by an apparent similar rate of crypt fusion to the extent that overall crypt numbers are maintained in the human colon (39). If a crypt fusion event occurs, there is an immediate increase in the number of stem cells in the newly formed crypt where each parent crypt has contributed 50% of the new fused crypt stem cells. The competition between these two distinct clones begins as if it were a normal crypt and niche succession eventually results in a clonal crypt with a similar number of stem cells to its neighbors with no remaining evidence of the loser half of the crypt.

While it is likely that the vast majority of somatic mutations that occur in human epithelial cells are neutral, there is evidence on how those with selective advantages behave. As discussed earlier in this review, in animal models, mutations in *apc*, *kras*, and *p53* can result in outcompeting WT intestinal stem cells via biased niche succession and crypts MT in these genes preferentially expanding via increased crypt fission as they confer proliferative and survival advantages to the individual crypt (23, 34). Using somatically acquired mutations as a means to lineage trace stem cell clones in the human colonic crypt, Nicholson and colleagues (34) showed a marked difference in the rate of niche succession and clonal expansion between neutral and pro-oncogenic mutations in morphologically normal colon. The average patch size of MT tumor suppressor STAG2 crypts is significantly larger than either the patch size of CCO-deficient (33) or MT period acid Schiff crypts (34) that have no selective advantage. It is highly unlikely that in truly normal human epithelium, even under conditions of natural selection that MT clones can expand through the entire tissue and it is therefore highly unlikely that individual clones are able to undergo clonal sweeps where the entire epithelium is clonal.

One of the most surprising observations of human clonal expansion in recent years was the discovery that using next-generation sequencing (NGS) technologies, normal epithelial cells particularly those taken from the eyelid (26) and esophagus (25) of aged individuals display a significant mutation burden and diversity of pro-oncogenic genes in the absence of cancer. Within the normal esophagus, the size of individual clones appeared to be affected through smoking and in the eyelid by sun UV exposure but time appears to be the predominant factor of clone size. This observation is not uniform across all human epithelial tissues. In the normal human bladder, while there is a similar diversity of mutations, most under positive selection are detected in chromatin remodeling genes, whereas comparison genes associated with bladder cancer are relatively rare (41). Overall, it is now clear that the normal epithelium represents a mosaic of competing clones that may or may not determine a patient's cancer risk.

