HYDRODYNAMIC DELIVERY FOR CANCER AND OBESITY RESEARCH

by

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(Under the Direction of Dexi Liu)

ABSTRACT

Hydrodynamic gene delivery was developed as an efficient and safe method for gene transfer into hepatocytes in mice via tail vein injection. The method then evolved into a versatile procedure to deliver various entities, including genes, proteins, and even living cells into various target organs in various animal models. In this dissertation, a series of studies on the application of hydrodynamics-based delivery method in cancer research and anti-obesity gene drug discovery are presented. In cancer research, the method was utilized to deliver tumor cells into mice liver, lungs, and kidneys for modeling of tumor metastasis. We have successfully established a multi-organ tumor growth model in mice using melanoma and colon carcinoma cell lines. The model was used for quantitative assessment of differential behavior of tumors growing in different organs. Our findings revealed that initial survival and growth rates of tumors vary in different organs. In addition, tumors respond to given chemo- and immunotherapies differently in different organs. The hydrodynamic method was also used deliver genes to overexpress the interferon beta (IFNβ1) gene in mice to assess the anti-obesity effects in mice. Our findings showed that IFNβ1 attenuates adipose tissue inflammation and blocks development of obesity and its related pathologies, such as insulin resistance. This dissertation comprises five chapters. Chapter one introduces the layout of the entire dissertation and provides a comprehensive overview of hydrodynamics-based delivery. Chapter two reviews theories and applications of

different methods of gene transfer in biomedical research. Chapter three details the use of hydrodynamics-based procedure for modeling cancer metastasis and assessing differential behavior of tumors growing in different organs. Chapter four details the use of the hydrodynamic method for the assessment of therapeutic activities of interferon beta gene in blockage of development of obesity and its related complications upon high fat-diet feeding. Chapter five provides a discussion of conclusions and future perspectives of hydrodynamics-based delivery, with a focus on remaining challenges and its applications in modern drug discovery.

INDEX WORDS: Drug discovery and development, Hydrodynamic gene delivery, Hydrodynamic cell delivery, Physical gene delivery, Cancer metastasis, Metastasis tumor models, Tumor heterogeneity, Obesity, Gene therapy, Interferon beta, Inflammation, Insulin resistance.

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DEDICATION

I would like to dedicate this dissertation to the best parents anyone could have: my father Hussein Alsaggar, and my mother Ibtisam Alsharo', for their endless love and support throughout my entire life. It is my obligation to thank them for giving me the confidence and faith to succeed. This dissertation is also dedicated to my lovely wife Samah Al-Shatnawi, who has been always there for me as instigator and spiritual supporter. I owe her a unique distinction for establishing a simple and sustaining way of life to bring joy and success even beyond our wildest dreams. To my kids, Taqi and Aleen, who always balance our stress with cuteness and stability, and convert our smiles to endless happiness. We thank them for being enough reason to get the best out of us.

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CHAPTER 1

INTRODUCTION

The Rise of Biopharmaceuticals

Until the 19th century, our concept of disease therapy was centered on the use of naturally available herbal and animal-derived remedies for disease cure, which all reflected the accumulated empirical experience over generations. While tremendous old remedies were developed, many of which are still in use today, such as senna, cannabis and opioid alkaloids [1], the scientific basis of drug development emerged in the late 19th century, along with sufficient advances in life sciences to drive the discovery of new drugs. Since then, the field of drug discovery and development has grown immensely, especially with the evolution of interdisciplinary sciences, such as pharmacology and synthetic chemistry. The 20th century witnessed the most important milestones in drug development. The emergence of DNA technologies and molecular biology tools in 1970s, and the sequencing of human genome were indeed breakthroughs that greatly altered drug discovery approaches through the introduction of high-throughput methods for exploring how living systems are governed by cell functions and identification of genes and related pathways that underlie various diseases, which allowed intervention in the biological system to tackle disease pathologies. Indeed, the proportion of biopharmaceutical therapeutics among newly registered drugs is steadily increasing. Nowadays, the use of human proteins, delivery of therapeutic genes and engineered peptides and nucleic acids drugs has become a daily practice in clinics for diagnostic, preventive, and treatment measures for variety of human diseases.

Gene Transfer Technologies for Gene Drug Development

Molecular biology has become a major part of drug development programs, and increasingly contributes to the development of new therapies for various human diseases. Over the past few decades, several molecular approaches combining multi-disciplines of genetics, life sciences and computer sciences have been introduced, and in particular, gene and cell-therapies have established new paradigms in drug discovery and development. Initially, gene drug development aimed to manage diseases by intracellular gene transfer to provide a lost gene function in diseased tissue [2]. The rationale, however, has recently evolved beyond treatment of diseases to include prophylactic strategies, such as DNA vaccines, as well as diagnoses and gene marking [3-5].

Certainly, efficient gene delivery is the cornerstone for biomedical applications of gene-based therapies. Current progress in gene drug development is challenged by the limited efficiency of gene transfer due to several intracellular and extracellular barriers that limit the amount of DNA reaching the nuclei of target cells where gene expression takes place [6,7]. Research has been devoted to develop methods for gene transfer utilizing biological, chemical or physical principles [8], with acceptable safety and efficiency to overcome barriers such as serum and tissue nucleases, and facilitate DNA entry across cell membrane. As of this writing, three different systems of gene transfer have been developed; viral methods, chemical methods, and physical methods. Viral methods remain the most efficient methods, but their use is limited due to their serious side effects. Chemical methods are safer alternatives to viral methods, but remain in need of significant improvement to enhance efficiency. Physical methods, including the hydrodynamic method, are vector-free methods with various efficiencies and safety profiles.

Different methods of gene delivery and their applications in biomedical research are discussed in chapter 2 of this dissertation.

Hydrodynamics-Based Delivery

Hydrodynamics-based delivery is currently considered among the simplest and most effective methods for intracellular delivery of membrane-impermeable macromolecules, such as DNA and RNA molecules, and proteins. The method was developed in the context of efforts aimed at enhancing the efficiency of intravascular delivery of "naked" DNA to avoid several barriers of carrier-based systems, i.e. viral and chemical vectors. In 1999, Liu et al. and Zhang et al. demonstrated successful intrahepatic gene transfer after rapid injection of plasmid DNA into tail vein in rodents [15,16]. Since then, hydrodynamic gene delivery has undergone several improvements, and has evolved into a robust and versatile method for transfer of various entities, such as genes, oligonucleotides, proteins and even live cells into a various target organs in different animal models. Indeed, the hydrodynamics-based procedure has gained wide appreciation, and now it is increasingly applied in drug discovery for therapeutic gene screening, assessment of gene expression regulation, and animal model establishment.

Basics of hydrodynamic gene delivery

The rationale of hydrodynamic delivery relies on thorough understanding of blood vessels anatomy and the dynamics of fluid passing through these vessels. Given the barrier function of capillary endothelium that limits permeation of cells and macromolecules, extravasation of delivered entities would necessitate breakage of the endothelial barrier, or formation of transient pores through which nucleic acids and other macromolecules can pass

through. In this direction, hydrodynamic delivery was developed to overcome the endothelial barrier by means of fluid dynamic pressure that acts directly on endothelial walls to push endothelial cells apart, resulting in transient holes that reseal shortly after the injection. Typically, efficient gene transfer into rodents' hepatocytes requires rapid injection of large volume of DNA solution via the tail vein to generate such pressure. The standard procedure comprises injection of fluid volume equivalent to 7-9% of body weight over 5-8 seconds. Mechanistically, the injected solution passes through the inferior vena cava and hits and stretches myocardial fibers, resulting in cardiac congestion and retrograde flow of solution into the liver and kidneys via the hepatic and renal veins, respectively. The accumulated fluid in these veins generates the dynamic pressure that forces out the DNA solution across the endothelium into surrounding parenchyma cells, such as hepatocytes in the liver. Microscopic examination of liver vasculature revealed disrupted sinusoids and enlarged fenestrae, along with the formation of intracellular vesicles and dilution of cytoplasm, suggesting fluid entry into cells [17-19]. It has been proposed that hepatocytes membrane perforation as a consequence of hydrodynamic impact is the underlying mechanism for DNA transfer into cytoplasm, as evidenced by electron microscopic examination [20]. Given the limited peri-cellular passage of the injected DNA solution between hepatocytes due to tight junctions, perforation of plasma membrane is likely due to pressure-induced invagination of the membrane, which in turn results in transferring the DNA solution into the intracellular compartment. Upon restoration of cardiac function, hepatic intravascular pressure is decreased, and cell membranes quickly reseal, trapping DNA molecules inside hepatocytes. The safety of the hydrodynamic procedure is well-characterized. Despite the short-term increase of serum concentration of liver-specific enzymes and animal discomfort, no long-term effects have been reported. Since the generated hydrodynamic pressure is a function of

volume and speed of injection, it is critical to finely adjust these parameters to maximize the efficiency of gene transfer while minimizing any potential tissue damage. It has been demonstrated that cell membranes reseal within 2 minutes after the procedure, the endothelial layer regains normal structure within 24 h, and liver enzymes levels decrease to normal ranges after 48 h [18,20].

In addition to overcoming the endothelial barrier, hydrodynamic delivery facilitates DNA transfer through protecting DNA from serum nucleases. Upon tail vein injection, DNA solution pushes pre-existing blood in the inferior vena cava and hepatic vasculature, resulting in minimal contact of DNA with blood nucleases. To date, the liver has been the primary organ targeted by hydrodynamic delivery, owing to the unique hepatic fenestrated capillary structure and the lack of basement membrane, compared to the continuous and basal-surrounded capillary structure of other organs. In addition, the anatomical location adjacent to the inferior vena cava makes the liver easily and non-invasively accessible via tail vein injection. Hydrodynamic delivery to other organs has also been described. Next to liver, muscles and kidneys are the most common organs that have been explored. In both organs, blood vessels are occluded using balloon catheter to prevent off-target distribution of DNA solution, allowing pressure build up in the targeted area, and minimizing the volume of DNA solution needed to generate necessary dynamic pressure. This approach is currently applied for hydrodynamic delivery in liver and muscles of large animals, such as dogs, pigs, and baboons, in which image-guided, computerized “hydrojector” is utilized for precise control of injection site and injection parameters [21].

Basics of hydrodynamic cell delivery

The principle of hydrodynamic delivery was recently utilized for delivery of live cells *in vivo*. Systemic delivery of tumor cells to establish metastatic tumor models has been challenged by the difficulty of extravasation of injected cells into the target organ. Apart from lung metastasis, in which physical entrapment of systemically injected cells is sufficient to drive metastatic growth, establishment of orthotopic tumors in various organs often requires an invasive procedure to precisely inject tumor cells into organ parenchyma. Given the potential of hydrodynamic delivery to overcome the endothelial barrier, it has been proposed that the dynamic pressure that mediates macromolecule transfer across the endothelium would be sufficient to drive cells in suspension outside capillaries in tissues impacted by hydrodynamic pressure. Indeed, hydrodynamic delivery of tumor cells via tail vein injection resulted in successful delivery, and subsequent growth of tumor cells in liver and kidneys (the primary organs affected by hydrodynamic method), and in lungs [22]. Similar to hydrodynamic gene delivery, cell delivery is mediated by transient breakage of the endothelial barrier primarily in the liver and kidneys, resulting in dispersion of tumor cells in organ parenchyma. Upon cardiac recovery and resuming normal blood pressure, the remaining tumor cells in liver vasculature and inferior vena cava circulate through the heart to the lungs, where they are physically trapped to establish a third tumor growth site. While the underlying mechanism of hydrodynamic gene delivery is well studied, additional work is needed to examine the fate of hydrodynamically delivered cells, particularly the initial interaction between tumor cells and tissue-resident cells.

Hydrodynamic gene delivery... advantages

HD is advantageous over other non-viral systems in that it is safe and simple, and doesn't require specialized instrumentation. In mice, all that is needed is a needle and syringe to perform

HD through the tail vein. The size of the needle depends on which vessel to be injected, and what animal model is used. In addition simplicity and convenience, HD has demonstrated outstanding versatility. As being driven by fluid physics, theoretically, HD should enable intracellular delivery of any cargo loaded in the injected fluid. Indeed, various macromolecule entities have been successfully transferred into target cells using HD, such as plasmid DNA [23], artificial chromosomes and large DNA fragments [24,2 5], genomic and synthetic RNA [26-28], oligonucleotides [29], proteins [29] and even live cells [22]. The applicability of HD in gene transfer studies has also been supported by its ability to be applied for gene transfer trials in different target tissues, particularly liver, muscles and kidneys in different small and large animal models, including rodents, pigs, dogs, monkeys, rabbits, chickens, and fish. Various injection routes have been explored for hydrodynamic delivery, including the tail vein, hepatic vein, portal vein, inferior and superior vena cava, jugular vein, femoral vein, renal vein, carotid artery and others [30]. Together, such profound versatility of the procedure greatly facilitates biomedical research by offering many options for various study designs. Another advantageous feature of HD is that liver is the primary organ targeted by HD. Added to large surface area of hepatocytes facing venous lumen, and the higher capacity of hepatocytes for gene expression, HD method would be the method of choice when large quantities of transgene product are needed.

Objectives of the Dissertation Research

This dissertation aimed to present two different studies on how the method of hydrodynamic delivery is applied in biomedical research for different purposes. In the first project, the method was utilized to deliver tumor cells into mouse liver, lungs and kidneys via tail vein injection for modeling of tumor metastasis. The model was used for quantitative

assessment of differential growth behavior and sensitivity profiles of tumors growing in different organs. Specific aims were:

- To establish a multi-organ tumor growth model in mice using the hydrodynamic cell delivery method.
- To quantitatively assess tumor cells' survival and growth in different organs.
- To assess the differential tumor cells' response to anticancer therapies when growing in different organs.

In the second project, the hydrodynamic gene delivery method was used to overexpress mouse interferon beta (IFNβ1) gene in a mouse model of diet-induced obesity to assess whether the anti-inflammatory properties of IFNβ1 would block the development of obesity and its complications in mice. Specific aims were:

- To assess the efficiency of IFNβ1 gene transfer into mice using the hydrodynamic gene delivery method.
- To assess the effects of IFNβ1on diet-induced adipose tissue inflammation.
- To assess the effects of IFNβ1 on development of obesity and related pathologies, such as insulin resistance and fatty liver.

Hydrodynamic delivery for cancer research

Cancer metastasis is defined as a complex cascade of dynamic biological events through which malignant tumor cells leave the primary tumor, travel to a distant site via the blood or lymphatic vasculature, and establish secondary tumors in distant organs. To do so, tumor cells have to detach from the extracellular matrix of primary tumor by a process called Epithelial-

Mesenchymal Transition (EMT), which results in loss of adhesion, remodeling of cytoskeleton and expression of proteases essential for invasion of surrounding parenchyma. Upon EMT, tumor cells change their epithelial properties into mesenchymal ones to liberate cells from adhesion interactions with surrounding cells and basement membrane, and to acquire motile properties to start migration. Getting access into blood or lymphatic vessels is the next step in the metastatic process which also requires cytoskeleton remodeling. Once in circulation, circulating tumor cells face challenges to survive until reaching distant organs. Circulating tumor cells have to survive immune surveillance, shear stress due to blood flow and lack of adhesion. If survived, they leave circulation (extravasation) at secondary organs to establish new metastatic tumors [31]. Metastatic cascade is a function not only of tumor cells but also the involvement of cooperative interactions of tumor cells with normal cells of the body, such as immune cells and endothelial cells. Tissue resident stromal cells, as well as tissue components (ECM) greatly affect the progress and efficiency of metastatic process, and the establishment of tumor colonies in the early metastatic niche. Thus, better understanding of the molecular mechanisms underlying the metastatic cascade, and the key molecular and cellular players in different organs will offer new opportunities for therapeutic intervention to inhibit metastasis, and cure of cancer after all.

Progress in anticancer drug discovery and development has made significant contributions to enhance survival of cancer patients. Several classes of anticancer therapies have been developed, such as small molecule and antibody therapeutics targeting selected receptors and pathways critical for proliferation and survival of tumor cells. Yet, there has been relatively little activity against molecules and pathways controlling the process of invasion and metastasis. Given that tumor metastasis accounts for the majority of cancer deaths [32], and that cancer mortality is largely defined by tumor invasion and metastasis, successful treatment of tumor

metastasis remains as an unmet need in clinic. It's been truly challenging to translate tremendous findings in cancer research into effective therapies, with a failure rate approaching 90% [33]. Since metastasis is a complex and multi-step process, it is likely that the process has many potential targets for intervention. However, our target validation and pre-clinical screening in anticancer drug development is challenged by the availability of *in vitro* and *in vivo* models that truly represent the natural metastatic cascade. The models that are currently used for screening of anticancer therapies, such as subcutaneous or orthotopic tumor models misleadingly assume tumors in different organs are the same in terms of growth and sensitivity. It is well-established that the formation of a clinically relevant tumor requires support from the surrounding stroma (microenvironment). Indeed, tumor microenvironment has a major role in modulating the metastatic capacity of most tumors. It remains unclear how the microenvironments in different organs affect tumor growth and its sensitivity to a treatment, and whether different regimens need to be used to inhibit and/or eliminate tumor cells growing in different sites. Therefore, an urgent need exists to develop an animal model that allows direct assessment of tumor cells behavior in different organs, as imposed by differential environmental inputs in these organs. Among primary objectives of this dissertation is to address these challenges, at least in part by using hydrodynamic cell delivery method to establish a multi-organ tumor growth model. I aimed to test the hypothesis that tumors in different organs behave differently, to explore whether the biological heterogeneity of metastatic tumors would influence the outcomes of anticancer therapies, and whether combined, organ-specific therapies are needed to act globally on metastatic tumors in different organs. Given that liver and lungs are the primary sites to be seeded with tumor cells upon hydrodynamic injection, and that the selected tumor models should be clinically relevant, murine metastatic melanoma cells (B16F1) were used because liver and

lungs, besides brain, are the most comon sites for metastasis of melanoma tumors in patients [34]. Results of assessment of melanoma tumors behavior were confirmed with murine colon carcinoma cells (C26). Similarly, liver and lungs are the primary sites for colon cancer metastasis [35]. Therefore, these cell lines were selected for the dissertation studies aiming at assessment of tumors in clinically relevant settings.

Hydrodynamic delivery for obesity research

The worldwide prevalence of obesity has increased in the past 30 years. As of 2014, the prevalence of obesity among age-adjusted US population was 36.5% [36]. In addition, obesity is closely related to a set of metabolic disorders, including cardiovascular diseases, type-2 diabetes, and fatty liver, and to various neoplasms, such as colon cancer [37]. Despite the severity of obesity and its complications, only a few treatment strategies are currently available to treat the disease and to prevent its complications with limited success. Therefore, new treatment strategies are urgently needed. While the exact molecular mechanism of obesity development remains elusive, mounting evidence suggests that chronic, low grade inflammation plays a major role in obesity development, and significantly contributes to obesity-related pathologies, such as insulin resistance [38]. Therefore, the second part of this dissertation research focuses on targeting inflammation as potential therapeutic strategy for obesity. The overall objective was to prevent development of high fat-diet-induced obesity by hydrodynamic gene transfer of interferon beta (IFNβ1) gene to mice to attenuate inflammation. The IFNβ1-based therapies represent new alternatives for management of various inflammation-driven pathologies.

IFNβ1 is an immunomodulatory cytokine that belongs to the type I interferon family, which also includes IFNα. IFNβ1 mediates its bioactivity through binding to ubiquitously

expressed IFNR1 and IFNR2, and signals through JAK1 and TYK2 pathway and downstream different members of STAT family to result in expression of different subsets of IFN-inducible genes [39]. Naturally, IFNβ1 is produced in response to viral infections or sensing nucleic acids like double-stranded RNA by cytosolic receptors. While all cell types can produce IFNβ1, dendritic cells are specialized to produce large quantities of IFNβ1 to activated natural killer (NK) cells to eliminate virally infected cells.

Interferons have long been believed to be a group of cytokines for interference with viral infection only. However, it has become evident that interferons mediate various pluripotent processes such as anti-inflammatory, anti-proliferative, anti-tumor and immunomodulatory effects. IFNβ1 signaling regulates important events in both innate and adaptive immune responses by acting directly or indirectly on NK cells, T cells, B cells and macrophages and dendritic cells. In adaptive immune response, IFNβ1 induces activation and expansion of cytotoxic T cells, and increases antigen presentation to T cells by antigen presenting cells (APCs) [40]. In addition, IFNβ1 is a potent activator of NK cells, particularly the anti-tumoral response. IFNβ1 also modulates immune responses toward an anti-inflammatory profile by suppressing T helper 17-mediated inflammatory responses through suppressing key-inflammatory pathways, such as NF-kB, down-regulation of Il-17 and induction of Il-10 expression. Moreover, it downregulates TNF-α and Il-1β expression to further enhance the anti-inflammatory response [41]. Therefore, the deregulated expression of IFNβ1 is highly linked to various inflammation-related pathologies, such as multiple sclerosis and ulcerative colitis. Besides immunomodulatory effects, IFNβ1 displays anti-proliferative properties, making it increasingly considered for cancer therapy. While the proapoptic effects of IFNβ1 are not fully clear, it is believed that IFNβ1 alone is not sufficient to drive apoptosis. Rather, proapoptic

effects are mediated by inducing activation of caspases, TRAIL and death receptors [42]. These antitumor effects are further enhanced by the antiangiogenic properties of IFNβ1. These effects are mediated by suppression of production of VEGF, FGF, and TGF-β in tumor microenvironment, along with suppression of the migration of endothelial cells [43].

Given its pleiotropic anti-inflammatory properties, IFNβ1 is increasingly being explored for therapeutic potential to treat inflammation-driven pathologies. Indeed, IFNβ1 has been approved as a disease-modifying agent for multiple sclerosis (MS). The beneficial effects of IFNβ1 in MS patients are attributed to suppressing the production of inflammatory mediators, such as IL-17, osteopontin and TNF-α, increasing the production of anti-inflammatory cytokines such as IL-10, attenuation of inflammatory cell migration across BBB, and increasing the production of nerve growth factor, which together lead to attenuation of neuronal inflammation and enhanced neuronal survival [44]. IFNβ1 is now commercially available as recombinant protein provided in lyophilized form that is reconstituted prior to injection, and given intramuscularly (IM) and subcutaneously (SC) once or twice a week. Clinical applications of IFNβ1 are currently being expanded with clinical trials investigating the use of IFNβ1 on patients with ulcerative colitis [45] and rheumatoid arthritis [46].

Overall, IFNβ1 therapy is well-tolerated, and reported side effects are commonly limited to skin reactions at the site of injection. Such reactions are more common with SC injections, and usually occur within the first month of therapy, and diminish after six months [47]. Flu-like symptoms, such as fever, headache and fatigue have also been reported, and commonly disappear after one day of injection. These effects have been attributed to transient increase in cytokines. Other side effects I include leukopenia and neutropenia, and functional deterioration

of liver [47]. While these effects are often not serious, continuous monitoring of blood count and liver function is highly recommended. So far, clinical data and continuous follow up indicate that the long term therapy with IFNβ1 is safe, and usually linked to better therapeutic outcomes [48].

Organization of the dissertation

The dissertation follows the standard format of the graduate school of the University of Georgia. With the exceptions of Chapter 1 (introduction) and Chapter 5 (conclusions and future perspectives, Chapter 2 is a book chapter published in "Drug Delivery", edited by Ashim Mitra, Deep Kwatra, and Aswani Dutt Vadlapudi, Jones & Bartlett Learning, Burlington, MA, USA, providing a comprehensive overview on principles, characteristics and applications of gene delivery systems that have been developed so far. Chapter 3 is a research paper published in "Clinical and Experimental Metastasis" presenting an example about how hydrodynamic delivery is used to establish a tumor metastasis model and to assess the influence of different environments in different organs on tumor growth, as well as tumor response to anticancer therapies. Chapter 4 is a research paper submitted to "Gene Therapy", presenting a study for assessment of preventive effects of hydrodynamic transfer of IFNβ1 gene on suppressing high fat diet-induced obesity. An appendix is included as additional work that I did during my training here in the University of Georgia. It is a review article summarizing the historical events and applications of physical methods for gene delivery. The overall objective of this dissertation is to provide not only the examples of using hydrodynamic delivery for drug discovery, but also the related work that I have contributed to the field of drug discovery and development using gene transfer approaches during my graduate training.

