Nanomedicine Based Approaches to Cancer Diagnosis and Therapy

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1. Introduction
Pharmaceutical nanoparticles were first described in 1970s, and the term “nanotechnology” is now commonly used to refer to the fabrication of new materials with nanoscale dimensions between 1 and 100 nm (Thrall 2004). Several types of nanometer scale systems such as nanoparticles, nanospheres, nanotubes, nanogels and molecular conjugates are being investigated (Lemieux et al. 2000; Liu et al. 2007; Ravi et al. 2004). The field of nanomedicine aims to use the properties and physical characteristics of nanomaterials which have been extensively investigated as novel intravascular or cellular probes for both diagnostic (imaging) and therapeutic purposes (drug/gene delivery). The sub-micron size of nanoparticle delivery systems confers distinct advantages as compared to large sized systems including targeted delivery, higher and deeper tissue penetrability, greater cellular uptake and greater ability to cross the blood-brain barrier (Kreuter et al. 1995; Vinogradov et al. 2002; Vogt et al. 2006). Therapeutic transgene(s) encoded by plasmid or chemically modified DNA can be dissolved, entrapped, chemically conjugated, encapsulated or adsorbed to the surface of nanoparticles. There are, broadly, two main types of nanosized particles with different inner structures: A. Nanoparticle/Nanosphere: Matrix composed of entangled oligomer or polymer units; and B. Nanocapsule: Reservoir consisting of a hydrophobic core surrounded by a polymer wall. Lipids can also be used to generate liposomes or micelles (discussed in detail later). These nanodevices can confer protection to the DNA against a variety of degradative and destabilizing factors, and enhance delivery efficiency to the cells while minimizing the toxic effects.
Nanoparticles are expected to play a critical role in the innovation and development of future cancer treatment modalities. Recent research has developed functional nanoparticles that are covalently linked to biological molecules such as peptides, proteins, nucleic acids, or small-molecule ligands (Alivisatos 2004; Chan et al. 2002; Michalet et al. 2005). Medical applications have also appeared, such as the use of superparamagnetic iron oxide nanoparticles as a contrast agent in the detection of lymph node prostate cancer (Harisinghani et al. 2003) and the use of polymeric nanoparticles for targeted gene delivery to tumor vasculatures (Hood et al. 2002). Target-specific drug/gene delivery and early diagnosis is currently a high priority R&D area, and one in which nanomedicine will inevitably make critical contributions. Current modalities of diagnosis and treatment of various diseases, especially cancer, have major limitations such as poor sensitivity or
specificity and high drug toxicities respectively. The success of nanoparticle delivery systems will ultimately depend on the ability to efficiently deliver the gene of interest and express a therapeutic gene(s) in tumor cells in a targeted manner in order to mitigate toxicity. This chapter examines current existing nanoparticle-based gene therapy approaches to cancer treatment, and assesses their therapeutic utility.

2. Nanobased cancer treatment strategies

Most neoplasms are derived from multiple mutations and rarely can be controlled through the targeting of a single mutation. The efficacy of gene therapy in tumor treatment will undoubtedly rely upon the simultaneous targeting of multiple cellular processes or the ability to invoke various antitumor responses. Current clinical trials in cancer exploit a variety of different treatment approaches. Tumor suppressor modalities compensate for a genetic mutation via gene transfer or replacement of an altered tumor suppressor gene (e.g. p53, BRCA1). Molecular chemotherapy, involves the transfer of a suicide gene (e.g. Herpes Simplex Virus-thymidine kinase [HSV-tk], CD::upp) targeted to specific tissues by extracellular tumor/tissue targeting strategies, and/or via tissue-specific expression. The delivered gene then makes the cell susceptible to a prodrug (e.g. gancyclovir, 5-FC) making tumor-specific expression critical. Tumor immunotherapy involves the gene transfer of cytokines (e.g. IL-2, IL-12) that impart antitumor immunomodulatory properties. Oncofactor inhibition strategies such as growth factor inhibition and oncogene inhibition (e.g. erb-B2 silencing) aim to prevent tumor progression by inhibiting key growth factors. Anti-angiogenesis therapy seeks to destroy the vasculature supplying the tumor in the hopes of starving it of essential nutrients to diminish or prevent its progression. Multi Drug Resistance associated genes strategies, involve knocking down gene associated with or conferring MDR, such as PRP-4 and survivin to improve chemosensitivity. We will examine in detail each of these strategies.

2.1 Tumor suppressor gene therapy

As a critical player in cancer onset, p53 has come to the forefront of oncological research and is now recognized to be the single most frequently inactivated gene in human cancers (Ning et al. 2011; Olivier et al. 2002; Olivier et al. 2010). The p53 gene enforces a variety of anticancer functions by encouraging cells to arrest or die in the face of DNA damage, hypoxia, oxidative stress, excessive mitogenic stimuli or denuded telomers. In addition, the protein influences several biological functions such as involvement in cell cycle regulation, programmed cell death, senescence, differentiation and development, transcription, DNA replication, DNA repair and maintenance of genomic stability. Thus, it’s not surprising that p53 is regarded as the “Guardian of the Genome” in preventing human neoplasia (Lane 1992). P53 protein has emerged as a key tumor suppressor protein in cellular stress response pathways. The p53 gene, mapped to chromosome 17p13.1, consists of 11 exons spanning over 20 kb of DNA encoding a 393 amino acid and 53 kDa nuclear protein. Its structure consists of an acidic N-terminus with a transactivation domain, a hydrophobic central DNA-binding core and a basic C-terminus with regulatory and oligomerization domains (Hainaut and Vahakangas 1997). Although greater than 90% of the isolated mutations in this gene have been localized to the DNA binding site of p53, coded by exons 5 to 8; some mutations have also been reported outside the evolutionary conserved regions (Hainaut and Hollstein
The p53 gene product is a sequence-specific nuclear transcription factor that binds to defined consensus sites within DNA as a tetramer and affects the transcription of its target genes. These target genes are involved in critical cell processes such as:

1. Growth arrest: P21, Gadd45 and 14-3-3σ.
2. DNA repair: p53R2.
3. Apoptosis: Bax, Bcl-xL, Fas, FasL, DR5/Killer, Apaf-1, Puma and Noxa.

The loss of p53 function is of relevance to a broad array of cancer types, including 15–50% of breast cancer cases, 25–70% of metastatic prostate cancers, 25–75% of lung cancers, 33–100% of head and neck cancers and 60–80% of ovarian cancers (Ruley, 1996). Furthermore, the null mutation of the gene imparts strongly unfavorable prognosis when associated with human ovarian, lung, colon and breast cancer cases. P53 also represses genes involved in tumor angiogenesis, and recent evidence suggests that tumor cells possessing a wild-type p53 allele are more sensitive to chemotherapeutic agents and radiation than p53 null mutants (Lowe et al. 1994). The pleiotropic abnormalities imparted by deficient p53 in a significant fraction of human cancers make it one of the primary candidates for cancer gene therapy, whereby the effective expression or replacement of p53 may re-establish cell growth control, restore appropriate responses to DNA-damaging agents (e.g. chemotherapy and radiotherapy) and preclude tumor angiogenesis. In human neoplasia’s that express the wild-type protein, aberrations of p53 regulators, such as MDM2, account for p53 inhibition. For this reason, improved understanding of the p53 pathway should lead to better diagnosis and treatment of cancer in the future.

The p53 gene therapeutic, Gendicine, is currently approved in China and its US counterpart, Advexin, has shown activity in number of clinical trials. In more conventional approaches a range of small drug like molecules targeting the p53 mediated system have been developed and several are now in clinical trials. Of critical importance has been the development of small-molecule inhibitors of the p53–Mdm2 protein interaction such as the Nutlins (Vassilev 2004), which have shown activity against human xenografts in preclinical models. Therefore, designing small-molecule inhibitors of the p53-MDM2 protein-protein interaction is a promising strategy for the treatment of cancers retaining wild-type p53 (Lauria et al. 2010). Advanced structural approaches have provided compelling support for the idea that some p53 mutants can be targets for small molecules that would cause them to regain wild-type function (Joerger et al. 2006). Many adenoviral vectors with cancer specific conditional replication properties have been preclinically evaluated to date. Most promising among these are: i) the Ad dl1520 that possesses a deletion of the 55-kd E1B gene limiting growth to only p53-deficient tumor cells. This strategy has shown preclinical efficacy against p53-deficient nude mouse-human ovarian carcinomatosis xenografts (Vasey et al. 2002); and ii) the integrin-targeted Ad5-D24RGD and serotype 3 receptor-targeted Ad5/3-D24 that possesses a 24 bp deletion in the retinoblastoma binding site of E1A, conferring selective replication in cancer cells that are deficient in the Rb/p16 pathway.

