Summary

Recombinant adenoviral vectors (Ad) have been employed for clinical gene therapy applications for cancer based on their unparalleled ability to accomplish in vivo gene transfer. Despite this capacity, current generation Ad are not fully suited for this application. From a conceptual standpoint, tumor selective targeting would enhance the specific delivery of anti-cancer genes to disease sites, thus improving the overall therapeutic index of any such approach. From a practical standpoint, tumors have been noted to exhibit a profound deficiency of the primary Ad receptor, coxsackie-and-adenovirus receptor (CAR). Both of these considerations predicate the need to alter the tropism Ad to address these points. In the first instance, the ability to target Ad to cancer-specific markers would be suggested. In the second instance, the ability to route the virus via non-CAR cellular pathways is required. Both of these key issues have been addressed in this thesis. We have sought to achieve these linked goals via a variety of strategies for Ad tropism modification. The fullest realization of the practical benefits of such efforts may ultimately be realized via genetic capsid modification approaches, which are the main focus of this thesis work. In the aggregate, we have established herein key principles with respect to adenovirus biology relevant to adenovirus vector utility. These principles have direct consequence for current human clinical gene therapy approaches for cancer and point clearly to the direction in which current vectorology efforts most focus to realize the full potential of cancer gene therapy.

In Chapter II of thesis we endeavored to alter the tropism of Ad via a strategy of capsid protein chimerism. Specifically, we configured the fiber knob of the Ad serotype 3 virus within the context of an Ad serotype 5 vector. This study established that major capsid protein engineering could be accomplished by genetic techniques. In addition, we showed that the fiber knob was the major dictate of vector tropism; mosaic Ad containing only the 3 knob exhibited a tropism pattern similar to an Ad 3 virus. Of note, this strategy allowed routing of the derived mosaic vector to non-CAR pathways. This study represented the first ever alteration of Ad tropism based on genetic capsid modification. Further, these findings were the first to establish the concept of “CAR-independent” gene delivery achievable via tropism alterations. Our subsequent studies have shown that the receptor of the serotype 3 Ad can be exploited for gene delivery in the context of tumor targets characterized by CAR deficiency. Dramatic augmentation in gene transfer efficiency were obtainable in these instances. On the basis of these studies we thus understand that: (1) it is possible to alter Ad tropism via genetic capsid modification (2) such modifications allowed CAR-
independent gene delivery (3) CAR-independent infection allowed dramatic efficiency enhancement of gene delivery in the context of CAR-deficient tumor targets.

In Chapter III we explored the possibility of more defined genetic capsid modifications consistent with the possibility of vector targeting. To this end we sought to identify locales within the Ad capsid capable of incorporation of heterologous targeting motifs. Based on available crystal structure analysis, we proposed the HI loop of the fiber knob as a candidate site which would embody the requirements for effective presentation of a targeting motif. Key to realization of these requirements would be the ability to retain the structural facets of the fiber in a trimeric context and the presentation of the incorporated ligand on an exposed surface locale. To this end we configured a FLAG epitope of the HI loop in the context of recombinant fiber and intact viral vector. Analysis of these substrates demonstrated that peptide incorporation at the HI loop did not abrogate fiber trimerization and/or particle assembly. Further, the peptide localized in this site presented a surface localization, potentially available for target cell receptor interaction. These key studies thus established a potential link between target definition technologies, such as phage biopanning of peptides/scFv, and vector targeting technologies. Subsequent work allowed the incorporation of the tumor targeting peptide RGD4C of the HI loop locale. This modification allowed the achievement of CAR-independent infection with dramatic efficiency enhancements of gene transfer to CAR-deficient targets. This specific modification allowed improvements of vector potency in the context of both Ad vectors and replicative adenoviral agents designed for tumor therapy (3,4). In addition, this particular targeting peptide provides a level of true tumor targeting in selected instances. On this basis, HI loop modified Ad are currently being advanced into human clinical trials for cancer. These trials represent the first ever employment of tropism modified viral vectors in the history of human gene therapy.

In Chapter IV we endeavored to establish the feasibility of true tumor targeting via tropism modification of Ad. To this end, we re-targeted Ad employing a bispecific adapter consisting of an anti-fiber knob mAb conjugated to an mAb for the tumor specific marker EpCAM. In these studies we could demonstrate that specific gene transfer to target cells was achieved via the targeted EpCAM pathway. Of note, the levels of retargeted Ad mediated gene transfer closely paralleled the levels of EpCAM characterizing each of the tested tumor targets. These findings clearly established that true tumor-specific targeting was achievable via tropism-modification of Ad. These studies were extended to the context of primary clinical material directly obtained from cancer patients. In these studies, the
EpCAM targeted Ad demonstrated augmented gene transfer to colon cancer specimens compared to unmodified Ad. These findings highlighted the concept that tumor targeted Ad may achieve gene transfer efficiency enhancements via the achievement of CAR-independent infection, over-and-above specificity gains. Of further note, the targeted vector exhibit a dramatic enhancement in targeting index compared to non-modified Ad when evaluated in the context of primary tumor versus primary normal tissue employing antibody retargeting moieties for the achievement of specific targeting. In addition, the dramatic improvement in targeting index obtained in studies of clinical specimens highlighted the shortcomings of current Ad vectors employed in clinical trials by suggesting the potential gains in therapeutic index obtainable via the approach. Of special note, the successful exploitation of available anti-tumor antibodies suggested the possibility of broader utility of these agents, or their derivatives, for tropism modification of Ad designed to achieve tumor targeting.

