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Opinion

Cellular Mechanisms Underlying Intertumoral Heterogeneity

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Intertumoral heterogeneity is driven by a combination of intrinsic and extrinsic mechanisms. Intrinsic mechanisms include the genetic/epigenetic mutational profile of cells and the nature of the 'cell of origin'. There is accumulating evidence that distinct 'cells of origin' within an organ can give rise to different subtypes of cancer. Tissue-specific stem and progenitor cells are the predominant targets exploited for tumor initiation. Extrinsic factors imposed by the microenvironment may also directly influence the cell of origin by eliciting dedifferentiation. Identification of these target cell populations is important for earlier diagnosis, the detection of premalignant clones during relapse, and the design of prevention therapies for high-risk cancer families. Here we review recent developments in deciphering the cellular origins of solid cancers.

Tumor Heterogeneity

Cancer embodies a collection of highly heterogeneous diseases. It seems intuitive that both the profile of acquired mutations and the cell of origin determine tumor fate and phenotype. For each organ, phenotypic and functional heterogeneity (intertumoral) is reflected in the presence of different subtypes of cancer characterized by their morphology and expression of histopathological markers. Molecular profiling studies have led to the stratification of tumors arising in many organs based on discrete expression signatures. The close association between cell lineage and cancer phenotype implies that lineage-specific mechanisms that normally coordinate development can contribute to oncogenesis. Diverse cancer phenotypes are often evident within individual tumors (intratumoral heterogeneity) and reflect both genetic and non-genetic mechanisms (reviewed in [1]).

For cancer, the 'cells of origin' refers to the normal tissue cell types that are prone to transformation through the acquisition of the first mutation(s) (reviewed in [2]). These cells need not be identical to cancer stem cells (CSCs) that reside in established tumors and sustain them [3,4]. Moreover, the phenotype of the cell of origin is likely to differ substantially from that of the CSC in most cases. Associations between mutation profiles and tumor subtypes have been revealed through several recent whole-exome sequencing efforts. For example, the mutation profiles of luminal and basal-like breast cancers differ substantially, suggesting that mutations can influence the differentiation state [5]. Interestingly, there is emerging evidence that the number of driver mutations in cancer may be fewer than originally thought, with only three sequential mutations being necessary for the development of lung and colorectal adenocarcinomas [6]. The recent discovery that pre-leukemic mutations in human acute myeloid leukemia persist in remission underscores the importance of precisely characterizing the cell population that is expanded in the pre-neoplastic phase as it may directly contribute to relapse [7,8]. Ultimately, it is the complex interplay between cellular and molecular mechanisms that influences tumor

Trends

Stem cell populations are generally more susceptible to transformation than transit-amplifying cells and differentiated cell populations. Stem cells are the likely cells of origin for intestinal and squamous cell carcinomas of the skin, whereas restricted progenitors have emerged as important targets for hereditary breast cancer and some glioma types. For several other solid tumors, multiple cell types have been implicated.

Tissue context can influence the propensity of specific cells to undergo malignant transformation.

Extrinsic factors, such as injury, inflammation, and signals from the microenvironment, can alter the fate of cells and convert them into stem-like cells capable of tumor initiation.

Understanding the cellular origins of cancer is crucial for identifying premalignant clones that may contribute to tumor relapse.

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behavior, whereby deregulated signaling promotes oncogenesis in a cell type-dependent manner.

Using the Differentiation Hierarchy to Decipher the 'Cells of Origin' of Cancer

The differentiation hierarchy that underpins the development of all cellular compartments is indispensable for understanding the cells of origin of cancer (Figure 1). For many tissues, however, the cellular hierarchies have not been adequately refined, thus making it impossible to definitively prove the cellular origins of cancer. Furthermore, features of the most abundant cell type in the tumor may not necessarily predict properties of the important functional population, and, if tumor cells exhibit phenotypic plasticity during neoplastic progression (e.g., in melanoma), then lineage markers of tumors will not reflect the *bona fide* cell of origin in normal tissue. Nevertheless, integrated genomic analyses and expression profiling of brain ependymomas [9] and breast tumors [10,11] have revealed striking correlations between the gene signatures of normal cells and specific tumor subtypes. Analysis of four recently identified subtypes of glioblastoma (GBM) indicated alternative cells of origin, and suggested that patients are unlikely to transition between different subtypes during tumor progression based on their characteristic genomic abnormalities [12]. Of note, functional proof for the relationships between normal and cancerous cells is still largely lacking and will be reliant on carrying out *in vivo* clonality studies.

