Targeting tumors with non-viral gene delivery systems

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Targeting therapeutic genes to tumors is an attractive concept in curing malignant diseases. Systemic gene delivery systems are needed for therapeutic applications in which the target cells are not directly accessible, and which can only be reached via the systemic route. Recent developments in the field of non-viral gene delivery have shown that, based on (poly)cationic carrier molecules, DNA can be efficiently targeted to tumors via the bloodstream. Tailor-made synthetic vectors can be used to achieve predominant gene expression in tumor tissue. Therapeutic concepts based, for example, on suicide genes or cytokines, showed encouraging results in preclinical and also in first clinical evaluations.

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▼ Cancer is responsible for >20% of all deaths and will soon overtake heart disease as the main killer in the Western world. Surgery is often the first course of action, but when the disease has already evolved and metastases are spread within the body, then systemic treatment with anticancer drugs is inevitable. The classical treatment of malignant diseases – radiation therapy, chemotherapy and combinations thereof – often have to face tumors that are already resistant to these kinds of treatment. Thus the development of new strategies for the cure of malignant diseases is an evolving field of research, with gene therapeutic approaches being a promising area. Almost two-thirds of all gene therapy trials undertaken, to date, have been for cancer, mostly based on genetically modified viruses to carry the gene of interest [1].

The treatment of cancer using gene therapies can be particularly advantageous in that a relatively short expression of therapeutically active proteins might be sufficient to eradicate tumors. In contrast, gene therapies for other genetic diseases (in which genes have to be replaced or added, such as in cystic fibrosis) can be confronted with additional needs, such as long-term gene expression and regulation of gene expression. Nevertheless, drawbacks such as the bloodstream's rapid clearance of virus-based gene transfer systems urges the development of new, synthetic gene delivery vectors, enabling the transport of foreign DNA from the injection site via the bloodstream to distant tumors or metastases thereof. Several cancer gene therapy approaches aim at the introduction of genes directly into the tumor tissue. Genes might be used that either kill the modified tumor cell directly (toxin genes) or indirectly (suicide genes), or block the cell cycle or induce apoptosis. Alternatively, genes that inhibit tumor cell migration and metastasis or block tumor neoangiogenesis could be used. A major challenge for non-viral gene delivery systems is the efficient targeting of these genes to the tumor tissue. Several tumor types are amenable to direct injection (e.g. melanoma or head and neck cancer) or other types of local application (e.g. peritoneal injection for bladder and ovarian cancers).

Nevertheless, metastatic tumor nodules, for example, can only be reached by administering therapeutic genes into the bloodstream and targeting the specific site. Systemic targeting of tumors via the bloodstream can be challenging: the delivery vectors have to survive the journey through the bloodstream without being degraded or captured by cellular defense mechanisms. Once at the tumor site, they have to extravasate into the tissue and bind specifically to the target cells. After their cellular internalization, intracellular barriers (release of the vector into the cytoplasm and then access to the nucleus to reach the cellular transcription machinery) present demanding tasks, in which each of the listed steps can be a major bottleneck for the efficiency of such a gene delivery system. In this review, the use of particle-based gene delivery

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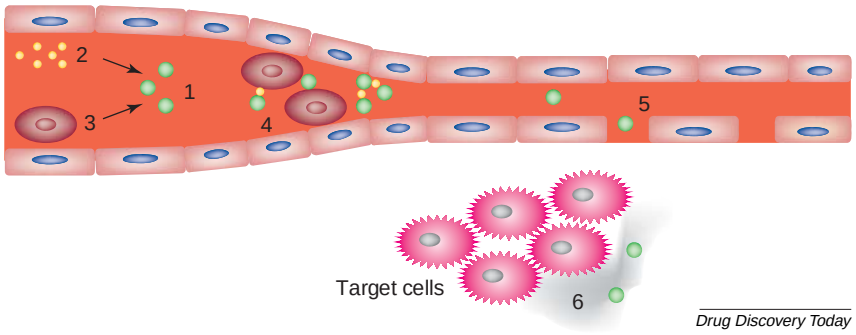

