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Engineering cancer selective virotherapies: are the pieces of the puzzle falling into place?

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Abstract

Advances in gene therapy, synthetic biology, cancer genomics and patient-derived cancer models have expanded the repertoire of strategies for targeting human cancers using viral vectors. Novel capsids, synthetic promoters and therapeutic payloads, are being developed and assessed via approaches such as rational design, pooled library screening and directed evolution. Ultimately, the goal is to generate precision-engineered viruses that target different facets of tumour cell biology, without compromising normal tissue and organ function. Here, we briefly review the opportunities for engineering cancer selectivity into viral vectors at both the cell extrinsic and intrinsic level. Such stringently tumour-targeted vectors can subsequently act as platforms for the delivery of potent therapeutic transgenes, including the exciting prospect of immunotherapeutic payloads. These have the potential to eradicate non-transduced cells through stimulation of systemic anti-cancer immune responses, thereby side-stepping the inherent challenge of achieving gene delivery to the entire cancer cell population. We discuss the importance of using advanced primary human cellular models, such as patient-derived cultures and organoids, to enable rapid screening and triage of novel candidates using disease-relevant human cellular models. We believe this combination of improved delivery and selectivity, through novel capsids and promoters, coupled with more potent choices for the combinations of immunotherapy-based payloads seems capable of finally delivering innovative new gene therapies for oncology. Many pieces of the puzzle of how to build a virus capable of targeting human cancers appear to be falling into place.

Introduction

Despite an array of improved surgical, chemotherapeutic and radiotherapy interventions, options for cancer treatment remain inadequate to meet the needs of all patients. Whilst new molecularly targeted therapies and immunotherapies have been successful in controlling certain tumour types, complete and durable remissions are often restricted to a disappointingly small subset of patients. Hence, cancer remains a leading global cause of death. There is an urgent need to establish innovative novel therapeutic strategies that are effective against tumours with the poorest survival rates**. As a fundamentally genetic disorder, developing gene-based therapies for cancer has long been a seductive notion.**

Thus far, the greatest accomplishments in the gene therapy field have come from the replacement or correction of single mutated genes to restore normal function in monogenic disorders. If the resulting protein operates via paracrine or systemic mechanisms, expression from only a subset of cells is needed to achieve meaningful clinical impact (e.g., a missing enzymatic activity). This can easily be achieved by means of viral-mediated gene transfer, including by vectors based on AAVs and adenoviruses. These have been effective across a range of monogenic disorders with licensed therapies approved for treatment of monogenic disorders including inherited retinal dystrophy (Luxturna™), spinal muscular atrophy (Zolgensma™) and hemophilia (Roctavian™). Nevertheless, the deaths of four patients in the recent Astellas AAV-based gene therapy clinical trial for X linked myotubular myopathy (XLMTM)[1] serves as an unfortunate reminder that improvements in viral selectively are required for dosing within appropriate therapeutic windows.

Similar strategies to correct cancers by delivering wild type tumour suppressor genes to tumours using a gene therapy approach has been the focus of a great deal of research. In 2003, a replication deficient adenovirus expressing wild type p53, Gendicine™, was approved in China for the treatment of cancer (reviewed in[2]). Despite the appeal of this strategy, adopting a similar approach to deliver functional tumour suppressor genes or correct oncogenic driver mutations in cancer is plagued by two perennial issues: firstly, the heterogeneity of genetic disruptions in most aggressive solid tumours leads to plasticity and redundancy in oncogenic signalling pathways. Hence, correction of no single key

mutation would suffice (in most circumstances) to restore normal cellular behaviour. Second is the delivery challenge, where prevention of tumour regrowth would only be accomplished if 100% of tumours cells we corrected. This is an impossible feat for even the most efficient methods of gene transfer. As such, despite decades of interest and exploration, development of gene-based therapies for cancer have lagged significantly behind those for monogenic disorders.

Nevertheless, there is growing excitement and optimism that gene therapies have matured sufficiently to be harnessed in oncology. Success will most likely require the delivery of therapeutic transgenes that target different facets of tumour cell biology, rather than correction of the underlying genetic driver mutations. Tumour-localised expression of therapeutic payloads that re-invigorate the anti-cancer immune response, inhibit angiogenesis, target the immunosuppressive tumour stroma, or convert pro-drugs into their active cytotoxic can target universal biological vulnerabilities of the cancer cells. The remaining challenge is how best to deliver such potent payloads to tumour cells whilst minimally impacting healthy tissue.

The potential of viral-based gene therapy to treat human cancers

Viruses are naturally pathogenic agents responsible for a broad spectrum of diseases and associated clinicopathological symptoms. Yet when harnessed appropriately, they enable highly efficient, targeted delivery of nucleic acids, and hence have been the favoured choice for the delivery of exogenous DNA transgenes to human tissues. The relative ease of genetic manipulation and capacity to be produced to high titers and degrees of purity further add to excitement regarding their potential application in cancer gene therapy.

The range of oncolytic and viral gene therapy vectors being developed in the cancer field is diverse, encompassing both RNA and DNA viruses. In this review, we focus our discussion on examples from adenoviruses and AAV-based vectors. However, the strategies and challenges outlined are applicable to other viral vectors.