2.2 Molecular chemotherapy

Suicide gene therapy/molecular chemotherapy is a new experimental form of cancer chemotherapy that is currently being evaluated in human trials (Bhaumik 2011; Onion et al. 2009; Xu et al. 2009). This approach involves intra-tumoral delivery of genes encoding enzymes that convert nontoxic prodrugs into toxic anti-metabolites. Two suicide genes that are being evaluated in the clinic are the Escherichia coli CD (codA cytosine deaminase) and
HSV-1 tk (thymidine kinase) genes, which confer sensitivity to 5-FC (5-flourocytosine) and GCV, respectively. The rationale behind the suicide gene therapy approach is that after "targeted" transfer of these genes to the tumor, only tumor and neighboring cells will be rendered sensitive to their cytotoxic action. A variety of tumor models have demonstrated that both the CD/5-FC (by inhibiting thymidylate synthase) and HSV-1 tk/GCV prodrug systems (by inhibiting DNA chain elongation) can enhance the efficacy of radiation therapy, resulting in significantly better tumor control and/or cure (Rogulski et al., 1997). Suicide gene therapy is a particularly attractive approach to the treatment of cancer as it is essentially a tumor-targeted chemotherapy. As such, the systemic toxicity commonly associated with, and a major limitation of, conventional chemotherapy is avoided. Using a pair of adenoviral vectors that express a CD/HSV-1 tk fusion gene without or with the wt human p53 gene, it has been found that co-expression of p53 did not enhance the cytotoxicity of CD/5-FC or HSV-1 tk/GCV suicide gene therapies using the SK-OV-3 and Hep3B tumor models in vitro or in vivo (Xie et al., 1999). This notion is consistent with the fact that CD/5-FC and HSV-1 tk/GCV suicide gene therapies have demonstrated effectiveness against a variety of tumors that lack functional p53 (e.g., SK-OV-3, Hep3B, 9L, WiDr, U251, DU145, PC-3, C33A, and many others). Azatian et al. (Azatian et al. 2009) investigated the effectiveness of HSV-tk activation as gene therapy for gastroesophageal junction and gastric adenocarcinomas using a stress-inducible Grp78 promoter. The gastric adenocarcinoma cell line MKN-74/tk cells were completely killed when cultured with 1 µg/ml GCV for 10 days. Cell viability was also significantly lower under glucose starvation conditions when HSV-tk expression was regulated by the Grp78 promoter. Furthermore, non-viral approaches are being investigated for use in combination with suicide gene therapy for treatment of various carcinomas. Using transferrin lipoplexes, prepared from cationic liposomes and cholesterol, significant tumor reduction was achieved upon intratumoral delivery of HSV-tk or CD genes, followed by intraperitoneal injection of GCV or 5-FC, respectively (Neves et al. 2009). Enhanced apoptosis, the recruitment of NK cells, CD4 and CD8 T-lymphocytes and an increase in the levels of several cytokines/chemokines were observed within the tumors. These observations suggest that suicide gene therapy with lipoplexes modifies the tumor microenvironment, and leads to the recruitment of immune effector cells that can act as adjuvants in reducing the tumor size.

2.3 Immunomodulatory strategies
Tumor cells alter antigen presentation on their surface compromising recognition by the immune system and immunosurveillance evasion. Tumor immunotherapy strategies aim to boost the anti-tumor immune response by stimulating the cell-mediated arm of the immune system (Wojtowicz-Praga 1997). Gene therapy approaches to enhance immune responses include delivery of cytokines to the tumor, administration of tumor vaccines based on tumor-associated antigens (TAAs), upregulation of Major Histocompatibility Complexes (MHC)(Nabel et al. 1993) or co-stimulatory molecules, and inhibition of immunosuppressive molecules. Many cytokines activate the immune system, including interleukins (IL) 2, 4, 7, 12 and 18, interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), and granulocyte–macrophage colony-stimulating factor (GM-CSF), which are among the most potent inducers of anti-tumor activity in a variety of preclinical studies. More recently, some exciting new cytokines have been characterized, such as IL-21, IL-23, IL-27, and their immunomodulatory and antitumor effects in vitro and in vivo suggest that they may have...
considerable promise for future immunotherapy protocols (Weiss et al. 2007). Recent studies using animal models have shown that genetically modified tumor cells expressing cytokines such as IL-2, IL-4, IL-6, IFN-γ or GM-CSF were capable of inducing an immune response against preexisting tumors (Connor et al. 1993). In preclinical models and clinical trials, cytokines have been delivered to melanoma lesions by intratumoral injection of naked DNA, recombinant viral vectors and transduced tumor cells and fibroblasts (Sun et al. 1998). Initial clinical trials evaluating the systemic delivery of IL-2 and TNF-α were complicated by significant systemic toxicity (Rosenberg 1999), thereby generating interest in gene transfer strategies that limit cytokine production to the tumor milieu. Preclinical studies employing TAAs to generate tumor vaccines have been promising, and seem to suggest that immunization with recombinant, virus-encoding TAAs can confer suppression of growth in pre-established tumors and the production of specific cytotoxic T lymphocyte responses. However, conversion to clinical trials has failed to show significant efficacy.

The cytokine IFN-γ has been shown to up-regulate MHC expression, antigen processing, antigen processing and presentation expression in many cell populations, including dendritic cells, B cells, macrophages, and endothelial cells, producing more potent antigen presenting cells (APCs), as IFN-γ can induce multiple gene expressions that are related to MHC processing and presentation. Dendritic cells (DCs) play a vital role in the initiation of immune response as professional antigen presenting cells to T cells promoting CD8+ T-cell-mediated cytotoxic responses. Reported research in animal studies indicates that vaccine immunity may represent a promising alternative therapy for cancer patients. However, broad clinical utility has yet to be achieved, owing to the low transfection efficiency of DCs (Chen et al. 2010b). Different formulations of liposomes have been designed to improve the uptake by DCs through different receptor-mediated routes. These formulations include liposomes prepared with mannosylated phosphatidylethanolamine (Man-PE), trimethyl ammonium propane, and phosphatidylserine targeted to mannose receptor (MR), negatively charged surface proteins and phosphatidylserine receptor (PSR) of DCs, respectively. Several other immunomodulatory clinical trials are under way with cytokines currently comprising 18.3% of immunotherapy clinical trials (http://www.wiley.com/legacy/wileychi/genmed/clinical/). Table 1 provides a list of various types of genes currently involved in various gene therapy clinical trials. A number of other monoclonal antibodies; namely EC90, Removab, and CNTO-95 which target FOLR1, EpCAM and α-integrin (CD51), respectively are in Phase I/II studies, and indicate that further clinical investigation in this area is warranted (Bell-McGuinn et al. 2011;Kowalski et al. 2010).

2.4 Oncofactor inhibition strategies

2.4.1 Growth factor inhibition

The epidermal growth factor receptor (EGFR) belongs to the proto-oncogene family, which consists of four structurally-related transmembrane receptors (i.e., EGFR, ErbB2, ErbB3, and ErbB4) and is a key therapeutic target in the treatment of many types of cancer. The most extensively studied growth factors are ErbB1 (Javle et al. 2010) and ErbB2 (Nihira 2003). These ErbB/EGF receptors are tyrosine kinases and play important physiologic roles in cell proliferation, survival, adhesion, motility, invasion, and angiogenesis. High expression of EGF receptor protein is observed in several types of cancer including breast, bladder, colon, lung and gastric cancers, making them potential targets for targeted therapies, which represent some of the most successful therapeutic approaches to date (Iqbal et al. 2011;Lai et al. 2009;Yarom and Jonker 2011).
| Gene type                                      | Gene Therapy Clinical Trials | Number | %   |
|-----------------------------------------------|-----------------------------|--------|-----|
| Adhesion molecule                             |                             | 10     | 0.6 |
| Antigen                                       |                             | 334    | 20.7|
| Antisense                                      |                             | 13     | 0.8 |
| Cell cycle                                    |                             | 8      | 0.5 |
| Cell protection/Drug resistance               |                             | 19     | 1.1 |
| Cytokine                                       |                             | 317    | 18.5|
| Deficiency                                    |                             | 136    | 7.9 |
| Growth factor                                 |                             | 128    | 7.5 |
| Hormone                                       |                             | 8      | 0.5 |
| Marker                                        |                             | 54     | 3.2 |
| Oncogene regulator                            |                             | 11     | 0.6 |
| Oncolytic virus                               |                             | 37     | 2.2 |
| Porins, ion channels, transporters            |                             | 12     | 0.7 |
| Receptor                                      |                             | 113    | 6.6 |
| Replication inhibitor                         |                             | 74     | 4.3 |
| Ribozyme                                      |                             | 6      | 0.4 |
| siRNA                                         |                             | 11     | 0.6 |
| Suicide                                       |                             | 144    | 8.4 |
| Transcription factor                          |                             | 28     | 1.6 |
| Tumor suppressor                              |                             | 150    | 8.8 |
| Viral vaccine                                 |                             | 6      | 0.4 |
| Others                                        |                             | 26     | 1.5 |
| Unknown                                       |                             | 49     | 2.9 |
| Total                                         |                             | 1714   |     |

Table 1. List of various types of genes presently involved in various gene therapy clinical trials (Source: http://www.wiley.com/legacy/wileychi/genmed/clinical/).

