The Question

*What is the origin of tumors?*

A tumor might be derived from a single rogue cell (monoclonal or uni-ancestral) or else it might be derived from multiple rogue cells (polyclonal or multi-ancestral).

Why Does It Matter?

*An ounce of prevention is worth a pound of cure.*

To develop strategies to effectively prevent cancers from forming, investigators must understand how tumors are initiated and established. If cancers have a multi-ancestral origin, disrupting interactions among multiple rogue cells might block the earliest stages of tumorigenesis.

*One size does not fit all.*

To develop strategies to effectively treat cancers, clinicians need to fully understand the composition of a tumor. If cancers have a multi-ancestral origin, discrete populations within a solitary cancer will need to be eliminated. Treatment might need to be a highly personalized combination of agents, each targeting a different population within the cancer. Moreover, treatment efficacy might be greatly impacted by interactions among the discrete populations.

Background

A longstanding belief in the field of cancer biology is that every tumor is derived from a single rogue cell. This belief was founded in assertions by Rudolph Virchow, whose cell theory published in 1855 stated “omnis cellula e cellula” (every living cell comes from another living cell) [1], and Julius Cohnheim, who in 1899 advanced the notion that tumors grow “from the multiplication of tumor-elements themselves” rather than “from a new formation proceeding from the tissue surrounding it” [2]. Early molecular studies seemed to support a single cell, or monoclonal origin, of tumors. In the 1960s, Mary Lyon hypothesized that one of the two X chromosomes is randomly inactivated during embryogenesis [3]. Ernst Beutler, Mary Yeh, and Virgil Fairbanks demonstrated that human females are mosaics consisting of some cells in which the maternal X chromosome is active and other cells in which the paternal X chromosome is active by studying the glucose-6-phosphate dehydrogenase gene (G6PD) that is on the X chromosome [4]. David Linder thought that G6PD could be used to explore the origin of tumors. At the time, the prevalent theory was the somatic mutation hypothesis that mutations transformed a normal cell into its neoplastic counterpart [5]. Stanley Gartler and Linder reported that uterine fibroids, tumors derived from smooth muscle, were likely derived from a single rogue cell because the tumors consisted entirely of cells with either the G6PD isoenzyme type A active or the G6PD isoenzyme type B active but not a mixture [6]. Using a similar approach, Philip Fialkow, Gartler, and Akira Yoshida found that chronic myelogenous leukemias (CML) were likely each derived from single rogue cell and that the progenitor was a multipotential hematopoietic cell [7].
The Philadelphia chromosome was also used as a marker to study the origin of CML. This abnormality in chromosome 22, identified by David Hungerford and Peter Nowell, dramatically shortens the chromosome and creates an oncogene by fusing the BCR gene to the ABL1 gene [8]. P.H. Fitzgerald, Alison Pickering, and Jenny Eiby found that CML was apparently derived from a single rogue cell carrying the Philadelphia chromosome by analyzing cell lines from a CML patient who was an XY/XXY mosaic [9].

The notion that tumors are monoclonal was further supported by studies that coupled restriction fragment length polymorphisms and methylation patterns of genes on the X chromosome. In the initial study, three different tumor types were analyzed. In each case, the tumor was composed entirely of cells with either the maternal X chromosome active or the paternal X chromosome active but not both (Figure 1). This approach was then used to assess the origin of human colorectal tumors. In all cases, the tumor appeared to be uni-ancestral [10]. Thus, the evidence supporting the belief that tumors are each derived from a single rogue cell primarily relied on X-inactivation to determine clonal architecture.

**The Problem**

Studies that rely on X-inactivation are heavily biased to conclude that neoplasms are derived from a single rogue cell. X-inactivation is a random process in which either the paternal copy or the maternal copy of the X chromosome is silenced in a cell during early embryogenesis. Once silenced, the X chromosome remains silent throughout the life of the cell and its descendants. Although silencing is random, sometimes the ratio of cells with an inactive maternal X to cells with an inactive paternal X deviates significantly from 50:50, exceeding 80:20 in some females. Moreover, the mosaicism within a tissue can be quite limited with very large patches of cells having either the maternal X or paternal X inactivated (Figure 2). Both of these phenomena would severely impair the ability to detect tumors derived from multiple rogue cells. For
example, Marco Novelli and colleagues estimated that every dysplastic crypt in over 430 colon polyps would need to be composed of cells that were monophenotypic to exclude the possibility that just 10% of tumors are multiancestral (95% confidence interval) [11]. The key to discovering multi-ancestral tumors is an increased number of cells on borders between distinct populations.

