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Abstract
Gene therapy is the use of genes to treat or prevent diseases. Diseases such as cancer, which are difficult to treat using conventional methods, can be treated using gene therapy. The transport of the therapeutic transgene can be accomplished using viral or non-viral methods. However, widespread use of viral vectors is limited due to its high cost of manufacture and safety concern. Non-viral vectors are limited in their effectiveness. The use of transposons such as the Sleeping Beauty transposon system can effectively deliver the transgene with less concern than viral vectors. This review discusses the various vectors and treatment strategies using gene therapy to treat cancer.

Introduction

The molecular basis for cancer is now well understood to involve the genetic control of multiple genes that control cell cycle and tissue growth. The random mutations that activate dominant oncogenes or inactivate tumor suppression genes result in a cell cloning itself with an abnormal pattern of growth control forming a tumor. Traditional cancer therapies aim to destroy or remove the tumor by chemotherapy, radiation or surgery. The problem with both radiation and surgery is the ability of tumors to metastasize and spread cancerous cells to areas inaccessible to these treatments or may not be noticeable at the time of treatment allowing a secondary tumor to appear. The trouble with chemotherapy is its low therapeutic index for many cancers and the rapid development of drug resistance in cancerous cells.

Gene therapy is a fundamentally different approach to the treatment of diseases. Originally proposed as treatment for inherited autosomal recessive Mendelian disorders, such as hemophilia, gene therapy is now being used to treat multiple acquired conditions including infections, degenerative diseases and cancer. Gene therapy is the use of genetic material (genes) to express a specific protein in a cell or to reduce the amount of protein by interfering with its synthesis. Replacing a defective gene with a functional one is the essence of gene therapy.

Cancer is widely believed to arise from mutations to the cell's DNA by either carcinogens or random mutations during cell division. This genetic basis coupled with the limitations of the traditional treatments, makes cancer a great candidate for gene therapies. More than 60 percent of all gene targeted therapy clinical trials since 1989 aimed to treat cancer (Ginn et al, 2013).

Strategies for Gene Therapy for Cancer

Cancer is a disease that involves multiple genetic changes to oncogenes, tumor-suppressor genes and modifier genes. There is also the intracellular interactions with multiple cells that regulate the body's immune response and the interaction with other cancer cells to maintain a solid tumor. Gene therapy can be used alone or together with conventional treatments. It can be used to sensitize cancerous cells to radiation or chemotherapy. It can increase the body's overall resistance to chemotherapy so larger doses can be used. Gene therapy can declump or shrink tumors to allow for surgical removal. Various approaches are being examined both in preclinical studies and in clinical trials for gene therapy for cancer.

Downregulation of Genetic Targets

To downregulate the expression of protein coded by oncogenes, antisense molecules are used. Antisense molecules are synthetic oligodeoxynucliotides (ODN) that hybridize with the coding (sense) mRNA of a specific gene. The antisense and sense molecules form double stranded RNA which cannot be translated destroying the mRNA. ODNs are designed to be highly resistant to nucleases which will destroy the mRNA (Stein et al., 1988). In a phase I–II clinical study using antisense ODNs to target BCL2 (an anti-apoptotic oncogene) mRNA combined with chemotherapy in patients with advanced malignant melanomas, the antisense ODN was found to successfully downregulate the target protein and has shown anti-tumor effects in 6 of the 14 patients (Jansen et al., 2000).

Immunomodulation by Gene Therapy

Because cancer cells originate from "self" cells, they generally do not cause a strong immune response. However, the immune system can be augmented by gene therapy to increase their function. Therapeutic genes can be introduced into tumor cells or into effector cells such as T lymphocytes.

To elicit a greater immune response, tumor cells have been modified with the insertion of cytokine genes. Cytokines are small cell signaling polypeptides involved in immunity and inflammation. Systemic and local administration of cytokines from the interleukin (IL) family has shown significant reduction in tumor size but have systemic side effects and short half-lives, making injection of interleukins deficient in long term tumor control. However, tumor cells modified to express IL-12 or IL-2 have shown the same size reduction as well as long term remission and metastasis control (Gao et al., 2005, Tahara et al., 1995).

