



# CANCER NANOTECHNOLOGY PLAN 2015



# Cancer Nanotechnology Plan 2015

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# SECTION II: UNIQUE MODALITIES FOR NANOTHERAPEUTICS

## Optimizing Nanoparticle Delivery of Chemotherapeutics

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### *Chemotherapeutics in Cancer Therapy*

Chemotherapy can be defined as the use of cytotoxic drugs that attack or interfere non-specifically with critical components of the cell. Chemotherapeutic drugs include at least 3 well-known categories: agents that damage the DNA template directly or indirectly; agents that damage microtubules; and, agents that inhibit DNA, RNA, or protein synthesis (antimetabolites). In addition to their lack of specificity, various pharmacologic factors seriously limit drug distribution and penetration to tumors and neutralize the activity of chemotherapy. This group of agents could tremendously benefit from a delivery system to improve its tumor specificity and reduce its toxicity to normal tissues. However, it is now often questioned whether chemotherapy will be abandoned and replaced entirely with biological and immunological therapies in the near future. While important advances have been made in the areas of biological therapy and immunotherapy of cancer, chemotherapy remains a critical tool of cancer treatment with a large contribution to cancer cures in the adjuvant setting and an important contribution to life extension in the metastatic setting. Improvements in safety and efficacy of chemotherapy are definitely a worthy endeavor since they will have a dramatic effect on the well-being of our patients, their quality of life during treatment, and their ability to face the hardship of therapy and complete successfully the protocol regimes. Moreover, chemotherapy is also likely to remain an important component of a multimodality therapeutic approach, together with biological therapy and immunotherapy, to improve the antitumor response rates in a broad array of cancer types. There are many examples of the continuing role of chemotherapy and its critical added value to biological therapy. One of them is exemplified by the combination of chemotherapy with anti-HER2 antibodies (Trastuzumab) in HER2-positive breast cancer, which is required for optimal antitumor response. From a tumor response rate of only 12% for single agent Trastuzumab, the response rate climbs to 56% when doxorubicin and cyclophosphamide are combined with Trastuzumab<sup>1</sup>. While this combination of doxorubicin with Trastuzumab was problematic because of a major rise in cardiac complications, a number of subsequent studies have shown that replacing doxorubicin with liposomal

doxorubicin can avoid or minimize cardiac toxicity<sup>2</sup>. This example emphasizes the valuable contribution of chemotherapy to targeted therapies and the need to refine the formulations of chemotherapy for optimal results.

### ***Towards “Smart” Chemotherapy with Nanoparticle Delivery***

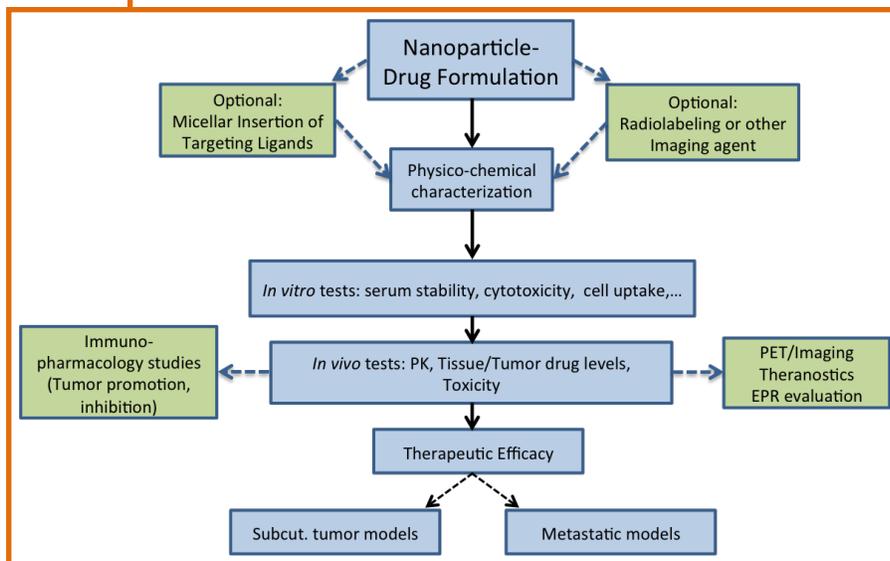
Nanomedicine is a platform to allow sophisticated and smart drug delivery within the size window of a submicroscopic system that enables delicate and complex interactions with cancer cells and their biological milieu. Nanoparticles and some macromolecules are the main tools of nanomedicine<sup>3</sup>. Pegylated liposomal doxorubicin (PLD) was the first nanoparticle-based cancer chemotherapeutic approved by the FDA. PLD together with nanoparticle albumin-bound paclitaxel (NAB-paclitaxel) are probably the cancer nanomedicines that have made, so far, the most important clinical impact<sup>4,5</sup>, excluding antibody-drug conjugates, generally considered to be a separate group of complex drugs.

Transforming the administration of a drug in free form, several angstroms across, into a 100-nm diameter nanoparticle loaded with thousands of drug molecules and with ~1 million-fold greater volume is a formidable pharmaceutical challenge that will have major pharmacological implications. However, from the clinical point of view, the only questions that have any significance when using nanopharmaceuticals are: Is the safety profile of the drug improved? Is the efficacy of the nano-engineered drug superior to the standard treatment or best performing comparator? To achieve these objectives, the nanoparticle-based approach should ideally fulfill two critical parameters:

- a. Stable association of drug and carrier in circulation, and release of active drug in tissues, at a satisfactory rate, for anti-tumor activity. This parameter appears to have been satisfactorily met by pegylated liposomal doxorubicin (PLD)<sup>6</sup>.
- b. Enhanced drug delivery to tumors via the nanoparticle formulation. For this to occur, first, the nanodrug or nanopharmaceutical must have a long circulation time to increase the number of potential passages through the tumor microvasculature. Second, the nanoparticle physical size has to be in the optimal size regime to allow extravasation across tumor blood vessels, which usually display higher permeability than normal blood vessels. The size window that will exploit the difference in permeability between normal and tumor blood vessels appears to be between 20 to 200 nm.

Successful control of these two parameters in the drug nano-formulation allows sparing normal tissues from toxicity and in boosting the antitumor effect with an overall increase of the therapeutic index. Some nanomedicines have failed to meet these requirements because

of either short circulation time, poor drug retention, or insufficient drug release<sup>7-9</sup>. Yet, other nanomedicines have been able to make a positive clinical contribution despite only minor changes in drug pharmacokinetics. This is the case of NAB-paclitaxel which avoids the acute toxicities associated with Cremophor EL<sup>®</sup> vehicle used in solvent-based paclitaxel, and has been found useful in various indications.



**Figure 1.** Schematic model of a work plan for rational development of nanoparticle-based chemotherapeutics.

High microvascular permeability is an important and frequent feature of tumors usually referred to as Enhanced Permeability and Retention (EPR) effect, and is a key component for nanoparticle transport into tumors<sup>10</sup>. EPR appears to be a particular feature of tumor-driven neoangiogenesis. While EPR is observed in most models of implanted experimental tumors,

large variations have been observed in human cancer depending on tumor type, tumor size, tumor site, and other factors, such as previous chemotherapy, antiangiogenic therapy, and radiotherapy. EPR may also be modulated by pharmacologic mediators. In some instances, tumors or their metastases derive their blood supply by a process known as co-option of normal blood vessels which results in blood vessels less permeable and less responsive to anti-angiogenic treatments and, consequently, less likely to display the EPR effect<sup>11</sup>. The high response rate of Kaposi Sarcoma, a tumor with high vascular permeability, to relatively low doses of PLD suggests that EPR is critical for the antitumor activity of nanodrugs. While this hypothesis has a strong pharmacologic rationale, it has not been tested rigorously, and we cannot discard that tumors with low EPR will still respond to nanodrugs better than to free drugs.

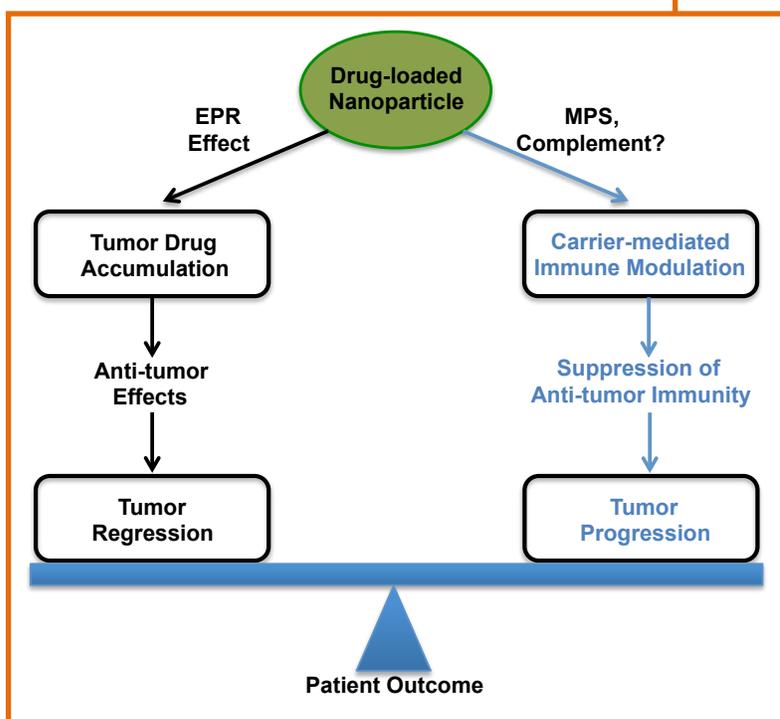
Smart delivery of chemotherapeutics may be simply achieved by controlling release rate of the active agent and by changes in tissue distribution, without necessarily including a targeting component specific for cancer cells. In fact, all the nanopharmaceuticals approved for clinical use belong to the non-targeted category. A scheme for development of nanoparticle-based chemotherapeutics is shown in **Figure 1**.

## Targeted Nanomedicines

Our understanding of the molecular processes underlying the pathologic behavior of cancer cells has progressed enormously in the last decade. Overexpressed receptors in the membrane of tumor cells, may offer a potential Trojan horse for targeting specific ligands or antibodies and delivering a cytotoxic drug cargo. Probably, the best example of a successful clinical translation of this approach is the antibody-drug conjugate known as T-DM1 which combines Trastuzumab, an anti-HER2 antibody, with emtansine, a potent and highly toxic chemotherapeutic, and has conferred a significant disease-free survival advantage to patients with HER2-positive breast cancer<sup>12</sup>.

Targeted delivery of a large payload of drug via ligand-directed nanoparticles to cancer cell-specific receptors is probably the most valuable objective of nanomedicine. A comprehensive and in-depth review of this subject has been recently published<sup>13</sup>. Indeed, the most logical improvement of nano-based drugs is the coupling of a ligand to the surface

of the nanoparticle to target to a specific cell-surface receptor. This would be followed by internalization and intracellular delivery of the small-molecule drug cargo. Examples in this direction are the targeting of PLD to HER2-expressing or folate-receptor expressing cancer cells using respectively a specific anti-HER2 scFv or a folate conjugate anchored to the liposome surface, or the targeting of polymeric nanoparticle of docetaxel to PSMA, a marker of prostate cancer<sup>14–16</sup>. Yet, another example is the tumor vascular targeting of liposomes with endothelium-specific peptides associated to liposomes<sup>17</sup>. A major advantage of targeted nanocarriers over ligand-drug bioconjugates is the delivery-amplifying effect of the former, which can deliver to the target cell at a ratio of ~1000 drug molecules per single ligand-



**Figure 2. Nanoparticle carrier interactions with the immune system may suppress antitumor immunity, thereby attenuating the antitumor effects of the drug cargo. A mechanistic understanding of the mechanisms of carrier-induced immune modulation will enable the development of systematic tools that may help to realize the full clinical potential of nanoparticle-based therapies.**

receptor interaction. In addition, the multivalent conjugation of targeting ligands on the surface of nanoparticles is presumed to enhance binding to the desired target. Targeting ligands, particularly small molecule ligands, can significantly enhance target-specific avidity of nanoparticles by several orders of magnitude through multivalent interactions<sup>13</sup>.

### ***Interaction of Nanoparticles with the Host***

Nanoparticles, including liposomes, are known to interact with the immune system to varying extents<sup>18</sup>. These interactions can affect drug pharmacokinetic parameters and may have significant clinical consequences. The majority of intravenously administered nanoparticles are rapidly cleared by the mononuclear phagocyte system (MPS) through internalization by phagocytic cells such as hepatic Kupffer cells and splenic macrophages. Notably, peripheral blood monocyte count and phagocytic function have been shown to correlate with PLD clearance rates in patients<sup>19</sup>, and similar correlations have been observed with other pegylated liposomal formulations (S-CKD-602, and SPI-077) in preclinical rodent and canine models<sup>20</sup>. Thus uptake and sequestration of nanoparticles in cells and organs of the MPS is a major barrier limiting the circulation half-life and, hence, tumor accumulation of carrier-mediated drugs.

In addition to interactions with the MPS, it is well established that nano-carriers interact with serum proteins such as IgG, IgM and the blood complement proteins, which contribute to opsonization of the carrier and enhance clearance by the MPS. Importantly, activation of complement proteins also generates anaphylatoxins (C3a, C4a, C5a) which can stimulate release of inflammatory mediators by immune cells leading to complement activation-related pseudoallergic reactions (CARPA) in swine and canine models, and several formulations of nanoparticles in clinical use (Doxil, DaunoXome, AmBisome, Abelcet, Amphocil) have been shown to cause hypersensitivity reactions consistent with CARPA. Clinically, it was shown that PLD activates complement in the peripheral blood of cancer patients and that the extent of complement activation correlated with the development of acute infusion reactions<sup>21</sup>. Therefore, undesired interactions with circulating serum proteins can also affect the pharmacokinetics and tolerability of carrier-mediated drugs.

Coating of nanoparticles with poly-ethylene glycol (PEG) (“pegylation”) has become widely used to reduce opsonization, improve stability in plasma, and prolong circulation time which are important requirements for effective tumor targeting. However, these approaches may not abolish immune reactions to nanoparticles. In addition, recent evidence suggests that PEG is not immunologically inert. Several groups have demonstrated that the initial systemic administration of pegylated nanoparticles induces production of anti-PEG IgM antibodies that enhance immune recognition and clearance of the second dose of nanoparticles in

preclinical models. Interestingly this “accelerated blood clearance” (ABC) phenomenon has not been reported in patients and its clinical relevance is currently unclear. In fact, the opposite has been observed in patients treated with PLD, where clearance rates decrease with repeat administration, up to 30% by the third cycle<sup>22</sup>.

