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REVIEW

Super competition as a possible mechanism to pioneer precancerous fields

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Cancer is the result of sequential genetic changes over time that transform a cell into a malignant and ultimately invasive entity. The insight that cancerous cells arise from a series of mutations in oncogenes and tumor suppressors, commonly known as multistep carcinogenesis, has been conceptually elaborated and proven in the last 20 years. Although knowledge about late steps of cancerogenesis and disease progression has greatly advanced, the initial molecular events remain largely unknown. Basic research in *Drosophila* has started the quest to find early markers that detect initial clonal expansion of precancerous cells. These efforts were spurred by novel findings demonstrating that certain mutations transform cells into super-competitors that expand at the expense of the surrounding epithelial cells without inducing histological changes. This mechanism, discovered as super competition in the fly, might also lie at the heart of a clinical observation termed 'field cancerization'. This review aims to bring together current understanding from basic research on cell competition and clinical studies that have analyzed field characteristics to highlight parallels and possible connections.

The journey of a thousand miles must begin with a single step (Chinese proverb).

Research focusing on 'early steps of cancerogenesis' shows that the definition of early steps is very stretchable. Studies range from characterizations of initial mutations in oncogenes to the analysis of benign precursor forms like adenomas or small primary tumors. Although a small tumor can be classified undoubtedly as 'early stage' with respect to a more positive prognosis outcome for such patients, the lesion may already comprise a mass of a billion (10^9) transformed cells, which represents the minimal size of a clinically detectable tumor (see Table I). By then, the tumor cells have already acquired several mutations in tumor suppressors (loss of function) and oncogenes (constitutive activating), in a process commonly referred to as multistep carcinogenesis (1). In a very simplistic model of tumor cell growth, it would require only 10 further doubling cycles to produce a tumor mass of 10^{12} cells, the maximal tumor size compatible with life (2). In reality, the dynamics of tumor growth are more complex. At the same time, as certain cells in the tumor overproliferate, other cells remain dormant or undergo apoptotic or non-apoptotic (necrosis, autophagy, senescence or mitotic catastrophe) cell death (3). The contribution of cell death to the dynamics of tumor growth can vary greatly depending on the genetic mechanisms involved in tumor development. In general, cell death is frequent when tumors have already reached a considerable size and oxygen supply, as well as waste disposal become limiting factors for further growth. Going back to the simplistic model, this would still imply that the later doubling cycles, which result in visible tissue transformation, represent merely the tip of the iceberg. The bulk of a tumor's doubling cycles occur before, undetectable for the pathologist's eye.

Abbreviations: Brk, Brinker; Dpp, decapentaplegic; HNSCC, head and neck squamous cell carcinoma; LOH, loss of heterozygosity.

The initial preneoplastic events and their consequences in a tissue, however, remain difficult to reveal because they are not yet accompanied by morphologic alterations. Therefore, many of the underlying molecular events are not well known. Consequently, there are few molecular markers available that would detect a cancerous process at such early stages, an achievement that could markedly improve therapeutic success of known agents, but might also lead to the development of new preventive treatments.

The number of mutations necessary and sufficient to give rise to a malignant cancer is unknown, but estimates range from 3 to 12 mutations depending on the cancer type, whereas organs with rapid turnover of cells require even more mutations (4). The spontaneous rate of somatic mutations is not high enough to account for such accumulation of mutations in a cell. Moreover, most damage is neutralized by effective DNA repair mechanisms. To explain the early events that ultimately lead to cancer, several hypotheses have been put forward: (i) mutations might cause a 'mutator phenotype' (5) (e.g. damage of DNA repair genes); (ii) reactivate a developmental program (including epigenetic changes) (6) or (iii) create a 'preneoplastic field', large enough to increase the likelihood of subsequent genetic hits in this population (7–9). In the latter case, such 'field cancerization' can arise due to independent hits in a large population of cells (caused by prolonged exposure to a carcinogen, polyclonal origin) or a specific mutation in a single cell, leading to subsequent clone spreading within a tissue (monoclonal origin).

If mutations occur in stem cells, patches of differentiated cells with the same genetic alteration will form in the differentiated tissue of an organ. This initial phase of tumor formation can be viewed from an ecological perspective: distinct subpopulations of cells are confronted in a 'habitat' of limited dimensions and resources. Variation among cells (genetic and epigenetic changes), competition (differential fitness) and replication provide the basis for an evolutionary process during carcinogenesis (10). At the end of this selection process, tumors often present with mutations in a limited number of genes that provide highest fitness as cancer development advances, whereas the heterogeneity among alleles of the same gene decreases, leaving left a dominant 'winner' allele.