Gefitinib and Erlotinib are small molecules that exert their function by inhibiting the intracellular tyrosine kinase domain of EGFR. Recently, the southwest oncology group
study S0413, a phase II trial of lapatinib was conducted as first-line therapy in patients with advanced or metastatic gastric cancer. Median (95% CI) time to treatment failure was 1.9 (1.6-3.1) months and overall survival (OS) was 4.8 (3.2-7.4) months. Lapatinib is well tolerated, with modest single-agent activity in advanced/metastatic gastric cancer patients. Potential molecular correlatives such as HER2, interleukin (IL)-8 and genomic polymorphisms IL-8, and vascular endothelial growth factor correlated with OS (Iqbal et al. 2011). Similarly, in other Phase II studies with single agent Gefitinib or Erlotinib only modest activity was observed. Gefitinib cytotoxic combinations are also currently under examination (Wagner et al. 2007). Further, very recently it was observed that direct covalent coupling of antibodies to glutaraldehyde activated nanoparticles is an appropriate method to achieve cell-type specific drug carrier systems based on polymeric nanoparticles that have potential to be applied for targeted chemotherapy in EGFR positive cancer (Aggarwal et al. 2011).

Trastuzumab (trade name Herceptin) is the first anticancer drug generated by Genentech, Inc. whose use as a treatment for breast cancer patients is decided based on the status of the HER2 gene amplification/HER2 protein over-expression. The development and standardization of an HER2 test was a key strategy in clinical development of this drug, since appropriate selection of patients with HER2 over-expression was critical for success. This also highlights the important and evolving era of personalized and targeted therapies. In the clinic, the success of imatinib (Gleevec®, STI571) and trastuzumab, both firsts of their kind, spurred further development of new, second-generation drugs that target kinases in cancer. Further, these targeted drugs combined with chemotherapeutic drugs are found effective and well tolerated in nanoparticle based drug delivery (e.g. albumin-bound paclitaxel combined with carboplatin and trastuzumab or as trastuzumab-dextran iron oxide nanoparticles) for the treatment of cancer (Conlin et al. 2010).

2.4.2 Signal transduction (PI3K/AKT/mTOR pathway) inhibition

Phosphoinositide 3-kinase (PI3K) is a major signaling component downstream of growth factor receptor tyrosine kinases (RTKs). PI3K catalyzes the production of the lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3) at the cell membrane. PIP3 in turn contributes to the recruitment and activation of a wide range of downstream targets, including the serine-threonine protein kinase Akt (also known as protein kinase B). The PI3K-Akt-mTOR signaling pathway regulates many normal cellular processes including cell proliferation, survival, growth, and motility—processes that are critical for tumorigenesis. Components of this pathway are frequently abnormal in a variety of tumors, making them an attractive target for anti-cancer therapy. In contrast to p53 and other tumor-suppressor pathways, the PI3K pathway is activated in cancer, making this an optimal target for therapy as it is easier to inhibit activation events than to replace lost tumor-suppressor function. Studies have demonstrated that phosphorylative activation via the mTOR pathway results in increased G1 cell cycle progression, cell survival, and tumor cell proliferation (Campbell et al. 2004; Vega et al. 2006). The mTOR pathway has been shown in preclinical studies to play a role in the carcinogenesis of breast, ovary and prostate as well as gastrointestinal malignancies. Novel analogs of rapamycin (temsirolimus, everolimus, and deforolimus), which have improved pharmaceutical properties, have been designed for oncology indications. Clinical trials of these analogs have already validated the importance of mTOR inhibition as a novel treatment strategy for several malignancies (Gibbons et al. 2009).
2.4.3 Oncogene inhibition

Gene therapy has also been employed in a reversed approach, namely by inhibition of oncogenes. The use of vectors to express anti-sense (ODN), ribozymes or small interfering RNAs (siRNA) to silence a variety of oncogenes has enabled genetic loss-of-function studies in tissue culture systems. Gene expression can be disrupted at the transcriptional (triplex DNA) or translational (antisense DNA or short inference RNA) level (Braasch et al. 2002). In the triplex DNA-based antigene approach, transcription is disrupted by the binding of a triplex-forming oligonucleotide at the promoter region of a target gene. In the antisense strategy, the oligonucleotide (ON) molecule corresponding to a target gene is delivered inside a cell where it binds to its complementary, targeted messenger RNA (mRNA), to produce a partially double-stranded ON/mRNA complex. Extensive work in this area has already led to one antisense ON product approved for local therapy of cytomegalovirus retinitis (Vitravene) and nearly twenty others in late-stage clinical trials (Cheung et al. 2010).

Silencing of specific mRNA using double stranded RNA oligonucleotides represents one of the newest technologies for suppressing a specific gene product. Small interfering RNA (siRNA) are 21 nucleotides long, double stranded RNA fragments that are identical in sequence to the target mRNA. Silencing of specific gene targets, such as cancer-associated mutations in oncogenes and their amplification in tumors therefore represents promising therapeutic approach in treating cancer, and strategies focusing on classic oncogenes (such as bcl-2, ras and HER-2/neu) have been extensively explored in esophageal, breast, ovarian and other carcinomas. Other candidate targets may include genes associated with cell proliferation, metastasis, angiogenesis, and drug resistance. Very recently, Liang et al., (2011) showed that DNA vector-based Stat3-specific RNA interference (si-Stat3) blocks Stat3 signalling which was delivered by hydroxyapatite nanoparticles and suppresses mouse prostate tumor growth in vivo. In this study, the Stat3 downstream genes Bcl-2, VEGF and cyclin D1 were also strongly downregulated in the tumor tissues that also displayed significant increases in Bax expression and Caspase3 activity (Liang et al. 2011). While the application of antisense technology to the treatment of human cancer is conceptually straightforward, selection of appropriate gene targets is an important parameter in the potential success of siRNA cancer therapies (Ashihara 2010), and in practice there are many complicated, mechanistically based questions that must be considered. Importantly, folding of target RNAs or their association with specific proteins in the cell often prevents the ON molecules from binding to their targets. In addition, silencing of such genes must not affect the functions of normal cells.

2.5 Anti-angiogenic therapy

The ability of solid tumors to grow locally, and subsequently disseminate to distant organs, is dependent upon the formation of new blood vessels (Azad et al. 2008) and involves angiogenic factors such as vascular endothelial growth factor (VEGF). VEGF plays an important role in the proliferation of cancer cells, angiogenesis and vascular permeability in peritoneal dissemination. BevacizumAb, the only United States Food and Drug Administration (FDA)-approved anti-VEGF agent, is a monoclonal antibody that inhibits the binding of VEGF to VEGF receptors. CBO-P11, a cyclo-peptide, has proven to specifically bind to receptors of VEGF and may be used as targeting ligand for tumor angiogenesis. Deshayes et al., (2011) investigated the conjugation of CBO-P11 on the surface of poly(vinylidene fluoride) nanoparticles using the copper(I)-catalyzed Huisgen 1,3-dipolar...
cycloaddition known as a “click” reaction. Nanoparticles were found to be spherical, dense, monodisperse and stable. No cytotoxicity was observed after four days of incubation demonstrating the biocompatibility of nanoparticles. Fluorescence highlighted the specific interaction of these functionalized nanoparticles for VEGF receptors, suggesting that the targeting peptide bioactivity was retained (Deshayes et al. 2011). Therapies based on mAb targeting and blocking of vascular endothelial growth factor, such as that conferred by the monoclonal bevacizumAb, have clearly demonstrated remarkable antitumor efficacy, although the mechanism of action here is not well understood. Combined treatment of bevacizumAb with Sorafenib (a small molecular inhibitor of several tyrosine protein kinases such as VEGFR and PDGFR) resulted in partial response or disease stabilization for ≥ 4 months (median, 6 months; range, 4 to 22+ months) in 22 (59%) of 37 assessable patients with advanced solid tumors.

Matrix metalloproteases (MMPs) are a family of more than 20 zinc and calcium dependent enzymes that degrade all major basement membrane and extracellular matrix components. These enzymes also promote tumor invasion and metastasis, regulating host defense mechanisms and normal cell function. They are well adapted to serve as signaling conduits in the tumor-stromal microenvironment given their crucial roles in the complex processes of tissue invasion, blood vessel homeostasis, and metastasis. Specifically, MMP-1, MMP-2 and MMP-9 mRNA expression in multivariate analyses has been correlated with a well-characterized clinical significance in solid tumors but have no such role in effusions, suggesting the clinical role of MMP is limited to solid lesions. MMP inhibitors (MMPIs) are expected to be useful for the treatment of diseases such as cancer, osteoarthritis, and rheumatoid arthritis. A vast number of MMPIs have been developed in recent years. With the failure of these inhibitors in clinical trials, more efforts have been directed to the design of specific inhibitors with different Zn-binding groups (Tu et al. 2008).

