Total deletion of in vivo telomere elongation capacity: an ambitious but possibly ultimate cure for all age-related human cancers

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Abbreviated title: Truly curing cancer by ablating telomere elongation

Abstract

Despite enormous effort, progress in reducing mortality from cancer remains modest. Can a true cancer "cure" ever be developed, given the vast versatility that tumours derive from their genomic instability? Here we consider the efficacy, feasibility and safety of a therapy that, unlike any available or in development, could never be escaped by spontaneous changes of gene expression: the total elimination from the body of all genetic potential for telomere elongation, combined with stem cell therapies administered about once a decade to maintain proliferative tissues despite this handicap. We term this therapy WILT, for "Whole-body Interdiction of Lengthening of Telomeres". We first argue that a whole-body gene-deletion approach, however bizarre it initially seems, is the only way truly to overcome the hypermutation that makes tumours so insidious. We then identify the key obstacles to developing such a therapy and conclude that, while some will probably be insurmountable for at least a decade, none is a clear-cut showstopper. Hence, given the absence of alternatives with comparable anti-cancer promise, we advocate working towards such a therapy.

Introduction

The reason cancer is so hard to combat is depressingly simple; it comes down to just two facts. First, cancer cells are very similar in most respects to the non-cancer cells from which they arose, making selective ablation of cancer cells intrinsically fraught. Second, unlike all other aspects of age-related degeneration cancer can acquire additional means of survival in response to whatever the body, or the clinician, may throw at it. Each cell in a tumour is a furnace of inventive potential, constantly experimenting with new combinations of gene expression as a result of its profound genomic instability and the availability of 6Gb of DNA to rearrange.

What therapies might, in principle, overcome this versatility? An answer is evident when we consider the mechanisms whereby tumours escape endogenous or medical attack, because those mechanisms have one very tangible thing in common: changes of gene expression. Immune stimulation is often escaped by loss of antigen presentation.[1] Angiogenesis inhibition is escaped by up-regulating alternative angiogenic pathways.[2] Chemotherapy may be escaped by numerous mechanisms, including up-regulating transporters that keep the cell free of the toxin, or specific DNA repair capacities.[3]

Telomerase inhibitors[4] are too new for clinical data to be available, but can in theory be escaped by over-expression of telomerase or degradation or export of the inhibitor.

We should therefore seek therapies that make such changes of gene expression impossible or at least highly improbable, even for the ever-inventive cancer cell. In principle, the **deletion** (not merely inhibition) of a gene whose function is essential for cancers to progress would present a major challenge to cancer cells. Creation of a new gene out of nothing does of course occur on evolutionary timescales, but it is many orders of magnitude rarer than mutations causing changes of expression, so tumours would never have enough cells or enough time to achieve it.

Unfortunately, cancer cells have the same genome as non-cancer cells. Selective deletion of a gene from cancer cells but not others faces the usual gene-expression problems: cancer cells can readily escape by down-regulating the DNA maintenance machinery on which targeted deletion depends, and the need to avoid killing too many non-cancer cells intrinsically limits any such treatment's therapeutic index.

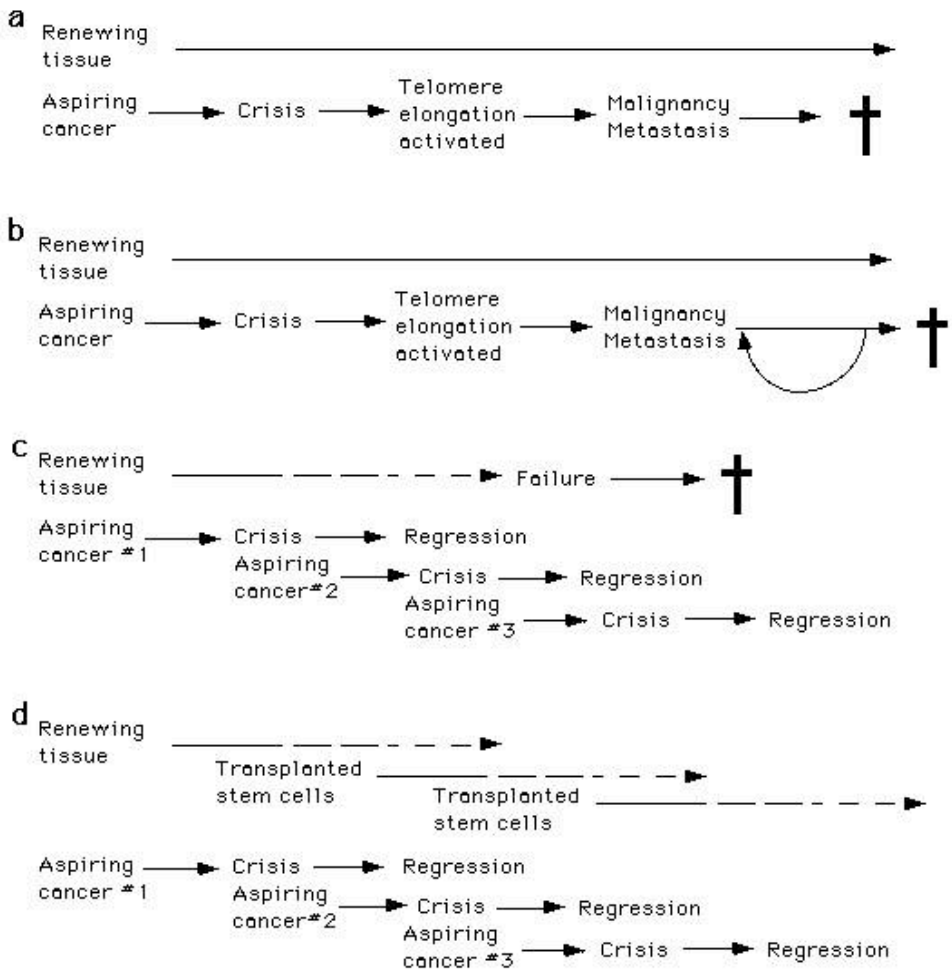

Figure 1. The WILT concept. An untreated cancer sufferer (a) dies rapidly once the cancer achieves malignancy and metastasis. Conventional treatments (b) delay death somewhat, but the cancer typically evolves gene expression changes that outmauoeuvre the treatment. A hypothetical telomere-elongation-negative human (c) could not develop metastatic cancer, but would die relatively young from failure of constantly-renewing tissues. A beneficiary of WILT (d) would maintain function of such tissues through periodic stem cell transplantation, so would not die of either cause.

