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Hypothesis

A Peptide-targeted, Hypoxia-responsive Adeno-associated Virus Platform for Tumor-selective Delivery of Chemokines and RNAi in Non-small Cell Lung Cancer: A Hypothesis

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Abstract

Adeno-associated virus (AAV) vectors have favorable safety and durable transgene expression but are limited in oncology by insufficient tumor specificity and off-target expression. Tumor hypoxia and non-small cell lung cancer (NSCLC)-associated surface ligands offer complementary layers of biological selectivity for more precise gene delivery. This study proposes an NSCLC-directed, hypoxia-responsive AAV architecture that integrates MGS4-guided capsid targeting with dual hypoxia-responsive element (HRE)-gated promoters driving glutamine-modified C-X-C motif chemokine ligand 9-fragment crystallizable region fusion protein (Q-CXCL9-Fc) expression and baculoviral IAP repeat containing 5 (BIRC5)-linked mesothelin (MSLN) silencing. We conceptually designed an AAV vector that combines three layers of NSCLC selectivity: MGS4-guided capsid targeting, hypoxia-gated transcription, and tumor-active promoter control. The capsid displays the MGS4 peptide, isolated by phage display biopanning as a high-affinity ligand for the lung squamous cell carcinoma cell line HCC15 and later shown to internalize into a substantial fraction of NSCLC cell lines and bind a subset of human NSCLC biopsy samples, indicating activity across multiple NSCLC subtypes. The genome encodes Q-CXCL9-Fc under a 4× HRE-cytomegalovirus promoter to sustain C-X-C motif chemokine receptor 3-dependent T-cell recruitment and a miR-30-based short hairpin RNA targeting MSLN under a 4× HRE-BIRC5 promoter to inhibit tumor progression. This architecture is hypothesized to enrich AAV entry into NSCLC lesions via MGS4 while restricting Q-CXCL9-Fc secretion and MSLN silencing to hypoxic, BIRC5-active tumor regions, enabling synergistic enhancement of antitumor immunity and suppression of tumor-intrinsic pathways. The proposed multimodal, hypoxia-responsive AAV platform represents a conceptual precision oncology strategy that couples environmental sensing, tumor-specific transcription, and peptide-defined tropism within a single vector and could inform next-generation NSCLC-directed AAV systems.

Introduction

Adeno-associated virus (AAV) vectors have become a leading platform for gene delivery due to their established safety profile, stable episomal expression, and broad tissue tropism.[1,2] In contrast to integrating retroviral systems, AAVs minimize the risk of insertional mutagenesis and exhibit comparatively low immunogenicity.[3] However, the application of AAV vectors in oncology is still hampered by inadequate tumor selectivity, inefficient systemic delivery, and unintended transgene expression in normal tissues.[4]

Hypoxia is a defining and pervasive feature of solid tumors, where dysregulated vasculature and rapid proliferation generate regions of chronic and intermittent oxygen deprivation that drive angiogenesis, metabolic reprogramming, immune evasion, and resistance to radiotherapy and chemotherapy.[5,6] In this context, hypoxia-inducible factor-1α (HIF-1α)-dependent transcription has been widely exploited to bias therapeutic activity toward malignant tissue, and hypoxia-activated prodrugs (HAPs) that undergo bioreductive activation in low-oxygen environments illustrate how oxygen tension can be used as a pharmacologic trigger, although their efficacy is often constrained by heterogeneous hypoxia and uneven drug penetration.[7] These limitations motivate gene-based

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strategies that couple durable expression to hypoxia-responsive regulatory elements as part of broader hypoxia-targeted therapeutic concepts.

Advances in synthetic biology and vector engineering have enabled the creation of conditionally active AAVs that respond to tumor-specific microenvironmental conditions. Among these cues, hypoxia can be harnessed as an especially attractive trigger for tumor-restricted gene activation. Incorporation of hypoxia-responsive elements (HREs) allows transgene expression to be restricted to hypoxic regions within tumors. In parallel, capsid engineering strategies permit surface display of targeting peptides that enhance tumor tropism.[8] The MGS4 peptide (also known as HCC15.2) was isolated by phage display biopanning as a high-affinity ligand for the lung squamous cell carcinoma cell line HCC15 and subsequently shown to internalize into approximately 54% of a panel of non-small cell lung cancer (NSCLC) cell lines and to bind 24% of human NSCLC biopsy samples, spanning both adenocarcinoma and squamous histologies.[9] These data indicate that MGS4 recognizes a surface determinant shared by a substantial subset of NSCLC rather than a single histologic subtype, supporting its use as a broadly NSCLC-reactive targeting motif. Its integration into the adeno-associated virus serotype 9 (AAV9) capsid, a serotype with broad systemic tropism and efficient transduction,[10] is expected to enhance NSCLC-specific uptake and improve tumor-directed delivery.

Within NSCLC, higher intratumoral levels of C-X-C motif chemokine receptor 3 (CXCR3) ligands such as chemokine C-X-C motif chemokine 9 (CXCL9) are associated with increased effector T-cell infiltration and improved responses to immune checkpoint blockade, whereas CXCL9 suppression characterizes "cold" tumors that poorly recruit cytotoxic lymphocytes. In contrast, mesothelin (MSLN) is a tumor-associated surface glycoprotein that promotes adhesion, invasion, epithelial-to-mesenchymal transition, and metastasis, and its overexpression correlates with poor prognosis. These observations provide a mechanistic rationale for pairing CXCL9-based immune recruitment with MSLN-directed inhibition of tumor aggressiveness within a single NSCLC-focused platform.