2.6 Multidrug resistance inhibition/gene transfer strategies

Cancer cells counter the influx of a chemotherapeutic by draining the drug from cells, deactivating the protein that transports the drug across cell walls, restoring DNA breaks, or developing some other mechanism to deactivate the drug. Multidrug resistance continues to be a major problem in the management of cancer and knockdown of drug resistance associated genes such as MDR1 (ABCB1), survivin, and pre-mRNA processing factor-4 (PRP-4) has been attempted in cancer cell lines. PRP-4 belongs to the serine/threonine protein kinase family, plays a role in pre-mRNA splicing and cell mitosis whereas survivin, a member of the inhibitor of apoptosis family of proteins, is implicated in both apoptosis inhibition and cell cycle control. Recently, Chen et al., (2010) developed two nanoparticle formulations, cationic liposome-polyacetylene-DNA (Whitmore et al. 1999) and anionic liposome-polyacetylene-DNA (LPD-II), for systemic co-delivery of doxorubicin (Dox) and a therapeutic small interfering RNA (siRNA) to multiple drug resistance (Hesdorffer et al. 1998) tumors. In this study, they have provided four strategies to overcome drug resistance. First, the investigators formed the LPD nanoparticles with a guanidinium-containing cationic lipid, i.e. N,N-distearyl-N-methyl-N-2-(N'-arginyl) aminoethyl ammonium chloride, which can induce reactive oxygen species, down-regulate MDR transporter expression, and increase Dox uptake. Second, to block angiogenesis and increase drug penetration, the authors have further formulated LPD nanoparticles to co-deliver vascular endothelial growth factor siRNA and Dox. An enhanced Dox uptake and a therapeutic effect were observed when combined with vascular endothelial growth factor siRNA in the
nanoparticles. Third, to avoid P-glycoprotein-mediated drug efflux, they further designed another delivery vehicle, LPD-II, which showed much higher entrapment efficiency of Dox than LPD. Finally, the authors delivered a therapeutic siRNA to inhibit MDR transporter. Three daily intravenous injections of therapeutic siRNA and Dox (1.2 mg/kg) co-formulated in either LPD or LPD-II nanoparticles showed a significant improvement in tumor growth inhibition (Chen et al. 2010a). Numerous other similar studies highlights a potential clinical use for these multifunctional nanoparticles with an effective delivery property and a function to overcome drug resistance in cancer.

3. Delivery systems

Over the past 20 years, a variety of techniques have been developed for encapsulating both conventional drugs (such as anticancer drugs and antibiotics) and new genetic drugs (plasmid DNA containing therapeutic genes, antisense oligonucleotides and small interfering RNA) within nanoparticles. Nanoparticle delivery systems for gene delivery possess useful inherent attributes, including a diameter of approximately 100 nm or less, a high drug-to-lipid ratio (in the case of lipid based systems), excellent retention of the encapsulated drug, and a long (> 6 h) circulation lifetime. These properties permit nanoparticles to protect their contents during circulation, prevent contact with healthy normal tissues, and accumulate at sites of disease.

3.1 Viral and non-viral delivery systems

A major impediment to the successful application of gene therapy for the treatment of a range of diseases is not a paucity of therapeutic genes, but the lack of an efficient non-toxic gene delivery system. Gene delivery is generally accomplished using one of two categories of delivery vector: 1) Viral delivery vectors derived from engineered retrovirus, adenovirus, herpesvirus, lentivirus or hybrid retro/adeno virus), and 2) Non-viral (synthetic) vectors comprised of polymers and lipids, naked DNA, plasmid-protein conjugates. Other (physical as opposed to chemical) methods include microneedle coating, gene gun, and ultrasound/microbubble-mediated gene delivery.

Viral vectors account for nearly 75% of all clinical trials conducted thus far (http://www.wiley.co.uk/genetherapy/ clinical). These vectors are essentially viruses that have been genetically modified to remove any replication/pathogenic genes, and instead encode a gene of interest (Hesdorffer et al. 1998). This strategy preserves the viruses highly efficient ability to infect cells, while eliminating/attenuating toxicity. The most commonly used viral vectors are retroviral, adenoviral, adeno-associated viral and herpes simplex viral-based (Young et al. 2006). Although Vitravene, an antisense oligonucleotide-based product, is the only gene delivery product approved so far by US-FDA, there are several other products in late stages of clinical trials. Gendicine, an adenovirus encoding the \( p53 \) gene, developed by SiBiono GeneTech Co., Ltd., was recently approved by China’s state Food and Drug Administration, for the treatment of head and neck squamous cell carcinoma. Virus-based treatments tend to maximize efficiency of gene transfer, often at the cost of safety, while non-viral options generally capitalize on a higher safety profile, but usually at the expense of Gene of Interest (GOI) expression efficiency. Many viruses including retroviruses, adenoviruses, herpes simplex viruses, adeno-associated viruses (AAV) and pox viruses have been modified to eliminate their toxicity, maintain their high gene transfer capability and long term gene expression.
However, despite these advantages, there are currently major limitations to the use of viruses as gene delivery vectors. These include the limited carrying capacity of transgenic materials (i.e. size of plasmid) since the packaging capacity of viral vectors (usually up to 5 kb) is constrained; the potential for oncogenesis due to chromosomal integration or activation of oncogenes/inactivation of oncogene regulators; the generation of infectious viruses due to recombination; and concerns regarding instability that challenge production and storage.

The potentially hazardous nature of viral vectors has been observed in a number of gene therapy-related patient mortalities in various clinical trials. Patient complications to date have included the rejection of DNA carriers, resulting in immune response that have led to already one death, Jesse Gelsinger, who died in 1999 from a rare metabolic disorder during a gene-therapy clinical trial at the University of Pennsylvania (Branca 2005; Ledford 2007; Stolberg 1999; Wilson 2010). DNA constructs need to be optimized to carry minimal immunogenic components without compromising both efficient and long-term transgene expression. In this context, technology employing minimal immunological defined gene expression technology (MIDGE) represents a promising future alternative to conventional plasmids in terms of biosafety, improved gene transfer, potential bioavailability, minimal size and low immunogenicity associated with these chemically engineered miniDNA vectors. MIDGEs are non-viral, lcc (linear covalently closed) miniplasmids synthesized in vitro via a patented chemical modification of linear open (lo). These plasmids confer the advantage of a minimal coding sequence that relieves complications from expression of additional unwanted genes and CpG motifs that have a 20-fold higher occurrence in bacterial cells. MIDGE lcc plasmid transfection expression was reported to increase luciferase transgene expression from 2.5 to 17 fold ex vivo compared to circular covalently closed (ccc), isogenic forms in a tissue-dependent manner (Schakowski et al. 2007). In addition, the mean numbers of MIDGE vector molecules per cell was also found to be significantly higher, suggesting that linear lcc plasmids transfect cells more efficiently. MIDGE technology has already been applied, with promising results, to the development of a Leishmania DNA vaccine and a colon carcinoma treatment. Here, therapy was based on IL-2 delivery to specific tumor cell lines ex vivo, and revealed 2 to 4 fold higher relative transgene expression compared to the ccc control (Schakowski et al. 2001). In combination, the existing studies employing lcc DNA systems suggest that lcc DNA is superior to lo DNA in transfection efficiency, and to ccc DNA in transgene expression. Purified TelN (closely related to Tel) was previously reported to generate lcc plasmids in vitro in (pal-related) telRL-dependent manner and was shown to successfully deliver EGFP to human embryonal kidney cells in vitro, as well as IL-12 in an untargeted manner to inhibit metastasis formation in B16F10C57BL/6 melanoma model mice in vivo (Heinrich et al. 2002).

At present, our laboratory is involved in design, and construction of bacteriophage-encoded recombination systems to generate linear covalently closed (lcc) DNA mini-vectors. Conditional expression recombinase systems in vivo in E. coli provide a one step production system for step minivectors from specialized parental plasmids. The exploitation of this recombination system allows us to generate lcc miniplasmids that should have a preferential safety profile, preventing viable vector-chromosome, single recombination products in mammalian cells. In theory, the integration of covalently closed linear exogenous DNA into double-stranded (ds) DNA breaks is unlikely due to the unavailability of terminal ends, and a vector single recombination event into a target
chromosome should theoretically result in the disruption of the chromosome, killing the cell (see Figure 1).

Fig. 1. Plasmid Integration Events: A miniplasmid that undergoes a single recombination event with the host chromosome should be rare due to the removal of all elements except the gene of interest. Any integration events by: A. A lcc miniplasmid should result in chromosomal disruption that is likely lethal and cannot be replicated or segregated; B. A ccc miniplasmid can integrate without disrupting the host chromosome.