The receptors on T lymphocytes can be modified to target tumor-associated antigens. The most common method of creating an artificial T cell receptor (TCR) is by protein fusion of single-chain

variable fragments derived from monoclonal antibodies that acts as the TCR ectodomain with the CD3-zetatransmembrane and endodomain. The endodomain can be further modified with co-stimulatory receptors to increase immunologic activity. Leukemias from B cell linages are suitable targets for this therapy. The CD19 antigen is expressed in differentiated cells with B cell linages and is rarely lost in cancerous cells. Anti-CD19 T cells can safely target and destroy cells expressing CD19 including cancerous cells, and they will later be replenished with healthy cells (Scheuermann & Racila, 1995).

For effective activation of T cells, non-specific signals are needed as well as the antigen-specific signal received by the T cell receptor. Co-stimulatory molecules interact with receptors, such as CD28, on the T cell to provide the non-specific signals. Martinet et al. (2000) has shown that transfection of both IL-12 and 4-1BB ligand genes into tumor cells resulted in long-term remission of liver metastases in mice. The 4-1BB ligand, a co-stimulatory molecule that binds with the 4-1BB receptor on T cells, synergizes with the CD28 pathway to increase the immune response (Melero et al., 1998).

'Suicide' Gene Therapy

A commonly used idea for gene treatment of solid tumors is 'suicide' gene therapy, where the cells expressing the therapeutic gene are killed. Suicide genes code for enzymes that can activate a drug with an otherwise low toxicity. When suicide genes are expressed only in the targeted cancer cells, the healthy cells avoid the drugs toxic effect. One enzyme commonly used is herpes simplex thymidine kinase, which converts the nontoxic drug Ganciclovir, into a toxic form by phosphorylation (Song et al., 2009). The suicide gene can be placed under the control of tumor specific promoters such as c-erbB2 in breast cancer, ensuring specific drug activation in the tumor (Pandha et al., 1999). Because the enzyme can bleed into and kill neighboring non-transduced cells in what is termed the bystander effect, suicide genes can be optimized to only require ten percent transduction in a solid tumor greatly increasing its efficiency (Xiong et al., 2012).

Apoptosis-inducing genes One of the problems involved in treating solid tumors by conventional therapies is that cancerous cells are often resistant to apoptosis and will not die with chemotherapy or radiation therapy alone. The gene that codes for the anti-tumor protein p53 is either mutated or deleted in more than 50 percent of human tumors (Hollstein et al, 1991). Inserting a copy of wild type p53 into cancerous cells has been shown to induce apoptosis and make the cells more susceptible to chemotherapy and radiation therapy.

Anti-angiogenesis

Tumors, just like any tissue, require a constant supply of nutrients, oxygen, hormones and growth factors for their growth and progression. This is provided by the formation of new blood vessels or angiogenesis. Therefore, inhibition of angiogenesis would stunt tumor growth. Patients with glioblastoma multiforme, a highly vascularized form of brain cancer that does not respond well to conventional treatments, would benefit greatly from an anti-angiogenic treatment. Even poorly vascularized tumors can be reduced by anti-angiogenic treatment (Beecken et al., 2001). Clinical trials involving systemic administration of angiogenic inhibitors such as angiostatin and endostatin showed no dose-limiting toxicity. However, they did not show marked signs of tumor regression due to the continuous release of pro-angiogenic factors released by the tumor (Ohlfest et al., 2005, Shepherd & Sridhar, 2003). Therefore, the delivery of angiogenic inhibitors by gene transfer is favored over systemic administration.

