8 Future directions

The aim of this thesis is to help develop a rational approach for the application of siRNA therapeutics for cancer treatment. Through a methodical and quantitative analysis of siRNAs and their formulation into nanoparticles for systemic delivery, several observations were made that can help direct future research in the field.

A consistent theme throughout the thesis work was the emphasis on studying the dynamics of biological processes instead of merely looking at individual snapshots in time. It is apparent that the information obtained from time-course studies, as exemplified by the live-cell and live-animal imaging studies, provides unique insights into the behavior of the biological systems. Additionally, the tumor growth inhibition studies highlighted how these insights can be applied to design more effective siRNA-based cancer treatments. Future studies examining the kinetics of the knockdown of therapeutic targets, and not just the luciferase reporter gene, will be critical for optimizing the dosing schedules of siRNA therapeutics. Moreover, these studies must correlate the target knockdown with the observed phenotypic changes. For example, determination of a threshold knockdown or duration of knockdown required to achieve efficacy, as mentioned in Chapter 7, would provide more rigorous criteria for achieving successful therapeutic response with siRNAs.

Additional studies are also needed to further probe the mechanism and in vivo behavior of nanoparticle carriers for nucleic acids such as siRNA. In Chapter 5, the use of PET and BLI to study the in vivo biodistribution and function of siRNA nanoparticles raised several intriguing questions concerning their biological activity. Even though a significant portion of the injected siRNA appears to dissociate rapidly from the

nanoparticles after systemic administration, nanoparticle formulation still appears to facilitate the delivery of functional siRNA to the target cells. This is confirmed by the observations in Chapters 6 and 7 where targeted siRNA nanoparticles were shown to be more effective at achieving tumor growth inhibition than non-targeted siRNA nanoparticles or naked siRNAs alone. Elucidating the mechanism for these differences will be essential for designing treatments based on the targeted siRNA nanoparticles.

In Chapter 5, a mechanism was proposed whereby nanoparticles formed by electrostatic interactions with nucleic acids can be dissociated in the high salt environment within the bloodstream, particularly in the kidney. Studies will need to examine the factors responsible for making some nanoparticles more susceptible to dissociation in the presence of competing electrolytes. The electrostatic interactions may be more stable for polycations with higher molecular weight, but these polycations also may exhibit greater toxicity through mechanisms such as complement activation (as shown in Chapter 3). The short length of siRNAs may also contribute to the reduced strength of the electrostatic interactions within the nanoparticles. Future work must be done to explore how these interactions can be stabilized to keep the nanoparticles intact until reaching the desired target cell population after intravenous administration. Methods such as reversible crosslinking, as mentioned previously, may be required to achieve long circulation times while still allowing intracellular release of the nucleic acid payload.

A more thorough examination of the intratumoral distribution of the nanoparticles after intravenous administration will be important to the field of nanoparticle delivery. A fundamental issue to address is whether or not intact nanoparticles are responsible for the

observed therapeutic effects, or whether the siRNA payload is first released within the extracellular environment of the targeted cells. The results from Chapter 3 comparing the activity of unmodified and nuclease-stabilized siRNAs may be utilized to explore this question. It was shown that nuclease-stabilized siRNAs show significantly greater efficacy than unmodified siRNAs only if the siRNAs must first be exposed to a nuclease-rich extracellular environment; once the siRNAs are internalized into the cells, there is no observable difference in the persistence or magnitude of gene inhibition. Therefore, comparison of gene inhibition after delivery with unmodified and nuclease-stabilized siRNAs can indicate whether or not the siRNA is released prior to cellular internalization. If the siRNAs are only released after cellular internalization, then their efficacies would be expected to be very similar. However, extracellular release of the siRNAs may lead to an enhanced potency observed for the nuclease-stabilized siRNAs owing to their reduced degradation upon exposure to the nuclease-rich extracellular environment. Incomplete nuclease protection of the siRNA payload despite nanoparticle encapsulation can confound the conclusions from these studies, but the magnitude of this complication will be unknown until such studies are performed.

If intact nanoparticles are observed to accumulate at the target site, then another question to be explored is the impact of surface decoration with different targeting ligands. In the field of antibody therapeutics, the binding-site barrier effect limits the penetration of high-affinity antibodies. This same barrier may be particularly relevant to targeted nanoparticles, especially in light of the avidity effects conferred by the multivalency of multiple targeting ligands decorating the nanoparticle surface. If such a barrier does exist, then modifications of the targeting ligand density or the affinity of the

attached targeting ligands may be used to modulate the tumor penetration of the injected nanoparticles. However, the size of nanoparticles may mask these binding-site barrier effects if diffusion limitations dominate. These questions remain to be answered for nanoparticle formulations.

Although not presented in this thesis work, initial studies were performed to develop a high-affinity targeting ligand based on a single-chain antibody fragment against the transferrin receptor. The antibody fragment was cloned from a parent plasmid containing the anti-TfR scFv donated by Dr. David FitzGerald at the National Cancer Institute. It was modified to contain a C-terminal cysteine residue to allow conjugation with PEG conjugates for attachment to the nanoparticle surface. Conjugation of a fluorophore to this C-terminal cysteine residue enabled the use of flow cytometry to examine the binding properties of the anti-TfR scFv. It was shown to strongly bind to human TfR on the HeLa human cancer cell line, but it exhibited no cross-reactivity to the mouse TfR on the Neuro2A mouse cell line. Furthermore, competitive uptake experiments showed that the binding of the anti-TfR scFv was not affected by the presence of Tf. As such, the nanoparticles targeted with anti-TfR scFv, unlike those targeted by Tf, will not be competed by endogenous Tf that is naturally present in the bloodstream. The parental 5e9 mAb from which the anti-TfR scFv is derived has a K_d of ~$2x10^{-9}$ M, whereas Tf has a K_d of ~$3x10^{-8}$ M. Therefore, the anti-TfR scFv also possesses a much higher affinity for the TfR than Tf. These scFv-targeted nanoparticles should exhibit extremely high binding affinities for cells expressing the TfR owing to the higher affinity of the anti-TfR scFv for the TfR, the absence of competition from free Tf, and the multivalency effects of multiple surface targeting ligands.

Altogether, these proposed studies will provide important information concerning the design of nanoparticle carriers for systemic siRNA delivery. The work presented in this thesis provides a foundation upon which these other studies can be built. The practical nature of the topics explored and their direct relevance to clinical application will hopefully expedite the development of more effective cancer therapies using siRNA.