Non-viral gene delivery is emerging as a realistic alternative to the use of viral vectors with the potential to have a significant impact on clinical therapies. Synthetic vectors provide flexibility in formulation design and can be tailored to the size and topology of the DNA cargo and the specific route of vector administration, and can be delivered selectively to a specific tissue type through the incorporation of a targeting ligand. Compared to viral vectors, synthetic vectors are potentially less immunogenic, relatively easy to produce in clinically relevant quantities, and are associated with fewer safety concerns (Liu and Huang 2002). As a result of these advantages, their use has rapidly moved from transfection of cell cultures to clinical cancer gene therapy applications.

Synthetic vectors designed for parenteral administration encompass a wide range of formulations. These include unmodified (naked) DNA, which is designed for direct intra-tissue injection, cationic polymer–DNA complexes, cationic lipid–DNA complexes, and cationic polymer–lipid–DNA ternary complexes (lipopolyplexes), which are mostly aimed at systemic administration.

3.1.1 Naked DNA

The simplest employed method of transgene delivery is the injection of naked DNA. It shows very little dissemination and transfection at distant sites following delivery and can be re-administered multiple times into mammals (including primates) without inducing an antibody response against itself (i.e., no anti-DNA antibodies generated) (Wolff and Budker, 2005). The simplicity of this approach is offset by serious limitations such as inefficient uptake of the therapeutic gene into the target cells and rapid clearance of the DNA from the circulation. Direct in vivo gene transfer with naked DNA was first demonstrated by efficient transfection of myofibers following injection of mRNA or pDNA into skeletal muscle (Wolff JA, 1990). This novel approach was heralded as a superior method of in vivo transfection and its application was later advanced to confer high level expression in hepatocytes in mice by the rapid injection of naked DNA in large volumes into the tail vein (Zhang et al., 1999). This hydrodynamic tail vein (HTV) procedure has proven very useful not only to gene expression studies, but also more recently for the delivery of siRNA (Lewis et al., 2002; McCaffrey et al., 2002). Ultrasound-mediated eruption of polyethylene glycol (PEG)-modified liposomes loaded with naked plasmid-DNA is also a feasible and efficient technique for gene delivery (Negishii et al. 2010).
3.1.2 Cationic liposomes

Cationic liposomes are one of the most efficient, and among the most widely used non-viral vector systems. They are composed of positively charged lipid bilayers that can be complexed to DNA through electrostatic interactions resulting in complexes, termed lipoplexes. The application of cationic liposomes to gene therapy was first described in 1987 by Felgner (Felgner et al. 1987). The lipoplexes are generally composed of a positively charged lipidic component (see Figure 2), such as dioleoylpropyltrimethylammonium chloride, dioleoyl triethylammonium propane (DOTAP), or dimethylaminoethane carbamoyl cholesterol (DC-Chol), that is capable of complexing and condensing the DNA (Giatrellis et al. 2009). Most lipoplex formulations include a “helper” lipid, such as dioleoylphosphatidyl-ethanolamine (DOPE, as seen in Figure 2), or cholesterol that provides added stability to the lipoplexes and enhances DNA release from endosomal compartments. While transfection activity of lipoplexes is shown to vary depending on their DNA/cationic lipid ratio, it is necessary for them to be positively charged to interact with the negatively charge cell membranes and exhibit transfection activity. Lipoplex uptake occurs through endosome formation (via a number of mechanisms including caveosomes, clathrin dependent, etc.), followed by disruption of the endosomal membrane by fusion with, or incorporation of the lipoplex lipids resulting in DNA release.

The advantages of liposomes in delivery system designs include their simplicity in preparation, ability to complex relatively large amounts of DNA, versatility for use with any size or type of DNA/RNA, ability to transfect non-dividing cells and overall stability (Dutta et al. 2010). In addition, lipids are non-immunogenic allowing for repeated administration without adverse immunologic reaction (Barron et al. 1999). The primary disadvantages of lipoplex delivery vectors include low tumor transfection efficiency and lack of tumor specificity.

In order to enhance gene transfection, based upon structure-activity relationship, various gemini surfactants (Gemini surfactants consist of two hydrophobic chains and two polar headgroups linked chemically by a spacer group) have been designed. In order to enhance gene transfection based upon structure-activity relationship, various gemini surfactants have been designed, synthesized and tested for gene delivery in our laboratory (Donkuru et al. 2010; Wang and Wettig 2011; Wettig et al. 2007a; Wettig et al. 2007b; Wettig and Verrall 2001). Recent reports have shown that obstacles can be overcome by exploiting receptor-mediated endocytosis for highly efficient internalization of ligands naturally employed by eukaryotic cells. Advances along this line include the conjugation of mAbs (“immunoliposomes”), ligands such as growth factors, or hormones to liposomes to confer targeting capability. Nanoparticle based anti-cancer gene/drug delivery first reached clinical trial in mid 1980s and the first nanomedicine Doxil® (liposomal encapsulated doxorubicin) was marketed in 1995. Other example of liposome-mediated drug delivery include daunorubicin (Daunoxome), which is also currently being marketed as liposome delivery systems. Numerous new nanoparticle based cancer gene therapy systems are under development.

3.1.3 Polyplexes

Polyplexes differ from lipoplexes in that they are comprised of charged complexes of plasmid DNA and a cationic polymer, such as poly-L-lysine (PLL), polyethylenimine, polyamidoamine (starburst) dendrimers, and chitosan with a net positive charge (See Figure 2). Cationic polymers differ from cationic lipids primarily in that they do not contain
a hydrophobic moiety and are completely soluble in water. A wide variety of cationic polymers that transflect cells in vitro have been characterized (Midoux et al., 2008). A key determinant of polyplex gene transfer efficiency is the positive (on amine nitrogen atoms in the polymer) to negative charge ratio or the related negative to positive (N/P) ratio. Given their polymeric nature, cationic polymers can be synthesized in different lengths, with different geometry (linear versus branched), and with substitutions or additions of functional groups with relative ease and flexibility, which opens the way to extensive structure/function relationship studies.

Fig. 2. Examples of commonly used lipids (DOPE and DOTAP) and polymers (PEI and poly-L-lysine) in gene therapy.

Polymer-based nanoparticles are now widely used for gene and drug delivery and targeted therapy. One of the most widely applied cationic polymers used for DNA transfections is polyethyleneimine (Choosakoonkriang et al. 2003). DNA complexation with PEI, has not been found to result in an alteration of DNA conformation, remaining essentially in the B form, and the utility of PEI as a gene delivery vector has been demonstrated in numerous studies (Jere et al. 2009; Moore et al. 2009). High molecular weight PEI has been shown to be one of the most successful polymeric vectors due to the large number of protonatable amine groups that result in an enhanced ability to escape from the endosome following uptake by the cell via the so-called “proton sponge” effect. This benefit is contrasted by the high level of cellular toxicity also imparted by the number of amine groups within the polymer. Attempts to overcome this increased level of toxicity have involved using low molecular weight PEI; however, transfection efficiencies are directly correlated with decreases in molecular weight while the tendency to aggregate can increase with decreasing polymer molecular weight. Another successful strategy has involved the shielding of the polyethylenimine/DNA core with a shell of polyethylene glycol (PEG). This approach results in the formation of a dense hydrophilic
outer surface of the complexes, reducing hydrophobic interactions with serum proteins and components of the reticulo-endothelial system (RES). In addition to decreasing toxicity of the polyplexes, this strategy also confers increased biocirculation times and therefore, further increases transfection efficiencies. [Please see van Vlerken et al. 2007 for a recent review of PEG modification of nanocarriers]. The presence of the terminal alcohol groups in this PEG outer shell also provides sites for further modification that have been conjugated to various types of targeting ligands, including glycoprotein, transferring, carbohydrates, folate and epidermal growth factors, facilitating tissue-specific gene delivery (Guo and Lee 1999; Han et al. 1999; Lai et al. 2009). Such complexes have been found to mediate efficient gene transfer into tumor cell lines in a receptor-dependent and cell-cycle-dependent manner. While the modifications described here have resulted in significant improvements in both the transfection efficiencies and toxicity profiles of polycation-based transfection vectors, a number of questions relating to mechanisms of endosome escape (Tros, I et al. 2010), structure-activity relationships, pharmacokinetics, and in-vitro vs in-vivo application remain (Jere et al. 2009). Overall, while proving to be a very promising strategy toward gene therapy design, polyplex systems still require much further testing and improvement prior to entering clinical trials (Midoux et al., 2008).