Similarly, tumor cells have to be able to survive and proliferate in a new microenvironment during metastasis. It seems probably that mutations that confer a competitive advantage during early expansion of the primary tumor are also important for metastasis. In fact, recent findings provide evidence that metastasis could occur during early stages of tumorigenesis, as opposed to the long-held view that dissemination represents the final deterioration in the cancerous process (11). This early metastasis model is supported by the fact that metastatic behavior is initially not selected for since it does not provide any growth advantage. Furthermore, new experiments have shown that normal or precancerous cells when injected into the bloodstream survived surprisingly well at ectopic sites.

In this review, we will contrast research on cell competition in *Drosophila* with clinical research focusing mainly on the concept of field cancerization for tumor development. Field effects are relevant for certain tumors types, especially epithelial tumors. In contrast, competition among cells is likely to play a role in most, if not all initial cancerous events and can take place as early as during development. Despite the more general nature of cell competition, we restrict the drawn parallels to field cancerization because the methodology used in those studies resemble the competition experiments performed in the fly epithelial wing disc or mammalian skin.

The genetic tools available for *Drosophila* allow mimicking the interaction of precancerous fields with the surrounding epithelium. Although the complete signaling pathways of cell competition are

Table I. Dimensions of field cancerization

	Diameter (mm)	Number of cells
One cell	0.01–0.1	1
Patch/cluster	2[a]	<200
Fields		
Lung		9×10^4
Bladder and gastric Epithelium	10	
HNSCC	>70	
Barrett's esophagus	10–90	
Tumor		
Histologically detectable		10^9
Incompatible with life		10^{12}

[a]Skin (patches of 10 mm diameter have been reported for bladder and gastric epithelia).

still unknown and the connection to cancer formation in mammals is not proven yet, we will discuss the current findings in this field, which might provide insight to advance understanding of the enigmatic early steps in carcinogenesis.

Cell competition—the losers quit the field

The phenomenon of cell competition was first discovered in *Drosophila* where several genetic tools are available to generate patches of mutant cells in an otherwise wild-type tissue (Figure 1A). These include the use of an inducible recombinase (flipase) that allows site-specific recombination at target flipase recognition target (FRT) sites in a process called mitotic recombination and direct flip-out of gene cassettes flanked by FRT sites in transgenic flies upon activation of the recombinase (equivalent to the Cre-lox system in mice) (Figure 1B) (12,13).

The first evidence of competition between cells was obtained in an experiment where mutant cells for ribosomal genes (*Minutes*) were apposed with wild-type cells and the proliferation of both cell types was monitored over time (14). *Minute* homozygous cells did not survive because they lacked functional ribosomes. Surprisingly however, *Minute* heterozygous cells, which were viable on their own and gave rise to normally sized flies in a homotypic background, proliferated less and were replaced by surrounding wild-type cells when both cell types were mixed (15,16). These experiments showed that the active purging of the slower growing 'losers' was dependent on the presence of the wild-type cells and therefore reflected a true case of cell competition (reviewed in ref. 17). It has been shown later that a similar process takes place during the development of chimeric mice, partly generated with *Minute* cells. When wild-type cells were injected into blastocysts heterozygous for a ribosomal mutation, the wild-type cells showed a growth advantage and ultimately contributed to a greater extent to diverse tissues of the adult mouse (18).

More recently, the transcription factor and proto-oncogene *myc* was identified as a key mediator of cell competition. Analogous to the *Minute* cells, clones mutant for *Drosophila myc* (*dmyc*) were outcompeted by wild-type cells when both cell types were confronted in the epithelial tissue of the developing wing imaginal discs (19,20). More intriguing even was the finding that cells overexpressing dMyc were able to eliminate 'optimal' wild-type cells (therefore named 'super-competitors') and take over the entire wing epithelia (20,21). Detailed analysis revealed that dMyc-overexpressing super-competitor cells proliferated at higher levels, but remarkably this excess growth was compensated by the concomitant death of wild-type cells in a way that overall cell numbers were not altered and no morphological malformations appeared neither in the developing larval discs nor later in the adult wing structure. Mutations inactivating the conserved Salvador–Hippo–Warts pathway, implicated in growth control (reviewed in ref. 22–24), were also proposed to trigger super competition (25). Cells expressing such mutations show overgrowth in the presence of wild-type cells. The fact that both deregulation of dMyc and the Hippo pathway are associated with tumorigenesis points to a possible link between cell super competition and cancer.

