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Research Article

Non-Random Selection of Cancer-Causing Mutations in Tissue-Specific Stem Cells Cause Cancer

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Abstract

Tissue-specific stem cells are the target for selected mutations in oncogenes or tumor suppressor genes that enhance the fitness of these cells, resulting in a self-limited clonal expansion and eventual cancer development. The initial or truncal mutations in the stem cell select for subsequent mutations that enhance their fitness, producing a reproducible order of mutations, selected for in each tissue type, during cancer development. Mutations in stem cells occur randomly, but the selection for increased fitness, occurs non-randomly, conferring a functional order on the selection of mutations. Tissue-specific stem cells are "units of natural selection" for somatic stem cells throughout life. This is why inherited cancer-causing mutations, which, by definition, are initial or truncal mutations, are observed to cause cancers with limited tissue specificities, even though the mutations are present in stem cells for all tissue types.

In future studies, we need to understand why the same signal transduction pathways function differently in different tissue-specific stem cells. We also need to understand the truncal mutations for each cancer type, so as to eradicate the stem cell clones for that cancer before they produce a malignant tumor. To accomplish these objectives, we need to carry out new kinds of clinical trials with drugs that target mutations in tissue-specific stem cells.

ABBREVIATIONS

CpG cytosine-3'5': guanosine monophosphate; LGR5: leucine-rich repeat containing G-coupled receptor-5; WNT: wingless; LPR 6: low density lipoproteion receptor-6; Fzd5: frizzled receptor d5; APC: adenomatous polyposis coli; SMAD: transcription factor for the TGF-beta pathway; TGF-beta transforming groth factor beta; Ras: rat sarcoma; BRCA 1/2: breast cancer gene; PTEN: phosphatase and tensin homolog; TSC1/2: tuberous sclerosis proteins 1,2; WT 1: Wilms tumor; ATM: ataxia telangiectasia mutant.

INTRODUCTION

A Stem Cell Classification

Throughout any complex organism's development, including sexual maturity, adult life, and aging, there are many different stem cell types that arise and populate the organism. The fertilized egg is the totipotent stem cell that is the forerunner of all cell types. In many multicellular animals, it produces a spherical blastocyst, where a subset of stem cells (inner cell mass cells in mammals) produce the organism and the outer trophoblast cells produce the membranes of the fetus. The symmetry and topology of the blastocyst is broken by gastrulation, forming anterior-posterior poles, dorsal-ventral sides and a sphere-to-torus transition. Three sets of stem cells, endoderm, mesoderm and ectodermal produce a subset of tissue-types in the body of the organism. As these tissues form, tissue-specific stem cells become responsible for producing the many different tissues of the organism and the many different cell types that populate a tissue. Tissue-specific stem cells function in the fetus to produce the organs. In mammals, they function throughout life to replace normal and damaged or aging tissues. In humans, tissue-specific stem cells in the crypt, reproduce a new endodermal lining every four days [1]. In skin, tissue-specific stem cells located in the hair follicle, produce new skin clones every 28 days [2]. In the hematopoietic system, tissue-specific stem cells located in the bone marrow, replicate to create a variety of progenitor cells that produce mesenchymal derivatives, which populate both the blood and tissues in a variety of organs [3]. In humans and mice, these tissue specific stem cells are the target cells for mutations that lead to tissue-specific cancers [4].

Changes, Tissue Regeneration and Tissue Replacement

In many invertebrates, such as the planarian flat worms, cutting the worm in half causes the frontend to regenerate a backend, and the backend to regenerate a frontend. This is accomplished by a differentiated cell in the area of the cut, that is reprogrammed epigenetically to dedifferentiate, losing epigenetic marks, and forming what is termed a blastema [5]. The blastema then regenerates a head or tail, depending upon its location in the worm. Similarly, vertebrates such as fish, amphibians and even some reptiles, can regenerate a tail or appendage as an adult