Gene Transfer Techniques

The success of gene therapy lies in the efficient delivery of the gene of interest to the target cell. The therapeutic gene is cloned into a vector together with appropriate regulatory regions (promoters/enhancers) as well as any supporting proteins needed. Selecting the right vector is a crucial part of gene therapy. The ideal vector will protect and easily deliver the genetic information across the cell membrane and into the nucleus. It should have the ability to regulate the expression of the gene of interest and be able to successfully target specific cells to minimize toxicity. It should be easy and inexpensive to produce in large quantities. Once the gene is cloned into the vector it can be introduced to the target cell. The gene can be delivered in vitro or in vivo. In in vitro transfer, cells from a specific tissue are removed from the patient and exposed to the gene-carrying vector. The transformed cells are selected by biomarkers and are reintroduced into the patient's body. In in vivo transfer, the vector is injected into the patient's body directly, usually into the target tissue or the tumor if a tumor is the target.

Viral Vectors

Currently, the most effective method of gene transfer is though viral vectors. Viruses have evolved over the years to enter the cell and efficiently hijack the cell's machinery to produce its own viral proteins. An ideal viral vector uses the viral infection pathway but avoids the expression of the viral genes that facilitate replication and the subsequent host cell death. This is achieved by deleting most of the viral genome, leaving intact the sequences (usually the long terminal repeats) required for capsid packaging and integration of the vector DNA into the host's chromatin. The most commonly used viruses for gene therapy are retrovirus, lentivirus and adenovirus.

Retroviruses carry their genetic material as RNA and integrate their genome into host DNA using the viral enzymes reverse

transcriptase and integrase. The integrated DNA acts as a provirus which replicates to make multiple copies of the virus and is released outside the cell. Therapeutic retroviral vectors are made replication-deficient by replacing the structural genes with the therapeutic gene. Since retroviruses only gain access to the host's DNA when the nuclear membrane is broken down, they only infect actively dividing cells and integrate the gene of interest into the target cell.

Lentiviruses, a subclass of retroviruses, have recently been adapted to be used as gene transmission vectors. Lentivirus vectors can naturally enter an intact nuclear membrane and integrate their genome into non-dividing cells, which a retrovirus vector cannot do. HIV-1 is the most common lentivirus used in gene transfer. Because of its dangerous nature, lentivirus vectors do not carry the gene required for replication. As an added precaution, self-inactivating (SIN) lentivirus vectors are being developed which contain deletions in the downstream LTR.

Adenoviruses carry their genetic material as double stranded DNA, but as opposed to the other viruses mentioned, the DNA is not integrated into the host's genome. However, adenovirus can successfully infect broad range of cell type and is not limited to dividing cells. In 2003 the first gene therapy to be approved for commercial production was a recombinant adenovirus-p53 gene therapy for head and neck squamous cell carcinoma approved by China's State Food and Drug Administration and is sold under the name Gendicine (Pearson et al., 2004).

Limitations of Viral Vectors

The obvious concern regarding the use of viral vectors is the possibility of the virus eliciting a strong immune response. In 1999, an 18-year-old male died due to an extreme immune response triggered by the administration of an adenoviral vector. He was participating in a phase I clinical trial to determine the safety of a gene therapy for ornithine transcarbamylase deficiency, an X-linked genetic disease of the liver (Raper et al., 2003). Since then most work with adenovirus vectors used genetically crippled constructs which would minimize the likelihood of an immune response.

The integration of the therapeutic gene when using retroviral or lentiviral vectors carries a possibility of oncogene activation. Since the point of insertion is mostly random, the transgene can insert into the upstream regulatory region of an existing gene and activate or upregulate the existing gene due to the proximity to the promoters in the downstream LTR of the transgene. The transgene can also insert itself into a transcriptional unit of an existing gene causing a loss of function in that gene. This was shown to be a real threat when four patients in a clinical trial that successfully treated X-linked severe combined immunodeficiency disease developed a form of leukemia. The cause in at least two of the patients appeared to be the integration of the therapeutic murine leukemia virus retroviral vector close to the LMO2 oncogene (Kohn et al., 2003).