Building on this rationale, we designed an AAV vector that combines HRE-mediated transcriptional control with MGS4-guided capsid targeting to enable coordinated delivery of glutamine-modified C-X-C motif chemokine ligand 9-fragment crystallizable region fusion protein (Q-CXCL9-Fc) and short hairpin RNA targeting mesothelin (shMSLN) to tumor sites. Q-CXCL9-Fc, a fusion of CXCL9 and a fragment crystallizable (Fc) domain and an N-terminal glutamine, is intended to amplify intratumoral T cell infiltration, sustain interferon gamma (IFN-γ)-CXCL9-CXCR3 signaling, and resist dipeptidyl peptidase-4 (DPP-4)-mediated truncation while benefiting from neonatal Fc receptor-mediated recycling and Fc gamma receptor engagement.[11–13] In parallel, shMSLN-mediated suppression of MSLN expression is expected to attenuate adhesion, invasion, and metastatic potential, thereby inhibiting tumor progression.[14–17]

Dual modulation of CXCL9-driven immune recruitment and MSLN-dependent invasion within the same lesion is anticipated to create a tumor microenvironment in which infiltrating T cells encounter cancer cells that are both more accessible and intrinsically less invasive, providing a mechanistic basis for potential synergy between these two therapeutic arms. Taken together, these considerations motivated the development of an NSCLC-directed AAV design that integrates hypoxia- and tumor-restricted promoters, MGS4-guided capsid targeting, and combined immune activation with MSLN silencing into a single, packaging-compliant genome.

Hypothesis

This study hypothesizes that a single, rationally engineered AAV9 vector can achieve highly selective and durable antitumor activity in NSCLC by combining three layers of tumor specificity with two complementary therapeutic functions that have not previously been integrated in one system. Unlike conventional AAV cancer vectors, which typically deliver a single immunostimulatory cytokine gene under one tissue-specific or broadly active promoter, the proposed construct assigns distinct hypoxia-responsive regulatory modules to two different transgenes within the same genome: a DPP-4-resistant Q-CXCL9-Fc fusion designed to enhance CXCR3-dependent T cell recruitment, and an MSLN-targeting miR-30-based short hairpin RNA (shRNA) intended to suppress intrinsic oncogenic signaling. The overall architecture of this NSCLC-directed AAV9 design, including the MGS4-displaying capsid and dual 4× HRE-gated cytomegalovirus (CMV) and baculoviral IAP repeat containing 5 (BIRC5) expression cassettes encoding Q-CXCL9-Fc and miR-30-based shMSLN, is schematically illustrated in Figure 1.

In contrast to existing hypoxia-regulated AAV designs that rely solely on HRE-gated transcriptional control, this vector couples dual 4× HRE-CMV and 4× HRE-BIRC5 promoters with MGS4-mediated capsid targeting, thereby linking environmental hypoxia sensing, tumor-specific transcription, and NSCLC-selective particle tropism in a single platform. Furthermore, whereas MGS4 has previously been used only as a soluble peptide ligand to deliver toxin conjugates to NSCLC cells, it is here repurposed as a capsid-displayed targeting motif on AAV9, representing a shift toward peptide-guided viral tropism engineering.

We therefore hypothesize that placing Q-CXCL9-Fc-mediated T-cell recruitment and BIRC5-gated MSLN silencing within the same hypoxia-responsive, MGS4-targeted vector will not only enhance NSCLC selectivity but also mechanistically couple immune attack and reduced invasive potential in the same lesions, so that effector T cells are concentrated in tumors that are simultaneously less able to adhere, invade, and metastasize, yielding a synergistic rather than merely additive constraint on tumor growth and dissemination.

Evaluation of the hypothesis

The proposed AAV platform builds on, but also extends beyond, prior work on hypoxia-responsive gene regulation and tumor-selective promoters. HIF-1α binding to HREs has long been used to bias transgene expression toward hypoxic tumor regions, and multimerized HRE motifs can sharpen hypoxia-dependent transcriptional switches. However, most existing systems implement a single promoter-HRE cassette, which limits the ability to differentially tune expression of distinct therapeutic payloads in heterogeneous tumors.[18] In this hypothesis, allocating 4× HRE-gated CMV and 4× HRE-gated BIRC5 promoters to Q-CXCL9-Fc and shMSLN, respectively, is intended to separate a broadly active chemokine arm from a more stringently gated RNA restriction within one vector. The overall mechanism, from MGS4-guided AAV9 entry into NSCLC cells through episomal persistence to Q-CXCL9-Fc secretion and miR-30-based MSLN silencing, is schematically illustrated in Figure 2.