Even with this heavy bias, some early studies that relied on X-inactivation claimed that some tumors have a multi-ancestral origin. The tumors types included those of the colon (Gardner syndrome) [12], nerve sheath (heritable neurofibromatosis) [13], parathyroid glands [14], and skin (heritable epithelioma adenoides cysticum) [15]. These findings were attributed to the fact that “heritable” cancers might be unique in this perspective because every cell in a tissue carried an “innate tumorigenic potential” or dismissed as “contamination” in which the samples contained a sufficient amount of normal cells so that the analysis indicated two cell populations were present.

The Solutions

One solution is use of the laboratory mouse, which allows investigators to unambiguously determine the origin of tumors. In this experimental system, mosaic animals can be readily created. One approach for creating mosaic animals is to generate aggregation chimeras by fusing two mouse embryos together. Initial studies fused one embryo with Rosa26, a lineage marker, to one embryo without Rosa26 [16]. When stained, cells carrying Rosa26 are blue whereas cells lacking Rosa26 are white. A tumor consisting of a mixture of blue cells and white cells likely had a multi-ancestral origin, being derived from at least two distinct rogue cells - one blue and one white. Another approach for creating mosaic animals is through the stochastic expression of lineage markers. Initial studies used a transgenic mouse in which cells could potentially express up to four fluorescent proteins [17]. When activated, the tissue was a patchwork of cells expressing different fluorescent proteins. A tumor consisting of a mixture of cells expressing different fluorescent proteins likely had a multi-ancestral origin.

The other solution is sequencing [18]. Advances in technology allow tumors from mice and humans to be sequenced at high resolution. Most colon tumors begin as a consequence of losing APC activity. Interestingly, several single nucleotide mutations in APC can lead to premature
truncation in contrast to other drivers where certain mutations are common, e.g., the vast majority of mutations in \textit{KRAS} occur in codons 12 or 13. If a tumor carries three or more APC mutations or different regions of a tumor carry distinct APC mutations, the most likely explanation is that a tumor has a multi-ancestral origin.

\textbf{What Have We Learned?}

\textbf{Major findings}

- Demonstrated that hereditary and carcinogen-induced tumors are often derived from multiple rogue cells. This finding refutes a longstanding paradigm in the field of cancer biology (\cite{Cancer_Prevention_Research_2011}).

- Discovered that multi-ancestral tumors form through recruitment in which a rogue cell transforms neighboring cells. Transformation might be mediated by paracrine oncogenic signaling (\cite{PNAS_2013}).

- Found that recruitment probably occurs over a relative short distance ranging from 43 to 109 microns (\cite{PNAS_2005}).

- Demonstrated that multi-ancestral tumors can be derived from as many as four distinct rogue cells (\cite{PloS_2016}).

- Found that tumor origin impacts tumor biology – growth, progression, and response to preventive agent and targeted therapies (\textit{Manuscript in preparation}).

- Identified a transcriptional signature that predicts the fate of early polyps. Elements of this signature have already been shown to be relevant to subcategorizing human colorectal cancers and predict survival (\cite{Cancer_Prevention_Research_2014}).

- Demonstrated that some tumors might be “born to be bad” because molecular events driving tumor progression occur early during tumorigenesis (\cite{Gut_2016}).

- Identified a new pathway to tumorigenesis in the colon and prostate (\cite{Cancer_Research_2012; PLoS_2017}).

- Developed and experimental platform to study the origin of tumors (\cite{PLoS_2106}).

- Developed an experimental platform to study how the mutation profile of a tumor impacts response to preventive agents and targeted therapies (\textit{Manuscript in preparation}).
Some hereditary colon tumors have a multi-ancestral origin.

Mice carrying the Min allele of the Apc gene develop tumors along the entire length of the intestinal tract [19]. Aggregation chimeras were generated by fusing an embryo carrying Min to an embryo carrying Min and Rosa26 (Figure 3) [16]. A significant number of intestinal tumors were a mixture of blue and white cells. This observation has several possible explanations. One possibility was random collision in which an existing blue tumor coalesced with an existing white tumor. However, some heterotypic tumors were observed in regions of the intestine where few tumors formed. In such areas, the probability of two independently formed tumors colliding is extremely low. In addition, some heterotypic tumors were quite small. One tiny tumor was discovered to consist of merely two blue dysplastic crypts and one white dysplastic crypt. The most likely possibility is that at least some hereditary intestinal tumors have a multi-ancestral origin.