3.1.4 Lipopolyplexes

Lipopolyplexes (lipid-polymer-DNA complexes or LPDs) combine plasmid DNA with both a cationic polymer and liposomes via electrostatic interactions. In general, these vectors are compact particles that exhibit superior colloidal stability, reduced cytotoxicity, and provide elevated transfection efficiency compared to either polyplexes or lipoplexes alone. The cationic polymer may be covalently linked to the liposomes (e.g. lipopolysine) or be non-covalently incorporated into a ternary lipid–polymer–DNA complex by a charge-mediated self-assembly process. The polycation component facilitates the optimal condensation of plasmid DNA, whereas lipidic components, to which targeting ligands can be attached, further stabilize the vector formulation and mediate the efficient endosomal escape of the vector following cellular internalization. LPD particles prepared using protamine as the cationic polymer and DOTAP/Chol cationic liposomes have been reported to inhibit tumor growth following i.v. administration in mice (Whitmore et al. 1999). Both in vitro and in vivo studies have demonstrated improved outcomes of (liposomes/protamine/DNA) LPD-mediated gene transfer over conventional liposomes (El-Aneeed 2004). It is believed that the small size of LPD (100 to 250 nm, which is almost three to five times less than conventional lipoplexes) will facilitate endocytosis and increase the in vivo circulating half life.

4. Challenges in nanoparticle based gene therapy

To facilitate efficient gene expression, delivered plasmid DNA must initially circumnavigate various barriers to cellular and nuclear entry as seen in Figure 3. The lipoplex/polyplex must first be internalized by the cell membrane, for which there are many different possible routes including receptor mediated endocytosis, pinocytosis and phagocytosis (Godbey and Mikos 2001). Receptor-mediated endocytosis or clathrin-dependent internalization is the most common of these and can be exploited to engineer polyplexes to express attached ligands to facilitate this process (Morille et al. 2008).
Pinocytosis is the process by which cells internalize liquids which contain suspended or soluble particles. Untargeted polyplexes that interact with the cell membrane electrostatically may be internalized via this pathway. Phagocytosis is another possible method of internalization involving the ingestion of larger particles greater than 0.5 μm in diameter.

Targeting and internalization of microspheres to phagocytic cells in vivo can be achieved through size exclusion. It is important to note that internalization mechanisms may also be largely dependent on the cell type, the vector used, and the process parameters of that a particular vector system (Duncan et al. 2006). On the cellular level, many complexes become buried in the endolysosomal compartment or are degraded in the cytoplasm. After internalization occurs, the lipoplex/polyplex is believed to be most commonly contained within an endocytic vesicle after which it is transferred to late endosomes and lysosomes. In these compartments the pH rapidly changes to the range of 4.5–6, and in order for successful transfection to occur the DNA must find a way to escape from these structures and reach the nucleus as DNA that remains in these cellular compartments is readily degraded (Pack et al. 2005). The final obstacle in the DNA vector’s journey is the double bilayer membrane surrounding the nucleus, or nuclear membrane. While small molecules may gain access to the nucleus directly through its network of pores, larger molecules must be internalized by specific nuclear import proteins. This process is largely dependent on the size of the DNA and its conformation. As such, in the absence of specialized enhancer sequences, nuclear import of plasmids is limited to actively dividing cells undergoing mitosis.

In gene therapy studies, nuclear localization signal (NLS) peptides have been investigated as facilitators of nuclear transport with the aim of enhancing transgene expression. In order to improve overall transfection specificity and efficiency it is necessary to optimize intracellular trafficking of the DNA complex as well as the performance after systemic administration (Schatzlein 2001). Properties of vector such as size, shape, and surface characteristics can also have a major impact on its pharmacokinetic properties and delivery efficiency. For most nanoparticles, it is unknown what size and/or charge of nanoparticles could lead to defects in DNA transcription and chromosomal damage and aberration. However, there are indications that certain types of nanoparticles are capable of causing DNA damage, where the composition and the coating of nanoparticles are likely the key factors imparting genotoxic effects.

With respect to systematic in vivo applications, nanocarrier approaches face additional hurdles. First, despite advances in using PEG or other hydrophilic polymers for extracellular stability to prolong their circulation in the blood stream, a large fraction of the injected dose of nanoparticles accumulates in the liver and is taken up by hepatic phagocytes. Second, due to the vascular endothelial barrier, nanoparticles can only reach certain tissues such as the liver, spleen, and some types of tumors as a result of enhanced permeability and retention (an effect due to the presence of fenestrated endothelium in tumor blood vessels i.e passive targeting) effect, where the nanoparticles tend to accumulate in tumor tissues much more than in normal tissues. However, nanoparticles cannot, or rarely access, parenchymal cells in most normal tissues as they are simply excluded by the endothelial barrier. Thus, many potential disease targets cannot at present be addressed by existing nanocarrier approaches.
Fig. 3. A schematic generalized diagram of folate receptor/ non-receptor mediated non-viral delivery system: The tethers connecting the folate to the lipid headgroups consist of 250 Å long polyethyleneglycol spacers. Condensation/ complexation of DNA-based therapeutics with DNA delivery vector; (B, C) Non-specific adsorption to cell membrane and cellular internalization via non-receptor mediated (passive entry) or folate-receptor (folate tittered PEGylated liposome’s shown in yellow balls and encapsulated drugs as red stars) mediated endocytosis pathway; (D) Monoclonal antibody-antigen complex mediated endocytosis pathway; (E) Uptake and degradation via endosome, lysosome; (F) Low pH dependent destabilization of endosome membrane; (G) Cytoplasmic release of plasmid DNA and drug efflux; (H) Nuclear targeting via nuclear localization signals, transcription and transgene expression.
4.1 Targeted nanomedicine

Construction of organ-targeted gene delivery vectors is a promising route to improve the safety and efficacy of nanomedicine based cancer gene therapy. There are a variety of ‘vector targeting’ strategies, that can be accomplished using transcriptional targeting, transductional targeting, or ideally, a combination of these. While transcriptional targeting refers to the use of gene regulatory elements (promoters and enhancers) to restrict gene expression to specific cells, transductional targeting refers to the delivery of DNA to specific cells. Targeted gene expression has been analyzed using tissue-specific promoters (breast-, prostate-, and melanoma-specific promoters) and disease-specific promoters (carcinoembryonic antigen, HER-2/neu, Myc-Max response elements, DF3/MUC). The addition of a ligand (i.e., folate, transferrin, RGD peptide, among others) to the nanoparticle surface, thus targeting the DNA to cells in vivo has been demonstrated quite successfully. Folate-receptor-targeted liposomes have proven effective in delivering doxorubicin in vivo and have been found to bypass multidrug resistance in cultured tumor cells (Immordino et al. 2006). Hong et al., (2010) exploited the possibility of combination of the functions of passive and active targeting by transferring-PEGlyated nanoparticles (Tf-PEG-NP), as well as sustained drug release in tumor by PEGylated drug for most efficient tumor targeting and anti-tumor effects enhancement. Such Tf-PEG-NP loaded with PEGylated drug conjugates could be one of the promising strategies in nanomedicine to deliver anti-tumor drugs to tumor (Hong et al. 2010). Further enhancement of the therapeutic index may also be achieved by overcoming barriers both at cellular and nuclear levels. In gene therapy studies, nuclear localization signal peptides have been investigated as facilitators of nuclear transport with the aim of enhancing transgene expression. Selective tumor targeting with minimal toxicity using folate modified, incorporating nuclear localization signal represents a popular approach. In recent years, Poly(ε-caprolactone)/poly(ethylene glycol) (PCL/PEG) copolymers which are biodegradable and amphiphilic, are also emerging as a potential nanoplatform for anticancer agent delivery (Gou et al. 2011).

5. Nanobased cancer diagnosis approaches

Current problems and unmet needs in translational oncology include (i) advanced technologies for tumor imaging and early detection, (ii) new methods for accurate diagnosis and prognosis, (iii) strategies to overcome the toxicity and adverse side effects of chemotherapy drugs, and (iv) basic discovery in cancer biology leading to new knowledge for treating aggressive and lethal cancer phenotypes such as bone metastasis. Advances in these areas will undoubtedly form the major cornerstones for a future medical practice of personalized oncology. Cancer detection, diagnosis, and therapy will be tailored to each individual's tumor molecular profile and used in predictive oncology, whereby genetic/molecular markers will play an essential role in the prediction of disease development, progression, and clinical outcomes.

The probability of a successful treatment modality increases dramatically if tumor cells can be selectively removed before they evolve to their mature stages and metastases production. As such, novel and more sensitive diagnostic tools like metallic and semiconducting nanoparticles are being developed with the aim of improving the early and noninvasive detection of rising malignancies and the accuracy of tumor tissue localization. Paramagnetic nanoparticles, quantum dots, nanoshells and nanosomes represent some of these new
technologies, used for diagnostic purposes (See Figure 4). Compared to conventional materials, inorganic nanomaterials provide several advantages such as simple preparative processes and precise control over their shape, composition and size. These systems provide promising potential not only in diagnostics, but also as delivery systems for therapeutic agents and are discussed in detail below.

Fig. 4. Nanoparticles used in cancer diagnosis and treatment. Liposomes contain amphiphilic molecules, which have hydrophobic and hydrophilic groups that self-assemble in water. Gold nanoparticles are solid metal particles that are conventionally coated with drug molecules, proteins, or oligonucleotides. Quantum dots consist of a core-and-shell structure (e.g., CdSe coated with zinc and sulfide with a stabilizing molecule and a polymer layer coated with a protein). Fullerenes (typically called “buckyballs” because they resemble Buckminster Fuller’s geodesic dome) and carbon nanotubes have only carbon-to-carbon bonds.