Classical shRNA expression systems are typically driven by RNA polymerase III promoters such as U6 or H1, which are op-

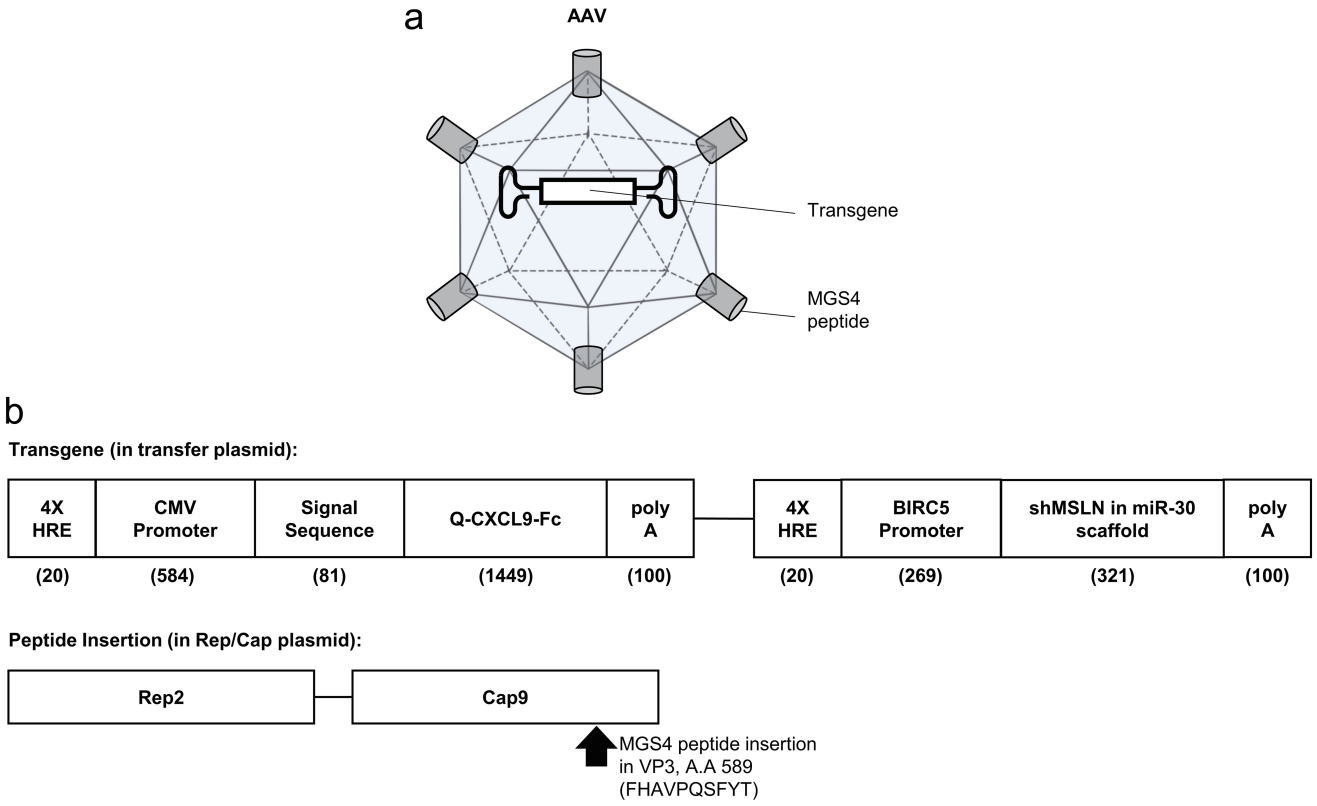

Fig. 1. Expected schematics of a hypoxia-inducible AAV vector encoding Q-CXCL9-Fc and shMSLN with MGS4 peptide inserted in VP3. (a) Expected schematic of an AAV capsid displaying MGS4 peptides and carrying a therapeutic transgene. (b) Schematic of the DNA construct design for a hypoxia-responsive AAV vector genome encoding Q-CXCL9-Fc and shMSLN in the transgene cassette, and MGS4 insertion in viral protein 3 (VP3) of the Rep/Cap plasmid. The dual-expression AAV transgene is designed so that the 4× HRE-CMV promoter drives secretion of Q-CXCL9-Fc, while the 4× HRE-BIRC5 promoter drives expression of shMSLN embedded in a miR-30 scaffold. The Rep2/Cap9 plasmid is modified to display the NSCLC-targeting MGS4 peptide (FHAVPQSFYT) in the VP3 capsid at amino acid 589, enabling peptide-guided AAV targeting to NSCLC cells. Together, this design may couple hypoxia-dependent transgene expression with MGS4-displayed capsid to enhance selective delivery of immunomodulatory and gene-silencing payloads to NSCLC cells. The numbers in parentheses indicate DNA sequence length in base pairs (bp). When an intergenic spacer of 500 bp is placed between the two genes, the total length of the transgene remains below 4.7 kb, indicating that the construct can be readily packaged as an AAV transgene. Detailed sequence information is provided in the supplementary materials. AAV, adeno-associated virus; BIRC5, baculoviral IAP repeat containing 5; Cap, capsid; CMV, cytomegalovirus; HRE, hypoxia-responsive element; miR-30, microRNA-30; MSLN, mesothelin; NSCLC, non-small cell lung cancer; Q-CXCL9-Fc, glutamine-modified C-X-C motif chemokine ligand 9-fragment crystallizable region fusion protein; Rep, replication; shMSLN, short hairpin RNA targeting mesothelin; shRNA, short hairpin RNA.

timized for high-level transcription of short non-coding RNAs but are not readily compatible with HRE-based hypoxia regulation.[19] Because HRE modules operate within RNA polymerase II promoter contexts, conventional RNA polymerase III-driven shRNA designs provide little flexibility for integrating hypoxia responsiveness into RNA interference (RNAi).[20] To overcome this constraint, the present hypothesis embeds an MSLN-targeting sequence in a miR-30 backbone and drives it from a 4× HRE-BIRC5 RNA polymerase II promoter,[21] thereby enabling HRE-mediated hypoxia gating while routing the hairpin through the endogenous microRNA (miRNA) biogenesis pathway for efficient processing and stable knockdown. This approach is consistent with established single-copy miR-30-based RNAi systems and, in this setting, specifically links MSLN silencing to a hypoxic, BIRC5-high transcriptional milieu characteristic of NSCLC lesions.

