OPINION

Inducing stable reversion to achieve cancer control

Scott Powers and Robert E. Pollack

Abstract | How can we stop cancer progression? Current strategies depend on modelling progression as the balanced outcome of mutations in, and expression of, tumour 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: tumour 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.

Tumour cell reversion is the re-establishment of all, or a significant fraction of, the normal growth control mechanisms that are lost in tumours. 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]. *In vitro* tumour cell reversion 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[3] (BOX 1). It was observed that the progeny of rare but stable revertant cells emerged as contact-inhibited fibroblast colonies if the dividing cells in a dense culture derived from a single viral oncogene-transformed cell were killed with the chemotherapeutic agent 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, which supports 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 (FIG. 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, tumour revertants had become a mainstay of cancer research. Partial revertants that had lost some but not all of the characteristics of fully transformed cells were isolated, and they were used to establish the relationships between the diverse cellular phenotypes of tumour cells[8]. Studies of partial revertants clearly established that anchorage-independent growth was the strongest indicator of tumorigenicity[8] (FIG. 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 tumour 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 tumour reversion.

Tumour 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 tumours. Although reports of tumour reversion in mouse models have been published, these revertants are either induced by an inhibitor[13] or detected by cloning tumour 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 tumour cell reversion, which begins as a genetically stable event occurring in individual tumour cells.

At around the same time as studies of tumour cell reversion 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 tumour suppressors between 1990 and 1995, including the ability to explore the phenotypic effects of tumour suppressor deletions *in vivo* using mouse models[15], research into tumour reversion dwindled.

Mechanisms of tumour reversion

The laboratory that has maintained a considerable active interest in studying tumour 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 tumour reversion that can be applicable to a range 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 translationally controlled tumour protein (*TCTP*; also known as *TPT1*), *SIAH1*, presenilin 1 (*PSEN1*) and *STEAP3* (also known as *TSAP6*)[16]. Telerman's hypothesis is that tumour 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 tumour suppressor genes that cause cancer.

Interestingly, normal developmental processes such as mesenchymal-to-epithelial transition have also been shown to induce tumour cell reversion in colon cancer models[17]. However, instead of 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 tumour 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 (REFS 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[18]. Activation of p53 or overexpression of *STEAP3* induces the production of exosomes, and these vesicles are likely to have a role in suppressing the malignant phenotype[20]. *SIAH1* encodes an E3 ubiquitin ligase induced by wild-type p53 that targets several proteins for degradation, including components of the shelterin complex, which protects telomeres and which when degraded promotes senescence[21].

The other two genes implicated in the tumour reversion process, *PSEN1* and *TCTP*, are downregulated in the revertants and downregulated by wild-type p53 (REFS 19,22). *PSEN1* is a component of the γ-secretase complex, which is responsible for Notch activation, and its downregulation by wild-type p53 suppresses Notch signalling[23,24]. Finally, *TCTP* encodes a protein that binds to p53–MDM2 complexes and promotes MDM2-mediated ubiquitylation and degradation of p53 (REF. 25). *Tctp*-haploinsufficient mice are sensitized to p53-dependent apoptosis[25], and this may underlie the ability of *TCTP* to promote tumour reversion when downregulated.

Telerman's laboratory is to be commended for continuing to study tumour reversion while the focus of most 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 tumour reversion studies with specific cancer genotypes in mind.

New approaches to studying 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 for pharmacologically active molecules that could achieve the same tumour 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 (FIG. 2).

Screening using CRISPR–Cas9 (clustered regularly interspaced short palindromic repeats–CRISPR-associated protein 9) is another new genetic method that could be used. 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 tumour cell should produce revertants. Libraries of single guide RNAs designed against the entire set of coding sequences could also be used[26]. Negative selection methods such as the treatment of cells cultured at a high density with FUdR, as described above, could be used to identify tumour 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 (FIG. 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 a cancer type that is both prevalent enough 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[27].

Choosing the right tumour system is one of two crucial decisions necessary to establish the utility of a reversion-based strategy for cancer treatment. The other

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 US immigrants whose collaboration in the 1940s 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[38]. 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[39]. 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 United States, they soon designed, carried out and analysed one of the most influential experiments of the twentieth century. They showed that resistance to bacteriophage infection in bacteria arose spontaneously as a process of random mutation[3]. Until that time this resistance 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 correct interpretation of 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.