Adenoviruses drive robust, transient, high-level expression of transgenes, making them ideally suited to cancer gene therapy applications. Furthermore, recently there has been

renewed interest in oncolytic virotherapy which utilises replication-competent vectors such as conditionally replicating adenoviruses. A partially intact E1-gene enables intratumoural viral replication, resulting in direct oncolysis of transduced tumour cells, as well as in situ amplification of the therapeutic agent[3]. Viral progeny can spread through the tumour, causing further destruction of malignant tissue and release of tumour antigens. This results in a multi-pronged attack on tumours that can synergise with other immunotherapeutic cancer targeting strategies and offers a clear advantage over non-viral means of gene transfer.

By contrast, AAVs are ideally suited to long term gene corrections strategies and form the platform of an ever-expanding number of licensed gene therapy products. In relation to cancer, AAVs offer the prospect of more durable gene expression that might be required for certain tumours to eliminate residual quiescent and dormant cells, since AAVs can transduce both dividing and non-dividing cells and the viral genome persists as an episome. Use of AAVs has therefore been explored in both preclinical and clinical studies using a range of payloads (reviewed in, Santiago-Ortiez et al.[4]) The physical size (~25nm) is smaller than adenovirus and may facilitate better biodistribution in certain tissues and there is evidence of good transduction from the natural serotypes across a range of cancer types, most likely due to the roles of heparan sulphate, integrins, proteoglycans and RTK as receptors/co-receptors – which will be elevated in many solid tumours. However, a potential limitation of AAVs application in oncology is the potential limitations of rapid loss from dividing cancer cells – and so the proliferative index of the tumour type of interest might dictate the best choice of vector. For example, in glioblastomas a significant proportion of the tumour is non-cycling and quiescent/dormant posing a challenge to transient adenovirus[5], while AAV would likely persist longer facilitating clearance of residual disease after chemo/radio therapy. The widespread delivery and persistence of AAV can of course pose risks if the anti-cancer payloads are detrimental to normal tissue; hence, ensuring limited off target expression or transduction of normal surrounding tissues is especially important for AAV compared to adenovirus.

A pre-requisite for any successful cancer therapy is the ability to target cancer cells selectively whilst sparing the body from local or systemic toxicities arising from off-tumour effects.

Below, we discuss progress and challenges in the approaches that can address this urgent need to restrict the activity of virotherapies to the malignant cell compartment. These encompass both cell extrinsic and intrinsic engineering opportunities (overviewed in Figure 1). We focus on how optimisation of delivery route, capsid design and promoter/enhancer regulation of viral gene expression can generate precision targeted vectors for cancer virotherapy. These can subsequently be armed with potent therapeutic payloads to ensure maximum efficacy with minimal off-target damage.

Cell extrinsic options for gaining selectivity

Extrinsic targeting strategies enable selectivity to be imposed before the virus has even entered the cell. The aim is to generate genuinely tumour-tropic viruses that exclusively infect tumour cells, thus avoiding sequestration in normal tissues. This reduces the required dose and risk of tissue toxicity, whilst simultaneously increasing the amount of payload that 'hits' the tumour. Directing viral vectors to tumour cells has been explored by several approaches, including optimising the route of delivery, choice of virus, and altering the array of cell entry receptors through capsid protein modifications (Figure 1).

Route of delivery: local or systemic?

Some level of control can be achieved through astute selection of the route of delivery of the virotherapy into the patient. The route of administration has a direct bearing on the range and order of tissues that will be encountered and is therefore an important determinant of vector tropism. Systemic delivery, via the bloodstream following intravascular administration necessitates trafficking of the vector to the tumour. Local delivery encompasses both direct intra-tumoral delivery and various site-specific injections that provide the vector near lesions at different anatomical locales (e.g., intraperitoneal injections for tumours localised within the abdominal cavity, intrathecal injection for tumours of the CNS).

Intra-tumoral injections are presently the most commonly relied upon approach for administering virotherapies to patients[6]. This is unsurprising given the stringent control over the exact dose reaching the tumour and more favourable safety profile resulting from restricting delivery to the site of need. Local delivery further avoids exposure to blood plasma proteins, including pre-existing neutralising antibodies[7]. For some viral serotypes, such as the heavily relied upon adenovirus 5 virus, seroprevalence rates in specific geographical cohorts can be extremely high (up to 80-90%)[8], potentially limiting the widespread effectiveness of systemically delivered therapeutics based on these viral backbones in specific populations. Other plasma factors, can also be problematic; the clotting factor FX, for example, can promote rapid and efficient liver sequestration of intravenously delivered Ad5-based therapeutics through cross-linking to HSPGs on hepatocytes[9,10].

Despite the advantages of local delivery, the ability to effectively administer virotherapies via intravenous injection remains a long-standing goal. Systemic delivery of virotherapies affords the opportunity of transducing the greatest number of tumour cells as intravenously injected viral vectors will travel through the bloodstream and can therefore potentially engage with all tumour cell populations, including disseminated metastatic or micro-metastatic lesions which may not be detectable via conventional imaging methods. At a practical level, intravenous injection is facile, subverting the need for the complex surgical procedures that are required to access difficult-to-reach tumours for direct injection. Furthermore, easily accessible tumours in non-critical organs are unlikely to benefit from intratumorally delivered virotherapies, since these are more amenable to conventional surgical resection – the preferred therapeutic stratagem. However, treating local tumours with oncolytic virotherapies may initiate immune priming and systemic abscopal effects[11]. Overcoming the systemic delivery challenge and identifying viral vectors that preferentially transduce tumour cells is therefore of the utmost importance; this will make trials easier and safer as well as increasing the opportunity to eradicate all tumour cells

There have been few studies investigating the clinical efficacy of intravenously delivered viral cancer therapy vectors[12]. Those that have completed have typically failed to

demonstrate clear improvements in primary endpoints, likely reflecting the significant challenges posed by immune-clearance and off-target sequestration. Nevertheless, these challenges are not insurmountable, and improvements may be made through modification of the vector capsid surface to evade immune recognition and increase the selectivity over target-cell transduction. With the tools of modern synthetic biology, it has been possible to develop strategies that facilitate the development of viruses with genetically and chemically modified capsid proteins. Successes in rodent models, discussed below, point to the potential for highly tumour-selective viruses to be engineered for human applications.