The choice of BIRC5 as a tumor-active promoter is supported by evidence that BIRC5 is minimally expressed in most differentiated normal tissues but strongly upregulated across many solid malignancies, with further induction under hypoxic conditions.[22] Positioning the miR-30-embedded shMSLN cassette under a 4× HRE-BIRC5 promoter is therefore expected to establish a dual gate requiring both malignant BIRC5-driven transcription and hypoxia for efficient MSLN silencing, reducing the likelihood of off-tumor knockdown in BIRC5-low, normoxic tissues that still express MSLN. In parallel, Q-CXCL9-Fc expression under a 4× HRE-CMV RNA polymerase II promoter is designed to ensure robust chemokine-Fc production in hypoxic tumor regions, maximizing T-cell recruitment and IFN-γ-mediated immune activation while keeping expression low in well-oxygenated normal tissues. At the same time, intratumoral hypoxia is spatially and temporally heterogeneous, so the effective hypoxia threshold, dynamic range, and basal leakiness of such composite promoters may vary between NSCLC models. As a result, the therapeutic window will depend on quantitative characterization of these HRE-CMV and HRE-BIRC5 cassettes in relevant experimental systems.

At the capsid level, the hypothesis leverages accumulated evidence that peptide insertion into AAV capsids can redirect tropism toward specific cell types while preserving manufacturability and packaging capacity. MGS4 has been reported to bind NSCLC cell lines such as H1299 and H2009 with high affinity and to mediate