With the advent of NGS and genome atlas projects, tumor and normal tissue sequencing established that many somatic mutations do not reliably result in cancer development and that most genetic changes within a particular cancer subtype are not shared across patients. While gradual, stepwise somatic mutations are essential to tumorigenesis, cancer sequencing studies demonstrated that punctuated bursts of genetic change at the karyotype level are necessary for cancer progression (42–44). Chromosomal instability (CIN),
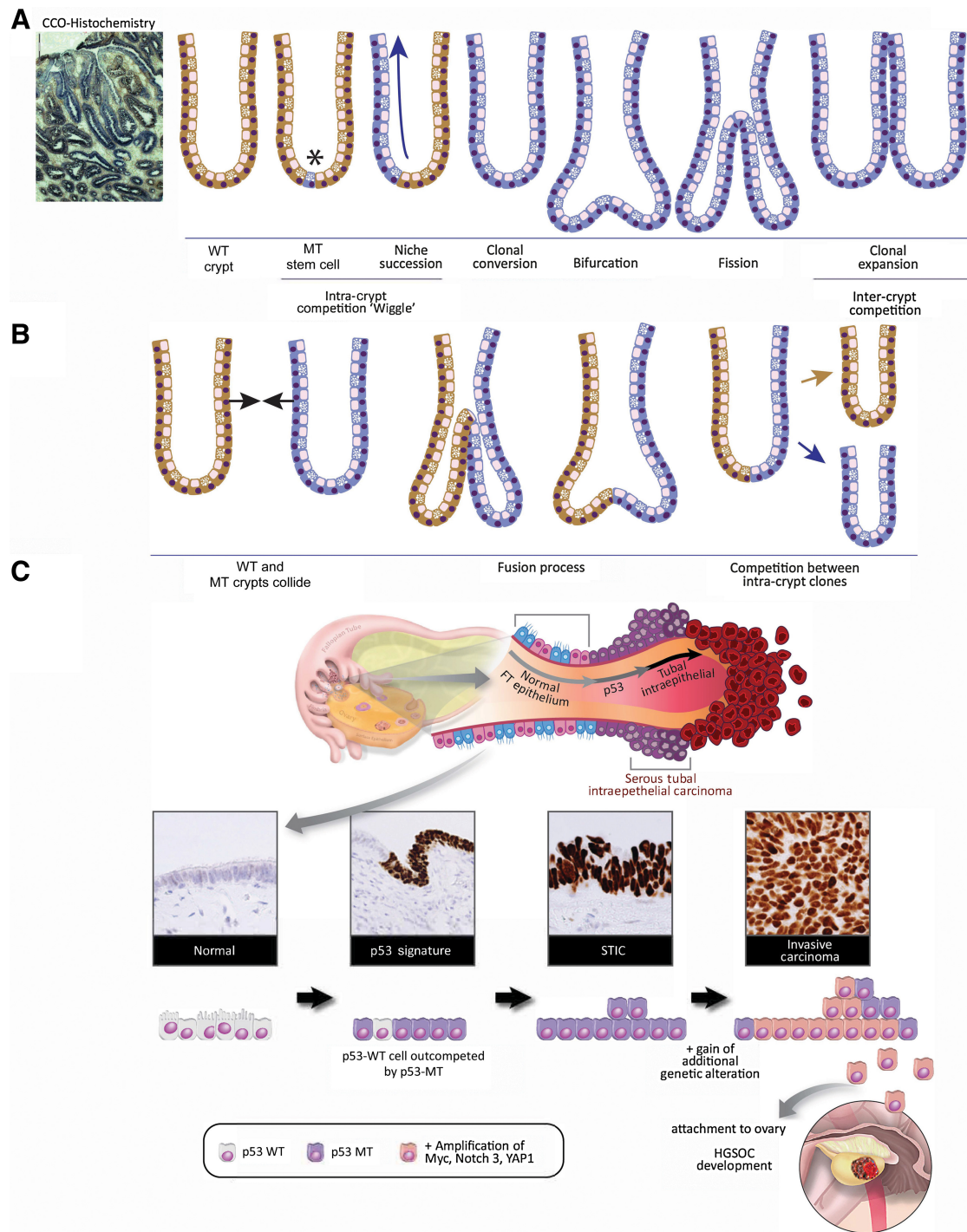
Figure 3.
A, CCO-deficiency as a result of mitochondrial DNA mutation. This process begins with a CCO-MT stem cell arising at the base of a CCO-WT colon crypt. Neutral stem CC results in niche succession and clonal conversion to a CCO-deficient crypt. MT crypts divide by fission creating a MT patch that competes for space with the colon mucosa. **B,** Crypts are able to fuse. Two phenotypically distinct crypts collide and begin to fuse in a process the reverse of fission. This results in two independent clones competing for the stem cell niche. The winner (in this scenario) is random but results in the removal of the loser from the colon. **C,** Mutations in *TP53* drive CC in early fallopian tube precursors of ovarian cancer. The majority of HGSCs arise in the distal fallopian tube. Histology: normal tubal epithelium is composed of ciliated and secretory cells. Acquisition of *TP53* mutations, through the repetitive release of inflammatory follicular fluid during ovulation, confirms an advantage to secretory cells. Their expansion leads to the formation of p53 signatures. These cells stain strongly for p53 by IHC. Subsequent alterations in oncogenes and tumor suppressors, like *MYC*, *PTEN*, *RB*, *YAP1*, or *NOTCH1*, among others, cooperate with MT *TP53* to drive the transformation of the p53 signatures into serous tubal intraepithelial carcinoma (STIC lesions). STIC lesions retain strong staining for p53. STIC cells outcompete neighboring WT cells and eventually invade through the basement membrane to establish an invasive HGSC. (Created with BioRender.com.)

classified as either structural or numerical, has typically been known to diminish cellular fitness. However, studies investigating chemotherapy resistance have demonstrated that CIN-driven macroevolution provides cancer cells with additional mechanisms to thrive under stressful environments (45). In particular, aneuploidy has been demonstrated to induce chemotherapy resistance in patient-derived xenografts (46) and promote metastatic progression (47) through increased epithelial-to-mesenchymal transition activity (48). Understanding the role of CIN in cell fitness will play a crucial role in evaluating cancer risk, as higher levels of CIN are typically associated with poorer prognosis (48).

