OPINION

Inducing stable reversion to achieve cancer control

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Preface

How can we stop cancer progression? Current strategies depend on modeling progression as the balanced outcome of mutations in, and expression of, tumor suppressor genes and oncogenes. New treatments emerge from successful attempts to tip that balance, but secondary mutational escape from those treatments has become a major impediment because it leads to resistance. In this Opinion article, we argue for a return to an earlier stratagem: tumor cell reversion. Treatments based on selection and analysis of stable revertants could create more durable remissions by reducing the selective pressure that leads to rapid drug resistance.

Introduction

Tumor cell reversion is the re-establishment of all or a significant fraction of the normal growth control mechanisms that are lost in tumors. For fibroblasts and epithelial cells, these include cell–cell contact inhibition, which is now known to be controlled by the Hippo pathway[1], and anchorage dependence, a characteristic that causes normal fibroblasts to become quiescent and epithelial cells to die in the absence of adhesion to extracellular matrix proteins[2]. Tumor cell reversion *in vitro* was first shown to occur in the absence of selective pressure in studies using the fluctuation test that was developed by one of the most interesting scientific partnerships of the twentieth century: Salvador Luria and Max Delbruck (Box 1)[3]. It was observed that the progeny of rare but stable revertant cells emerged as contact-inhibited fibroblast colonies when the dividing cells in a dense culture derived from a single viral-oncogene-transformed cell were killed with the chemotherapeutic 5-fluoro-2'-deoxyuridine (FUdR)[4]. Normal fibroblasts cultured at that same density had stopped growing and would not have been affected by FUdR. These early studies found some revertant clones in which the viral transforming genes were lost, supporting the idea that their function was required to maintain the transformed state[5, 6]. More frequently though, viral oncogenes were retained and continued to be expressed, while unknown cellular genetic or epigenetic alterations returned the revertant cell line back to a state of stable, normal growth control (Figure 1). In certain cases, reversion was

accompanied by a change in chromosomal composition, specifically hyperploidy, which suggested that understanding DNA-based changes in revertant genomes could lead to a treatment strategy[7].

By the mid-1970s, tumor revertants became a mainstay of cancer research. Partial revertants were isolated that lost some but not all of the characteristics of fully transformed cells, and they were used to establish the relationships between the diverse cellular phenotypes of tumor cells[8]. Studies of partial revertants clearly established that anchorage-independent growth was the strongest indicator of tumorigenicity[8] (Figure 1). 'Flat' revertants of viral *kras*-transformed cells — the term 'flat' meaning that the cells were cell-contact inhibited and did not pile on top of each other when growing in culture — were found to be selectively resistant to retransformation by some but not all viral oncogenes tested, establishing a functional relationship between previously unlinked oncogenes[9].

Attempts to elucidate the molecular mechanisms of reversion included characterization of reversion-induced proteins and genes[10, 11]. The most widely studied reversion-associated gene, *KREV1* (also known as *RAP1A*), was isolated from a cDNA library constructed from a flat revertant of cells transformed with viral *Kras*, and its overexpression was shown to partially induce reversion [11]. *KREV1* was heralded as an 'anti-oncogene' at the same time as the first human tumor suppressors, originally called anti-oncogenes, were being characterized[12]. However, the relevance of *KREV1* overexpression to the phenotype of the original flat revertant was never established, nor did molecular analysis of the *KREV1* gene lead to any generalizable insights about tumor reversion.

Tumor reversion is a relatively rare event *in vitro* and, based on its rarity, it is not surprising that pathologists have failed to notice it in human tumors. Although reports of tumor reversion in mouse models have been reported, they are either induced by an inhibitor[13] or detected by cloning tumor cells in vitro and looking for variants[14]. Inhibitor-induced reversion temporarily affects an entire population of cells, and so it is conceptually distinct from tumor cell reversion, which begins as a genetically stable event occurring in individual tumor cells.

At around the same time as studies of tumor cell version failed to generate much traction on the mechanistic side, there was a major shift in cancer research from *in vitro* to *in vivo* studies, coinciding with the development of transgenic mouse models. Compared with the extraordinary progress that was being made in the molecular characterization of tumor suppressors between 1990 and 1995, including the ability to explore the phenotypic effects of tumor suppressor deletions *in vivo* using mouse models[15], research into tumor reversion dwindled.