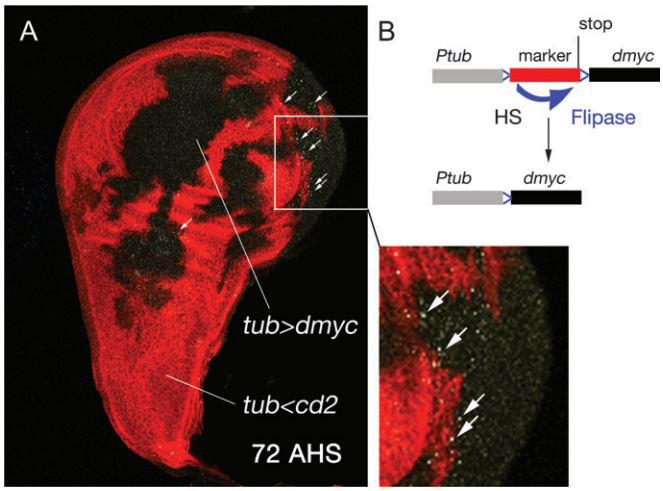

Fig. 1. Precancerous fields in the fly. (**A**) Patches of cells that overexpress *Drosophila* Myc (black cells) under the *tubulin* promoter are generated in the imaginal disc epithelium with wild-type levels of dMyc (red cells). The image depicts the extension of dMyc-overexpressing cells 72 h after clone induction by heat shock (72 after heat shock). The precancerous patches have grown at the expense of the surrounding wild-type cells, which undergo apoptosis at the borders of cell competition (see inset). Apoptosis was detected by staining against activated Caspase 3 (arrows). (**B**) Scheme depicting a transgene used to generate super-competitor clones. The transgene consists of the *tubulin* promoter (Ptub, gray box) followed by a marker gene (here *cd2*, red box) flanked by FRT sites (blue triangles). In addition, the transgenic flies carry a heat-shock inducible Flipase on a different chromosome (data not shown). A short heat shock (HS) will activate the recombinase and cause the flip-out of the marker cassette and stop signal in a subset of cells, leading to expression of *dmyc* (black box) driven by the *tubulin* promoter.

In a short-lived fly, the consequences of replacing most tissue with dMyc-overexpressing clones might be small. In humans, however, the successful expansion of such super-competitor cells could create patches and eventually large fields of precancerous cells, where the probability of secondary and tertiary genetic hits will be more likely (26). The most challenging aspect of this process is that the conquest of the super-competitors is accompanied by the loss of neighboring cells that give way to the invaders in a form that no disturbance of the tissue becomes visible.

Induced apoptosis and engulfment pave the way

The disappearance of the loser cell population is characterized by continuous apoptosis of loser cells along the borders of cell competition, where winners and losers physically interact (20,21,27,28) (Figure 1A, inset). If the apoptotic machinery is blocked, the super-competitor behavior of the winner cells is contained and both groups coexist, although the super-competitors still show a proliferation advantage. These experiments show that cell competition relies on the killing of surrounding cells to allow the expansion of potential competitors. Further genetic analysis that aimed to determine what occurs upstream of apoptosis demonstrated that loser cells upregulate the repressor Brinker (Brk). In the fly, upregulation of Brk can activate the c-jun N-terminal kinase pathway, leading to apoptosis (27). In addition, the induction of the proapoptotic gene *hid* also seems to precede the death of loser cells (21). In winner cells, Brk expression is repressed by high decapentaplegic (Dpp)/ bone morphogenetic protein (BMP)-signaling levels (20,29). Dpp is a secreted morphogen produced by a stripe of cells in the center of the fly wing disc. If Dpp signaling or endocytosis is artificially elevated in loser cells by genetic manipulations, the cell competition response seems to be hindered and a rescue of the loser cells is observed (20). This indicates that winner cells compete more efficiently for survival factors compared with their neighbors. One of these factors seems to be Dpp, but

other signaling molecules might also be required. On the other hand, the expansion of super-competitors depends on the ability of dMyc to maximally activate the protein synthesis machinery (30). dMyc-overexpressing super-competitors that are heterozygously mutant for a ribosomal protein gene are no longer able to outcompete surrounding cells (20). This demonstrates that the highly competitive behavior of dMyc super-competitors depends on increased metabolic activity, which probably allows highly efficient endocytosis of survival factors such as Dpp/BMP.

