Molecular therapeutics of cancer by means of electroporation-based transfer of siRNAs and EBV-based expression vectors

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1. ABSTRACT

A variety of gene transfer technologies have been devised as tools for functional analysis of genes as well as gene therapy and regenerative medicine. Electroporation enables efficient genetic transfection *in vivo* into various organs as well as tumors without the aid of any infectious viral vector, and has been applied to preclinical nonviral gene therapy approaches. The efficacies of transfection and expression can be further elevated when the electroporation procedure was used to deliver Epstein-Barr virus (EBV)based plasmid vectors that have characteristic artificial chromosome-like functions. Electroporation also facilitates delivery of synthetic short interfering RNA (siRNA) into some organs and tumors, potentially providing feasible strategies of RNAi-based molecular targeting therapy against various diseases including malignancies.

2. ELECTROPORATION AS AN EFFICIENT METHOD FOR GENETIC TRANSFECTION BOTH *IN VITRO* AND *IN VIVO*

Basically, gene delivery systems so far devised can be classified into two categories, viral and nonviral vector systems. The former consist of recombinant viral particles that are capable of infecting target cells, while the latter do not use any genetically modified infectious viruses. Compared with viral vector systems, nonviral vectors are devoid of virus-associated adverse effects, including induction of strong immune responses (e.g. adenovirus), potential generation of replication-competent viruses, and oncogenesis due to proviral integration into host chromosome (e.g. retrovirus). Thus, nonviral gene delivery systems may offer promising measures to treat patients (1). Another advantage of nonviral systems is large-scale, affordable manufacture. The major hurdle that should be overcome is the low efficiency of nonviral systems in transferring and expressing exogenous genes in target cells. In this regards, technical breakthrough is required to develop nonviral gene delivery systems that are feasible for molecular therapeutics.

A nonviral gene delivery system consists of two components: (i) nucleic acids carrying genetic information, most typically plasmid DNA (pDNA), and (ii) gene delivery methods/materials (nonviral vectors). Both of which should be taken into account to develop superior nonviral systems. The former will be discussed in the following sections, while we first briefly discuss the nonviral vectors.

A variety of nonviral vectors have been devised, including particular chemical compounds and physical methods. Chemical compounds (carrier molecules) include synthetic and natural macromolecules such as cationic lipids (2-4) and cationic polymers (5-7) that interact with nucleic acid and form complexes (lipoplex, polyplex etc.) to be endocytosed into cells. Physical methods such as electroporation (8-10), particle bombardment (gene gun) (11), sonoporation (12, 13) and other procedures (14) promote transfer of nucleic acid into cells in an endosome pathway-independent manner. Naked DNA methods are another means to transfect exogenous gene into some tissue/organs (8, 15).

Among them, the electroporation is one of the most promising methods to facilitate transfection of naked DNA, not only into cultured cells *in vitro* but also into organs/tissues *in vivo* (9, 10, 16, 17), including skeletal muscle (18-20), liver (21, 22), skin (23-25), cornea (26), blood vessels (27), cardiac muscle (28), and synovium of joints (29).

Application of an electric field forms pores in the lipid membrane, allowing influx of extracellular substances such as nucleic acids into the cell (30, 31). Methods, equipment and parameters for electroporation should be devised and optimized for each target organ. The efficiencies of electric gene transfer are highly affected by the electric field strength and pulse shapes. It is generally believed that square wave pulses are superior to exponentially decaying pulses, because the pulse duration and amplitude can be controlled independently (32), so that most electroporation devices are designed accordingly. Square wave pulses of low field strength have been popularly employed in electroporation in vivo, while exponentially decaying wave pulses have been used rarely (32, 33). However, some reports showed that an exponentially decaying wave pulse was also suitable for electrotransfection in vitro (34), probably because it provides both the initial high voltage required for poration and the following low voltage "tail" for electophoretical transfer of DNA molecules into the cell, while the feasibility of another waveform has also been presented (35). We have shown that transcutaneous direct current (DC) shock that is commonly used for cardiac delibrillation in clinical situations is capable of enhancing delivery, into

cardiac muscle of canine, of plasmid DNA that is simultaneously infused into coronary artery and vein via cardiac catheter (28). Such a procedure is quite simple and inexpensive, providing a new strategy for myocardial gene therapy without any special devise or technique other than cardiac catheterization and DC cardioversion that are general performed in ordinary hospitals. DC shock may be applied to gene or drug delivery to other organs, due to the high penetration of the devise, although parameters such as voltage should be optimized for each application.