Mechanisms of tumor reversion

The laboratory that has maintained a considerable active interest in studying tumor reversion since the beginning of the twenty-first century has been that of Adam Telerman in France (see Further information). The approach of the Telerman laboratory has been to look for a mechanism of tumor reversion that can be applicable to variety of cancer types by studying genes for which expression is

commonly dysregulated in a set of revertants derived from several different cancer cell lines. Such dysregulated genes include *TCTP* (also known as *TPT1*), *SIAH1*, *PSEN1* (which encodes presenilin 1) and *STEAP3* (also known as *TSAP6*)[16]. Telerman's hypothesis is that tumor reversion is defined at the molecular level by a cellular reprogramming mechanism caused by altered expression of these genes, which in turn overrides the genetic changes in oncogenes and tumor suppressor genes that cause cancer.

Interestingly, normal developmental processes such as mesenchymal-to-epithelial transition have also been shown in colon cancer models to induce tumor cell reversion[17]. However, rather than invoking a cellular reprogramming mechanism, an alternative interpretation of the reversion genes that Telerman has identified is that they all restore key aspects of the tumor suppressive function of p53 (encoded by *TP53* in humans). Indeed, the revertants obtained by Telerman were all derived from cancer cell lines expressing mutant p53. The two genes *STEAP3* and *SIAH1* are upregulated in the revertants and upregulated by wild-type p53[18, 19]. *STEAP3* encodes a transmembrane protein that localizes to the trans-Golgi–endosome compartment. This protein is activated by p53 and is required for exosome production in cells undergoing a p53-mediated response to stress[20]. Activation of p53 or overexpression of *STEAP3* induces the production of exosomes, and these vesicles are likely to play a role in suppressing the malignant phenotype[21]. *SIAH1* encodes an E3 ubiquitin ligase induced by wild-type p53 that targets several proteins for degradation, including components of the shelterin complex that protects telomeres and that when degraded promotes senescence[22].

The other two genes implicated in tumor reversion process, *PSEN1* and *TCTP*, are downregulated in the revertants and downregulated by wild-type p53[19, 23]. *PSEN1* is a component of the gamma-secretase complex, which is responsible for Notch activation, and its down-regulation by wild-type p53 suppresses Notch signaling[24, 25]. Finally, *TCTP* encodes a protein that binds to p53–MDM2 complexes and promotes MDM2-mediated ubiquitination and degradation of p53[26]. *Tctp*-haploinsufficient mice are sensitized to p53-dependent apoptosis[26], and this may underlie its ability to promote tumor reversion when downregulated.

Telerman's laboratory is to be commended for their continuing the study of tumor reversion while the focus of the majority of research in cancer biology shifted away from this topic. In their work, they looked at genes dysregulated in revertants of p53-mutant cancer cells and found many connections between those revertant genes and the p53 pathway. In this way, they highlighted the potential importance of designing future tumor reversion studies with specific cancer genotypes in mind.

New approaches to study reversion

Up until the advent of next-generation sequencing in 2004, it was impossible to identify the particular mutation underlying the generation of relatively rare phenotypic revertants of fully transformed cells. Today, with the advent of massively parallel sequencing, it is no longer impossible.

A panel of revertants could be sequenced to look for recurring mutations. The identification of these genes could be the start of a systematic search to find pharmacologically active molecules that could achieve the same tumor reversion effect. Whole-genome sequencing is becoming increasingly affordable and could be used to search for mutations in regulatory regions as well as the entire set of expressed genes (Figure 2).

Screening using CRISPR–Cas9 (clustered regularly interspaced short palindromic repeats–CRISPR-associated protein 9) is another new genetic method that could be employed. For example, because we know that two genes, *SIAH1* and *STEAP3*, are upregulated in the revertants and upregulated by wild-type p53, we predict that CRISPR–Cas9-mediated repair of mutant p53 in a tumor cell should yield revertants. One could also use libraries of single guide RNAs designed against the entire set of coding sequences [27]. Negative selection methods such as the treatment of cells cultured at a high density with FUdR previously described could be used to identify tumor revertants. The candidate gene(s) knocked out in these revertants by the specific single guide RNAs of the CRISPR–Cas9 library would be subjected to further validation tests (Figure 2).

Telerman's results strongly suggest a particular strategy for starting a new approach to the study of revertants: target a specific cancer genotype and cancer type that is both prevalent enough such that several cancer cell lines exist and highly relevant for potential translational impact. Two obvious choices that come to mind are tumours that express both mutant *KRAS* and mutant *TP53*. This double-mutant genotype is detected in a subset of pancreatic cancers and lung adenocarcinomas that are particularly aggressive and in dire need of new therapeutics[28].