A way around this is to eliminate the gene from **all** cells, and somehow to make it non-essential to the non-cancer cell (or, at least, to the tissue of which that cell is a part) while preserving the cancer cell's

absolute requirement for it. This is the approach discussed here. The genes in question are those responsible for maintaining telomere length through large numbers of cell divisions by telomere elongation. Tissue integrity would be preserved by, about every ten years, re-seeding the stem cell compartments of all tissues reliant on continuous cell division with stem cells whose telomeres had been lengthened ex vivo but whose telomere elongation machinery was deleted (Figure 1). Transformation of a living organism—ultimately, a human—to a totally telomere elongation-incompetent state would be performed gradually, using a variety of techniques discussed below. We suggest that, if (as we foresee) it could be implemented without serious side-effects, this therapy would almost completely eliminate cancer as an age-related cause of death, something which no other present or currently contemplated therapy would do. In fact, one's risk of cancer would actually **decline** with age as telomere elongation-competent cells were progressively depleted.

Here we discuss the plethora of obstacles to the development (even on a multi-decade timescale) of such therapy, which we term WILT, "Whole-body Interdiction of Lengthening of Telomeres". We conclude that, while many of those obstacles are daunting, none is so insurmountable as to justify dismissing this approach. We foresee a considerable risk that, as sophisticated cell and gene therapy technologies mature in the next decade or two, progress in reducing age-specific cancer death rates will lag behind that for other major killers,[5] and that the proportion of people dying from cancer will thus rise sharply. It may be that only by developing ambitious but extremely powerful anti-cancer therapies, such as WILT, can this be prevented.

Efficacy

Requirement of telomere elongation for human cancer progression

A typical clinically relevant cancer contains around 2^{40} (10^{12}) cells. Thus, at least 40 cell generations have occurred in that cancer starting from the initiating non-cancer cell. In fact, however, this is a gross underestimate: (a) there is abundant cell death in cancer, and (b) the development of cancer is multi-stage. Suppose, for simplicity, that a cancer develops as a result of two mutations—one to escape growth control and, later, one to induce angiogenesis. The former may allow it to grow to, say, 10^6 cells; the second, to 10^{12} cells. But that second mutation will have occurred in just one of the 10^6 cells which harboured the first. Thus, starting from the cell in which the first mutation occurred, there will have been not 40 but 60 divisions—even ignoring the contribution of cell death.

In practice, since cancer progression in humans requires many more than two mutations (except some rare childhood cancers), there are probably at least a few hundred cell generations between the originating non-mutant cell and the clinically relevant cancer.[6] It is this that makes prevention of telomere elongation a realistic way to prevent cancers from ever reaching an advanced stage. No human cell has ever been observed to divide more than 100 times without telomere elongation machinery. Moreover, even though the "end-replication problem"[7,8] that originally inspired the telomere-based explanation of this "Hayflick limit"[9] suggests that telomere loss per cell generation could be much slower than is typically seen in vitro,[10] only a modest extension of replicative capacity results from growth in low oxygen.[11] Direct evidence for the absolute requirement of telomere elongation for progression of human cancers is widespread.[12-14]

Telomerase

All mammals maintain telomere length in rapidly-dividing cells by reverse transcription of a 6-base RNA template of which the telomere is a many-copy DNA tandem repeat. The RNA including the template (hereafter "TERC") and the reverse transcriptase (hereafter "TERT") form a heterodimer called telomerase, which adds copies of this sequence to the end of one strand; the other strand is elongated by standard DNA replication machinery.[15] TERC is ubiquitous in human tissues, but TERT is expressed only at trace levels in tissues that require telomere elongation and is undetectable in quiescent and postmitotic cells.[16,17] In about 90% of human cancers, however, TERT is highly expressed and telomere

length thereby stabilised.[18] Many non-cancer human cell types that normally senesce (have a finite replicative capacity) in culture have been "immortalised" (given indefinite replicative capacity) by introducing constitutively active TERT.[19-21]

Though telomerase may have cytoprotective properties not directly related to cell division-associated telomere elongation,[22] it seems that neither telomerase subunit has any essential physiological function except telomere elongation. For TERT in humans this is shown by its absence in nearly all cells and very low levels in any cell type. In laboratory mice, which maintain their telomere length several organismal generations "ahead of the game" (allowing serial inbreeding of telomerase knockout mice, in which telomeres in the germ line progressively shorten), knockout of either TERC or TERT confers no detectable phenotype for three generations.[23,24] Thus, these genes are prime targets for WILT.

ALT (alternative lengthening of telomeres)

However, about 10% of human cancers—predominantly mesenchyme-derived ones such as sarcomas, in which the proportion approaches 50%—do not express telomerase but nonetheless maintain telomere length indefinitely both in vivo and in vitro.[25] They do so by a mechanism termed ALT, for "Alternative Lengthening of Telomeres". Moreover, cancers of epithelial tissues may express ALT rarely only because they have the easier option to activate telomerase (which may be suppressed less thoroughly in epithelial than in mesenchymal tissues). Hence, epithelial-derived cancers might turn ALT on as easily as sarcomas do if the telomerase route were denied them. A therapy that eliminated cancers' ability to activate telomerase but left ALT untouched might thus only modestly reduce age-specific cancer mortality rates.

The mechanism of ALT clearly involves a recombination-like process but the molecular details have not been determined. Short telomeres are the recipients of ALT events (but perhaps not exclusively) but it is unclear whether the telomere repeats added are chromosomal or extra-chromosomal in origin. One model proposes that t-loop structures initiate intra-telomeric rolling circle replication in ALT cells, though there is currently no supporting evidence for this mechanism in human cells. A second model [which has some supporting evidence[26]] proposes strand invasion and copying of telomere repeats from a donor to a recipient telomere in a BIR (breakage-induced repair)–like process.[27]

Even with our limited current understanding of ALT, however, we can make one observation that gives cause for optimism that, once its underlying genetic etiology is discovered, it will be amenable to the same sort of manipulation as for telomerase. There are formally three types of possible explanation for why ALT is seen in certain cancers but not in normal tissues. One is that, like telomerase, ALT is an activation of a gene or genes that are normally turned off in the cell type in which the cancer arises. (Candidate genes might be ones involved in meiosis or in a form of DNA repair that is only activated under certain circumstances, for example.) The second is that telomere elongation by recombination is a side-effect of a constitutive process (a ubiquitous DNA repair process, for example): that is, that it is happening all the time in normal cells but is counterbalanced by a shortening process and so progressive telomere lengthening is not seen in such cells. Indeed, there seems to be a system for actively shortening telomeres that have been lengthened by ALT.[28] If this second mechanism were the basis of ALT it would be a blow to the potential efficacy of WILT, because no gene would be available to be deleted without rapidly deleterious effects in normal cells. This seems unlikely, however, because a constitutive system in which telomere elongation by recombination is balanced by shortening of over-long telomeres would maintain telomere length by default in all tumours and there would be no pressure to activate telomerase. Additionally, telomeric recombination has not been detected in normal cells.[26,29] Finally there is the possibility that the active players in ALT are indeed active in normal cells (performing non-telomeric DNA maintenance) but that, rather than being constantly lengthened and re-shortened, some system protects telomeres from being adventitiously lengthened by this machinery in the first place, and this is lost in ALT cells. In this scenario, however, since the hypothetical constitutive function of the ALT machinery is certainly not inter-chromosomal recombination, it seems plausible that (once it had

been identified) judicious site-directed mutagenesis could delete its capacity for such recombination while preserving its constitutive function.