Capsid engineering: capitalising upon natural viral variation

Human adenoviruses comprise a diverse phylogenetic tree containing 57 canonical serotypes. This diversification arises from natural selection pressures, resulting in viruses that differ in their tropism, cytotoxic capacity and cellular infection kinetics[13]. Adenoviruses are classically divided across 7 different human adenoviral species from A-G; these engage diverse cell entry receptors, ranging from the tight junction localised coxsackie and adenovirus receptor (CAR) for Species A, C, E, F and (to a lesser extent) D, to CD46 and DSG-2 for Species B viruses and sialic acid for certain members of Species D[14,15]. This variation can be capitalised upon for improved vector design by enabling the selection of serotypes that possess more favourable properties in the context of anti-cancer therapeutics.

Despite its prevalent use as a gene therapy vector, the species C adenovirus type 5 (HAdV-C5) has a broad tropism resulting from its reliance on CAR as a primary entry receptor, expression of which has further been shown to be downregulated on numerous cancer sub-types. The ability of Ad5 hexon to bind FX protein and traffic to the liver, coupled with high seroprevalence rates in certain populations make the investigation of alternative serotypes with lower seroprevalence rates and unstudied tropisms worthwhile for virotherapy applications. In this regard, species D adenoviruses (the largest human adenoviral species) represent a relatively untapped pool of potential backbones with far lower seroprevalence rates[10,16] which do not bind FX in the blood[10]. HAdV-D10 has recently been shown to form weak interactions with known adenoviral receptors, therefore providing a compelling starting point for engineering more cell type selective infection and

lysis of cells (*in vitro* and *in vivo*)[17]. HAdV-D 26, 28, 45 and 48 infect and replicate in several cancer cell lines, although cytotoxicity was lower than adenovirus 5-based vectors[18]. Moreover, a replication-competent species B Ad11-derived vector was demonstrated to be capable of infecting and inducing oncolysis in colon cancer cell lines which expressed high levels of the CD46 receptor[19]. These studies highlight the feasibility of generating virotherapies from non-canonical human adenoviral serotypes.

Efficient cellular transduction, potent cytotoxicity and wealth of knowledge surrounding the use of HAdV-C5 remain attractive features of this vector, hence it remains a leading contender in cancer virotherapy development. To take advantage of these desirable traits whilst simultaneously adjusting the cellular tropism to one more suitable for cancer targeting, a heavily adopted approach has been to generate pseudotyped viral vectors. This involves the replacement of either the C-terminal fiber knob domain or the entire fiber protein with that from another adenoviral serotype[20]. This radically changes the receptors that can be engaged for cellular transduction[21]. For example, pseudotyping the fiber knob of adenovirus 5 for that of the species B adenovirus 3 alters primary receptor usage to render cellular infection CAR-independent, with the resulting Ad5/3 pseudotype virus displaying enhanced transduction and oncolysis of ovarian carcinoma cell lines *in vitro* and in immunocompromised murine xenograft models[22,23]; this was due to cell entry through Desmoglein-2 (DSG-2), the primary receptor for the HAdV-B3 virus[24]. Pseudotyping the CD46-binding HAdV-B35 fiber has also proved particularly promising, enabling enhanced gene delivery to human smooth muscle, CD34+ haematopoietic cells, gliomas and various other tumour cell lines[21,25,26,27]. The length of the adenovirus fiber has been shown to be important in impacting interaction with cell surface receptors and subsequent αv-integrin mediated virus internalization[28]. Care must therefore be taken to consider the impact of any alteration in fiber length when exchanging the entirety of this capsid protein. Nevertheless, the successful generation of several HAdV-C5 fiber pseudotyped viruses demonstrates the feasibility of this approach.

For AAV, there are around 13 different serotypes that are widely available, each with distinct capsid proteins that underpin their distinct tropisms. Natural serotypes have strikingly different cell type selectivity, for example, when comparing these in parallel in

the primate central nervous system (CNS)[29]. The reader is directed to Samulski et al., for a comprehensive review of AAV biology, the history of its discovery and its importance in gene therapy[30]. It is relatively straightforward to engineer altered capsids (encoded by the polycistronic viral *cap* gene) and produce recombinant AAV (rAAV) that can be tested with distinct genes of interest flanked by the common AAV-2 ITRs. The AAV capsid is responsible for the binding to various proteoglycans and both primary and secondary receptors. With improved knowledge of the key biochemical structural features required for receptor interactions, opportunities have opened for rational capsid engineering, often focussing on a specific region of the VP2 loop[31].