Choosing the right tumor system is one of two critical choices necessary to establish the utility of a reversion-based strategy for cancer treatment. The other is to choose a system that yields revertants that are stable in the laboratory: that is, those that show the lowest frequency of cells that are able to 'escape' back to malignancy. Initial descriptions of flat revertants did not detect any perceptible frequency of 'back-mutation' to the transformed phenotype, but this will have to be tested in each case for which reversion from malignancy is used as a potential therapeutic approach.

Can reversion prevent resistance?

Why should induction of reversion be any better than any other method used to treat cancer? Our argument is based on evolutionary theory: mainstay cancer treatments seek to kill as many cancer cells as possible. Unfortunately, if there is a pre-existing mutant cell that is resistant to that treatment, it will survive and become a much larger component of the recurrent tumor. After repeated rounds of treatment, the resistant clone will dominate the tumor population, and the cancer will no longer respond to that particular treatment. Many studies have demonstrated the existence of pre-existing mutants that emerge following targeted treatment[29-31]. Now consider a treatment scenario inducing reversion. Cells now re-establish normal growth control such as cell-contact inhibition, but there is no

cell death and therefore no chance for enrichment of resistant clones. Similar to the effect of cytostatic drugs, reversion therapy would leave behind cells to take up space and resources. This should help to slow the clonal expansion of cells resistant to reversion therapy, but it may not totally prevent resistance. Indeed, this treatment might uncover the existence of 'escaper cells': cancer cells that overcome the reversion and go back to being full-blown tumor cells. In this setting, one can mitigate this possibility by examining a large set of mutations that induce reversion and picking the one that is the most stable, and thus measure the rate at which these escaper cells appear.

The strategy of achieving stabilization of cancer by inducing permanent differentiation is a tantalizing option similar to induction of reversion, and it was first demonstrated by Beatrice Mintz in a study involving microinjection of fully malignant teratocarcinoma cells into blastocysts[32]. Additionally, permanent differentiation of fully transformed cancer cells has been achieved in colon cancer cell lines treated with sodium butyrate[33]. Although often singled out as a clinical success for differentiation therapy, treatment of promyelocytic leukemia (PML) with all-trans retinoic acid (ATRA) and arsenic, which effectively cures the disease, is in fact a highly specific targeted therapy that induces complete protein degradation of the transforming fusion oncogene *PML–RARA*. Although treatment with the ATRA does induce differentiation, this is in effect a 'passenger' event that obscures the action of ATRA in inducing *PML–RARA* degradation[34].

Also related to reversion is senescence[35]. Although in some cases this is accompanied by removal of the senescent cells by the immune system[36], and therefore may be subject to the same selective pressures that allow for rapid resistance to pro-apoptotic agents, in other cases — such as in moles comprised of pre-malignant melanocytes[37] — senescent growth arrest could form the basis for stable remission.

Our opinion is that stability is the most critical feature to achieve and that inducing stable tunour reversion offers great advantages over related strategies that aim to induce senescence or differentiation; these cell states represent modulations of cell behavior that need not have any mutational component but rather are more likely to require the ongoing presence of the senescing or differentiating signal. Strategies that aim to achieve a specific type of cell growth arrest such as differentiation or senescence may or may not be stable. It is worth considering that the rate of copy number alterations and other genetic alterations may be different in different cell states and this could influence evolvability (the ability of cells to escape back to malignancy) (Box 2).

It is important to stress that complete genetic stability of revertants, though desirable, is not an absolute requirement. Consider that when a person is infected with a bacterium, the prescribed 5 to 10 days of antibiotic treatment[38] required to clear the infection do not kill the last bacterium, but rather causes a reduction in bacterial load that is sufficient to enable the immune system to kill and clear the pre-existing but low numbers of resistant mutants. These mutants are the bacteria that will present as a drug-resistant infection if a person stops taking the antibiotic prematurely. By analogy, we would

expect reversion-inducing drugs to lower the load of dividing tumor cells, so that the immune system or conventional cancer treatments could kill the remaining tumor cells. At a minimum this would considerably improve current protocols, as it would lower the required dose of toxic drugs and lower the frequency of escaping tumor cells.