Hence, in summary, until the molecular basis of ALT is understood we cannot state how or whether it can be ablated along with telomerase as part of WILT, but there is reason for optimism.

Chemotherapy and chemoresistance

The characteristic that most centrally defines a cancer cell is its high division rate. Thus, the cell types that are hardest to distinguish from cancer cells, when designing a therapy, are those that themselves divide fast, such as in the bone marrow, skin and gut. The blood, in particular, is maintained by the rapid division of transit amplifying cells in bone marrow whose ablation as a result of anti-cancer therapy is highly prejudicial to the welfare or even survival of the patient.[30]

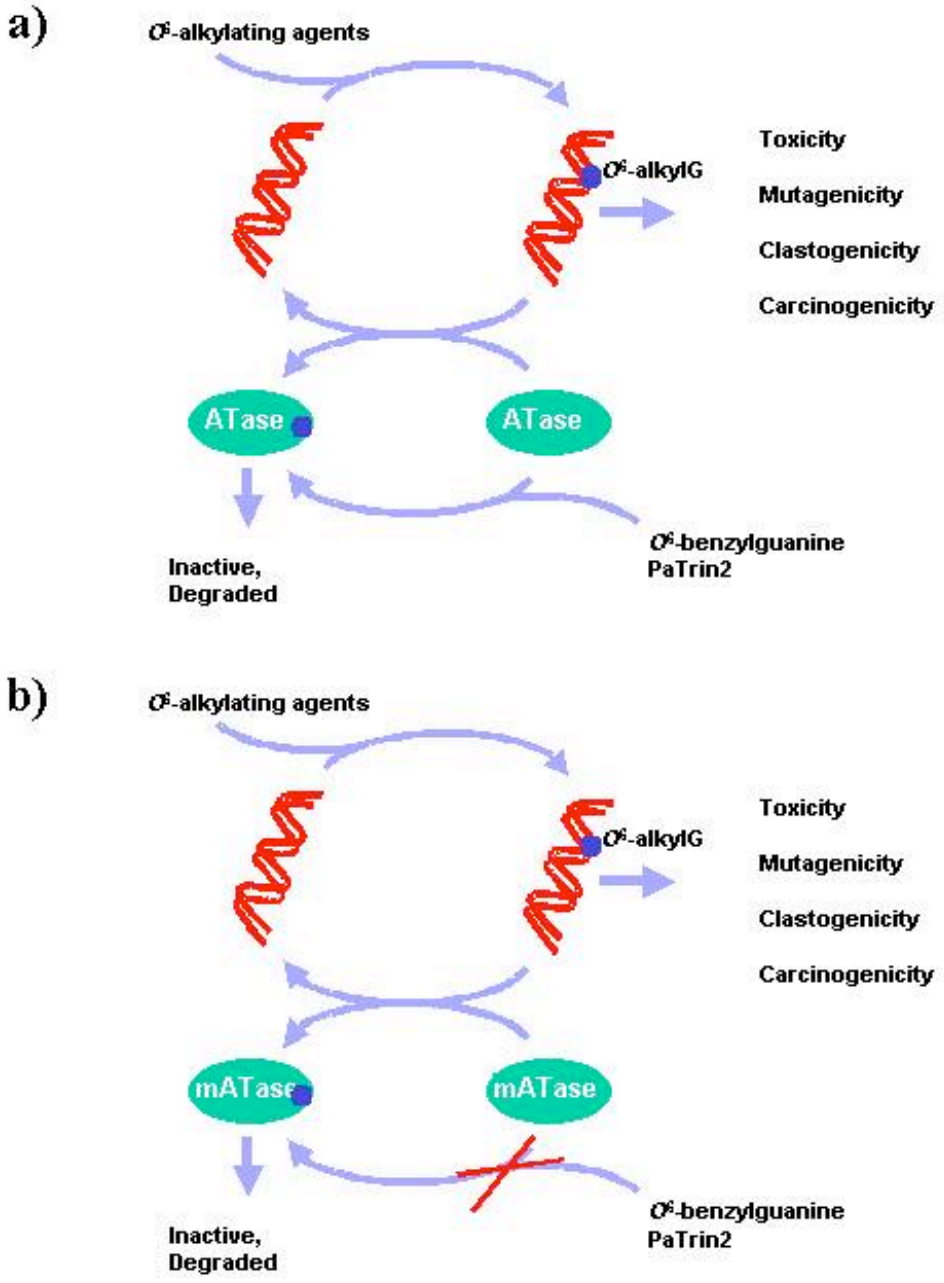

Figure 2. Mechanism of ATase activity and inactivation. (a) O^6-alkylating agent modification of DNA leads to incorporation of alkyl groups at the O^6-position of guanine and to subsequent genotoxic events.

ATase repairs O^6-alkylguanine lesions in a stoichometric and autoinactiavting manner. Small molecule psueodosubstrates of ATase lead to inactivation and thus to sensitivity of cells to O^6-alkylating agents. (b) Certain point-mutated versions of ATase are unable to react with pseudosubstrates, yet retain their DNA-repair capacity.

Conveniently, the tissues most at risk in this regard are just those that would also—again because of their rapid division—be eventually compromised by WILT. Such tissues must be maintained by the periodic introduction of new cells that have been engineered ex vivo. This ex vivo manipulation can potentially include manipulations to diminish sensitivity to anti-cancer agents.

In fact, many groups have been exploring such an approach (independently of WILT, of course).[31,32] A prominent stratagem has been to exploit the cell's spectacularly laborious mechanism for reversing a particular type of DNA damage, alkylation of guanine at position 6; this is done by a protein that transfers the alkyl group to itself and is then ubiquitinated and destroyed, rather than acting catalytically. This protein, O^6-alkylguanine-DNA-alkyltransferase (ATase) is a first-class target for chemotherapeutic agents because of two additional features: first, there are small molecules that mimic O^6-alkylation in DNA and act as pseudosubstrates of the protein leading to its inactivation, and second, there are single-amino-acid changes to the protein that render it almost completely resistant to such inactivation (Figure 2). Hence, before chemotherapy with a combination of inactivator and O^6-alkylating agent, the patient can be transplanted with haemopoietic stem cells engineered to express inactivator-resistant ATase; then, a dose of the inhibitor/O^6-alkylating agent combination that the cancer cell cannot survive will ablate native bone marrow but leave the transplanted cells (and hence the patient) unscathed.[33]

This approach possesses an inherent shortcoming: engineered marrow—which must necessarily contain haemopoietic stem cells—could give rise to new cancers, which would be resistant to some chemotherapeutic challenges. Since haemopoietic stem cells can repopulate many tissues other than the blood,[34,35] this problem might be severe if the patient lives many years after the treatment. However, when implemented in combination with WILT, no such concern exists: such cancers might arise, but they could not reach a life-threatening stage.