Electroporation *in vivo* has the following advantages over other methods: 1) genes may be introduced into any tissues or cells, 2) the procedure is easy and very rapid, requiring application of the electric field for only a few seconds, 3) the amount or size of DNA used is not stringently restricted, compared with the limitation in other gene delivery procedures, 4) introduction of genes can be limited to the region charged with an electric field, 5) repeated DNA administration is possible because of low immunogenicity, and 6) the procedure requires no special skill and is inexpensive. Due to these advantages, electroporation has been considered a promising mean of nonviral gene therapy to eradicate malignancies (36).

3. ELECTROPORATION-BASED GENE THERAPY AND CHEMOTHERAPY OF CANCER

The above mentioned features of electroporation provoked scientist to study its application to medicine, especially treatment of malignant diseases. Tumors such as malignant melanoma (36-39), glioma (40), and hepatocellular carcinoma (41) are good targets of electrogene therapy in vivo. In some studies, tumors were given toxic genes such as the A fragment of diphtheria toxin that directly kills the cancerous cells (40, 42, 43) or so-called suicide genes encoding, e.g., herpes simplex virus thymidine kinase and bacterial cytosine deaminase (CD) genes whose products show toxicity to cells in the presence of the corresponding prodrugs (ganciclovir and 5fluorocytosine that are substrates for HSV-tk and bacterial CD, respectively)(43, 44). In this kind of cancer gene therapy, the delivery process must be accurately controlled to localize gene expression exclusively in tumors, to reduce possible adverse effects of such toxic genes. Electroporation may be quite suitable for this purpose.

Several studies have focused on electro-immunogene-therapy approaches, particularly cytokine gene therapy (45). A potential advantage of these strategies is that the transfection efficiency of 100% is not always required, because small number of successfully transfectedtumor cells may induce anti-tumor immune responses, which in turn destroy metastatic tumor cells that reside in various organs. Another important feature of the cytokine gene therapy is that long-term expression of transgene is not always required. Electroporation may be quite suitable for cytokine gene transfer into tumor cells. Some investigators including us previously demonstrated the effectiveness of electroporation-mediated delivery of genes encoding IL-2 (38), IL-12 (37-39, 46-48) and IL-18 (37) into tumors including melanoma (37-39, 48), hepatocellular carcinoma (46), as well as colon and rectal carcinomas (47). By some protocols, complete regression of preestablished melanomas has been observed (39, 48). More recently, potential toxicity of electro-IL-12-gene therapy has been intensively studied, demonstrating that adverse effects caused by the procedure is very low, if any (49).

Electroporation was also applied to the DNA vaccination approach, another category of cancer immunotherapy. Quaglino *et al.* vaccinated plasmid vector encoding extracellular and transmembrane domains of HER2/neu (c-ErB2) oncogene into mice that had displayed multifocal progressive mammary carcinomas (50), showing that repetitive vaccination resulted in complete clearance of the tumors and tumor free survival. Without electroporation procedure, plasmid DNA vaccine alone significantly delayed, but failed to prevent, tumor growth in their experimental setting.

The B7-1 and B7-2 are important adhesion molecules expressed on the surface of antigen presenting cells, and interact with the CD28 on the T cells that subsequently receive costimulatory signal through this molecule (45). CTLA4 is another ligand for B7-1 and 2, while this T cell surface molecule antagonizes CD28 stimulation to suppress T cell activation. Chakrabarti *et al.* recently co-transfected, via electroporation, a DNA vaccine encoding carcinoembryonic antigen (CEA) gene and a mutant form of B7-1/Ig fusion protein that specifically binds to CTLA-4 and cancels T cell-suppression via this negative regulatory molecule (51). The treatment ameliorated the effect of the anti-tumor DNA vaccine.