Capsid modifications

A major limitation of relying on the natural viral variation to achieve cell extrinsic selectivity is that the array of receptors available is restricted to those naturally encountered and selected for during viral evolution. Rational design may be a way to expand viral tropism usefully to enable targeting of transformed cells. As such, the extensive body of knowledge surrounding viral capsid structure and interaction with host receptors can be leveraged to generate precise tropism-modifying alterations that improve the tumour selectivity of vectors (overviewed in Figure 2). This approach has great potential to yield highly selective novel anti-cancer virotherapies; not only can it aid in the identification of mutations that ablate interactions with broadly expressed native receptors, but it can also reveal permissive sites for the insertion of new targeting moieties that redirect viral tropism towards cancer cell populations of interest. An example of this is the insertion of peptides that support binding to ECM or their receptors that are enriched in solid tumours, such as tenascin, LeX, or RGD peptides[32]. This combination of viral de- and re-targeting is a pre-requisite for the development of precision-targeted virotherapies that traffic exclusively to tumour cells, especially following systemic administration.

Attempts have been made to non-genetically alter viral tropism such as through chemical coupling of capsid proteins to polymers such as polyethylene glycol (PEG)[33], and poly-[N-(2-hydroxypropyl)methacrylamide] (pHPMA)[34]. These decorate the viral surface in a way that abrogates interaction with native receptors as well as plasma proteins, potentially enabling evasion of immune-mediated clearance by pre-existing anti-viral antibodies.

Targeting towards specific cellular subpopulations is subsequently achievable through coupling of the capsid to multivalent polymers, antibodies, peptides or other high affinity moieties that bind the receptor of interest[35,36]. Other non-genetic targeting strategies utilize bi-specific adaptors such as Fab-based fragments, scFv diabodies[37], bi-specific DARPins[38], and single-chain diabodies to simultaneously engage capsid proteins and cancer-associated receptors via opposite poles of the molecule[39]. These non-genetic strategies have the limitation of an increased complexity associated with the manufacturing, quality controls and delivery of such multi-component systems. Furthermore, this retargeting strategy lacks heritability of tropism, which is of concern for replication-competent viral vectors where non-targeted daughter virions produced *in situ* could mediate off-target effects. As such, genetic modification of adenoviral tropism is a more desirable strategy.

Noteworthy for adenovirus has been the application of rational design to generate genetically targeted vectors that lack affinity for the ubiquitously expressed CAR receptor[40]. Binding can be abolished through two amino acid substitutions (S408E, P409A; referred to as KO1 mutation) in the HAdV-C5 fiber knob AB loop[41], or a Y477A substitution and TAYT deletion in the FG loop[42]. These have been widely incorporated into cancer-targeted adenoviral-based therapy vectors to prevent CAR-dependent cellular transduction in off-target healthy bystander tissues. Despite these tropism alterations, Alemany et al., showed that liver sequestration remains an issue[43]. Further modifications within the remaining two major capsid proteins – the hexon and penton – eliminate the residual interactions with FX and αvβ3/5 integrins respectively[44]. These encompassed modification of the hexon hypervariable region 7 and an RGD to RGE mutation in the penton base. The resulting vector, $Ad5_{NULL}$ is totally devoid of native means of uptake. Such vectors provide invaluable 'blank canvases' on which further re-targeting modifications may be superimposed to generate precision-targeted virotherapies that are exquisitely tuned to cancer cells transduction.

The site of insertion of targeting moieties must be carefully considered to avoid disruption of viral packaging and nuclear trafficking, whilst facilitating presentation to tumour-associated receptors of interest. Several capsid locales have been evaluated, including the

hypervariable loops of the hexon[45,46], fiber[47], and minor structural protein pIX[48]. Whilst the high copy number of the hexon and pIX proteins (720 and 240 copies per virions respectively) makes these ostensibly appealing sites. However, a general concern is always that artificially increasing the strength of receptor interactions may compromise the downstream release from endosomes following internalisation.

By far the most common site for rational insertion of targeting scaffolds has been the knob domain of the adenovirus fiber[47]. The surface exposed HI loop of the HAdV-C5 fiber-knob has been shown to be particularly tolerant of heterologous insertions, with peptides in excess of 100 amino acids being incorporated without detriment to viral fitness[49,50]. Homology modelling has revealed the DG loop of the Species D adenoviruses 10 and 48 to be similarly permissive to insert engineering[17,51,52]. The C-terminus of the HAdV-C5 knob domain has also previously been shown to enable virus re-targeting without compromising capsid assembly[53], whilst another approach has been to remove the fiber-knob entirely and replace this with domains that facilitate incorporation of larger targeting molecules without structural clashing such as the T4 bacteriophage-derived fibritin protein[54].

An extensive range of scaffold proteins with variable affinities have been rationally engineered into the adenoviral capsid structure to facilitate retargeting. These range from peptides, and scFvs, to TCRs, DARPins and nanobodies. Of note are RGD-motif containing peptides that facilitate viral retargeting to integrins. Integrins are frequently over-expressed on aggressive carcinomas whilst displaying relatively limited presence on healthy human tissues and this interaction could therefore be harnessed to achieve highly specific cancer targeting. Incorporation of the FMDV2 derived, αvβ6 integrin targeted A20 peptide into the HI loop of the fiber knob of the $Ad5_{NULL}$ platform has been shown to facilitate tumour-restricted transduction and transgene expression immunocompromised mouse xenograft model[44,55]. Similarly, DARPins for cancer overexpressed receptors, such as HER2/neu have also been demonstrated to facilitate cancer specific delivery for AAV[56].

Unbiased screening with directed evolution

A complementary approach to rational engineering discussed above is to use directed evolution strategies that do not require any a priori knowledge of the underlying receptor-

ligand interaction – although often do focus on specific structural regions. Highly diverse initial libraries are subjected to iterative rounds of *in vitro* or *in vivo* screening for transduction of the cancer cells of interest with normal cell controls, to select for those virions that have improved transduction profiles and/or infection kinetics (Figure 2).