Telomerase capture: a possible cancer escape route from WILT?

The WILT concept assumes that creating a new gene is far harder than changing expression patterns. However, genomes can gain genes by lateral gene transfer. Could tumours escape WILT that way?

A formal possibility is that the reverse transcriptase of a retrovirus might be recruited for telomere elongation; these work very differently from TERT, however,[36] so this seems remote. Alternatively, a cell engineered to lack telomerase but to be chemoresistant might fuse, in vivo, with one that was present natively and hence retained telomerase genes. Cell fusion (and phagocytosis of apoptotic bodies, which could be equivalent) has been demonstrated in vitro,[37-39] but its in vivo relevance remains unknown. Such fusion products would, anyway, still be susceptible to chemotherapeutic agents to which resistance had not been transgenically conferred, so this would not seriously subvert WILT.

Feasibility

Stem cell technology

We purposely say little here about the prospects for developing the stem cell technology needed for WILT. Clearly WILT could not be implemented until we can transform adult cells into stem cells of all rapidly-renewing tissues, expand them very substantially in vitro and introduce them into the person from whom they came;[40] these are major advances. But numerous other advances necessary for WILT are also very considerable, so that we do not foresee any prospect of its being developed in under ten years even in the best case. Progress in stem cell technology is presently so rapid that nothing can meaningfully be predicted about where it will stand in ten years, let alone thereafter. Hence, we merely

note that the necessary sophistication of stem cell therapy is likely to exist by then, and focus here on the many other prerequisites for implementing WILT.

Ex vivo genetic manipulation

Three genetic alterations are entailed in WILT (plus, perhaps, adding an inducible suicide gene for safety):

- Deletion of telomere-elongation genes;
- Introduction of chemoresistance;
- Elongation of telomeres to the length seen in a typical stem cell.

Of these, the last is probably easiest, because it does not require genomic integration. Expression of telomerase from an extrachromosomal transgene, in a cell that has already undergone the other required genetic changes, would extend telomeres to somewhat more than the desired length. The cells would then be grown for enough generations to allow confirmation of loss of the extrachromosomal transgene. Additionally, by incorporating a 'suicide gene' such as HSVTk into the extrachromosomal construct it should be possible to selectively eliminate (using ganciclovir) any cells that retain the construct.[41]

Chemoresistance could be conferred by introducing a transgene (e.g. encoding a drug-resistant ATase) at a random position in the genome. This approach, however, in common with most gene addition strategies for gene therapy, faces the severe problems of transgene silencing and instability after transplantation (not to mention potentially deleterious effects on genes close to the integration site), even if pre-transplant selection is used to enrich for transgene-expressing cells. Such problems can be avoided by modification of an endogenous gene; the fact that chemoresistance can sometimes be conferred by one or two amino-acid changes[31] to an endogenous gene is particularly valuable in this context. The best-established technique for endogenous gene modification is gene targeting by homologous recombination between a target locus and a plasmid carrying several kbp of homologous dsDNA.[42-45] Other, less developed methods involve triplex-forming oligonucleotides,[46] single-stranded oligonucleotides[47,48] or RNA/DNA oligonucleotides (RDOs).[49,50] The choice of technique would be driven mainly by the incidence of accompanying random mutations, rather than of the desired alteration, because selection for (e.g.) ATase inhibitor resistance can be used to enrich for rare events. Although the incidence of accompanying random integrations is easier to assess (by simple Southern analysis) in standard gene targeting methods, oligonucleotides have the attraction that they are relatively easy to prepare and deliver. It is therefore important that oligonucleotide-based methods be thoroughly validated, characterised and optimised.

A problem with altering endogenous genes is that the target tissues are often sensitive to chemotherapeutic drugs because they naturally express low levels of drug-resistance mechanisms such as ATase. Thus, small changes to endogenous genes might be more appropriate for ubiquitously expressed enzymes such as thymidylate synthetase or dihydrofolate reductase, where specific mutations confer resistance to specific anti-metabolites.[51,52] Other alternatives are to combine point mutations in the coding regions with genomic changes to alter expression patterns, or to deliver the drug resistance transgene on a mammalian artificial chromosome.[53,54]

Deletion of telomere-elongation genes is more challenging. First, deletion of both copies of at least two to four genes will be desirable. Second, these deletions will not confer an inherent selectable advantage on the cells (though with gene targeting this might be addressed by replacing the gene by a selectable marker). Third, the alterations must delete large portions of the relevant genes, because less drastic changes (such as single-base-pair changes to introduce a stop codon or frameshift) might be too easily reverted in a cancer. This may constrain the choice of technique: targeted deletions (see Figure 3) of several kbp (or Mbp if Cre/lox technologies are used) are a well established capability of standard gene targeting methods,[45,55-57] while oligonucleotide-mediated approaches have so far been limited to base changes or deletions of only one or two base pairs (although multiple modifications could be introduced sequentially). This situation may change, however, as oligonucleotide-based methods are developed in

mammalian cells. In *E. coli*, for example, single-stranded oligonucleotides can generate a deletion of 3.3kb as efficiently as a single base-pair change.[58]

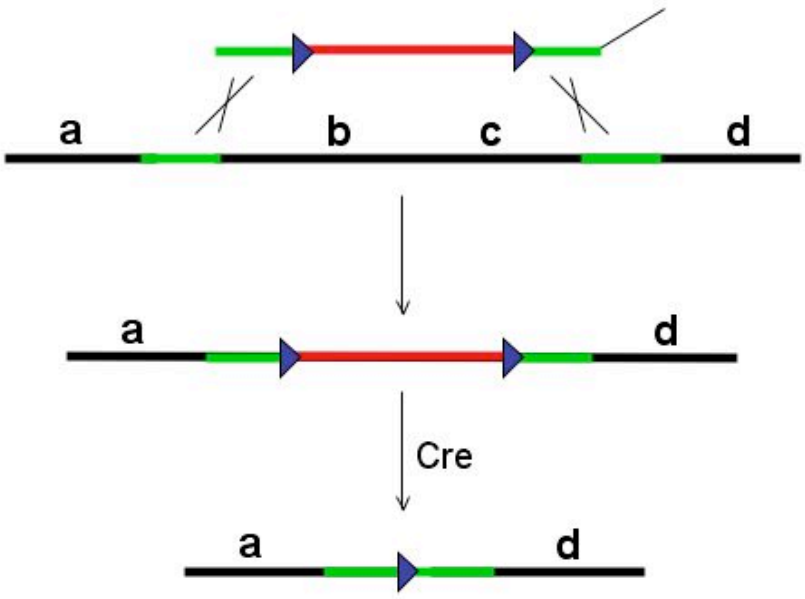

Figure 3. Making a genomic deletion by gene targeting. Regions of homology between the targeting construct (top) and the target locus are at the ends of the construct. In the first step, homologous recombination between the target gene and target locus deletes a portion of the target gene, replacing it with a drug resistance cassette (central part of the construct). If desired, the latter can be removed by the action of a site-specific recombinase (e.g. Cre) on flanking sequences (triangles; e.g. LoxP).