Electroporation has been used for transferring chemotherapeutic agents into tumor cells (electrochemotherapy)(reviewed in (52, 53)). This strategy enables a local and effective administration of chemotherapeutic agents into tumor cells, avoiding severe toxicity that is usually caused in patients by systemic high dose chemotherapy. Clinical trials of electrochemotherapy have been conducted using bleomycin (54-62) or cisplatin (63-65) against cutaneous or superficial tumors including malignant melanoma (54, 56, 59, 62-65), head and neck carcinoma (55, 58, 60, 61), basal cell carcinoma (54, 57, 62-64), squamous cell carcinoma (61-63) and adenocarcinoma (54). Lack of toxic effect in electrochemotherapy suggests that in vivo electroporation might be safely applied to patients when nucleic acids will purposes. be administered for therapeutic Electrochemotherapy and electro-gene therapy have been shown to synergistically inhibit tumors in some animal experiments (see below) (66).

4. IMPROVEMENT OF ELECTROPORATION AND OTHER NONVIRAL GENE DELIVERY SYSTEMS BY EBV-BASED PLASMID VECTORS

As described above, a nonviral gene delivery system consists of (i) nucleic acids carrying genetic information, most typically pDNA, and (ii) gene delivery methods/materials (nonviral vector). Although a variety of nonviral vectors have been devised, the other component of the nonviral gene delivery systems, pDNA, has not been very intensively studied, in an effort to improve nonviral gene transfection. Indeed, nucleotide sequence drastically affects not only intensity of transgene expression but also efficacy of gene delivery and longevity of expression. If ideal sequence elements are devised, potentially every nonviral vectors may be significantly improved in efficacy by combining them with plasmid vectors containing such elements.

We have proposed that the transfection/expression efficiency of nonviral vectors can be significantly improved by employing the EBV nuclear antigen 1 (EBNA1) gene and oriP sequence in the plasmid construct (EBV-based plasmid vector) (67-70). They were originally known as elements to support replication of EBV genome in latently infected human cells (71-73). The EBNA1 is a nuclear phosphoprotein consisting of 642 amino acids and capable of binding to specific motifs in the oriP sequence that contains dyad symmetry (DS) approximate 110 bp in length and family of repeat (FR) spanning approximately 620 bp. DS and FR contain 4 and 20 copies of EBNA1 binding consensus sequence, respectively. EBNA1 is believed to conduct DNA replication in concert with cellular cofactors, so that EBVbased plasmid vectors are maintained in transfected human cells as an autonomously replicating episome that duplicates in synchrony with host chromosomal DNA (74-80).

The facilitation of DNA replication, however, is not the only function of EBNA1/oriP. The EBNA1 also promotes transfer of oriP-bearing plasmid DNA from cytosol to the nucleus (81, 82) and its maintenance in the nucleus (83). Another function of the EBNA1 is to activate transcription through binding to oriP (84-88). These characteristics may explain the reason why plasmid vectors with EBNA1 gene and oriP enable gene transfer and expression at high rates (89).

In this regard, we estimated contribution of each activity of the multifunctional viral elements to the high efficiency of transfection. Bottom line is that transfer of pDNA from cytoplasm into the nucleus is actually the most critical step of which efficacy is quite different between conventional and EBV-based plasmid vectors (Kishida et al., manuscript in preparation). This is consistent with previous reports indicating that most pDNA molecules introduced into a cell is entrapped and degraded in the cytoplasmic and endo-lysosomal compartments without successfully transported into the nucleus, and this barrier is the critical obstacle of conventional nonviral gene delivery strategies (reviewed in (90)). We also found that replication of pDNA does not essentially contribute to the high rate transfection of EBV-plasmids ((89) and Kishida et al., manuscript in preparation), although this function may play key roles in prolonged transgene expression in cultured human cells (76, 77).