The starting viral pool may be composed as a mixture of naturally occurring viral serotypes which are encouraged to undergo recombination during serial passaging, producing chimeric viruses optimised for target cell killing. Proof of principle of this was demonstrated by Kuhn et al, in a study in which culture of a mixture of adenoviral serotypes from across species B-F with the colon cancer cell line HT-29 led to isolation of an Ad3/11p chimeric virus termed ColoAd1[57]. This was demonstrated to have increased lytic capacity and selectivity for transduction of colon cancers when compared to the parental serotypes. These approaches, conducted in cell culture *in vitro* tend select for subtle advantageous recombinants with alterations in early proteins enhancing the intrinsic properties– i.e., for alterations that enhance viral replication in and killing of tumour cells, rather than extrinsic tumour selectivity *per se*. Potentially, the most promising approach may combine rational design with the high-throughput screening afforded by directed evolution.

By inserting large libraries of diverse protein or peptide domains (e.g. DARPins or scFvs) it is possible to screen iterative rounds of selection, recovery, with mutagenesis by synthesis of random variants for further diversification. By starting with a pool of initial viruses whose design was informed by pre-existing knowledge of structural biology and using protein sequences with anticipated activity, the likelihood of identifying a lead candidate vector for the desired purpose at the end of this process is greatly increased. This semi-rational approach has been adopted within the AAV field[58]. However, increased complexities surrounding the generation of in-context adenoviral display libraries mean this approach is yet to produce significant breakthroughs for these vectors[59].

For AAV, an example of the power of screening random peptides to yield capsids with improved binding properties has been achieved in the nervous system, reviewed by Challis et al[60]. Using elegant genetic screening in mice, Deverman et al were able to identify the PHPeB capsids, which displayed remarkable tropism for the adult CNS, crossing the blood-

brain barrier[61,62]. Although these have not proven effective in primate studies, due to mouse strain specific molecular mechanism (SCA-1 binding) underlying the activity[63]. These studies nonetheless show the power of using sophisticated genetic screening strategies to recover desirable capsids.

Other innovative approaches for delivery, such as exosomes, nanoparticles or synthetic cells may emerge in coming decades. However, at present these are unlikely to supersede engineered viruses. Discussion of these non-viral methods for delivery are beyond scope of this review. For the moment evolution has resulted in very efficient gene delivery vehicles for human cells in the form of viruses; tapping into their vast potential for engineering selectivity remains a priority for the field.

Cell intrinsic options for gaining selectivity: tumour-selective replication and cell type selective promoters

Following internalisation, additional control can be exerted by regulating expression of payloads and/or critical viral replicative genes (for adenovirus) using promoters and enhancers that restrict their transcription to the malignant cellular compartment. Arguably, the selectivity which can be achieved through engineering of promoter/enhancers may far outstrip that which could ever be achieved through capsid engineering, since capsids are likely constrained by limitations inherent in the biology and biochemistry of available interactions. By contrast, modular regulatory elements and 'tuning' of expression for specific cell types and states can be spectacularly selective as there is a combinatorial logic in the transcription factor (TF) 'code' that underpins the identity of many thousands of diverse cell types and states that make up human tissues.

Historically, ubiquitous viral promoters have been favoured for cancer gene therapies. These provide constitutive, high-level expression of the transgene. However, there are two broad choices that might give desirable selectivity by ensuring transcription in only tumour cells: 1) use of a tissue/developmental specific elements that are reactivated or highjacked in the tumour cells but not normally present in adult tissues (e.g., alphafetal protein for hepatocellular carcinoma[64] or carcinoembryonic antigen (CEA) for colon cancer[65]); 2) promoters/enhancers which are the endpoints in classic oncogenic signalling pathways

(e.g. E2F) (reviewed in, Nettlebeck et al[66]), cancer-specific mutations (e.g. telomerase, TERT promoter[67]) or hypoxic elements[68]. Other examples are oncogenic pathways including the use of the oncogene associated promoters. These genes and hence their associated promoters would display higher activity in cancer cells than surrounding tissues, providing therapeutic window.

These early studies focussed on proximal promoter regions; yet, for many cell type-specific genes it is the long range cis-regulatory elements (enhancers) that provide the opportunity for selectivity[69]. Since ~2000, there has been an explosion of technologies and methods that now enable rapid identification of candidate enhancers (cell type specific) through mapping of peaks for key transcription factors using ChIP-seq, enhancer chromatin markers such as H3K27 acetylation, or chromatin accessibility (ATAC-seq). This has resulted in thousands, or tens of thousands, of candidate cell type specific regulatory elements might be useful in adenoviral or AAV to provide selective expression. Moreover, advances in single cell profiling to map enhancers – both RNA-seq, DNA methylation and chromatin accessibility – means we will soon have a comprehensive atlas of diverse human cell types and cell states including tumour cells. Functional annotation of these regulatory elements alongside computational tools and machine learning strategies to identify the 'rules' underlying their activity should transform our ability to rapid garner optimal synthetic promoters for gene therapy. As we learn more about the key TFs that define cell type identity, their specific motifs and the grammar that leads to their selective activity[70], these can be readily synthesized and screened (as they are often short sequences) for specific activity. Quicker, cheaper and scalable methods to read, write, synthesize and assemble DNA underpins the relatively new field of synthetic biology (e.g., automation, golden gate, Gibson assembly, massively parallel DNA synthesis). Novel synthetic promoter and enhancers are therefore increasingly very easy to design, build and test.