Stem cells are favored candidates for targets of transformation because they have an inherent capacity for self-renewal and are long-lived, thus allowing the accumulation of requisite genetic or epigenetic mutations for transformation. Intriguingly, a link between the lifetime risk of cancer and the number of stem cell divisions over an average lifespan was recently reported [13]. Most tissue stem cells, however, have not been sufficiently purified to derive precise calculations as yet. Nonetheless, the long-term protection against breast cancer conferred by a single early pregnancy, and the increased incidence associated with radiation early in life, underscore the importance of long-lived tissue stem cells [14,15]. Progenitor and mature cells can also serve as target cells in cancer, provided they acquire mutations that confer self-renewal capacity and prevent terminal differentiation. Furthermore, although the crucial mutation(s) may originally be incurred by stem cells, they may not manifest as neoplasia until stem cells have differentiated into a more restricted progenitor cell type.

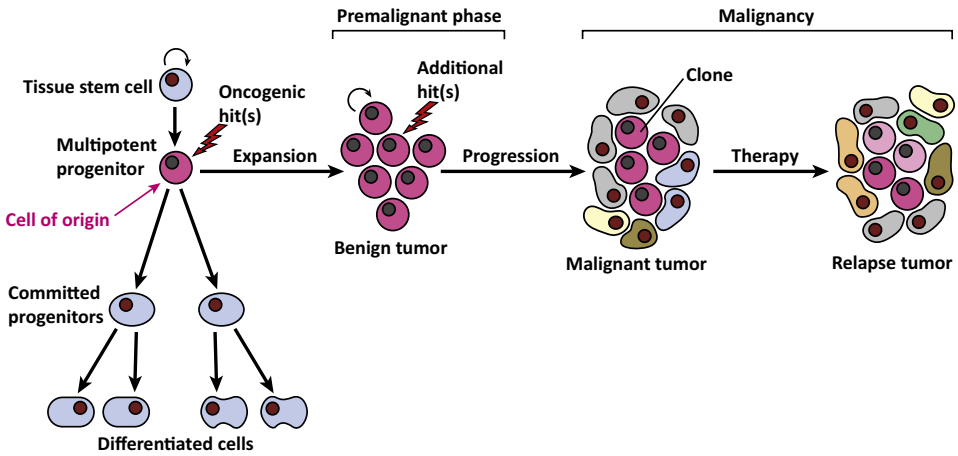

Figure 1. The 'Cell of Origin' Model of Tumorigenesis. The normal cellular hierarchy comprises stem cells that progressively generate common (multipotent) and more-restricted progenitor cells, yielding all the mature and differentiated cell types that constitute a particular tissue. The cell of origin, depicted here as a progenitor cell, is the cell that acquires the first genetic alteration(s). The accumulation of further genetic and/or epigenetic modifications occurs during neoplastic progression. As recently shown for leukemia [7,8], cells present at the premalignant stage (premalignant clone) may persist during therapy, and possibly contribute to relapse of the tumor.

The 'field cancerization' effect first postulated by Slaughter *et al.* (1953) also has bearing on the cells of origin of cancer, particularly in the case of carcinomas. The development of an expanding field of preneoplastic cells appears to be an important step in the genesis of epithelial cancers, whereby the preconditioned field of genetically altered cells is thought to directly contribute to tumor persistence or recurrence. Such field effects could facilitate the cellular plasticity of progenitor cells towards a stem cell identity and culminate in an increase in the absolute number of possible cells of origin.