Taken together, the work in the *Drosophila* imaginal discs over the last years has shown that cell competition is a multistep process, characterized by at least six distinguishable events. At first, an insult (i.e. mutation in *Minute* or *dmyc* genes) alters the fitness of a particular cell or subpopulation within the imaginal disc epithelium. Second, this gain or loss in fitness translates into imbalances in morphogen and survival factor signaling (i.e. differences in uptake or signal transduction of the BMP2/4 homolog Dpp). Third, through an unknown mechanism, cells are able to monitor the signaling levels of their neighboring cells and recognize distortions in the morphogen gradient (i.e. the Dpp gradient). Fourth, several lines of evidence suggest that once such discrepancies in signaling levels are detected, a secreted signal is sent by the winner cells in order to kill the 'loser' cells. Such a molecule might be encountered at the cell surface or in a secreted form to allow communication between cells. Recently, the conditioned medium of cocultured winner and loser cells was found to contain factors able to trigger apoptosis when transferred to naive cells (31), whereas the factors were not produced in homogenous cell populations. This indicates that a secreted molecule might be involved in conveying information, at least *in vitro*.

Fifth, this cell-to-cell communication ultimately leads to c-jun N-terminal kinase signaling and weak caspase activation in the loser cell. Lastly, weak caspase activation in the loser cell activates an engulfment response in the winner cell, which helps killing and finally removes the corpse of the outcompeted loser cell and/or accompanies its extrusion from the epithelial layer (28). Ultimately, this process results in a novel type of proliferation, where the winner cells proliferate by replacing (killing) the loser cells. Consequently, this type of proliferation requires the killing of the surrounding cells and has been termed 'apoptosis-dependent proliferation'.

It becomes evident that many key mediators of cell competition are still unknown. Current efforts in the field center on the identification of upstream players, such as the receptors, that might allow cells to compare their signaling levels or the killing signal produced by the winners. Recent approaches include genetic screens and microarray techniques, which allow to detect genes specifically upregulated (or downregulated) during cell competition. The discovery of such cell competition-dedicated components, if conserved in humans, might yield useful markers to trace early expansion of precancerous clones or offer completely new targets for cancer therapy.

The concept of field cancerization

In contrast to cell competition systems in the fly, clinical research on field cancerization has a straight connection to tumor formation, but faces more difficulties to trace back the events to the early cellular changes that had initiated the cancerous process based on the tissue samples available from patients. The concept of field cancerization was developed by Slaughter *et al.* based on histological examinations. One of the first observations was that cancer seemed to arise out of 'an anaplastic tendency involving many cells at once' (32). In 1953, they introduced the term field cancerization in a study on oral cancer to describe the presence of epithelial areas with precancerous characteristics, from which multifocal tumors developed with abnormal tissue surrounding the primary tumor, which might explain the appearance of second field tumors due to persistence of abnormal tissue after surgery (33). Up to now, field cancerization is best studied in head and neck squamous cell carcinoma (HNSCC), but precancerous fields have been subsequently found in numerous epithelial tumors, confirming Slaughter's early observation.

New molecular techniques have allowed to determine the relationship between field and the emerging tumor, which helps to distinguish between the different proposed theories (monoclonal versus polyclonal origins) (reviewed in ref. 26,34). It is now thought that in an initial phase, a cell acquires a mutation that allows it to multiply and form a patch of altered daughter cells. Stem cells are considered likely candidates for this initial transformation because they continuously give rise to differentiated progeny that incorporates into the tissue. With the accumulation of further mutations, the patch might eventually convert into an expanding field by gradually replacing the surrounding normal tissue, however, without showing invasive growth. Clonal divergence within the contiguous field will ultimately lead to the emergence of a primary tumor, which will share many genetic alterations with the surrounding cells of the precancerous field. Such clonally related fields can be much larger than the actual carcinoma. Remaining parts of the field after tumor resection can give rise to secondary field tumors, which show related genetic alterations to the primary tumor. Therefore, they are not independent second primary tumors. On the other hand, secondary field tumors are not identical to the primary tumor cells, as it would be the case for local recurrent carcinomas that develop from residual cancer cells left behind after surgery.