Screening approaches for synthetic promoters and enhancers follow a similar strategy to capsids and involves similar steps: building complex libraries of DNA parts, designing strategies to identify the 'winners', and recovery of those regulatory sequences with desired activity using appropriate human cells or animal models. An example of what is possible is the screening in mice for 230 different enhancer sequences in AAV, which

uncovered many novel elements that are specific for neuronal and glial cell subtypes. Furthermore, Mich et al., show an elegant *in vivo* screening strategy searching for neuronal subtype enhancers revealed by scATAC-seq and identify those which can operate in AAV across species[71,72]. Single cell profiling technologies therefore not only provide the input set of candidate enhancers, but also can be used to functionally annotate and rank these within their appropriate rAAV context[73].

Natural enhancers may have the wrong size, selectivity, or strength to be useful in gene therapy applications. For fully synthetic enhancers, most efforts initially have focused on transcription factor binding motifs (5-10bp) (TFBMs) and concatemerisation of these to gain stronger activity[74]. This approach has been used to search for glioblastoma promoters using screening of libraries[75]. However, this assumes that the key TFBMs are known and ignores the need for the appropriate 'grammar' which will undermine either activity (strength) or selectivity. Future trends will likely combine use of natural elements screened at scale that can be minimised (to retain the key natural grammar of motifs) but combined in ways to rationally designed cell state or type expression.

Cancer selective payloads

Whilst replication-competent adenoviral-based therapies have some intrinsic anti-cancer activity through their oncolytic capacity, potency can be greatly potentiated by the incorporation of additional transgenes that confer novel therapeutic functionalities – effectively enabling them to act as Trojan horses that deliver anti-cancer payloads to the tumour cells. Whilst an array of different transgenes have been explored within the context of cancer virotherapy vectors (overviewed in Figure 3), these are united by the fact that their anti-cancer effects can extend beyond the cell from which they are expressed to impact upon neighbouring untraduced cells. This "bystander effect" is critical for ensuring clearance of the entire tumour rather as opposed to exclusively the transduced cell subset. The ability to induce an immunological memory response is further desirable since this may enable protection against recurrence. How close are we to achieving this? Certainly, great steps towards achieving this 'holy grail' have been enabled by the emergence of immunotherapeutic payloads; with the successes of checkpoint inhibitors for melanomas and CAR-T approaches for leukaemia, the immunotherapy field has blossomed and is

stimulating new discoveries and likely a wealth of candidate payloads that might be suitable for local delivery.

Arming with immune-stimulating anti-cancer transgenes

Many solid tumours develop, via immunoediting, to acquire mechanisms that are immunosuppressive – most obviously, through down regulation or deletion of MHC or associated antigen presenting apparatus, as well as evolving a myelosuppressive microenvironment involving increasing concentrations of immunosuppressive immune cells such as regulatory T cells (Tregs), tumour associated M2 polarized macrophage and likely multiple other pathways. Hence, educating or unleashing the immune system through local tumour specific immune modulators is an appealing strategy. The successes of immunotherapy for certain cancers and massive interest and investment in this area means there is likely to be a wealth of new candidate payloads[76]. Furthermore, encoding such immune modulators within the framework of a cancer selective viruses bypasses some of the issues associated with their systemic application such as immune system hyperactivation, which in the worst cases can lead to systemic cytokine storms and ultimately death. Restricting the activated immune response to the tumour and its local microenvironment is one of the appealing prospects for viral gene therapies.

Proof of principle of the ability of immune transgenes to provide therapeutic efficacy when encoded as part of cancer virotherapies comes from the first oncolytic virus to be approved in the US and Europe in the form of Talimogene laherparepvec (Imlygic™). This is an HSV-1 based virotherapy that incorporates a transgene encoding GM-CSF to promote the proliferation and differentiation of the bone marrow myeloid precursor cells that give rises to important effector cells of the immune system. Additional examples of immune-activating transgenes that have been encoded within the viral genome include checkpoint inhibitors, cytokines, chemokines, and bi- or tri-specific NK and T-cell engagers[3,76]. Typically, the latter of these possess two scFv domains that enable them to engage with tumour-associated antigens at one end and the relevant immune cell subset at the other, thus bringing the two into proximity for immune-cell activation and subsequent tumour clearance. IL-12 has been explored as a potential anti-tumour cytokine, with extremely promising preclinical studies that were subsequently undermined by toxicities when

systemically delivered in clinical trials[77]. However, use of IL-12 in a more restricted manner with viral delivery has shown some signs of success in glioblastoma[78].

Finally, viruses with oncolytic capacity, such as adenovirus, have the potential to provide particularly powerful synergies with immune-stimulating anti-cancer transgenes, since the immunogenic cell death that follows vector self-amplification and subsequent cell lysis releases DAMPS and tumour cell antigens that lead to recruitment and activation of immune effector cells that potentiate the effects of any transgenes[79]. Oncolytic activity in adenoviral constructs is commonly conferred by subtle changes in viral early genes that enable replication to proceed in tumour cells, yet attenuated in non-transformed cells. One such modification is the deletion of the adenoviral E1B protein, and this modification is the basis of Oncorine™ which has been licensed in China since 2005 for the treatment of nasopharyngeal carcinoma and head and neck cancers (reviewed in[80]).