Recently, it was shown that nanoparticle-induced complement activation could promote C5a-dependent tumor growth in tumor bearing mice, presumably through the recruitment and activation of immunosuppressive leukocytes. Yet, the nanoparticles used in these studies were intentionally designed to activate specific complement pathways<sup>23</sup>. It is not known whether clinically relevant nanoparticulate carriers, which activate complement in the peripheral blood, also induce complement activation in the tumor tissue, or how this impacts tumor growth. However, new evidence with a pegylated liposomal carrier similar to the PLD carrier, showed that these liposomes significantly enhanced tumor growth in an immune competent murine tumor model<sup>24</sup>. This was associated with suppression of antitumor immunity as indicated by blunting of cytokine production in tumor-associated macrophages and cytotoxic T cells, and diminished tumor antigen specific immune responses. Moreover, tumor microvessel density was significantly increased, consistent with enhanced angiogenesis. Collectively, these findings suggest that carrier-induced immune modulation could attenuate therapeutic efficacy of the nano-encapsulated drug (**Figure 2**), which may partially explain why there has been an insufficient improvement in anticancer efficacy in many of the clinical studies with nano-drugs despite their major pharmacologic advantages over free drugs<sup>25</sup>.

It is possible that during preclinical development, the prevalent use of rodent models with immune defects and the dearth of *in vivo* immune functional studies may have downplayed the consequences of the interactions between drug carriers and the immune system. It is also possible that manufacturing of the nanomedicines themselves were not as pure as initially thought with various solvents left behind in the formulations. Either way, incorporation of fully immune competent tumor models along with systematic immune functional studies may yield more accurate insight and analytical tools, that may help to realize the full clinical potential of nanoparticle-based therapies<sup>26</sup>.

### ***Cancer Nanodrugs in Clinical Use or Clinical Testing***

**Table 1** shows a list of nanoparticle-based drugs approved for cancer treatment by the FDA and/or the EMA. As seen in Table 1, the number of nanopharmaceuticals in clinical use has been slowly albeit steadily rising and includes chemotherapeutics of various classes, such as anthracyclines, taxanes, vinca alkaloids, and DNA topoisomerase-1 inhibitors. Most of these formulations are liposome based. Two of them, Depocyt and Mepact, are large

liposomes above the ultrafilterable range and probably should not be considered *bona fide* nanomedicines. Also included in Table 1 is NaL-Iri, which has not yet been approved although it has completed phase 3 trials for the 2nd line therapy of pancreatic cancer and met its primary objective of improved survival rates.

The early and positive preclinical and clinical experience with liposomal delivery of anthracyclines is probably one of the reasons for the dominance of liposomes in the field. Liposomes still remain as one of the most attractive particulate systems for cancer nanomedicine applications. A liposome formulation of doxorubicin, PLD (known as Doxil/Caelyx or Lipodox in generic version), is currently approved for various indications and in wide clinical use<sup>4</sup>. PLD has significantly reduced acute toxicity, as well as cardiac toxicity as compared to free doxorubicin precisely because of its unique pharmacokinetic characteristics. Probably the most significant clinical value added of PLD is the evidence of a major (~3-fold) risk reduction of cardiotoxicity as compared to free doxorubicin enabling risk-free, extended treatment<sup>2</sup>.

In addition, many other promising nanochemotherapeutic products are under clinical testing or about to be clinically tested. These include: polymeric nanoparticles of docetaxel in targeted and non-targeted form which have a significantly different pharmacological profile from the solvent-based docetaxel formulation; pegylated liposomal formulations of various cytotoxic drugs including eribulin and a prodrug of mitomycin C; a HER2-targeted version of PLD (MM-302); a low-temperature, release-sensitive, liposomal doxorubicin formulation; and a liposome formulation of co-encapsulated cytarabine and daunorubicin at fixed molar ratio<sup>16,27–32</sup>.

<i>Product</i>	<i>Indication in cancer</i>
Pegylated Liposomal Doxorubicin	Kaposi Sa., Ovary, Breast, Myeloma
Liposomal Daunorubicin	Kaposi Sa.
NAB-Paclitaxel (Abraxane)	Breast, Lung, Pancreas
Liposomal Doxorubicin	Breast
Liposomal Vincristine (Marqibo)	Adult A.L.L.
Low-pegylated Liposomal Irinotecan (NAL-IRI)	Pancreas (Phase 3 completed, awaiting NDA)
Liposomal Cytarabine (DepoCyt)	Lymphomatous meningitis
Liposomal Mifamurtide (Mepact)	Osteosarcoma

## ***The Future of Nanoparticle-Based Chemotherapeutics - Quo Vadis?***

Two fundamental aspects of nanomedicines remain to be clarified in upcoming years: we need an improved understand of the interaction of nanoparticles with the immune system and to learn how to manipulate it for the benefit of the patient; and, we need to understand how relevant is the EPR effect in human cancer, particularly in metastases, and what role does it play in the performance of nanopharmaceuticals.

It is likely that we will witness a more extensive use of the currently approved nanotherapeutics at the expense of conventional use of chemotherapeutics. In addition, other nanodrugs in clinical development may be approved in the coming years, expanding the classes of drug available in nanopharmaceutical form. Nanodrugs designed to exploit the EPR effect best, with optimal stability and drug release profiles, are likely to perform better although safety improvements will remain a key aspect dictating clinician preference. The use of targeted nanomedicines is probably going to be on the rise, particularly when there is a need to improve the cell uptake of a specific pharmaceutical agent.

The use of nanoparticles to deliver therapies, other than chemotherapeutic drugs, is also foreseeable, especially for agents with problematic *in vivo* delivery. In the case of siRNA, the nanoparticle protection is crucial. Recently published studies suggest that for some biologic agents such as tyrosine kinase inhibitors<sup>33</sup>, or, immunomodulators such as aminobisphosphonates<sup>34</sup>, nanoparticle-based delivery may also improve their *in vivo* performance in combination with chemotherapy or adoptive lymphoid cell therapy respectively.

Another area where nanoparticles could have a future impact is co-encapsulation of drugs<sup>35</sup>. Synchronized co-delivery of drugs co-encapsulated in the same particle or encapsulated separately in particles with identical physico-chemical and pharmacokinetic characteristics. Ideally, the drugs chosen should have synergistic or complementary anti-tumor effects with minimal overlap of toxicity profiles.

The co-administration, on the same nano delivery platform, of a therapeutic and a diagnostic or tracking agent, such as a PET-emitting radionuclide, is referred to as a Theranostic. This approach could enable real-time monitoring of the fate of a nanoparticle and its drug

**Two fundamental aspects of nanomedicines remain to be clarified in upcoming years:...**

payload. In essence, providing an insight as to the degree of cancer targeting achieved in each specific cancer individual. By imaging the nanoparticle, the EPR effect can then be predicted in each specific case and correlated with clinical response. This would provide direct clinical data to determine whether selecting patients based on their EPR tumor activity could lead to improved therapeutic benefit of nanoparticle based therapy<sup>36</sup>.

Finally, the use of nanomedicines in conjunction with loco-regional approaches to therapy (e.g., hyperthermia, radiofrequency ablation, radiotherapy) is a small niche, but has potential opportunities in specific applications that will increasingly attract clinical testing and adoption<sup>37</sup>.

## RNAi Therapeutics

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### *RNAi as a Tool for Precision Cancer Medicine*

**P**recision cancer medicine, i.e., the design of therapeutic regimens informed by tumor genotyping, continues to be a central paradigm in modern cancer research.

The most recent FDA approval of crizotinib and vemurafenib for the treatment of ALK-translocated lung cancer and BRAF-mutated melanoma, represents the latest proof-of-concept that oncogenomics-driven drug design can improve cancer prognosis<sup>38,39</sup>. High-throughput interrogations of cancer genomes have evolved with unprecedented pace. Bioinformatics, functional cancer biology and genetics continue to identify oncogenes and tumor suppressors that drive or contribute to the pathogenesis of cancer. The design and clinical testing of small molecules inhibiting 'druggable' targets, such as BRAF or ALK, embodied the initial promise of precision medicine, but the vast majority of the dauntingly complex oncogene has yet to be translated into meaningful therapeutic strategies. How can the activity of multiple unprecedented, non-enzymatic targets with unknown *modi operandi* be modulated?

RNA interference (RNAi) comes to mind, as a potent mechanism to silence aberrant oncogene expression by blocking the translation of their encoding mRNAs. Without prior knowledge of oncogene function, sequence-specific microRNAs (miRNAs) or small interfering (si) RNAs can be designed to selectively target oncogenic pathways, which drive unabated growth, apoptosis resistance, neo-angiogenesis and enhanced migration/invasion of tumor cells. siRNAs are generated by cleavage of long double-stranded (ds) RNAs into ~20 nucleotide-containing siRNAs by the enzyme Dicer. Unwinding of siRNAs into two single-stranded (ss) RNAs, incorporation of the guide strand into the RNA-induced silencing complex (RISC), and binding of siRNAs to complementary mRNAs triggers the degradation of endogenous mRNA by Argonaute, the catalytic component of the RISC complex (reviewed by Hannon and Rossi 2004)<sup>40</sup>. Structurally similar to siRNAs, mature miRNAs are non-coding RNAs, which typically exhibit incomplete base pairing to the target mRNA, and inhibit translation of multiple mRNAs via binding to their untranslated regions (reviewed by Di Leva et al. 2014)<sup>41</sup>. Thus, the level of expression of single miRNAs can influence multiple biologic processes. In contrast, siRNAs bind the coding portion of the mRNA with complete base-pair match and induce mRNA cleavage only in a single, specific target. Due to the negative charge of the RNA backbone, siRNA or miRNA oligonucleotides require delivery systems to

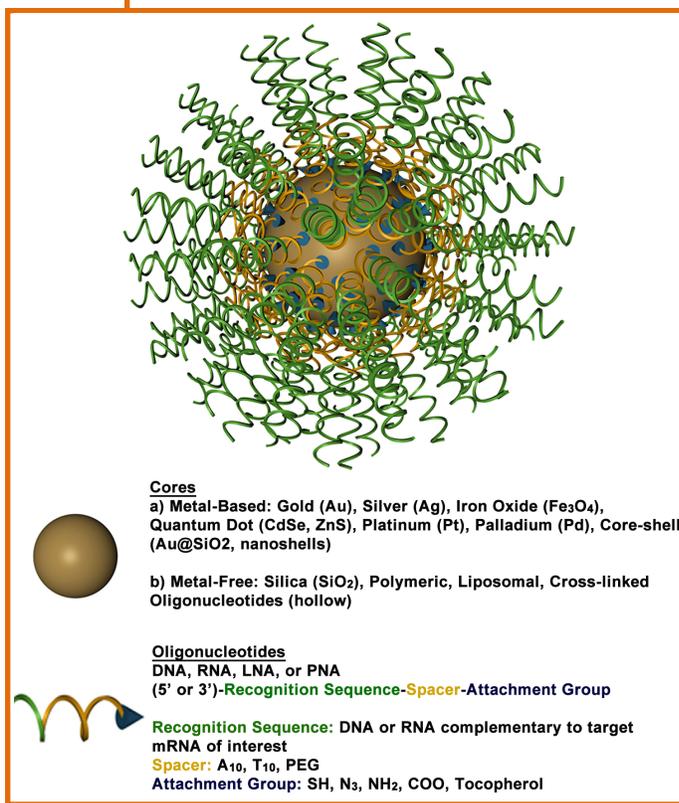
overcome negatively charged membranes, and to prevent rapid renal and hepatic clearance, the degradation of si/miRNAs by nucleases, and toxicity and immunogenicity of the RNA payload.

### ***Preclinical Evaluation of RNAi-Based Therapeutics – Recent Developments Utilizing Nano-Enabled Approaches***

The first clinical proof-of-concept that systemically delivered siRNA reduce oncogene expression via an RNAi mechanism in humans<sup>42</sup> motivated the development of several RNAi delivery platforms, which target a wide array of oncogenes in many different cancers.

Spherical nucleic acids (SNAs) (i.e., 13 nm polyvalent gold nanoparticles functionalized with siRNAs or miRNAs) were preclinically evaluated to deliver Bcl2-Like12 (Bcl2L12)-targeting siRNAs (**Figure 3**) and mature miR-182 sequences to intracranial glioblastoma<sup>43,44</sup>. Bcl2L12

is potent caspase and p53 inhibitor with near ubiquitous expression in primary GBM specimens<sup>45–49</sup>. miR-182 is a tumor suppressive miRNA, which regulates apoptosis, growth and differentiation programs via transcriptional repression of Bcl2L12, c-Met, and Hypoxia Inducible Factor 2 alpha (HIF2α) to enhance therapeutic susceptibility, and to decrease expansion and multipotency of glioma-initiating cells<sup>44</sup>. siBcl2L12 and miR-182-based SNAs robustly penetrated glioma-initiating cells via scavenger receptor-mediated endocytosis. In an *in vitro* blood-brain barrier (BBB) model involving the co-culture of human primary brain microvascular endothelial cells separated from astrocytes by a semi-permeable filter insert, Cy5.5-labeled SNAs passed through the endothelial cell layer and filter, and rapidly entered the astrocytes. Systemic administration into Sprague-Dawley rats and non-human primates have not resulted in SNA-related differences in body or organ weight, nor in an inflammatory response in the brain



**Figure 3. Schematic representation of a Spherical Nucleic Acid (SNA) nanoconjugate.** The surface of a variety of different core materials including metal nanoparticles (e.g., Au, Pt), liposomes and polymers, can be functionalized with highly oriented nucleic acids (Reprinted with permission from Barnaby et al., 2015)<sup>54</sup>.

or in reticuloendothelial system (RES) organs, as shown in published<sup>43</sup>, and unpublished data. Importantly, si/miRNA-based SNAs crossed the blood-tumor barrier and accumulated in glioma elements relative to normal brain tissue likely via enhanced permeability and retention of the tumor-associated vasculature. Accumulation and pervasive dissemination into extravascular tumor parenchyma translated into robust intratumoral protein knockdown, increased intratumoral apoptosis, impaired tumorigenicity, and prolonged survival of GIC-derived xenogeneic mice<sup>43,44</sup>.

Jacks and colleagues developed a combinatorial RNAi regimen using lung-targeting polymeric nanoparticles made of low-molecular-weight polyamines and lipids to deliver siRNA and miRNA mimetics to lung adenocarcinoma cells *in vitro* and to tumors in a genetically engineered mouse model (GEMM) driven by KRas activation and p53 deletion<sup>50</sup>. The lead compound is a nanoparticle with multilamellar structure, which was synthesized by reacting with a 15-carbon lipid tail in ethanol<sup>51</sup>, mixed with C<sub>14</sub>PEG<sub>2000</sub>. Delivery of miR-34a and siRNAs targeting KRas reduced lung cancer progression more effectively than either small RNA alone, and synergized with cisplatin-based chemotherapy to prolong survival of animal subjects<sup>50</sup>.

Bhatia and colleagues developed a tumor-penetrating nanocomplex (TPN) with siRNAs specific for the ovarian cancer oncogene inhibitor of DNA binding 4 (ID4)<sup>52</sup>. For tumor delivery, the nanoconjugate was co-functionalized with a tandem tumor-penetrating and membrane-translocating peptide, which enabled robust and pervasive delivery of siRNA to the tumor parenchyma. Subsequently, treatment of ovarian tumor-bearing mice with ID4-specific TPN suppressed growth of the established tumors and significantly improved survival. Similar to TPN-mediated ID4 knockdown, inhibition of the DNA repair enzyme poly(ADP-ribose) polymerase 1 (PARP1) with siRNA-based lipoids is an effective treatment for ovarian cancer. Intraperitoneal (i.p.) administration of siPARP1 lipoids promoted apoptosis, and increased animal subject survival in BRAC1-deficient, but not the wildtype allografts *in vivo*<sup>53</sup>.