In Chapter V we sought to extend our findings of the previous study by improving the technical means to exploit anti-tumor antibodies for Ad tropism modification. In this regard, earlier reports of Ad retargeting had exploited bispecific retargeting adapters to cross-link Ad to non-CAR receptors. These molecular species were, in the main, derived from chemical conjugates of anti-fiber knob mAb and a cellular ligand or anti-receptor mAb. This approach was limited by the heterogenous nature of such preparations deriving from these methods. Of note, these technical limits represented legitimate barriers for clinical translation of antibody retargeted Ad. On this basis we endeavored to derive the benefits of tumor selective targeting provided by anti-cancer mAbs in a manner which circumvents the inherent limits noted above. To this end we derived bispecific single chain antibodies to serve as bispecific retargeting adapters for Ad tropism modification. A single chain antibody (scFv) directed against the fiber knob was genetically fused to an scFv against the tumor marker EGF-R. Our studies demonstrated that the designed bispecific antibody could be expressed in mammalian cell systems and could be purified to homogeneity via an incorporated tag system. Of note, the bispecific antibody functioned successfully as a bispecific retargeting adapter, allowing tumor selective gene delivery specifically to cells expressing EGF-R. These studies thus established a practical means to exploit scFv for Ad retargeting. This represents a highly relevant consideration in the context of the available anti-tumor scFv which can presently be exploited in this fashion. Further, the functional utility of scFv for Ad retargeting to tumor establishes the biologic basis of future uses which might be based on direct incorporation of scFv into the Ad capsid.
In Chapter VI we sought to define additional methods for genetic capsid incorporation of targeting ligands commensurate with our tumor targeting goals. In this regard, defined fiber locales for incorporation of targeting ligands were all limited by the structural constraints imposed by the fiber knob domain. On this basis, only small peptide ligands were of potential utility. Given the limited available repertoire of peptide ligands, and in consideration of the availability of specific anti-tumor scFv, we sought to define a capsid locale for ligand incorporation potentially free of knob-imposed structural constraints. To this end we advanced an approach based on substitution of fiber shaft and knob with an alternate protein which would provide a trimerized structure to compensate for loss of the fiber domains associated with this key structural aspect. We showed that chimeric fiber molecules could be designed, and derived, which contained the bacteriophage T4 Pol protein fibrin as a fiber knob/shaft substitute. Recombinant forms of these molecules trimerized and presented ligand in a surface accessible manner. Importantly, viable virus could be rescued which incorporated the chimeric fiber replacement protein. Further, such virions could function as vectors with successful gene transfer noted to target cell accomplished in a targeted manner based on Ad-incorporated ligands. The main intent of this work was to establish the feasibility of fiber replacement as a means to achieve capsid incorporation of desirable targeting ligands, such as scFv. Established feasibilities in this study provided a practical system to evaluate this concept. In further work we have shown that the fiber replacement approach is indeed compatible with capsid incorporation of larger/more complex targeting ligands than possible in the context of knob structure imposed constraints. Current work is focused upon demonstrating the functionality of anti-tumor scFv configured into the Ad capsid via the fibrin approach.

In Chapter VII we sought to identify additional capsid sites compatible with incorporation of heterologeous targeting motifs. In this regard, preceding work had focused largely on the major capsid proteins fiber, hexon and penton to achieve these ends. In intrinsic limits of these locales restricted the type and range of targeting ligands exploitable and thus led us to explore a novel locale. The pIX protein is one of the so called “cement proteins” of the capsid and is thought to function as a stabilizer for intra-hexon assembly. Based on the recognition that its C terminus was in an ectodomain locale with respect to the capsid, we explored it utility as a site for incorporation of targeting ligands. Studies employing tag peptides established that heterologeous peptide could be incorporated at pIX without any deleterious effect on viral rescue. In addition, C terminus tag was localized on the virion ectodomain, accessible for surface-to-surface
interactions. Incorporation of small peptide ligands at the C terminus of pIX allowed altered Ad tropism with the achievement of CAR-independent gene delivery. These studies thus established the potential utility of pIX as a candidate locale for ligand incorporation of the context of genetic capsid modification strategies for altering Ad tropism. We have extended the studies described in this chapter by evaluating the feasibility of scFv incorporation of the C terminus of pIX. These data have established that scFv can be incorporated at this locale with the rescue of viable virus. Further, the incorporated scFv contributed to Ad tropism, indicating, scFv function in the context of capsid incorporation. This represents the first such demonstration of genetic scFv incorporation within the Ad capsid with altered vector tropism and represents a field milestone.

In Chapter VIII we explored the initial basis of a novel targeting paradigm based on the exploitation of multiple tropism alterations within a targeted Ad vector. Such a strategy of “complex mosaicism” would offer theoretic advantages – 1) distinct infectivity enhancement modifications could operate additively, or synergistically, to maximize vector gene transfer potency 2) distinct targeting motifs could operate in a cross-supportive manner to offer more precise target cell selectivity based on highly individualized profiling. The starting point for realization of these ends is the demonstration that an Ad5 vector can incorporate more than one genetic capsid modification which embodies a tropism modification principal. To this end, we sought to derive an Ad which was chimeric with respect to the fiber knob (serotype 3 substituted for native 5) and containing a C terminus fiber addition. Such a complex mosaic Ad was designed and successfully rescued. Of note, both distinct capsid modification were capable of functional operation in the context of the presence of the other. This key first step has thus established the practical basis of deriving complex mosaic Ad. This study represents the first ever demonstration of this concept. The means to incorporate multiple, and distinct, targeting motifs within a single vector particle will allow study of vector gains with respect to gene transfer efficiency and target cell specifically. Both of these considerations are of key relevance in consideration of the requirements of cancer gene therapy approaches.