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CHAPTER 2

GENE DELIVERY: AN ESSENTIAL COMPONENT FOR SUCCESSFUL GENE THERAPY[*]

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Introduction

The concept of human gene therapy emerged in the 1960s as a consequence of advancing research in molecular genetics [1]. Earlier experiments showed that the Shope papilloma virus is capable of inducing arginase activity in infected cells of rabbits [2] and the tobacco mosaic virus is effective in expressing viral genome along with the added sequence in tobacco leaves after infection [3]. Therefore, it was suggested that viral genomes can be used to deliver therapeutic effects mediated by viral endogenous or added genes. Initially, this idea was applied clinically in the first human gene therapy trial conducted in 1970 with the intention of treating arginase deficiency using Shope papilloma viruses [4]. Three German siblings with severe arginase deficiency received systemic injections of purified Shope papilloma virus as a means of supplementation for the missing enzyme activity. Unfortunately, the study failed to provide any benefit to the patients, largely due to flaws in the experimental design, an incomplete understanding of the disease mechanism, and the lack of gene expression. Nevertheless, this study provided a direct path toward the use of viruses as a gene carrier in disease treatment. In September 1990, the first approved clinical trial for gene therapy using retroviral vectors was conducted in the treatment of severe combined immunodeficiency disease (SCID) caused by adenosine deaminase deficiency [5]. Although the trial was considered a great success and inspired significant efforts in applying the gene therapy concept to the treatment of other diseases, it was later realized that retroviral vectors used in the protocol had a tendency to activate the oncogene through insertional mutagenesis and induced leukemia in four of nine patients treated [6]. This finding raised a serious concern about the safety of viral vectors and

welcomed efforts in improving them and developing nonviral alternatives. Since then, many methods of gene delivery have been developed.

Methods of Gene Delivery

The site of gene expression is in a cell's nucleus. Since nuclear acid sequences encoding a therapeutic gene in the form of either RNA or DNA are polyanion and nonpermeable to cell membranes, the primary objective of gene delivery is to apply the principles of cell biology, chemistry, and physics to facilitate gene transfer from the site of administration to the nuclei of intended cells. Three systems have been studied in the past. The first system uses the power of viral infection as a means for gene delivery. The second takes advantage of the cellular function of endocytosis to facilitate gene internalization, whereas the third system simply uses physical force to overcome the membrane barriers and allow gene-coding sequences to enter the cells.

Virus-based Gene Delivery

Gene transfer using viral vectors has evolved as an advanced technology for efficient gene delivery. Preparation of viral vectors follows a common procedure, summarized in **Figure 2-1**. Virus-based gene delivery aims to harness the natural viral infection pathway for efficient gene delivery while avoiding the subsequent expression of viral genes associated with replication and toxicity, an approach achieved using replication-deficient viruses harboring the gene of interest, with viral virulent genes deleted [7]. Current viral vectors found in research and clinical uses are based on RNA viruses and DNA viruses that have different genomic structures and host ranges. **Table 2-1** summarizes the main features of commonly used viral vectors.

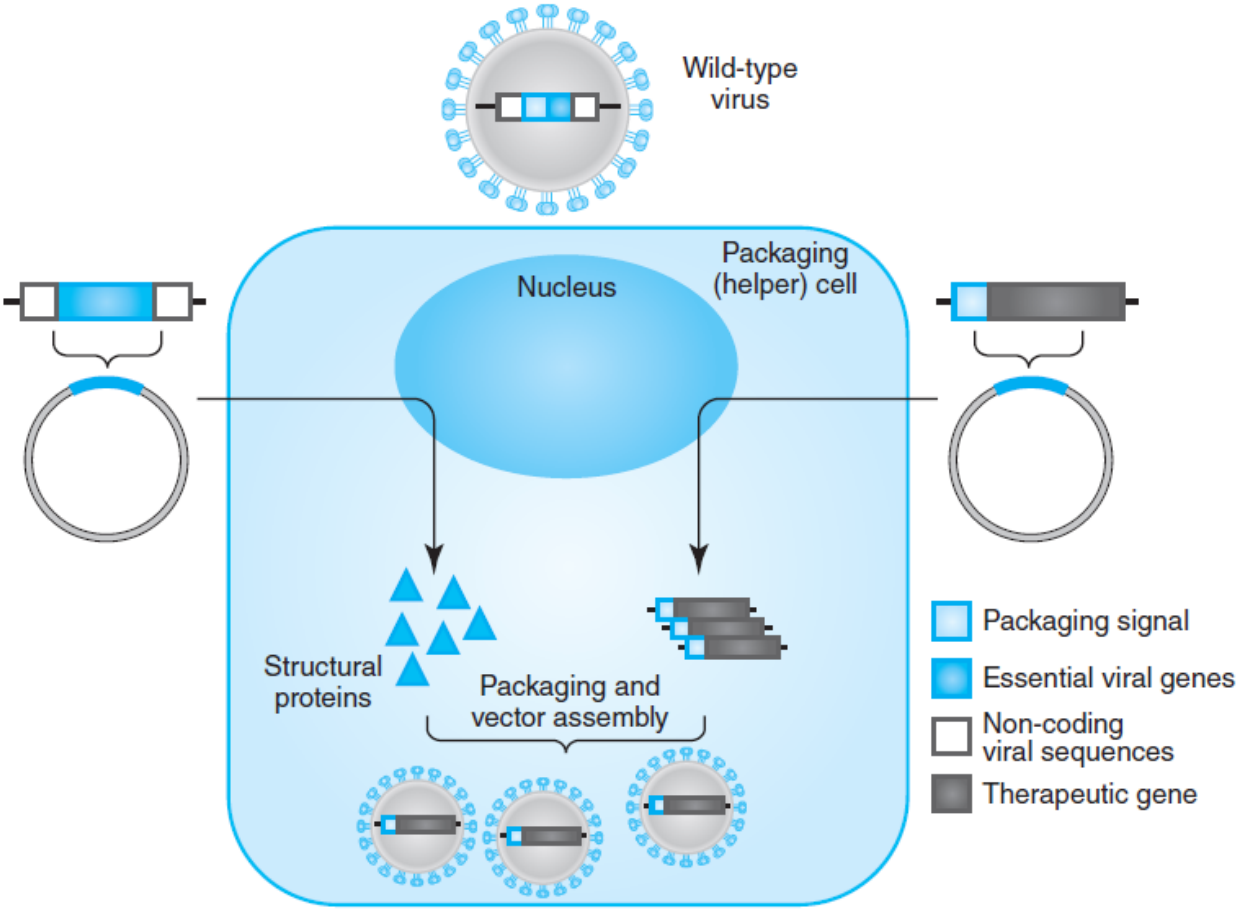

Figure 2-1: Production of viral vectors. For safety reasons (see text), viral vector production involves two unrelated DNA contexts: the first context aims to produce viral structural proteins while lacking their packaging signal, and the second one aims to generate viral RNA or DNA harboring the transgene of interest. The helper (packaging) cell is co-transfected with two plasmids intended for each purpose. Viral proteins and RNA are generated independently. Viral structural proteins recognize the vector (having packaging signal) but not the helper nucleic acid, resulting in packaging of the vector genome into a particle along with the transgene.

Table 2.1: Summarized features of most commonly used viral vectors

Vector*	Genetic material	Capacity (kb)	Titer	Vector genome	Immune induction	Limitations	Advantages
ORV	ss-RNA	8	10^6	Integrated	Low	Random integration, Efficient for dividing cells only	High transduction efficiency
LV	ss-RNA	8	10^8	Integrated	Low	Random integration	Efficient and sustained gene expression in non-dividing and dividing cells
FV	ss-RNA	9	10^3	Integrated	Low	Random integration, low titer	Efficient and sustained gene expression in dividing cells
Ad	ds-DNA	8-10	10^{12}	Episomal	High	Transient gene expression, highly immunogenic	High transduction in dividing and non-dividing cells
HDAV	dsDNA	35	10^{10}	Episomal	High	Significant immunogenicity	High transduction, enhanced safety
AAV	ss-DNA	4-5	10^6	Both	Low	Low loading capacity	Non-pathogenic, low immunogenicity
HSV	ds-DNA	30	10^{10}	Episomal	High	Immunogenicity, transient expression in non-neuronal cells	High loading capacity, highly efficient in neuronal gene delivery

*: ORV: Oncoretrovirus, LV: Lentivirus, FV: Foamy virus, Ad: Adenovirus, HDAV: Helper dependent-adenovirus, AAV: Adeno-associated virus, HSV: Herpes simplex virus.

Retroviral (RNA-based) Viruses: Efficient integration of the viral genome into host DNA is a key feature of the retroviral life cycle, making replication-deficient retroviral vectors for sustained expression of transgenes in target cells [6]. Retroviral vectors have been the second most commonly used gene delivery vehicles after adenoviral vectors [8], and their therapeutic potential has been demonstrated impressively in clinical gene therapy of SCID using an ex vivo approach [9–12]. Retroviruses are enveloped viruses with capsid encapsulating viral genome and two key enzymes: reverse transcriptase generates DNA from viral RNA and integrase incorporates DNA into the host genome [13]. The retroviral genome contains four genes (gag, pol, pro, and env) that encode structural and envelope proteins, as well as cis-acting sequences such as long terminal repeats and the viral packaging signal (ψ). Long terminal repeats contain elements required to drive expression, reverse transcription, and integrate into the host genome, whereas the packaging signal (ψ) sequence interacts with viral proteins, allowing specific packaging of viral RNA [14]. With a genome size of 8 through11 kb, retroviral vectors can harbor exogenous gene inserts of 7 through10 kb [13]. As noted in **Table 2-1**, retroviral vectors have shown high transduction efficiency and long-term gene expression, with relatively lower immunogenic potential than most DNA viruses. Importantly, they lead to stable gene transfer due to integration of the viral genome into chromosomes of target cells. Retroviruses exploited for gene delivery include oncoretroviruses, lentiviruses, foamy viruses, and spumaviruses. Oncoretroviruses, like the murine leukemia virus, were the first to be used as vectors for gene transfer, and their efficiency was demonstrated in several clinical trials. However, transduction of these vectors is limited to dividing cells [7]. Lentiviral vectors, another group of retroviruses, have been most commonly used for gene delivery due to their ability to transduce nondividing cells [15], due to the additional proteins they have to facilitate transport through the nuclear

membrane and eliminating the need for membrane breakage during mitosis to get into nucleus [16]. Lentiviral vectors showed an enhanced safety profile compared with oncoretroviruses [17, 18]. In addition, they have demonstrated huge successes and much promise in several clinical trials [19–22].

Adenoviral vectors: Adenoviruses (Ad) evolved as highly effective gene expression vectors in the early 1980s [23]. As of 2007, Ad became the most commonly used DNA-viral vector in clinical trials, accounting for almost 25% of clinical gene therapy trials [8]. In fact, the use of Ad has significantly increased as an alternative to retroviruses because of a unique set of attributes: highly efficient gene transduction in dividing and nondividing cells, high titer (10^{12}–10^{13} virus particles/mL) of recombinant viruses can be produced, can accept up to 8 kb of exogenous sequence, and lacks insertional oncogenesis associated with retroviruses [24, 25].

Ad viruses are un-enveloped, double-stranded DNA viruses with an icosahedral protein capsid of 70 to 100 nm in diameter encasing a viral genome of 36 kb [26]. The Ad genome consists of nine major transcription units termed as early (E1–E4) and late (L1–L5) transcription units relative to the onset of viral DNA replication, and inverted terminal repeat sequences (ITRs) located at each end of the genome. E1 products, subdivided into E1A and E1B, play essential roles in viral replication, with E1A proteins serving to activate all remaining viral transcription units [27].

Ad infection involves capsid protein interaction with multiple host cell receptors, including coxsackie and the Ad receptor [28, 29], CD46 [30], and sialic acid [31]. These interactions promote sequential steps in cell entry, including attachment and receptor-mediated internalization, followed by endosomal escape [32]. After escaping to the cytosol, the viral

capsid migrates toward the nuclear membrane for disassembly. Nuclear entry is completed upon dissociation of the capsid and release of the viral genome within the nucleus where it is episomally transcribed and replicated. Similar to retroviral vectors, production of recombinant Ad vectors involves the viral DNA construct having the exogenous gene replacing E1 and E3 genes and packaging cells expressing the viral proteins. Deletion of the E1 region essential for replication and the nonessential E3 region allows cloning of the exogenous gene insert with size up to 8.2 kb [33]. E1, E3-deleted Ad vectors, referred to as first-generation vectors, have shown high levels of transient gene expression and have been widely used in gene transfer in vitro and in vivo, including preclinical studies on different animal models and clinical trials on human patients [24, 34]. The use of first-generation Ad vectors, however, was limited by strong immune and inflammatory responses to the vector itself resulting in vector loss [35, 36].

Deletion of all viral coding sequences was the next logical improvement in enhancing the safety profile of Ad vectors. Indeed, helper-dependent or "gutless" Ad vectors were developed, having all viral sequences deleted except the ITR and packaging signal, allowing accommodation of up to 35 kb of exogenous genes. Products of deleted genes are supplied from the replication incompetent (helper) viruses in packaging cells. Gutless vectors have shown reduced immunogenicity and more sustained gene expression [37, 38].

Adeno-associated Viral Vectors: Adeno-associated virus (AAV) is a nonpathogenic human parvovirus that has attracted considerable interest in gene therapy applications where sustained gene expression is required as a gene transfer vector over the past several years [39]. The therapeutic value of AAV vectors has been attributed to many features, including the lack of pathogenicity and minimal immunogenicity of the virus, as well as efficient and sustained gene

expression in dividing and nondividing cells [40]. AAV has the ability to specifically integrate to establish latent infection. Current AAV vectors do not have this ability, and this site-specific integration would ensure long-term transgene expression in tissues with a minimal risk of insertional mutagenesis.

AAV is an un-enveloped parvovirus with a capsid of 22 nm in diameter, packaging a single-stranded DNA of 4 to 5 kb. The AAV genome consists of two major genes, rep and cap, encoding proteins Rep40, Rep52, Rep68, Rep78, and VP1, VP2, VP3, respectively. The two genes are flanked by palindromic sequences (ITR) at each end. These ITRs are essential for AAV DNA replication, genome packaging and transcription, and site-specific integration [41]. AAV vectors are naturally replication-deficient because viral promoters are inactive in the absence of the helper virus. Viral replication can be facilitated by proteins derived from Ad or herpes simplex virus (HSV) genomes [42]. Production of AAV vectors involves co-transfection of packaging cells with the AAV vector plasmid where the exogenous gene replaces viral rep/cap genes and flanked by ITRs and helper plasmids express AAV rep/cap proteins. The adenoviral helper function is provided by superinfecting packaging cells with Ad or transfection with a third plasmid expressing Ad proteins required to facilitate AAV replication [43]. After infection, most of the AAV genome remains in episomal form in the host cell nucleus with almost 10% integrated into host DNA [44]. AAV vectors are increasingly used in gene transfer applications, and its efficacy has been demonstrated in clinical trials [45–48], suggesting AAV as reliable a gene transfer vector, particularly in cases where sustained gene expression is needed.

Other Types of Viral Vectors: Although most gene-transfer studies and gene therapy applications involve the previously mentioned viral vectors, many other viruses have been considered and

have successfully demonstrated potential for gene delivery. HSV, a double-stranded DNA virus with theoretical loading capacity of >100 kb [49], has been an attractive vehicle for gene delivery, particularly in studies including gene transfer to neuronal tissues using neurotropic features of HSV [50]. HSV vectors have been successfully used in clinical trials for gene therapy of brain tumors, chronic pain, and other neuronal disorders [50–52]. Other viral vectors that are being developed and used in gene transfer studies include, but are not limited to, vaccinia virus [53], baculovirus [54], members of alphavirus genus [55], and others. More advanced vectors contain selected components of different viruses to yield hybrid viral vectors possessing the advantages of the original viruses. An example of a hybrid viral vector is Ad/AAV, an Ad vector having the transgene flanked by AAV ITRs, Ad packaging signals, and Ad ITRs. AAV ITRs added the feature of integration to Ad vectors [56]. Alternative hybrid viral vectors include HSV/AAV and HSV/Epstein-Barr virus [57].

Chemical-Based Gene Delivery Systems

Chemical methods (also called nonviral vectors) for gene transfer using synthetic compounds began in the 1960s when diethylaminoethyl dextran was first shown to enhance the transfer of RNA into mammalian cells in culture [58]. The rationale of this approach is to formulate DNA into particles to protect DNA from nuclease-mediated degradation and to facilitate DNA internalization by endocytosis. Being synthetic, chemical carriers are generally less immunogenic and safer than viral vectors. They are also amenable for modifications and inclusion of desirable features such as target specificity and controlled release of DNA. The most studied nonviral carriers are cationic liposomes.

Cationic Liposomes: Liposomes were first described as a model of cell membranes in the 1960s [59] and became increasingly used as a vehicle for delivery of drugs and nutrients. Efficient gene delivery using cationic liposomes was first demonstrated in 1987 using N-(2,3-dioleyloxypropyl)-N,N,N-trimethyamonium chloride [60]. Cationic liposomes spontaneously interact with negatively charged DNA to form stable DNA–liposome complexes (lipoplexes) (**Figure 2-2**). Liposomes have shown significant transfection efficiency and minimal toxicity in animal studies and clinical trials [61]. Cationic liposome formulations with varying lipid structures and chemical compositions are now commercially available. Among many, lipofectamine appears to be the most commonly used as a transfection reagent in vitro.

Cationic Polymers: Cationic polymers are also shown to be effective in gene delivery. Similar to cationic liposomes, highly water-soluble polycations form complexes with DNA (polyplexes) (Figure 2-2) by means of electrostatic interaction, condensing both DNA and the cationic polymers. Polyplexes have comparable in vivo efficiency to lipoplexes; however, they tend to have a higher risk of toxicity. Polyethyleneimines emerged as efficient chemical carriers in 1995 and became the most extensively used cationic polymers in gene transfer experiments [62]. Efficiency of polyethyleneimines as a delivery vehicle is a function of molecular weight. Lower-molecular-weight polyethyleneimines are associated with higher efficiency and lower toxicity [63]. Different polymers have been used as gene carriers, including dendrimers, polyallylamines, polyamidoamines, cationic peptides, and chitosan, as well as many others [64].

Other Chemical Methods: Alternative nonviral carriers have been developed for over 30 years. For example, small insoluble Ca2+-DNA precipitates, now called calcium phosphate nanoparticles, are effective in delivering plasmid into cells in vitro [65]. Nanoparticles prepared

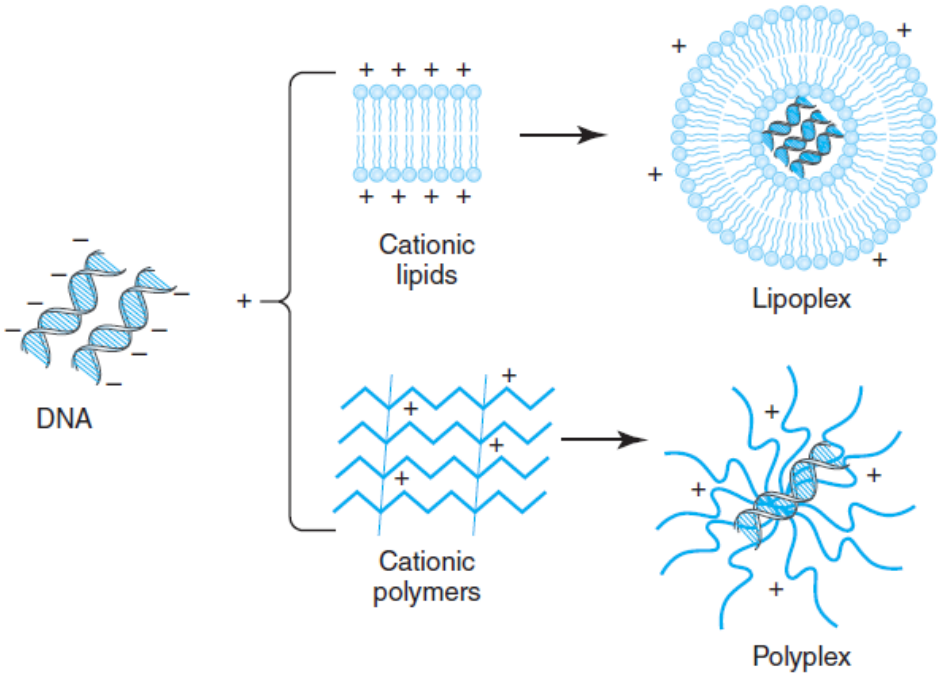

Figure 2-2: DNA complexation with cationic liposomes and polymers. DNA spontaneously makes complexes with cationic species like cationic lipids and polymers. The purpose for this complexation is to protect DNA from serum and tissue nuclease enzymes. DNA forms these complexes by means of electrostatic interactions with oppositely charged lipids and polymers to produce lipoplexes and polyplexes, respectively.

from metal-like gold or inorganic salts-like carbonate salts of magnesium or calcium have also demonstrated promising potential for efficient and safe gene delivery [66]. Efforts have been made to combine polymers with liposomes for better transfection activities.

Physical Methods for Gene Delivery

Physical methods of gene delivery have emerged recently as a simpler and safer approach for gene transfer using mechanical forces (pressure, sound waves, shocks, or electric pulses) to overcome the physical barriers of cells and tissues. These methods facilitate gene delivery to cells or tissues by inducing transient injuries or defects on cell membranes without the aid of cellular functions like endocytosis or pinocytosis.

Needle Injection: The simplest way to deliver genetic material is the direct injection of DNA into target tissues. Gene expression was achieved in vivo by direct injection of reporter plasmids into the skeletal muscles of mice [67] as well as other tissues. The mechanism of DNA uptake is not clear, but data collected so far suggest that the physical damage induced by needle penetration plays an important role. Due to its low transfection efficiency, applications of direct needle injection are limited to DNA vaccination [68] where small amounts of gene product produced are sufficient to stimulate an immune response. It is worth noting that microinjection using glass micropipettes to deliver genetic material into living cells is the most commonly used method for creating transgenic animals [69].

Gene Gun: Another physical approach is the use of a gene gun, also called ballistic DNA transfer or DNA-coated particle bombardment [70]. A gene gun is mostly applied in gene transfer to exposed tissue like skin and mucosa or to tissues surgically exposed like liver and muscle [71].

DNA is deposited on the surface of spheres made from gold or tungsten, and these DNA-coated particles are propelled against tissues and cells by the aid of accelerating forces such as pressurized inert gas (helium) or high-voltage electronic discharge. Particles penetrate a few millimeters deep into the tissue and release DNA into cells on the path. Due to limited gene expression achieved, gene gun–based gene transfer has been used in vaccination [72, 73] and immune therapy [74].

Electroporation: Electroporation is based on the fact that the application of a pulsed electrical field into living cells enhances the permeability of cell membranes by creating transient pores across the membrane, resulting in cellular uptake of DNA. Electroporation has been introduced as an efficient method of gene transfer for in vitro experiments in 1982 [75] and later became a versatile method extensively used for in vivo gene transfer to skin, muscle, and other tissues [76]. The efficiency of gene transfer is a function of voltage, pulse duration, and number of cycles from two electrodes applied. Electroporation has gained increased attention for applications in DNA vaccination [77] and is currently involved in clinical trials for treatment of various cancers and infectious diseases [78]. In vivo application of electroporation is challenged by its limited effective range of ~1 cm between the electrodes, and the need for surgery to place the electrodes deep into the internal organs [79].

Sonoporation: Application of an ultrasound to cells for gene delivery (sonoporation) was reported initially in 1990 for plant cell transfection [80]. Mechanistically, ultrasound waves applied to cells create plasma membrane defects by cavitation-induced bilayer disordering [81]. Sonoporation applied in gene transfer experiments was reported to significantly enhance transfection efficiency of plasmid DNA both in vitro and in vivo [82]. The presence of gas-filled

microbubbles has been suggested to enhance gene transfer efficiency upon ultrasound exposure [83].

Photoporation: Photoporation is a laser-assisted method of gene delivery resembling that of electroporation and sonoporation mechanistically. Laser pulses serve as a physical force to create transient pores in cell membranes, allowing DNA to enter [84]. This method has demonstrated a significant potential for gene transfer [85] and has been successfully used in in vitro gene transfer to human hepatocarcinoma cells [86]. Additional efforts are needed, however, for further optimization of various parameters involved.

Magnetofection: Magnetofection uses magnetic nanoparticles made of iron oxide with or without coupling with nonviral [87] or viral vectors [88] to enhance gene transfer into target cells or tissues in the presence of an external magnetic field [89]. Magnetofection has been successfully applied to gene transfer studies in vitro and in vivo and has successfully delivered small interfering RNA to tumors in a mouse model [90]. However, there is an increased safety concern regarding the fate of iron oxide in the cell, especially when multi-dosing is needed. It is worth noting that success in magnetofection-mediated gene transfer requires endocytosis. Magnetofection can be considered as a modified procedure for viral and chemical methods of gene delivery with an advantage of trapping gene carriers at the tissue where the magnetic field is applied.