Inducible lineage-tracing studies using genetically engineered mouse models have substantially advanced our understanding of potential cells of origin in various cancer types. These mouse genetic models have predominantly relied on a cell-specific promoter to govern spatial expression and a tamoxifen-regulatable *cre-ER* cassette to control temporal activation. Whilst several studies have identified the lineage in which the cancer originates, the precise cell type along the hierarchy in which transformation occurs has not been proven in the vast majority of cases. At present, firm evidence exists for the cells of origin of intestinal cancer, but data are still being gathered for most other solid tumors. Moreover, an increasing number of examples indicate that tumor histology or lineage marker expression do not necessarily reflect the cell of origin, thus emphasizing the requirement for *in vivo* studies to define the initiating population.

Stem Cells as the 'Cells of Origin' in Intestinal Cancer

For solid tissues, the intestine represents the paradigm in which stem cells serve as the cell of origin for cancer. This may be a characteristic of tissues with high turnover, such as the gastrointestinal tract, because progenitor cells presumably do not live long enough to acquire the full mutational spectrum required for transformation. Intestinal cancers largely arise from activating mutations in the Wnt signaling pathway, either via loss of the tumor-suppressor adenomatous polyposis coli (*Apc*) or by activating mutations in β-catenin [16]. Two types of crypt stem cells have been identified in the intestine: cycling Lgr5-positive columnar cells [17], and Bmi1-positive stem cells in the +4 position of the crypt [18], which have been postulated to represent active stem cells for general homeostasis and a reserve pool to replenish $Lgr5^+$ cells following injury, respectively. The finding that $Lgr5^+$ cells can express markers of +4 cells has somewhat blurred the distinction between these two stem cells [19]. Nonetheless, *Apc* deletion in either $Lgr5^+$ or $Bmi1^+$ cells within the stem cell compartment has demonstrated that stem cells, and not short-lived transit-amplifying cells, are the predominant cell of origin for intestinal adenomas [20–22] (Figure 2). It seems possible that the multiple intestinal cancer types observed clinically originate in distinct types of intestinal stem cells. The precision of promoters selected for lineage-tracing studies to address such issues is paramount and could confound identification of the true cell of origin if they fail to recapitulate endogenous gene expression. Studies on the dynamics of mutated versus wild-type stem cells in tumor initiation have suggested that while common genetic mutations in intestinal cancer confer an advantage on these clones, they remain subject to replacement by the rapidly dividing pool of normal stem cells, invoking a complex process of clonal evolution [23,24].

The Cellular Origins of Prostate, Skin, and Brain Cancers: Stem Cells or Restricted Progenitors?

Prostate cancer is relatively uniform in its pathology, and has been long presumed to originate from luminal and not basal cells owing to its luminal phenotype. The primary cell(s) of origin for prostate carcinomas in mouse and human is an area of considerable debate. In mouse models, distinct cells in either the basal or luminal compartments can initiate prostate cancer, mediated through loss of *PTEN* or PI3K activation [25,26], or through the tracking of castration-resistant Nkx3.1-expressing cells (CARNs) in the regressed prostate [27]. Histologically-similar tumors were generated irrespective of their cell of origin. Fate-mapping studies in normal prostate have pointed to the existence of multipotent cells in the basal cell compartment and independent