Adenovirus vectors, since they do not integrate the gene into the host's genome, carry no risk of insertional mutagenesis. However, since adenovirus vectors to not integrate the gene into the cell's DNA they are only expressed transiently and require multiple administrations to be effective and repeated delivery may compromise efficacy and might induce a severe immune response (Hartman et al, 2008).

While self-inactivating (SIN) retroviral vectors may reduce the risk of insertional mutagenesis (Ellis, 2005), the clinical use of retroviral vectors is curtailed due the limited packaging capacity of viral vectors. Most retroviral vectors can carry a transgene of up to 8 kb, as larger genes would compromise the efficiency of viral reverse transcription (Thomas et al,. 2003). This excludes the transport of multiple or large transgenes. Finally, the high costs involved in the manufacture of clinical-grade retroviral vector and regulatory issues keep viral vectors from widespread translation into clinical practice.

Non-viral Methods

The simplest method of gene delivery is injecting naked DNA directly into the target tissue. Since naked DNA lacks any mode of transport through the cell membrane, there have been various methods developed to increase the efficiency of cellular uptake.

In smaller animals, hydrodynamic injection can overcome the low efficiency of cellular uptake. This procedure involves the injection of a large volume, about 10% the weight of the mouse, of DNA/saline solution through the tail vein in less than 10 seconds, with most of the protein being expressed and accumulating in the liver (Liu et al., 1999). In larger animals, the volumes required for hydrodynamic injection become prohibitive. This limitation can be overcome by isolating an organ's blood flow using catheters or part of a limb using external tourniquets.

The efficiency of delivery can be enhanced by physical methods such as microinjection directly into the cell, electroporation, sonoporation and the use of microparticle in gene guns and magnetofection. Most of these methods are only feasible in in vitro gene delivery. Electroporation has been used successfully in gene delivery to mice skeletal muscle in vivo (Miyazaki et al., 2002), but the larger energy required to increase the permeability of the cell membrane across a human limb risks destroying too many cells. Sonoporation is limited to acoustically accessible organs. Although these methods are efficient, they require expensive specialized equipment reducing their benefit over viral vectors.

The efficiency of delivery can also be increased by chemical methods. The negatively charged phosphate on DNA can bind to a variety of cationic polymers to form a DNA-polymer complex called a polyplex. The polyplex interacts with the cell membrane and is absorbed into the cell by phagocytosis where the polyplex is released and the DNA can migrate into the nucleus. Polyethylenimine (PEI) is one of the most commonly used non-viral vectors based on polycations for DNA delivery both in vitro and in vivo. PEI is a polymer with repeating units composed of an amine group and two carbon spacers. PEI can be linear, branched or as a highly branched dendrimer, though linear PEI is more commonly used. The exact mechanism my which the DNA-PEI polyplex escapes the endosome is unknown but is thought to be a result of the increased influx of protons, chloride ions, and water during endosome acidification causing it to rupture from the high osmotic pressure. Using confocal laser scanning microscopy, Merdan et al. (2002) observed the dispersal of the genetic cargo soon after the rupture of the endosomes on living cells.

Cationic lipids are also used to condense the DNA into a liposome. These liposomes can protect the DNA from damage during transport, something that the other non-viral delivery methods fail to do. Suzuki et al. (2010) successfully transfected murine ovarian tumors in vivo with IL-12 using self-prepared bubble liposomes combined with ultrasound applied to the tumor. The bubble liposome protocol was more effective than using the more expense commercially available transfection agent, Lipofectamine 2000. However, the Lipofectamine control in the study was not conducted together with ultrasound.

Non-viral methods present many advantages over viral vectors. Most non-viral methods are simpler and cheaper to produce and elicit a smaller host immune response. The transfer capacity of non-viral methods is functionally unlimited compared to viral capacity. Low levels of transfection used to be the limiting disadvantage, but advances have resulted in methods with transfection rates that are clinically viable and can compete with viral vectors. However, the efficient delivery of DNA to the nucleus is not enough for long-term transgene expression if it cannot integrate into the host genome. Transposable elements (transposons) could potentially offer such an alternative.