Using a genetically engineered breast cancer model, driven by SV40-large T antigen under the control of the C3(1) component of the rat prostate steroid binding protein (PSBP) to direct SV40 expression to the mammary gland, computational gene network modeling identified HoxA1 as a putative driver of early breast cancer progression. RNAi-mediated

**Accumulation  
and pervasive  
dissemination  
into extravascular  
tumor parenchyma  
translated into  
robust intratumoral  
protein  
knockdown...**

suppression of HoxA1 in mammary tumor spheroids increased acinar lumen formation, reduced tumor cell proliferation, and restored normal epithelial polarization. *In vivo*, intraductal delivery of siRNA-based lipid nanoconjugates targeted to HoxA1 into FVB C3(1)-SV40Tag mice triggered robust reduction of breast cancer progression associated with reduced cell proliferation rates, and sustained expression of estrogen and progesterone receptors<sup>55</sup>.

### ***Future Challenges and Directions***

The confluence of progress in many different areas of cancer research, i.e., high-throughput oncogenomics, the development of physiologically relevant cell and animal models as testing platforms for gene function and gene-specific therapeutics, and the emergence of RNAi-based nanotechnological strategies, have positioned the field well to implement precision cancer nanomedicine into clinical practice. With currently 24 different RNAi-based therapeutics in 43 different clinical trials, critical questions and challenges for the next 5 to 10 years have become very apparent, i.e., to identify the most critical target genes that drive or contribute to cancer initiation, progression, metastasization and therapy refractoriness, as well as to further improve and comprehensively evaluate efficacy, specificity, and biocompatibility of RNAi nanotherapeutics in the most relevant cell and animal models. Specifically, several important areas for development include the following.

#### **RNAi Nanoconjugates as Tools for Discovery Sciences**

With the number of gene aberrations ranging from thousands to hundreds of thousands, the genomic and genetic landscape of cancer is complex. Only a subset of genes drive the initiation and maintenance of cancer. In addition, tumors show specific, spatially and temporally controlled genetic changes, which are influenced by cooperative oncogenic and tumor suppressive signatures, and further modulated by heterotypic tumor-stroma interactions, and patient-specific germline mutations. Genome-wide RNAi and cDNA complementation screens are constantly evolving to determine cancer gene function and their genetic context, and will continue to provide lists of candidate genes that require further in-depth testing in cell and animal models. For preclinical evaluation, established or patient-derived cancer cells, together with murine cancer cell lineages are engineered to over- or underexpress the gene of interest, and these cell systems are then channeled into a variety of functional assays determining the impact of gene dosage on cellular transformation, growth, apoptosis sensitivity and migration/invasion. By orthotopically injecting these cell systems into immunocompromised or syngeneic hosts, subsequent *in vivo* experiments then evaluate the impact of cancer gene overexpression and knockdown on tumor progression. Nano-RNAi should be developed as a tool for discovery science to

evaluate gene function and its impact on cancer progression in cells *in vitro* and in animal models *in vivo*. Instead of generating cell transfectants stably or transiently expressing small hairpin (sh) RNAs and siRNAs, or engineering cells with a gene-specific knockout harnessing the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 technology, RNAi-based nanoconjugates can be administered to cells, graft and genetically engineered cancer models, to determine cancer gene function *in vivo*.

### Further Developing RNAi-Based Nanotherapeutics

While a plethora of RNAi-based nanoconjugates have emerged in the past 10 years as fundamentally novel classes of therapeutics that can robustly and safely delivery RNAi to tumor sites, structure-activity relationships that dictate nanomaterial activity (RNAi delivery to cells, target gene knockdown) are only beginning to emerge. This incomplete understanding is based in part on the difficulty in generating structurally defined materials, and in rapidly evaluating the cellular impact of these nanomaterials in a massively parallel fashion. Design rules have to be determined that

optimize the development of RNAi nanoconjugates for therapeutic applications. Unlike small molecule-based therapeutics, where millions of compounds are surveyed in an initial high-throughput screen, and thousands are tested under optimized conditions in various cell culture models, nanomedicinal evaluations typically focus on a defined subset of candidates only. Furthermore, deep mechanistic and biological studies are required to fully understand some of the fundamental properties underlying gene knockdown (is gene knockdown truly mediated by an RNAi mechanism, or is it due to rather unspecific toxic effect of the conjugate?) cellular entry, endosomal escape, tissue dissemination, and low-level cellular and organismal impact. With more comprehensive screenings of cancer cell-specific surface markers, the modification of RNAi nanoconjugates with ligands or antibodies to facilitate tumor-specific uptake, beyond the EPR effect, has to be optimized to further increase conjugate efficacy while reducing the potential for adverse side effects associated with systemic administration. Due to the dependence of the cancer phenotype on multiple deregulated pathways, co-extinction strategies have to be developed that concomitantly silence multiple oncogenes and oncogenic pathways. In particular, the concept of therapeutic synergy between siRNAs and miRNAs has to be exploited further, as recent study in ovarian and lung cancer showed significant cooperativity in reducing tumor progression when compared with either monotherapy alone<sup>50,56</sup>. The design of such combination therapies, and the development of multimodal si/miRNA nanoconjugates have to be optimized, and evaluated *in vivo* for efficacy, pharmacokinetics, pharmacodynamics,

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**...nanomedicinal evaluations typically focus on a defined subset of candidates only.**

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and toxicology in the relevant grafts and GEMMs. Finally, we have to understand and harness synthetic lethal interaction of si/miRNAs with conventional chemotherapy (e.g., DNA-damage-inducing agents), targeted pharmaceuticals that inhibit critical driving oncogenes, such as (receptor) tyrosine kinases, and possibly immunotherapies. It will be critical to determine the molecular mechanisms that act as roadblocks preventing chemo- and RTK-targeted therapies from inducing tumor-specific apoptosis and regression, and enabling cancers to escape immune surveillance. We then can target these roadblocks using RNAi-based nanomaterials, and can envision using hybrid conjugates co-functionalized with chemotherapeutics, small molecules, biotherapeutic antibodies and si/miRNA sequences to concurrently target driving oncogenes and their downstream signaling.

Milestones to address these critical areas that researchers should be able to be achieved over the next 5-10 year time frame include many aspects. In the next 5 years, researchers will comprehensively determine structure-function relationships of RNAi nanoconjugates with high-throughput methods; determine the potential synthetic lethal interaction between cancer genes and extant chemo-/targeted therapies to identify those genes required for therapy resistance; develop and preclinically evaluate multimodal nanoconjugates for the concurrent delivery of small RNAs and chemo-/targeted therapies; preclinically develop combination regimens of immunotherapies and RNAi-based nanomaterials; and develop RNAi nano-conjugates as tools for discovery sciences to characterize oncogene function in cells and animal models. Looking further ahead over the next 10 years, researchers will perform clinical testing of multiple RNAi-based nanoconjugate combinations, in conjunction with established therapies; and potentially there should be FDA approval of several RNAi conjugates and RNAi-based combinatorial regimens.

## X-ray Induced Photodynamic Therapy

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### *Introduction to X-PDT and its Importance to Oncology*

Photodynamic therapy (PDT), as a relatively new cancer treatment methodology, has attracted wide attention. PDT uses a photosensitizing drug that is activated by exposure to light of a specific wavelength. While they display minimal toxicity in the dark, photosensitizers, upon light activation, produce cytotoxic reactive oxygen species such as singlet oxygen ( $^1\text{O}_2$ ) and hydroxyl radicals, leading to cancer cell death. PDT is minimally invasive and highly selective. Unlike ionizing radiation, PDT can be applied repeatedly to the same diseased sites without causing incurred resistance. PDT can also be applied in conjugation with other treatment modalities to facilitate tumor management. For instance, PDT is being evaluated in the clinic to treat prostate cancer patients who have failed radiotherapy.

One major limitation to PDT, however, is the shallow penetration depth. Even with new generations of photosensitizers, it is challenging for PDT to treat tumors of large volumes ( $> 1\text{cm}^3$ ) or ones located deep under the skin. This restraint is a major cause behind the limited impact and current role of PDT in the clinic. To address the issue, there have been many efforts on developing two-photon PDT and upconversion nanoparticle-mediated PDT. However, because the excitation source is near-infrared light, their potential therapeutic outcomes are still heavily surface-weighted.

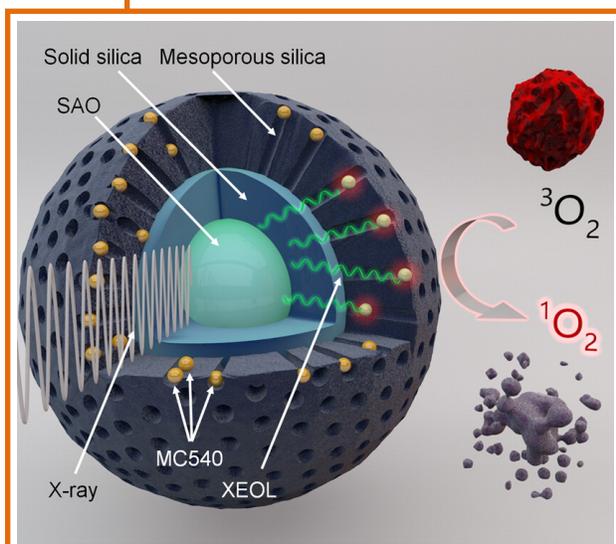
Very recently, our group and others have exploited the possibility of using X-ray as an energy source to activate PDT. We termed this methodology X-ray inducible PDT, or X-PDT. Unlike visible or near-infrared light, X-ray affords excellent tissue penetration ability and is widely used in clinical diagnosis and therapy. X-PDT can thus, to a large degree, transcend the depth limitation of conventional PDT ( $\sim 1\text{ cm}$ ), permitting deep-tissue therapy<sup>57</sup>. For X-PDT to work, there are several requirements. First, a scintillating transducer, which converts X-ray photons to visible photons. Second, a photosensitizer, whose excitation wavelength is well matched to the emission of the scintillator. Third, a carrier, which can co-deliver the scintillator and photosensitizer, and ensure that the two components are spatially close enough for efficient energy transfer. As simple as it sounds, it is difficult to meet all three requirements using conventional methods.

This puzzle is solved by advances in nanotechnology, which allow for preparation of nanoscale scintillators and carriers. **Figure 4** shows an example of such an integrated nanosystem, consisting of a nanoscintillator core made of  $\text{SrAl}_2\text{O}_4:\text{Eu}$  (SAO), a photosensitizer merocyanine 540 (MC540), and a silica capsule that encapsulates the two. Upon X-ray irradiation, the SAO core converts X-ray photons to visible photons via a physical phenomenon known as X-ray excited optical luminescence (XEOL). Due to excellent spectral overlap between the emission and the excitation of MC540, the photons emitted by SAO are absorbed by MC540 deposited in the silica matrix. This produces reactive oxygen species, including hydroxyl radicals and singlet oxygen ( $^1\text{O}_2$ ), causing death of cancer cells.

### ***Current State of the Art in X-ray Inducible PDT***

The number of studies on X-PDT is relatively small but is increasing. In addition to this group's work, other groups have exploited different scintillator materials using similar or

different designs. For instance, the Chen group has investigated X-PDT with Cu-cysteine<sup>58</sup>,  $\text{LaF}_3:\text{Ce}$ <sup>59</sup>, and  $\text{ZnS}:\text{Cu},\text{Co}$ <sup>60</sup>. The Shi group reported that Ce(III)-doped  $\text{LiYF}_4@\text{SiO}_2@\text{ZnO}$  nanoparticles upon ionizing irradiation can generate hydroxyl radicals to kill cancer cells<sup>61</sup>. Recently, Kotagiri et al. observed that Cerenkov radiation from radionuclides can be harnessed to activate  $\text{TiO}_2$  nanoparticles, an oxygen-independent nanophotosensitizer, to produce radicals and kill cancer cells<sup>62</sup>.



**Figure 4. X-PDT, mediated by MC540 loaded and silica coated SAO nanoparticles (or M-SAO@SiO<sub>2</sub> nanoparticles).** Upon X-ray irradiation, SAO works as a transducer, relaying energy in the form of X-ray excited optical luminescence (XEOL) to MC540 to activate it and produce cytotoxic  $^1\text{O}_2$ . M-SAO@SiO<sub>2</sub> nanoparticles can be conjugated with a tumor targeting motif to further enhance the selectivity against cancer cells (*Reprinted with permission from Chen et al, 2015*).

X-PDT treated cells often display blebbing, swelling, and morphology changes, suggesting PDT-induced necrosis as the dominant cell killing mechanism. This is different from ionizing irradiation, in which cell death is often caused by apoptosis. However, it does not mean that there is no contribution of ionizing irradiation in X-PDT. While  $^1\text{O}_2$  is produced in nanoparticle-rich compartments such as the cell membrane and endosomes/lysosomes, other organelles are under the impact of ionizing irradiation. Hence, X-PDT is essentially a combination therapy of PDT and ionizing irradiation. Previously, several groups

have studied PDT and radiation combination therapy and observed a synergistic effect between the two<sup>63-66</sup>. This is because the two modalities act on different targets: PDT often damages cell membranes whereas ionizing irradiation targets DNA. Due to distinctive cell killing routes, each modality suppresses the cell repair mechanism of the other, leading to enhanced treatment outcomes. The same synergy is believed to play a role in X-PDT.

From this perspective, X-PDT is not only a PDT derivative, but also a type of radiation therapy derivative. It however, affords several benefits over conventional ionizing irradiation. First, X-PDT can kill cells that are resistant to radiotherapy (e.g., glioma cells<sup>57</sup>). This is because the main cell killing mechanism of X-PDT is PDT-induced cell damage rather than radiation caused DNA damage. Second, low irradiation doses. Like PDT, X-PDT achieves good tumor control within in a few or even single treatment sessions<sup>57</sup>. The total irradiation dose is often less than 10 Gy. The dose is much lower than traditional radiotherapy, in which case a total dose of 60-80 Gy is often needed<sup>67,68</sup>. Third, low irradiation dose rates. It is known that irradiation induced toxicities are positively correlated to dose rates<sup>69</sup>. In X-PDT, irradiation doses per fraction are often comparable to conventional radiotherapy (e.g., 2-5 Gy); however, the irradiation is given out over a span of 15-30 min (typical for PDT), as opposed to minutes or even less in radiotherapy. This leads to dramatically lowered dose rates and potentially reduced toxicities. Fourth, high selectivity. In X-PDT, the treatment is mediated by not only irradiation but also the respective nanotransducers. With proper surface coating and by conjugating with a tumor targeting ligand, nanotransducers may accumulate in tumors with high efficiency. This dual selectivity, in conjugation with low irradiation doses and dose rates, are expected to minimize normal tissue toxicities, a major concern in radiotherapy.