Oncogenic mutations alone are not necessarily sufficient to generate large-scale field cancerization, and microenvironment factors such as inflammation play an essential role in clonal expansion. Inflammation appears to strongly select for p53-MT crypts, and this is reflected in human cancers associated with inflammation such as ulcerative colitis and Crohn's disease (49). The evolution of cancer begins long before the emergence of a detectable malignancy. As described above, ostensibly healthy tissues accrue mutations throughout life, and a subset of the mutation bearing cells have an altered phenotype that experiences evolutionary selection leading to an expansion of the fitter clones. Sometimes the clonal expansion is the first step towards cancer. Studies in Barrett's esophagus and inflammatory bowel disease are excellent examples of this precancer evolution. In both conditions, MT cells, often carrying alterations in critical cancer-associated genes including *CDKN2A* (p16) and *TP53* (p53), experience positive selection and expand over huge extents. For example, in Barrett's, alterations to *CDKN2A* are often present throughout the entire Barrett's lesion (50), and in general, the expansion of MT clones is positively correlated with cancer risk (51). In IBD, we documented in one remarkable Crohn's colitis patient the occurrence of multiple *TP53* clones, one of which grew along the entire colon length and crossed into the small intestine during 4 years of surveillance (52). Large clonal expansions are found more commonly in people with IBD who progress to colorectal cancer (53).

However, in many examples, cells bearing cancer-associated mutations do not progress to cancer in a person's lifetime. For example, in Barrett's esophagus mutations of many cancer-associated genes are also found in never-dysplastic Barrett's; only *TP53* and *SMAD4* mutations are stage-specific to cancer (54). In the colon, epithelial cells within crypts contain thousands of somatic mutations by middle age and 1% of crypts reportedly carry a driver alteration (55), yet less than 10% of people will develop colorectal cancer in their lifetime (56). Thus, it appears that the initial stages of cancer evolution involve many 'false starts' where the acquisition of a so-called cancer mutation is not, in fact, the first step along the evolutionary trajectory to cancer, or at least not the first step upon a trajectory that is rapid enough to lead to cancer in a patient's lifetime. Moreover, whether 'false starts' are a result of the wrong combination of genes being simultaneously activated or a consequence of induction of counteracting genomic changes remains a possibility to be explored.

CC could play a role in preventing cells with a cancer-associated mutation from forming cancer. As described above for neutral competition, to retain a place in the stem cell niche is commonplace across tissues. Competition between stem cells for a place in the intestinal crypt niche is a clear example. While a cancer-associated mutation may give an advantage in the competition for a place in the niche, it may be insufficient to lead to the growth of the niche itself, or alternatively be insufficient to allow the MT cells to grow without reliance on the stem cell niche. In the intestine, for example, *KRAS* mutations are positively selected in the intestinal crypt (23, 40), but alone are insufficient to

produce neoplasia. In other words, CC could conceivably act as a 'red herring' for somatic evolution, providing selective pressure for a phenotype that is competitive for initial expansion (e.g., within the intestinal crypt), but not necessarily adapted for subsequent development beyond the confines of the initial growth (e.g., to drive the growth of the crypt population).