Transposon Systems for Gene Therapy

Transposons or transposable elements (TE) are mobile regions of DNA that can change their position in the genome. DNA transposons rely on a transposase enzyme to cut the TE from the donor site and insert it at the receiving site in a 'cut-and-paste' like manner. The transposase gene is the trans-acting element of the transposon system. The cis-acting element is a pair of inverted repeats at either end of the gene which are the target sites for the transposase as well as associated enhancers and promoters. Most regions coding for transposase also contain the cis-acting elements and are considered autonomous TEs. While transposons seem to have played a large role in evolution with close to fifty percent of the human genome made up of TEs, most species have accumulated mutations in the transposase genes.

One family of transposons, the mariner/tc1-like superfamily, has members in a wide range of species including nematodes, insects, fish, and humans with analogs reported in prokaryotes. This is a result of horizontal gene transfer between species. Unfortunately, not a single autonomous element has been isolated from vertebrates. Ivics et al. (1997) reconstructed the first man-made transposon system called the Sleeping Beauty (SB) transposon system based on the consensus sequence of members of the mariner/tc1 family isolated from fish. The reconstructed SB transposase was successfully able to transfer elements not only in fish but also in other species including human cells. The use of a SB transposon based system of gene transfer was proposed in 2000 when successful integration and long tern expression using SB was shown in mice (Yant et al., 2000).

Because transposons have to survive along with the host cell, traits that posed less of a threat to the cell were selected evolutionarily. A transposase with a high activity increases the chance of insertional mutagenesis. Therefore, early iterations of the SB tranposase had lower activity that could not compete with the fast acting retroviral enzymes. It was known early on that exchanging certain amino acids in SB transposase could increase its efficiency, but guessing the right combination of changes needed to construct a hyperactive transposase would be near impossible. Mates et al. (2009) conducted a large scale genetic screen of SB mutants and yielded a transposase (SB100X) that was ~100 times more efficient than first generation SB transposases. This hyperactive transposase allows the SB transposon system to compete with viral vectors and was awarded the title of "Molecule of the Year, 2009."

Since SB transposon based vectors integrate the transgene into the host's DNA, just as retroviral and lentiviral vectors do, they also pose the risk of insertional mutagenesis. Statistical analysis of the integration sites of both viral integrase and SB transposase suggests the SB vectors are safer. SB transposons integrate at TA dinucleotides and shows a preference ($p<0.01$) for a short TA palindromic consensus sequence. This preference can be explained by the greater bendability at TA sites that is possibly required to allow the transposase access, but with ~108 TA sites in the human genome this can still be considered random on a genomic level. A study mapping over 1,300 SB integration sites found 39 percent ($p=0.02$) integrated into known genes with a weak preference for regions slightly upstream or within 5kb of the start site (Yant et al., 2005). These results were compared to

10,000 computer-simulated insertions that showed truly random insertions would only result in 33 percent of genes. It is hard to avoid any preference for transcriptionally active genes since those are accessible due to chromatin modification such CpG islands. Even though SB transposon shows a statistically significant preference for genes, the percentages are low compared to viral vector insertions. Schröder et al. (2002) reported 69 percent ($p<0.0001$) of in vivo integration sites using HIV-1 vectors were in known genes. Adding insulator sequences to the ends of transposable element can further reduce the risk of unwanted activation of neighboring genes (Walisko et al., 2007).

Long term expression of the transposase gene can result in the transposon being excised and reinserted in a different location in what is known as "re-hopping", increasing the risk of insertional mutagenesis. To limit the amount of time in which transposase will be produced the transposase gene can transported on a second plasmid that will be transcribed ectopically and destroyed. The use of mRNA as a source of transposase in also being explored (Wilber et al., 2006).