The above described clonal expansion model is regarded as the most probable explanation for field cancerization observed in contiguous epithelial tissues, such as skin, colon, esophagus, bladder, cervix, vulva and the stomach. However, it cannot be excluded, that some fields may be generated during development due to the overproportional expansion of a mutant cell. In the case of multiple tumors in glandular tissues, such as lung, breast, ovary, pancreas and prostate, the 'putative field effects' are rather attributed to the spread of polyclonal stem cells or in some cases to the homogenous exposure of a carcinogen that would provoke similar mutations simultaneously in a large group of cells.

Marking the field at risk

In order to delineate a field, molecular techniques are utilized that identify an early common genetic alteration that is unique to the tumor and surrounding tissue, but not found at distant sites in 'healthy' organs. The drawbacks of this approach are that many tumors show genetic instability, which complicates the tracing of mutations and that only known mutations can be searched for (35). Field cancerization is not analyzed yet with a fixed panel of markers, but more and more studies emerge that allow comparison of different targets for the respective cancer type (see Table II and reviewed in ref. 47).

Alterations found in patches (<200 cells), which are field precursor lesions, are thought to belong to the class of 'early markers'. The most commonly analyzed markers to reveal patches are mutations in TP53. Clusters of TP53-mutated cells are already found in normal human skin, especially in the sun-exposed parts (66). In addition, studies have shown that surgical margins of more than half of all HNSCC

Table II. Current markers—field analysis in different tissues

Tumor	Markers analyzed	References
HNSCC	TP-53, mtDNA, LOH, proteomics	(36–41)
Esophagus	TP-53, LOH (INK4a), methylation	(42–44)
Stomach	Methylation, C-erb	(45,46)
Skin	TP-53, mtDNA	(47–49)
Cervix	LOH	(50)
Vulva	X-chromosome inactivation	(51)
Bladder	TP-53, LOH	(52–54)
Colon	Methylation	(55,56)
Lungs	TP-53, LOH	(57–59)
Breast	LOH	(60–62)
Ovary	Methylation	(63)
Pancreas	K-ras	(64)
Prostate	Methylation	(65)

patients showed TP53-mutated cells clonally related to the primary tumor (36). Genetic damage in the mitochondrial DNA is regarded as another early marker. Mitochondrial DNA is more prone to genetic damage because of the presence of reactive oxygen species and the lack of an efficient repair mechanism. Therefore, it might represent a 'sentinel' indicating potential field cancerization even before nuclear alterations have occurred (47).

At this moment, it is not known which genetic alterations are involved in the conversion of a patch into field. It is conceivable that newly identified cell competition genes will eventually provide an explanation for this step, although they are also likely to act even earlier during the formation of a patch of an aberrant cell derived from a single clone.

During field progression, mutations that enhance the proliferative capacity seem to be involved in the transition from a patch to a preneoplastic field. Increased cyclin D1 expression and later duplication of the cyclin D1 locus are often found during field progression in HNSCC (67). Further genetic hits in the field lead to subsets of cells that show in addition loss of heterozygosity (LOH) for certain genes depending on the tumor type. In HNSCC, staining for Ki-67, a protein present during all active phases of cell cycle, proved to be a useful surrogate marker for LOH (68). In Barrett's esophagus, a preneoplastic condition of esophageal cancer, the field expansion correlated with LOH at 9p21 (p16 locus), where p16−/− clones extended over a region of 8 cm, whereas p16+/+ cells covered areas of only 1.5 cm (42). However, in the absence of LOH of p53, p16-deficient clones were not probably to develop into tumors (69). Large precancerous fields in Barrett's esophagus are also evident from hypermethylation of various genes such as APC, CDHI, ESR1 and p16 (43). In colorectal cancer, promoter methylation of the DNA repair gene *MGMT* has been found to create preconditioned genetic fields (55). Such epigenetic gene silencing patterns represent an additional way to analyze field changes that usually occur at an intermediate time point in the cancerous process. However, epigenetic modifications are less reliable markers than mutations since they represent reversible structural changes in the genome. Late changes, associated with a high risk of the lesion to develop cancer, are chromosomal and microsatellite instability.