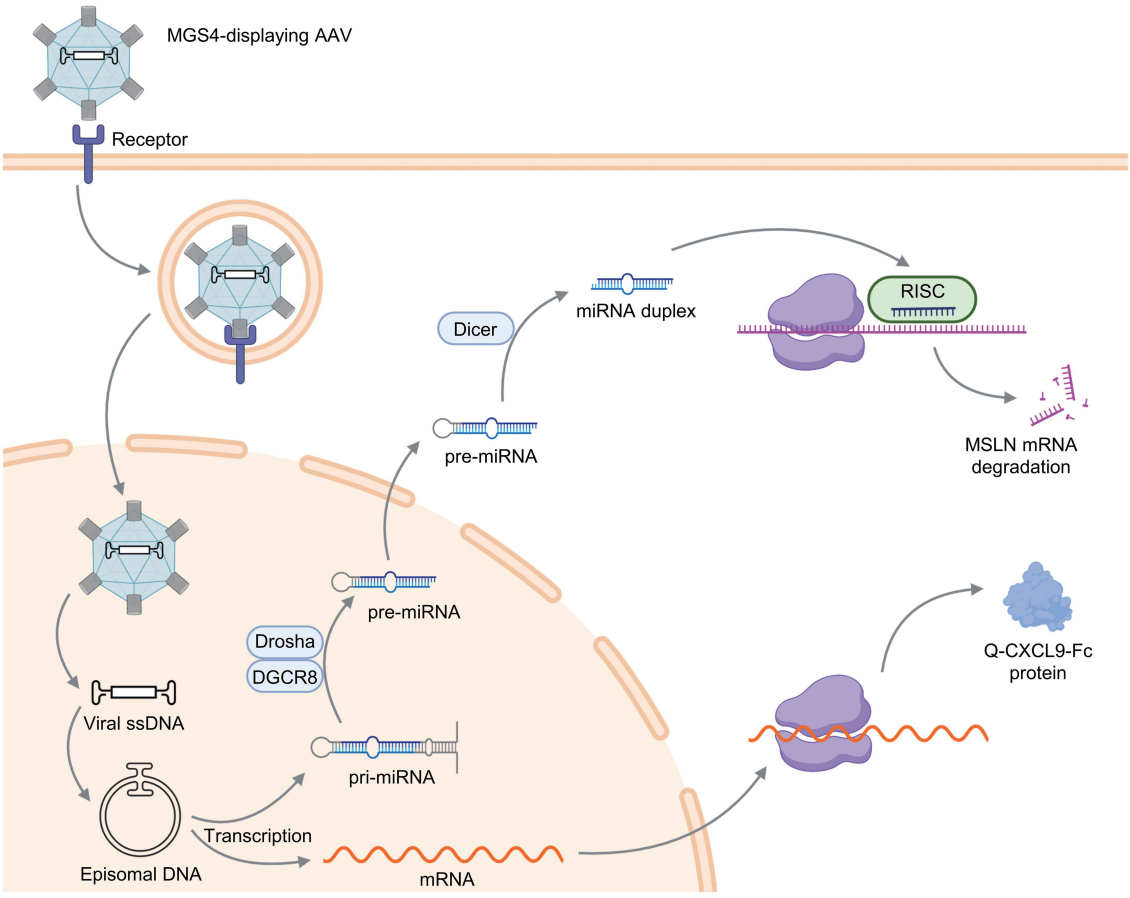

Fig. 2. Schematic of the expected mechanism of MGS4-displaying AAV-mediated Q-CXCL9-Fc production and miR-30 scaffold-based MSLN silencing in NSCLC cells. MGS4-displaying AAV vectors are hypothesized to preferentially target and bind NSCLC cells, which may internalize the vectors via receptor-mediated endocytosis. The vectors then undergo capsid uncoating to expose the viral single-stranded DNA (ssDNA), which is converted into episomal DNA that can drive transcription of Q-CXCL9-Fc mRNA together with a miR-30 scaffold-based pri-miRNA. The Q-CXCL9-Fc mRNA is translated into a secreted fusion protein, and its persistent expression from the AAV episomal genome is expected to enhance T cell infiltration into tumors. In parallel, the pri-miRNA is sequentially processed by Drosha/DGCR8 and Dicer into a mature miRNA duplex that is incorporated into the RISC complex, which may result in sequence-specific degradation of MSLN mRNA and consequent suppression of MSLN gene expression in NSCLC cells. AAV, adeno-associated virus; CMV, cytomegalovirus; DGCR8, DiGeorge critical region 8; HRE, hypoxia-responsive element; miR-30, microRNA-30; miRNA, microRNA; mRNA, messenger RNA; MSLN, mesothelin; NSCLC, non-small cell lung cancer; pre-miRNA, precursor microRNA; pri-miRNA, primary microRNA; Q-CXCL9-Fc, glutamine-modified C-X-C motif chemokine ligand 9-fragment crystallizable region fusion protein; RISC, RNA-induced silencing complex; ssDNA, single-stranded DNA.

efficient intracellular delivery of saporin-based toxin conjugates *in vivo*, supporting its use as a lung tumor-targeting ligand.[9] Repurposing MGS4 as a capsid-displayed motif on AAV9 is therefore mechanistically plausible, although it remains to be determined whether this modification will preserve native AAV9 fitness, vector yield, and biodistribution while providing sufficient gain in NSCLC selectivity over off-target organs. These uncertainties highlight the need for head-to-head comparisons between MGS4-modified and unmodified AAV9 capsids in future studies.

The dual therapeutic module is grounded in emerging data on the CXCR3-axis chemokines in antitumor immunity and on MSLN as a clinically relevant oncogenic driver. DPP-4-resistant CXCL9-Fc and CXCL10-Fc variants have been shown to sustain CXCR3-dependent chemotactic activity and to elicit potent antitumor immune responses, and intratumoral CXCL9 is generally associated with increased effector T-cell infiltration, improved survival, and enhanced responsiveness to immune checkpoint blockade.[11] In parallel, MSLN overexpression contributes to proliferation, survival, epithelial-mesenchymal transition, and metastasis, and MSLN-targeted approaches, including immunotoxins and CAR-T cells, have demonstrated antitumor effects in preclinical and early clinical studies.[23,24] Embedding an MSLN-directed sequence into a miR-30 backbone under an HRE-BIRC5 RNA polymerase II promoter aligns with these data by coupling MSLN knockdown to the same hypoxic, transcriptionally malignant context in which Q-CXCL9-Fc is produced. Together, these observations suggest that a strategy which concentrates CXCR3-expressing effector T cells within hypoxic, BIRC5-active NSCLC lesions while simultaneously diminishing MSLN-driven adhesion, invasion, and metastatic potential could provide synergistic tumor control, as infiltrating T cells would be poised to attack cancer cells that are both densely surrounded by immune effectors and intrinsically less capable of escape and dissemination.

Finally, the selection of AAV9 as the backbone reflects its proven capacity for systemic gene delivery with broad biodistribution, including to the lung, while underscoring the need for multi-

layer safeguards against off-tumor expression.[10] By combining MGS4-guided capsid targeting with HRE- and BIRC5-restricted RNA polymerase II promoters, the proposed architecture aims to mitigate the off-target effect concerns raised in some AAV applications, yet retain the advantages of durable, episomal transgene expression.[25] Overall, the available literature supports the feasibility of each individual module—HRE-based transcriptional control, BIRC5-driven tumor selectivity, peptide-directed capsid retargeting, CXCL9-Fc-based immune activation, and miR-30-guided MSLN RNAi—whereas their integration into a single NSCLC-focused AAV9 vector remains untested and constitutes the central hypothesis that requires empirical evaluation.

Discussion

This study describes an NSCLC-directed AAV platform that treats cancer gene therapy as a multi-layered, conditionally active system rather than a single-promoter, single-payload vector. The conceptual novelty does not come from any single element, because HRE modules, BIRC5-responsive promoters, miR-30 scaffolds, and peptide-retargeted AAV capsids have all been reported separately. Instead, the innovation lies in their architectural integration into one packaging-compliant genome that links environmental sensing, tumor-selective transcription, and capsid tropism within a unified design. In this sense, the platform is intended to complement existing AAV and antibody-based approaches by offering an alternative way to organize established building blocks into a multi-layered scaffold.

Mechanistically, the construct departs from conventional AAV cancer vectors that usually connect a single therapeutic gene to one tissue-specific or broadly active promoter. Here, two RNA polymerase II promoters (CMV and BIRC5) are arranged in parallel under a shared HRE cassette. This layout is designed to drive both a secreted Q-CXCL9-Fc fusion and a miR-30-based shRNA against MSLN within the same hypoxic tumor microenvironment. The goal is that CXCR3-biased T-cell recruitment and local RNAi against a tumor-promoting antigen occur together, so that the immune context and tumor-intrinsic signaling are modulated in a coordinated way. In principle, this could give broader therapeutic scope than single-gene cytokine or suicide constructs, although true synergy between the Q-CXCL9-Fc and shMSLN arms still needs to be tested with appropriately controlled vectors.