Figure 1. Barriers for non-viral gene delivery *in vivo*: gene delivery vehicles **(1)** can interact with plasma proteins **(2)** or blood cells **(3)**, and the resulting aggregates **(4)** can clog capillaries. Extravasation into the surrounding tissue can be possible via leaky vasculature or incomplete blood vessels **(5)**, although tumor access can be still limited by the extracellular matrix **(6)**.

systems, especially those based on polycationic carrier molecules for the delivery of therapeutic genes to tumors after systemic delivery, will be discussed.

Tumor architecture

A prerequisite for tumor growth is a constant supply of nutrients. Metastases with a diameter >2 mm are reliant on blood supply and, therefore, induce the formation of new blood vessels. Blood vessels inside a tumor are often incomplete, with small capillaries ending bluntly inside the tissue. Furthermore, different areas within a tumor have to be distinguished in terms of accessibility and nutrient supply [2]. The outer core is usually well vascularized and easily accessible via the bloodstream. Adjacent to the inner core are the semi-necrotic and necrotic regions. Here, tumor cells are not sufficiently supplied with nutrients and oxygen, and become necrotic. The area of interest for a therapeutic gene is usually the outer region, where the cells are actively dividing. Besides the tumor cells, the newly developed tumor vasculature can be targeted by gene therapies to cut off the tumor's blood supply [3]. Direct killing of the tumor endothelium with suicide genes or cytokine secretion can help to eradicate malignancies.

All of these possibilities encourage the development of molecular medicines that can be delivered systemically via the bloodstream. The tumor matrix, consisting of collagen and other proteins, is a further barrier to gene delivery as the target cannot be easily reached because of limited diffusion of the vector within the tissue [4]. Depending on the type of tumor, the influence of the extracellular matrix on mobility can vary significantly.

Interaction(s) with blood and blood components

Blood can be a harsh environment for drugs injected into it and it acts as an important barrier against infection. Usually, plasmid DNA is used for non-viral gene therapy approaches: after its injection into the bloodstream, nucleases can rapidly degrade this 'naked' DNA within seconds [5]. Also, the extended structure of the molecule (e.g. a plasmid of several kilobases has a dimension of micrometer scale) will cause the molecule to arrive in the first capillary bed encountered (e.g. the lung capillary bed after intravenous injection into the tail vein of mice).

To reduce its size and to protect it against nucleases, the polyanionic DNA molecule can be condensed with (poly)cationic carrier molecules (for review see Ref. [6]). Usually, such polyplexes are generated with an excess of positive charge to enable sufficient compaction of the complexed DNA. The resulting net positive surface-charge of the gene-transfer particles can be advantageous for binding to a negatively charged cell surface when used in cell culture; by contrast, this positive surface charge can cause a disadvantageous situation when injected into the bloodstream. Positively charged particles can bind to (mostly negatively charged) plasma proteins such as immunoglobulin M, complement C3 and proteins of the coagulation cascade [7], but can also activate the complement system [8], an innate defense mechanism against 'foreign' particles within the bloodstream. Further, the cellular component of blood (i.e. thrombocytes, leukocytes and erythrocytes) also leads to rapid clearance from the blood. Particles electrostatically bound to negatively charged erythrocyte membranes will usually end up in the lung [9], whereas particles opsonized with complement, albumin, immunoglobulins or proteins of the coagulation cascade will be recognized by macrophages and will ultimately be relocated to the liver [5,10]. Figure 1 summarizes these barriers for gene delivery vectors after systemic application.

Delivery techniques

(Poly)cationic carriers

The first and crucial question is how to deliver the gene to the target site, such as a distant or inaccessible tumor. Recombinant viruses were developed as highly efficient vectors for gene delivery. However, their immunogenic and inflammatory potential, together with certain safety concerns, reduces their applicability for systemic gene delivery [11]. These negative side effects can be prevented using non-viral vectors based on synthetic molecules, although their drawback is the low efficiency of transgene expression.