### ***Future Scientific and Clinical Developments***

While X-PDT has demonstrated good efficacy and benefits, there is a lot that we don't know about this new therapeutic modality. As discussed above, X-PDT is essentially a combination therapy of PDT and ionizing irradiation. However, exactly how the two modalities interplay and whether we can improve the synergy by tuning irradiation parameters and/or changing nanotransducer targets is largely unknown. These need be elucidated in future studies.

The nanoscintillator is the key to X-PDT. It will be important to exploit ways to improve their energy conversion and safety profiles. These include: (1) change scintillator materials to ones that have a larger X-ray absorption cross-section and higher X-ray-to-visible-photon conversion efficiency as well as optimized spatial positioning of the molecular entities involved; (2) reduce the overall size of the nanotransducers; this however, should be balanced against the loss in energy conversion efficiency. It is noted that many of the

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**One solution to the problem is to use coatings to coat hydrolytic scintillator cores so as to slow down, but not prohibit hydrolysis.**

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reported nanotransducers in X-PDT have a relatively large size, which is suboptimal to tumor targeting; and (3) strike a balance between short-term stability and fast biodegradation of nanoparticles. Many scintillator materials are hydrolytic, quickly reducing to constituent ions when exposed to water. Water resistant scintillators do exist, but then the issue becomes the too slow degradation *in vivo*. One solution to the problem is to use coatings to coat hydrolytic scintillator cores so as to slow down, but not prohibit hydrolysis. Taking  $\text{SrAl}_2\text{O}_4:\text{Eu}$  nanoparticles for instance, it was found that after silica coating, the particles can maintain stability in physiological environments for 3-7 days and are then gradually degraded. Other materials/coating strategies should be exploited to modulate the stability and degradation of scintillators *in vivo*.

So far, X-PDT has been demonstrated mostly *in vitro* or with subcutaneous models. In future studies, it is important to evaluate the methodology in more clinically relevant tumor models. X-PDT holds the potential of clinical translation as an alternative to irradiation therapy in the next 10-15 years. It is important to compare the two modalities in the clinic to assess benefits and drawbacks of X-PDT with regard to treatment efficacy and side effects. It is also interesting to evaluate the capacity of X-PDT to treat tumors refractory to or ones that have failed radiotherapy. In radiotherapy, pre-treatment functional imaging (e.g., PET) is often performed to stage tumors and guide irradiation planning. However, functional imaging is not permitted in an irradiation room, and a change in patient position from prescans may occur, leading to setup errors. Many scintillator materials contain high-Z-value elements, making them visible under on-board CT. It is thus possible to use these nanoscintillators to not only regulate PDT but also guide the irradiation so as to minimize normal tissue damage. These possibilities should also be investigated to facilitate clinical translation of X-PDT.

## Targeting Undruggable Targets

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### *The Importance of Targeting Undruggable Targets to Cancer Research/Oncology*

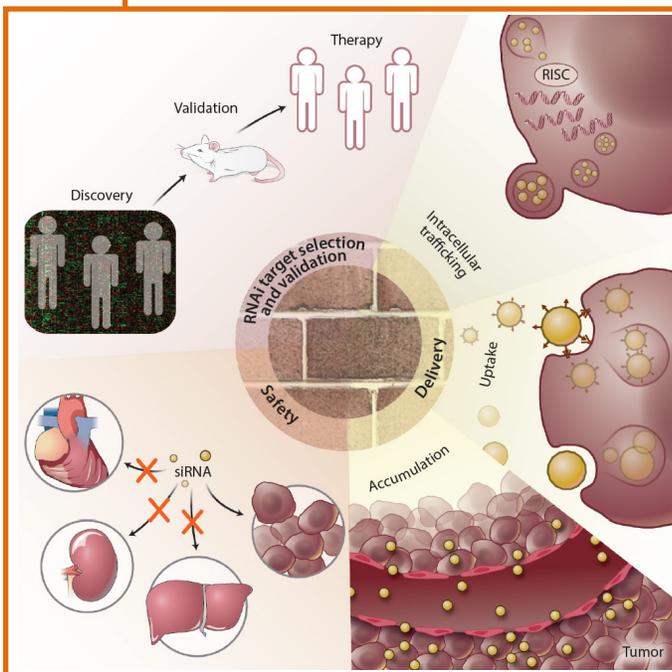
Over the last few decades, advances in surgery, chemotherapy, and targeted drugs have led to improvements in progression-free and overall survival increases for many cancer types<sup>70</sup>. However, cure rates have remained largely unchanged. To accelerate the gains in clinical outcomes, large-scale efforts such as the Cancer Genome Atlas (TCGA), Clinical Proteomic Tumor Analysis Consortium (CPTAC), Cancer Target Discovery & Development (CTD<sup>2</sup>), and others were launched. These efforts have produced very high quality data due to the stringent requirements for sample quality and have clearly increased the pace of discovery for novel targets. However, to date, most of the knowledge is correlational in nature and large-functional data are needed. Challenges to rapid translation include the need for rapid, reliable, and effective functional data. While genetically engineered mouse models (GEMMs) remain a key tool in our armamentarium to determine the effects of various molecular pathways on biological processes, such models can have limitations (e.g., lengthy time, expense) and do not always reflect the biology of advanced stage human tumors. Therefore, other approaches such as 3-D, patient-derived xenografts, and orthotopic model systems remain an important component of biological validation and drug development.

The growing knowledge from the large-scale “omics” efforts has produced highly complex maps of genetic dysregulation in cancers. Moreover, these functional and biological systems have produced a plethora of targets that appear attractive for therapeutic development. However, many of the targets are not druggable by conventional strategies. Many important targets are difficult to inhibit with small molecules and furthermore require lengthy development phases that often fail. In addition, many small molecule inhibitors lack specificity and can be associated with intolerable side effects. While monoclonal antibodies have shown substantial promise against specific targets (e.g., VEGF, EGFR), their use is limited to either ligands or surface receptors. Some oncogenic proteins (e.g., Ras) activate pathways leading to altered transcription while others (e.g., Myc) are themselves transcription factors that directly control the expression of genes essential for proliferation, survival, and metastasis. Attempts have been made to develop pharmaceutical inhibitors against some of these factors, but many are still widely considered “undruggable”.

Collectively, these and other observations have led many investigators to consider alternative strategies, such as RNA interference (RNAi), for inhibiting these targets.

### ***Current Status in the Targeting of Undruggable Targets***

Since the first report of RNAi in the late 1990s, there has been a massive expansion in efforts to apply it for therapeutic applications. Among these, short interfering RNA (siRNA) allows for highly selective silencing of target(s) of interest. Non-coding RNAs such as microRNAs (miRNA) can be used to target a larger array of targets. Moreover, combinations of siRNA and miRNA offer opportunities for “co-extinction” to maximize therapeutic efficacy while avoiding activation of redundant/compensatory pathways. While the promise of RNAi-based therapeutics is enormous, challenges (e.g., potential off-target effects and toxicity, requirement for delivery, endosomal uptake, activation of adaptive pathways) also exist<sup>71</sup>. Among these, perhaps the biggest challenge is achieving efficient systemic delivery. Naked siRNA becomes degraded rapidly and cannot be delivered into the tumor efficiently.



**Figure 5.** Strategies for targeting undruggable targets that rely on careful target discovery followed by developing nanoparticle systems that allow for highly efficient systemic delivery into the tumor microenvironment while sparing delivery into normal organs such liver, kidneys and heart (Reprinted with permission from Wu et al., 2014).

However, these are precisely the kinds of concerns that can be overcome with biocompatible nanotechnology platforms. Already, several such platforms have yielded promising results in both pre-clinical and clinical settings for oncological and other clinical needs. For example, Davis and colleagues demonstrated in a landmark paper the ability of a cyclodextrin-based nanoparticle (CALAA-01) to deliver RRM2-targeted siRNA in patients with melanoma<sup>42</sup>. Other studies with delivery of miR-122 for HCV infection<sup>72</sup> and lipid nanoparticles for delivery of siRNAs targeting VEGF and KSP in cancer patients have also demonstrated promising clinical results<sup>73</sup>. The DOPC nanoliposomal platform has already shown promise for delivery of Grb2-targeted anti-sense nucleotides<sup>74</sup> and has also been introduced into phase 1 testing for EphA2-targeted siRNA. Additional platforms are likely to build on these initial experiences and allow for robust delivery of RNAi-therapeutics.

The success of RNAi-therapy depends, in part, on careful selection of targets for such approaches and delivery to the appropriate sites. Several key targets (e.g., KRAS, MYC) are already widely considered to be important. Additional efforts in the selection of targets, have incorporated systems biology approaches where genomic and proteomics screens can be merged with functional and clinical data to identify the highest priority targets<sup>75,76</sup>. In such an approach, following a systematic effort aimed at target selection, validation studies are carefully carried out (**Figure 5**). The biological validation studies are ideally carried out in a portfolio of model systems that can recapitulate human disease and hopefully inform success and potential for toxicity in subsequent clinical studies. The nanoparticle systems should be selected based on several criteria including biocompatibility, efficiency of delivery, safety profile and pharmaceutical feasibility (e.g., ability to scale-up, nucleotide incorporation and cost efficiency).

### ***Future Scientific and Clinical Developments***

We are clearly at a crossroads of a massive amount of information and a need to converge disciplines to understand the biological and clinical significance of such data. The ability to convert such data into personalized medicine regimes is still in its infancy. Success will require multi-disciplinary teams that include biomedical engineers, cancer biologists, pharmacologists, and translational as well as clinical scientists.

The achievements so far have demonstrated important proof-of-concept studies for RNAi-based therapeutics and have identified opportunities for future work. One major future opportunity will be in improving frequency of dosing and careful planning of clinical trials. Most of the current delivery platforms require frequent dosing to maintain sustained gene silencing. While such therapies are feasible to deliver in clinical trials, sustained delivery methods could ideally reduce the number of clinic visits required for treatment. Some of these delivery methods (e.g., multistage vectors, dual-assembly nanoparticles) have shown preclinical evidence of sustained delivery. But, additional work will be required to refine these approaches for clinical testing.

Given the genomic chaos and instability present in many solid tumors, it is not surprising that bypass or redundant molecular pathways are activated following many of the current therapeutics. Such adaptive mechanisms require an iterative process whereby careful preclinical testing and information-rich early-stage clinical trial designs utilize systems

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**One major future opportunity will be in improving frequency of dosing and careful planning of clinical trials.**

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biology approaches. Either Phase 0 or Phase 1 trials with pre- and post-treatment biopsies are an important avenue to learn about adaptive changes. Moreover, Phase 0 studies offer another unique opportunity for assessing the delivery of nanoparticles directly to the tumor site. Then, using sophisticated model systems, rational combinations could be rapidly developed. Adaptive trial designs can further help to limit the number of patients in the inactive-dose cohorts with the test article and allow faster transition to phase 2 clinical trials. Nanotechnology-enabled RNAi therapies are ideally suited for carrying out “co-extinction” of adaptive pathways. Questions related to packaging multiple RNAi molecules in same nanoparticles vs. loading them separately, but co-administering them is similarly worthy of additional future investigation.

It is unlikely that biologically-targeted drugs will replace the existing therapies such as chemotherapy and radiation. Opportunities exist, however, to identify and block targets that can amplify the anti-tumor response to these traditional therapies. These combinatorial approaches will likely offer new avenues for not only improving response rates, but perhaps even cure rates. Another opportunity resides in enhancing immune therapies. Check-point inhibitors (e.g., anti-CTLA-4, anti-PD-1) have resulted in remarkable efficacy in a fraction of patients with various tumor types, in particular melanoma<sup>77</sup>. There are many reasons why others do not respond to such therapies at present, but silencing “undruggable targets” among others related to immune-tolerance represents an opportunity for expanding the reach of immunotherapies.

Many of the existing delivery methods result in a fraction of the payload being deposited into the tumor with a large fraction going to other organs, especially liver. Understanding the physico-chemical properties that allow for enhanced delivery into the tumor represents an important area of investigation. Moreover, exploiting targeted delivery of nanoparticles decorated with peptides, aptamers or other approaches might enhance therapeutic ratios. Clinical regulatory pathways are needed to allow these targeted delivery methods to move into clinical testing.

## Drug Reformulation

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### *Reformulation via Nanotechnology*

Reformulation of legacy drugs offers an efficient pathway for commercialization of nanotechnology platforms. Nanotechnology-based medicine, as a relatively new area of science, does not have the well-defined regulatory path of traditional drugs. Since the development of a new chemical entity utilizing nanotechnology further compounds regulatory scrutiny, the reformulation of existing drugs represents a logical first step toward market. An alternate formulation of an existing drug that is no longer under patent can be developed under the FDA 505(b)(2) regulatory path that utilizes existing safety data, and has less associated development cost and time than that of a new chemical entity under the traditional 505(b)(1) application process. The 505(b)(2) regulatory path was codified in the “Drug Price Competition and Patent Term Restoration Act” (1984) statutes with the specific goal of offering cheaper alternatives to the branded products, but has had the, perhaps, unintended consequence of expediting commercialization of new drug formulation technologies that offer therapeutic improvement of existing drugs.

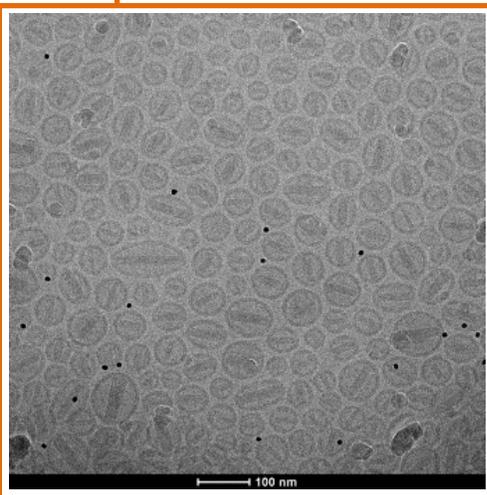
Nanotechnology reformulation can overcome many of the liabilities of current oncology drugs, including insolubility, rapid metabolism, poor bioavailability and off target toxicity. The earliest successful commercialization of nanotechnology was encapsulation of doxorubicin in a nanoscale liposome, approved by the FDA in 1995 (**Figure 6**). Liposomal doxorubicin, Doxil® (Janssen Biotech, Inc.), decreases systemic free doxorubicin concentrations, reducing cardiac exposure and associated cardiotoxicity<sup>78</sup>. The success of this formulation is highlighted by the recent approval of the first Doxil generic, Lipodox® (Sun Pharmaceutical, FDA approval 2013). Liposome reformulation strategies are also being used to deliver synergistic combinations of oncology drugs, an example being Celator’s combination cytarabine-duanorubicin liposome (CYT 351) that is currently in phase III clinical trials for treatment of acute myeloid leukemia.