Hydrodynamic Gene Delivery: Hydrodynamic gene delivery was developed in 1999 as a simple and effective method of gene transfer [91, 92] using a tail vein injection of plasmid DNA. DNA transfer is mediated by a rapid injection of a large volume of DNA solution into the tail vein, resulting in subsequent structural defects of vascular endothelia (fenestrations) and cell

membranes of nearby hepatocytes [93]. The method was recently approved as an efficient cell delivery technique for the establishment of a metastatic tumor model in mice [94]. Since its development, hydrodynamic delivery has gone through several improvements, bridging the achieved success in animal models to real application in the clinic. A computer-controlled injection device has been developed for hydrodynamic gene delivery in large animals [95,96] with great success in gene transfer to pig livers, kidneys, and muscles.

Remaining Challenges in Gene Delivery

In practice, success of gene therapy is largely dependent on the amount of protein expressed toward the desired therapeutic outcome, which in turn depends on the quantity of therapeutic gene that is successfully delivered to target cells. Each method developed thus far has advantages and disadvantages and faces a series of challenges that limit its applications in research and clinical applications. Therefore, the development of a delivery system that effectively and safely delivers therapeutic genes into target cells is in an urgent need. Future efforts need to focus on the following aspects depending on the method of delivery.

Viral Gene Delivery

Viral vectors have the advantage of achieving highly efficient gene transfer in vivo. Although replication-deficient vectors are used, viral vectors still pose significant safety concerns. Induction of an immune response is the main obstacle associated with viral vectors, and it greatly limits the application of viral vectors in successful human gene therapy. Ad is the most potent immunogenic vector among all viral vectors. Ad induces multiple components of the immune response. Cytotoxic T-lymphocyte responses can be induced against viral proteins,

transgene products that are expressed by transduced cells, and/or against the viral capsid itself. Humoral virus-neutralizing antibody responses and potent cytokine-mediated systemic inflammatory responses are also induced against Ad vectors and, to a lesser degree, other viral vectors [44, 97]. Integration into a host genome is the hallmark for long-term expression obtained with retroviral vectors. Random integration of retroviral vectors, however, can result in inactivation of tumor suppressor genes or activation of oncogenes, both of which are associated with tumorogenesis. Indeed, incidences of T-cell leukemia were reported after retroviral gene therapy for SCID [98, 99], and the uncontrolled proliferation of T cells were attributed to expression deregulation of (LMO2) oncogene as a consequence of retroviral integration [100, 101].

Nonviral Gene Delivery

For gene delivery with nonviral carriers, DNA complexes with cationic lipids and polymers significantly protect DNA against nucleases. The colloidal stability of these lipoplexes and polyplexes in extracellular environments, however, is a major problem to be solved. Aggregation of these complexes is frequently observed with most systems involving complexes prepared near charge neutrality [102, 103]. The (+/–) charge ratio of the cationic polymer/liposomes to DNA greatly affects the size and structural geometry of the complexes, and larger size aggregates have been observed with lower charge ratios. Excess positive charge further increases the colloidal stability of complexes in serum as a result of interactions with negatively charged serum proteins that compete for pDNA binding to cationic complex [104]. Introduction of these systems into biological compartments is accompanied by an increased ionic strength of the media that significantly affects the physical stability and increases the tendency

for aggregation of these complexes [97]. Increased ionic strength decreases electrostatic interactions between polycation and DNA while shielding inter-particulate electrostatic repulsive forces, resulting in aggregation of complexes. Physical stability of these systems was improved by shielding the particle surface with hydrophilic, uncharged polymers such as polyethylene glycol (PEG). Surface PEG coating sterically hinders aggregation and the interaction and binding of serum components with the complex surface [105, 106]. It has been reported that cationic polymers with high charge densities are most resistant to polyanion-mediated particle disintegration. Indeed, serum polyanion-mediated instability of lipoplexes was significantly improved by incorporation of helper lipids like 1,2-dioleoylphosphatidyl-ethanolamine and cholesterol [107]. Furthermore, these helper lipids stabilize complexes against interactions and fusion with erythrocytes in case of intravenous administration [108}.

An additional challenge in nonviral carrier-mediated gene delivery is associated with the interaction between the DNA complexes and blood components that trigger their clearance from blood and uptake by the reticuloendothelial system (**Figure 2-3**). Binding of plasma proteins (opsonization) is the major mechanism for the reticuloendothelial system in recognizing circulating particulate substances and consequently clear them from the circulation [109]. Macrophages, such as the Kupffer cells in the liver and histiocytes in spleen and lymph nodes, recognize the opsonized nanoparticles via the scavenger receptor. Escape from the reticuloendothelial system is currently achieved by coating particulate systems with PEG to counteract opsonization of these systems by blood proteins and minimize their macrophage recognition and uptake [110, 111].

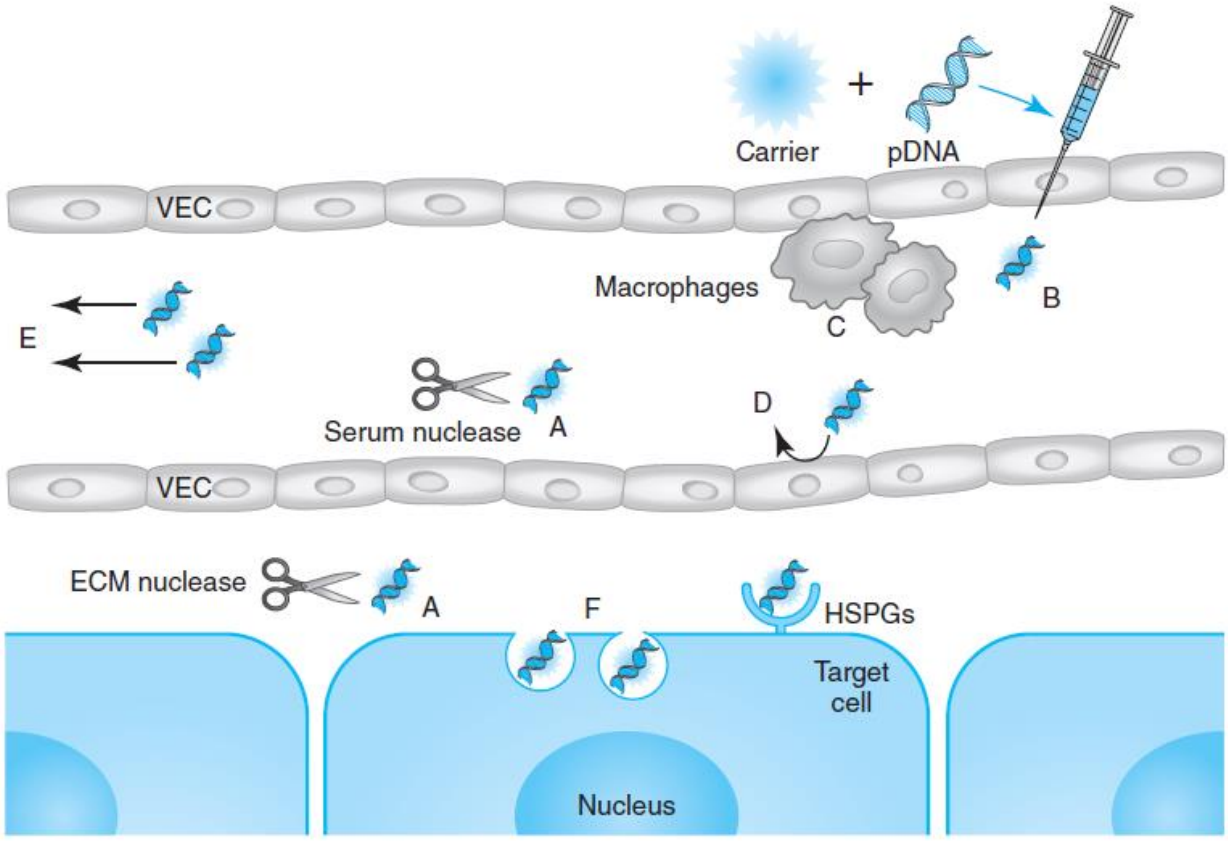

Figure 2-3: Extracellular barriers of gene transfer. DNA is delivered as naked plasmid or in complex with carrier system. Transfer of DNA is challenged with serum and extracellular matrix (ECM) nucleases (a), physical stability of the DNA complex in the biological compartment (b), reticuloendothelial uptake by macrophages and organs of reticuloendothelial system (c), extravasation across vascular endothelial cells (VEC) (d), distribution and nonspecific targeting (e), and interactions with cell surface and subsequent internalization (f).

Extravasation of gene-carrying particles through capillary walls is controlled by several biological factors, such as regional differences in capillary structure and disease state of the tissue as well as physicochemical properties of macromolecules like size, shape, and permeability through the vascular walls they encounter. The transfer of DNA complexes through capillary walls into target tissues after systemic administration is another challenge for efficient gene transfer, because it limits deposition of DNA complexes in target tissues and greatly affects the bioavailability and distribution of these complexes in the body. Blood capillaries in most tissues have continuous endothelium acting as a barrier for macromolecules, and those materials of up to 6.0 nm in diameter can extravasate. On the other hand, liver and tissue of the reticuloendothelial system have fenestrated and discontinuous endothelium with fenestrations of up to150 nm, and particles of 100 nm in diameter can pass through [112]. Tumor endothelium has a leaky and discontinuous vasculature structure, allowing easier extravasation in the tumor region, an effect known as enhanced permeability and retention [113]. Direct tissue injection of DNA complexes can overcome the extravasation barrier by direct deposition of complexes in target tissue [114].

Targeted gene delivery is a most desirable method for gene therapy. It seeks to concentrate therapeutic gene delivery systems in the tissues of interest while reducing the relative concentration of these systems in the remaining tissue, aiming to improve efficacy while reducing side effects. Systemically administered gene carriers are distributed throughout the body through the systemic blood circulation, and a small portion of these systems reaches the desired tissue. Biodistribution of DNA complexes is a function of morphological properties of complexes like size, shape, and charge. Therapeutic effects can be specified for target tissue through two strategies: targeted gene delivery, where gene delivery system is preferentially

deposited in target tissues and taken up by target cells, and targeted gene transcription, where gene transcription takes place only in target cells despite gene delivery to several tissues and cell types [115]. Strategies to achieve tissue-specific gene delivery include conjugation of DNA complexes with targeting ligands of receptors overexpressed on target cells, like conjugation of DNA lipoplexes with folate to specifically targeting cells overexpressing folate receptors, or using synthetically modified complexes capable of releasing DNA content under specific stimuli providing means of spatial and temporal release control [116]. **Table 2-2** summarizes some of these strategies. Targeted gene transcription is achieved using synthetic promoters that are activated by signaling factors abundant in target cells, like specific targeting of colon cancers using promoters activated by β-catenin and T-cell factor; both are highly abundant in colon cancer cells [117].

At the cellular level, internalization of therapeutic genes is one of the most critical steps for successful gene delivery. For chemical carrier-mediated gene delivery, DNA complexes are taken up by cells through endocytosis (**Figure 2-4**). The fate of internalized materials is either to be returned to the cell surface, like in the case of recycling endosomes, or proceed via late endosomes with a progressive increase of acidity to lysosomes to be degraded in enzymatic-degrading environment [118]. In both cases, failure of the DNA complex to escape from the endosome results in a significant reduction in intracellular bioavailability of DNA, which greatly affects the efficiency of therapeutic gene delivery and subsequent gene expression. Therefore, efficient endosomal escape is critical for efficient gene delivery and successful gene therapy.

DNA lipoplexes possess enhanced endosomal escape and DNA release as a result of lipid mixing between endosomal and cationic lipid membranes, which results in membrane

disruption of endosome and DNA release into cytoplasm [119]. The escape and release of DNA complexed with cationic polymers are mediated by interactions of cationic polymers with negatively charged lipids of the endosomal membrane to form neutral ion pairs that destabilize and promote disassembly of the endosomal membrane and consequent release of DNA [120]. Polymers with ionizable amine groups, like polyethyleneimine, have enhanced endosomal escape and DNA release. Protonation of these amine groups in acidic environments within the endosome is associated with the extensive inflow of protons, ions, and water into the endosomal lumen that leads to the rupture of the endosomal membrane and release of the entrapped components; this process is known as the proton sponge effect [62].

Destabilization of the endosomal membrane was also achieved by stimuli-responsive lipids or polymer derivatives that are responsive to sulfhydryl reduction [121] and enzymatic cleavage [122]. Endosomal enzymes and/or reducing environment activate these derivatives to fuse with the endosomal membrane and, finally, release the entrapped cargo. Lysosomotropic agents that accumulate preferentially in the lysosomes of cells, like chloroquine and polyvinylpyrolidone, are commonly used in conjunction with plasmid vectors, viral vectors, and in combination with polymeric systems to improve the efficiency of gene delivery. It is believed that chloroquine inhibits endosomal DNA degradation by preferential accumulation and protonation in lysosomes and subsequent ions and water influx and raising pH within lysosomes, thereby providing a suboptimal pH environment for enzymatic degradation of DNA [123]. In addition, being large in molecular weight and negatively charged, DNA was reported to have minimal association with the cell membrane as a result of electrostatic repulsion with negatively charged components that cover the cell surface, namely heparin sulfate proteoglycans (HSPGs) that possess negative charges [97]. Neutralization of the negative charge of DNA by means of

Table 2-2: Strategies Currently Employed to Achieve Target Specific Gene Delivery

Strategy		Principle	Example	Ref.
Conjugation with targeting ligand		Complex binds to target cells expressing receptors for the ligand	EGF to target cancer cells overexpressing EGFR	[1]
Coating with Ab against cancer specific antigens		Complex recognizes target cells by antibody binding to antigens on target cell surface	Antibody against tumor cell surface nucleosomes	[2]
Stimuli- responsive DNA complexes	pH-sensitive complexes	Acid-sensitive complex that disassembles at certain pH values of tissue environment	Tumor specific gene delivery using pH-sensitive polymer	[3]
	Thermo-sensitive complexes	Enhanced aggregation and endosomal escape of DNA complexes and DNA release	Enhanced gene transfer after heat application	[4]
	Redox-sensitive complexes	Change in complex architecture under different redox potentials with controlled DNA release	Enhanced DNA release in tumor-relevant redox environment	[5]

EGF, epidermal growth factor; EGFR, epidermal growth factor receptor.

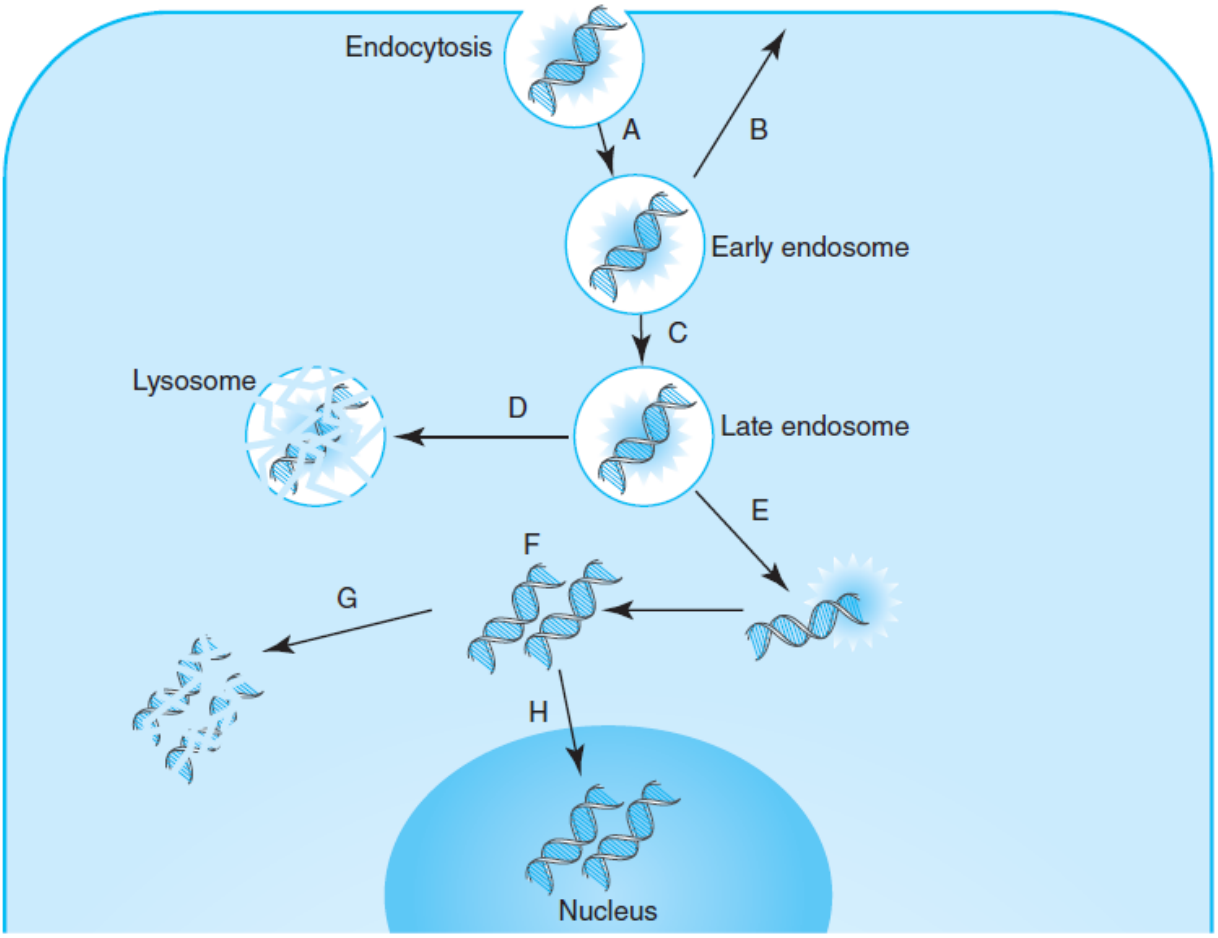

Figure 2-4 Intracellular trafficking of DNA. After internalizing the DNA complex via endocytosis, the complex is trafficked into an early endosome (a), which may be recycled to the cell surface (b), or proceed to late endosome (c); this results in either trafficking of the complex to the lysosome for degradation (d) where DNA degradation takes place, or escape of the DNA with or without the carrier (e) into the cytoplasm. DNA became free in cytoplasm (f), which might be degraded by the cytoplasmic nuclease (g). Free DNA is then transported into the nucleus (h).

complexation with cationic lipids and polymers enhances cellular association and internalization of DNA by increasing interactions between positively charged DNA complexes with HSPGs. Therefore, efficiency of gene transfection is greatly dependent on the expression level of HSPGs on the cell surface, and reduced expression of these HSPGs was associated with reduced levels of transgene expression [124]. Increased DNA complex binding with HSPGs helps induce internalization of DNA complexes by different processes of endocytosis like adsorptive endocytosis [125], clathrin-mediated endocytosis [126], phagocytosis [127], and, rarely, micropinocytosis [128].

Efficient delivery of DNA from the cytosol to the nucleus, where the transcription takes place, is another area that needs additional work. The DNA complex dissociation seems to occur in the endosomal compartment to release DNA into the cytosol [129]. Therefore, after endosomal escape, DNA should make its way through the cytosol to reach the nucleus. The cytoplasm is crowded with organelles, solutes, soluble macromolecules, and a network of skeletal proteins that maintain cell structure like microtubules and actin filaments, and this crowding significantly hinders diffusion of macromolecules through the cytoplasmic compartment [130, 131]. Diffusion of noncomplexed DNA in the cytoplasm is significantly lower than in water, with a diffusion coefficient <1% of that in water for DNA >2000 bp [132]. Smaller plasmids are preferred for higher transfection efficiency, because the diffusion coefficient inversely correlates to the size of the plasmid.

Metabolic instability of DNA in the cytoplasm is another challenge in gene delivery [133]. Cytosolic calcium-sensitive nucleases rapidly degrade microinjected DNA, with a half-life of 50 to 90 minutes [134, 135]. Encapsulation of microinjected plasmids into stabilized lipid

particles delays the degradation of DNA with enhanced efficiency [134]. The nuclear envelope is the ultimate barrier to the nuclear entry of plasmid DNA. Evidence of inefficient nuclear uptake of plasmid DNA from the cytoplasm was further demonstrated when compared with the transfection efficiency of microinjected plasmid DNA either into the cytosol or the nucleus [136]. It has been suggested that possible routes through which DNA can translocate to the nucleus include passive (or active) transport through nuclear pores, physical association with chromatin during mitosis, or traverse nuclear envelope; the last seems less likely as it lacks experimental evidence [97].

Nuclear pores embedded in the nuclear envelope allow passage of particles <26 nm [137] but do not allow typical nonviral gene delivery complexes. In dividing cells, the nuclear envelope disassembles and breaks down during mitosis; nuclear translocation can occur at this stage after the permeability barrier is eliminated. In nondividing cells, developing mechanisms of DNA nuclear transport is of critical importance. Nuclear translocation of DNA was improved by attachment with nuclear localization signal peptides (or sequences), which tags cargo for import into the cell nucleus by nuclear transport. Nuclear localization signal peptides bind to cytoplasmic receptors known as importins; together, the complex moves through the nuclear pore. The most well-known and popularly used nuclear localization signal is from the large tumor antigen of simian virus 40; plasmids cloned with the simian virus 40 enhancer region showed significant nuclear localization. Some DNA sequences themselves have nuclear import activity based on their ability to bind to cell-specific transcription factors, such as the SMGA promoter and flk-1 promoter [138].

Physical Gene Delivery

Physical methods were designed to overcome barriers of gene transfer by means of physical forces that create defects in cell membranes and essentially enforce cellular uptake of DNA. Physical methods of gene transfer are generally well tolerated. Results reported on cell or tissue toxicity are inconsistent. Targeted cell type, type of physical force used, and physical parameters of the procedure (pulse strength and frequency, acoustic pressure, exposure duration, etc.) are the key determinants of the degree of cytotoxicity, and local tissue damage may be associated with this procedure [139]. Physical methods have some practical issues that limit their applicability in gene therapy trials, along with relatively lower in vivo efficiency compared with viral and nonviral methods. Microinjection, for example, is challenged by its fastidious technique that requires a skilled person to do injections one by one to individual cells. The gene gun uses a special device that is quickly and easily operated; however, in vivo efficiency is limited by the shallow penetration of particles into biological tissue. Electroporation-mediated gene transfer, on the other hand, is limited to the area where the electrodes were positioned. These issues are also encountered by other methods of gene transfer. Other challenges associated with most physical methods include the requirement of special hardware to run the procedure. This hardware adds an extra challenge regarding the technical aspects of these devices. Improving the efficiency of physical methods for gene transfer, while minimizing the collateral damage to the tissue being treated, represents the major focus for future development.

Summary

The potential and promise for gene therapy in treating diseases have grown exponentially, paralleling advances in molecular biology that offer powerful tools to address the very basis of disease pathogenesis. One can think of genes that may ultimately be used as other

small molecule drugs. When given as simple intravenous injections, gene transfer vehicles seek out target cells for stable and regulated gene expression. Delivery of transgenes in target cells is currently achieved using three different strategies: viral vectors, nonviral vectors, and physical methods. So far, viral vectors remain the most prevalent in human clinical trials despite the increased safety concerns associated with their in vivo applications. Synthetic or nonviral vectors, on the other hand, offer safer, though less efficient, alternatives to viral vectors. Physical methods use physical forces to enforce cellular uptake of DNA. With the exception of hydrodynamic gene transfer, in vivo efficiency of physical methods has not been fully demonstrated thus far. Current research is devoted to the engineering of more efficient, safer, and targeted systems for gene delivery and is aimed at overcoming challenges associated with current methods of gene delivery. What diseases will ultimately be curable by gene therapy and which strategies will be developed and successfully applied remain to be seen. At this point, with the progression of science, it seems safe to predict that gene therapy and DNA-based therapeutics will account for a large part of our next generation tools to fight against human diseases, prominently cancers and genetic disorders.