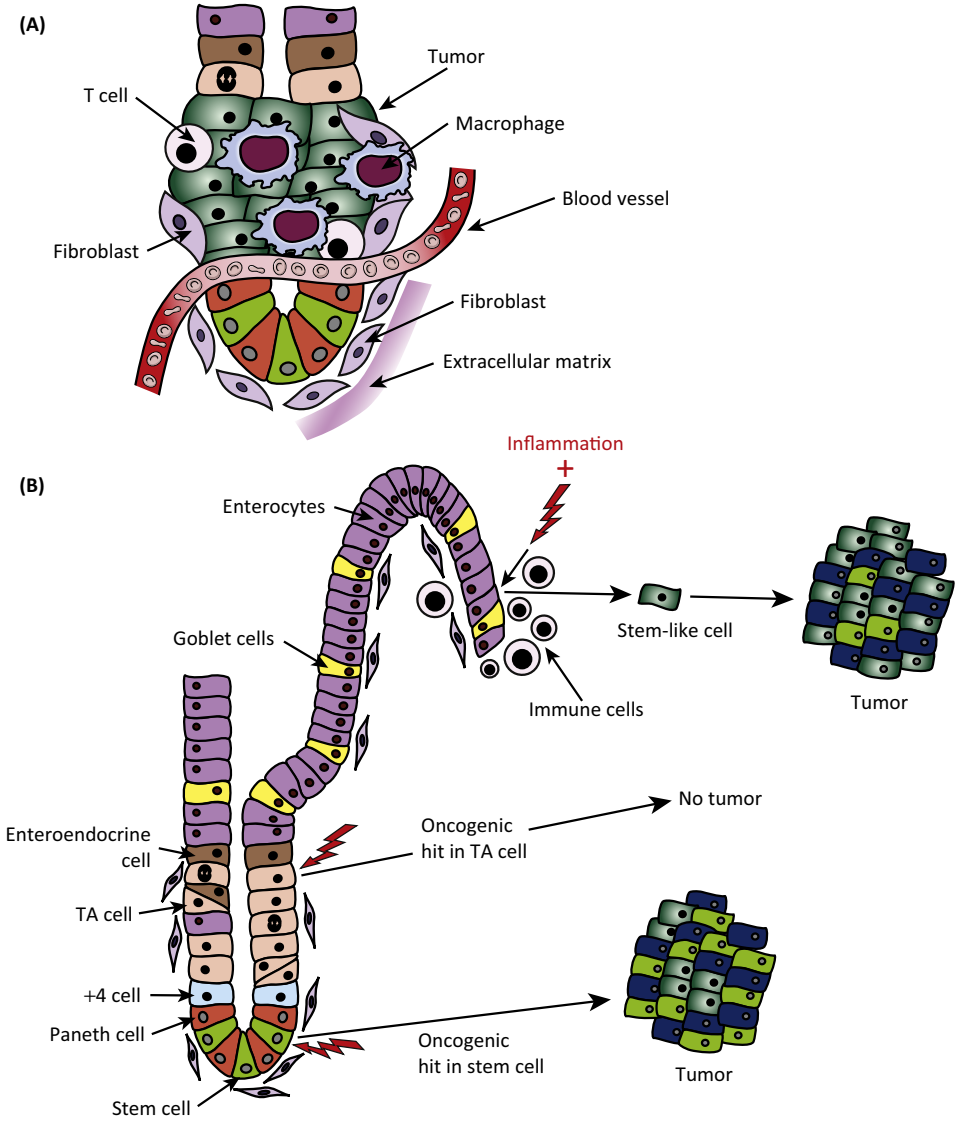

Figure 2. Cellular Plasticity and Influence of the Microenvironment on the 'Cells of Origin' of Cancer. (A) The tumor microenvironment, including endothelial cells, fibroblasts, immune cells, and the extracellular matrix (ECM), plays an important role in determining the behavior and characteristics of a tumor. (B) In the intestinal crypt, intestinal tumors generally arise from stem cells and not from transit-amplifying cells. However, under inflammatory conditions and elevated Wnt pathway signaling, mature enterocytes in the villi can dedifferentiate to stem-like cells, which then initiate tumorigenesis [66]. Abbreviation: TA cell, transit-amplifying cell.

unipotent progenitor cells in the luminal lineage [28], but it has yet to be established which of these populations is prone to transformation. By contrast, studies on human prostate tissue have demonstrated that only the basal subset is capable of initiating the development of prostate cancers, based on transduction of sorted subpopulations with oncogenic lesions and tissue recombination *in vivo* [29]. Resolution of these differences demands a more comprehensive analysis of the lineage hierarchy in the prostate and an understanding of the different micro-environments to which the initiating cells are exposed in mouse versus human assays. Even though different target cells may exist for prostate cancer, β-catenin inhibition could serve as a

therapeutic option for certain prostate carcinomas because pharmacologic inhibition of this pathway was recently shown to suppress basal cell-derived (but not luminal-derived) cancers [30].