### *Current Enabling Technologies*

Liposomal doxorubicin commercialization was followed by cremophor-free formulations of the highly insoluble drug paclitaxel, initially as an albumin nanoparticle, Abraxane® (Abraxis BioScience), approved in the US 2005, and later a polymeric nanomicelle,

Genexol-PM<sup>®</sup> (Samyang Genex Company), approved in Korea 2007<sup>79</sup>. Abraxane is a 130 nm nanoparticle composed of human donor-derived albumin, while Genexol-PM is a 25 nm micellar particle composed of monomethoxy poly(ethylene glycol)-block-poly(D,L-lactide) (PEG-PDLLA) copolymer. By removing cremophor from the legacy paclitaxel formulation, Taxol<sup>®</sup> (Bristol-Myers Squibb), these nanotechnology reformulations demonstrated dramatic improvements in dose tolerability, as cremophor-dependent dose-limiting hypersensitivity reactions were no longer observed. This allows maximum tolerated doses of >300 and 260 mg/m<sup>2</sup> for Cynviloq and Abraxane, respectively, in comparison to 175 mg/m<sup>2</sup> for the legacy Taxol formulation. In addition to eliminating unwanted hypersensitivity side effects, these new cremophor-free formulations are effective against malignancies that the legacy Taxol formulation was not. Abraxane received orphan drug status for treatment of late-stage pancreatic cancer in the US in 2013 and has projected sales of \$1.5-2 billion (Celgene Presentation at UBS Global Healthcare Conference, May 19, 2014 pp.9)<sup>80</sup>. Genexol-PM is currently in development in the US under the brand name of Cynviloq<sup>™</sup> (Sorrento Therapeutics, Inc.) as an alternate formulation of Abraxane under the 505(b)(2) regulatory pathway for the treatment of advanced pancreatic cancer<sup>81</sup>. This use of the 505(b)(2) pathway for development of an alternate formulation of a marketed

nanotechnology formulation is an example of how approval of nanotechnology formulations can further expedite approval of other nanotechnology formulations.



**Figure 6.** Cryo-transmission electron microscopy image of Doxil liposomal doxorubicin (courtesy of Dr. Ulrich Baxa, Electron Microscopy Laboratory, Frederick National Laboratory for Cancer Research, 2015).

The success of these reformulation efforts have solidified the advantages that nanotechnology offers the pharmaceutical industry, driving the implementation of nanotechnology earlier in the discovery phase of drug development. Many pharmaceutical companies now have in house nanotechnology formulation efforts underway, or are partnering with nanotechnology companies to optimize leads and even resurrect failed molecules. For example, a nanotechnology reformulation technique that has become so commercially acceptable that it is now used routinely in development of oral drugs is the Nanocrystal<sup>™</sup> technology first developed by the Elan Corporation. The first commercial nanocrystal formulation was a reformulation of sirolimus, Rapamune<sup>®</sup> (Wyeth Pharmaceuticals, Madison, NJ), approved in 2000<sup>82</sup>. Nanocrystal formulation can increase bioavailability of oral formulations by reducing drug particle size, resulting in a dramatic increase in

surface area, and therefore drug dissolution rate (**Figure 7**)<sup>83</sup>. Other advantages can include enhanced dose linearity and consistency. The Elan nanocrystal technology is also being used for parenteral drug delivery, and an intramuscular nanocrystal reformulation of the schizophrenia drug paliperidone palmitate was approved in 2009.

### Future Developments

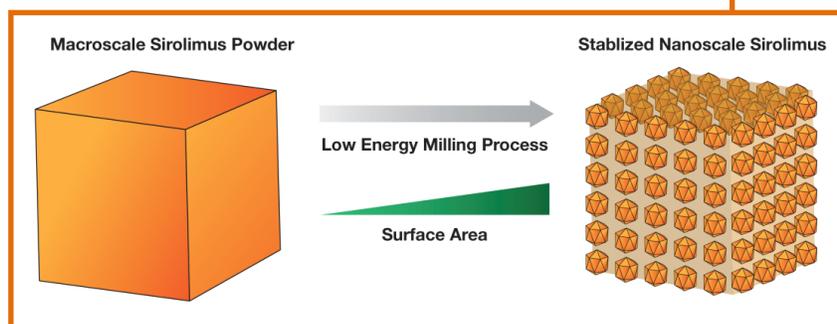
As described above, the earliest use of nanotechnology to improve oral bioavailability was for incremental increases

in the bioavailability of drugs already approved for oral administration through the use of nanocrystal technology. Recent formulation efforts are now focusing on the more difficult challenge of overcoming biological

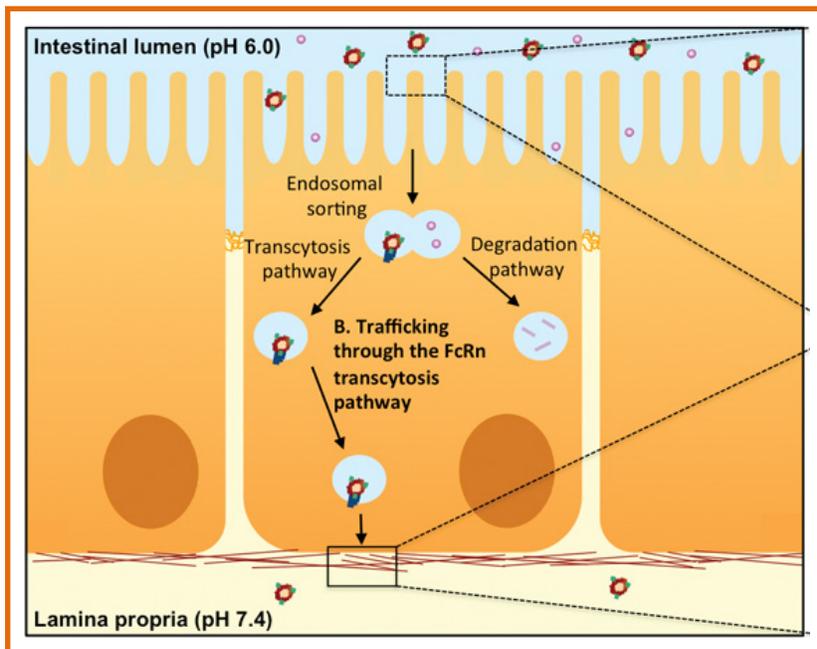
barriers, formulating

molecules with little or no inherent bioavailability, such as protein therapeutics. One such example is the work of Robert Langer's lab on oral insulin, utilizing receptor mediated transport to overcome the gastrointestinal mucosal barrier<sup>84</sup>. These researchers utilized a polymeric nanoparticle construct targeting gastrointestinal FcRN receptors to stabilize and deliver insulin to the systemic circulation (**Figure 8**). Optimization of this uptake pathway could revolutionize both protein and small molecule therapeutics, no longer requiring costly and invasive intravenous administrations. Another example of utilization of receptor-mediated transport to cross biological barriers is glutathione-targeted doxorubicin liposome designed to increase uptake across the blood-brain barrier. These glutathione-targeted doxorubicin liposomes developed by BBB Therapeutics are currently in phase II clinical trials for treatment of brain metastasis and glioma<sup>85</sup>.

Clearly, the future of nanomedicine resides in targeted therapies that allow for exquisite selection of diseased over healthy tissues. This was and continues to be the unrealized potential of this technology. The most notable advance in this area has come from Bind Therapeutics' progression of PMSA-targeted polymeric nanoparticles containing paclitaxel, Bind-014, to the clinic<sup>16</sup>. Bind's Accurin™ platform consists of a PMSA targeting S,S-2-[3-[5-amino-1-carboxypentyl]-ureido]-pentanedioic acid small molecule, attached to a mixed pegylated poly(d,l-lactide) (PLA) and poly(d,l-lactide-co-glycolide) (PLGA) nanoparticle. In addition to paclitaxel, Bind also has a vincristine formulation under late stage development,



**Figure 7.** The Elan Nanocrystal™ technology.



**Figure 8.** FcRN receptor-mediated nanoparticle uptake. (Reprinted with permission from Pridgen et al., 2013).

and is partnering with several pharmaceutical companies, including Pfizer, AstraZeneca, Roche, Merck, and Amgen, for development of their proprietary small molecules. Success of the Accurin platform will undoubtedly lead to further development of targeted therapies and new avenues for targeted reformulation. As has been the case in the past, reformulation will continue to lead commercialization of novel nanotechnology platforms.

With the joint efforts of investigators at academic institutes and within industry, several advances should come to

fruition over the upcoming 5-10 year time frame. In the next 5 years, researchers will have begun streamlining of drug reformulation by identification of optimal drug physicochemical properties that result in successful reformulation for each nanomedicine class; and begin commercialization of actively targeted-nanoparticle reformulations. Looking further ahead over the next 10 years, researchers will generate reformulation of intravenously administered small molecule and protein-based therapies for oral and inhalation administration.

## Nanotherapeutic Solutions for Metastatic and Disseminated Cancers

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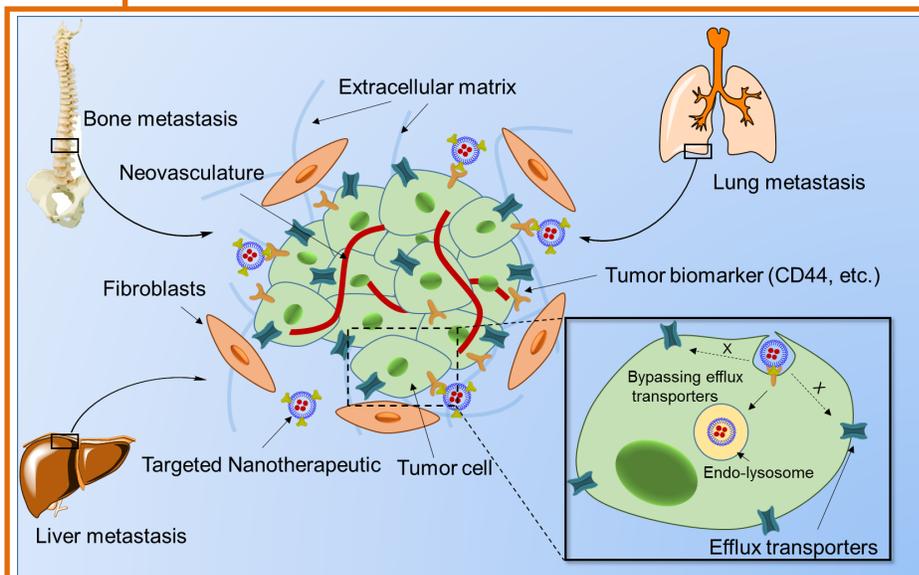
### *Metastasis Remains the Bane of Successful Cancer Therapy*

Cancer metastasis accounts for over 90% of all cancer associated death and suffering, representing the single biggest challenge to the management of cancer<sup>86</sup>. Although the advent of novel therapies and effective combination regimens has increased overall patient survival, many of these interventions are only palliative and an overwhelming number of cancer patients succumb to the disease<sup>87</sup>. Several factors can be attributed to this undesirable outcome, including the inefficiency of using conventional chemotherapeutics to treat small clusters of disseminated malignant cells or therapy-resistant metastases<sup>88</sup>. The three major sites of most cancer metastasis are the lungs, liver, and bone marrow (**Figure 9**).

Although small drugs and nanotherapeutics are readily delivered to the liver and lungs, the protective bone marrow niche provides a conducive environment for metastatic cells to undergo intrinsic genetic and epigenetic cellular changes that eventually lead to drug resistance<sup>88</sup>. When present in small clusters, the small tumor surface area relative to surrounding uninvolved tissue reduces the efficacy of treatment at the typically low concentrations of drugs that reach the metastatic tumor cells. Further complicating the treatment response is the high expression of cell membrane-based efflux transporters, such as P-glycoprotein 1 and multidrug resistance-associated protein 1, which effectively expel the drugs before they can exert therapeutic effects on the cellular machinery<sup>89</sup>. Moreover, the serious side effects caused by conventional chemotherapeutics, particularly to the bone marrow stem cells, are limiting factors. As efforts to uncover the biological mechanisms of cancer metastasis and resistance to therapies continue to provide new insight into the metastatic niche, it is obvious that new therapeutic approaches are needed to increase treatment efficacy, prevent relapse, and provide a cure with minimal off-target toxicity. These goals can be accomplished by harnessing the multivalent and multifunctional attributes of nanoparticles to design novel nanotherapeutics with the capacity to irreversibly trigger cancer cell death.

## Cancer Nanotherapeutic Strategies for Metastatic and Disseminated Tumors

Nanotherapeutics have considerable advantages over conventional chemotherapeutics, including the ease of controlling their circulation times in blood, as well as their *in vivo* stability, bioavailability, and bioactivity. These properties can be employed to address some fundamental limitations of small molecule chemotherapeutics in treating metastatic tumors. For example, nanotherapeutics are frequently used to improve the bioavailability and local concentration of existing drugs that are highly effective against metastatic cancer cells via passive targeting. This approach is most effective in large metastases of the liver and lungs, where an enhanced permeability and retention (EPR) effect is achievable. However, EPR uptake is ineffective for small and poorly vascularized micrometastases (tumors <2 mm in size), which are frequently found in the bone marrow and at early stages of metastasis elsewhere. Efforts to address this challenge have focused on nanoparticle formulations designed to target cancer biomarkers selectively. Although the mechanism of tumor uptake is not fully understood at this point, albumin-bound paclitaxel (Abraxane), represents an interesting coupling of EPR and cancer-targeted approaches to deliver drugs to tumor cells. Clinical studies demonstrate that this nanoparticle-bound drug exhibited a blood circulation half-life more than 100 times longer than that of the small molecule paclitaxel alone. Response rate (74% vs 39%) and progression-free survival (14.6 vs 7.8 months) using the nanotherapeutics were higher than for the unbound drug in patients with metastatic breast cancer<sup>90</sup>.



**Figure 9.** Major sites of cancer metastasis and the respective nanotherapeutic targeting strategies.

Some disseminated tumors, such as multiple myeloma, which can serve as a model of bone marrow metastasis, and particularly drug resistant phenotypes, commonly found in niches such as the bone marrow microenvironment, are not responsive to Abraxane nanotherapy. For example, adhesion of multiple myeloma

cells to the bone marrow stroma results in cell-adhesion-mediated drug resistance (CAM-DR). Thus, a dual-function ligand that simultaneously targets the tumor cells and inhibits adhesion to surrounding stroma would improve treatment outcome. This goal was achieved in a recent study by loading self-assembling micellar nanoparticles with doxorubicin and functionalizing the micelle surface with very late antigen-4 (VLA-4) peptide, which served as an anti-adhesion molecule. This formulation not only selectively delivered doxorubicin to the tumor cells, but also overcame CAM-DR. The micellar nanoparticles preferentially homed to tumors in the bone marrow with ~10-fold higher drug accumulation and tumor growth inhibition with a reduced overall systemic toxicity compared to the small molecule drug alone<sup>91</sup>. An alternative approach incorporates antisense drugs into polymeric nanoparticles for targeting the genes of osteopontin and bone sialoprotein, which are overexpressed in bone metastases of mammary carcinomas. These nanoparticles protect the drugs against nuclease degradation, thereby enabling sustained release of antisense therapeutics and a significant decrease in the incidence of bone metastasis<sup>92</sup>.