Modification of multiple non-selectable genes is daunting because of the generally very low efficiencies of modification. Gene targeting frequencies of 1 per million transfected cells are not atypical, for example, and random integration frequencies are often 1-3 logs higher. Furthermore, the initially reported[59] high frequencies of RDO-mediated gene modification have often not been reproduced.[50] The efficiency of standard gene targeting and of oligo-based methods in general will therefore need to be improved. Numerous approaches are being explored,[45,60,61] many based on promoting and impairing the machinery for homologous recombination and non-homologous end-joining, respectively, and many improvements have already been reported. Other approaches can be envisaged, however. For example, methods for in vitro evolution of target-specific endonucleases may well emerge within the next decade, and these should be powerful tools as it is known that double-strand breaks in the target gene can improve gene targeting efficiencies by 2-3 logs.[62] Adeno-associated viruses also seem promising.[63] Aside from WILT, there are already powerful incentives for developing such approaches: as a means of determining gene function and as cures for single-gene disorders such as cystic fibrosis and thallassaemia. Our understanding of the important variables in gene modification should thus continue to grow and lead to better efficiency, and modification of multiple non-selectable target genes should become realistic.

In vivo genetic manipulation

Genetic alteration of rapidly-dividing cells for WILT will almost certainly always be easiest by an ex vivo procedure. Apart from the intrinsic difficulties of somatic gene therapy, the result of deleting telomere elongation function in such cell types would be the long-term inviability of the tissue and the need to replenish it (discussed below), so the cells whose genetic engineering is required will already have to be manipulated in other ways ex vivo to give them the desired properties. However, several important cancer types, including sarcomas and gliomas, derive from relatively quiescent cells whose

total requirement for cell division in a lifetime is low. Repopulating such tissues with engineered cells appears very challenging.

It may therefore be necessary to alter these cell types in situ. Unfortunately, this seems unlikely to be achievable soon by gene targeting, given the rate of random integration noted above. However, several groups are working to improve the reproducibility of high-efficiency oligonucleotide-mediated gene targeting, and other approaches are also on the horizon.[61]

Finally, we must remember that a therapy so advanced and technically difficult as WILT is not likely to become an attractive option until considerable progress has been made against all other major causes of death and debilitation—particularly cardiovascular disease, neurodegeneration and diabetes—because only then will the limitations of more conventional anti-cancer approaches become apparent as a steep rise in the proportion of people dying of cancer. Such advances will surely also require effective in vivo genetic manipulation.[5] Thus, this aspect of WILT is not in fact a substantive obstacle to its development by the time it is needed.

Side-effects

Cancer promotion?

Cells with very short telomeres are genetically unstable. Indeed, in humans it is considered likely that an initial phase of inadequate telomere maintenance early in tumorigenesis confers a "mutator" phenotype, accelerating the occurrence of further mutations—including activation of telomerase or ALT—that allow the tumour to progress.[64]

This phenomenon should not affect the efficacy of WILT, however. When telomere elongation cannot be activated, the mutator phenotype will if anything hasten the tumour's demise by increasing the accumulation of mutations that prevent cell division.

Telomerase knockout mice

The best laboratory models currently available to explore the WILT concept are mice engineered to lack telomerase. Even though mouse ES cells lacking telomerase acquire an ALT-like character,[65] this is not seen in vivo—perhaps because it is too rare, or perhaps because ALT is incompatible with the normal differentiated state of some mouse tissues. Thus, mice lacking telomerase can be inbred for successive generations to yield mice with telomeres too short to sustain highly proliferative tissues for the animal's normal lifetime.[66] The most prominent phenotypes observed are in the gonad, blood and skin. Interestingly, mice lacking p53 as well as telomerase show a delay in the major phenotype that can be measured at an early enough age not to be masked by the cancers that result from lack of p53, namely sterility: $TERC^{-/-}$ $p53^{-/-}$ mice can be bred for two generations longer than the simple TERC knockouts.[67]

It is important to understand why p53 ablation accelerates death from cancer even in late-generation telomerase knockout mice.[68] The number of cell divisions needed for a mouse cancer to grow big enough to kill it is considerably fewer than in a human, for several reasons: (1) the cancer need not grow as big; (2) it need not metastasise, whereas most human cancers only become life-threatening after metastasis; and (3) the telomere damage-detection system in mice is simpler—in particular, the Rb pathway does not exert a strong protective effect[69]—so fewer mutations are required. Also, as noted earlier, mouse cells can activate ALT quite easily in vitro; it has not been determined whether cancers developed by $TERC^{-/-}$ $p53^{-/-}$ mice (or, indeed, by $TERC^{-/-}$ mice) are phenotypically ALT-like, though they do become ALT-like after serial transplantation.[70] The anti-cancer effect of short telomeres is therefore challenging to assay in mice. However, when these confounders are minimised, the effect is dramatic: in $TERC^{-/-}$ Apc^{Min} mice, a mild reduction in telomere length increased the incidence of cancer at a given age, but severe telomere shortening reduced that risk to the point where no deaths at all occurred by the age at which all $TERC^{+/+}$ animals had died.[71] Other models of cancer in $TERC^{-/-}$ mice also show resistance to cancer progression.[72]

Dyskeratosis congenita

Humans have shorter telomeres than most mammals [including non-human primates[73]], and their gestation is longer. This is probably why humans lacking telomerase activity are not found: they would not develop to term. However, a disease has been known for nearly 100 years[74] that exhibits symptoms quite like those of late-generation telomerase knockout mice: it is called dyskeratosis congenita (DC). Indeed, the genetic basis for nearly half the known cases of DC is a mutation in one of two genes whose product is telomere-associated, and in both cases patients have shorter telomeres than age-matched controls (Figure 4).