For squamous cell carcinomas (SCCs) arising in the skin, stem cells in the hair follicle are more susceptible to transformation by *K-ras* and *p53* mutations than transit-amplifying cells [31,32], despite the hypothesis that SCCs arise from the interfollicular epidermis (IFE). Interestingly, however, UV-B irradiation of p53 mutant epidermis led to the exponential growth of preneoplastic cellular clones that exhibited a stochastic fate and appeared to be short-lived [33]. The activation state of the stem cell also appears to be important as quiescent hair follicle stem cells are more refractory to transformation than cycling stem cells [34]. Conversely, for BCC, there are discrepant data on whether the stem or progenitor cell is the more relevant target cell. BCCs frequently display aberrant hedgehog (Hh) signaling and were originally proposed to derive from the hair follicle based on phenotypic similarities. While initial lineage-tracing studies reported that cells within the IFE serve as the cells of origin in a mouse model of Hh activation [35], analysis of targeted *Ptch* mice [36,37], and those with constitutive activation of *Gli2* [38], has indicated that BCCs could originate in either different hair follicle stem cell populations, or progenitor cells within the IFE, particularly those innervated [37]. The multiple cell types implicated in the genesis of BCC and the histological subtype of BCC tumor formed may be a function of the number and type of oncogenic lesions as well as of the degree of Hh signaling [38]. Furthermore, the different tumors arising from quiescent stem cells versus activated stem cells in growing follicles [38] highlight the importance of cellular/developmental context.

Analogous to prostate and skin, different cell types in the brain have the capacity to initiate malignant glioma, including multipotent stem cells, unipotent progenitor cells, and mature astrocytes (reviewed in [39]). Analysis of preneoplastic tissue from mice harboring *Tp53/Nf1* mutations (initiated sporadically) revealed oligodendrocyte precursor cells (OPCs) as the key target cells for glioma, and transcriptome analysis confirmed the OPC-like nature of the tumor cells [40]. Importantly, these findings suggest that OPCs are the crucial cell of origin for gliomas even when the initial mutations occur in neural stem cells (NSCs). NSCs may lie dormant and only trigger malignant transformation once they have differentiated into daughter cells. These cells are of particular interest because they are more numerous than NSCs in the human brain and divide slowly throughout the adult. Although mature astrocytes may serve as the cell of origin for glioma, the stem/progenitor cell population demonstrated a greater propensity for transformation [41]. Moreover, developmental context matters since less-differentiated neuro-glial progenitors were shown to be more prone to malignant transformation at early stages of ontogeny [42]. Despite numerous lineage-tracing studies, the true identity of cells (within the subventricular zone of the brain) that are susceptible to oncogenic transformation remains to be determined.

A similar picture applies to the cells of origin for ependymoma [9] and medulloblastoma. In medulloblastoma, there is evidence that the desmoplastic subtype is linked to committed granule neuron precursors [43,44], whereas the classic subtype of medulloblastoma originates from precursor cells in the dorsal brain stem [45]. One important point to emerge from these studies is that each subtype is also uniquely sensitive to a given set of mutations. It will be important to determine whether different epigenetic mechanisms operate in the cells of origin for distinct subtypes of cancer, in view of recent findings that differential DNA methylation of specific loci occurs in different ependymoma subtypes [46,47].

'Cells of Origin' in Hereditary Cancers

Cancer chemoprevention will be most applicable to individuals within high-risk cancer families such as *BRCA1/2* mutation carriers [48,49] and colorectal cancer families with defects in mismatch repair deficiency (*MLH1* or *MSH2* mutations) [50] or germline mutations in the *APC* gene. If crypt stem cells prove to be the cell of origin for colorectal cancers, reminiscent

of intestinal tumors, then neutralizing antibodies against biomarkers on these stem cells could be effective in driving these cells into a dormant state or promoting apoptosis.