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CHAPTER 3

DIFFERENTIAL GROWTH AND RESPONSIVENESS TO CANCER THERAPY OF TUMOR CELLS IN DIFFERENT ENVIRONMENTS[†]

[†] Mohammad Alsaggar, Qian Yao, Houjian Cai and Dexi Liu (2015) Differential growth and responsiveness to cancer therapy of tumor cells in different environments. *Clinical and Experimental Metastasis*. 2016 Feb; 33(2):115-24.
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Abstract

Tumor metastasis often confers poor prognosis for cancer patients due to lack of comprehensive strategy in dealing with cells growing in different environment. Current anticancer therapies have incomplete effectiveness because they were designed assuming metastatic tumors behave similarly in different organs. We hypothesize that tumors growing in different sites are biologically heterogeneous in growth potential, as well as in tumor response to anti-cancer therapies. To test this hypothesis, we have developed a multi-organ tumor growth model using the hydrodynamic cell delivery method to establish simultaneous and quantifiable tumor growth in the liver, lungs and kidneys of mice. We demonstrated that growth rate of melanoma tumor in the liver is higher than that of the lungs and kidneys. Tumors in the lungs and kidneys grew minimally at the early stage and aggressively thereafter. Tumors in different organs were also heterogeneous in response to chemotherapy and immune gene therapy using dacarbazine and interferon beta gene, respectively. Lung tumors responded to chemotherapy better than tumors in the liver, but showed minimal response to interferon beta gene therapy, compared to tumors in the liver and kidneys. We also confirmed differential tumor growth of the metastatic colon cancer in mice. Our results point out the importance of a better understanding of the differences in tumor growing in diverse environments. The biological heterogeneity of metastatic tumors demonstrated in this study necessitates establishing new drug screening strategies that take into account the environmental difference at the sites of tumor growth.

Introduction

While an increasing number of human cancers have become treatable, especially with early detection, the presence of tumor metastasis often confers poor prognosis because successful treatment of metastatic tumors remains as an unmet need in clinic [1]. Current preclinical evaluation of anticancer therapies is generally focused on subcutaneous or orthotopic tumor models, and incompletely addresses the differential behavior of metastatic tumors in different environments. In addition, the lack of a full understanding of biological heterogeneity of metastatic tumors, particularly tumor-stroma interactions within the tumor microenvironment, limits our ability to predict the therapeutic outcomes of a given anticancer therapies [2,3]. Therefore, appropriate tumor models are urgently needed for reliable anticancer drug screening and development, and for the assessment of tumor-environment interactions of the metastasized tumors.

Hydrodynamics-based delivery was initially established for gene transfer into mouse hepatocytes via tail vein [4,5]. This method involves a rapid injection of a large volume of DNA solution into the tail vein to generate high intravascular pressure within the inferior vena cava (IVC), causing back flow into the blood vessels with connection to IVC, and consequently, increasing the permeability of vascular endothelium, resulting in the influx of DNA into parenchymal cells in the liver, and to a lesser extent, in the kidneys [6]. Hydrodynamic gene delivery is presently among the most efficient non-viral methods for gene transfer, and it is increasingly applied for gene therapy, gene drug discovery, and animal model establishment [7].

Recently, the method was utilized for successful delivery and subsequent growth of tumor cells into mouse liver, lungs, and kidneys simultaneously [8].

In this study, a systematic approach was employed to achieve a quantitative assessment of the differential behavior of tumors growing in different organs. We aimed to investigate whether tumors grow and respond differently to anticancer therapies when seeded into different organs. We show that tumors grow differently in different organs, despite originating from the same cell population. We further demonstrate that tumors are also heterogeneous in response to selected anticancer therapies. Our results have significant clinical implications since most current anticancer approaches do not consider the heterogeneity of tumor behavior in different organs, leading to profound failure to treat cancer once metastasis has developed. In addition, our data suggest that more complex regimens are needed to treat metastatic tumors residing in different organs.

Materials and Methods

Materials. The pLIVE plasmid vector was purchased from Mirus Bio (Madison, WI). Mouse IFNβ1 gene was sub-cloned into pLIVE plasmid using complementary DNA sequences. DNA sequencing was used to confirm the sequence of the constructed plasmid. Plasmid was prepared using the method of cesium chloride-ethidium bromide gradient centrifugation, and kept in saline at −80 °C until use. The purity of the plasmid preparation was examined by absorbency at 260 and 280 nm and 1% agarose gel electrophoresis. Dacarbazine (DTIC) (purity ≥ 98%), and 5-fluorouracil (5-FU) (purity ≥99%), were purchased from Sigma-Aldrich (St. Louis, MO).

Cells. B16-F1 (murine melanoma) and C26 (murine colon carcinoma) cells were obtained from ATCC. B16-F1 cells were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin, and C26 cells were cultured in RPMI with 10% FBS and 1% penicillin/streptomycin. Luciferase-tagged cells were created using Lenti viral vectors containing luciferase reporter gene. At 80% confluence, the medium was removed, and each plate was treated with 4 ml of Trypsin/EDTA solution (0.25% Trypsin, 2.21 mM EDTA) at room temperature for 4 min. Cells were washed twice with serum-free medium and filtered through a membrane filter (40 μm pore size) and centrifuged at 1,400 rpm for 5 min at room temperature. Cell concentration was determined using a hemocytometer and diluted with serum-free medium to the desirable concentration. Standard calibration curves were established for B16F1 and C26 cells correlating tumor cell number and luciferase activity.

Mice and treatment. Female Balb/c (6–8 weeks old, 18–22 g) mice were purchased from Charles River Laboratories and housed in a pathogen-free environment in the Animal Facility of the University of Georgia. The animal procedures used were approved by the Institutional Animal Care and Use Committee of the University of Georgia (protocol #: A2011 07-Y2-A3). For growth quantification, 12 mice were used and three at each time point. For treatment studies, animals were divided into control and treatment groups (5 mice each). Tumor cell suspension and plasmid solution were injected hydrodynamically via tail vein. Chemotherapy with DTIC or 5-FU was administered intra-peritoneally with vehicle as control. Tumor sensitivity to chemotherapy and immunotherapy was quantified using the equation:

$$\text{“}Sensitivity = (\#cells_C - \#cells_T)/\#cells_C\text{”}$$

where $cells_C$ and $cells_T$ refer to tumor cell number in control and treatment groups.

Hydrodynamic injection. The procedure of hydrodynamic delivery has been previously reported for gene [4,5] and cell delivery [8]. Briefly, for hydrodynamic cell delivery, a volume equivalent to 8% body weight of cell suspension in serum-free medium was injected into the tail vein over 5–8 sec. For conventional cell injection, the same number of cells was injected into tail vein in a volume of 200 μl over 10 sec. For gene delivery, saline solution of plasmid DNA was injected via tail vein following the procedure of hydrodynamic gene delivery [4].

H&E staining. Tissue samples were fixed in 10% neutrally buffered formalin and dehydrated using increasing ratios of ethanol/water (v/v). Tissue samples were embedded into paraffin for 16 hrs. Paraffin-embedded tissue samples were cut into sections at 6 μm in thickness and dried at 37 ℃ for 1 hr before incubation in xylene, followed by a standard H&E staining using a commercial kit (BBC Biochemical, Atlanta, GA).

Analysis of luciferase activity. After animal euthanasia, tissue samples from the selected organs were collected and immediately frozen in liquid nitrogen and kept at −80 °C until use. For the luciferase assay, 1 ml of lysis buffer was added to each sample (~150 mg) and kept on ice. The thawed tissue was homogenized using a tissue homogenizer (1 min, max speed). The tissue homogenate was centrifuged in a microcentrifuge (10 min, 10,000 rpm at 4 ℃), and the supernatant was collected. Ten μl of supernatant was taken for luciferase and protein assay according to the previously established procedure [4].

In vitro assessment of IFNβ1 activity. Animals were hydrodynamically injected with 20 μg of pLIVE-IFNβ1 plasmid (empty plasmid as control). Animals were euthanized 24 hr later, and serum samples were obtained, mixed with saline at different dilutions and added to cultured

B16F1 cells with regular media. The cells were treated for 0 -72 hours and stained with crystal violet (0.5% w/v) as previously described [9].

Analysis of gene expression. Total mRNA was isolated from collected tissues using TRIZOL reagent purchased from Invitrogen (Carlsbad, CA). One μg of total RNA was used for first strand cDNA synthesis using a Superscript RT III enzyme kit from Invitrogen (Carlsbad, CA). Quantitative real-time PCR (qPCR) was performed using SYBR Green as the detection reagent on the ABI StepOnePlus Real-Time PCR system. The data were analyzed using the ΔΔCt method [10] and normalized to internal control of GAPDH mRNA. Primers employed were synthesized in Sigma (St. Louis, MO) and their sequences are summarized in **Table 3.1**.

Statistics. All results are expressed as means ± SD, and statistical significance was determined using student t-test and analysis of variance. A value of $P < 0.05$ was considered significant difference.

Results

Establishment of tumor growth

To establish tumor growth in multi organs, luciferase-tagged B16F1 cells (1×10^6) in serum-free medium were hydrodynamically injected into a mouse via the tail vein. Tissue distribution of injected tumor cells was assessed by measuring luciferase activity in different organs six hr after cell injection using cell type specific standard curves (**Fig. 3.1**). The results showed that hydrodynamic injection efficiently delivered cells into the liver, the lungs and the kidneys, but not into the other organs (**Fig. 3.2A**). Among these three organs, liver was the primary recipient organ, receiving approximately one third of the injected cells. The lungs

Table 3.1: Primer sequences for qPCR experiments

Gene	**Forward primer**	**Reverse primer**
IFNβ1	CAGCTCCAAGAAAGGACGAAC	GGCAGTGTAACTCTTCTGCAT
Mx1	GACCATAGGGGTCTTGACCAA	AGACTTGCTCTTTCTGAAAAGCC
Granzymb	AACCAGCCACATAGCACACAT	GCCCACAACATCAAAGAACAG
Perforin	AGCACAAGTTCGTGCCAGG	GCGTCTCTCATTAGGGAGTTTT
IFNγ	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
GADPH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

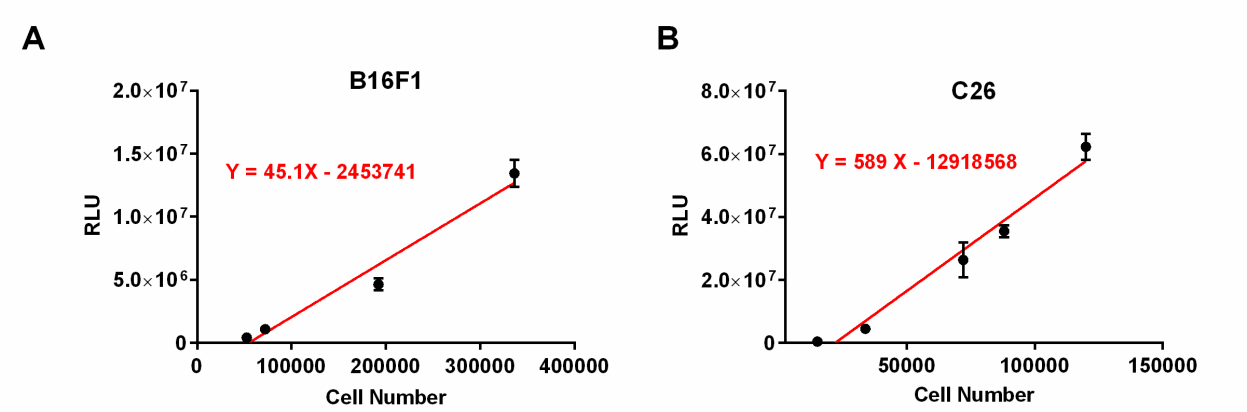

Fig. 3.1: Standard calibration curves correlating cell numbers and luciferase activity (RLU). B16F1 cells (**A**) and C26 cells (**B**) were seeded in 24-well plates at different serial dilutions (1.2 X 10^5 maximum), 4 wells for each cell number. After 24 hrs, one well was used to count cells, and the other wells were used for luciferase activity measurement by adding 200 µl cell lysis buffer, incubate for 5 min, cell lysate centrifuged (10 min at 10000 rpm), and luciferase activity measured. Fitting line and the equation correlating RLU to cell number are shown in red. (Experiment was done in triplicate).

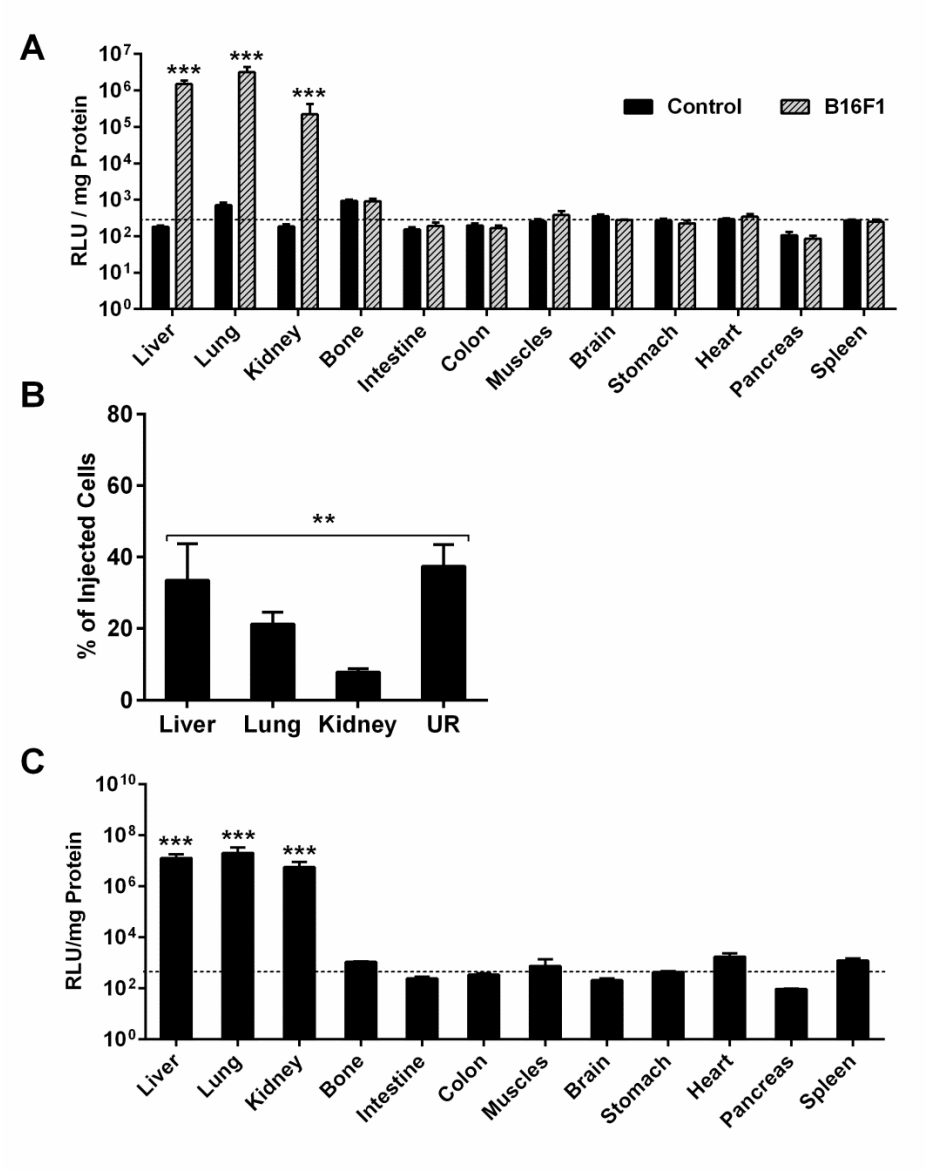

Fig. 3.2: Distribution of hydrodynamically injected tumor cells in the liver, lungs, and kidneys. 1x10^6 cells/mouse (serum-free medium as control) were injected via tail vein by hydrodynamic injection. Animals were euthanized six hr after injection, and tissues were collected and analyzed for luciferase activity. (**A**) Tissue distribution of injected tumor cells. Dotted line represents background level. (*** *P* <0.001, calculated by student t-test) (**B**) Proportion of cells distributed in the liver, lungs, and kidneys. UR: Unrecovered fraction. (** *P* <0.01, calculated by ANOVA test). (**C**) Relative luciferase activity in different tissues collected from animals received 1x10^6 cells/mouse hydrodynamically via tail vein, 12 days after tumor cell injection. Dotted line represents background level. (*** *P* <0.001, student t-test). (n= 5, experiment was repeated for reproducibility).

and kidneys received 21% and 8% of injected cells, respectively (**Fig. 3.2B**). Up to 62% of injected cells were recovered, as calculated by luciferase activity, indicating that approximately 38% of injected cells died, adopted dormancy/senescence phenotype, or both during or after cell delivery into the mice. Hydrodynamic injection of the tumor cell suspension resulted in tumor growth in the liver, the lungs, and the kidneys (**Fig. 3.3A**). In contrast, conventional tail vein injection of the same number of cells in a volume of 200 μL resulted in tumor growth solely in the lungs (**Fig. 3.3B)**. Tumors were macroscopically visible on the surfaces of the three organs. Tumor growth was also confirmed by H&E staining of tissue sections (**Figs. 3.3C, 3.3D**). Assessment of luciferase activity after 12 days of tumor cell injection revealed that tumor growth remained confined to these three organs (**Fig. 3.2C**).

Differential tumor cell survival and growth in different organs

Given the key roles of organ stroma to provide the supporting niche for the initial survival of the tumor cells [11], we examined whether different organs support tumor growth differently by measuring luciferase activity in target organs one day after hydrodynamic cell delivery. The results showed that tumor cells survived differently in the targeted organs (**Fig. 3.4A**), suggesting that different organs have different environments for tumor survival. We also assessed tumor growth rate in different organs using the standard curve and the measured luciferase activity at days 1, 6, and 12 after tumor cell injection to quantify tumor growth. The results in **Figs. 3.4B** and **3.4C** show that the liver is most receptive to melanoma growth. The tumor grew steadily from day one and continued to grow throughout the course of experiment. However, tumors in lungs and kidneys grew very minimally initially, but aggressively thereafter.

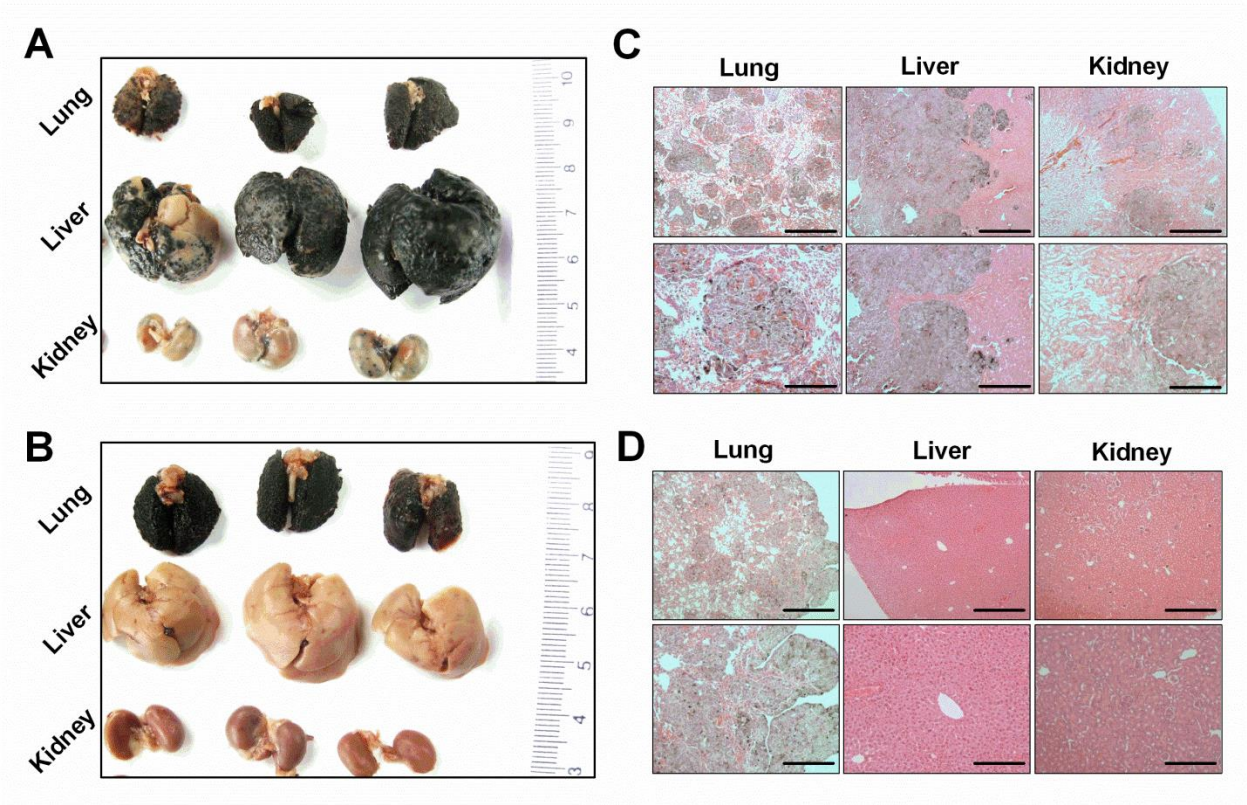

Fig. 3.3: Establishment of multi-organ growth of B16F1 cells in mice. $1x10^6$ cells/mouse were hydrodynamically injected via tail vein (injection volume, 8% of body weight, injection time: 8 sec), or using conventional method (injection volume: 100 µl, injection time, 10 sec). Animals were sacrificed 14 days after the injection. (**A)** Photo images of organs from hydrodynamically injected animal. (**B**) Photo images of organs from animals received conventional injection. (**C**) and (**D**) Photo images of H&E staining of tissue sections from animals received hydrodynamic or conventional injections, respectively. Bars represent 50 µm (upper panel) and 10 µm (lower panel). (n=5, experiment was repeated for reproducibility).

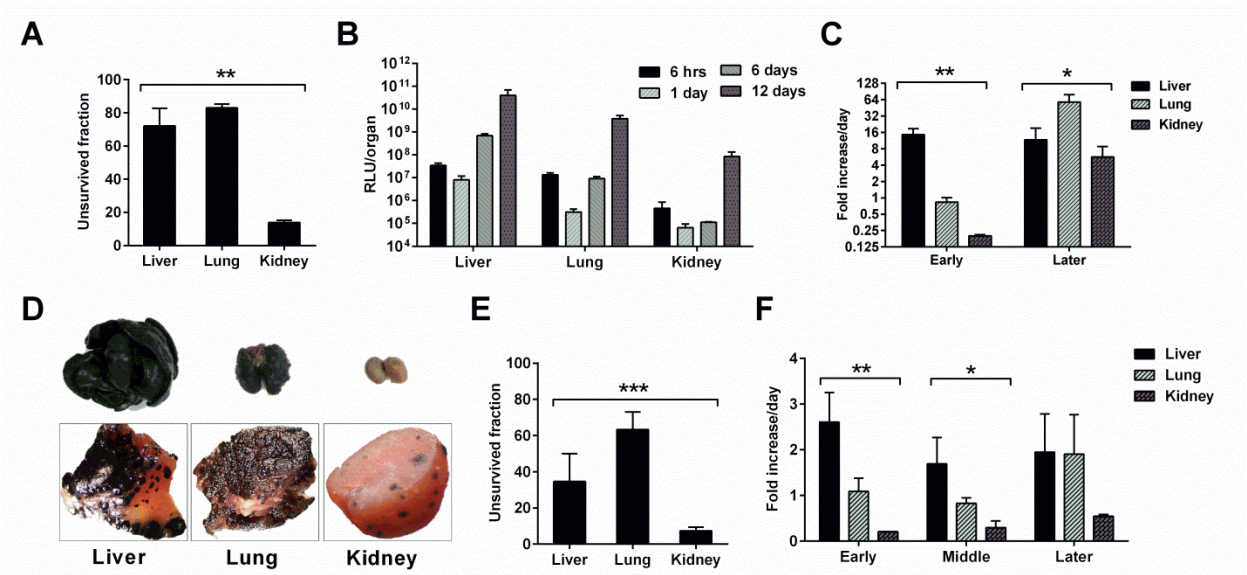

Fig. 3.4: Differential survival and growth rates of tumor cells in different organs. Luciferase-tagged B16F1 cells ($2x10^6$) were hydrodynamically injected into mice. Animals were euthanized at different time points, and organs were collected and analyzed for luciferase activity. (**A**) Fractions of reduced luciferase activity from the injected cells one day after injection in different organs. (**B**) Luciferase activity per organ at different time points. (**C**) Fold increase in cell number per day at early (day 1 to day 6) and late (day 6 to day 12) phases. (**D**) Upper, macroscopic tumor visible on organ surfaces. Lower panel, photo images of organ surfaces at 20X. (**E**) Differential survival of C26 cells in different organs one day after hydrodynamic cell injection ($1x10^6$). (**F**) Fold increase in C26 tumor cell number per day at early (day 1 to day 6), middle (day 6 to day 12) and later (day 12 to day 18) phase. (* P <0.05, ** P <0.01, ANOVA test). (n=12).