In principle, the most common DNA condensing agents can be divided into two groups, namely cationic lipids and polycationic molecules. Both agents bind to DNA because of electrostatic interactions and finally lead to hydrophobic collapse with particle formation in the nanometer range. This compaction protects the DNA from nucleases and reduces its dimensions. Cationic lipids usually consist of a cationic headgroup for electrostatic binding to DNA and a lipid tail, which enables the hydrophobic collapse of a DNA–lipid complex (for review see Ref. [12]). Polycationic carriers are either naturally occurring proteins such as histones or protamines, or chemically synthesized compounds, such as the poly-amino-acids, polylysine, polyarginine or polyhistidine (for review see Refs [13,14]).

More recently, a compound initially used in the chemical industry, namely polyethylenimine (PEI), has been described as a carrier that condenses DNA and enables its efficient delivery into cells (for review see Ref. [15]). The chemical synthesis of these carriers can be easily scaled up and produced at relatively low costs. Based on such carriers, these molecules can be used as a type of 'work bench' to add additional components to the system. Usually, the presence of primary amino groups within polycationic carriers enables the chemical attachment of further components with additional properties for gene delivery (see later).

Surface shielding

Masking the net positive charge to reduce non-specific interactions with blood components and to enable the circulation of complexed DNA in the bloodstream can be accomplished by 'shielding' the complex surface with hydrophilic polymers, such as (poly)ethylene glycol (PEG), (poly)hydroxypropylmethacrylamide (pHPMA) or (poly)vinylpyrrolidine. This type of modification inhibits non-specific interaction with blood components and enables both a definite circulation of complexes after systemic application and expression of the delivered gene in a distant tumor [7,9].

In the case of PEG, the polymer was attached to the complex surface after complex formation via a single amine reactive group per PEG molecule. Oupicky and colleagues described the use of pHPMA, in which several amine reactive groups were present in the polymer [16]. This strategy resulted in particles with circulation times of several hours and passive accumulation in a subcutaneous tumor. This passive accumulation of macromolecular drugs in tumors can be achieved because of the incomplete vasculature within the tumor (Fig. 1). A mechanism described as the enhanced permeability and retention (EPR) effect [17] enables the influx of macromolecules from the blood into the tumor via leaky or incomplete blood vessels. As the efflux is hampered, the particles can reach a considerable concentration within the tumor tissue.

An alternative approach for shielding the surface charge was used by Kircheis and colleagues. The plasma protein transferrin was applied both to shield and to target polyplexes to distant tumors via the transferrin receptor [18] (discussed later). The net negative charge of the protein and the fact that transferrin is a plasma protein, enabled the shielding of the complex surface against non-specific interactions with other plasma proteins or blood cells.

Active targeting of DNA polyplexes

Targeting cell-surface receptors is an attractive concept to achieve specific binding and internalization using the incorporation of cell-binding ligands into a macromolecular delivery system. The iron-transporting plasma protein transferrin has been used as targeting agent for a wide variety of cell lines and types (for review see Ref. [19]). Although the transferrin receptor is expressed on almost all cell types, its expression is upregulated in many rapidly dividing tumors. This enables greatly increased gene delivery to tumor cells in cell culture [20] and also *in vivo* [18].

Epidermal growth factor (EGF)-receptor expression is often increased in breast or prostate cancers, making it a good candidate for targeting gene-transfer complexes. Blessing and colleagues recently used such an EGF-targeted PEI-based system and found highly increased reporter gene expression *in vitro* [21]. Lee and colleagues also demonstrated a 50-fold increase in gene transfer efficiency into hepatocellular carcinomas compared with normal liver tissue in nude mice using a reporter gene and a therapeutic gene [22]. Specific antibodies for certain cell-surface markers can also be used for targeting: a monoclonal antibody directed to the CD3 surface marker in human T-cell leukemia enabled efficient gene delivery *in vitro* [20]; ErbB2, a tumor marker that is highly upregulated in many human breast and prostate cancers, was targeted with a delivery system containing a single-chain antibody [23]. The second application has the additional advantage of lacking the Fc-fragment of the antibody, which could otherwise be recognized by macrophages and thus cleared from the bloodstream.