The greatest weakness of the SB transposon system is its inability to infect cells. Any of the non-viral delivery methods mentioned above can be used to transport the SB-containing plasmid. There is also the possibility of using hybrid vectors that transport the transposon packaged into virions. These hybrid vectors may prove more effective than either method alone. The integration sites of an HIV-1/SB hybrid was shown to be closer to truly random integration profile with only 30 percent inserting into known genes (Staunstrup et al., 2009), and a herpes simplex virus/SB hybrid has successfully increased the capacity of the SB transposon (de Silva et al., 2009). SB transposon have also been successfully delivered to target tissue using modified liposomes that used Asialoglycoprotein receptor (ASGPR)-mediated endocytosis to target hepatocytes in vivo (Wang et al., 2009) and hybrid vectors may be employ similar targeting techniques being researched for viral vectors (Waehler et al., 2007).

Since transposons simply 'cut-and-paste', they do not have to rely on reverse transcriptase which has a tendency to incorporate mutations. The transposase can possibly be modified in the future to target a specific region of DNA using a synthetic zinc finger domain (Yant et al., 2007). SB transposons are easier and cheaper to manufacture than replication-incompetent viral vectors, allowing researchers without the resources required to produce viral vectors to continue exploring transposon based systems.

Conclusion

Non-viral vectors with the ability to integrate the transgene are far more efficient than viral vectors or non-integrating DNA plasmids. The increase safety risks associated with viral vectors and resultant bureaucracy has limited the widespread use of commercially licensed gene therapy treatments. There are only two approved gene therapies worldwide in over 25 years of clinical trials, mostly due to safety and toxicity concern and not effectiveness. There is a need for a safer delivery system and the SB transposon system may be it. Most of its early limitations have been resolved to the point that it can compete with viral vectors. SB-mediated gene transfer has been shown to be effective in a variety of treatment strategies including anti-angiogenesis (Ohlfest et al., 2005), suicide therapy (Song et al., 2009), and others.

The first human clinical trial using SB transposons is under way. The trial will attempt to transfect T cells ex vivo with engineered receptors against the CD19 antigen as described above (Williams, 2008). Regardless of what the results of this trial will be in terms of efficacy, the trial will prove the safety of this method and provide the data necessary for further improvement in future trials.

References

Beecken, W. D. C., Fernandez, A., Joussen, A. M., Achilles, E. G., Flynn, E., Lo, K. M., ... & Shing, Y. (2001). Effect of antiangiogenic therapy on slowly growing, poorly vascularized tumors in mice. Journal of the National Cancer Institute, 93(5), 382-387.

de Silva, S., Mastrangelo, M. A., Lotta, L. T., Burris, C. A., Federoff, H. J., & Bowers, W. J. (2009). Extending the transposable payload limit of Sleeping Beauty (SB) using the herpes simplex virus (HSV)/SB amplicon-vector platform. Gene therapy, 17(3), 424-431.

Ellis, J. (2005). Silencing and variegation of gammaretrovirus and lentivirus vectors. Human gene therapy, 16(11), 1241-1246.

Gao, J. Q., Sugita, T., Kanagawa, N., Iida, K., Eto, Y., Motomura, Y., ... & Nakagawa, S. (2005). A single intratumoral injection of a fiber-mutant adenoviral vector encoding interleukin 12 induces remarkable anti-tumor and anti-metastatic activity in mice with Meth-A fibrosarcoma. Biochemical and biophysical research communications, 328(4), 1043-1050.

Ginn, S. L., Alexander, I. E., Edelstein, M. L., Abedi, M. R., & Wixon, J. (2013). Gene therapy clinical trials worldwide to 2012–an update. The journal of gene medicine, 15(2), 65-77.

Hartman, Z. C., Appledorn, D. M., & Amalfitano, A. (2008). Adenovirus vector induced innate immune responses: impact upon efficacy and toxicity in gene therapy and vaccine applications. Virus research, 132(1), 1-14.

Hollstein, M., Sidransky, D., Vogelstein, B., & Harris, C. C. (1991). p53 mutations in human cancers. Science, 253(5015), 49-53.