Similarly, in ovarian cancer, mutations in *TP53* are obligatory (57), occurring in nearly 100% of cases, and are detected in the earliest recognized precursor lesions in the fallopian tube called 'p53 signatures' (58). These early precursors are composed of stretches of contiguous nonproliferative secretory cells that maintain a near-normal morphology yet harbor somatic *TP53* mutation and accumulation of DNA damage (**Fig. 3C**). The development of these precursors is epidemiologically and experimentally linked to ovulation and the associated inflammation triggered by the repetitive release of monthly follicular fluid that contains cytokines, chemokines, and reactive oxygen species (59, 60). These p53 signature cells are flanked by benign WT secretory cells. Next-generation whole-exome sequencing shows that p53 signatures are not only the precursors to high-grade serous carcinoma (HGSC; ref. 61), the most common subtype of ovarian cancer, they can also persist for decades before acquiring additional alterations that lead to an *in situ* carcinoma (61, 62). This suggests that MT p53-expressing secretory cells have a transient fitness advantage that enables the MT cells to expand and develop into a p53 signature. However, in the absence of additional mutations, these cells can persist but are kept in check by neighboring WT cells. This is relevant because nearly a third to 50% of all women have 'p53 signatures' in their fallopian tubes regardless of risk (e.g., *BRCA1/2* mutation status) yet ovarian cancer is a relatively rare disease.

Interestingly, these molecular studies also showed that multiple tubal precursors could arise in the fallopian tube, and they are not always related. Specifically, in a fallopian tube with multiple 'p53 signatures', each signature may harbor a different *TP53* mutation, presumably due to the stochastic effects of ovulation, and only one of these signatures may ultimately develop into an *in situ* lesion and invasive carcinoma. Presumably, the *TP53* mutations that were unsuccessful in progressing to carcinoma were limited by competition with a more aggressive *TP53* mutation that is detected in the subsequent *in situ* lesion and invasive carcinoma. It also suggests that not all *TP53* mutations are created equally and that WT cells can often impose a restraint on their impact, an example of EDAC.

The ability of some 'p53 signatures' to progress into *in situ* lesions and HGSC is often accompanied by the acquisition of *MYC*, *NOTCH3*, or *YAP1* amplification. As seen in other organs, these oncogenes enable the nascent p53 signature cells to outcompete and likely eliminate their adjacent WT cells. Although the acquisition of these activated oncogenes may take decades, once acquired, the progression from a tubal cancer to an ovarian cancer with widespread dissemination to the peritoneal cavity can occur rapidly within 6 years (61, 62).

Conclusion

Advanced sequencing technologies that allow identification of extensive cellular heterogeneity coupled with intricate clonal lineage tracing techniques to analyze the temporal and spatial regulation of clonal behavior is rapidly advancing our understanding of mechanisms underlying normal tissue growth versus aberrant growth, i.e., cancer. Cancer clonal evolution is dependent on cell-extrinsic interactions between other cells as well as cues from the microenvironment. Observational findings from various human tissues and experimental

models suggest CC may be a crucial mediator of clonal dynamics in normal tissues and throughout tumorigenesis, as it has been implicated in initial tissue transformation (both permissive and suppressive to tumor formation), field cancerization, and tumor outgrowth at the expense of normal tissue. Additionally, CC offers a distinct lens into the evolution of premalignant lesions into malignant cancers. As our understanding of CC increases, new potential therapeutic targets will be uncovered and specific genomic changes allowing more accurate staging of premalignant and metastatic cancers will lead too enhanced prognostication of therapeutic responses.

Authors' Disclosures

R.A. Winn reports personal fees from Genentech and grants from Bristol-Myers Squibb Foundation outside the submitted work, and reports being a board member for LUNGevity Foundation and American Cancer Society. R. Drapkin reports personal fees from Repare Therapeutics and other support from VOC Health outside the submitted work. P.B. Fisher reports being a cofounder and having ownership interest in InVaMet Therapeutics Inc. (IVMT); Virginia Commonwealth University and the Sanford Burnham Prebys Medical Discovery Institute have ownership interest in IVMT. P.D. Fisher is also a cofounder and has ownership interest in InterLeukin Combinatorial Therapies (ILCT); Virginia Commonwealth University has ownership interest in ILCT. Both companies focus on cancer and cancer therapies. None of the work discussed in this review directly relates to research currently being performed at IVMT or ILCT, or through sponsored research agreements with IVMT or ILCT. No disclosures were reported by the other authors.

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