Intracellular barriers

When reaching the target cells, synthetic gene-transfer particles still face several severe obstacles to reach the cell nucleus for transcription of the delivered DNA (see Fig. 2 for summary). After internalization by receptor-mediated or adsorptive endocytosis, the vector is enclosed within the endosomal or lysosomal membrane and is, therefore,

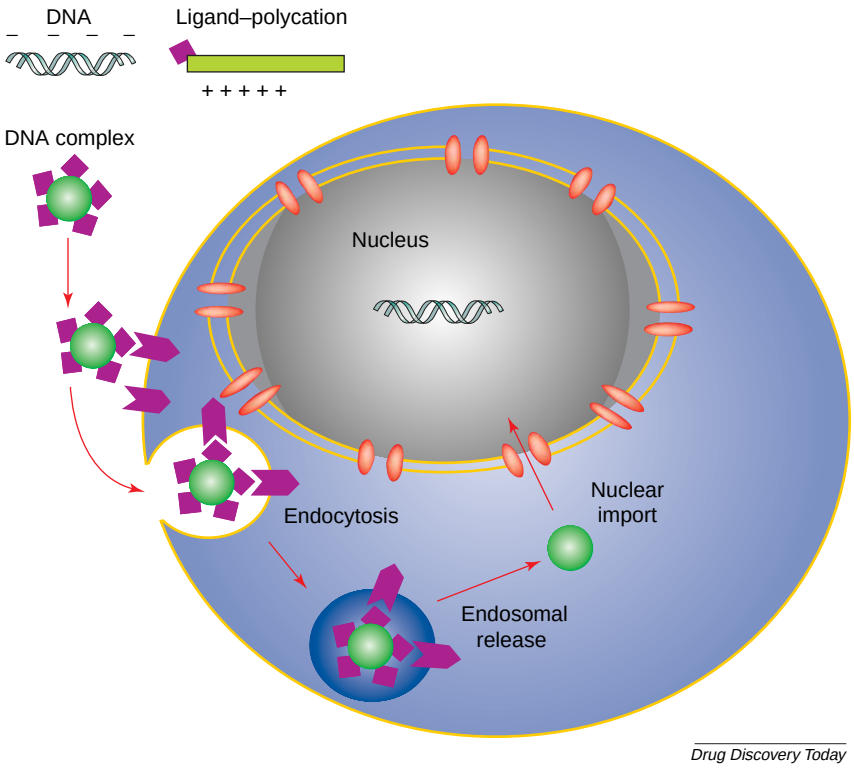

Figure 2. Intracellular bottlenecks for gene delivery. Each step, such as release into the cytoplasm after (receptor-mediated) endocytosis and transport into the nucleus to access the nuclear transcription machinery, can limit transgene expression.

separated from the cytoplasm; this bottleneck in gene delivery can be responsible for the degradation of >99% of the internalized DNA. The intracellular proton pump acidifies endosomal or lysosomal compartments and lysosomal nucleases can degrade the enclosed DNA. Viruses have developed clever mechanisms to overcome the endosomal barrier: viral proteins often contain membrane-active domains mediating the delivery of the viral genome to the cytoplasm after their activation in the endosome (for review see Ref. [24]). These membrane-active domains can be chemically synthesized as peptides and incorporated into polyplexes. Also, other naturally occurring peptides and small proteins, such as those in the venoms of vertebrates and invertebrates, can be highly membrane-active (for review see Ref. [25]). Both types of compound have already been used to enhance the intracellular delivery of transferred DNA, such as using influenza-derived peptides [26] or melittin, which is derived from bee venom [27]. The DNA-carrier PEI offers an intrinsic mechanism that enables the release of endocytosed DNA into the cytoplasm under certain conditions. The buffering capacity of the molecules can hamper the acidification of the endosomes, causing it to burst [28].