The effectiveness of some drugs is hampered by the high efflux rate in drug resistant phenotypes of metastatic cells expressing P-glycoprotein 1 and multidrug resistant transporters. Despite several studies demonstrating the efficacy of Vincristine sulfate (VS) in cancer therapy, the high efflux rate by these transporters decreases the intracellular resident time for effective therapy. To overcome this impediment, VS was encapsulated in polymeric nanoparticles, causing it to be taken up through clathrin and caveolae mediated endocytotic pathways and allowing it to bypass the efflux transporters. The ensuing accumulation and retention of VS nanotherapeutics in metastatic cancer cells resulted in a ~21-fold increase in cytotoxicity compared to VS alone<sup>93</sup>.

### ***Future Challenges***

Cancer is a highly heterogeneous disease with distinct cell subpopulations that are phenotypically and biochemically diverse. Given their different capacities to grow, differentiate, develop drug resistance, and form metastases, understanding tumor biology is critical for the development of successful therapies. Biomarker discovery and identification is an important aspect of this progress and an indispensable step in the development of targeted nanotherapeutics. However, significant variations between primary and metastatic cancer from the same patient further complicate the development of a consensus strategy to

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**Cancer is a highly heterogeneous disease with distinct cell subpopulations that are phenotypically and biochemically diverse.**

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treat the disease. The ability to target multiple cancer biomarkers and deliver combinatorial therapy favors the use of nanotherapeutics to maximize treatment outcome. An emerging frontier in cancer therapy is in understanding the contribution of tumor environment to its survival and metastasis. Some studies suggest that several factors alter a secondary site before the homing of migrating tumor cells. Sometimes the metastatic tumor cells remain dormant and undetectable after the primary cancer is removed, leading to relapse. With current knowledge of cancer-type specific metastatic patterns, it will be possible to develop nanotherapeutics that can reside in the secondary tissue for prolonged periods to achieve preventive or augmented nanotherapy. In addition, this treatment paradigm could be enhanced by other forms of therapy, such as gene silencing and immunomodulatory techniques to provide a multipronged strategy to combat cancer, with minimal morbidity effects to the patient. Phototherapy appears to be effective in treating metastasis, but the limited penetration of light has hampered the use of this technique in clinics. A recent study postulates that Cerenkov radiation from radionuclides used in positron emission tomography could serve as a depth-independent light source for cancer therapy in the presence of photo-sensitive nanomaterials that generate cytotoxic radicals upon exposure to light<sup>62</sup>. Application of this concept to the treatment of circulating tumor cells and metastases could improve treatment outcome, especially for chemotherapy resistant metastasis.

## Nanotechnology Solutions to Overcome Plasticity and Resistance Using Epigenetic and MicroRNA-Based Reprogramming

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### *Tumor Plasticity and Therapeutic Resistance*

Plasticity is an inherent characteristic of cancer and plays a vital role in cancer initiation and sustenance. The cellular changes that transition a normal cell into a cancer cell can be defined as cellular plasticity; likewise the perpetual adaptations that cancer cells undergo to survive can be classified as cellular plasticity. In this sense, tumor plasticity enables therapeutic resistance and could be considered a survival response. As cells that continually transform to maintain their immortalization, cancer cells are the ultimate biological representation of “survival of the fittest,” through their inherent plasticity they are able to adapt and survive in inhospitable conditions (low oxygen, nutrient deprived) and even evade the effects of cytotoxic drugs and biologics. In 2000 and in a 2011 follow-up review, Hanahan and Weinberg took a comprehensive approach to characterizing cancer and defined the six hallmarks of cancer as; the ability to sustain proliferative signaling, the ability to evade growth suppressors, activation of invasion and metastasis, replicative immortality, induction of angiogenesis, and resistance to cell death<sup>94</sup>. An important feature of solid tumor masses is their cellular heterogeneity, this is caused by survival adaptations of cells (plasticity) and the inherent genome and proteome dysregulation characteristic of cancer cells; tumor heterogeneity undoubtedly contributes to drug resistance. Multi-drug resistance (MDR) can be innate (biologically inherent to the cancer cell) or acquired (after drug exposure); as discussed below, epigenetic factors and microRNA contribute to both innate and acquired MDR as well as to tumor plasticity. Cancer cells employ a variety of mechanisms of MDR including decreasing drug influx into the cell, increasing drug efflux, increasing DNA repair, increasing drug metabolism, and decreasing apoptosis<sup>95</sup>. Tumor heterogeneity is a challenge to the clinical treatment of solid tumors as tumor sub-populations of cells respond differently to treatment, which can increase the development of acquired MDR and metastasis. Tumor plasticity enables drug resistance and cell survival despite aggressive therapeutic treatment.

## *Epigenetic and Phenotypic Reprogramming*

In recent years, the role of epigenetics in genotype expression has been elucidated and we are beginning to understand the significance of epigenetics in cancer development and regulation. Epigenetics refers to a heritable (mitotic and meiotic), stable change in gene expression without a modification of the DNA sequence<sup>96</sup>. The most common epigenetic changes include direct chemical modifications of DNA (methylation), histone modifications, and chromatin remodeling. Epigenetic modifications regulate cell differentiation, maternal and paternal inheritance patterns, gene expression responses to environmental factors and stress, seasonal gene expression, and cancer development<sup>97</sup>. When the human genome project completed in 2003, there were still many questions that the vast “decoding” could not seem to answer; how do our experiences, the food we eat, the environment we are exposed to, and daily stress exert a genetic effect? How can these variables lead to cancer?

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**...the role of epigenetics in genotype expression has been elucidated and we are beginning to understand the significance of epigenetics in cancer development and regulation.**

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How does parental imprinting occur? The epigenome has evolved as an answer to these questions. If DNA is thought of as the same set of ingredients that every cell has, the epigenome can be thought of as the recipe – what each cell makes with those ingredients; an old, memorized family recipe that is passed down from generation to generation. Given the governing role of the epigenome in gene expression, the contribution of epigenetic changes to cancer initiation, progression, plasticity, and resistance is not surprising<sup>97</sup>. Although tissue-specific and patient specific epigenetic variations have been noted in tumors, in general, the cancer epigenome displays hypomethylation and hypermethylation at site-specific CpG islands (cytosine clusters) within gene promoters<sup>97</sup>.

Also in recent years, the powerful contribution of microRNAs (miRNAs) to cancer has been discovered. MicroRNAs are 18-25 nucleotide, noncoding RNAs that negatively regulate gene expression at the post-transcriptional level. RNA polymerase

II or III transcribes a primary microRNA (pri-miRNA) in the

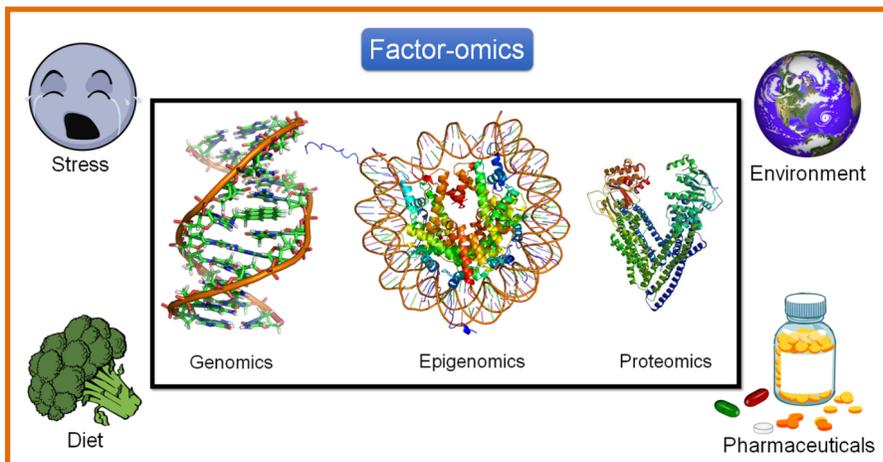
nucleus, the pri-miRNA is cleaved by a Drosha/DGCR8 complex to form precursor miRNA (pre-miRNA) which is transported into the cytoplasm, then Dicer processes the pre-miRNA into mature miRNA for incorporation with RISC (the Argonaute containing RNA-induced silencing complex)<sup>98</sup>. It is this miRNA-RISC complex that blocks gene expression by either degrading target mRNA or by hybridization to the 3' untranslated region of the target mRNA<sup>98</sup>. Over 2,500 miRNAs have been identified and many have multiple targets; although

many miRNAs are down regulated in different cancers (such as the miR-34 family), miRNAs that are overexpressed in many cancers have been coined “onco-miR’s;” these oncogenic microRNAs include miR-155 and miR-21<sup>99</sup>. Validated oncogenic miRNAs such as miR-21 have been demonstrated to contribute to drug resistance, as has miR-19 and the miR-221/222 family<sup>100</sup>.

There is a dynamic feedback circuit between epigenetics and miRNAs where the epigenome regulates the expression of miRNAs and certain miRNA’s control mediators of the epigenome such as histone deacetylases, DNA methyltransferases, and polycomb group proteins (regulate lineage delineation)<sup>101</sup>.

### ***Nanotechnology-Based Delivery Strategies for Reprogramming***

A recent study validated epigenetic targeting with nanoparticle based therapies as an approach to reverse MDR. The study combined decitabine (a DNA hypermethylation inhibitor) loaded nanoparticles with doxorubicin loaded nanoparticles and demonstrated that combination therapy improved the efficacy of treatment and decreased the expression of DNA methyltransferase isoforms in the tumor bulk and in cancer stem cell populations in an MB-MDA-231 xenograft model in mice<sup>102</sup>. Using nano-based delivery systems to co-administer epigenome modifiers with standard chemotherapeutics has clinical potential as a strategy for reducing tumor plasticity and stem-like properties while reversing drug resistance. Likewise, combination therapy with chemotherapeutics and microRNA mimetics delivered in nanoparticle based formulations have demonstrated reversal of MDR through down regulation of ABC transporters (drug efflux pumps)<sup>103</sup>. MicroRNAs demonstrated to down regulate ABC transporters include miR-451, miR-27a, miR-223, miR-331, miR-326, miR-297, miR-487a, and miR-181a<sup>103</sup>. A variety of nanoparticle platforms have been explored for miRNA mimetic delivery, nanoparticles are ideal for nucleic acid delivery as they offer levels of protection as well as the ability to surface functionalize the vector for active targeting to tumor tissue. In April of 2013, the first clinical trial (phase 1) of a microRNA mimetic began in patients with liver cancer and hematological malignancies<sup>104</sup>. MRX34 consists of a miR-34 mimetic administered in “Smarticles”; pH responsive liposomes that exploit the lower pH of tumors to facilitate uptake<sup>104</sup>. As endogenous miR-34 regulates over 20 oncogenes, pre-clinical studies have demonstrated MRX34’s ability to restore tumor suppression<sup>104</sup>. Cationic liposomes have been used to deliver miR-29b in pre-clinical lung cancer models, as miR-29b targets the cyclin dependent protein kinase 6 oncogene in lung cancer, treatment with the liposomes resulted in sixty percent tumor growth inhibition in a mouse model<sup>105</sup>. A variety of lipid and cationic polymer based nanoparticle systems have been developed for miRNA delivery in pre-clinical pancreatic cancer models<sup>106</sup>. More elaborate systems such as a liposome-polycation-hyaluronic acid nanoparticle system surface modified with



**Figure 10. Emergence of “factor-omics” as a field, classifying and studying the environmental, dietary, physiological, and pharmacological factors that influence the epigenome, post-transcriptional gene expression, and the proteome.** Genomics is the foundational field, proteomics is the translational product of the genome, the epigenome regulates gene expression (and hence, proteomics), and factor-omics will detail the environmental, nutritional, physiological (such as stress), and pharmacological factors that influence the genome, epigenome, and proteome.

a single chain antibody fragment to actively target GC4 (a metastatic melanoma epitope) for combination delivery of siRNA and miRNA have been developed and have demonstrated efficacy in reducing tumor growth and inhibiting metastasis<sup>107</sup>. Nucleic acids require delivery vectors such as nanoparticles to avoid immune system clearance and degradation and achieve therapeutic concentrations at the target site; the clinical application of microRNA relies on nanotechnology to enable therapeutic delivery. In addition to therapeutic applications,

nano-based sensors are also being explored for cancer biomarker detection of circulating microRNAs and circulating tumor DNA<sup>108,109</sup>. In a 2011 article in *Nature Nanotechnology*, Li-Qun Gu and fellow researchers reported the development of a nanopore sensor capable of sub-picomolar detection of target microRNA in the plasma of lung cancer patients<sup>109</sup>. The nanopore used in this study was the  $\alpha$ -haemolysin protein pore; synthetic nanopores are sure to follow in coming years<sup>109</sup>. More recently, researchers have developed a gold nanoparticle based sensor with peptide nucleic acid probes that exploit localized surface plasmon resonance to detect tumor-specific epigenetic variations in human serum samples<sup>108</sup>. Profiling a patient’s disease from their plasma sample is a remarkable advancement in clinical oncology and could provide a powerful means of assessing and tailoring treatment.

## *Future of the Field*

In this era of “omics” we anticipate the development of the next “omics” field; a field we will dub “*factor-omics*” for now (**Figure 10**), a field studying and classifying the factors that affect the epigenome, post-transcriptional gene expression, and the proteome. This field has already begun although has yet to be unified in a cohesive way, as with genomics, proteomics and epigenetics, this will occur naturally as the science progresses. Studies detailing the genetic, epigenetic, and post-translational effects of environmental, nutritional, physiological, and pharmacological factors have been well under way for some time, yet the key to evolving this field will be reviewing the results of the studies and making collective observations that can form the foundational science of the field. A second significant anticipated advancement in this arena will be the clinical application of nanotechnology-based sensors for microRNA and epigenetic cancer biomarkers.

With the joint efforts of investigators across the spectrum, several advances should come to fruition over the upcoming 5-10 year time frame. In the next 5 years, researchers will have performed scientific studies/reviews to classify and interpret the environmental, physiological, and pharmacological factors that influence the epigenome and proteome; perform clinical evaluations of microRNA nano-sensors for cancer biomarker screening; and research investigational nano-therapeutics that reverse MDR using microRNA and epigenetic approaches. Looking further ahead over the next 10 years, the establishment of “*factor-omics*”; a field classifying and studying the environmental, physiological, and pharmacological factors that influence the epigenome, post-transcriptional gene expression, and the proteome will occur. As genomics is the foundational field, proteomics is the translational product of the genome, and the epigenome regulates gene expression (and hence, proteomics), *factor-omics* will detail the environmental, physiological, and pharmacological factors that influence the epigenome and proteome; clinical application of microRNA nano-sensors for cancer biomarker screening; and clinical testing of nano-therapeutics that reverse MDR using microRNA and epigenetic approaches.