5.1 Quantum dots
Quantum Dots (QDs) are a unique class of light emitting semiconductor nanoparticles ranging from 2-10 nanometers in diameter and are becoming highly popular for biological imaging due to their high intensity and stable fluorescence profile (most QDs are approximately 10-20× brighter than organic dyes). QDs usually consist of a CdSe core surrounded by an inorganic shell composed of ZnS (Pinaud et al. 2010). For biological imaging applications, they are given hydrophilic coatings of PEG or multiple carboxylate groups.
Compared to the commercially available organic dyes and fluorescent proteins used in medical imaging, QDs provide many advantages (Park et al. 2009; Pic et al. 2010; Rogach and Ogris 2010; Yong et al. 2009). The first and foremost important feature is the long-term photostability of QD imaging probes, which opens the possibility of investigating the dynamics of cellular processes over time, such as continuously tracking cell migration, differentiation, and metastasis. In addition, QD emission wavelengths are size-tunable and extend from visible to near infrared (NIR) (650 nm to 950 nm), to take advantage of the improved tissue penetration depth and reduced background fluorescence at these wavelengths. For example, CdSe/ZnS QDs of approximately 2 nm in diameter produce a blue emission, while QDs approximately 7 nm in diameter emit red light. While fluorescence imaging is often limited by the poor transmission of visible light through biological tissue, there is a NIR optical window in most biological tissue that is suitable for deep tissue optical imaging, where only a few organic dyes emit brightly in this region and generally suffer from photobleaching. In contrast, the novel optical properties of QDs allow the synthesis of bright and stable fluorescent labels that emit in the near infra red spectrum by adjusting their size and composition.

Surface functionalization using peptides, proteins and antibodies, confers the ability of QDs to provide high biological compatibility and capacity to target and image tumors in living subjects through the rapid readout of fluorescence imaging. Moreover, QDs allow imaging of deeper tissues and is also used to image lymph nodes and blood vessels in tissues. A key property for in vivo imaging is the unusual QD Stokes shift (measured by the distance between excitation and emission peaks), which can be as large as 300-400nm depending on the wavelength of the excitation light, which can be used to further improve the detection sensitivity. Organic dye signals with a small stoke shift are often buried by strong tissue autofluorescence, whereas QD signal with large Stokes shift are clearly detectable above the background. Unlike traditional dyes which usually show a broad emission band, QDs exhibit narrow sharp emission peaks and broadband absorption, which are ideal for multiplexed multicolor imaging. QDs are thus able to increase the number of labels that can be used simultaneously in a single system. The effective brightness per probe particle is also superior with quantum dots as evidenced by their large molar absorption cross-sections which are a consequence of their nanometer size and composition. Different from “soft” organic nanoparticles (e.g., polymers, micelles, liposomes), inorganic nanomaterials with rigid cores usually show inefficient extravasation inside tumors. A number of reports suggest that QDs tend to stay within the tumor vasculatures without getting into the interstitial space or tumor cells, reducing the nonspecific tumor cell labeling in angiogenesis imaging (Liu and Peng 2010). The long term photostability and superior brightness of QDs make them appealing for live animal targeting and imaging. These properties have made QDs a topic of intensive interest in cancer biology, molecular imaging, and molecular profiling.

The ability to functionalize as well as control the surface of quantum dots with specific linkers and multi-functional molecules is critical for nanoparticle-based gene therapy. Currently, QDs are used both as a transfection vector as well as a fluorescence label in RNA interference research. Quantum dot conjugates have been successfully used for targeted silencing of bcr/abl gene by RNA interference in human myelogenous leukemia K562 cells (Zhao et al. 2010). In addition, Derfus et al., (2007) using PEGylated quantum dot core as a scaffold, and conjugating siRNA and tumor-homing peptides (F3) to functional groups on the particle's surface found that the homing peptide was required for targeting.
internalization by tumor cells, and that siRNA cargo could be co-attached without affecting the function of the peptide (Derfus et al. 2007). Using an EGFP model system, the role of conjugation chemistry was also investigated, with siRNA attached to the particle by disulfide cross-linkers showing greater silencing efficiency than when attached by a non-reducible thioether linkage. Delivery of these F3/siRNA-QDs to EGFP-transfected HeLa cells and release from their endosomal entrapment led to significant knockdown of EGFP signal. By designing the siRNA sequence against a therapeutic target (e.g., oncogene) instead of EGFP, this technology may be ultimately adapted to simultaneously treat and image metastatic cancer. These nanoprobes could be used for both active and passive targeting. Therefore, in future research, QDs can be seen as multi-functional platforms focusing on targeted delivery, high transfection efficiency, and multi-modal imaging/tracking and treatment of cancer as shown in Figure 5.

Fig. 5. (A): Quantum dots as a multi-functional nanoplatform to deliver siRNA and to elucidate of EGFR knockdown effect of PI3K signaling pathway in brain tumor cells. (B): Detailed structural information of multifunctional siRNA-QDs: CdSe as core with ZnS capping along with thiol reactive linker for siRNA conjugation as well as RGD peptides as shown in red and TAT peptides as shown in green. In order to make QD constructs water-soluble and suitable for conjugating with siRNA, hydrophobic ligands are displaced with a dihydrolipoic acid (DHLA) derivatized with an amine terminated polyethylene glycol (PEG) spacer. (C): Two different strategies for the siRNA-QD conjugate. (C1) Linker for attaching siRNA to QDs through a disulfide linkage which are easily reduced within the cells to release the siRNA. (C2) Linker for covalently conjugating siRNA to QDs which enable tracking of siRNA-QDs within the cells. (Taken from: Jung et al. 2010).
However, there have been some QD concentration dependent toxicity and distribution concerns. QDs have been shown to remain in liver, lymph nodes and bone marrow of mice for 1 month after tail injection, despite of its low affinity to cells and tissues (Ballou et al. 2004; Ballou et al. 2009). Recently, the hydrophilic QDs (diameter <10 nm) have attracted more attention for in vivo applications, due to the rapid renal clearance of QDs, minimizing the potential toxicity to the system (Park et al. 2009). Recently, Peng and co-workers developed InAs/InP/ZnSe core/shell/shell QDs with high quantum yield (76%) of NIR fluorescence, ultrasmall hydrodynamic sizes (< 10 nm), and the biocompatibility desired for in vivo applications (Liu and Peng 2010). These novel QDs have obviously lower intrinsic toxicity compared to commercial Cd-containing NIR emitting QDs and showed significantly improved circulation half-life with reduced RES uptake. NIR-emitting QDs demonstrate exceptional brightness and fluorescent quantum yields. Also, once capped with a chemically stable shell, QDs can exhibit remarkable photostability, providing continuous fluorescent singles for long-term imaging applications. Other types of novel QDs with entirely different compositions and photoluminescence mechanism such as silicon QDs and carbon dots have also emerged as potential probes in bioimaging applications. Further, typical fluorescence images of a single QD shows changes in emission intensity. The intensity time trace illustrates the random alternation between “on” and “off” states, which is known as blinking and it is a signature feature of an individual QD. Also, reducing size of QDs has proven difficult because of decreased colloidal stability and increased nonspecific interactions. Other major limitations include reproducibility in production, proper control of surface functionality, bulky surface coatings (PEG, multiple antibodies, amphiphilic molecules) which leads to restrictions on studying spatially confined, crowded regions of the cell and may also perturb the behavior of the labeled molecules. Quantum dots are not yet approved for use in humans and much more research is needed in future for this growing field.

5.2 Carbon nanotubes

Molecular imaging exploits the specific recognition of labeled probes to their biological targets in conventional imaging techniques to monitor biological processes at the molecular level with improved specificity and sensitivity. Conventional clinical cancer imaging techniques, such as X-ray, CT and MRI, do not possess sufficient spatial resolution for early detection of the disease. Positron emission tomography (PET) is a highly sensitive and accurate imaging technology that relies on changes in tissue biochemistry and metabolism. It is the most valuable means we have so far to identify early-stage alterations in molecular biology, often before there is any morphologic change. Nevertheless, fluoro-deoxy-glucose (FDG), the most commonly used PET tracer in clinical oncology (more than 95% of the molecular imaging procedures make use of FDG at present), is not a specific tracer for malignant diseases but for increased metabolism. Therefore, it is imperative to develop new tools for early cancer diagnosis.