The proposed interaction between Q-CXCL9-Fc and MSLN knockdown builds on known roles of MSLN in solid tumors. MSLN expression has been linked to increased survival, invasion, and chemoresistance, and to a more aggressive phenotype at the invasive front in several cancers, including NSCLC subsets. Silencing MSLN in hypoxic, BIRC5-positive cells could therefore reduce the intrinsic fitness and adhesive capacity of MSLN-high cells. At the same time, Q-CXCL9-Fc is intended to enrich the lesion with CXCR3-positive effector T cells. Together, these effects may make the microenvironment more permissive to T-cell-mediated cytotoxicity, so that MSLN knockdown not only debulks tumor cells directly but also facilitates their immune clearance. This provides a mechanistic rationale while keeping the synergy claim at a hypothesis level.[26]

The use of HRE-based regulation places this vector within the broader field of hypoxia-targeted cancer therapy. HAPs, oxygen-sensitive gene switches, and hypoxia-responsive viral vectors have all been explored to exploit low oxygen tension in poorly perfused tumor regions. Tumor hypoxia promotes malignant progression, immune exclusion, and therapy resistance, and HAPs show that these features can be turned into therapeutic entry points when hypoxia is used as a gate for drug activation. In this context, the present AAV system can be viewed as a gene-based extension of hypoxia-targeted strategies, where the hypoxic trigger is used not only to gate cytotoxic pressure but also to coordinate immune recruitment and RNAi-based modulation of tumor antigens. Rather than replacing small-molecule HAPs, such a vector would more realistically act alongside them as a complementary modality.

However, intratumoral hypoxia is highly heterogeneous over space and time. Oxygen gradients and fluctuating perfusion create shifting hypoxic niches within and between lesions. This has direct implications for hypoxia-gated expression systems. If the HRE threshold is too strict, moderately hypoxic regions may be under-treated; if it is too permissive, expression may leak into better-oxygenated tumor rims or stressed normal tissues. In the current platform, this could lead to uneven Q-CXCL9-Fc and shMSLN expression across the tumor volume and occasional activation in non-target sites during transient hypoxia. These issues highlight the need for quantitative characterization of HRE promoter sensitivity, dynamic range, and oxygen dependence in relevant NSCLC models, and support future work on combinatorial gating or additional tumor-specific promoters to buffer against hypoxia heterogeneity.

The platform also contrasts with antibody and antibody-drug conjugate (ADC) therapies that target MSLN. Antibodies and ADCs recognize antigen on the cell surface, require repeated systemic dosing, and can cause off-tumor binding and dose-limiting toxicities when MSLN is shared with normal mesothelial tissues.[27,28] By contrast, HRE- and BIRC5-gated expression of shMSLN is intended to restrict gene-silencing activity to hypoxic, transcriptionally malignant cells even if MSLN is expressed more broadly. In addition, sustained intratumoral production of Q-CXCL9-Fc and shRNA from episomal AAV genomes may help overcome heterogeneous penetration and narrow exposure windows that limit many ADC regimens, especially in poorly perfused tumor cores.[29] For these reasons, MSLN-directed AAV vectors are best positioned as complements to MSLN antibodies and ADCs, with modality choice guided by disease stage, lesion accessibility, and tolerability. Clinically, this platform could be well suited for combination with immune checkpoint inhibitors such as anti-programmed cell death protein 1 (PD-1) or anti-programmed death-ligand 1 (PD-L1) antibodies (e.g., pembrolizumab and atezolizumab), in which CXCR3-mediated T cell recruitment by Q-CXCL9-Fc may synergize with checkpoint blockade to further enhance T cell activation.[30]

From a clinical perspective, the architecture aligns with precision oncology principles. MSLN, BIRC5, and hypoxia are enriched in aggressive NSCLC subsets and other solid tumors, and the MGS4 peptide has been reported to increase binding and internalization in NSCLC cells by binding to surface targets preferentially expressed in these malignancies. By combining capsid tropism, microenvironmental sensing, and transcriptional selectivity, the platform aims to confine effective expression to tumors that are both MSLN-positive and hypoxic/BIRC5-high. This multi-parameter targeting moves AAV gene therapy toward a precision oncology model in which vectors are matched to tumors based on antigen profile, hypoxia signatures, and BIRC5 expression levels, and are deployed with immune checkpoint blockade, radiotherapy, or hypoxia-modifying agents in rational combinations. If validated experimentally, such an approach could help turn long-term AAV-mediated expression into a controllable asset by keeping durable transgene activity largely restricted to the most clinically relevant tumor niches.

Future directions

Future studies will be essential to translate this multi-modal AAV platform from a conceptual framework into an experimentally validated strategy and to assess its broader applicability in NSCLC and beyond. First, each module of the system—MGS4-mediated capsid targeting, hypoxia-responsive 4× HRE gating, BIRC5-driven tumor selectivity, Q-CXCL9-Fc secretion, and MSLN silencing by miR-30-based shRNA—should be dissected and validated stepwise *in vitro*. Researchers could, for example, generate modular AAV or plasmid constructs in which individual elements are selectively removed or substituted, and then compare transduction efficiency, hypoxia dependence, and on-off tumor selectivity across NSCLC and normal lung cell lines. A proposed stepwise study design summarizing these *in vitro*, *in vivo*, and translational evaluation steps is outlined in Figure 3.