Ivics, Z., Hackett, P. B., Plasterk, R. H., & Izsvák, Z. (1997). Molecular Reconstruction ofSleeping BeautyaTc1like Transposon from Fish, and Its Transposition in Human Cells. Cell, 91(4), 501-510.

Jansen, B., Wacheck, V., Heere-Ress, E., Schlagbauer-Wadl, H., Hoeller, C., Lucas, T., ... & Pehamberger, H. (2000). Chemosensitisation of malignant melanoma by BCL2 antisense therapy. The Lancet, 356(9243), 1728-1733.

Kohn, D. B., Sadelain, M., Dunbar, C., Bodine, D., Kiem, H. P., Candotti, F., ... & Glorioso, J. (2003). American Society of Gene Therapy (ASGT) ad hoc subcommittee on retroviral-mediated gene transfer to hematopoietic stem cells. Molecular Therapy, 8(2), 180-187.

Liu, F., Song, Y., & Liu, D. (1999). Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene therapy, 6(7), 1258-1266.

Martinet, O., Ermekova, V., Qiao, J. Q., Sauter, B., Mandeli, J., Chen, L., & Chen, S. H. (2000). Immunomodulatory gene therapy with interleukin 12 and 4-1BB ligand: long-term remission of liver metastases in a mouse model. Journal of the National Cancer Institute, 92(11), 931-936.

Mátés, L., Chuah, M. K., Belay, E., Jerchow, B., Manoj, N., Acosta-Sanchez, A., ... & Izsvák, Z. (2009). Molecular evolution of a novel hyperactive Sleeping Beauty transposase enables robust stable gene transfer in vertebrates. Nature genetics, 41(6), 753-761.

Melero, I., Bach, N., Hellström, K. E., Aruffo, A., Mittler, R. S., & Chen, L. (1998). Amplification of tumor immunity by gene transfer of the co-stimulatory 4-1BB ligand: synergy with the CD28 co-stimulatory pathway. European journal of immunology, 28(3), 1116-1121.

Merdan, T., Kunath, K., Fischer, D., Kopecek, J., & Kissel, T. (2002). Intracellular processing of poly (ethylene imine)/ribozyme complexes can be observed in living cells by using confocal laser scanning microscopy and inhibitor experiments. Pharmaceutical research, 19(2), 140-146.

Miyazaki, J. I., & Aihara, H. (2002). Gene transfer into muscle by electroporation in vivo. Gene therapy protocols, 49-62.

Ohlfest, J. R., Demorest, Z. L., Motooka, Y., Vengco, I., Oh, S., Chen, E., ... & Largaespada, D. A. (2005). Combinatorial antiangiogenic gene therapy by nonviral gene transfer using the sleeping beauty transposon causes tumor regression and improves survival in mice bearing intracranial human glioblastoma. Molecular Therapy, 12(5), 778-788.

Pandha, H. S., Martin, L. A., Rigg, A., Hurst, H. C., Stamp, G. W., Sikora, K., & Lemoine, N. R. (1999). Genetic prodrug activation therapy for breast cancer: A phase I clinical trial of erbB-2–directed suicide gene expression. Journal of clinical oncology, 17(7), 2180-2180.

Pearson, S., Jia, H., & Kandachi, K. (2004). China approves first gene therapy. Nature biotechnology, 22(1), 3-4.

Raper, S. E., Chirmule, N., Lee, F. S., Wivel, N. A., Bagg, A., Gao, G. P., ... & Batshaw, M. L. (2003). Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. Molecular genetics and metabolism, 80(1), 148-158.

Scheuermann, R. H., & Racila, E. (1995). CD19 antigen in leukemia and lymphoma diagnosis and immunotherapy. Leukemia & lymphoma, 18(5-6), 385-397.

Schröder, A. R., Shinn, P., Chen, H., Berry, C., Ecker, J. R., & Bushman, F. (2002). HIV-1 integration in the human genome favors active genes and local hotspots. Cell, 110(4), 521-529.