The importance of patient-derived tumour models

Having designed precision-engineered viral vectors and armed them with therapeutic payloads for efficacy, screening and testing in appropriate models must be performed to identify the most promising candidates for clinical translation. A key concern of any screen or cell line/rodent-based studies for a human therapeutic agent, is that the experimental model will reveal highly selective promoters or capsids, but these only operate in that specific model due to its unusual biology – and may have limited utility across species. The advent of primary human cancer models provides a timely experimental advance that can help address these limitations[81]. These patient-derived cancer models can provide improved *in vivo* models when orthotopically transplanted into mouse tissues (immunocompromised mice). These xenografts have more realistic histology and virus activity can therefore be explored via different delivery routes.

Patient-derived monolayers or organoids enable regulatory elements and viral vectors to be tested in the most relevant human disease context. 3D organoids that have the heterogeneity, hypoxic microenvironments and diversity of differentiated states seen in primary tumours make them a more realistic model for screening and head-to-head comparisons can be made with normal non-transformed tissue control organoids[82]. Solid

tumours including patient-derived models are now widely available and often are shared to the community and industry[83].

There are of course caveats. Patient-derived cultures lack vasculature and immune cells; they are often not easy to scale up and engineer genetically. Furthermore, they may lack other features of the tumour biology such as appropriate extracellular matrices or structural features/mechanical forces. With long term passage there is the risk of genetic drift. However, as a complementary approach to murine studies, human primary cell cultures, organoids and slice cultures should always be part of the triage process for identifying optimal engineered viruses for gene therapy.

This ability to move back and forth between *in vitro* and *in vivo*, with normal matched tissue stem cell controls, should be harnessed in all discovery research to optimise and triage candidate lead products before entering the clinic. A major limitation, however, is the difficulties of modelling human immune interactions with the organoid derived tumours. While strategies have been developed, these are often technically challenging, artificial, and costly; co-cultures of organoids with vascular cells and/or human immune cells may be more tractable.

Concluding remarks

In conclusion, viral-mediated therapeutic gene delivery could achieve cancer selective killing or cytostatic effects if the virus preferentially transduced the cancer cells, only replicated within the cancer cells, or delivers payloads that are only expressed or active within the cancer cells (or a combination of these features). Over the past two decades proof of concept for each of these different facets has been obtained and we now are armed with a wealth of new technologies and experimental models to develop these innovative therapeutics. Arguably, the vectors with greatest chance of success in the clinic will combine desirable features, from both cell-extrinsic and intrinsic selectivity mechanisms, to generate advanced virotherapies that specifically seek out tumour cells, and only in these unleash their full potency through oncolysis and/or therapeutic transgene expression. The parts of the puzzle of how to build an exquisitely selective

virotherapy are therefore emerging. This will surely ultimately guide us towards advanced and effective new gene based therapies for cancer patients.

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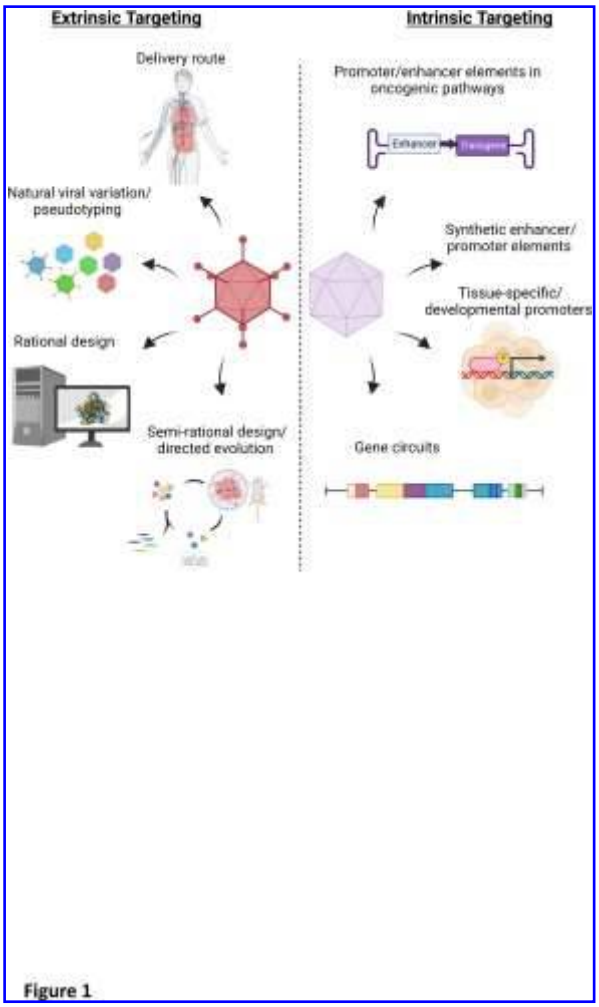

Figure 1: Summary of extrinsic and intrinsic strategies for engineering cancer selective virotherapies. Extrinsic methods may encompass combinations of route of delivery, pseudotyping approaches, as well as knowledge guided rational or semi rational vector engineering approaches. Intrinsic selectivity may be achieved through combinations of tissue or tumour specific enhancer or promoter sequences, gene circuits, or alterations in early viral genes that enable selective viral replication within tumour cells.