CNTs have been explored in almost every single cancer treatment modality, including drug delivery, lymphatic targeted chemotherapy, thermal therapy, photodynamic therapy, and gene therapy. Based on their structure, CNTs can be classified into two general categories: single-walled (SWNTs), which consist of one layer of cylinder graphene (diameter 0.4-2 nm) and multi-walled (MWNTs), which contain several concentric graphene sheets (diameter 2-100 nm). CNTs have unique physical and chemical properties such as high aspect ratio,
ultralight weight, high mechanical strength, high electrical conductivity, and high thermal conductivity (Ji et al. 2010). Carbon nanotubes are the strongest and stiffest materials yet discovered in terms of tensile strength and elastic modulus respectively. As CNTs are intrinsically not water soluble, modification through chemical functionalization can increases the solubility of carbon nanotubes in aqueous solutions.

Imaging functionalities and therapeutics can be incorporated on the same nanoparticle for multifunctional cancer imaging and treatment. SWNT-paclitaxel (PTX) conjugates also showed higher efficacy in suppressing tumor growth than clinical Taxol alone in a murine 4T1 breast cancer model, owing to prolonged blood circulation time and enhanced permeability and retention (EPR) in the tumor (Feazell et al. 2007). Besides, with very high surface area per unit weight, SWNTs provide higher capacity of drug loading, compared to that reported for conventional liposomes and dendrimer drug carriers. Doxorubicin, a commonly used cancer chemotherapy drug, can be loaded on the surface of PEGylated SWNTs with remarkably high loading, up to 4 g of drug per 1 g of nanotube, owing to the ultrahigh surface area of SWNTs. Further, the intrinsic stability and structural flexibility of CNTs may prolong the circulation time as well as improve the bioavailability of drug molecules conjugated to them. Surface-enhanced Raman spectroscopy of carbon nanotubes opens up a method of protein microarray with detection sensitivity down to 1 fmol/L. In vitro and in vivo toxicity studies reveal that highly water soluble and serum stable nanotubes are biocompatible, nontoxic, and potentially useful for biomedical applications. However, nonfunctionalized nanotubes are toxic to cells and animals and therefore one has to be cautious about the safety aspects of CNTs. If well functionalized, nanotubes may be excreted mainly through the biliary pathway in feces.

Carbon nanotube-based drug delivery has shown promise in various in vitro and in vivo experiments including delivery of small interfering RNA (siRNA), paclitaxel and doxorubicin (Liu et al. 2009). Multiwalled PEGylated carbon nanotubes are found to be successful, effective and do not alter particle sizes and zeta potentials of carbon nanotubes after PEGylation (Ilbasmis-Tamer et al. 2010). In addition, the propensity to absorb the body transparent NIR radiation also envisages photothermal and photoacoustic therapy using nanotubes.

5.3 Gold and other nanoparticles for cancer diagnosis

Thermal ablation therapy is one the most promising of methods in cancer treatment but is limited by incomplete tumor destruction and damage to adjacent normal tissues. Current radiofrequency ablation techniques require invasive needle placement and are limited by accuracy of targeting. Use of nanoparticles has refined noninvasive thermal ablation of tumors, and several nanomaterials have been used for this purpose. These include gold nanomaterials, iron nanoparticles, magnetic nanoparticles, carbon nanotubes and affisomes (thermosensitive liposomes). Heating of the particles can be induced by magnets, lasers, ultrasound, photodynamic therapy and low-power X-rays. The clinical trials include studies of designed nanoparticles such as the thermosensitive liposomal doxorubicin (Thermodox®) as a novel activated therapy using radiofrequency ablation (Wang and Thanou 2010). As gold nanoparticles have evolved other gold structures have also been suggested. Nanorods, with the appropriate PEG stealth layer, are being developed as an improved means of hyperthermia. By attaching monoclonal antibodies (mAbs), which can recognize a specific cancer cell, to gold nanoparticles or nanorods are also used in cancer detection. Gold nanoparticles conjugated to anti-epidermal growth factor receptor (El-Sayed et al. 2006)
mAbs specifically and homogeneously bind to the surface of the cancer cells with 600% greater affinity than to the noncancerous cells. This specific and homogeneous binding is found to give a relatively sharper surface plasma resonance (Hinz et al. 2006) absorption band with a red shifted maximum compared to that observed when added to the noncancerous cells. Surface plasma resonance scattering imaging or SPR absorption spectroscopy generated from antibody conjugated gold nanoparticles may be useful in molecular biosensor techniques for the diagnosis and investigation of cancer cells in vivo and in vitro.

These inorganic nanoparticles represent a different class of nanoparticles that are usually much smaller, 5–40 nm and they do not have the flexibility observed in liposomes and polymeric nanoparticles. Inorganic nanoparticles have made their appearance in cancer therapy during the last decades in a number of applications. The main type of inorganic nanoparticles—the iron oxide nanoparticles, has been used for imaging tumor (Wang and Thanou 2010). The main advantage of magnetic nanoparticles is their ability to be visualised by Magnetic Resonance (MR) imaging. Additionally, iron oxide nanoparticles can be guided to target sites (i.e. tumor) using external magnetic field and they can be also heated to provide hypothermia for cancer therapy. Yu et al. reported thermally cross-linked superparamagnetic iron oxide nanoparticles that could carry a Cy5.5 near infra-red probe (dual imaging) and doxorubicin for the imaging and treatment of cancer. The nanoparticles substantially diminished tumor size and provided the proof of concept that they can combine several modalities for maximum antitumor effect (Yu et al. 2010). Magnetic nanoparticles have been used in the development of dual purpose probes for the in vivo transfection of siRNA. The iron nanoparticles deliver siRNA at the same time as imaging their own accumulation in tumor sites. Hence, multifunctional nanoparticles have emerged that are capable of cancer targeting and simultaneous cancer imaging and therapy.

Metal nanoshells are another class of nanoparticles with tunable optical resonances. Metal nanoshells consist of a spherical dielectric core nanoparticle, in this case silica, which is surrounded by a thin metal shell, such as gold. These particles possess a highly tunable plasmon resonance, a resonant phenomenon whereby light induces collective oscillations of conductive metal electrons at the nanoshell surface. Nanoshells derived from gold provide an attractive system for imaging applications owing to the established ease of preparation, chemical inertness, good biocompatibility, and surface functionalization. Further, nanoparticle based near infrared imaging (NIR) is steadily presenting itself as a powerful diagnostic technique with the real potential to serve as a minimally invasive, nonionizing method for sensitive, deep tissue diagnostic imaging that are not prone to the rapid photobleaching and instability of their organic counterparts. NIR laser treatment of the bulk tissue selectively heats and destroys the nanoshell-laden tumor regions within the tissue, while leaving surrounding tissue intact. Nanoshells are currently evaluated in a number of clinical settings after a 5-year period of intensive preclinical development. Such development of nanoshells included the combination of nanoshells with cancer antibodies. Anti-HER2 antibody conjugated onto nanoshells provides the potential of combining antibody therapy with imaging and hyperthermia. NIR dye-encapsulating nanoparticles also demonstrate improved optical performances compared to unencapsulated organic fluorophores. Specifically, the encapsulation shields the dye molecules from unfavorable environmental influences that normally hinder fluorescence signals, thereby enhancing quantum yields, emission brightness, and fluorescent lifetime. While, at present, these NIR
nanoparticulates appear to be superior in terms of optical performances, they are marred by their heavy metal composition and high propensity for toxicity. It is therefore reasonable to be concerned about the ineffectual clearance and long-term accumulation in untargeted organs and tissues of these particulates for in vivo use.

6. Conclusion

With the understanding of the genetic origins of certain cancers, an entirely new approach to the treatment of this disease has evolved, employing nanoparticle-based gene therapy. Numerous nanoparticle based cancer gene therapy strategies are already in clinical trials. The key to the success of any new therapeutic is to maximize safety without compromising efficacy, which has led to growing interest in non-viral gene delivery systems (such as liposomes) over the viral gene delivery systems. Grafting biorecognition molecules (ligands, antibodies) onto the nanoparticles (i.e active targeting) aims to improve targeting by specific cell uptake and using hydrophilic polymer coating, PEG, which aims to further enhance biocompatibility. To overcome other challenges of gene therapy, such as escape from endosome and other nuclear and cytosolic barriers, next generation vectors are being designed with use of gene regulatory elements (promoters and enhancers) to restrict gene expression to specific cells, along with nuclear localization signal peptides for nuclear targeting.

There has been substantial interest in dual purpose nanoparticle based gene therapy for both diagnostic (imaging) and therapeutic purposes (drug/gene delivery). Newer technologies for cancer detection/diagnosis using metallic and semiconducting nanoparticles are also under intense investigation. These nanoparticles for in vivo application targeting cancer are amenable to different size structures and possess tunable properties. Quantum dots possess unique size- and composition-dependent optical and electrical properties. In addition to quantum dots, carbon nanotubes, paramagnetic nanoparticles, nanoshells and nanosomes represent just a few of these novel technologies, used for both diagnostic and delivery purposes.

The imminent research challenge facing investigators moving forward is the expansion of the knowledge and understanding of the chemical and physical properties associated with these nanoparticle systems toward the design of superior cancer therapy modalities that maximize efficiency of treatment, while maintaining a superior safety profile.

7. References

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