Some tumor-specific or inducible (e.g., blood glucose level-inducible) promoter/enhancer sequences can



Figure 1. Chemotherapeutic agent and Th1 cytokine may synergistically induce anti-tumor immunity in electro-chemo-gene therapy approach. See text and Ref 66 for detail.

be inserted into an EBV-based plasmid vector to drive either or both transgene and/or EBNA1 gene, so that transcriptional targeting as well as inducible gene regulation can be achieved more stringently compared with using a conventional plasmid vector (91, 92).

It has been showed that the EBV-based plasmid vectors improves delivery and expression efficiencies, both *in vitro* and *in vivo*, of variety of nonviral vectors including cationic lipid (89, 93-95), cationic polymer (91, 94, 96-99), gene gun (100), naked DNA method (89, 101, 102), and electroporation (28, 29, 37, 103, 104). Taking advantage of the high transfection efficiency, we performed preclinical gene therapy studies with EBV-based plasmid vectors in a variety of animal model systems, as reviewed in elsewhere (67-69). In brief, significant therapeutic or prophylactic outcomes were obtained against benign disorders (92, 101, 105, 106) as well as subcutaneous (37, 66, 95, 96, 98, 99, 107) and metastatic (66, 95, 108-110) tumors.

Other investigators have also employed EBVbased plasmid vectors for *in vivo* gene therapy against various diseases (reviewed in (67, 68)). Briefly, Tsukamoto *et al.* delivered dystrophin gene-encoding EBV plasmid into muscle and succeeded in prolonged expression of the transgene (111). Shibata *et al.* transfected EBV-plasmid carrying diphtheria toxin A gene into mammary carcinoma by means of electroporation (42). Yoo *et al.* used cationic emulsion and transferred the EBV-based plasmid vector encoding proinsulin gene into diabetic animals, resulting in sustained correction of hyperglycemia (112). More recently Mei *et al.* obtained sustained expression of factor VIII in muscle by transfecting the EBV based plasmid vector via electroporation (113).

To demonstrate electroporation-mediated cancer gene therapy using the EBV-based plasmid vector, we previously transfected subcutaneous melanoma in mice with EBV-plasmids carrying IL-12 and IL-18 genes, showing that the serum concentrations of the cytokines reached significantly higher levels in comparison with those obtained by transfection with conventional (non-EBV type) plasmid vectors. The cotransfection of IL-12 and IL-18 genes elevated cytotoxic T lymphocyte (CTL) killing activity against the melanoma as well as natural killer (NK) cytolytic activity, which in turn resulted in drastic suppression of the preestablished tumors (37). Another example is the electro-chemo-gene therapy approach in which we co-delivered bleomycin and IL-12 gene into subcutaneous melanoma in mice and found the treatment extremely effective in suppressing the malignancy, while significant retardation of pulmonary metastasis was also obtained by the combination therapy (66). The synergistic action between the chemotherapeutic agent and cytokine gene may be mediated through activation of dendritic cells (DCs) that may preferentially engulf apoptotic tumor cells and present tumor antigen derived from them, subsequently leading to the induction of tumor-specific T cell responses, although precise immunological mechanisms elicited by the combination treatment remain unrevealed (Figure 1).

5. THERAPEUTIC MOLECULAR TARGETING FOR CANCER BY ELECTROTRNAFERING RNAi MOLECULAR THERAPEUTICS FOR TREATMENT OF CANCER.

In contrast to the gene therapy trials that aim at eradicating cancers by adding therapeutic genes to patients (gain-of-function), loss-of-function approaches, or therapeutic molecular targeting, may provide another promising measure to treat malignancies.

RNA interference (RNAi)-based gene silencing offers a powerful means of functional analysis of genes as well as therapeutic molecular targeting for treatment of a variety of diseases. Fire et al. first demonstrated that posttranscriptional knockdown of genetic expression can be induced by double-stranded RNA in nematodes (114). RNAi has also been demonstrated in other organisms, including fungi (115), flies (116), trypanosomes (117), hydra (118), plants (119), and amphibians (120), suggesting its important roles in the development and antiviral defense mechanisms. Although RNA interference is evolutionarily conserved in mammals (121), introduction of long doublestranded RNA molecules into mammalian cells is problematic due in part to induction of the interferon response that causes general inhibition of protein synthesis and growth arrest of the cells. Elbashir et al. (122) demonstrated that RNA interference can be achieved without triggering the interferon response using two complementary oligoribonucleotide chains approximately 21 bases in length with a 2-base overhang at the 3' end of each chain (short interfering RNA; siRNA). Expression of reporter (122, 123), enzyme (124), and viral (125-127) gene could be specifically inhibited in human (122-127) and rodent (122, 123, 127) cells in culture.