using these same dedifferentiating strategies. In experiments carried out by Yamanaka and Takahashi, using mouse or human fibroblasts in cell culture, which were epigenetically reprogrammed with four transcription factors, produced inner cell mass-like stem cells that could differentiate into many or all mouse tissues in vivo or diverse cell types in vitro [6]. It is curious that this is the case with mammalian fibroblasts, even though these animals are not capable of regenerating limbs, and utilize tissue-specific stem cells to regenerate selected tissues. However, the efficiency with which the Yamanaka factors reprogram differentiated mammalian cells is poor (0.1-1%) and the time it takes to accomplish this is long (weeks to months) in culture. This makes a good deal of sense because epigenetic marks, and cell and tissue types, must be stable and resistant to change. The mechanisms that form the basis for this stability have been explored by Jaenisch and his colleagues [7]. They made a conditional mutation in the DNA methyl-transferase-1 gene of mice. This is the copy DNA methyl-transferase gene, whose protein will identify a methylated CpG dinucleotide on a DNA template strand and methylate a cytosine on the newly synthesized DNA strand so that the DNA epigenetic marks are inherited in a tissue-specific fashion. In this experiment, the DNA methyl-transferase-1 activity is destroyed by the Cre-Lox mediated mutation placed in this gene (similar to the cancer-causing mutation in that gene). The resultant cells divide twice and then undergo apoptosis [7]. A few cells can escape the apoptosis and go on to be transformed and cause a cancer [8]. When the p53 gene is also deleted, along with the DNA-methyl-transferase-1 gene, apoptosis fails to occur, and the cell produces a cancer. Most interestingly, when the Yamanaka factors reprogram a differentiated cell carrying a temperature sensitive p53 mutation at the restrictive temperature (non-functional p53), they very efficiently produce stem cells (up to 80% efficiency) in a short period of time, while at the non-restrictive temperature (p53 is active), the efficiency is low and the time to reset the epigenetic program is long [9]. Thus, it appears that a p53-regulated activity is important for epigenetic stability [10]. This is just one of the reasons why the p53 gene is the most commonly mutated gene in human cancers [11], and why there is epigenetic instability and epithelial to mesenchymal transitions, in cancerous tissues.

Tissue-Specific Stem Cells

Tissue-specific stem cells are the targets of mutations that produce tissue- and cell- type-specific cancers. This was demonstrated by the isolation of tissue-specific stem cells from normal tissues, which are cloned and produce organoids in cell culture, and are shown to produce many or all of the cell types in normal tissues [4]. When these stem cells are cloned from cancerous tissues, they harbor the mutations that cause these cancers [4]. These stem cells from many diverse tissues have in common that they all express Lgr5 G-protein coupled receptors on their cell surface. This receptor is coupled to the Wnt ligand receptors LRP-6 and Fzd5, and when internalized into the cell together, potentiate the Wnt pathway, increasing its downstream transcription and replicating the stem cells that then differentiate into the many different cell types of that tissue [12]. Over a life-time, these tissue-specific stem cells replicate and accumulate about 40 mutations per year (both cancer-causing and non-cancer-causing mutations) [13]. This was demonstrated by obtaining stem cells from organoids (small intestine, colon and liver), derived from individuals 3 to 87 years-old, and sequencing whole genome DNA from the stem cells. These tissue-specific stem cells reproduce themselves in a specific location in a tissue, producing a pool of stem cells, some of which become committed to differentiate into tissue components. These stem cells thus become Darwinian units of selection for reproductive fitness [3]. When mutations occur in pathways that have an impact upon cell division, cell death, or DNA repair processes (the loss of which increases the mutation rate), these mutations affect the fitness of the stem cell, in some cases increasing the concentration of stem cells carrying the mutation, producing a self-limited benign tumor [14]. Such initial or truncal mutations in a tissue-specific stem cell produce a small clone of cells in the tissue, as has been observed with notch mutations in skin [15] and esophagus [16, 17] and leukemias [18-23] harboring DNA methyltransferase 3 beta (epigenetic) and p53 (DNA damage response) mutations. In cells shed in uterine fluid from women who develop ovarian cancer, ultra-deep sequencing has enabled the detection of p53 mutations. Ultra-deep sequencing has also enabled detection of age-related p53 mutations in normal healthy women [14]. Not every oncogene product or tumor suppressor gene mutation is functional in a tissue-specific stem cell [24, 25]. Different tissue-specific stem cells have different mutations in tumor suppressor genes or oncogenes that result in observed phenotypes, such as increased fitness or self-limited clonal cell growth. This could be due to a lack of transcription of the causal gene in a tissue-specific stem cell or to a lack of activity of the gene product [25]. In fact, this is why inherited tumor suppressor gene mutations or inherited defective DNA repair gene mutations, which are by definition initial or truncal mutations, demonstrate a distinct tissue- or cell-type phenotype [24,26]. Table 1 lists a number of inherited mutations of tumor suppressor or DNA repair genes that demonstrate an early age of onset of a tissue-specific cancer, even though many of these genes are expressed in a wide variety of tissue types.