**A second significant anticipated advancement in this arena will be the clinical application of nanotechnology-based sensors for microRNA and epigenetic cancer biomarkers.**

## Exosome-Mediated Communication in the Tumor Microenvironment and Metastasis

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### *Tumor Exosomes and Content*

Although exosomes were first discovered in 1987<sup>110</sup>, it wasn't until recent years that the importance of exosomes in cellular communication has been elucidated. Exosomes are 30-100 nm vesicles shed by cells as a process of cell signaling and communication. In recent years it has been discovered that cancer cells produce and shed more exosomes than normal cells<sup>111</sup>. Exosomal release is one of three possible fates for multivesicular bodies (MVB). Multivesicular bodies are formed when plasma membrane receptors are marked for recycling or degradation through ubiquitination; early endosomes are formed through plasma membrane internalization and as internal vesicles form within the endosome, the endosome transitions to multivesicular bodies<sup>111</sup>. The three fates for multivesicular bodies are; recycling through the trans-Golgi network, lysosomal degradation, or secreted through exocytosis or through fusion with the plasma membrane (exosome release). Exosome secretion through exocytosis is mediated through intracellular  $Ca^{2+}$  levels while factors such as extracellular/intracellular pH gradients can effect release and uptake<sup>112,113</sup>. Much investigation has focused on exosome content and determining if exosome content is a deliberate process in cell signaling; exosome content is rich in enzymes, microRNA, transcription factors, heat shock proteins, MHCs, cytoskeleton components, signal transducers, and tetraspanins (transmembrane proteins). It is most commonly accepted that exosome content is determined non-specifically under multivesicular formation and not through a deliberate sorting and packaging process<sup>111</sup>. But is this really the case? Are most biological processes not deliberate? From a metabolic perspective, it would be a vast waste of cellular energy for exosome content *NOT* to be deliberate. Perhaps there is a missing piece we have not had insight to yet, indeed, the function of the endosomal sorting complex required for transport (ESCRT) in sorting ubiquitinated proteins provides insight to a possible sorting process<sup>114</sup>. Perhaps in healthy cells exosome release is one of three cellular fates for MVB, but in cancer cells, exosome release is exploited as a deliberate means of cell communication and to specifically achieve metastasis. The existence of this missing piece – the confirmation that cancer cells use exosomes as a deliberate mechanism of communication is likely to be proved or disproved within the next five years.

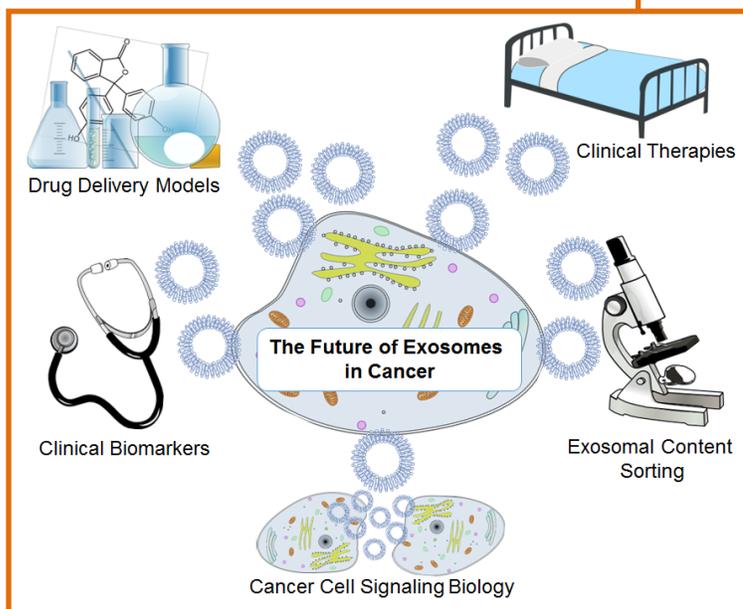
## Exosome-Mediated Cell-Cell Communication

Exosomes are taken up by recipient cells through receptor-mediated endocytosis, pinocytosis, phagocytosis, or through fusion with the cell membrane resulting in direct release of contents into the cytoplasm. If cancer cell exosomal content is not selected randomly, but is a deliberate process, then exosomes can be thought of as the cancer cells elevator pitch to the outside world – *this is what I want you to know and why*. On the other hand, if the current paradigm is correct where exosomal content is not selective, and is just a random sample of the cellular content then exosomes can be thought of as an informational press release to the public – *this is the news, this is what I am doing right now*. Either way, it is a powerful means of communication that is utilized by cancer cells more than normal cells. Despite the intent of the message, what is the result of these messages?

Among other effects, such as transferring drug resistance, a demonstrated result of exosomal communication is metastasis. The metastatic process consists of a series of events that include the epithelial-mesenchymal transition (EMT; mobilizing cells) and the mesenchymal-to-epithelial transition (MET; establishing a secondary tumor site). Cancer exosomes have been demonstrated to deliver functional proteins, complexes, and RNA that promote both EMT (such as HIF-1 $\alpha$ ) and MET (such as miR-200).

### Metastasis: Epithelial-Mesenchymal Transition (EMT)

Hypoxia Inducible Factor-1 $\alpha$  (HIF-1 $\alpha$ ) has gained attention over the past ten years as a powerful transcription factor contributing to oncogenic, aggressive, and drug resistant phenotypes in cancer. Under hypoxic conditions and under conditions of cell stress HIF-1 $\alpha$  translocates from the cytoplasm to the nucleus where it forms an active transcription complex with HIF-1 $\beta$  binding to hypoxia responsive



**Figure 11.** The future of exosomal research in cancer will entail fast-tracked clinical therapies and diagnostics for clinical biomarkers, deeper insight into cancer cell signaling particularly from highly heterogeneous tumors, studying exosomes as a model for drug delivery, and answering the highly debated question of exosomal content sorting and selection as a deliberate or non-selective process.

elements on over fifty target genes including growth factors, drug efflux pumps, glucose transporters, cadherins, and factors that promote invasion and metastasis<sup>115</sup>. Our own studies have demonstrated a correlation between HIF-1 $\alpha$  expression, multidrug resistance, and aggressive tumor phenotypes<sup>115</sup>. HIF-1 $\alpha$  also contributes to epithelial-mesenchymal transition (EMT)<sup>116</sup>. A recent study by Pagano and Shackelford demonstrated that HIF-1 $\alpha$  is excreted in a functional form from nasopharyngeal carcinoma cells infected with Epstein-Barr virus<sup>116</sup>. The study illustrated that transfection of nasopharyngeal carcinoma cells with latent membrane protein 1, the primary oncogene of Epstein-Barr virus, increased HIF-1 $\alpha$  in secreted exosomes<sup>116</sup>. Using HA-tagged HIF-1 $\alpha$  expression vectors in a series of *in vitro* studies the researchers demonstrated that exosomal HIF-1 $\alpha$  was transcriptionally active in recipient cells. This, and similar studies, have demonstrated that exosome content can be altered through genetic and phenotypic modifications in the donor cell and these alterations can have profound effects on cell signaling through exosomal release and uptake.

### ***Metastasis: Mesenchymal-to-Epithelial Transition (MET)***

One of the most groundbreaking exosomal studies in recent years was the eloquent investigation conducted by Judy Lieberman at Boston Children's Hospital. Lieberman et al demonstrated that exosomes and ectosomes (larger vesicles formed by cell membrane budding) released from metastatic cancer cells can transfer metastatic capability to non-metastatic cells and this capability appears to be mediated through the microRNA-200 family, known regulators of mesenchymal-to-epithelial transition (MET)<sup>117</sup>. The study used extensive *in vitro* and *in vivo* techniques and through the meticulous selection of experimental conditions, resulted in a foundational exosomal and microRNA study. For example, the study selected cells with distinct metastatic capabilities (metastatic 4T1E mouse cells and metastatic human cells CA1a and BPLER cells and poorly metastatic 4T07 mouse cells and poorly metastatic human mesenchymal MB-231 cells) to study *in vivo* metastatic induction in mouse and human xenograft models. The study optimized the use of fluorescent cell labeling in many experiments; for example, to distinguish between metastatic lesions formed from circulating tail-vein injected cells from primary tumor cells, GFP-expressing primary orthotopic breast cancer tumors were developed in mice and firefly luciferase and mCherry expressing tumor cells were injected via tail-vein-injection<sup>117</sup>. Collectively, the *in vitro* and *in vivo* analysis demonstrated that exosomes and ectosomes from highly metastatic cells can increase the metastatic capabilities of local and distal poorly metastatic cells through the uptake of MET regulating miR-200<sup>117</sup>.

## ***Exosome Content Modulation and Application***

An interesting phenomena that was noted in the Lieberman study was that micro-RNA's delivered in exosomes are sometimes associated with Ago2, indicating these miRNA's may be contained in RNA-induced silencing complexes (RISC) which results in their immediate activity in recipient cells<sup>117</sup>. In the Pagano and Shackelford's studies of HIF-1 $\alpha$  exosomal delivery, HIF-1 $\alpha$  was delivered both as an inactive (uncomplexed) and active (complexed) form<sup>116</sup>. Our current understanding of exosomal content is that it is non-specific and dependent on the cellular content. It may be, just as years ago introns were considered to be "junk DNA", that we just do not have a complete understanding of this process yet. It may be that as we learn more about exosome formation and communication that the process is revealed as a deliberate and selective mechanism of cellular communication.

From a drug delivery perspective, exosomes are nature's own nanoparticles delivering an array of functional proteins and nucleic acids. Exosomes are innate "stealth" carriers that can have profound effects on recipient cells. Exosomes can benefit the field of medicine and therapeutics in two ways; studying exosomes as a biological model for "drug" delivery and manipulating exosomes for therapeutic outcomes and as diagnostic tools (**Figure 11**).

The methods for altering exosome content are electroporation, direct chemical transfection of exosomes, transfection of exosome donor cells, activation of exosome donor cells, and direct incubation of exosomes with loading cargo<sup>118</sup>. Elaborate investigational studies, such as Lieberman's miR-200 exosomal study are being conducted, and this exosomal research has been so exciting and promising, exosomes seem to have fast-tracked their way into clinical trials. Several clinical trials have already completed globally to explore the medical promise of exosomes as cancer therapeutics. The most recently completed exosome clinical trial in the United States was a pilot study of an immunotherapy vaccine for malignant gliomas<sup>119</sup>. The Phase I trial was conducted by David Andrews at Jefferson University Hospital and consisted of extracting the patient's own tumor cells, treating them with an antisense oligodeoxynucleotide against insulin-like growth factor type 1 receptor (IGF-1R/AS-ODN), placing the treated cells in a biodiffusion chamber, implanting the device in patients abdomens and relying on exosomes released from the chamber to communicate and initiate an immune response (T-cell activation) against the tumor<sup>119</sup>. A second Phase 1 trial of this therapy is underway as the majority of patients (8/12) in the first trial elicited a positive clinical response<sup>119</sup>. Other clinical trials recruiting

**...exosomes are nature's own nanoparticles delivering an array of functional proteins and nucleic acids.**

patients in the US include a study investigating the use of plant derived exosomes to deliver curcumin to colon tumors and normal colon tissue and a study evaluating circulating exosomes as prognostic and predictive biomarkers for gastric cancer patients. Exosomes are indeed proving to be effective, innate, *cellular nanoparticles* that can be manipulated for therapeutic applications, used as cancer biomarkers, and studied as ideal models for drug delivery.

Several milestones should come to realization over the upcoming 3-10 year time frame. In the next 3-5 years, researchers will have standardized methods for isolation and study of Exosome communication in the immune/tumor interface, intra-tumoral communication, extracellular matrix composition, and metastasis; should have a definitive answer, is exosomal content deliberately selected in cancer cells as a mechanism of cell communication, invasion, and metastasis?; be studying exosomes as “native” nanoparticles as a model for drug delivery; and clinical trials for therapeutic and biomarker applications of exosomes. Looking further ahead over the next 10 years, the establishment of tools and methods for biomarker screening; began therapeutic intervention at the immune/tumor interface, intra-tumoral communication, extracellular matrix composition, and metastasis; studied exosome signaling from distinct cancer cell populations, MDR cells, cancer stem cells; and clinical approval and marketing of exosomal therapeutics and diagnostic tools.

## Measuring Therapeutic Response to Cancer Immunotherapy via Nanotechnology

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Cancer Immunotherapy was the Science Breakthrough for the Year 2013<sup>77</sup>, with tremendous promise and excitement surrounding two immunotherapy classes. Class 1 is comprised of immune checkpoint inhibitors<sup>120,121</sup>, such as for the programmed death (PD)-1/L1 blockade, or anti-CTLA-4. These drugs can increase the susceptibility of cancer cells to immune system attack. Class 2 is adoptive cell transfer (ACT)<sup>122,123</sup>, which seeks to strengthen the anti-tumor immune system function. ACT of chimeric-antigen-receptor (CAR) engineered T cells is now being pursued within a number of major pharmaceutical companies as an effective treatment for leukemias and lymphomas. The clinical testing of PD-1/L1 blockade has been carried out in multiple cancers, but has been led by work in melanoma<sup>124</sup>, and has demonstrated a new era in cancer treatment<sup>125,126</sup>. It is fair to say that cancer immunotherapy has, in just the past two years, altered the conversation around cancer therapies from that of ‘treatments’ to that of ‘cures.’ However, it is still in its very early days yet, and immunotherapies have only been shown to provide powerful treatments for a subset of cancers, and even within those subsets, only for specific patient populations. Even for those patients who exhibit strong anti-tumor responses to immunotherapies, only a fraction (albeit a large one) exhibit durable responses. Thus, in order for the profound benefits of cancer immunotherapy to be extended to increasingly larger patient populations, there are a number of technological challenges to be addressed, and there are important roles for cancer nanotechnology to play. Here we outline two of many such challenges.

### *In Vivo Biomarkers*

As with any therapy, it is challenging to identify potential immunotherapy responders from non-responders. The most promising prognostic biomarker is that of a pre-therapy anti-tumor immune response, in the form of CD8+ T-cells infiltrating into the growing margins of the tumor. Patients that exhibit such a baseline immune response are significantly more likely to respond to PD-1/L1 blockade therapies<sup>127</sup>, and it is an absolute requirement for patients seeking ACT therapies that utilize *in vitro* expanded populations of tumor-infiltrating lymphocytes<sup>122</sup>. For melanoma patients, obtaining tissue biopsies for the analysis of CD8+ T cell infiltrates is straightforward, but for many tumors, such biopsies are not readily obtained. Thus, an *in vivo* imaging probe of CD8+ T cells would provide a powerful diagnostic tool for stratifying patients. If it is a positron emission tomography (PET) probe, then

antibodies are unlikely to serve this purpose, as their retention time in the body provides unwanted competition for the half-life of the <sup>18</sup>F-radiolabels commonly used. In addition, commercially available anti-CD8+ monoclonals do not exhibit particularly high affinities for the target. A high affinity, and a low off rate, are both important metrics, because many patients who exhibit a baseline anti-tumor immune response only have a low number of CD8+ T cell infiltrates. Other *in vivo* biomarkers include the emerging list of immune checkpoint molecules that are being explored for expanding immunotherapy to cancers such as prostate or breast. Thus, there is a unique opportunity here for nanotech solutions that can provide for rapid clearance, high target avidity, and tumor penetration.