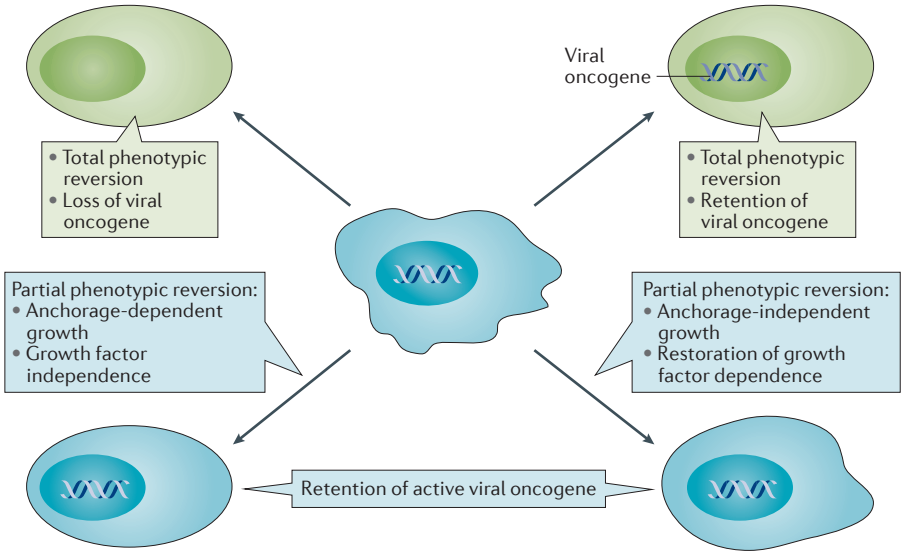

Figure 1 | **Mechanisms of stable reversion.** Revertants isolated from viral oncogene-transformed cells can either be phenotypically normal (green cells) or have only partially regained normalcy (blue cells). For example, certain revertants that regain normal growth factor dependency can still form colonies in suspension[46]. 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]. Blue indicates cells with either a malignant or partially malignant state, and green indicates a normal cell or a cancer cell that has fully reverted its phenotype back to normal growth control.

is to choose a system that produces 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 tumour. After repeated rounds of treatment, the resistant clone will dominate the tumour 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[28–30]. Now, consider a treatment scenario in which reversion is induced. 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 tumour 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 Mintz and Illmensee[31] in a study involving microinjection of fully malignant teratocarcinoma cells into blastocysts. Additionally, permanent differentiation of fully transformed cancer cells has been achieved in colon cancer cell lines treated with sodium butyrate[32]. Although often singled out as a clinical success for differentiation therapy, treatment of promyelocytic leukaemia (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 ATRA does induce differentiation, this is in effect a passenger event that obscures the action of ATRA in inducing *PML–RARA* degradation[33].

Also related to reversion is senescence[34]. Although in some cases this is accompanied by removal of the senescent cells by the immune system[35], 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 comprising pre-malignant melanocytes[36] — senescent growth arrest could form the basis for stable remission.

Our opinion is that stability is the most crucial feature to achieve and that inducing stable tumour reversion offers

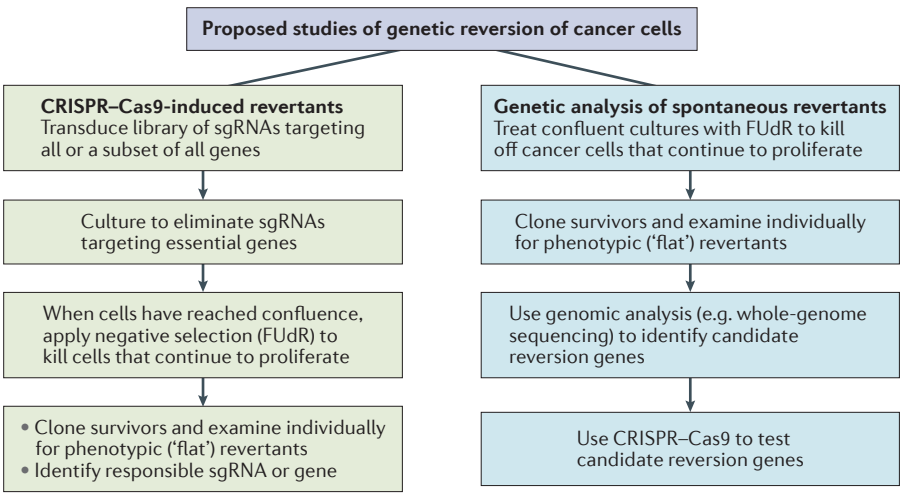

Figure 2 | **Proposed studies of genetic reversion of cancer cells.** The steps in our proposed studies are outlined. CRISPR–Cas9, clustered regularly interspaced short palindromic repeats–CRISPR-associated protein 9; FUdR, 5-fluoro-2'-deoxyuridine; sgRNA, single guide RNA.

great advantages over related strategies that aim to induce senescence or differentiation; these cell states represent modulations of cell behaviour that need not have any mutational component, but instead are more likely to require the ongoing presence of the senescence or differentiation 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, although desirable, is not an absolute requirement. Consider that when a person is infected with a bacterium, the prescribed 5–10 days of antibiotic treatment[37] required to clear the infection does not kill the last bacterium, but instead 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 reduce the load of dividing tumour cells, so that the immune system or conventional cancer treatments could kill the remaining tumour cells. At a minimum this would considerably improve current protocols, as it would lower both the required dose of toxic drugs and the frequency of escaping tumour cells.

Conclusions and perspective

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

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*, which established that DNA polymerase I, the enzyme previously purified by Nobel Laureate Arthur Kornberg, was in fact not involved in normal DNA replication[40,41]. In 1963, while he was Director of Cold Spring Harbor Laboratory (New York, USA), he also established 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[42]. 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 the accumulation of mutations[43]. He was also the only scientist brave enough to challenge the dogma established by the experiments of 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[44]. This result suggested that bacteria might mutagenize their own genome when growth is blocked. Although now understood to be a result of 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[45].

Although 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-inducing drugs will give 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:
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