The Order of Mutations Producing a Tumor Matters

Vogelstein and his colleagues [27,28] were the first to suggest that in colon cancers, mutations occur or are selected for in a specific order, with APC mutations occurring first, producing a small benign tumor, activating RAS mutations occurring next, producing a larger benign tumor, inactivating SMAD4 mutations occurring third, producing a large benign tumor, and inactivating p53 mutations occurring last, producing a malignant tumor. They did this by collecting colonoscopy samples from many different individuals and reconstructing the events by the size and state of the tumor. This idea was confirmed by obtaining normal colon organoids and introducing the mutations for APC, RAS, SMAD4 and p53, using a CRISPR/Cas9 mutational mechanism, to introduce mutations into the tissue-specific stem cells in different orders of addition. The most efficient order to produce an organoid that was tumorigenic in nude mice was APC, followed by RAS, followed by SMAD4, and then by p53. P53 mutations introduced first, or before the other oncogenes, had no impact alone [29]. The concept that the order of mutations mattered was proven experimentally by introducing a transposon into a mouse genome that moved around the genome causing insertion mutations and abnormal gene expression. The transposon

Table 1: Examples of Mutations that Cause Inherited Tissue Specific Cancers at Early Ages.

Mutated Gene	Tissue Specific Cancers
Retinoblastoma	Retinoblasts, followed by Osteogenic sarcomas
P53	Medulary blastoma, Adrenal corticocarcinoma, Rhabdomyosarcoma, Sarcomas
BRCA-1/2	Ovarian cancers, Breast cancers
PTEN	Cowden Disease, Hamartomas, Breast cancers
Lynch Syndrome - Mismatch repair APC	Polyps of the colon
TSC-1/2	Astrocytomas
WT-1	Nephroblastomas
ATM	Lymphomas, Leukemia

was expressed in the cells of the intestine, inserting randomly throughout the genome. Under these conditions, 50% of the mice developed an intestinal tumor and survived for 85 days. When homozygous inherited p53 mutations were present in the germ line of these mice, half the mice survived for 65 days. When SMAD4 mutations were present in the germ line of these mice, half the mice died in 55 days. When a conditional Kras mutation was activated just after birth in the intestine, half the mice lived for 40 days, and when a homozygous inherited APC mutation was present in the germ line, half the mice died with intestinal tumors in 20 days [30]. The APC gene was therefore the most efficient initial or truncal mutation in producing an intestinal tumor most rapidly. The order of mutations is random, but the selection for these mutations is non-random in each particular tissue-specific stem cell (Table 1). In slim stem cells in the colon, APC mutations enhance the fitness of the cell, producing a clonal expansion of stem cells, whereas Kras, SMAD4, and p53, which are required after APC for a malignant tumor to arise, do not increase the fitness of the stem cell unless APC is present. That the initial truncal mutation can affect the order of the genes contributing to a tumor received further support when the inherited initial truncal mutation was Smad4. In this case, two thirds of the intestinal tumors had activating mutations in R-spondin-1 (Rspo-1) and Rspo-2, which enhance the WNT pathway by targeting a different gene than APC [30]. Thus, having an initial truncal mutation in the TGF-beta pathway in a slim stem cell seems to have changed the gene that preferentially is selected for mutation in the order of selection for fitness of a tumor [30]. This is observed in about 10% of human colon cancers. Although this is the best studied example of the observation that the order of mutational selection is important for the optimal selection of a tumor, there are a large number of other examples where mutational order matters [24, 26].

Inherited p53 mutations, Li-Fraumeni Syndrome

The simplest way to determine which tumor suppressor genes or DNA repair gene defects are functional in tissue-specific stem cells is to examine the phenotypes of inherited mutations that act as initial truncal mutations in humans. By determining the tissue types and frequencies of different tumors, the age interval in which tumors arise and the excess risk of developing a tissue-specific tumor compared to the general population, one can begin to classify the oncogenes and tumor suppressor genes that initiate cancers in humans [26, 31]. For example, some individuals who inherit a cancer-causing p53 mutation may develop an adrenal cortical carcinoma at 6 months to 4 years of age, a choroidplexis papilloma/carcinoma at 6 months to 3 years of age, a medullary blastoma at 2-9 years of age, and a rhabdomyosarcoma at 1-4 years of age, and these tumors have an excess risk of 100-200 times the population not carrying an inherited p53 mutation. Liposarcomas are observed from 1-50 years of age and leiomyosarcomas at 20-60 years of age, whereas breast cancers (ER+/PR+) are commonly observed at 18-45 years of age in 50-70% of the women with inherited p53 mutations [26, 31]. These tumors appear at 50-100-fold excess risk. Colon tumors, lung cancers, pancreatic tumors and ovarian tumors all occur very late in life, 50-70 years of age, at an excess risk of 2-4-fold, from that observed in the general population (Table 2). It is of some interest that ectoderm and mesoderm derived stem cells produce tissue-specific stem cells with inherited p53 mutations that lead to tumors much earlier in life and at much higher excess risk, than endoderm-derived tissue-specific stem cells with p53 mutations. This is not occurring because endodermal tissue-specific stem cells do not require p53 mutations to form a tumor (table 2). Spontaneous p53 mutations occur at very high frequencies in ovarian, colon, stomach, pancreatic and lung cancers, but in those cancers, a p53 mutation is selected for as one of the last mutations in a series leading to the transition from benign to malignant tumors. In those tissues, p53 mutations have no impact upon tissue-specific stem cells as initial truncal mutations, as seen in the low excess risk of these tumors in inherited patients, and the very late time of development of these cancers in inherited forms of mice and humans [26] [30-31].