Novelli and colleagues demonstrated that hereditary colorectal tumors in humans could also have a multi-ancestral origin [20]. They analyzed tumors from a patient carried a germline mutation in APC and who was also an X0/XY mosaic. Some tumors were a mixture X0 and XY cells indicating that the tumors could be derived from multiple rogue cells.

Christina Thirlwell and colleagues also concluded that hereditary colorectal cancers in humans could have a multi-ancestral origin [18]. They sequenced different regions of polyps determine whether the regions carried the same APC mutations or different APC mutations (Figure 4). In three of the five tumors that were tested, different regions carried different mutations. Interestingly, in two of the five tumors, one region carried a somatic mutation in APC, but other region did not even though the cells were transformed.

Figure 3. Aggregation chimeras permit the origin of tumors to be determined. A 4-8 cell embryo carrying the Rosa26 lineage marker (blue) was fused to a 4-8 cell embryo lacking the Rosa26 lineage marker (white). The fused embryos were implanted in a pseudo-pregnant female. Aggregation chimeras were born and allowed to mature. The intestinal tract was removed and stained. The intestine is a patchwork of blue and white cells. The ratio of blue to white varied from 90:10 to 10:90. The ideal ratio is 50:50 with every other cell alternating between blue and white as this increases the opportunity to detect multi-ancestral tumors. Images adapted from Halberg and Dove, Cell Cycle 6:44-51, 2007.
Spontaneous colon tumors can have a multi-ancestral origin.

We used two approaches to determine the origin of carcinogen-induced colon tumors [21]. The first was to test tumors for mutations in \textit{Apc} using an assay for \textit{in vitro} synthesis of protein. A few tumors (3/145) had three or more mutations in \textit{Apc}, indicating these tumors had a multi-ancestral origin. The percentage could be low for a number of reasons, including the possibility that tumors might be initiated through mutations in other genes and pathways. Some tumors (6/29) were composed of \textit{Apc}-positive and \textit{Apc}-negative cells; both types of cells were neoplastic. The second approach was to assess the clonal architecture of tumors from aggregation chimeras treated with ENU. Several tumors (9/19) were composed of both blue and white neoplastic cells indicating the tumors were derived from multiple rogue cells.

A multi-ancestral origin likely reflects recruitment in which an initiated progenitor facilitates the transformation of neighboring cells.

Multi-ancestral tumors could form through different mechanisms [22]. One possibility is that two existing tumors merely coalesce to form a single tumor. To test this possibility, aggregation chimeras were created that developed relatively few intestinal tumors. In these animals, several tumors were still a mixture of blue and white cells. The percentage of multi-ancestral tumors was independent of the density of the tumors indicating coalescence was an unlikely explanation.

Another possible explanation is that multi-ancestral tumors form through recruitment (Figure 5) [23]. To test this possibility, aggregation chimeras were created from embryos with stark differences in tumor susceptibility. For example, one embryo carrying Min (maximal susceptibility) was fused to an embryo that was wildtype except that it carried the Rosa 26 lineage marker (negligible susceptibility). These aggregation chimeras still developed several multi-ancestral tumors (8/54).

\textbf{Figure 4. High resolution sequencing of tumors permits the origin of tumors to be determined.}
Multi-ancestral tumors can carry different APC mutations in different regions because several different mutations in APC can lead to the loss of APC activity. In this study, Thirlwell and colleagues sequenced two different regions (crypts 1-8 and crypts 9-10) of a colorectal polyp from a patient with Familial Adenomatous Polyposis. The different regions carried different truncating mutations indicating the tumor was derived from two different rogue cells. Similar results were obtained with all colorectal polyps from FAP patients and a few (2/12) colorectal polyps with patients with sporadic disease. Images adapted from Thirlwell et al., \textit{Gastroenterology} 138:1441-54, 2010.
Recruitment occurs over a relatively short distance. Recruitment might be limited to neighboring cells [22]. By knowing the geometric pattern of mosaicism and the frequency of multi-ancestral tumors, the distance between rogue cells contributing to a single tumor can be estimated through statistical modeling (Figure 6). Interactions among rogue cells that are one to two crypt diameters apart is sufficient to account for the frequency of multi-ancestral tumors.