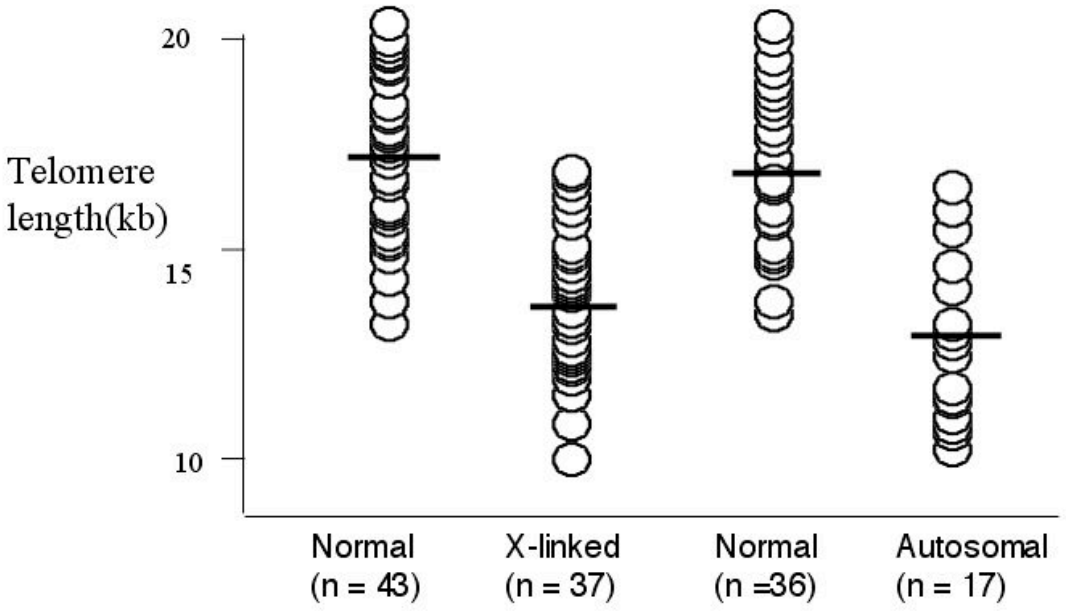

Figure 4. Both X-linked (dyskerin) and autosomal (TERC) DC cause shortened telomeres.

One of these genes is TERC.[75] Several TERC mutations have been identified in different DC families. These mutations are present in only one of the TERC alleles and families display autosomal dominant transmission of the DC phenotype. Of interest is that many sufferers only acquire symptoms in middle age, and in particular are fertile, since pedigrees exist. Moreover, the age of onset of DC in offspring tends (though the small number of sufferers so far identified precludes firm conclusions) to be considerably earlier than in parents, and grandchildren tend to develop symptoms in childhood[75] (also I.D., unpublished data). This suggests cumulative telomere shortening in the germ line.

The other gene is named dyskerin. Unlike TERC, most dyskerin mutations so far seen in humans are missense alterations, presumably hypomorphic. Intriguingly, dyskerin knockout mice die at embryonic day 6.[76] Dyskerin must thus have a non-telomeric function (it seems inconceivable that telomere maintenance defects could be so rapidly lethal); there is suggestive evidence that it is pseudouridylation of ribosomal RNA.[77] Mutations in dyskerin, which on average cause an earlier age of onset of symptoms than those in TERC, cause depletion of TERC to around 20% of normal,[78] so the dominant phenotype of TERC mutations is probably simple haploinsufficiency. DC promotes cancer,[79] but insufficient tissue has been available thus far to address key questions, such as whether DC tumours express telomerase or ALT.

DC sufferers are potential beneficiaries of the techniques to prevent side-effects of WILT that will be discussed below. One, bone marrow transplantation, is a standard treatment already. With anticipated advances in stem cell manipulation, such treatments should become more feasible and effective and DC may become a truly curable disease.

Avoidance of side-effects

The problems that would certainly arise from ablating telomere elongation can, in principle, be avoided by periodically reseeding all highly proliferative tissues with stem cells whose telomeres have been lengthened ex vivo (but whose autonomous telomere elongation competence has not been restored, or has been restored but then removed again). While this is conceptually straightforward, in practice it faces major obstacles, which vary from tissue to tissue. Below we consider three key tissues needing this restoration and the difficulties to be overcome.

Bone marrow reseeding

The haemopoietic system is a rapidly-renewing tissue for which replenishment techniques already exist: bone marrow transplantation (BMT) has long been routine. New problems would arise, however, in BMT for WILT.

Firstly, for complete re-seeding of the haemopoietic system, substantial expansion of the transplantable stem cell population ex vivo would be required. Current efforts at expanding stem cell numbers utilising combinations of cytokines[80] have been limited by several factors. Firstly, the most primitive stem cells, the so-called long term repopulating stem cells, are typically not expanded and are frequently lost by differentiation following prolonged culture in ex vivo expansion conditions. These are the key stem cells for long term maintenance of a transplant[81] so this largely explains the failure of transplantability of ex vivo expanded stem cell populations. Thus, there is much current interest in characterising factors which will allow self-renewal of these cells against a block in differentiation. Recently, many researchers have focused on members of the Notch ligand family in this respect.[82] However, although results are promising, these analyses are at an early stage.

A further complication is that following such culture, these cells will typically be proliferating. Under normal circumstances, most stem cells are not proliferating and there is abundant evidence for a requirement for a G_0/G_1 state of haemopoietic stem cells for proper homing and engraftment following transplantation.[83] Thus, pre-transplantation resetting of this 'quiescent' status will be essential. This may be achieved using combinations of the well characterised inhibitors of stem cell proliferation appropriate for long term repopulating stem cells.[84] The same applies to molecules involved in the in vivo homing and engraftment of haemopoietic stem cells: expression of these molecules, such as the chemokine receptor CXCR4, may be altered by culture conditions[85] and need to be reset before transplantation.

Thirdly, BMT works best when the stem cell niche has already been denuded of native cells.[86] In certain diseases of the haemopoietic system, however, this is not seen,[87] indicating that functionally compromised stem cells are less resistant to displacement by incoming, more robust ones. For second and subsequent WILT reseedings, it may thus be possible to rely on the fact that many of the previous reseeding's stem cells will be nearing exhaustion in terms of telomere length. The first treatment, however, might need chemotherapeutic or radiation-mediated depletion of the marrow to allow the engineered cells to engraft.

Gut reseeding

The gut lining consists of a juxtaposition of finger-shaped structured, termed villi, and invaginations, termed crypts. It is maintained by stem cells at or near the crypt base, which generate rapidly dividing cells that migrate up out of them and along the villi, eventually being shed at the tip.

Several years ago, one of us (F.C.C.) and his colleagues developed a technique for surgically repopulating the mouse colon with cells extracted from the small intestine. Despite considerable ex vivo handling, cell aggregates that had been plated onto freshly denuded colon developed in vivo into morphologically normal crypts containing all four of the differentiated cell types normally observed.[88] Clearly this augurs well for maintaining the gut in the context of WILT.