Second, the central hypothesis that dual delivery of Q-CXCL9-Fc and shMSLN provides superior antitumor activity relative to single-modality vectors should be directly tested in appropriate *in vivo* models. Comparative studies using AAVs expressing only Q-CXCL9-Fc, only shMSLN, or the full dual cassette in syngeneic or humanized mouse models will help determine whether combining immune activation with targeting of intrinsic tumor vulnerabilities yields merely additive effects or true synergy in controlling tumor growth and metastasis. In addition, the impact of Q-CXCL9-Fc on T-cell phenotype, exhaustion markers, and spatial distribution within the tumor microenvironment could be profiled by flow cytometry and spatial transcriptomics, providing a framework for mechanistic interrogation of chemokine-Fc-based gene therapies.

Third, the modular design of this platform makes it well suited for adaptation to other solid tumors characterized by hypoxia, BIRC5 overexpression, and MSLN or alternative tumor-associated antigens. Future work could replace MGS4 with peptides selected by *in vivo* phage display or rational design for different tumor types and substitute shMSLN with miR-30-based RNAi modules targeting oncogenic drivers relevant to each disease, while retaining the same 4× HRE-CMV/BIRC5 dual promoter backbone. As these variants are explored in ovarian, pancreatic, mesothelioma, or other hypoxic, BIRC5-high tumors, it will be important to frame them explicitly within a precision oncology context by defining biomarker panels (e.g., antigen expression, hypoxia signatures, BIRC5 levels, and pre-existing anti-AAV antibodies) that can guide indication selection and future patient stratification.

Finally, translational and regulatory considerations will need to be addressed to support potential clinical development. Rigorous studies in immunocompetent models should assess off-tumor transduction and transgene expression, characterize pre-existing and treatment-induced anti-AAV and anti-MGS4 immune responses, and define dose-response relationships under systemic administration. In parallel, establishing standardized manufacturing and analytical protocols for peptide-displaying AAV vectors, along with head-to-head comparisons against ADCs or recombinant antibodies in relevant models, would provide a practical roadmap for other investigators to benchmark and further test the proposed platform. These preclinical efforts could naturally lead into early-phase trials that use enrichment or basket designs and add-on cohorts to test the vector alongside PD-1/PD-L1 blockade or other standard regimens. In an enrichment design, only patients whose tumors meet predefined biomarker criteria (e.g., MSLN-positive, hypoxic, BIRC5-high NSCLC) are enrolled, whereas a basket trial would test the same AAV platform across multiple biomarker-defined tumor types in parallel cohorts. In add-on cohorts, the vector would be layered on top of an existing standard-of-care regimen such as anti-PD-1 therapy to assess incremental benefit and safety without redesigning the entire treatment backbone.

Limitations

This hypothesis article is based on a conceptual AAV design rather than completed experimental data, so many of the proposed advantages remain unproven. The functions of key modules, including MGS4-mediated retargeting, 4× HRE-CMV/BIRC5 regulation, Q-CXCL9-Fc secretion, and miR-30-based shMSLN silencing, have not yet been validated in NSCLC-relevant models, and it is unclear whether the full multi-component cassette can be packaged and manufactured at high titer without compromising capsid integrity, tropism, or immunogenicity.

Clinical translation may also be limited by biomarker and patient factors. The strategy assumes tumors that are concurrently MSLN-positive, hypoxic, and BIRC5-high, but the prevalence and assay standardization of this profile are not well established, which could complicate biomarker-enriched trial designs. In addition, pre-existing or treatment-induced immune responses against AAV or MGS4 may restrict systemic dosing, re-administration, and multi-lesion targeting, especially in heavily pretreated patients.

Finally, biological and safety uncertainties remain substantial. The proposed synergy between Q-CXCL9-Fc and MSLN knockdown has not been demonstrated, and the oxygen threshold, dynamic range, and leakiness of the HRE-BIRC5 module have not been quantified in heterogeneous tumors, raising the risks of both under-treatment and unintended activation in transiently hypoxic normal tissues. Potential adverse effects, such as excessive T-cell recruitment or effects of MSLN modulation in normal mesothelial tissues, have not yet been evaluated. For these reasons, the current platform should be regarded as a framework that requires systematic experimental testing and comparison before any clinical application is considered.

Conclusions

We propose a multi-layered, NSCLC-directed AAV platform as a conceptual framework for next-generation, tumor-selective gene therapy in oncology. Rather than introducing completely new components, this hypothesis emphasizes the architectural integration of established elements—re-directed capsid tropism, hypoxia- and BIRC5-gated transcription, secreted immune activation, and miR-30-based RNAi against a tumor-associated antigen—within a single, packaging-compliant vector. In doing so, it aligns AAV design with principles of precision oncology, in which patients are selected on the basis of MSLN expression, hypoxia signatures, and BIRC5 expression levels and treated with rational combinations that can include immune checkpoint blockade. If confirmed experimentally, this multi-layered architecture could serve as a template for configuring similarly gated AAV platforms across a wider range of hypoxic, BIRC5-positive solid tumors, while the limitations and future directions outlined above provide a roadmap for rigorously testing, refining, or reformulating the hypothesis before any clinical application is pursued.

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None.