RNAi in living mammalian organs was first documented by McCaffrey et al. (128) and Lewis et al. (129), who intravenously injected siRNA into post-natal mice *via* the tail vein under high pressure. Because these intravenous administration methods may allow transfer of siRNA into multiple organs (102, 130, 131), organ-specific delivery methods were required. Thus, technologies were devised to deliver siRNA or siRNA-expressing vectors into various organs, such as the skeletal muscle (132), central nerves system (133), articular synovial cells (134, 135), cardiac vessels (13), etc., suggesting that RNAi-mediated gene knockdown might be useful for molecular targeting for human diseases. Indeed, preclinical trials of therapeutic molecular targeting have been conducted against various (136-140). disorders including infectious neurodegenerative (133, 138, 141), inflammatory (134, 135), and malignant (137, 138, 142) disorders.

In therapeutic experiments for malignancies, a lot of target genes have been proposed. Cell surface growth factor receptors and signal transduction molecules are crucially involved in oncogenesis as well as survival and proliferation of neoplastic cells, so that several preclinical therapeutic RNAi trials have been conducted to knockdown these signaling molecules, including bcr-abl (143), EGF receptor (144), and ErbB2/HER2/neu (145, 146). Other important molecules for cancer cell survival are cell cycle regulatory molecules. Therefore, some studies have targeted viral proteins, i.e., human papillomavirus (HPV) E6 (147, 148) and E7 (148, 149) that interfere with p53 and Rb, respectively, while other cell cycle regulators also served as targets (150-153). Because catepsin L interacts with p21^{WAF1}, RNAi-mediated silencing of catepsin L rendered tumor cells sensitive to chemotherapeutic drugs and promoted tumor cell sensecence (154). Premature cellular senescence was also observed when expression of the helix-loop-helix transcriptional factor, Id1, was silenced to increase p16^{INK4a} (155).

But the most frequently used targets are probably the proteins involved in apoptosis signal transmission. Because tumor cells frequently express anti-apoptotic proteins, silencing of these molecules may induce apoptosis of tumor cells and/or sensitization of tumor cells to chemoand radiation therapies. These include the Fas-associated death domain-like interleukin-1beta-converting enzymelike inhibitory protein (FLIP) (156), Bcl-2 (157-160), BclxL (157, 161, 162), Mcl-1 (163), survivin (157, 164-169) and X chromosome-linked IAP (XIAP) (160, 170). Genes involved in protein stability and degradation are other molecular targets for RNAi intervention. Cks-1 (171), Skp-2 (172, 173) and E3-ubiquitin ligase receptor subunit betaTRCP1 (174) are classified into this category.

Neoangiogenesis is essentially required for growth *in vivo* of tumors. Thus, inhibition of angiogenesis provides a different RNAi therapy strategy. For this purpose, VEGF (175, 176) and VEGF receptor 2 (177) have been knocked down. In the meanwhile, proliferation, invasion, and migration of tumors are dependent on degradation of extracellular matrix. In this context, some trials have been conducted to silence urokinase-type plasminogen activator (u-PA) (178), catepsin B (179, 180), urokinase plasminogen activator receptor (uPAR) (181), matrix metalloprotease (MMP)-9 (179, 181), and heparanase (182). Tumor invasion and metastasis may also be inhibited by targeting other molecules (183-187).