However, daunting problems remain. One is fibrosis. The mouse work involved only a small area of colon; when similar surgery was done on newborn pigs, the resulting fibrosis precluded restoration of

functional intestine (F.C.C., unpublished data). Further, for application to humans there would be a requirement to avoid surgery. The gut may, like the bone marrow, resist engraftment of new stem cells in tissue already replete with them, so denudation may be required; this might be possible using endoscopy technology, but that would risk short-term impairment of gut function. Finally, gut stem cells cultured in vitro lose proliferative capacity after only a few divisions. This sensitivity may or may not be alleviated by more sophisticated culturing technology (such as low oxygen).

Skin reseeding

DC sufferers and late-generation TERC$^{-/-}$ mice both show severe epidermal dysfunction, and this will undoubtedly be a tissue needing replenishment in the context of WILT. However, epidermal function depends critically on the underlying tissue—the dermis.[89] The dermis-epidermis interaction seems to be central to epidermal maintenance and regeneration. Also, most of our skin grows hair, and it may be the hair follicles, from which several important types of skin cancer are believed to originate, that hold the key to effective skin regeneration.[90]

As elsewhere, an important requirement for skin reseeding with a frequency of around a decade will be to control the differentiation process so that stem cells divide rarely enough to survive. Several factors have been reported to exert appropriate influences. One is 14-3-3□, which inhibits differentiation.[91] Reliable identification of stem cells is also important; high levels of □1 integrin expression,[92] high □6 integrin with low CD71 expression,[93] Keratin 19,[94] and p63[95] are among markers championed as being diagnostic of the keratinocyte stem cell state. A definitive marker would permit refinement of techniques already developed for isolating a pure population of these cells for tissue engineering purposes.[96]

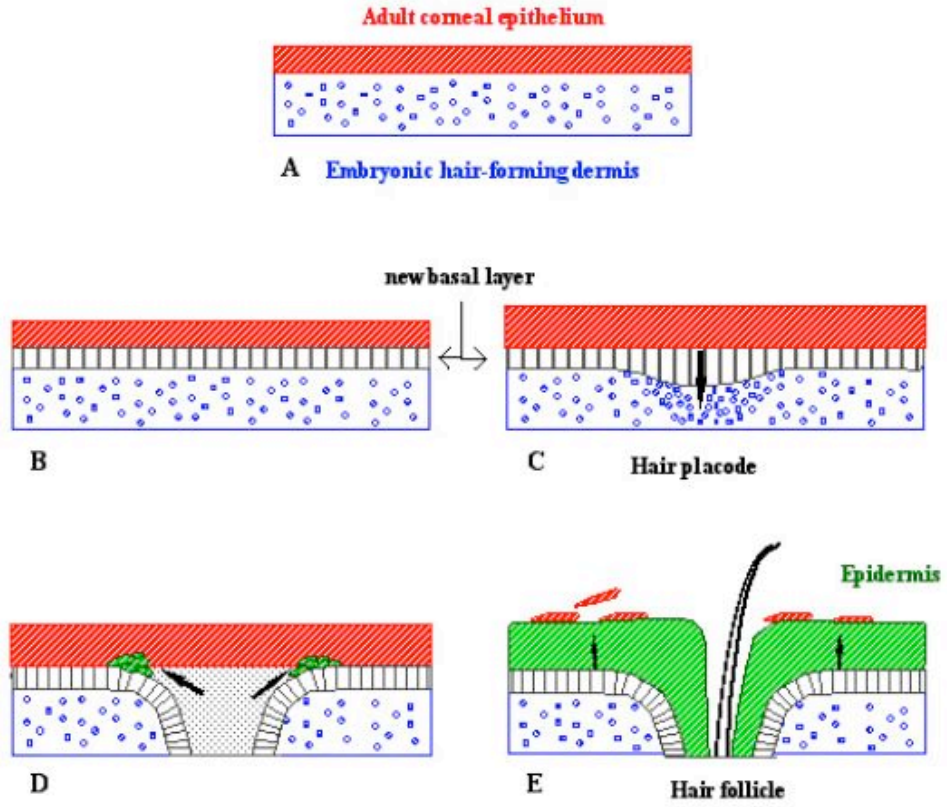

Figure 5. Corneal epithelium is reprogrammed by the dermis as hair-forming epidermis, starting with the hair follicle. Epidermis formation after grafting of corneal epithelium (A) proceeds through basal layer formation (B), placode formation (C), epidermis formation at the follicle edge (D) and finally hair growth (E).

A particularly useful tool for studying skin regeneration is the corneal epithelium. Its central region can be removed without removing the stem cells from the surrounding germinal region (the limbus) and grafted onto denuded dorsal dermis; the corneal cells are rapidly reprogrammed to a follicular state, including development of normal hair follicles.[97] Importantly, hair follicle formation actually precedes formation of new epidermis, and epidermal differentiation spreads from the neck of the follicle (Figure 5), reinforcing the idea that the follicle can be a repository of stem cells for the epidermis.[90] Also, this type of transdifferentiation work illustrates the possibility of using stem cells from an organ like the eye to repopulate another like skin.

Taken together, these and other observations give cause for optimism regarding skin regeneration, whether for WILT or for more traditional applications such as treatment of burns or DC. Since the dermis is a very slowly-renewing tissue, its capacity to orchestrate the behaviour of its epidermal coating affords us great flexibility in the introduction of WILT cells. There is still some uncertainty as to the numbers and exact location of stem cells in hair follicles but the most widely held view is that they undergo bursts of activity corresponding with the periodic initiation of the follicle growth cycle.[98] Certainly a follicle (not to mention its epidermal neighbourhood) contains more cells than a villus. Moreover, matrix epithelial cells at the base of actively growing follicles are among the fastest dividing cells in the body: in human scalp the matrix replaces itself every 23 hours, and corresponding mouse cells are thought to divide every 13 hours.[99,100] Since the active growing phase in scalp follicles can last several years this represents many generations at the transit amplifying stage. Thus, the skin should not only be relatively straightforward to repopulate: it should also not need reseeding very often.

As a practical consideration and on the question of whether periodic reseeding of skin cells would be an acceptable practice and taken up if available, it is worth noting that many people currently pay for (often very painful) chemical or laser "peels" of skin merely for cosmetic anti-ageing purposes.

Reseeding of other tissues

Similar techniques would presumably have to be developed for many other epithelial tissues in order to make WILT a viable therapy. These include the lung, on which stem cell therapy is already an active area of research given its potential to treat diseases such as cystic fibrosis.[101] Though several such tissues may present specific difficulties, we feel that the techniques developed for the three tissues discussed above will probably be sufficiently versatile to be relatively easily adapted to these other tissues.