Access to the nuclear transcription machinery in dividing cells can be achieved after the nuclear membrane degradation during mitosis and the subsequent inclusion of the transferred DNA. Hence, in slowly or non-dividing cells, efficient nuclear entry can only be achieved via the tightly controlled import or export machinery located within the nuclear pore complex. Usually, non-viral gene delivery systems are highly inefficient in crossing this barrier [29], whereas the incorporation of proteins or peptides containing nuclear localization signals (NLS) can help to overcome this bottleneck (for review see Ref. [30]). Recently, the membrane-active peptide melittin was used for enhancing the intracellular delivery of a transgene [27]. Cell culture experiments revealed that melittin enables the efficient release of polyplexes from intracellular vesicles, and also uses a nuclear transport mechanism to enhance reporter gene expression in slowly or non-dividing cells.

Predominant gene expression in tumors

Several groups have achieved targeted gene expression in distant tumors after systemic application of particulate gene delivery systems (Table 1). Our group has used a system based on PEI and transferrin, and complexes were shielded with PEG. Within this setting, efficient gene transfer was observed in a well-vascularized, subcutaneous neuroblastoma tumor in inbred mice. Luciferase reporter gene expression was increased up to 1000-fold in the tumor compared with other organs such as the liver or lung [7,9] (Fig. 3). The use of a liposomal system known as stabilized plasmid–lipid particle (SPLP), which contained PEG as a shielding agent, enabled circulation of the particles in the bloodstream for >6 h after systemic tail-vein injection and tumor-targeted gene expression in subcutaneous growing B16 mouse melanoma [31].

In another method that was lipid-based, but using transferrin instead of PEG as the shielding and targeting agent, therapeutically active DNA was delivered to head and neck tumors in a nude mouse model [32] (see also next paragraph). Further enhanced gene expression was observed with a polyplex system based on a linear PEI derivative shielded with transferrin [18]. With this serum protein

in the complex, erythrocyte aggregation was inhibited and high reporter gene expression was found in different tumor models.

The same system was also used to deliver therapeutic genes to different tumors (R. Kircheis, unpublished; see also next paragraph). Polyplexes based on PEI, with EGF covalently attached to the complex surface as a targeting ligand, were applied systemically to achieve gene expression in HUH7 hepatoma in nude mice (E. Wagner *et al.*, unpublished results). Luciferase reporter gene expression in tumors was >1000-fold higher compared with other organs. A delivery system that combines both polycationic carrier molecules (which condense DNA within an inner core) and cationic lipid (which coats the core's surface with a lipid film) (for review, see Ref. [33]) enabled the efficient delivery of therapeutic genes to ovarian carcinoma after tail-vein injection in nude mice [34].

Table 1. Preferential gene expression in distant tumors after systemic application of particulate gene delivery systems

System used	Center	Refs
PEGylated transferrin–PEI–DNA	Boehringer Ingelheim Vienna; University of Vienna, Austria	[7,9]
Transferrin-coated liposome–DNA	Lombardi Cancer Center, Washington, DC, USA	[32]
PEG-coated liposome–DNA	Inex Pharmaceuticals, British Columbia, Canada	[31,47]
Lipid–Polymer–DNA	Targeted Genetics, Seattle, WA, USA	[34]
Vaccinia vector	National Cancer Institute, Bethesda, MD, USA	[41]
Recombinant *Salmonella*	Vion Pharmaceuticals, New Haven, CT, USA	[43]

Therapeutic concepts

Choosing the therapeutic gene

After the particles' long journey inside the body to reach the distant tumor (and the laborious steps to make such a system work), the transcription of the gene into the relevant mRNA and its subsequent translation into the therapeutic protein will determine the success or failure of the treatment. Therefore, the right choice of therapeutic gene is essential.