Conclusions and perspective

In summary, we are calling for a return to the study of tumor reversion because we think that it will lead to effective new treatments for highly lethal cancers. By not killing tumor cells, pharmacologically induced tumor reversion could theoretically avoid selective pressures that drive the evolution of drug-resistant clones. There are likely to be many mechanisms of tumor reversion for a specific cancer genotype, just as there are many mechanisms of tumorigenesis. Most critical will be to determine which genetic reversion mechanism achieves the lowest frequency of cells escaping back to malignancy. In this way, powerful counteracting forces of evolution that cause the high rate of resistance to current cancer treatments can be prevented.

While a cure through reversion is certainly the most desirable outcome of this strategy, we do not think that the bar needs to be set that high. We predict that even if not a cure, reversion-derived drugs will yield a better survival rate with fewer side effects. If such improvements are achieved to a large enough degree, a cure then emerges *de facto* if not by initial design.

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Competing interests statement

The authors declare no competing interests.

Further information

Adam Telerman's laboratory homepage: http://www.tumor-reversion.org

Box 1. Theory by Max Delbruck, experiments by Salvador Luria. One of the most influential reports in the history of genetics resulted from the combined effort of two U.S. immigrants whose 1940s collaboration was a harbinger of the resurgence of quantitative biology in the 2000s. Max Delbruck was an accomplished theoretical physicist whose German ancestors were important academics. Delbruck entered the world of biology and introduced the novel concept that the gene was a molecule, which could be seen as the birth of the field of molecular genetics[39]. He fled Nazi Germany in 1937 and met Salvador Luria 4 years later at Vanderbilt University (Tennessee, USA). Luria was from a Sephardic Jewish family in Turin, Italy. He had heard of Delbruck's ideas and, as a result, started developing methods for testing these ideas with bacteriophages[40]. Increasing discrimination against Jews forced Luria to escape Italy in 1938, and he travelled all the way to Marseilles (France) on a bicycle. When he finally met Delbruck in the U.S., they soon designed, performed, and analyzed one of the most influential experiments of the twentieth century. They showed that in bacteria, resistance to bacteriophage infection arose spontaneously as a process of random mutation[3], which until that time had largely been thought to occur by some inductive mechanism, rather than being a process that paralleled natural selection and evolution. In that classic report, a footnote stated "Theory by M.D., Experiments by S.L."; it acknowledged the equal importance of experimental design (the elegant fluctuation design) along with a theoretical basis for

correctly interpreting the results. In this case, Delbruck derived a probability distribution that correctly predicted the results and established that the mutations giving rise to bacteriophage resistance were indeed random rather than directed.

Box 2. Induced changes in evolvability. John Cairns had an unusually productive scientific career that included major contributions to both molecular biology and cancer research. In molecular biology he is best known for isolation of the *polA* mutant lacking DNA polymerase I enzymatic activity in *Escherichia coli* and which established that DNA polymerase I, the enzyme previously purified by Nobel Laureate Arthur Kornberg, was in fact not involved in normal DNA replication[41,42]. He also established in 1963, while he was Director of Cold Spring Harbor Laboratory (New York, USA), that *E. coli* DNA was a single molecule that is replicated at a moving locus (the replication fork) at which both new DNA strands are being synthesized[43]. He thought very deeply about the implications of the standard hypothesis that cancer was caused by mutagens, which led him to be one of the very first scientists to consider tissue stem cells as the origins of cancers, and he proposed the immortal strand hypothesis for how tissue stem cells minimized accumulation of mutations[44]. He was also the only scientist brave enough to challenge the dogma established by the experiments by Luria and Delbruck that selective pressure and evolvability (rates of mutation) were totally independent of each other. He came out of retirement to perform a classic experiment in which he grew a leaky *lac* mutant of *E. coli* on lactose medium (*lac* mutants cannot grow on lactose medium, but leaky mutants show partial growth and observed the accumulation of revertant (lac^+) colonies over time above a non-growing lawn[45]. This result suggested that bacteria might mutagenize their own genome when growth is blocked. Although now understood to be due to gene amplification rather than nucleotide sequence alteration, this line of analysis established that natural selection can operate without cell division when variability is generated by local over-replication of a genome subregion[46].

Figure Legends

Figure 1. Mechanisms of stable reversion. Revertants isolated from viral oncogene-transformed cells can be either phenotypically normal (green cells) or have only partially regained normalcy (blue cells). For example, certain revertants that regain normal growth factor dependency can still form colonies in suspension[47]. Completely normal revertants can arise either through loss of the viral oncogene or by other genetic changes that in some cases include dramatic alterations of chromosomal composition[7]. Green indicates cells with either a malignant or partially malignant state, and blue indicates a normal cell or cancer cell that has fully reverted its phenotype back to normal growth control.