Mutations in the tumor-suppressor gene *BRCA1* are associated with basal-like breast cancer (often exhibiting a triple negative phenotype) and high-grade serous ovarian cancer [49]. In the case of *BRCA1*-associated breast cancer, there is substantial evidence from both human studies and mouse models indicating that the committed luminal progenitor represents the cell of origin [10,51,52]. Even though stem cells harbor mutations in this gene, they may not play a role in tumor initiation because *Brca1*-deficient mouse stem cells exhibit reduced repopulating capacity upon transplantation [53]. Instead, it is the daughter cell that undergoes expansion in the premalignant phase and shows greatest susceptibility to mutagenic insults [10,51]. Pertinently, based on lineage-tracing studies, the likely mouse counterpart of this progenitor cell is relatively long-lived [54]. Analysis of perturbed progenitor cells in preneoplastic tissue has the potential to unveil therapeutic targets that could be used to eradicate or 'switch off' aberrant progenitor cells before the onset of disease in these individuals. Finally, although luminal progenitor cells have been implicated in *BRCA1*-associated cancers, other cancers within this heterogeneous subtype of cancer may emanate from different cells in the hierarchy, such as the stem cell.

Remarkably, triple-negative breast cancers are considerably closer to serous ovarian cancers (SOC) in their genomic and expression portraits than they are to luminal breast cancers [11]. The cell of origin of this aggressive ovarian cancer type [55] is the subject of intense debate, but has been speculated to be either the ovarian surface epithelium (OSE) or the distal fallopian tube. A fallopian tube origin is consistent with tumor morphology and the presence of precancerous lesions frequently observed at the ends of the fallopian tube in human patients. Both *BRCA1* and *BRCA2* mutation carriers are prone to the high-grade SOC subtype. Lineage-tracing studies in fallopian secretory epithelial cells targeted for *Brca1/2, Tp53*, and *Pten* have shown that these cells can initiate high-grade SOC that recapitulates human disease and arises with short latency [56]. However, these findings do not exclude the possibility that SOC could also emanate from stem/progenitor epithelial cells in the OSE [57]. Pancreatic adenocarcinomas and high-grade prostate cancer also develop in *BRCA2* mutation carriers, but the cells of origin for these aggressive diseases have not been interrogated as yet.

Cellular 'Plasticity' and Extrinsic Influences on the 'Cells of Origin' of Cancer

In the classical stem cell hierarchy model, tissue homeostasis and regeneration following extrinsic insults are coordinated by resident stem cells that have the capacity to self-renew and generate all cell types within the tissue. Emerging evidence from lineage-tracing studies has added another layer of complexity through the findings that differentiated epithelial cells have the potential to adopt an alternative fate and function as reserve stem cells under conditions of tissue damage (reviewed in [58]). The level of plasticity within differentiation hierarchies may be more prevalent than anticipated, with important implications for tumorigenesis. For example, differentiated endocrine cells in the pancreas appear to alter their fate to serve as the cells of origin for pancreatic ductal adenocarcinoma under conditions of chronic injury and the presence of an initiating lesion [59]. Dedifferentiation may also occur in the case of small cell lung cancer (SCLC). Sporadic loss of the tumor-suppressor genes *Tp53* and *Rb1* in distinct cell lineages of the adult mouse lung revealed that neuroendocrine ($CGRP^{+}$) cells were the predominant cells of origin of SCLC [60,61]. Therefore, in tissues where the turnover rate is low, such as the lung, differentiated cells may have the capacity to act as tumor-initiating cells through the acquisition of deleterious mutations. Interestingly, however, inactivation of *Tp53* and *Rb1* in alveolar type 2 (SPC-expressing) cells also gave rise to SCLC, but at a much lower frequency, suggesting that these genetic insults either induce a cell fate change from alveolar type 2 cells to neuroendocrine cells or render a bipotent progenitor population susceptible to transformation. When oncogenic

K-ras was used as the driver mutation, adenocarcinomas arose in both the club ($CC10^+$) and alveolar type 2 (SPC^+) cell lineages, indicating that this genetic lesion is dominant over the cell of origin in determining tumor initiation [62,63]. However, formal proof for dedifferentiation during the development of pancreatic or lung cancers is yet to be obtained.