Therapeutic concepts (Box 1) can be subdivided into relatively few groups. A common form is the development of a so-called prodrug or enzyme strategy. In this case, the delivered gene encodes an enzyme that specifically converts an inactive prodrug into the active compound at the site of gene expression – the tumor (for a recent review, see Ref. [35], for a recent book, see Ref. [36]).

The most common combinations, such as herpes simplex virus 1 (HSV1) thymidine kinase with ganciclovir and cytosine deaminase with 5-fluorocytosine are already used in clinical trials. The anticancer drug cyclophosphamide is an alkylating agent that requires bioactivation by liver cytochrome P450 (CYP450) enzymes. Because tumor cells do not usually express CYP450, the delivery and expression of CYP450 into tumor cells efficiently sensitizes them to treatment with cyclophosphamide [37]. In parallel to CYP450 gene therapy, the expression of liver-specific CYP450 can be efficiently suppressed by liver CYP450 inhibitors to prevent liver toxicity [38]. The plant-derived enzyme linamarase hydrolyzes the cyanogenic glucoside substrate linamarin into glucose, acetone and cyanide. Using a retroviral vector, large glioblastomas could be eradicated, helped by a cyanide bystander effect [39].

Another approach is the expression of highly potent cytokines, which usually show strong side effects when being applied systemically, but can be used for local killing

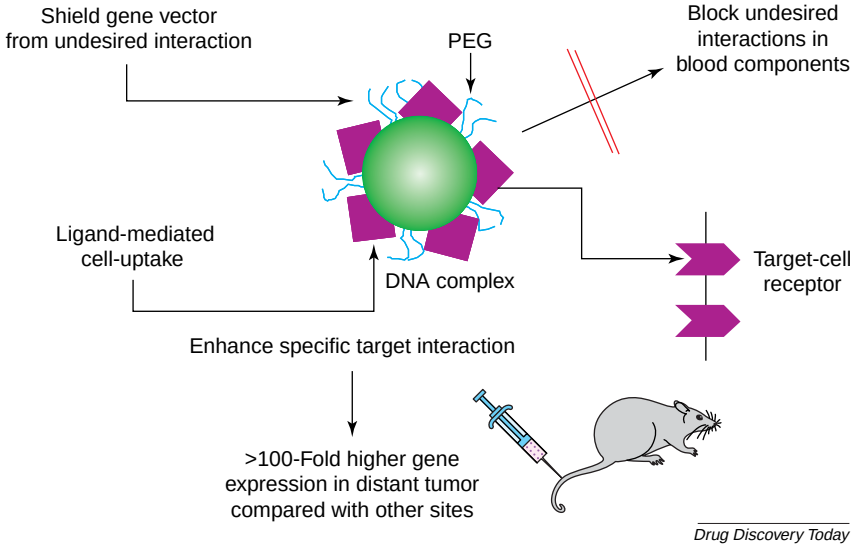

Figure 3. Strategies to achieve tumor-targeted gene delivery. Inhibiting blood interactions by surface shielding with the hydrophilic polymer polyethylene glycol (PEG) and active targeting to cellular ligands (e.g. transferrin) can enable preferential transgene expression in distant tumors.