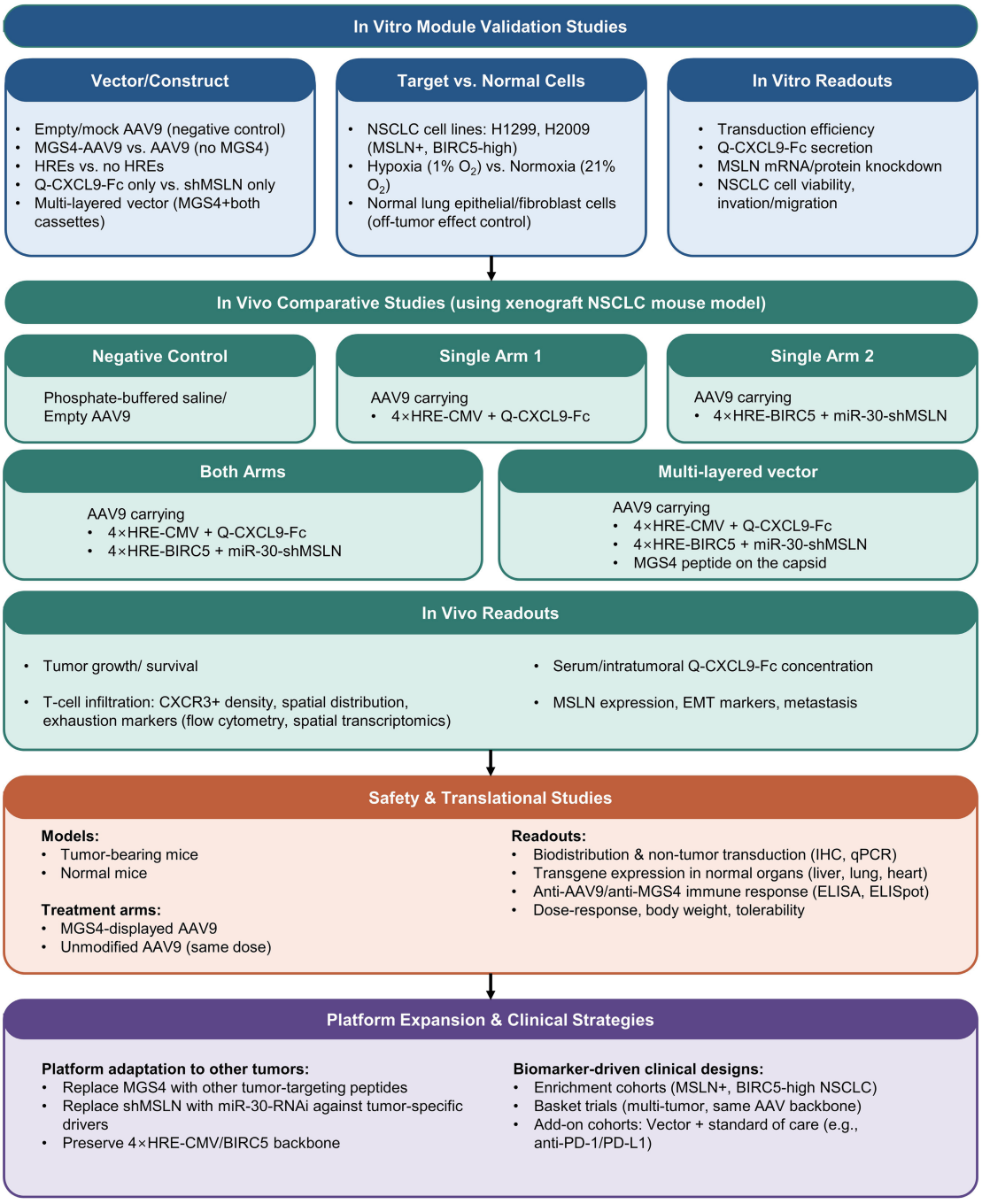

Fig. 3. Conceptual study design for evaluating the multi-modal MGS4-AAV9 platform in NSCLC. The diagram outlines a stepwise framework beginning with *in vitro* module validation, in which empty or unmodified vectors, single-arm constructs, and the full MGS4-displaying dual-cassette vector are compared in hypoxic versus normoxic conditions in NSCLC and normal lung cells to assess transduction, Q-CXCL9-Fc secretion, MSLN knockdown, and tumor-cell behavior. In xenograft NSCLC mouse models, PBS/empty AAV9, single-arm, and full dual-cassette vector arms are then evaluated for effects on tumor growth, survival, intratumoral CXCR3$^+$ T-cell density and spatial distribution, Q-CXCL9-Fc levels, and MSLN-related invasive features. Dedicated safety studies in tumor-bearing and non-tumor mice characterize biodistribution, non-tumor transduction, transgene expression in normal organs, immune responses to AAV9 and MGS4, and dose-limiting toxicities, while the modular design is finally positioned for adaptation to other hypoxic, BIRC5-high tumors and for integration into biomarker-driven clinical trial strategies such as enrichment, basket, and add-on cohorts. AAV, adeno-associated virus; AAV9, adeno-associated virus serotype 9; BIRC5, baculoviral IAP repeat containing 5; CMV, cytomegalovirus; CXCR3, C-X-C motif chemokine receptor 3; ELISpot, enzyme-linked immunospot assay; ELISA, enzyme-linked immunosorbent assay; EMT, epithelial-mesenchymal transition; HRE, hypoxia-responsive element; IHC, immunohistochemistry; miR-30, microRNA-30; MSLN, mesothelin; NSCLC, non-small cell lung cancer; PBS, phosphate-buffered saline; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; Q-CXCL9-Fc, glutamine-modified C-X-C motif chemokine ligand 9-fragment crystallizable region fusion protein; qPCR, quantitative polymerase chain reaction; RNAi, RNA interference; shMSLN, short hairpin RNA targeting mesothelin.

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Conflict of interest

None.

Author contributions

Conceptualization and design of the hypothesis (DH), literature review (DH), manuscript writing (DH), critical revision of the manuscript (DH, EL), and study supervision (EL). All authors have made significant contributions to this study and have approved the final manuscript.

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