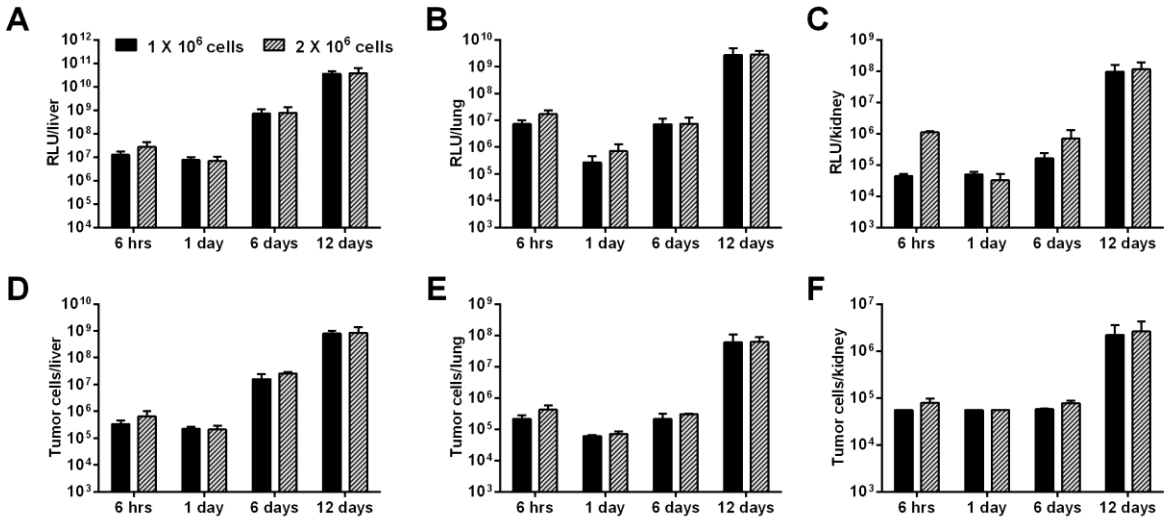

Fig. 3.5: Differential growth of B16F1 melanoma cells in the liver, lungs, and kidneys. Two independent experiments were performed with 1 x 10^6 or 2 x 10^6 cells/mouse to demonstrate similar trend of tumor growth in different organs. (**A**), (**B**), (**C**) Luciferase activities at different time points after tumor cell injection in the liver, lungs, and kidneys, respectively. (**D**), (**E**), (**F**) Tumor cell number per organ at different time points as calculated using the standard calibration curve. (n=12).

By day 12, melanoma tumors had grown massively, and macroscopic tumors were visible on the surface of the examined organs (**Fig. 3.4D**). Tumor growth was confirmed with additional experiments using different number of cells (1 x 10^6, 2 X 10^6 cells/mouse) (**Fig. 3.5**). Differential growth of tumor cells in different organs was also confirmed with luciferase-tagged C26 murine colon carcinoma cells. Similar to B16F1 tumors, C26 cells showed distinct survival when seeded into different organs (**Fig. 3.4E**). In addition, C26 cells grew in different rates in different organs (**Fig. 3.4F**). These results point out the heterogeneity of supportive environmental inputs among different organs, resulting in differential survival and growth potential of tumor cells in these organs.

Differential response of tumors in different organs to anticancer therapy

Beyond the pivotal role that tumor environment plays in tumor growth, it has profound effects on therapeutic efficacy. Therefore, we looked at the sensitivity of tumors in different organs to anticancer therapies by assessing the response of melanoma tumors to chemotherapy and immunotherapy using DTIC and interferon beta (IFNβ1) gene therapy, respectively. Animals were injected hydrodynamically with 10^6 cells, and received 50 mg/kg DTIC intraperitoneally. Similar to distinctive growth, melanoma tumors in different organs showed differential sensitivity to DTIC treatment, as quantified by luciferase activity (**Fig. 3.6A**). While significant antitumor activity was seen in the lungs and the kidneys, tumors in the liver responded modestly to DTIC in comparison to other organs (**Fig. 3.6B**). Differential response of metastatic tumors was also verified with the gene therapy approach. Animals were injected hydrodynamically with 10^6 cells, and three days later, received hydrodynamic injection of 10 μg plasmid expressing mouse IFNβ1 gene. The control animals received empty plasmid. Hepatic mRNA levels of *IFNβ1* gene was checked three days after gene transfer and was more than 30-fold higher in

treated animals, suggesting efficient gene transfer into mouse hepatocytes (**Fig. 3.6C**). IFNβ1 signaling was induced in the three organs, as evidenced by the induction of expression of Mx1 gene (**Fig. 3.6D**), the biomarker of IFNβ1 activity [12]. In contrast to chemotherapy, tumors in the liver and kidneys were the most responsive to IFNβ1 gene transfer, while tumors in the lungs were practically resistant to IFNβ1 (**Figs. 3.6E**, **3.6F)**. Tumor load reduction was obvious judging by the reduction of the number of nodules visible from organ surfaces (**Fig. 3.6G**), consistent with H&E staining of tissue samples from different groups (**Fig. 3.6H**). We also examined the response of colon cancer tumors in different organs to 5-FU and IFNβ1 gene therapy. Upon treatment, luciferase activity and tumor load were decreased in all organs (**Fig. 3.7**). However, tumor suppression effect varied between organs (**Figs. 3.6I, 3.6J**), albeit to a lesser extent than melanoma tumors. Therapeutic outcomes of 5-FU and IFNβ1 on C26 tumors were also visible on organ surfaces (**Fig. 3.6K**). These results suggest that in addition to differential growth rates, tumors in different organs are also heterogeneous in response to anticancer therapies. Since genetically identical tumor cells were seeded into different organs, the observed differential growth and sensitivity of tumor cells are largely explained by distinct environmental clues tumors receive in different organs.

We sought to confirm the differential efficacy of IFNβ1 gene therapy on melanoma tumors in different organs using various doses IFNβ1-expressing plasmid DNA. The therapeutic effect of IFNβ1 on tumor growth in the liver was dose dependent (**Fig. 3.8A**). In contrast, the tumor load was similar in the lungs of treated and control animals, regardless of the amount of DNA injected (**Fig. 3.8B**), suggesting that tumor cells are minimally responsive to IFNβ1 in spite of dose increase. Visual assessment on the kidneys could not be made because the tumor load was too low (**Fig. 3.8C**). Histochemistry was used to verify tumor growth and the results showed

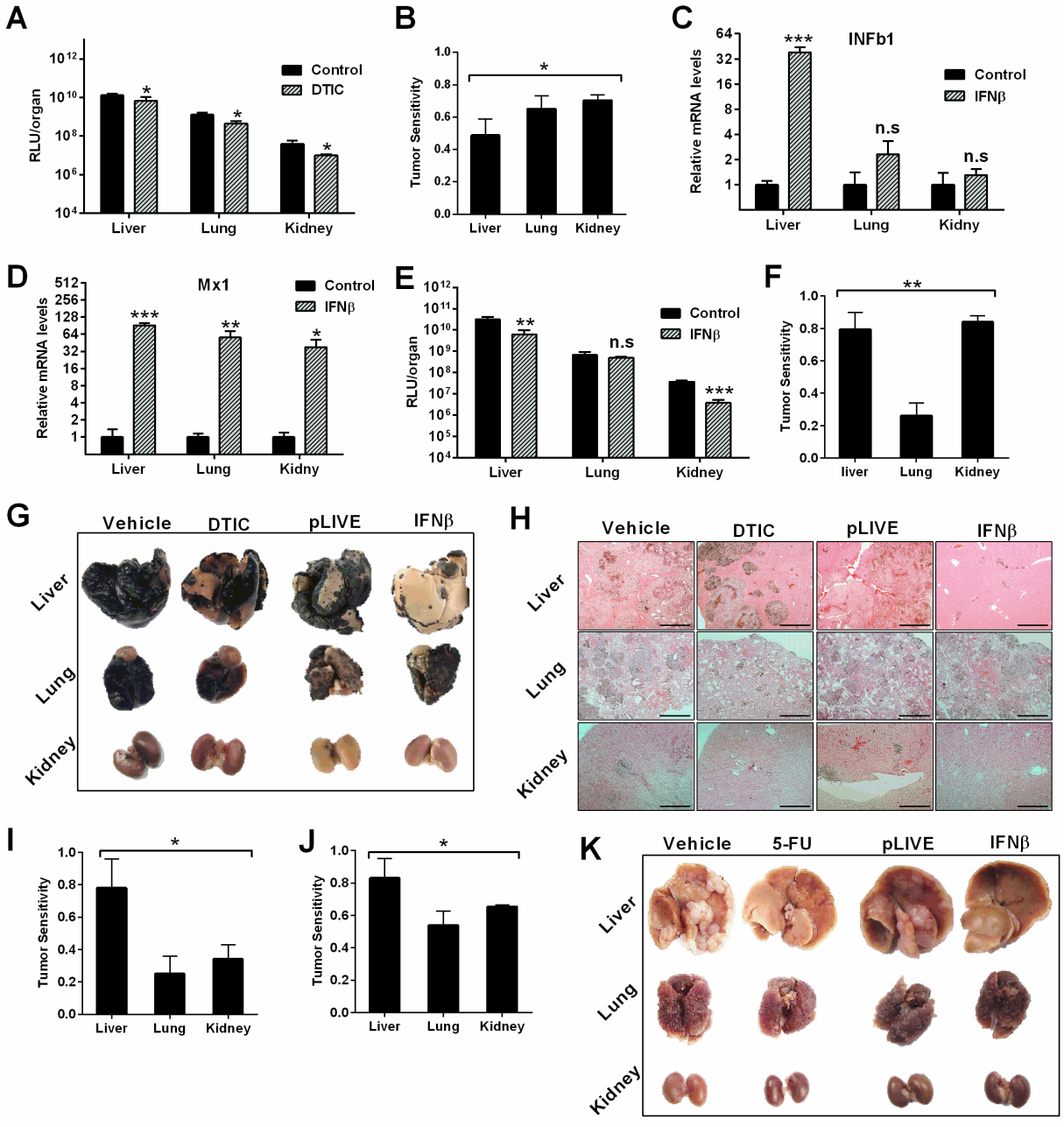
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Fig. 3.6: Differential response of tumors in different organs to anticancer therapies. B16F1 cells (10^6 cells/mouse) were hydrodynamically injected via tail vein. Three days after the injection, animals received 5 intraperitoneal injections of 50 mg/kg DTIC (vehicle as control), or hydrodynamic transfer of 10 μg IFNβ1 plasmid (empty plasmid as control). Animals were euthanized on day 12. (**A**) Luciferase activity per organ with or without DTIC treatment. (**B**) Quantified B16F1 tumor sensitivity to DTIC in each organ. (**C**) and (**D**) Relative mRNA levels of *IFNβ1* and *Mx1* genes in each organ after hydrodynamic transfer of IFNβ1 plasmid, compared with control group. (**E**) Luciferase activity per organ with or without IFNβ1 treatment. (**F**) Quantified B16F1 tumor sensitivity to IFNβ1 gene transfer in each organ. (**G**) Images of the organs with B16F1 tumor, with or without treatment. (**H**) H&E staining of tissue sections from liver, lungs, and kidneys in all groups. Bars represent 50 μm. (**I**) Quantified C26 tumor sensitivity to 5-FU treatment in each organ. C26 cells (10^6 cells/mouse) were hydrodynamically injected via tail vein. Five days after the injection, animals received 5 intraperitoneal injections of 20 mg/kg 5-FU (vehicle as control), or hydrodynamic transfer of 10 μg IFNβ1 plasmid (empty plasmid as control). Animals were euthanized at day 15. (**J**) C26 tumor sensitivity to IFNβ1 in each organ. (**K**) Images of the organs with C26 tumors, with or without treatment. (* P <0.05, ** P <0.01, *** P <0.001, calculated by student t-test and ANOVA test). (n=5).

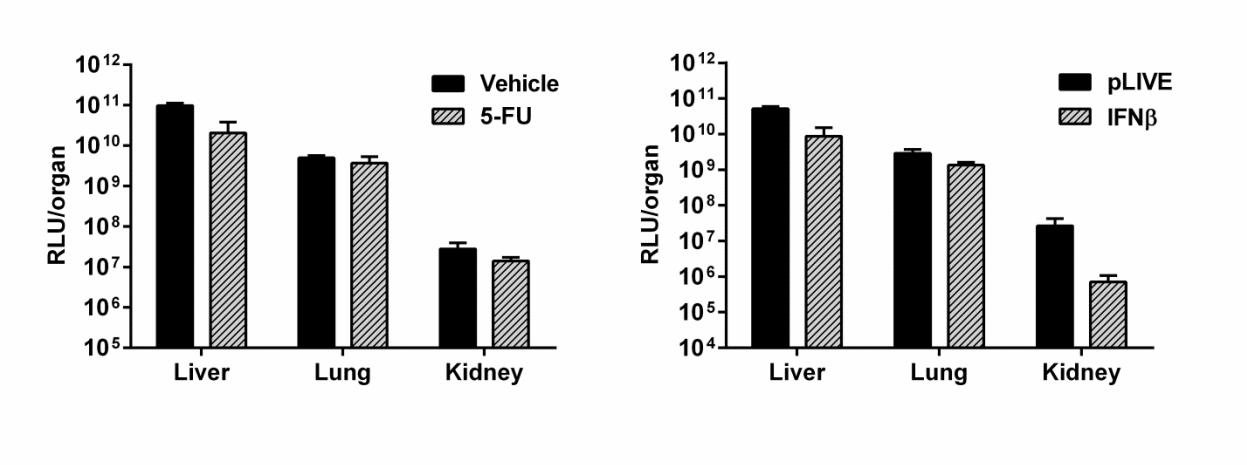

Fig. 3.7: Differential response of C26 tumors to anticancer therapies when grown in different organs. C26 cells (10^6 cells/mouse) were hydrodynamically injected via tail vein. Animals received 5 intraperitoneal injections of 20 mg/kg 5-FU (vehicle as control), or hydrodynamic transfer of 10 μg IFNβ1 plasmid (empty plasmid as control) on day 5. Animals were euthanized on day 15. (**A**) Luciferase activity per organ with or without 5-FU treatment. (**B**) Luciferase activity per organ with or without IFNβ1 gene transfer. (n=5).

significant effect of tumors in the liver with 5 μg of plasmid DNA, but not in the lung (**Fig. 3.8D**). Microscopic tumors were observed in kidneys of the control mice and animals injected with low dose of plasmid DNA at 0.5 μg per mouse (arrows in **Fig. 3.8D**). To exclude the possibility that the differential response to IFNβ1 gene therapy is due to the lack of access of therapeutic protein into the lungs, B16F1 cells were treated *in vitro* with diluted sera of animals received hydrodynamic injection of IFNβ1 expressing plasmid DNA (**Fig. 3.8E**). Dose-dependent inhibition of melanoma cell growth was observed similar to *in vivo* experiment, suggesting that IFNβ1 was not confined to the liver, but instead, had access to the other extra-hepatic tissues, through blood circulation.

Besides direct anti-proliferative activity, type 1 interferons like IFNβ1 exert antitumor immune response primarily through natural killer cell-mediated production of perforin and granzyme b, and immune-activating cytokines. Therefore, we looked at the expression levels of these target genes in tumor-loaded organs upon IFNβ1 gene transfer. Results showed that the treatment markedly induced the expression of granzyme b (**Fig. 3.8F**) and perforin (**Fig. 3.8G**) in the three organs. However, the level of induction in the lungs was lower than that of the liver and kidneys, in parallel with the treatment efficacy in these organs. IFNγ expression was also induced in the three organs upon treatment with IFNβ1 (**Fig. 3.8H**), but to a lesser degree than the effector cytolytic molecules. Together, these results suggest that the environment in the liver is highly conducive to IFNβ1 activity against tumor growth. Similarly, minimal efficacy against lung tumors indicates that the environment in the lungs is not favorable for IFNβ1-mediated tumor suppression, since effector molecules were not sufficiently induced, indicating an environment of immune suppression, in spite of IFNγ induction.

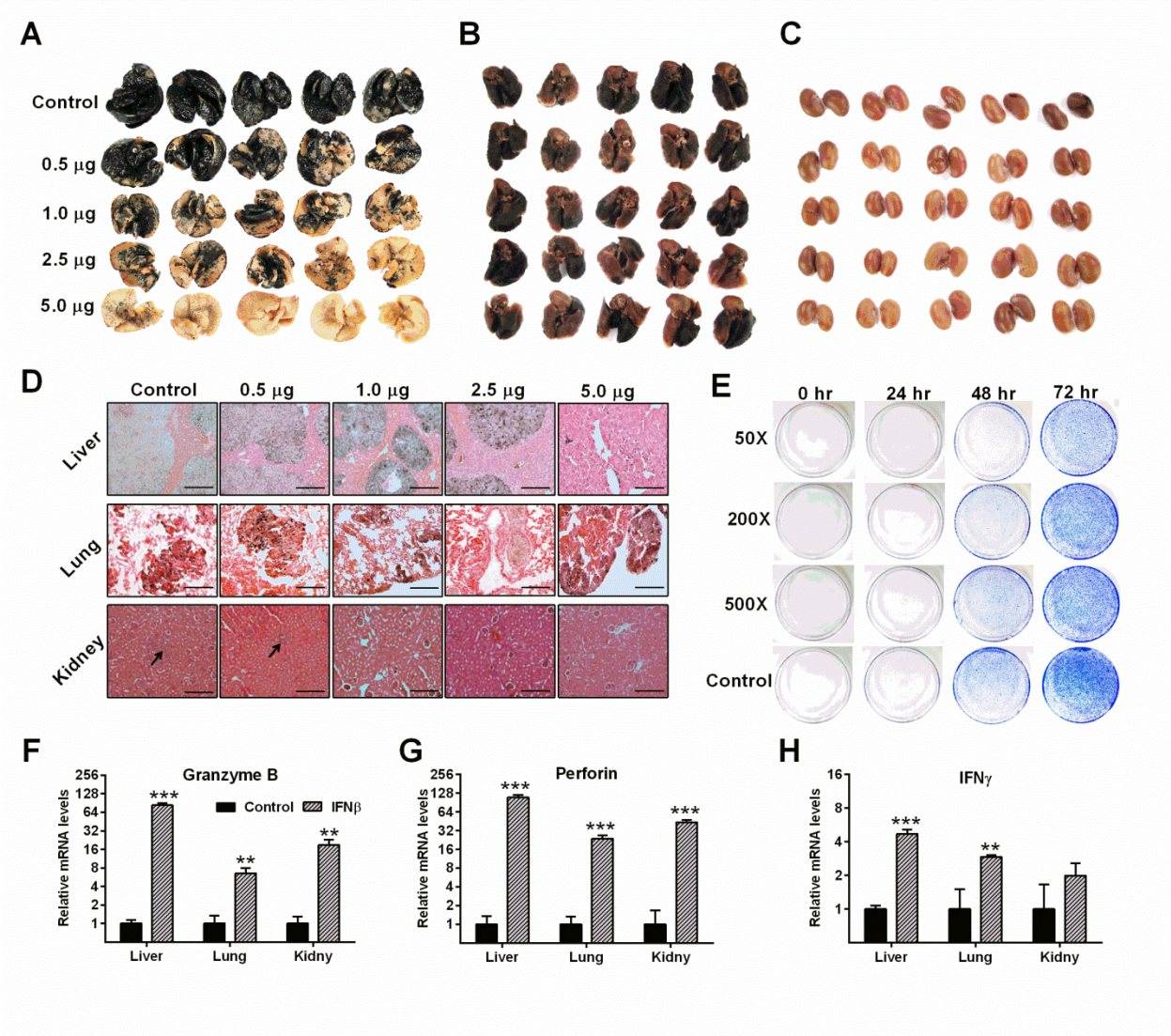

Fig. 3.8: Liver, but not lung environment, is in favor for IFNβ1 antitumor activity. B16F1 cells (10^6 cells/mouse) were injected hydrodynamically via tail. On day 3, animals received hydrodynamic transfer of various doses of pLIVE-IFNβ1 plasmid (0.5, 1.0, 2.5, and 5 μg DNA/mouse), or empty plasmid as control. Animals were sacrificed 9 days after tumor cell injection. (**A**) Tumor load in the livers of animals injected with same number of tumor cells but different amount of pLIVE-IFNβ1 plasmid DNA. (**B**). Tumor load in the lungs of same groups of mice. (**C**). Nonvisible tumor load in kidneys of the same groups of animals. (**D**) H&E staining of tissue sections from the liver, lungs, and kidneys of animals received increasing doses of IFN-β1 plasmid. Arrows in kidney sections show microscopic tumor growth. Bars represent 50 μm. (**E**) Effect of IFNβ1-containing serum on B16F1 cell growth *in vitro*. **(F), (G), (H)** Relative mRNA levels of *Granzyme B, Perforin,* and *IFNγ* genes in each organ after hydrodynamic transfer of IFNβ1plasmid, compared to control group. (** *P* <0.01, *** *P* <0.001, calculated by t-test) (n=5).

Discussion

Modeling tumor metastasis for reliable assessment of anticancer therapies has been a major challenge for the development of efficient anticancer therapies. In this study, we took advantage of the hydrodynamic delivery method to establish a multi-organ tumor growth in mice. We demonstrated that tail vein injection of tumor cell suspension in a volume of 8% of body weight over 5-8 seconds results in simultaneous delivery, and subsequent growth of tumor cells in the liver, lungs, and kidneys. Extravasation of tumor cells upon conventional injection is often inefficient due to limited vascular permeability [13]. However, upon hydrodynamic injection, the permeability of vasculature in the liver and kidneys is dramatically increased due to retrograde dynamic pressure [8], resulting in extravasation of tumor cells into the milieu of these organs. This mechanism has been recently verified using fluoroscopic imaging of injected phase contrast medium to visualize the back flow of injected phase contrast medium from the inferior vena cava to the liver and kidneys in real time [14]. Upon restoring normal circulation, cells remaining in blood vessels will move through the heart and then to the lungs, where cells are trapped and filtered out in the lung vasculature [15] to establish tumor growth. This would explain the confined cell delivery and tumor growth in the liver, lungs, and kidneys, but not in the other organs. Given the liver plasticity and the fenestrated vasculature, the liver is the primary organ impacted by hydrodynamic procedure [6]. In contrast, the lack of intra-vascular pressure in conventional injection resulted in entrapment and sole growth of tumor cells in the lungs because cells had no access to the other organs due to embolic effect of the tumor cells passing through the lung endothelium with blood flow. Similar to other transplantable tumor models involving direct injection of tumor cells into organ parenchyma, our model differs from the process of natural tumor metastasis, and only recapitulates the last step in metastatic cascade,

i.e. the colonization into secondary organs. Hydrodynamic cell injection is advantageous over other orthotopic injections in being more convenient and non-invasive. In addition, tumor cell distribution and subsequent tumor growth is fully reproducible using hydrodynamic injection. We believe that this procedure is convenient and appropriate for examining tumor cell survival and growth in different organs, exploring tumor-stroma interaction, and assessing therapeutic activity of anticancer regimens.

Tumor metastasis is a highly inefficient process because, among millions of disseminated tumor cells, very few cells successfully engraft, survive and proliferate to form macro-metastatic tumors at distant sites [15,16]. It is well recognized that efficient tumor metastasis and growth competency are not random, rather, there is an emerging pattern of organotropism, *i.e.* organ specificity [17]. Although the blood flow pattern contributes to organ specific metastasis, the propensity of tumor cells to metastasize to, and grow in specific organs is largely controlled by local homing mechanisms that involve coordinated interactions between tumor cells and stromal components of the organ environment. Consistent with these theories, we have demonstrated that different organs have different supportive potential for initial tumor survival and subsequent growth upon hydrodynamic delivery. In spite of initial significant cell death in the liver, melanoma cells recovered and proliferated aggressively to form macroscopic tumors as early as six days after injection. This would suggest a supportive environment for tumor growth in the liver, which can be attributed to the abundance of growth factors and active growth signaling, along with high vasculature and nutrients availability, making it the second most common target for metastasized tumor cells [18]. Despite tumor growth in the liver, there was a trend of decreased growth rates with time, largely due to increased tumor size. Recently, it has been hypothesized that the reduction in growth rate with increased tumor size is due to systemic

inhibition of angiogenesis [19]. In contrast, tumor cells in the kidneys and lungs had minimal growth in the first six days, *i.e.* no apparent increase in tumor load in these organs. The lung is a very common metastatic site, largely because it is the first capillary bed encountered by circulating tumor cells after passing the vena cava and heart. However, mounting evidence suggests that not all physically entrapped cells will successfully establish tumor growth in the lungs because of the need for cell adhesion and vascular remodeling molecules to mediate extravasation and interaction with lung stroma [20,21]. In current study, we show that in spite of a significant number of tumor cells delivered into the lungs, approximately only 20% of the cells survived and initiated tumor growth. Significant cell death is broadly due to the failure to extravasate, and consequent vulnerability to the shear stress of blood flow, immune checkup and anoikis (lack of adhesion apoptosis) [22]. However, tumor cells may acquire a protective shield through pulmonary tumor embolism by co-opting blood platelets, using them as shields to protect from shear stress, as well as immune cells [2], permitting survival and adaptation of tumor cells to establish macroscopic tumors at later stages. Overt metastatic melanoma in the kidneys, on the other hand, is clinically uncommon. However, microscopic metastases of melanoma have been detected in up to 50% of patients [23]. This suggests that the local environment in the kidneys is not favorable for tumor growth and explain the infrequency of metastasis of most human cancers in the kidneys. In this study, we demonstrated that initial melanoma growth in the kidneys is inefficient, and a significant increase in the cell number was seen only at later stage of tumor progression. Given the minimal initial tumor cell death in the kidneys, it would indicate that the apparent lack of increased tumor cells is likely attributed to the adoption of dormancy phenotype, rather than an active proliferative status that is counter-balanced by active apoptotic events. Indeed, it has been reported that melanoma cells remained

solitary with modest aggressiveness, even long after treatment [23,24]. The marked increase in growth at later stage may suggest remodeling and the activation of local stroma in the kidneys and lungs, boosting tumor cell growth. Together, these finding conclude that tumor cell behavior varies in different organs, and thus, treatment regimens targeting cell proliferation machinery should accordingly be modified. Notably, differential growth behavior is not cell line-specific, because this trend was also shown with colon carcinoma cells, in which tumors grew at various rates, with more aggressive growth in liver; the primary target organ for colon cancer metastasis.