DISCUSSION AND CONCLUSIONS

The target cells for spontaneous mutations that lead to cancer development are tissue-specific stem cells [4]. Because of exposure to mutagens and the replication of these stem cells over time, they accumulate about forty spontaneous mutations per year of life [13]. There are hundreds of oncogenes and tumor suppressor genes, and it takes mutations in only about 2-5 of these genes to initiate a malignant cancer. There are also hundreds of genes that encode different DNA repair functions. Inherited or spontaneous mutations in these DNA repair genes contribute to an enhanced mutation rate and an earlier onset of cancer. These are the reasons why the age of a person is such an important variable for cancer development. Cancer rates rise logarithmically with age [24], although in cases where an inherited mutation contributes to cancer, the incidence commonly occurs at a younger age. Each tissue-specific stem cell type that contributes to the repair and renewal of each tissue of the body,

Table 2: Selected Examples of Somatic p53 Mutations that occur at high frequencies and Function as Late mutations in the order of Cancer Development.

Tissues	Frequency
Serous Ovarian	100%
Colorectal adenocarcinoma	70%
Pancreatic	60%
Non Small Cell Lung Adenocarcinoma	70%

responds to a random mutation in only a small subset of tumor suppressor genes or oncogenes, by increasing its fitness or clonal replication, producing a self-limiting benign clonal expansion [24-26]. Although mutations are random in their occurrence, selection of a mutation for an increased fitness is not random among the oncogenes and tumor suppressor genes. Functional mutations are specific to tissue stem cell types. This means that there are a limited number of mutations that can act as an initial or truncal mutation for a specific tissue type [24,26]. Inherited mutations function, by definition, as initial or truncal mutations in a subset of tissue-specific stem cells, and, as such, inform us as to what gene mutations are functional for increased fitness in the human body. This explains why inherited tumor suppressor genes demonstrate a clear tissue tumor specificity in causing cancers, but the same gene mutations are found at very high frequencies in different tissues when they occur as spontaneous mutations. Tables 1 and 2 demonstrate the tissues that develop tumors at a high excess risk with inherited p53 mutations (and other tumor suppressor genes) because they function as initial or truncal mutations in that tissue-specific stem cell, whereas the very same mutations function, in a series of mutations, as late onset spontaneous mutations in other tissues with a very low excess risk as inherited tumors. They only function later in life after other mutations have accumulated in the tissue-specific stem cell. This is not to say that these mutations occur in a specific order, they do not, mutations occur randomly. It is functional selection for increased fitness that occurs in a specific order, ultimately producing a malignant cancer. Both the type of tissue-specific stem cell and the order of functional mutations, modulate the development of a cancer [24-26].

An increasing number of examples of these ideas are being documented in the literature [24-26]. These observations help to explain why drug development for cancers was, from the beginning, limited by tissue specificity and why drug approvals came with an associated tissue type. More interesting have been the drugs that target a specific gene or even a specific mutation in a gene (BRAF, RAS, ABL, etc.). They are functioning differently in different tissue types of cancers even with the exact same mutation. They are, of course, functioning in different tissue-specific stem cells. The signal transduction pathways we draw in thousands of publications need to be understood as approximations, which can function differently in their circuitry or feed-back or feed-forward mechanisms, in different stem or progenitor cell populations. Drugs focused upon targeted gene mutations or even allele-specific mutations, have a good deal to teach us as they are explored in different human cancers with the same mutation in a different tissue type. This interpretation of what is happening in a cancer stem cell may call for a new kind of drug and a new kind of clinical trial, carried out to understand more about cancer development rather than to get to registration first. The comparison of the same mutant allele in inherited mutations versus spontaneous mutations (in the same or different tissues) is not something that drug companies would normally pursue. Perhaps, there is a role for the NCI or private foundations to explore this by setting up small clinical trials at cancer centers that do not necessarily develop drugs, but use them to understand the basis of cancer development, and, in the end, learn how to cure cancers before they ever arise as malignant or metastatic tumors.

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CONFLICTS OF INTEREST

The author is the founder of a Biotech company that is attempting to synthesize small molecule reactivators of p53 mutations. He is also the chair of the Janssen Scientific Advisory Board.

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