Multi-ancestral tumors can be derived from many distinct progenitors. Multi-ancestral tumors are quite complex [24]. The experimental models that we employed to identify these tumors allowed us to distinguish two distinct lineages (blue versus white or else green versus red) so we could only claim that the tumors were derived from two different rogue cells. To begin to determine whether multi-ancestral tumors were derived from more than two rogue cells, whole tumors were imaged using a method that allowed 3D reconstructions. These 3D reconstructions revealed that multi-ancestral tumors had up to four discrete populations within a single tumor (Figure 7). Moreover, sequencing revealed that these populations carried different Apc mutations. Multi-ancestral tumors appear to be derived from several rogue cells and subsequently be composed of several distinct populations. Our results are consistent with those from another study that indicated that some tumors could be derived from three discrete rogue cells [25]. This complexity would likely make successful treatment more difficult, especially if interactions among populations alter drug efficacy.

Figure 5. A rogue cell and its descendants might emit a signal that facilitates the transformation of neighboring cells. Reade Roberts and David Threadgill found that rogue cells in intestinal crypts create a bulge. (Roberts et al, PNAS 99:1521-6, 2002.)

Figure 6. Statistical modeling allows the distance between interacting rogue cells to be estimated. Areas around tumors in aggregation chimeras was imaged (A) and digitized (B) with each circle representing a crypt and possible interactions between crypts represented by lines. Blue circles are blue crypt, white circles are white crypts and interactions between crypts of different colors are represented by red lines (C). Interactions between rogue cells that are one crypt diameter apart (green) or two crypt diameters (yellow) are sufficient to explain the number multi-ancestral tumors that were observed so the interactions occur over as little as 150 microns (D). C and D are enlargements of the area outlined in B. Images adapted from Thliveris and Halberg et al., PNAS 102:6960-5, 2005.
Multi-ancestral tumors remain heterotypic as tumors progress from a benign to a malignant state.

The clonal architecture of tumors may change as tumors progress [24]. One possibility is that tumors might have a multi-ancestral origin but eventually one population would outcompete the other(s). Our animal models allowed us to test this possibility because the clonal architecture could be followed over time by fluorescence endoscopy as some tumors progressed from a benign to malignant state (Figure 8). Tumors that had a multi-ancestral origin remained heterotypic throughout the study. Moreover, benign adenomas (62%) and invasive adenomas (68%) both can have a multi-ancestral architecture (Figure 9). These observations indicate that interactions among different populations may be vital, favoring the coexistence of multiple populations as tumors grow and progress.

Figure 7. Multi-ancestral tumors are complex. A tumor was isolated from a mosaic animal in which some cells express green fluorescent protein whereas others express red fluorescent protein, cleared and imaged through and through (A). The images were reconstructed to identify discrete areas of red and green to begin to ascertain the number of rogue cells (and clones) contributing to the tumor (B). In this particular tumor, three discrete green clones (C) in a primarily red tumor. This observation is consistent with the notion that tumors may be derived from more than just two rogue cells. From Zahm et al., PLoS One 11:e0150170, 2016.

Figure 8. The clonal architecture of heterotypic tumors persists over time. Mosaic mice develop heterotypic tumors composed of red cells and green cells. These tumors can be visualized using white light (upper row) or a laser that excites the green fluorescent protein (lower). This example shows a heterotypic tumor that is roughly half red (dark) and half green. This ratio and architecture appears to unchanged over a period of four months.
Multi-ancestral tumors can form even when one clone carries a strong driver mutation.

Uni-ancestral tumors were thought to progress owing to new mutations in key genes that drove clonal sweeps because those cells possessed a growth advantage. This view failed to account for intratumoral heterogeneity, which is a feature common in colorectal cancers. We sought to determine whether a strong driver mutation altered the clonal architecture of intestinal tumors in mice. Mosaics were generated in which red fluorescent cells lacked Apc activity and green fluorescent cells lacked Apc activity while expressing activated PI3K, a strong driver. Some tumors were composed solely of either red or green cells, whereas other tumors were a mixture of red and green cells. The multi-ancestral tumors grew faster and were more aggressive than the homotypic red or homotypic green tumors. The presence of a strong driver did not preclude a multi-ancestral origin. Moreover, interactions between different populations presumably altered the tumor biology.

The efficacy of preventive and therapeutic agents depends on the clonal architecture of tumors.

Our novel animal models allow us to test efficacy understanding clonal architecture and its potential impact. Xiaoyun Liao and colleagues found that patients with colorectal cancers carrying mutations in PIK3CA survived longer if they regularly used low-dose aspirin [26]. To test whether our animal models mimic this effect, we generated mosaic animals that developed red tumors (Pik3ca wildtype), green tumors (Pik3ca mutant), and heterotypic tumors (mixed). The red tumors did not respond to treatment but the heterotypic tumors did. Mosaics given low dose aspirin developed fewer green tumors but the difference was not statistically significant (Figure 10A). The effectiveness of low-dose aspirin appears to depend on the mutation profile of a tumor as it does in humans.