Shepherd, F. A., & Sridhar, S. S. (2003). Angiogenesis inhibitors under study for the treatment of lung cancer. Lung Cancer, 41, 63-72.

Song, J. S., Kim, C. W., & Ochoa, E. R. (2009). Sleeping Beauty-mediated suicide gene therapy of hepatocellular carcinoma. Bioscience, biotechnology, and biochemistry, 73(1), 165-168.

Staunstrup, N. H., Moldt, B., Mátés, L., Villesen, P., Jakobsen, M., Ivics, Z., ... & Mikkelsen, J. G. (2009). Hybrid lentivirus-transposon vectors with a random integration profile in human cells. Molecular Therapy, 17(7), 1205-1214.

Stein, C. A., Subasinghe, C., Shinozuka, K., & Cohen, J. S. (1988). Physicochemical properties of phospborothioate oligodeoxynucleotides. Nucleic Acids Research, 16(8), 3209-3221.

Suzuki, R., Namai, E., Oda, Y., Nishiie, N., Otake, S., Koshima, R., ... & Maruyama, K. (2010). Cancer gene therapy by IL-12 gene delivery using liposomal bubbles and tumoral ultrasound exposure. Journal of Controlled Release, 142(2), 245-250.

Tahara, H., Zitvogel, L., Storkus, W. J., Zeh, H. J., McKinney, T. G., Schreiber, R. D., ... & Lotze, M. T. (1995). Effective eradication of established murine tumors with IL-12 gene therapy using a polycistronic retroviral vector. The Journal of Immunology, 154(12), 6466-6474.

Thomas, C. E., Ehrhardt, A., & Kay, M. A. (2003). Progress and problems with the use of viral vectors for gene therapy. Nature Reviews Genetics, 4(5), 346-358.

Waehler, R., Russell, S. J., & Curiel, D. T. (2007). Engineering targeted viral vectors for gene therapy. Nature Reviews Genetics, 8(8), 573-587.

Walisko, O., Schorn, A., Rolfs, F., Devaraj, A., Miskey, C., Izsvák, Z., & Ivics, Z. (2007). Transcriptional activities of the Sleeping Beauty transposon

and shielding its genetic cargo with insulators. Molecular Therapy, 16(2), 359-369.

Wang, X., Sarkar, D. P., Mani, P., Steer, C. J., Chen, Y., Guha, C., ... & Roy-Chowdhury, J. (2009). Long-term reduction of jaundice in Gunn rats by nonviral liver-targeted delivery of Sleeping Beauty transposon. Hepatology, 50(3), 815-824.

Wilber, A., Frandsen, J. L., Geurts, J. L., Largaespada, D. A., Hackett, P. B., & McIvor, R. S. (2006). RNA as a source of transposase for Sleeping Beauty-mediated gene insertion and expression in somatic cells and tissues. Molecular Therapy, 13(3), 625-630.

Williams, D. A. (2008). Sleeping beauty vector system moves toward human trials in the United States. Molecular Therapy, 16(9), 1515-1516.

Xiong, T., Li, Y., Ni, F., & Zhang, F. (2012). Monitoring of bystander effect of herpes simplex virus thymidine kinase/acyclovir system using fluorescence resonance energy transfer technique. Journal of biomedical nanotechnology, 8(1), 74-79.

Yant, S. R., Huang, Y., Akache, B., & Kay, M. A. (2007). Site-directed transposon integration in human cells. Nucleic acids research, 35(7), e50.

Yant, S. R., Meuse, L., Chiu, W., Ivics, Z., Izsvak, Z., & Kay, M. A. (2000). Somatic integration and long-term transgene expression in normal and haemophilic mice using a DNA transposon system. Nature genetics, 25(1), 35-41.

Yant, S. R., Wu, X., Huang, Y., Garrison, B., Burgess, S. M., & Kay, M. A. (2005). High-resolution genome-wide mapping of transposon integration in mammals. Molecular and cellular biology, 25(6), 2085-2094.