The environmental factors that dictate the tumor growth profile in different organs are also critical determinants of tumor response to anticancer therapy. Given the heterogeneity of environmental components among different organs, the outcomes of a given anticancer treatment will vary among different organs. In agreement, we brought to light two cases of differential tumor response to DTIC chemotherapy and INFβ1 gene therapy, when grown in different organs. IFNβ1 gene therapy approach was chosen based on the pleiotropic activities of interferons regulating direct antitumor activities, such as apoptosis induction, and regulating cell immune responses, allowing depiction of cell-intrinsic, and environment-mediated antitumor response. In addition, gene therapy approach will provide sustained levels of IFNβ1 in mice to maximize therapeutic outcomes, given the very short half-life of IFNβ1 protein. The differential efficacy profile among different organs was more prominent with IFNβ1 treatment, rather than DTIC. While the efficacy of IFNβ1 was limited to tumors in the liver and kidneys, tumors in all organs responded to DTIC, albeit to different degrees. The discrepancy between the two therapies is largely attributed to their mode of actions. DTIC works directly on tumor cells as an alkylating agent [25], and therefore, reflects the intrinsic resistance of tumor cells. Since tumors in all organs evolved from a genetically identical cell population, it is expected to see less variation in

response to therapy. On the other hand, IFNβ1 treatment reflects both intrinsic and acquired mechanisms of resistance because it mediates antitumor effects through direct cytotoxicity on melanoma cells [26], activation of natural killer cells and cytotoxic T cells [27], and antiangiogenic effects [28]. Thus, the heterogeneity of IFNβ1 efficacy among different organs is attributed, at least in part, to differences in local immune cell infiltration and activation, and/or the extent of dependence on active angiogenesis in these organs. The higher expression levels of perforin and granzyme B in the liver and kidneys may suggest a superior induction of antitumor immune response in these organs in comparison to the lungs. In addition, pulmonary embolization with tumor cells mentioned earlier may further contribute to the resistance of lung tumors because it protects tumor cells from cytotoxic drugs and effector immune cells. Consistent results were obtained in the dose-response study, which further supports our idea of heterogeneous response to IFNβ1 among different organs. These results indicate that the environment in the lungs is generally immunosuppressive and in favor of tumor survival, whereas liver and kidneys are better candidates to be considered for immunotherapy for melanoma. Therefore, alternative and more potent regimens are needed to overcome immunosuppressive events and to eradicate tumors in lungs.

In summary, we demonstrated in this study that the hydrodynamic cell delivery is efficient for reliable establishment of multi-organ tumor growth in mice. This model provides a convenient tool for examination of tumor growth, tumor-stroma interactions in different anatomical locations, and for therapeutic screening of anticancer regimens. The observed diverse tumor growth and sensitivity to anticancer therapies in different organs highlights the impacts of the tissue environment on tumor cell behavior, and the need for a serious consideration of environmental factors for proper design of anticancer regimens. Functional dissection of the

tumor microenvironment may reveal an organ-specific signature of stromal components that dictate distinct cell behavior in different organs. Such profiling will certainly help predict progression and sensitivity of tumors in different organs to selected anticancer therapies. To date, targeting single pathway with monotherapy has been insufficient in spite of initial response, and tumor resistance is often developed with subsequent tumor relapse and regrowth. Therefore, combined therapies tackling multiple targets and pathways potentially maximize the efficacy against tumors in different organs. Paralleling the progress in "personalized" medicine for cancer patients, we now have the tools for the development of therapeutic strategies for the treatment of tumors residing in different organs and for maximal therapeutic outcomes.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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CHAPTER 4

INTERFERON BETA OVEREXPRESSION ATTENTUATES ADIPOSE TISSUE INFLMMATION AND HIGH FAT DIET-INDUCED OBESITY AND MAINTAINS GLUCOSE HOMEOSTASIS [‡]

[‡] Mohammad Alsaggar, Michael Mills and Dexi Liu (2016) Interferon beta overexpression attenuates adipose tissue inflammation and high fat diet-induced obesity and maintains glucose homeostasis. *Gene Therapy*. Submitted

Abstract

The worldwide prevalence of obesity is increasing, raising health concerns regarding obesity-related complications. Chronic inflammation has been characterized as a major contributor to the development of obesity and obesity-associated metabolic disorders. The purpose of the current study is to assess whether interferon beta (IFNβ1), an immune-modulating cytokine, will attenuate high fat diet-induced adipose inflammation and protect animals against obesity development. Using hydrodynamic gene transfer to elevate and sustain blood concentration of IFNβ1 in mice fed a high fat diet, we showed that overexpression of *Ifnβ1* gene markedly suppressed immune cell infiltration into adipose tissue, and attenuated production of pro-inflammatory cytokines. Systemically, IFNβ1 blocked adipose tissue expansion and body weight gain, independent of food intake. Increased energy expenditure in adipose tissue further contributed to blockade of weight gain. More importantly, IFNβ1 improved insulin sensitivity and glucose homeostasis. These results suggest that targeting inflammation represents a practical strategy to block the development of obesity and its related pathologies. In addition, IFNβ1-based therapies have promising potential for clinical applications for the treatment of various inflammation-driven pathologies.

Introduction

The increasing prevalence of obesity represents a global health concern, largely due to its related co-morbidities. Obesity is closely associated to many metabolic disorders, including cardiovascular diseases, type-2 diabetes, and fatty liver; and to various neoplasms such as colon carcinoma[1]. Increasing number of studies suggests that low grade chronic inflammation is a driving force for obesity-related pathologies.[2] It has been shown that inflammation in adipose tissue is accompanied by enhanced immune cell infiltration, particularly macrophages, due to prolonged nutrient overload.[3] Induced inflammation appears essential for adipose tissue remodeling and modulation of adipocyte functions, and responsible for development of insulin resistance, inhibition of adiponectin secretion, and alteration of local and systemic cytokine profiles. As such, more research is being focused on further investigating inflammation's contribution to obesity pathogenesis, and developing strategies to target inflammation for treatment of the related diseases.

Interferon beta (IFNβ1) is a cytokine with pleiotropic activities, including antiviral and antitumor activities, as well as immunomodulatory effects.[4] Recombinant IFNβ1 is used in clinic for treatment of multiple sclerosis,[5] owing to its disease-modifying anti-inflammatory properties. Mechanistically, IFNβ1 effects are attributed to increasing production of anti-inflammatory cytokines such as IL-10, decreasing production of inflammatory mediators, such as IL-17, osteopontin and TNFα, and impairment of inflammatory cell migration across BBB.[6] Therefore,

IFNβ1 is increasingly being investigated for the therapeutic potential to treat inflammation-driven pathologies.

In this study, we explored the therapeutic potential of IFNβ1 to suppress adipose tissue inflammation and to block obesity development in mice. We showed that IFNβ1 overexpression attenuates obesity-induced adipose inflammation, while modulating adipose tissue hypertrophy. These effects were associated with blockade of weight gain, and restoration of glucose homeostasis. Together, our findings suggest that IFNβ1 has beneficial effects on lipid metabolism in an obesity model, and that IFNβ1 is a novel therapeutic target for prevention and treatment of obesity and insulin resistance.

Methods

Materials. The pLIVE plasmid vector was purchased from Mirus Bio (Madison, WI). Mouse *Ifnβ1* gene was sub-cloned into pLIVE plasmid using complementary DNA sequences. DNA sequencing was used to confirm the sequence of the constructed plasmid. Plasmid DNA was prepared using the method of cesium chloride-ethidium bromide gradient centrifugation, and kept in saline at −80 °C until use. The purity of the plasmid preparation was examined by absorbency at 260 and 280 nm and 1% agarose gel electrophoresis.

Mice and treatments. Male C57BL/6 mice purchased from Charles River Laboratories (Wilmington, MA) were housed under standard conditions with a 12-h light–dark cycle. All animal procedures used were approved by the Institutional Animal Care and Use Committee at the University of Georgia, Athens, Georgia (protocol number, A2011 07-Y2-A3). HFD (60% kJ/fat, 20% kJ/carbohydrate, 20% kJ/protein) used in this study was purchased from Bio-Serv

(Frenchtown, NJ). The procedure of hydrodynamic gene delivery has been previously reported.[7,8] Briefly, 10 μg of plasmid DNA expressing mouse *Ifnβ1* gene in saline solution with volume equal to 9% body weight was injected into a mouse tail vein over 5–8 s. Plasmid expressing mouse *Seap* (secreted alkaline phosphatase) gene was used as a control. Body weight and food intake were measured weekly, and body composition analysis was performed at the end of the experiment using EchoMRI-100 (Echo Medical Systems, Houston, TX).

Evaluation of glucose homeostasis. Intraperitoneal glucose tolerance test (IPGTT) was carried out in mice that fasted for 6 h. Glucose solubilized in phosphate-buffered saline was injected (*i.p.*) at 2 g/kg, and the time-point was set as 0 min. Blood glucose was measured at predetermined time-points (0, 30, 60, and 120 min) using glucose test strips and glucose meters. Intraperitoneal insulin tolerance test (ITT) was performed in mice that fasted for 4 h. Insulin (Humulin, 0.75 U/kg) purchased from Eli Lilly (Indianapolis, IN) was injected (*i.p.*), and blood glucose was measured at predetermined time-points identical to IPGTT. Blood insulin was measured using an ELISA kit (#10-1113-01) purchased from Mercodia Developing Diagnostics (Winston Salem, NC).

H&E staining. Tissue samples were collected, fixed in 10% neutrally buffered formalin and dehydrated using increasing ratios of ethanol/water (v/v). Tissue samples were embedded into paraffin for 16 h. Paraffin-embedded tissue samples were cut into sections at 6 μm in thickness and dried at 37 ℃ for 1 h before incubation in xylene, followed by a standard H&E staining using a commercial kit (BBC Biochemical, Atlanta, GA).

Oil-red O staining. Freshly collected liver samples were immediately frozen in liquid nitrogen. Tissue sections were cut at 8 μm in thickness using a Cryostat. Sections were placed on slides

and fixed using neutrally buffered formalin for 30 min. The sections were washed with 60% isopropanol before being stained with freshly prepared Oil-red O working solution (#26079-05, Electron Microscopy Sciences) and counterstained with haematoxylin.

Determination of liver triglyceride. Liver samples (200–300 mg) were homogenized in 1 ml of phosphate buffered saline, and protein concentration was determined. Total lipids in homogenate were extracted by addition of 5 ml of chloroform-methanol (2:1, vol/vol) mixture and incubated overnight at 4 ℃. The tissue homogenates were then centrifuged at 12,000 rpm for 20 min, and the supernatants were dried and the contents re-dissolved in 2% Triton X-100. Hepatic triglyceride level was determined by using a commercial kit from (Thermo-Scientific, PA).

Analysis of gene expression. Total mRNA was isolated from collected tissues using TRIZOL reagent purchased from Invitrogen (Carlsbad, CA). One μg of total RNA was used for first strand cDNA synthesis using a Superscript RT III enzyme kit from Invitrogen (Carlsbad, CA). Quantitative real-time PCR (qPCR) was performed using SYBR Green as the detection reagent on the ABI StepOnePlus Real-Time PCR system. The data were analyzed using the ΔΔCt method, and normalized to internal control of GAPDH mRNA. Primers employed were synthesized in Sigma (St. Louis, MO) and their sequences are summarized in **Supplementary Table 1**.

Statistics. All results are expressed as means ± SD, and statistical difference was determined using student t-test and analysis of variance. A value of $P < 0.05$ was considered significant difference.

Table 4.1: Primers sequences used in PCR experiments.

Gene	Forward primer	Reverse primer
Acc	ATGGGCGGAATGGTCTCTTTC	TGGGGACCTTGTCTTCATCAT
Adiponectin	TGTTCCTCTTAATCCTGCCCA	CCAACCTGCACAAGTTCCCTT
Cd11c	CTGGATAGCCTTTCTTCTGCTG	GCACACTGTGTCCGAACTCA
Cd36	ATGGGCTGTGATCGGAACTG	GTCTTCCCAATAAGCATGTCTCC
Cidea	TGACATTCATGGGATTGCAGAC	GGCCAGTTGTGATGACTAAGAC
Dio2	AATTATGCCTCGGAGAAGACCG	GGCAGTTGCCTAGTGAAAGGT
Elovl3	TTCTCACGCGGGTTAAAAATGG	GAGCAACAGATAGACGACCAC
F4/80	TGACTCACCTTGTGGTCCTAA	CTTCCCAGAATCCAGTCTTTCC
Fas	GGAGGTGGTG ATAGCCGGTAT	TGGGTAATCCATAGAGCCCAG
Gadph	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Ifnβ1	CAGCTCCAAGAAAGGACGAAC	GGCAGTGTAACTCTTCTGCAT
Il-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
Il-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
Il-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
Leptin	GAGACCCCTGTGTCGGTTC	CTGCGTGTGTGAAATGTCATTG
Mcp1	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
Mx1	GACCATAGGGGTCTTGACCAA	AGACTTGCTCTTTCTGAAAAGCC
Pgc1a	TATGGAGTGACATAGAGTGTGC	CCACTTCA ATCCACCCAGAAAG
Scd1	TTCTTGCGATACACTCTGGTGC	CGGGATTGAATGTTCTTGTCGT
Srebp1c	GCAGCCACCATCTA GCCTG	CAGCAGTGAGTCTGCCTTGAT
Tnf-a	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
Ucp1	AGGCTTCCAGTACCATTAGGT	CTGAGTGAGGCAAAGCTGATTT
Ucp2	ATGGTTGGTTTCAAGGCCACA	CGGTATCCAGAGGGAAAGTGAT
Ucp3	CTGCACCGCCAGATGAGTTT	ATCATGGCTTGAAATCGGACC

Results

Hydrodynamic injection resulted in efficient gene transfer in mice.

We first assessed the efficiency of hydrodynamic injection to transfer the *Ifnβ1* gene into mouse hepatocytes. Hydrodynamic injection of pLIVE-IFNβ1 plasmid resulted in a successful delivery, and subsequent expression of the *Ifnβ1* gene in the liver but not in white (WAT) and brown (BAT) adipose tissue (**Fig. 4.1A**). IFNβ1 signaling was induced and sustained in all three tissues for more than nine weeks post plasmid injection, evidenced by induction of *Mx1* gene (**Fig. 4.1B**). There was no change in the serum levels of the liver enzymes ALT and AST, suggesting that neither the hydrodynamic injection nor IFNβ1 activity causes liver damage (**Figs. 4.1C, 4.1D**). These results prove hydrodynamic injection is an efficient and safe method of gene delivery to the liver.

IFNβ1 attenuates HFD-induced adipose hypertrophy and inflammation.

HFD-induced obesity is often accompanied by WAT hypertrophy, fat accumulation, and induction of inflammation of adipose tissue, as evidenced by an increased expression of inflammatory cell marker genes such as *F4/80, Cd11c,* and *Mcp1*. IFNβ1 overexpression efficiently blocked hypertrophy and expansion of WAT tissues (**Figs. 4.2A, 4.2B**). Suppressed fat accumulation in adipose tissue, upon treatment, was demonstrated with H&E examination of WAT and BAT tissue sections (**Fig. 4.2C**). More importantly, IFNβ1 decreased adipose expression of inflammatory cell marker genes (**Fig. 4.2D**), suggesting attenuated trafficking of inflammatory cells into adipose tissue. In parallel, IFNβ1 reversed cytokine profiles toward anti-inflammatory phenotype by down regulating common pro-inflammatory signals *Tnf-α, Il-1β* and *Il-6* (**Fig. 4.2E**), and upregulating the anti-inflammatory cytokine *Il-10* (**Fig. 4.2F**). Given the

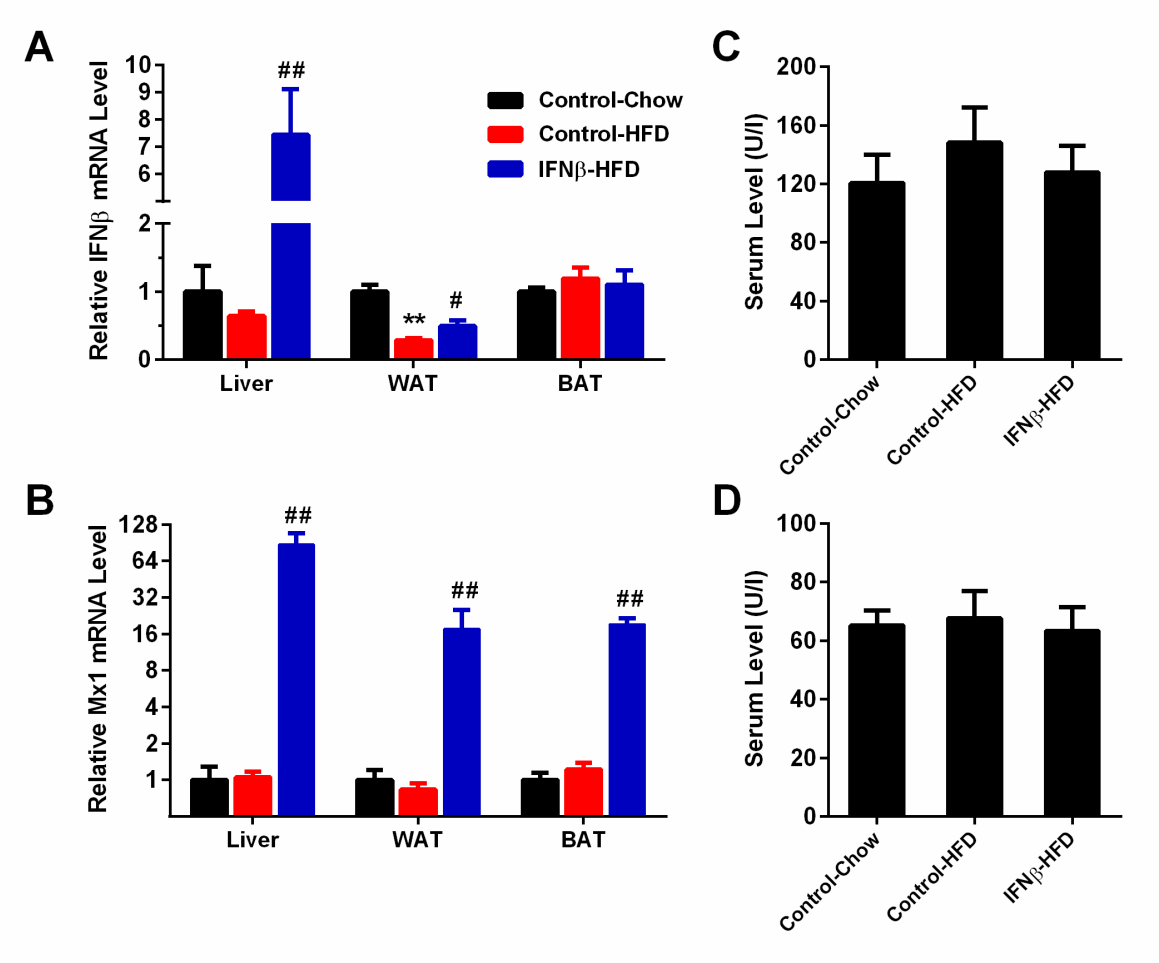

Figure 4.1: Efficiency and safety of IFNβ1 gene transfer using hydrodynamic delivery method. Mice received hydrodynamic injection of plasmid DNA expressing *Seap* (control) or *Ifnβ1*, and fed with HFD or Chow diet for 9 weeks. (**A**) Relative mRNA levels of *Ifnβ1* in liver, WAT and BAT. (**B**) Relative mRNA levels of *Mx1* in liver, WAT and BAT. (**C**) Serum level of aspartate aminotransferase. (**D**) Serum level of alanine aminotransferase. Values represent average ± SD (n=5). ** $P < 0.01$ compared with chow-fed *Seap*-injected mice. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with HFD-fed *Seap*-injected mice.

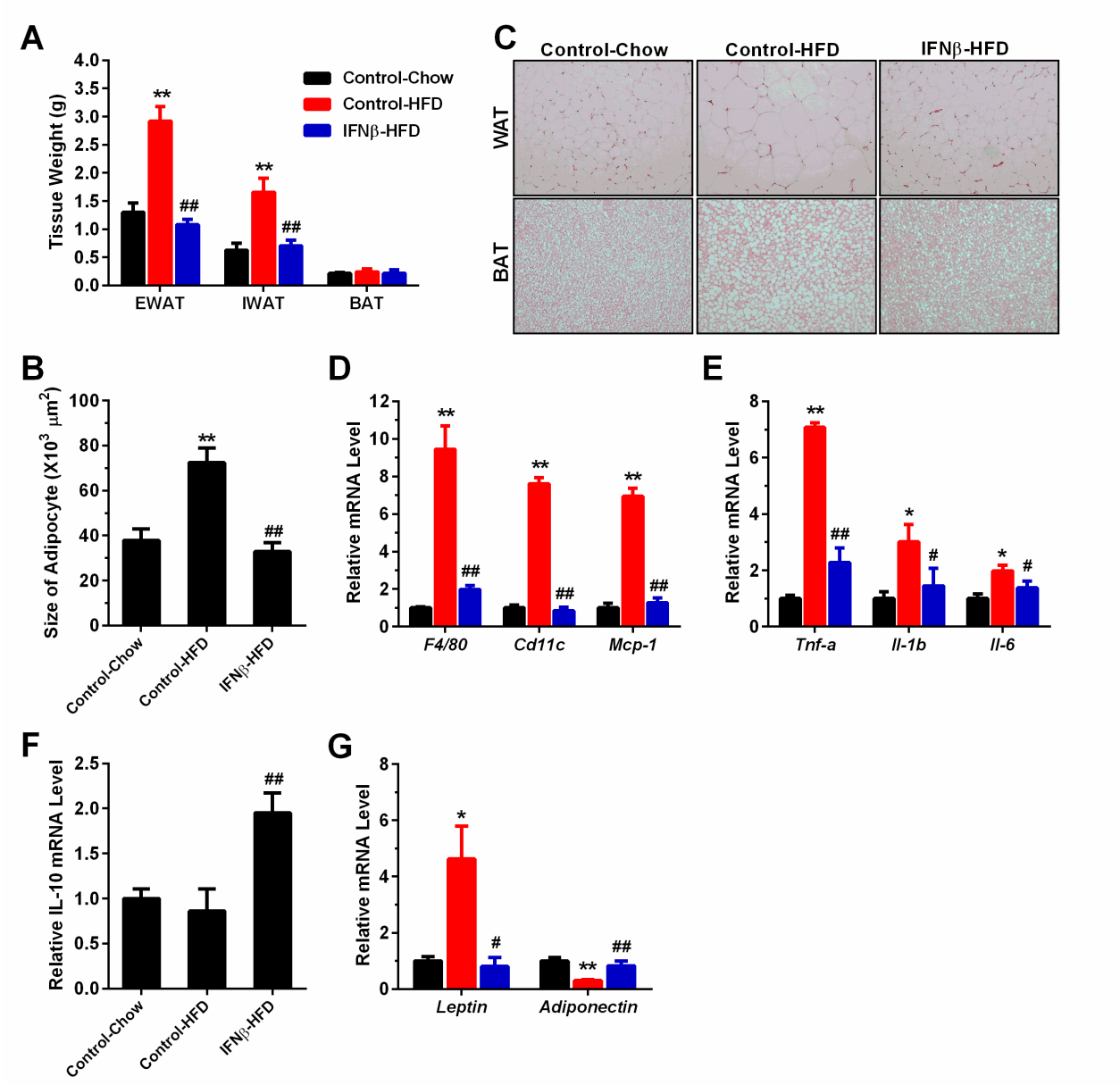

Figure 4.2: IFNβ1 effects on HFD-induced adipose tissue inflammation and adipocyte hypertrophy. (**A**) Average weights of epididymal WAT, inguinal WAT and BAT. (**B**) Average areas of adipocytes in epididymal WAT. (**C**) Representative images of H&E staining of WAT and BAT. (100X magnification) (**D**) Relative mRNA levels of *F4/80*, *Cd11c* and *Mcp1* in WAT. (**E**) Relative mRNA levels of *Tnf-α*, *Il-β* and *Il-6* in WAT. (F) Relative mRNA levels of *Il-10* in WAT. (**G**) Relative mRNA levels of *Leptin* and *Adiponectin* in WAT. Values represent average ± SD (n=5). * $P < 0.05$, ** $P < 0.01$ compared with chow-fed *Seap*-injected mice. $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$ compared with HFD-fed *Seap*-injected mice.

impact of inflammation on adipokine production, we assessed expression levels of leptin and adiponectin upon HFD feeding. While the leptin level was increased, suggesting leptin resistance, adiponectin expression was significantly decreased (**Fig. 4.2G**). IFNβ1 restored the expression of these adipokines to normal levels. Overall, IFNβ1 suppressed HFD-induced adipose inflammation, hypertrophy, and ameliorated the dysregulated adipokines back to a normal level.