Figure 9. Advanced cancers have a multi-ancestral origin. Tumors from mosaic were isolated, embedded, and sectioned. Some sections were stained with H&E for pathological assessment (Panel A, C, and D) whereas others were imaged to determine origin (Panels B, E, and F). Many advanced adenomas with high-grade dysplasia and intramucosal carcinomas were shown to be composed of green and red neoplastic cells. A representative image of a multi-ancestral adenoma with high dysplasia (Panels A and B) and a multi-ancestral intramucosal carcinoma (Panels C-F). Panels A and B are shown at the same magnification; size bar = 1mm. Panels C and E are the same magnification; size bar = 1mm. Panels D and F are 4x enlargements of the area outlined in C. From Zahm et al., PLoS One 11:e0150170, 2016.
We also tested whether GDC-0941 affected Pik3ca-mutant intestinal tumors. This drug specifically targets the kinase that is encoded by Pik3ca. Mosaic mice treated with GDC-0941 developed a higher percentage of red tumors and heterotypic tumors but a much lower percentage of green tumors (Figure 10B). This observation indicates that clonal architecture impacts tumor response to targeted therapy. It appears that the unresponsive red cells in a heterotypic tumor protect the potentially responsive green cells. Additional studies need to be performed to more fully understand how the clonal architecture of tumor impacts the effectiveness of preventive strategies and treatment.

Molecular analyses reveal that some tumors are born to be bad.

About 5% of early intestinal tumors are thought to progress to cancers, however it unknown which will progress. Using our animals, we identified a 68-gene signature that distinguished between benign adenomas and malignant cancers [27]. Importantly, this signature was evident when comparing tumors that first formed. Two of the genes, MUC2 and TFF3, are being utilized clinically to subcategorize human colorectal tumors into one of six different subtypes [28]. Five other genes predict survival of patients diagnosed with colorectal cancer. Genes that were identified in mice indicate that some tumors are “born to be bad” and have prognostic value in the clinic.

To further explore the possibility that some tumors are “born to be bad”, we have analyzed a unique set of human polyps that were followed over time by CT colonography (Figure 11) [29]. Our preliminary analysis revealed that early tumors often carry mutations in genes that were thought to drive the malignant transformation of a tumor. Moreover, tumors with mutations in two or more key genes were more likely to grow than those with a mutation in a single gene. These observations are also consistent with the notion that some intestinal tumors are “born to be bad”. Current studies are focused on determining whether CT textural features or molecular features including the mutation profile, methylome, or transcriptome of the tumor can predict fate or clinical outcome.
What Questions Remain?

How are neighboring cells recruited to form a tumor?

Are recruited cells alone tumorigenic?

Does cooperativity among different populations within a tumor affect response to preventive or therapeutic agents?

Do colon polyps arise from a field of mutant cells?

Are multi-ancestral tumors more likely to grow or progress in humans?

**Figure 11.** Early human polyps can carry mutations that drive tumor progression. The growth of human colorectal polyps was monitored over time by CT colonography. The polyps had different fates: some grow and some regress. Samples from tumors were analyzed to determine whether fate could be predicted based on mutation profile (A) or pathology (B). Mutation profile was assessed by performing targeted sequencing in which 50 cancer-related genes were surveilled. Some mutations were present in all cells (public), whereas some mutations were present in only a few cells (private) (C). From Sievers et al., *Gut* 66:2132-40, 2017.
Are multi-ancestral tumors more difficult to treat in humans?

Are some tumors “born to be bad”?

Can CT textural parameters or molecular features predict tumor fate and consequently facilitate developing better screening guidelines?

Summary

Our group has made a number of important findings by developing new animal models and analyzing a unique set of colorectal polyps from humans. Contrary to a longstanding paradigm in the field of cancer biology, we found that tumors are often derived from multiple rogue cells. Our goal is to fully understand the recruitment process in which one rogue cell transforms neighboring cells and consequences of early intratumoral heterogeneity on tumor biology. In addition, we found that some tumors are “born to be bad” as they carry mutations in genes that drive progression and they express a transcriptional program that foretells a pathologic fate. Our work has significant implications for the development of personalized screening guidelines and personalized treatment strategies.
References