Frequency of reseeding

The utility of WILT depends on all rapidly-renewing tissues surviving for at least several years with stem cells that lack all telomere extension function. This implies stem cell generation times of at least two months. That may be the natural rate in blood;[102] what about other tissues?

The tissue ostensibly of greatest concern here is the gut. In both DC sufferers and $TERC^{-/-}$ mice, gut phenotypes tend to arise contemporaneously with those of other tissues such as the blood. [In DC there is an important exception—certain sufferers of Hoyeraal-Hreidarsson syndrome, a severe allelic variant of DC,[103] show gut abnormalities before other problems.] Yet, mouse haemopoietic stem cells divide only every few weeks,[102] whereas gut stem cells have been calculated to divide once a day.[104] [This calculation depends on how many stem cells are present per crypt, which remains unclear: crypts are monoclonal,[105] but cells at an early stage of differentiation can return to the stem cell niche,[106] so maybe stem cells can also sometimes divide symmetrically to form two stem cells, as in the haemopoietic system.[107]] If blood and gut stem cell division frequencies differ by an order of magnitude, how can the age at which telomere maintenance deficiency affects those two tissues be comparable? In DC there might be tissue-specific factors—for example, the gut might express more TERC—but no such loophole seems available for $TERC^{-/-}$ mice. Resolution of this paradox is of high priority, as it may reveal aspects of stem cell population dynamics that can be exploited for many therapeutic purposes. For WILT, however, the implication is inescapable that the gut should survive as long between transplants as the blood.

Senescent cell ablation

Telomere elongation-incompetent stem cells, when compromised by over-short telomeres, may not fall obligingly upon their swords: they may need to be actively eliminated. Cultured cells with over-short telomeres remain alive in a distinctive "senescent" state long after losing the ability to divide; similar cells have been observed in vivo.[108,109] They seem to be very rare except in cartilage,[109] but might be deleterious even so;[110] WILT might raise their abundance considerably, with unknown consequences.

Luckily, however, the possibility that even rare senescent cells may do harm in vivo has prompted work on their removal.[111] One strategy being pursued is to incorporate into cells a "suicide" gene that induces apoptosis if the cell adopts a senescent gene expression profile. Vaccination against cell surface markers of the senescent state is also a plausible approach. This work is at an early stage, but the fact that it is already being aggressively attempted gives cause for optimism that it will be perfected within the ten-year minimum timeframe that we foresee for WILT.

Immune memory

Unlike the gut lining and epidermis, the blood comprises not only short-lived cells but also a small minority of long-lived ones. The immune system relies on two types of long term memory, and the possibility and consequences of loss of this memory as a result of WILT must be considered.

One type is the memory of previous infections. While memory cells are undoubtedly good for us, they may be of diminishing importance as we make progress against the many other aspects of aging that make the elderly more susceptible to infection in the first place. The immune system of a young person contains relatively few memory cells, but one would not replace it with that of a centenarian. Additionally, to the extent that it may be desirable to preserve memory cell abundance and function, the same technology proposed here for stem cell renewal may also be applicable to memory cells, which are relatively oligoclonal and could be amplified along with telomere lengthening ex vivo.

The other type of memory is self/non-self discrimination. Here we must bear in mind that lymphocytes cannot participate in an immune response until maturation in either the thymus or the bone marrow. One part of this maturation process is the pre-emption of an autoimmune response, mainly by clonal deletion of cells that could react to self antigens.[112] The memory of which cells to delete is thus not stored in the haemopoietic system itself but in the stromal cells that vet immature lymphocytes. In the thymus, some of these cells are epithelial in origin and are maintained by stem cells,[113] so retention of their self/nonself memory during ex vivo manipulation would be needed. Critically, however, these cells—and also those of other provenance, such as bone marrow-derived dentritic cells at the cortex/medulla boundary within the thymus—do not undergo the genomic reorganisation that occurs in the lymphocytes themselves. Thus, since the cells to be used for reseeding will be autologous, the selective pattern that their descendents in the thymus and bone marrow subsequently impose on maturing lymphocytes will be the same as that imposed by native cells—that is, a faithful self/non-self divide. Autoimmune consequences of WILT thus seem unlikely.

Putting it all together: development and implementation

Developing WILT in mice

Reseeding of rapidly-renewing tissues can be explored not only in normal mice, but also in telomerase-negative ones. Sixth-generation TERC$^{-/-}$ mice have a shortened lifespan as a result of failure of highly proliferative tissues;[66] successful reseeding of such tissues should, in principle, restore these animals' longevity. Similarly, mice with constitutive p53 activity exhibit shortened lifespan due to an accelerated decline of function of various tissues, even though they are markedly protected from cancer;[114] such mice, when made TERC$^{-/-}$ in addition, would be ideal subjects for testing the benefit of reseeding relevant tissues.

WILT in humans: when should it be initiated?

Several issues of clinical judgement would arise when and if WILT became safely and affordably available. The first is whether it would be advisable to use WILT pre-emptively, on individuals who did not yet have cancer. This may not be generally advisable, as the main motivation would be to use high-dose chemotherapy to deplete "native" (telomere-elongation competent) stem cells, and the side-effects of chemotherapy are considerable. However, factors such as a family history of early death from cancer may alter this judgement.

A related issue concerns the frequency of administration of chemotherapy after a patient's first WILT treatment. If the reseeding of all relevant tissues is efficient, it should be possible to raise chemotherapy doses to a point that kills the large majority of "native" stem cells as well as the patient's cancer itself, thus greatly reducing the risk of subsequent cancers. But this will ultimately be limited by the toxicity of chemotherapy to cell types that are not replenished from stem cells, as well as by the degree of chemoresistance of the engineered cells. Thus, especially at advanced ages when new tumours may be appearing more often, it may become appropriate to administer chemotherapy at milder doses but higher frequency.

The question of subsequent fertility must also be considered, since the proposed high doses of chemotherapy might well cause permanent sterility, at least in males. This should be surmountable, however, by a variety of assisted reproduction technologies—not least by the option of introducing engineered germline stem cells in the same way as is proposed here for other constantly renewing tissues. Germ-line manipulations raise unique ethical and safety issues, of course, but these can only be evaluated when what the therapy might offer is clearly delineated.

Conclusion

The idea of eliminating from the body a function known to be essential for survival is a conceptual leap that takes substantial justification even to contemplate, let alone implement. However, here we have examined its ramifications in detail and found that none is so clear-cut as to preclude the possibility that WILT might be feasible. Given (a) the acknowledged inadequacy of present cancer therapies, (b) the persuasive logic that no foreseeable therapy that could be escaped by changes of gene expression will do much better, and (c) the likelihood that progress against other aspects of age-related decline will make cancer a progressively bigger menace,[5] we conclude that serious consideration should be given, even at this early stage, to development and refinement of the many techniques that would be necessary to make WILT work.

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