IFNβ1 blocked HFD-induced weight gain without impacting food intake.

IFNβ1 not only generates attenuation of adipose tissue inflammation, but also blocks development of obesity in spite of HFD feeding (**Fig. 4.3A**). While control HFD-fed animals gained approximately 20 g in nine weeks, *Ifnβ1* gene transfer completely blocked body weight gain (**Fig. 4.3B**). Analysis of body composition showed IFNβ1 overexpression had no significant impact on lean mass, confirming that the difference in body weight gain was primarily due to the increase in fat mass, and precluding toxicity-related weight loss (**Fig. 4.3C**). The anti-obesity effects of IFNβ1 were independent of food intake since both groups of animals had comparable food intake rates over the nine weeks of HFD feeding (**Fig. 4.3D**).

IFNβ1 altered gene expression in adipose tissues toward thermogenic phenotype.

Given the critical role of adipose tissue, particularly BAT in thermogenesis and overall energy balance, we further examined the anti-obesity effects of IFNβ1 by the assessment of thermogenic genes in WAT and BAT. IFNβ1 significantly upregulated the expression of various isoforms of uncoupling proteins (UCP) in both WAT (**Fig. 4.4A**) and BAT (**Fig. 4.4B**), suggesting enhanced energy expenditure in these tissues, which further contributes to anti-obesity effects. In addition, IFNβ1 upregulates the expression of genes involved in mitochondrial

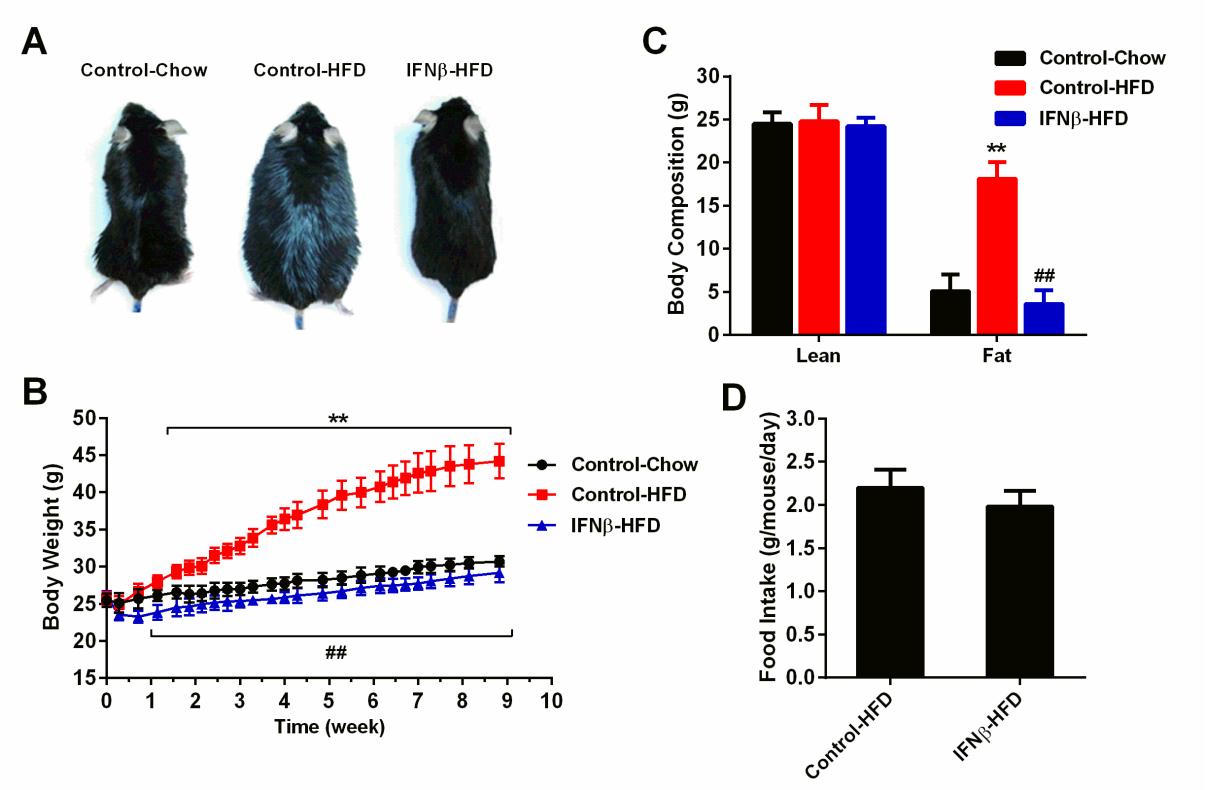

Figure 4.3: IFNβ1 effects on body weight and composition, and food intake. (**A**) Representative images of mice at the end of experiment. (**B**) Growth curves of control and IFNβ1-treated mice over a 9-week period. (**C**) Body composition of mice from the three groups. (**D**) Average food intake over the 9-week period. Values represent average ± SD (n=5). ** $P < 0.01$ compared with chow-fed *Seap*-injected mice. $^{\#\#}$ $P < 0.01$ compared with HFD-fed *Seap*-injected mice.

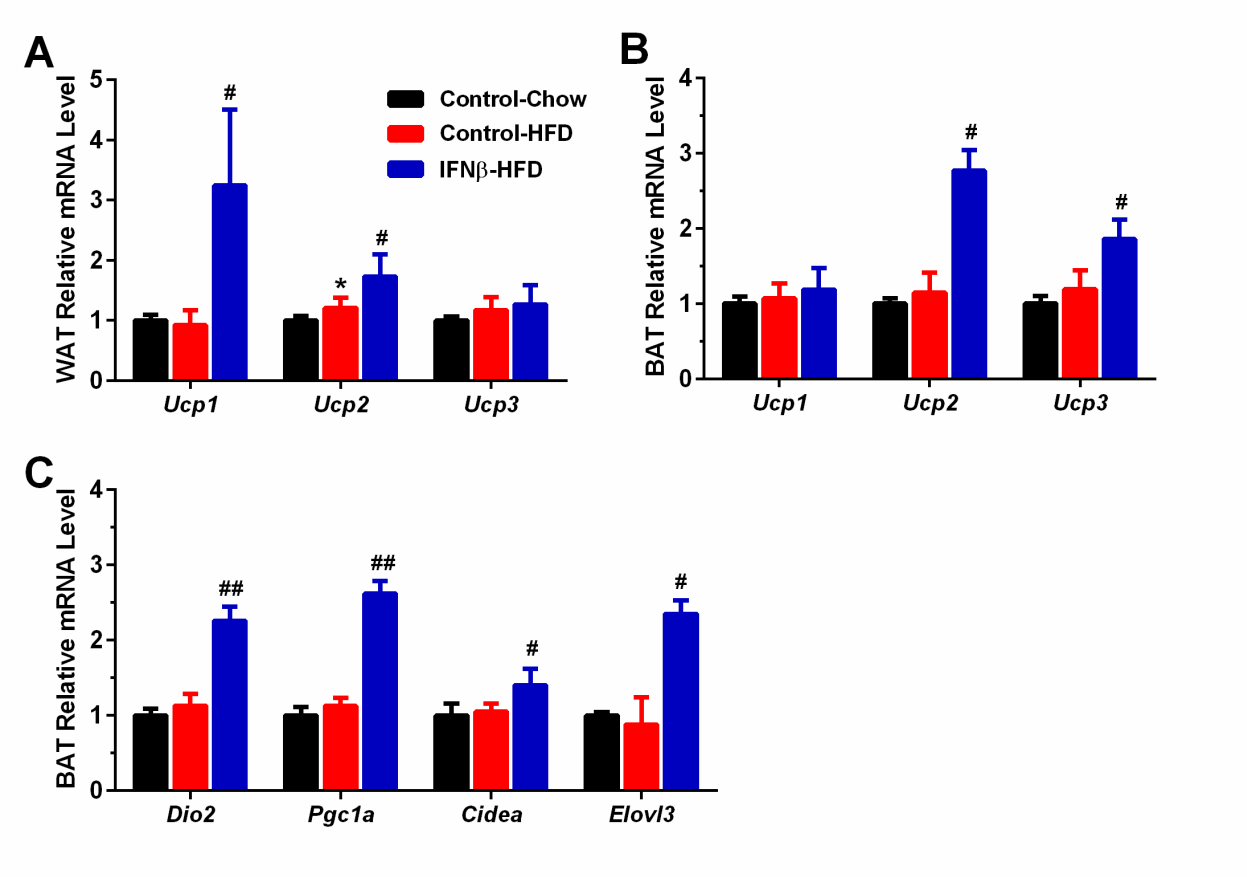

Figure 4.4: IFNβ1 effects on thermogenic genes in WAT and BAT. (**A**) Relative mRNA levels of *Ucp1*, *Ucp2* and *Ucp3* in WAT. (**B**) Relative mRNA levels of *Ucp1*, *Ucp2* and *Ucp3* in BAT. (**C**) Relative mRNA levels of *Dio2*, *Pgc1-α*, *Cidea* and *Elovl3* in BAT. Values represent average ± SD (n=5). * $P < 0.05$ compared with chow-fed *Seap*-injected mice. $^{\#}$ $P < 0.05$, $^{\#\#}$ $P <$ 0.01 compared with HFD-fed *Seap*-injected mice.

biogenesis and energy utilization in BAT (**Fig. 4.4C**), increasing the overall energy expenditure in these tissues.

IFNβ1 did not reverse HFD-induced fatty liver.

Fatty liver is a common manifestation of diet-induced obesity. We examined the effects of IFNβ1 on liver *de novo* lipogenesis and ectopic fat accumulation. Liver analysis showed increased liver weight upon HFD feeding, in spite of IFNβ1 treatment (**Fig. 4.5A**). Assessment of triglyceride content showed comparable levels of liver triglycerides in treatment and control animals (**Fig. 4.5B**) suggesting that IFNβ1 did not protect against fatty liver development. These results were confirmed by H&E analysis of liver sections, which showed vacuole structures in both HFD-fed groups, but not chow-fed animals (**Fig. 4.5C**). To explore the underlying mechanisms of fat accumulation in the liver, we assessed the expression of genes involved in lipogenesis and lipid uptake. While control animals possessed an increased expression of lipogenic genes *srebp1c*, *fas*, and *scd1*, suggesting increased *de novo* lipid biosynthesis, IFNβ1-treated animals showed lower levels of these genes, albeit higher than the normal levels (**Fig. 4.5D**). On the other hand, the expression level of *cd36*, the major fatty acid transporter in the liver, was significantly increased in IFNβ1-treated animals, even higher than that of control animals. These results suggest that while IFNβ1 downregulated the expression of lipogenic genes and partially attenuated lipid biosynthesis, these effects were counterbalanced by an increased uptake of ectopic fat, as evidenced by increased level of cd36, resulting in fat accumulation in the liver.

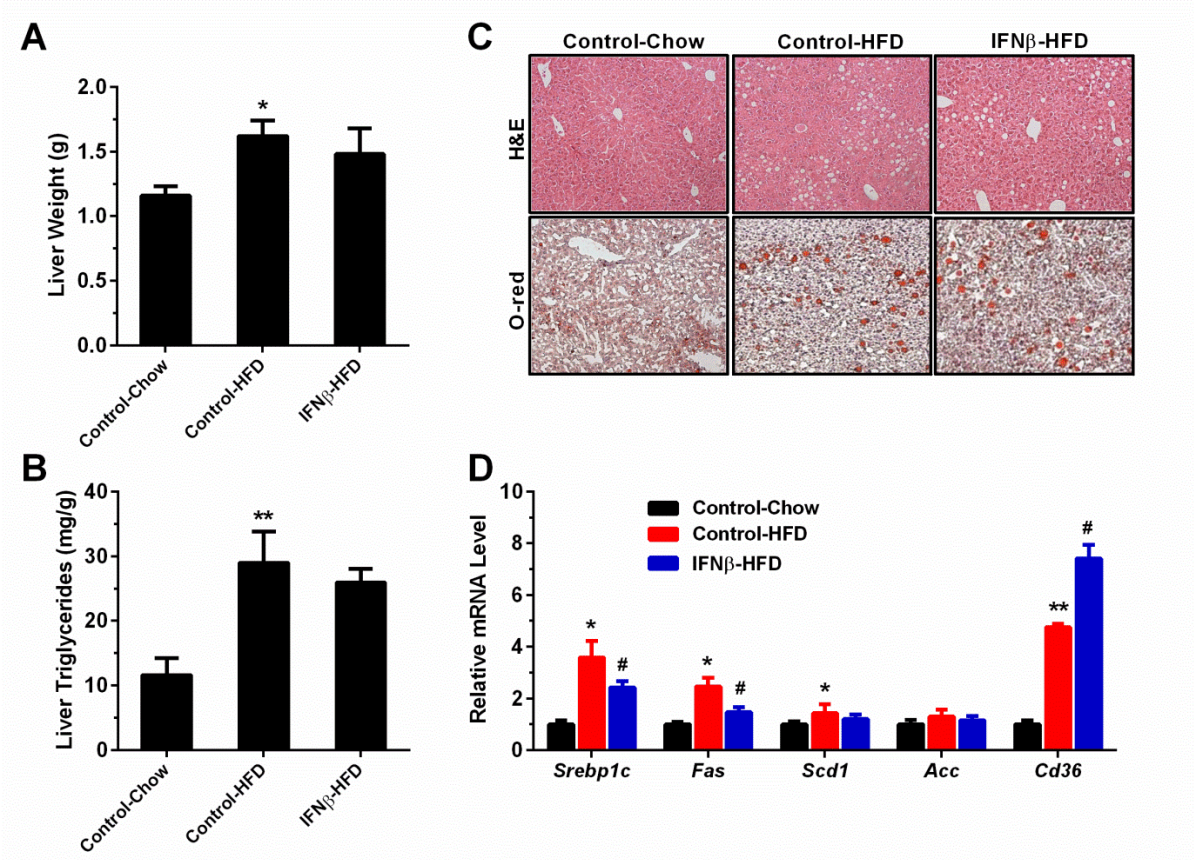

Figure 4.5: IFNβ1 effects on HFD-induced fatty liver and hepatic lipogenesis. (**A**) Average liver weight in chow- and HFD-fed mice. (**B**) Average liver triglyceride content in these mice. (**C**) Representative images of H&E staining (upper panel) and O-red oil staining (lower panel) of liver sections (100X magnification). (**D**) Relative mRNA levels of *Srebp1c*, *Fas*, *Scd1*, *Acc* and *Cd36* in liver. Values represent average ± SD (n=5). * $P < 0.05$, ** $P < 0.01$ compared with chow-fed *Seap*-injected mice. $^{\#}$ $P < 0.05$ compared with HFD-fed *Seap*-injected mice.

IFNβ1 restores insulin sensitivity and improves glucose homeostasis.

It has been well established that obesity is a risk factor for diabetes since obese individuals often display a decreased sensitivity to insulin-stimulated glucose uptake. To assess IFNβ1 effects on glucose homeostasis, we conducted a glucose tolerance test to examine systemic insulin sensitivity upon a glucose challenge. Results showed impaired tolerance to glucose in the control group; whereas IFNβ1-treated animals demonstrated the same efficient glucose clearance as did chow-fed animals (**Fig. 4.6A**). These results were verified by calculation of the area under the curve (**Fig. 4.6C**). Insulin sensitivity was also assessed by an insulin tolerance test showing similar results, in which HFD-fed IFNβ1-treated animals demonstrated improved insulin sensitivity compared to HFD-fed control animals (**Fig. 4.6B**). Assessment of fasting glucose and insulin levels showed consistent results as in IPGTT and ITT. IFNβ1-treated animals remained within normal ranges of glucose (**Fig. 4.6D**) and insulin (**Fig. 4.6E**), compared to hyperglycemic, hyperinsulinemic HFD-fed control animals.

Discussion

It is evident that adipose tissue inflammation is a hallmark for obesity development, and a critical contributor to obesity-related pathologies. Targeting adipose tissue inflammation, therefore, is a potential therapeutic approach that could block development of obesity and its related disorders. Results presented here demonstrated that efficient IFNβ1 overexpression (**Fig. 4.1**) attenuated adipose tissue inflammation (**Fig. 4.2**), blocked the development of HFD-induced obesity (**Fig. 4.3**), and alleviated insulin resistance (**Fig. 4.6**). Anti-obesity effects of IFNβ1 were also linked to increased adipose tissue thermogenesis (**Fig. 4.4**). However, IFNβ1 overexpression did not protect against fatty liver, which is attributed to an increased liver lipid

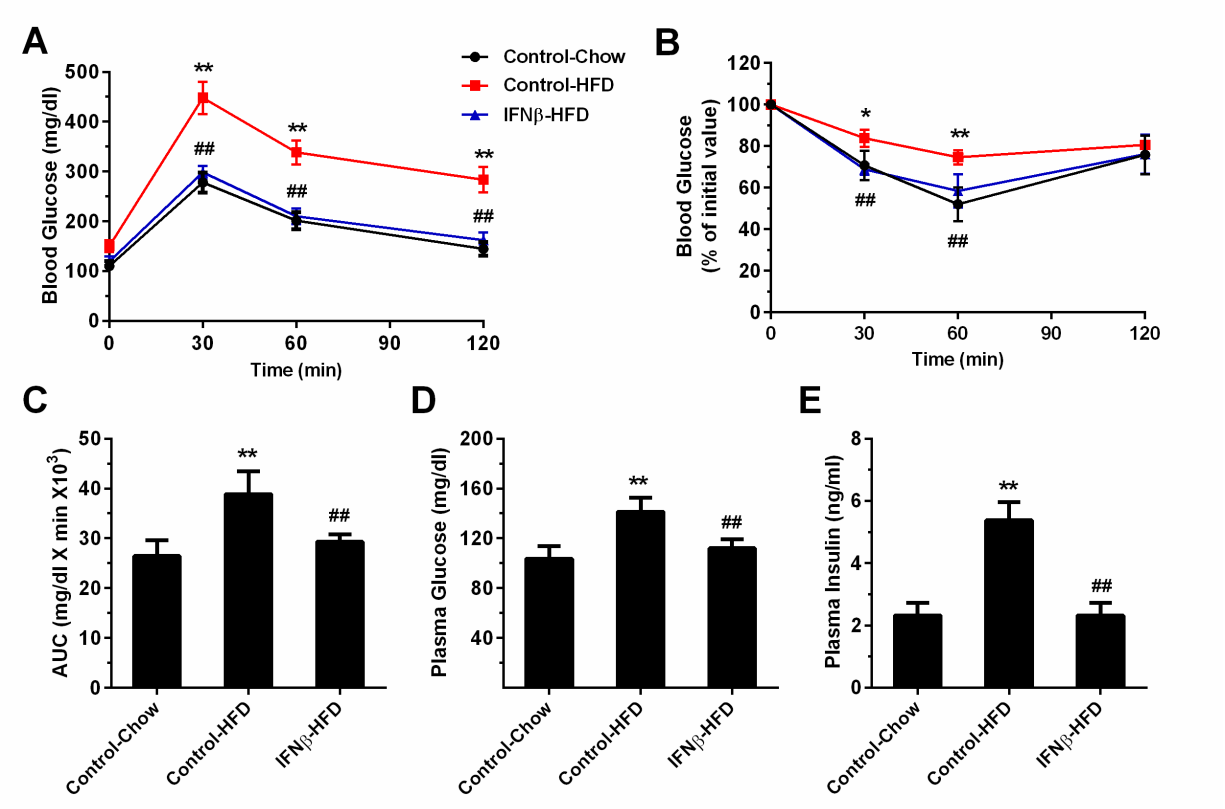

Figure 4.6: IFNβ1 effects on insulin sensitivity and glucose homeostasis. (A) Blood glucose level as a function of time in intraperitoneal glucose tolerance test (IPGTT). (**B**) Blood glucose level as a function of time in insulin tolerance test (ITT). (**C**) Calculated area under the curve for IPGTT. (**D**) and (**E**) Blood levels of fasting glucose and insulin, respectively. Values represent average ± SD (n=5). * $P < 0.05$, ** $P < 0.01$ compared with chow-fed *Seap*-injected mice. $^{\#\#}$ $P <$ 0.01 compared with HFD-fed *Seap*-injected mice.

uptake rather than lipid biosynthesis (**Fig. 4.5**).

Earlier studies reporting increased TNF-α expression in adipose tissue of obese mice and its role in insulin resistance provide the first evidence of the contributing factor of chronic inflammation in obesity and its complications.[9] Additional studies have confirmed the elevated levels of various inflammatory mediators in different obesity models.[10] It has been proposed that fat accumulation and adipose hypertrophy induces a hypoxia response, which accounts for the various oxidative and inflammatory stress events caused by chronic activation of several inflammatory pathways, particularly NF-kB.[11-13] The local inflammation is translated into systemic events, such as low-grade systemic inflammation, insulin resistance, suppression of adiponectin release, and ectopic fat accumulation through cytokines and free fatty acids released from adipose tissue. In agreement with these theories, we observed a significant increase in adipose tissue inflammation (**Fig. 4.2**) accompanied by ectopic fat accumulation in the liver (**Fig. 4.5**) and exacerbated insulin resistance (**Fig. 4.6**) upon HFD feeding. Thus, our results present new evidence supporting the use of anti-inflammatory therapies to prevent obesity and its related pathologies, such as insulin resistance.

Type I interferons, including IFNβ1, are widely expressed cytokines with profound antiviral and immune modulating effects. IFNβ1, in particular, is well recognized for its anti-inflammatory activity and has been proven effective in treating inflammatory diseases, such as multiple sclerosis[5] and ulcerative colitis.[14] IFNβ1 signals through a heterodimeric and ubiquitously expressed IFNα/β receptor (IFNAR), and mediates downstream events through several STAT family members. While the pro-inflammatory IFNγ acts through STAT1 to promote production of pro-inflammatory mediators and enhances antigen processing, IFNβ1 acts through STAT3 which suppresses pro-inflammatory responses, and directly inhibits STAT1

activation.[15] In addition, IFNβ1 enhances the production of IL-10 independently of STAT3 by activating the PI3K signaling pathway.[16] Consistent with these mechanisms, we have demonstrated that IFNβ1 overexpression attenuated HFD-induced inflammation in adipose tissue (**Fig. 4.2**), and thus blocked obesity development (**Fig 4.3**). IFNβ1 likely acts directly through suppression of inflammatory cell infiltration into adipose tissue and subsequent production of inflammatory mediators, and indirectly through promotion of IL-10 in adipose tissue, which in turn represses various TNF-α and IL-1β-mediated inflammatory events.[17] Beneficial effects of IFNβ1 may also be attributed to the inhibition of the NF-kB pathway, a major pathway underlying inflammation-driven metabolic disorders.[18,19]

The anti-inflammatory effects of IFNβ1 were translated systemically into the blockage of adipose hypertrophy and weight gain without affecting food intake (**Fig. 4.3**). Adipose tissue has the capacity to expand under conditions of energy surplus. Macrophage infiltration is critical for adipose tissue expansion due to the tissue remodeling properties of macrophages, such as stimulation of angiogenesis,[20] and production of growth factors essential for adipose tissue growth.[21] Thus, the observed anti-obesity effects of IFNβ1 are likely mediated through inhibition of macrophage infiltration and/or activation, and blockage of adipose tissue remodeling. Increased energy expenditure in adipose tissue (**Fig. 4.4**) also contributes to anti-obesity effects of IFNβ1. Increased thermogenesis in IFNβ1-treated animals may also be related to the restoration of adiponectin expression, which acts centrally and peripherally to increase thermogenic hormones, lipid oxidation, and glucose utilization.[22]

Insulin resistance in obesity is a consequence of fatty acid release from adipocytes and accumulation in insulin target organs. This fatty acid release and accumulation inhibits glucose uptake and activates pro-inflammatory pathways in these organs by adipose-derived cytokines.