Some tumors are capable of escaping from immune surveillance through particular mechanisms that play quite important roles for the survival and growth of the tumor cells in the hosts (45, 188). RNAi technology may be also useful in canceling tumor escape machinery from immune surveillance. In this regard, silencing of some tumor-derived immunoregulatory cytokines have been reported (189, 190). Other strategies aim at increasing susceptibility of tumors to chemotherapy or radiotherapy. Multidrug resistant proteins (191-196) and the molecules involved in DNA repair (197-201) are silenced in these studies.

6. THERAPEUTIC RNAI FOR MALIGNANCIES TAKING ADVANTAGE OF ELECTROPORATION

The biggest problem of RNAi cancer therapeutics is the lack of ideal methods to transfect siRNA or siRNAexpression vectors into tumors *in vivo* (137, 138, 142). Indeed, therapeutic potential against malignancies has been tested in *in vitro* experiments in most reports mentioned above (143, 144, 146-157, 159-165, 167-171, 173, 174, 178, 182-187, 189-201). Effectiveness and safety of each strategy should be assessed by *in vivo* administration experiments to achieve feasible RNAi cancer therapy. Also the most appropriate delivery system should be selected for each RNA-based therapeutic application depending on the target cell type, location of the target organ, number of treatment, etc.

Some investigators used retroviral (145, 202-205), adenoviral (166, 172, 206), adeno-associated viral (207, 208) and lentiviral (139, 172) vectors engineered to express siRNA or shRNA, while others tested nonviral transfection in vivo into tumors to deliver synthetic siRNA (158, 175-177, 209) or plasmid vectors carrying expression units for siRNA (179-181). Yano et al. used cationic liposome to administrate bcl-2-specific siRNA into the murine models bearing liver metastasis through intravenous bolus injection, as well as into the mice with subcutaneous transplant of prostate cancer via subcutaneous injection around the tumor (158). Takei et al. delivered VEGFspecific siRNA into xenograft prostate cancer model by means of atelocollagen, showing dramatic suppression of tumor growth and angiogenesis (176). Schiffelers et al. devised ligand-targeted, sterically stabilized nanoparticles to administer intravenously VEGF R2-targeting siRNA into the mice bearing tumor burdens, demonstrating selective tumor uptake of the siRNA and inhibition of tumor growth and angiogenesis (177).

Electroporation may also provide a quite powerful means to transfect the functional RNA molecules *in vivo* into tissues and tumors. First application of siRNA electro-delivery into vertebrates was performed *in ovo* into chick embryos (210, 211), as well as into postimplantation mouse embryos (212). Thereafter, electroporation has been widely used to transfer siRNA into embryos to examine functional characterization of target genes in development and morphogenesis (213-215).

We first demonstrated electrotransfer of siRNA into a postnatal mammalian organ in vivo (132). In this study, siRNA duplexes specific for luciferase GL3, enhanced green fluorescence (EGFP), or glyceraldehyde-3phosphate dehydrogenase (GAPD) genes was injected, alone or in combination with GL3 expression plasmid, into skeletal muscle of wild type or EGFP transgenic mice, immediately followed by transdermal electric pulsation. It was found that expression of the target genes was almost completely silenced in vivo in the muscle when the GL3 plasmid and GL3-specific siRNA were cotransfected, and the silencing effect sustained for more than 7 days. Endogenous genes (EGFP in EGFP transgenic mice, and GAPD) were also significantly suppressed, albeit to a lower extent in comparison with the suppression level of GL3 reporter gene (132). Thereafter it was also shown that electroporation enables transfer of siRNA into other tissues, including glomerulus (216), intestinal epithelium (217), articular synovium (134, 135) and brain (218) (reviewed in (17)).

As a molecular therapy application of electrotransfer of siRNA, Schiffelers *et al.* (134) and our group (135) succeeded in sequence specific silencing of tumor necrosis factor-alpha (TNF-alpha) in joint articular synovium of mouse (134) and rat (135) collagen-induced arthritis models, respectively, demonstrating that the joint disorder was remarkably ameliorated by silencing the inflammatory cytokine.