Ultimately, X-inactivation patterns in female patients allow field characterizations in retrospective, once the signature of the aberrant tumor cells has been identified and clonally related cells can be traced. Although the catalog of utilized field markers is constantly expanding, it is unlikely that absolute proof of the common origin of distinct cell clones may be achieved in humans. At best, a set of highly predictive markers may be distilled that allows to trace the most relevant precancerous changes.

Plowing the field: new markers and their clinical relevance

Current research in cell competition and clinical field cancerization follows a common strategy, searching the field systematically to identify new markers or targets. Often, these screenings rely on high-throughput technology platforms to uncover specific RNA or protein signatures specific to field cancerization or cell competition.

The use of laser capture microdissection allows a more efficient and precise sampling of cells at the tumor borders versus more distant sites. In a recent study, protein profiles of HNSCC, tumor-adjacent, tumor-distant and healthy squamous mucosae were analyzed by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (37), which allows high-throughput analysis of proteins in diverse clinical samples such as biopsies, urine and saliva. A total of 48 protein forms were found to be differentially expressed between healthy mucosa and HNSCC. The protein profiling proved to be more accurate to identify precancerous tissue than histopathological classification that fails to detect aberrant cells if the morphology of the cells is not affected. On the other hand, mutational load distribution analysis has proven an efficient means of monitoring a wide array of alleles for a given (onco)gene. Although the targets need to be known in this case, results obtained with pancreatic juice suggest that mutational load distribution analysis may become a suitable tool to detect early cancerous changes (10).

In *Drosophila*, a study is underway that compares differential RNA expression in the fly wing epithelia under competition versus non-competitive conditions (C.Rhiner, J.López-Gay, M.Portela, D.Soldini and E.Moreno, unpublished data). Baker *et al.* have taken yet a different approach. They screened the *Drosophila* genome for mutations that permit survival of loser cells in the fly eye (25). In addition to various known tumor suppressors of the Hippo–Warts pathway, they also identified two non-hyperplastic mutations that are necessary for the cell competition response. The characterization of these new genes will show if a conserved target, ideally a receptor, can be identified that sheds more light on the mechanisms of cell competition.

dMyc-overexpressing super-competitor cells in the fly are thought to have increased metabolic activity due to the property of Myc to stimulate protein synthesis (30). If a connection between abnormally elevated metabolic activity and super-competitor behavior should be confirmed in mammals, future approaches to detect precancerous alterations may include non-invasive methods such as functional Magnetic Resonace Imaging, although more specific signals would be desirable.

One can argue that there is already a confusing amount of diagnostic markers available, which are known to be upregulated or downregulated in certain tumors, whereas they do not seem to matter for other cancers. The advantage of the above-presented strategies is that they depart from genome-wide scans and focus on early changes in the cancerous process. In this way, they are more probably to identify markers that point to the origin of the disease than markers of the disease. Such new biomarkers might not only be more accurate in defining tumor margins but most importantly might allow to detect aberrant cells at an earlier stage. This could be especially useful to monitor tissues, where non-invasive samples can be obtained, such as saliva, urine, blood or nipple aspirate fluids.

However, early detection does not only bear advantages. The study of early tumor markers may also pose serious moral problems. As earlier and earlier markers are tested, the number of subjects with abnormal findings will rise and the definition of cancer may change. Who should be treated then (and how) and who will probably never develop the disease? This is why early detection is only desirable if an effective treatment can be offered to patients with a higher risk to develop cancer. Therefore, the possible development of preventive therapies that target precancerous alterations is of foremost interest. It is conceivable that, for example, targeting of potential cell competition receptors might yield new therapeutic approaches in the future. Blocking cell competition and thereby preventing apoptosis of wild-type cells might already be a means of containing precancerous super-competitor clones, which usually rely on the killing of surrounding tissue for their expansion (2,70). Myc, the most potent inducer of cell competition known so far, has recently been shown to be a promising target for cancer therapy. Evan *et al.* demonstrated in a mouse model that targeting Myc with a dominant-interfering Myc version prevented the formation of Ras-induced lung adenocarcinoma and led to regression of established lung tumors (71).

Collaboration of basic and clinical research will be crucial to advance our knowledge on underlying molecular events at early stages of cancers and select the best possible markers and targets for new therapies.

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