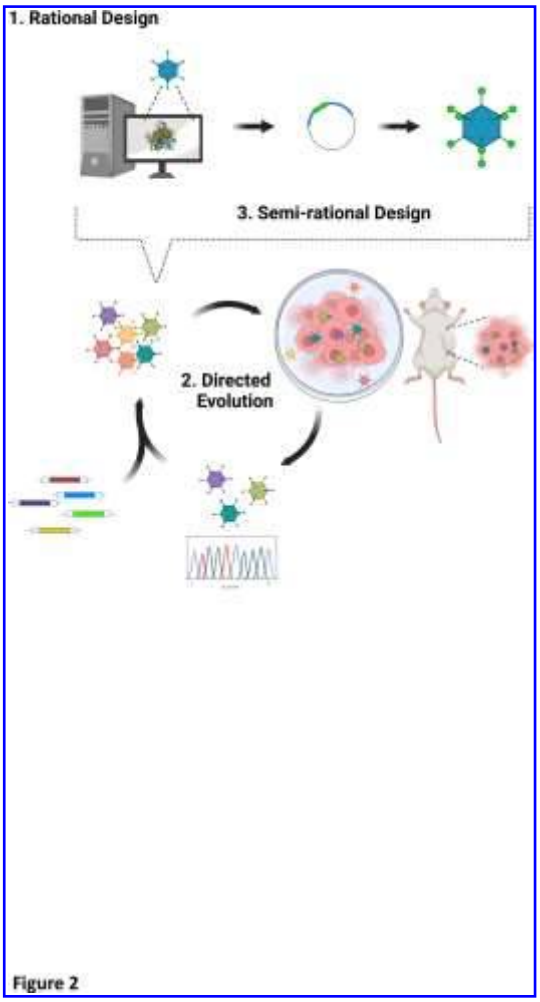

Figure 2: Strategies for the development of genetically re-targeted cancer virotherapy vectors. Knowledge of capsid protein structure can be harnessed to aid in the rational design of virotherapy vectors with novel binding interactions (1). Alternatively, a structure-free directed evolution approach may be employed, in which initial diverse pools of potential viral vectors are subjected to selection on cell populations of interest, either in vivo or in vitro. Virions recovered from this can be sequenced and further diversified (e.g. by error-prone PCR) followed by use as the input for subsequent rounds of selection. After multiple rounds, viruses with improved replication and infection kinetics in the cell line of interest can be isolated (2). These two approaches can also be combined in a powerful semi-rational design approach. Here, structural knowledge can guide the insertion of random targeting molecule libraries into permissive regions of the viral capsid. Insertions that improve cancer selective targeting can be selected for by subjecting the resulting viral pool to high throughput directed evolution screening (3).

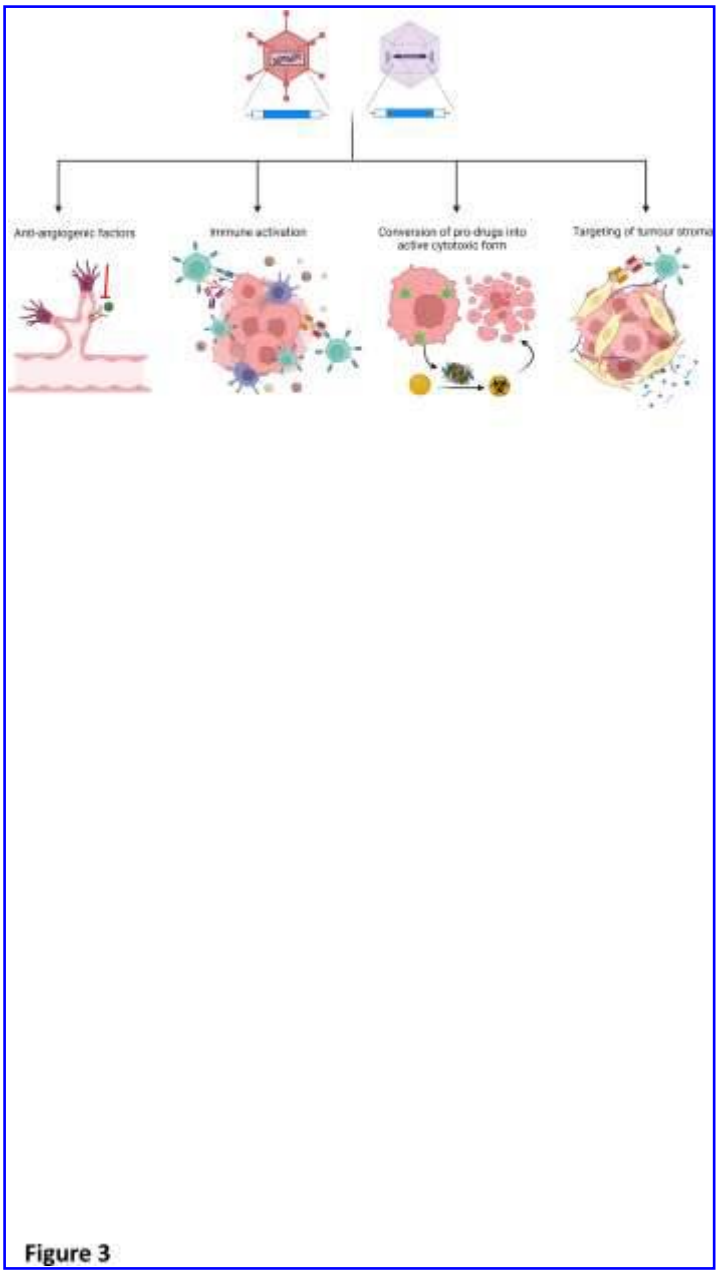

Figure 3: Arming cancer virotherapies with therapeutic payloads. Cancer virotherapy vectors can mediate efficient delivery of therapeutic transgenes that target multiple different aspects of tumour cell biology. This includes factors that inhibit angiogenesis, re-invigorate the anti-cancer immune response, promote tumour cell lysis through localised activation of cytotoxic drugs and target the dense immunosuppressive stroma.