With regard to RNAi therapy of malignancies, we have recently reported an electroporation-mediated cancer molecular therapy approach, using synthetic siRNA targeting the microphthalmia-associated transcription factor (Mitf) that is the transcriptional factor with helix-loop-helix leucin zipper structure responsible for induction of several enzymes of melanin biosynthesis. Mitf is also crucially involved in the growth and differentiation of melanocytes (219-221). Role of Mitf in growth and survival of melanoma, however, remained controversial; some studies indicated that Mitf essentially contributed to survival and proliferation of melanoma (222-224), whereas other reports documented that Mitf induced growth suppression of, or even apoptosis in, melanoma (225-229).

We synthesized some siRNA duplexes corresponding to the Mitf cDNA sequence and transfected them into B16 melanoma in vitro and in vivo (209). When B16 cells in culture were treated with the Mitf-specific siRNA duplex that had been coupled with a lipid vehicle, the tumor cells showed a remarkable decrease in viability as well as significant apoptosis, accompanying with a reduction in the expression level of the transcriptional factor. In vivo transfection of the siRNA was performed into subcutaneous B16 transplant in syngenic mice by means of electroporation. Repetitive treatment resulted in significant growth retardation of the preestablished melanoma. Moreover, we found that a considerable proportion of the tumor cells underwent apoptosis in vivo, as demonstrated by TUNEL staining of the tumor sections. Compared to more commonly used target genes for cancer RNAi therapy, such as cell cycle regulator, signaling molecule for growth control, and cytokines, Mitf, especially Mitf-M isotype, is specific for melanomas, which may guarantee the safety of patients when the Mitf RNAi will be put into a clinical trial. Another advantage of the Mitf knockdown therapy is that Mitf silencing may induce apoptosis of tumor cells rather than cytostatic effects, while specific silencing of angiogenic or cell cycle-regulatory genes may have transient and reversible influence on tumor cells. A combination therapy targeting more than two genes may elicit more promising results.

Electroporation may be repetitively applied to cancer patients without causing any serious adverse effects. The repetitive treatment should be quite important, because transfection efficiencies achieved by a single trial hardly reach 100%. The tumor cells that fail to be transfected with siRNA may survive the treatment, but when repetitive therapy is conducted the residual tumor cells may be killed by subsequent treatments.

Category	Example
Growth factor receptors/signal transducers	bcr/abl, EGFR, Her2/neu
Viral genes that affect p53 or RB pathways	HPV E6, E7
Genes involved in cellular senescence	Id1, p16INK4a, catepsin L
Anti-apoptotic genes	FLIP, Bcl-2, Bcl-XL, Mcl-1, survivin, XIAP
Genes involved in protein stability and degradation	Cks-1, Skp-2, betaTRCP1
Genes involved in angiogenesis	VEGF, VEGFR2
Genes involved in ECM degradation	u-PA, catepsin B, uPAR, MMP-9
Immunoregulatory cytokine genes	TGF-β, IL-10
Lineage specific transcriptional factor	Mitf

Table 1. Early Research on RNAi Therapeutics of Cancer. Several genes were proposed as the targets for RNAi-based cancer molecular therapy. See text for detail and citations

7. CONCLUSIONS

The electroporation procedures may offer powerful tools to eradicate malignancies, by inducing anti-tumor immune responses, apoptosis of tumor cells, etc., through delivery of therapeutic molecular agents that exert specific gain-of-function and/or loss-offunction activities in the neoplasms. The unsolved issues include technologies to regulate the location, intensity and duration of transgene expression, and to guarantee safety for patients. As far as RNAi therapy is concerned, possible off-target effects should be taken into consideration, while safety issue of EBV-based plasmid vectors should be carefully examined. To treat the tumors in non-superficial regions, as such gastrointestinal, respiratory, and urinary tracts, peritoneal and intracranial cavities, as well as reproducible organs, appropriate devises such as catheter-equipped electrodes and corresponding pulse generators should be developed. Finally, multidisciplinary approaches should be important to eradicate malignancies in patients, and electroporationassisted gene- and RNAi-therapies may play an important part in this context.

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Abbreviations: EBV: Epstein-Barr virus; siRNA: short interfering RNA; pDNA: plasmid DNA; RNAi: RNA interference; EGFP: enhanced green fluorescence

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