Figure 2. Proposed studies of genetic reversion of cancer cells. The steps in our proposed studies are outlined.

Author biographies

Scott Powers received his Ph.D. in Biological Sciences from Columbia University in 1983, where he began his career in cancer genetics as a student in the laboratory of Robert Pollack. He is currently Director of Cancer Genomics and Professor of Pathology at Stony Brook University and Research Professor at Cold Spring Harbor Laboratory.

Robert E. Pollack received his Ph.D. in microbiology from Brandeis University in 1966. He isolated the first non-tumorigenic revertant cell lines as a postdoctoral Fellow in Pathology at New York University in 1968. He subsequently did research at Cold Spring Harbor, and has been Professor of Biology at Columbia University since 1978**.**

TOC blurb

Current cancer therapies exert selective pressures that drive the evolution of drug-resistant clones. In this Opinion article, the authors argue that inducing stable tumour reversion represents an alternative strategy that could reduce resistance and thus effectively and durably treat cancer.

Subject categories

Biological sciences / Cancer / Cancer therapy
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Figure 1

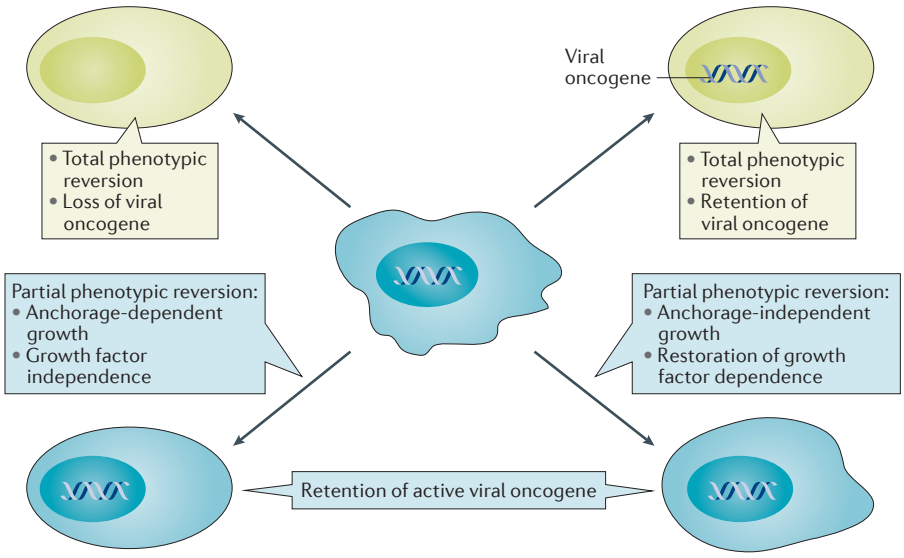

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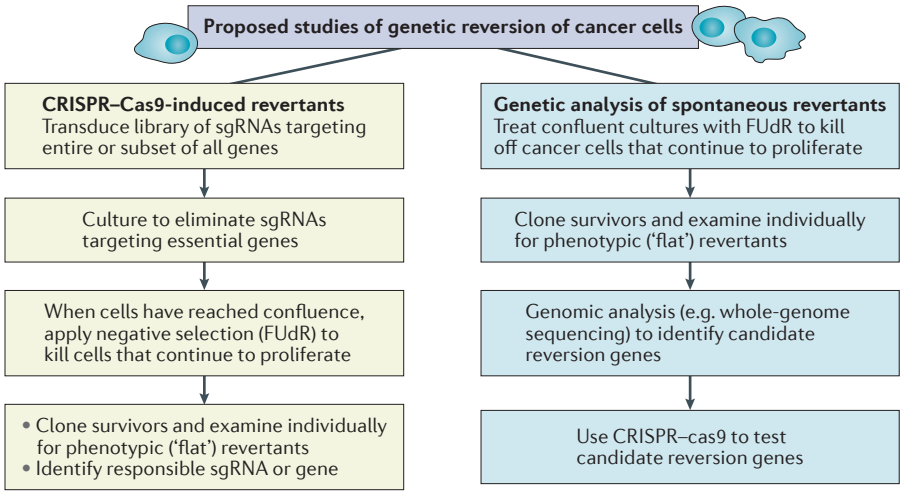
Figure 2



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