**The most promising prognostic biomarker is that of a pre-therapy anti-tumor immune response, in the form of CD8+ T-cells infiltrating into the growing margins of the tumor.**

### ***Neoantigens and the Design of ACT Therapies***

In any cancer immunotherapy, the major tumor cell killers are CD8+ T cells. The killing function of those T cells is activated following a highly specific interaction between the T cell receptor (TCR) and a tumor antigen presented by tumor cells (**Figure 12**). Very recent findings are pointing to the importance of neoantigens in eliciting strong and highly specific anti-tumor T cell responses<sup>128–131</sup>. Neoantigens are fragments of proteins from the cancer cells that contain

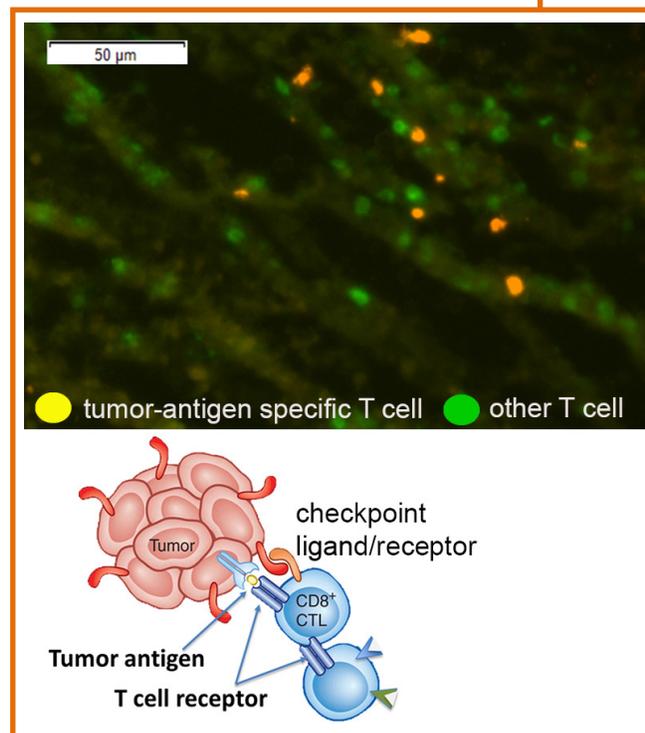
genetic mutations, and so differ from self-antigens. The very strong implication is that if one knows the tumor antigens present within a patient's tumor, and one knows sequence of the TCR  $\alpha/\beta$  chain gene that encodes a TCR that recognizes those antigens with high avidity, then one can design a personalized, and potentially highly effective ACT therapy for that patient. In terms of guiding this technology discussion, we'll assume that one has access to tumor tissue from the patient. The key information for designing a personalized ACT therapy regimen for the patient is the following:

- *Which T cell populations, as defined by specific TCR receptors, have clonally expanded within the tumor?* That information identifies the cells that have 'seen' tumor antigen.
- *What are the tumor antigens that are promoting this clonal expansion?* If the tumor antigens are neoantigens, then they are likely safe immunotherapy targets. If they are not, then they must be evaluated with great caution.

- *What are the TCR  $\alpha/\beta$  gene sequences that encode recognition for the specific neoantigens?* This is the information that is required for genetically engineering the T cells for the actual ACT.

There has been a recent flurry of activity in this area, but no approach has come close to yielding all three pieces of information, and most only yield one of the three pieces<sup>132,133</sup>. As such, here are the major challenges.

First, the tumor exome may be mined to identify potential neoantigens using existing software, and the number of neoantigens for a given tumor is likely on the order of 20-200. One can build a tetramer library based upon these 20-200 neoantigens<sup>134</sup>, but the best cytometry approaches for tetramer-based T cell sorting based are 20-plex, and so barely touch the required range of multiplexing<sup>133</sup>. Even those methods require that the T cells infiltrates from the tumor be expanded *in vitro*. Next, identification of those T cell populations that have clonally expanded within the tumor requires analysis of infiltrating lymphocytes directly from the tumor – i.e., without expansion *in vitro*. One may obtain only  $10^4$ - $10^5$  T cells from a tumor biopsy. This is not enough for standard cell analysis tools, but may be enough for nanotech tools. Finally, once the T cells that recognize a specific neoantigen are identified, the TCR  $\alpha/\beta$  genes must be sequenced at the single cell level. The TCR gene is very challenging to sequence, but methods for TCR gene sequencing with reasonable (~50%) yield have been reported<sup>135-137</sup>. No existing technology can *simultaneously* solve these three challenges. This should motivate a challenge to the cancer nanotechnology community, specifically, for an analytical/diagnostic modality that can help provide such a solution, in the next 5-10 years.



**Figure 12.** Tumor antigen-specific T cells are imaged in this fluorescence micrograph of a tumor from an *in vivo* immunotherapy model. Details of tumor/T cell interactions are shown in the drawing below.

## Enhancing Cancer Immunotherapy with Nanotechnology

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### *Cancer Immunotherapy*

Cancer immunotherapy utilizes the patient's own immune system to treat cancer, now a powerful novel strategy in cancer treatment. Antibodies blocking negative immune regulatory pathways, such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death 1 (PD-1), have substantially improved clinical outcomes in patients with metastatic melanoma<sup>125,138,139</sup>. Moreover, these agents have been shown to be effective in many other cancers, including head and neck, lung, kidney, bladder, and liver cancer<sup>140</sup>. In addition to checkpoint blockade agents, dendritic cell therapy and chimeric antigen receptor (CAR) T-cell therapies have also achieved clinical success<sup>141,142</sup>. Lastly, recent clinical data suggest that some cancer vaccines may also provide survival benefit. Such successes have generated high interest in developing strategies to further improve cancer immunotherapy.

While highly effective, the major limitation of checkpoint inhibitor therapeutics is the low rate of long-term, durable responses. Most patients eventually develop resistance and progressive disease. CAR-T cells are difficult to engineer and have high toxicity (frequently fatal) if the targeted antigens are also present on normal cells. Lastly, current dendritic cell therapy has low potency and the therapeutic benefit is only realized several years after treatment. Thus, there is ample opportunity for the development of novel therapeutics and strategies to improve cancer immunotherapy.

### *Nanoparticles and Cancer Immunotherapy*

Nanoparticles, because of their virus-like size, readily elicit an immune response upon local or systemic administration. Without pegylation or other anti-fouling surface modification, nanoparticles are rapidly taken up by macrophages and other antigen presenting cells (APCs) and lead to immune activation. While this innate nanoparticle property has been detrimental to drug delivery applications, it is highly favorable for cancer immunotherapy. Taking advantage of this property, nanoparticles can be utilized to deliver tumor antigens to APCs. Moreover, immune responses to NPs can be modulated by adjusting the size and shape of nanoparticles<sup>143,144</sup>. Nanoparticle-bound antigens have been shown to elicit greater

immune responses than free antigens. In addition, nanoparticles can also act as immune adjuvants, enhancing response when given together with cancer vaccines.

Cancer immunotherapy can also capitalize upon the drug delivery property of nanoparticles. Nanoparticles can be formulated to deliver pro-inflammatory/pro-immune molecules with tumor antigens to enhance immune reactions. Such co-delivery is more likely to activate APCs and thus result in robust immune responses.

### ***Current Approaches using Nanotechnology to Enhance Cancer Immunotherapy***

Despite being a new area of investigation, nanotechnology has been explored by a number of research groups to improve cancer immunotherapy. A common approach has been the use of nanoparticles to improve tumor antigen presentation by APCs *in vivo*<sup>145</sup>. Using mouse tumor cells (such as B16 melanoma cells) overexpressing ovalbumin (OVA) protein, several groups have shown that nanoparticle-delivered OVA is more effective than OVA itself in eliciting immune responses. Such data suggest that nanoparticle-antigen combinations can be effective cancer vaccines. To further enhance immune responses, immune-activating molecules such as CpG have been co-delivered with tumor antigens<sup>146</sup>. The investigators showed that co-delivery of antigen and adjuvant are several-fold more effective than each agent given separately.

Another strategy to improve cancer immunotherapy has been the use of nanoparticles to activate immune cells. Fadel et al. recently reported the use of carbon nanotubes containing immune activating molecules (e.g., IL-2) to activate T-cells<sup>147</sup>. Such activated T-cells were then able to delay tumor growth. In a separate study, Perica et al. engineered nanoparticles that mimic APCs and utilized these nano-APCs to activate T-cells<sup>148</sup>. Nanoparticles have also been used to directly activate dendritic cells (APC)<sup>149</sup>. These studies suggest a role for nanoparticles in cell-based cancer immunotherapy.

In addition to improving antigen presentation, nanoparticles have also been used for their drug delivery properties. Tumor microenvironments are frequently immune suppressive, and nanoparticles can deliver therapeutics to overcome immune suppression. Park et al. demonstrated the proof-of-principle of this approach by delivering a TGF- $\beta$  inhibitor and IL-2 and showing that these drugs delayed tumor growth and improved survival using a mouse model of melanoma<sup>150</sup>. Xu et al. further demonstrated this approach using nanoparticles

**...nanotechnology holds great potential in improving cancer immunotherapy.**

to deliver a TGF- $\beta$  inhibitor to the tumor microenvironment to enhance tumor vaccine effects<sup>151</sup>. These studies suggest that drug delivery approaches can be combined with vaccine and immune activation approaches described above.

### ***Future Directions***

Nanoparticle-based cancer immunotherapy is a new and exciting field. It holds high potential in making direct impact on cancer care. To fully realize the potential of this approach, studies are needed to systematically characterize nanoparticles properties (e.g., size, shape and surface properties) that are optimal for immune activation and cancer immunotherapy.

Immune activation against tumor cells is a highly complex process (**Figure 13**). Because of unique properties of nanoparticles, they can be applied to improve each of these steps. Nanoparticle therapeutics can induce tumor cell death and in turn increase antigen release. They can be utilized to improve antigen presentation and activation by the APCs. Nanoparticles can also deliver pro-immune/pro-inflammatory agents to tumors and tumor microenvironments to enhance the cancer immunotherapy response. Lastly, nanoparticles can be utilized to “train” dendritic and cytotoxic T-cells *ex vivo* for cancer immunotherapy.

Given the exciting clinical data with checkpoint blockade inhibitors, approaches that combine nanomedicine and checkpoint blockade inhibitors are most likely to make immediate clinical impact. Future studies should focus on which checkpoint blockade agents and regimens are synergistic with nanoparticles and how nanoparticle-based agents can be integrated into checkpoint blockade treatments (e.g., timing of nanoparticle administration).

Cancer vaccine is another application where nanomedicine can make immediate impact. Nanoparticles can be formulated using biodegradable and biocompatible GRAS (generally regarded as safe) materials, which enables rapid clinical translation. However, existing clinical

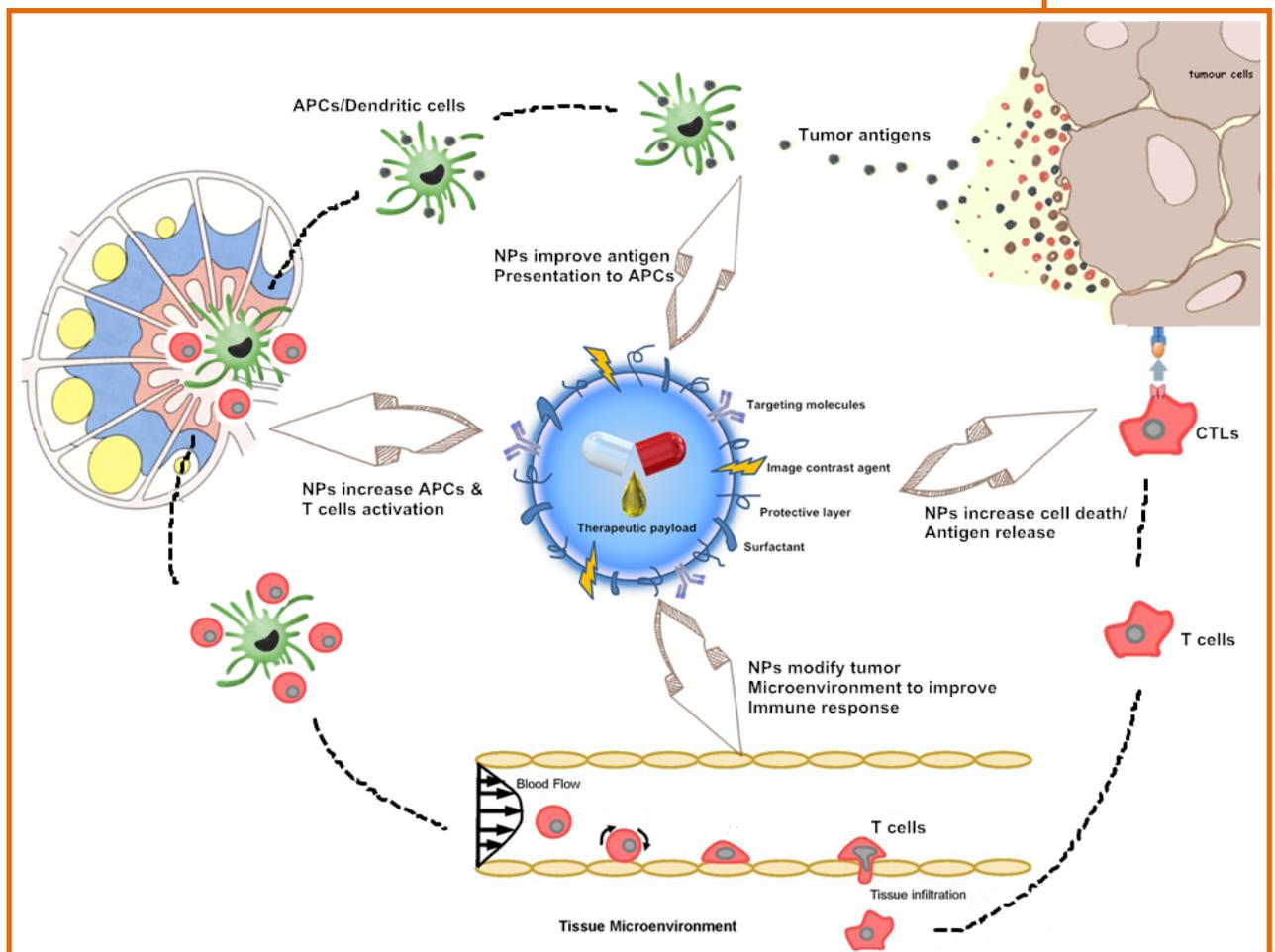
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**Cancer vaccine is another application where nanomedicine can make immediate impact.**  
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literature suggest that cancer vaccines targeting a single tumor antigen have limited benefits. Therefore, future work should focus on the development of multi-antigen cancer vaccines.

Other applications for nanoparticles in immunotherapy include the development of tumor-targeting T cells as well as CAR-T cell treatments. In addition, they can also improve dendritic cell treatments. These applications require better understanding of nanoparticle properties as well as tumor immunotherapy (e.g., which tumor antigens more likely to elicit antitumor responses). As the field of cancer

immunology evolves, nanomedicine approaches will likely become more effective and more clinically relevant.

In summary, nanotechnology holds great potential in improving cancer immunotherapy. There are many known and potential applications of nanoparticles in immunotherapy. We also expect many novel applications for nanoparticles in cancer immunotherapy that have not been discussed given the rapidly evolving field of immunology. Future success in this field will depend on the full integration of cancer biology, cancer immunology and nanomedicine in this research space.



**Figure 13.** Depiction of the complex pathway involved in cancer immunotherapy. Nanoparticle delivery vehicles can play a role at multiple points along this pathway.

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