It is well accepted that the tumor microenvironment [64,65], comprising stromal fibroblasts, inflammatory and other immune cells, neuronal cells, the vasculature, and the extracellular matrix, influences tumorigenesis, but the potential impact of the microenvironment on the cells of origin of cancer has only come to light recently. Strikingly, inflammation can alter the fate of cells that are normally refractory to cellular transformation and convert them into stem-like cells capable of tumor initiation (Figure 2). This may be particularly relevant to colorectal cancer, which is strongly influenced by chronic inflammatory conditions [66]. Although stem cells are considered the cell of origin of intestinal [20] and probably colorectal cancers, non-stem $Lgr5^-$ cells (enterocytes) could dedifferentiate into cancer-initiating cells when triggered by an inflammatory signal under the control of the key proinflammatory transcription factor NF-κB [67]. This process was also highly dependent on the level of Wnt pathway activation, the most common genetic event associated with colorectal cancer. Other $Lgr5^-$ cells, such as secretory progenitors, may be susceptible to inflammation-induced changes given that they can regenerate the stem cell compartment upon damage [68]. The ability of inflammation to alter the fate of cell populations may be intertwined with the degree of plasticity inherent within specific cell subtypes under non-homeostatic conditions, and this is likely to vary considerably between different organs. Importantly, the propensity of committed cells to dedifferentiate was shown to be inversely proportional to the maturation state of cells [69].

The notion that inflammation can create alternate cells of origin may explain some of the discrepancies that have arisen between cell of origin studies in mouse and human tissues. Indeed, it has recently been proposed that inflammation or an altered microenvironment may account for the disparate data obtained for the cells of origin of prostate cancer [70]. Genetically engineered mouse models for lineage-tracing studies harbor an intact immune system, whereas the prostate tissue recombination approach used to recapitulate human prostate cancer does not. Luminal cells may be more susceptible to inflammation, with expansion of a luminal stem/progenitor population, or, conversely, the inflammatory stimulus may induce a basal to luminal cell switch [71]. Furthermore, the profoundly different mesenchymal microenvironments in the mouse and human assays invokes the actions of different growth factors on candidate prostate cancer-initiating cells [70].

Concluding Remarks

Mouse models of oncogenesis combined with lineage tracing have been pivotal in unraveling the cellular origins of cancer. However, one of the most significant limitations that the field currently faces in defining these cells is the nature of the *cre* that drives expression of the initiating event (see Outstanding Questions). The different *cre* drivers that have been employed for a given cell lineage often yield disparate results, leading to substantial confusion. More rigorously defined promoters to drive expression of the oncogenic insult should help resolve these matters, but this is dependent on further refinement of the differentiation hierarchies within distinct tissues. It is equally important to continue analysis of premalignant human tissue because human tumors are more heterogeneous than their mouse counterparts, and the cellular architecture of a particular organ may even differ (e.g., in contrast to human lung, the more-distal airways in mouse lung lack basal progenitor cells). With the advent of human tissue organoids grown *ex vivo*, it may be possible to infer the cells of origin of cancer and their sensitivity to mutations [72,73]. Single cell genomic, expression, and cellular analyses of samples from patients at different stages of disease should provide insight into useful molecules that mark the cells of origin of cancer. Finally, identification of these target cells is integral to devising new strategies for prevention and

Outstanding Questions

How does developmental context (e.g., initiation in the prenatal versus postnatal period, or in young versus old adults) influence the propensity of specific cell types to undergo malignant transformation?

Do different cells of origin exhibit a unique susceptibility to genetic mutations? This will require a systematic analysis of the *in vivo* effects of oncogenic lesions within different cellular compartments that have been defined using 'precision' tools.

How can the field move forward in developing more sophisticated tools for lineage tracing? Future strategies could incorporate activation at the single cell level to mimic sporadic gene loss or activation *in vivo* using a light-activated *cre* recombinase, or a Sleeping Beauty mutagenesis approach in combination with well-characterized cell-restricted promoters.

How prevalent is cell interconversion in the early preneoplastic phase?

How much heterogeneity has yet to be uncovered within purportedly well-defined lineage hierarchies?

What is the role of epigenetics in determining the susceptibility of cells to oncogenic transformation?

How do cell-extrinsic factors, including the microenvironment, injury, and inflammation, influence the cell of origin and serve as pivotal drivers of tumor initiation? The generation of more suitable mouse models to replicate these different facets is pivotal to improved disease modeling and understanding the mechanisms by which extrinsic factors act.

early detection, as well as for identifying potential pre-malignant clones that might contribute to relapse.

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