Box 1. Different concepts for cancer gene-therapy applicable with non-viral gene delivery methods

- Direct killing of tumor cells (e.g. enzyme or prodrug therapy)
- Block cell cycle or induce apoptosis
- Inhibit tumor cell metastasis or migration
- Inhibit or revert tumor neoangiogenesis
- Induce chemoprotection (in bone marrow)
- Stimulate anti-tumor immunity (cytokines or vaccines)

of tumors when delivered as a gene. The tumor necrosis factor-α (TNF-α) gene has also been used (R. Kircheis *et al.*, unpublished). The systemic application of plasmid DNA encoding TNF-α and condensed within transferrin and PEI-containing complexes [18] led to significant expression of the cytokine within the tumor, whereas no elevated levels of TNF-α were observed in liver or plasma. The system was shown to be effective in all the tumor models tested in immune-competent mice. Pronounced hemorrhagic tumor necrosis and inhibition of tumor growth without systemic TNF-related toxicity was observed because of the localization of the activity of the cytokine to the tumor. Polyplexes were repeatedly administered over two weeks and tumor growth was significantly reduced in the murine neuroblastoma Neuro2A, and total tumor regression was observed in some cases in a MethA fibrosarcoma tumor model. DNA encoding the immune stimulatory cytokine, interleukin-2 (IL-2), was applied intratumorally as a lipoplex in the treatment of head and neck cancer patients in a Phase I clinical trial [40]. It is hypothesized that the local cytokine release will attract or induce anti-tumor immune responses. Head and neck cancer xenografts in nude mice have been successfully treated by delivering the tumor suppressor gene p53 after systemic application of transferrin-targeted lipoplexes [32]. The combination of gene therapy and radiation resulted in complete tumor regression and inhibition of their recurrence even six months after the end of all treatment.

The expression of other immunostimulatory cytokines, such as interferon-γ or IL-12, can be induced by the delivered DNA *per se*. Using bacterial plasmid DNA, the unmethylated CpG sequences in the molecule can activate immune cells, inducing proinflammatory responses (for review, see Ref. [41]). Although this so-called 'empty vector effect' is a problem for gene replacement therapy, it can be a powerful adjunct in anticancer therapies. Taken together, these data indicate that targeted gene delivery to tumors might be an attractive strategy applicable to highly active yet toxic molecules in cancer treatment.

Other strategies

In addition to synthetic carriers and active viruses, other particulate gene delivery vectors based on recombinant bacteria or inactivated viruses have been successfully used for tumor targeting. Puhlmann and colleagues used recombinant *Vaccinia* virus carrying the luciferase reporter gene and inactivated the virus with the DNA-photocrosslinking agent psoralen before intravenous application [42]. Reporter gene expression was up to 180,000-fold and 80,000-fold higher in tumor compared with liver or lung, respectively, using different tumor models either in nude or immune-competent mice. Zheng and colleagues used the ability of recombinant *Salmonella* to replicate selectively in tumor tissue and express considerable amounts of therapeutic protein within the tumor [43], a concept that is already used in a clinical Phase I trial for cancer treatment [44].

On the basis of molecular biology, the specificity of transgene expression can be further increased by using specific promoters, such as a hypoxia-responsive element (a promoter responding to the typically low concentration of oxygen in tumors) [45] or heat-inducible promoters for local hyperthermia [46]. This approach can help to further increase the specificity of tumor-targeted gene therapies.

Conclusions

Gene delivery to distant tumors for therapeutic approaches is a demanding task that urges the development of delivery vectors capable of overcoming many barriers. In recent years several laboratories have successfully developed non-viral delivery vectors, which are now capable of delivering recombinant DNA to distant tumors after systemic delivery. Initially, non-viral gene delivery systems were thought to be far too inefficient to achieve any therapeutic effect. After several years of intense research within this interdisciplinary field, including chemistry, biochemistry, cell biology and pharmacology, these initial criticisms have been refuted. Preclinical models showed the applicability of such systems to express sufficient amounts of therapeutically active compounds within tumors, enabling reduced tumor growth or even complete regression. These promising results encourage clinical evaluation for tumor therapy and provide hope that new and powerful therapies will be realized within this decade.

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NOTICE

Please note the following updated contact details for Jason Tierney, coauthor of 'The impact of microwave-assisted organic chemistry on drug discovery' by Wathey *et al.*, published in the 15th March issue of *Drug Discovery Today*.

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