Together, this leads to inhibition of the insulin pathway and impaired glucose homeostasis. Mounting evidence suggests that targeting inflammatory pathways in obesity efficiently restores insulin sensitivity and improves glucose tolerance.[23] Moreover, restoring adiponectin levels boosts insulin signaling and ameliorates glycemic control.[24] Here we have shown similar findings. IFNβ1 overexpression resulted in improvement of insulin sensitivity and glucose homeostasis (**Fig. 4.6**) indirectly, through suppression of local and systemic inflammation and restoration of adiponectin expression. IFNβ1 can also improve glucose homeostasis directly, via activation of the PI3K/Akt pathway leading to enhanced glucose uptake.[25]

Fuel mobilization from adipose tissue, in the form of free fatty acids deposited in non-adipose cells, often results in various pathologies such as fatty liver and atherosclerosis. Despite reversing several obesity-related pathologies, IFNβ1 failed to protect against fatty liver (**Fig. 4.5**). While fatty liver may result from *de novo* lipogenesis or lipids mobilization, fatty liver in IFNβ1-treated animals appears to be linked to increased lipid uptake into hepatocytes, rather than increased hepatic lipogenesis, as evidenced by a substantial increase of *Cd36* expression, the major fatty acid transporter, along with down regulation of lipogenic genes, such as *Srebp1c*, *Fas* and *Scd1*.

In summary, we demonstrated that targeting adipose tissue inflammation by IFNβ1 overexpression is a promising therapeutic approach to protect against obesity and its related complications. Our data provide additional evidence to support the rationale to use IFNβ1 as an immune modulator to treat various inflammatory diseases.

Author Disclosure:

The authors have no conflicts of interest to disclose.

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CHAPTER 5

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Drug discovery and development employ robust tools and methods, and clinically relevant models to develop safer and effective medications for diagnostic, treatment and preventive measures for medical practice. Recombinant DNA technologies and methods of gene transfer have become cornerstones in drug discovery, and are increasingly contributing to the development of new biopharmaceutics. Indeed, it would be far more difficult to conduct high throughput screening of protein drugs without essential technologies for production of large quantities of proteins and antibodies, such as hybridoma and recombinant DNA technologies. Advances in DNA sequencing technology made it possible to sequence genomes of species, including human, for comprehension of disease etiology and identification of druggable targets. Transgenic animal models that have provided significant insights about disease pathology and monitoring therapeutic efficacy would never be in hand without the novel techniques developed for genetic manipulation. These applications encompass gene transfer as a cornerstone to achieve the designated goal. Among gene transfer technologies is the hydrodynamic method which is increasingly applied in various gene delivery trials in different animal models.

Hydrodynamics-Based Delivery… Current Status

The development of efficient and safe methods of gene transfer will undoubtedly pave the way for breakthroughs in gene-based drug discovery, and for fruitful applications of gene therapy approaches to human disease management. While viral vectors remain the major type of

methods for clinical studies [1] owing to its high transduction efficiency, insertion mutagenesis and immune responses associated with viral vectors represent serious safety concerns. Plasmid DNA (pDNA) systems stepped in as a safer alternative gene medicine. However, systemic delivery of naked pDNA is pharmacologically inactive due to series of extracellular and intracellular barriers that limit the bioavailability of pDNA [2]. Therefore, research has been devoted to develop methods of gene transfer that facilitate pDNA delivery into target cells, particularly *in vivo*. Hydrodynamic delivery is among the physical methods that has attracted significant interest, and increasingly applied for gene transfer trials.

Because of its demonstrated reproducibility, efficiency and convenience, hydrodynamic-based delivery method has been widely used in various studies, including therapeutic screening of genes in different disease models, assessment of functions of DNA regulatory elements in plasmid vectors, and establishment of disease models in animals. Protein drug discovery is among the most common application for hydrodynamic method. Progress in protein drug development has always been challenged by resource- and time-consuming methodologies for production, characterization, and formulation of recombinant proteins for screening purposes. In addition, post-translational modifications, such as glycosylation pose another challenge for recombinant protein when the prokaryotic expression system is used for protein production. [3]. Efficient in vivo gene transfer technologies like hydrodynamic method offer the tools to bypass the laborious protein production, and allow simultaneous examination of specific genes or gene products for their pharmacological and toxicological properties directly in animal models. Moreover, protein overexpression in animal models eliminates the need for multiple administration of candidate protein because the protein could be continually produced in the animal after gene transfer, which also provides a better pharmacokinetic profile, as there would

be no fluctuation in protein level. Increasing numbers of studies are utilizing hydrodynamic gene delivery for identifying genes with therapeutic potentials. Gene transfer approach has been explored using hydrodynamic method in different disease models, such as hemophilia, obesity, different cancers, Fabry disease, diabetes, myocarditis, liver diseases and many others [4]. In addition assessment of therapeutic potential, hydrodynamic method was used for target validation, such as evaluation of pathogenic potential of certain overexpressed genes coding for receptors or enzymes in development of hepatocellular carcinoma [5-8]. Importantly, therapeutic gene-assessment was not limited to overexpression in the liver only, as other organs have also been explored, such as muscles and kidneys [9]. In addition, other gene therapy approaches have also been explored with hydrodynamic method, such as DNA vaccination [10] and gene knock out [11]. The later became possible with efficient hydrodynamic transfer of short-interfering RNA (siRNA) as intact molecules [12], or as siRNA expression cassettes, which demonstrated up to 20 weeks of suppressed expression of the target gene [13]. Along with gene drug discovery, hydrodynamic method is widely used to assess transcriptional regulation of gene expression. This includes construct optimization of expression vectors [13], as well as identification of regulatory elements in promoter region that control expression of various receptors and enzymes, such as CYP enzymes [14]. Generation of animal disease models has also been achieved using hydrodynamic method. Having animal disease models of many viral infections has been challenging due to receptor incompatibility or safety concerns. Modeling hepatitis B infection in mice was made possible through hydrodynamic injection of viral genome and, subsequent liver expression of viral proteins [15]. In line with these applications, this dissertation includes a series of studies utilizing hydrodynamic delivery for gene and cell delivery in mice. Hydrodynamic cell delivery was used to establish multi-organ tumor growth

model in mice to study tumor behavior in different organs, as imposed by different environmental inputs in different organs. Hydrodynamic gene delivery was used to evaluate therapeutic activity of the interferon beta gene in attenuation of diet-induced obesity and its related complications, such as insulin resistance.

Given the unpracticality of tail vein injection procedure in large animals, a catheter-based injection procedure has been developed [16]. The procedure comprises intravascular insertion of balloon catheter through which a computer-controlled injection into the target organ is performed. This procedure offers targeted delivery not only at the organ level, but also at specific area within the organ, such as lobe-specific, liver targeted injection. A pressure sensor is located at the tip of the catheter that allows live monitoring of pressure throughout the injection period, which in turn sends the data back to the computer. Based on the recorded pressure, the computer controls the injection by manipulating the opening and closure of the valve through which the DNA solution is propelled into target organ. Earlier injection devices use pressurized CO_2 tanks as a driving force to propel DNA solution, while newer devices employ electrical motor to drive DNA solution [17]. These systems have demonstrated efficient gene transfer into liver and muscles of pigs, dogs, and baboons [18], and importantly, parameters for hydrodynamic injection in human-sized pig liver has been optimized, bringing the procedure closer to potential clinical application.

Hydrodynamics-Based Delivery… Remaining Challenges and Future Directions

Physical methods of gene transfer have been developed to overcome barriers of viral and chemical vectors. However, these methods seem to be a long way from clinical applications, largely because the use of mechanical forces to disrupt cell membranes is not risk free. The

method of hydrodynamic delivery utilizes fluid pressure to facilitate delivery, and this pressure has resulted in liver enlargement to double its size [19], and subsequent elevation of liver enzymes. While these effects are transient, alternative strategies should be explored to reduce the volume injected for enhanced safety, while maintaining efficient gene transfer. The observed local tissue damage, albeit transient, cuts off the applicability for more delicate tissues that will not tolerate pressure impact, like brain and eyes. Moreover, the robustness of the method is another limitation, especially when repeated injections are needed. An additional challenge for hydrodynamic method, as for all non-viral methods of gene delivery, is the limited fraction of transfected cells per targeted tissue. It has been shown that transfection efficiency of around 30-40 % of live cells [20], and 22% of muscle fibers [21] has been achieved so far. This would explain the bias in applying physical methods in gene therapy of secreted proteins over intracellular ones, where limited local expression of the protein is sufficient to drive systemic effects, such as DNA vaccination and enzyme replacement therapies.

Nonetheless, hydrodynamics-based delivery has advanced enough to ensure feasibility to implement minimally invasive strategies for gene transfer into various target tissues in various animal models, with adequate levels of transfection, and minimal side effects. This is clearly illustrated in the establishment of an image-guided computerized hydrodynamic method for large animals. The procedure comprises tunable parameters with facile application into specific organ, and specific area within the organ, and thus, holds promise for gene therapy trials on human patients. Moreover, the automated, computerized procedure will also enhance the reproducibility and reduce variations commonly associated with manual procedures.

Future work should consider further improvements in hydrodynamic method to enhance safety and efficiency to meet clinical demands. Reducing the injected volume, while maintaining delivery efficiency, would be of particular importance. Regional hydrodynamic delivery, such as liver-targeted lobe-specific delivery by means of catheterization, will drive the future applications of hydrodynamic method. Innovative integration of physics, chemistry, biology, and computer engineering will be essential to develop strategies for precise control of vasculature pressure for maximal efficiency and minimal side effects. Getting adequate and persistent levels of transgene expression has been truly challenging, largely due to rapid shutting down of promoter activity. Therefore, future research will also need to focus on optimization of plasmid DNA constructs for better kinetics of transgene expression. Such improvements will pave the way for the urgently needed breakthroughs in the field of non-viral gene delivery, and ultimately trigger the initiation of clinical trials using hydrodynamic method for human gene therapy after all.

Anti-Metastatic Drug Development

Because metastasis is the main cause of death from cancer, it is critically important to explore the insights and findings resulting from basic laboratory research and translate them into useful diagnostic and therapeutic measures in clinical practice.

Since metastasis is a complex, multi-step process, it is likely that the process would have many potential targets for intervention. However, pre-clinical trials to develop effective anticancer therapies are challenged by the availability of *in vitro* and *in vivo* models that represent biological features of metastatic tumors, particularly tumor-stroma interactions within the tumor microenvironment, which are considered major determinants of tumor growth and

survival, as well as therapeutic outcomes of anticancer therapies [22, 23]. In addition, the current preclinical testing of drugs is generally focused on subcutaneous or orthotopic tumor models that don't recapitulate the multi-organ nature of metastatic tumors, as to what is commonly observed in clinic, and thus rarely compare differential effects on tumors in different organs. Despite the fact that metastasis accounts for the majority of cancer deaths [24], there are increasing restrictions on research funding and efforts devoted toward invasion and metastasis to bring new and effective therapies to cancer patients. These restrictions are possibly due to the failure to translate tremendous findings in cancer biology into therapies, with a failure rate approaching 90% [25]. This is imposed by the obvious difficulty of treating heterogeneous disseminated tumors, along with the challenges of formulation and delivery of treatment to disseminated tumors. Moreover, clinical trials evaluating anti-metastatic therapies would necessitate earlier introduction of treatment to a relevant animal model.

The vast majority of the currently used anticancer small molecule drugs and antibodies are focused on cell proliferation, survival, and angiogenic pathways, the essential pathways in primary as well as in metastatic tumors. Despite resulting in many successes in clinical practice, it becomes evident however, that this approach has significant defects as there was limited efficacy when metastasis has already developed, as these regimens didn't count the environmental factors. Targeting angiogenesis, for example, with an anti-VEGF antibody resulted in beneficial effects in metastatic regions depending on active angiogenesis, without affecting micro-metastatic regions because of relying on the pre-existing vasculature for survival [26], suggesting that the current anticancer therapies have been inappropriately designed by assuming that different metastatic tumors in different organs behave the same as each other, or as the same as the primary tumor. Indeed, we have demonstrated that genetically-identical tumor

cells behave differently when growing in different environments within different organs. Tumor cells demonstrated distinct survival and growth when seeded in different organs. Moreover, the outcomes of anticancer therapies vary among different anatomical locations. Thus, future drug development programs aiming at proper design of anti-metastatic therapies should consider the differences in tumor microenvironment in different organs, and rethink the possibility of organ-specific therapies for better outcomes. Obviously, this would not be possible without development and optimization of clinically relevant metastatic models for reliable screening of anticancer therapies, taking into account the heterogeneous behavior of tumors growing in different organs. Coupled with advancing bio-imaging techniques and reporters of target inhibition, such models would certainly trigger robust progress in anti-metastatic drug development. Another consideration in designing anti-metastatic treatment is that the ultimate goal is not the blockage of tumor dissemination from primary site. Instead, it should target the invasive phenotype and proliferation and of already-disseminated tumor cells, as most cancer patients are already having circulating tumor cells in their blood, and likely having metastatic colonies at distal sites at the time of diagnosis [27]. This would minimize morbidity and mortality of advanced cancers by inhibiting local tissue destruction by invasive tumor cells. Designing anti-metastatic treatment should also consider the anatomical features of the metastatic organs that reflect barriers related to drug delivery, such as poor vascularization in certain tissues, and inaccessibility to certain organs, such as BBB that might protect brain metastasis from systemically administered agents [28]. The proliferative status of metastatic tumors is another factor to be considered. We have demonstrated that tumors in different organs grow differently, and hence, the outcomes of cytotoxic treatment were different. It became evident that micro-metastases (minimally proliferative and dormant lesions) are commonly

resistant to cytotoxic drugs which act on cell division machinery [29]. This would explain, at least in part, the higher drug-resistance commonly observed in micro-metastatic, often dormant sites in comparison with primary tumors and macro-metastatic ones. Taken together, future work in cancer research should seriously consider these factors for development of effective anti-metastatic therapies to be introduced into clinical practice.

Anti-Obesity Drug Development

The prevalence of obesity is increasing, raising a major health concern because of the serious comorbid complications of the disease, such as insulin resistance that ultimately leads to diabetes, fatty liver disease, cardiovascular diseases and many cancers.

Despite the well-established risks of obesity, management of the disease remains challenging. Among the first options considered is a life style modification, including diet restriction, physical exercise and behavior change. Nonetheless, these measures often result in insufficient improvement and it is difficult to maintain outcomes. Bariatric surgery is also considered but is usually reserved for severe obesity conditions, because of operative complications and cost. Therefore, development of effective pharmacotherapies is critical for disease management. Despite the well-recognized need, few anti-obesity agents have been developed, suggesting a major challenge in the field. Development of effective anti-obesity drugs has to meet several regulatory requirements, such as a minimum of 5% reduction on body weight maintained for at least one year, improvement in obesity-related pathologies such as hyperlipidemia and hyperglycemia, and long term post-marketing safety data [30]. Given that obesity treatment is lifelong therapy, multiple administration would be required, and importantly the threshold of tolerance for side effects has been reduced, resulting in withdrawal of many

agents, such as Dinitrophenol, Sibutramine, Amphetamines and others [31]. Anti-obesity drug development is also challenged by availability of animal models that recapitulate all pathological aspects of obesity [32], such as the effect of age, hyperlipidemia and the development of complications such as liver fibrosis and carcinoma. This would provide incomplete visualization about disease pathology, and limit the value of preclinical screening strategies using these models. Advanced research in obesity has identified various molecular targets to block obesity through different mechanisms, such as controlling appetite, increasing energy expenditure, and targeting inflammation. However, targeting these processes has resulted in several adverse events, often central nervous and cardiovascular effects that limit their applicability. In addition, such strategies failed to generate sustainable weight reducing effects due to neuroendocrine feedback mechanisms that promote weight regain through regulating appetite, minimizing energy expenditure and controlling fat storage [33]. Thus, progress in anti-obesity drug development is in urgent need of exploring additional therapeutic targets and strategies.

Mounting research suggests that low-grade chronic inflammation due to nutrition overload is a driving force for obesity-related pathologies. Therefore, more studies are currently exploring inflammation's contribution to obesity pathogenesis, and developing strategies to target inflammation for treatment of the related diseases. In this dissertation, I used a gene therapy approach employing hydrodynamic delivery method to overexpress mouse interferon beta gene as an anti-inflammatory strategy to block obesity. The results obtained support that attenuation of adipose tissue inflammation, is indeed a valid approach to block development of high fat diet-induced obesity and its related disorders. While diet-induced obesity model didn't recapitulate all obesity related pathologies, such as hyperlipidemia, the model did depict most inflammation-driven pathologies like insulin resistance and immune infiltration into adipose

tissue, and how these events were blocked by anti-inflammatory therapy. The current study design comprises three groups of animals; control chow-fed, control HFD-fed and IFNβ1-treated HFD-fed group. The chow-fed control group was added to ensure the establishment of obesity phenotype in HFD-fed animals, such as weight gain, insulin resistance and adipose inflammation. While the current three-groups design provides reliable assessment of IFNβ1 to block development of diet-induced obesity, a fourth group of chow-fed IFNβ1 treated animals would help to assess the metabolic outcomes of IFNβ in non-obese settings. This would also provide a clearer visualization of IFNβ1 safety. Treatment with IFNβ1 restores most phenotypes of obesity to the normal levels. The presence of chow-fed IFNβ1 group would help preclude the possibility that IFNβ1toxically induces weight loss that was counterbalanced or masked by the HFD feeding. Future work should be directed toward further investigating how interferon beta attenuates inflammation events, and whether interferon beta could reverse already established obesity. Additional work is also needed to address any potential acute toxicity of hydrodynamic procedure and/or treatment. In our study, while no change in liver enzymes in IFNβ1-treated mice indicated the long term safety of gene therapy approach, i.e. the procedure and the treatment itself, additional work is needed to assess the acute effects of IFNβ1, especially within the first week of therapy. The observed lack of gain in body weight within the first week might be attributed to the flu-like symptoms commonly associated with the first 24 hrs of IFNβ1 treatment; therefore, such acute symptoms should be further assessed, with monitoring body temperature and serum biochemistry. As demonstrated in this dissertation, a gene transfer approach using hydrodynamic method would be an important tool for preclinical screening of anti-inflammatory gene drugs for obesity management to overcome barriers related to protein therapies and the need for frequent, long term administration. Using gene transfer approach, it is

now possible to target inflammation through nature's own anti-inflammatory mediators, such as overexpressing interleukin 10 and 13, rather than targeting inflammatory molecules by means of neutralizing antibodies, like anti-IL1β and anti-TNFα, or small molecule inhibitors of receptors and kinases.

Given the multi-factorial nature of obesity pathology, future therapies should encompass combined therapies whereby multiple modes of action would better address the counter-regulatory neuroendocrine mechanisms to attenuate weight regain and sustain anti-obesity effects, while relying on lower doses of individual agents to help minimizing adverse events. An alternative, recently developed technology is to design peptide molecules that possess different mechanisms of action through targeting several pathways simultaneously, such as a peptide triagonist targeting glucagon-like peptide 1, glucose-dependent insulinotropic polypeptide and glucagon receptor. This triagonist has shown impressive improvement in body weight, glucose homeostasis control and fatty liver compared to individual agonists [34]. Such advances would drive the future of obesity pharmacotherapies.

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APPENDIX A

PHYSICAL METHODS OF GENE TRANSFER[§]

[§] Mohammad Alsaggar and Dexi Liu (2015) Physical methods for gene transfer. In "*Non-Viral Vectors for Gene Therapy: Physical Methods and Medical Translation.*" L. Huang, D. Liu, and E. Wagner (eds), *Advances in Genetics* 89: pp1–24.
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Abstract

The key impediment to the successful application of gene therapy in clinics is not the paucity of therapeutic genes. It is rather the lack of nontoxic and efficient strategies to transfer therapeutic genes into target cells. Over the past few decades, considerable progress has been made in gene transfer technologies, and thus far, three different delivery systems have been developed with merits and demerits characterizing each system. Viral and chemical methods of gene transfer utilize specialized carrier to overcome membrane barrier and facilitate gene transfer into cells. Physical methods, on the other hand, utilize various forms of mechanical forces to enforce gene entry into cells. Starting in 1980s, physical methods have been introduced as alternatives to viral and chemical methods to overcome various extra- and intracellular barriers that limit the amount of DNA reaching the intended cells. Accumulating evidence suggests that it is quite feasible to directly translocate genes into cytoplasm or even nuclei of target cells by means of mechanical force, bypassing endocytosis, a common pathway for viral and nonviral vectors. Indeed, several methods have been developed, and the majority of them share the same underlying mechanism of gene transfer, i.e., physically created transient pores in cell membrane through which genes get into cells. Here, we provide an overview of the current status and future research directions in the field of physical methods of gene transfer.

Introduction

The concept of gene therapy has been first introduced in 1960s (Lederberg, 1963), and since then, the field has grown immensely despite the disappointing outcomes of the early clinical trials. The original concept of gene therapy has been redefined by the ever-expanding research in the field to include recently emerged therapeutic "molecular" strategies that center on the use of various forms of nucleic acids as agents for disease treatment, such as RNA interference (siRNA) and antisense oligonucleotides. At first, gene therapy aimed to treat diseases through intracellular gene delivery to restore missing gene function in patient's cells. However, the rationale has recently evolved beyond the treatment of diseases to include prophylactic strategies, such as DNA vaccine, as well as diagnoses and gene marking (Barese & Dunbar, 2011). Clearly, the biomedical applications of gene therapy mentioned thus far encompass "gene delivery" as a cornerstone to accomplish the designated goal. Therefore, efficient gene delivery is essential in successful implication of gene therapy for human disease management. Gene delivery refers to the strategy in which genes or oligonucleotides are purposely introduced into cells in culture, animals, or humans to express the encoded information.

Current progress in gene therapy is challenged by the limited efficiency of gene delivery as a result of a series of intracellular and extracellular barriers limiting the amount of DNA that reaches the nuclei of target cells where gene expression takes place. This is because nucleic acids are large anionic molecules, rendering them hard to permeate through cell membranes.

Moreover, biodegradation of naked DNA by serum and tissue nucleases poses another challenge to overcome (Wiethoff & Middaugh, 2003). Therefore, the development of delivery systems that are able to effectively and safely introduce DNA into host cells in vitro, and importantly, in vivo is critically needed. Immense research has been conducted to optimize gene transfer with acceptable safety and efficiency, and different strategies have been developed utilizing biological, chemical, or physical principles. **Table A-1** summarizes the characteristics of the methods employed thus far including viral and synthetic vectors and physical methods.

Viral vectors harness the natural infectivity of viruses to deliver genetic material to cells. Retroviruses and adenoviruses represent the most employed viral vectors in clinical trials (Edelstein, Abedi, & Wixon, 2007). The major limitations of viral vectors are their intrinsic properties of immunogenicity and potential harm of random insertion, such as activation of naturally silenced genes like oncogenes (Kay et al., 2001). Nonviral methods, on the other hand, utilize natural or synthetic compounds to deliver genetic material into target cells. Chemical methods aim to formulate DNA in complexes to protect DNA from nuclease degradation and facilitate gene transfer by triggering internalization function of cells such as endocytosis, phagocytosis, and pinocytosis. Chemical systems are generally less immunogenic and safer than viral vectors and are increasingly employed in gene therapy studies, despite lower transfection efficiency (Al-Dosari & Gao, 2009; Gao et al., 2007; Mintzer & Simanek, 2009). Chemical systems are also amenable for modifications to enhance targeting specificity. Yet, further optimization is needed to enhance the delivery efficiency. Apart from vector-based systems, physical methods of gene delivery are unique in eliminating the need for a special carrier to transfer DNA into cells. The physical methods employ physical forces to create transient pores in plasma membrane so that “naked” DNA molecules can pass through. Among the forces that have

Table 1: Characteristic features of major classes of gene delivery systems.

Method	Principle	Advantages	Disadvantages
Viral Methods	Transfer of DNA or RNA through the natural viral infectious pathway using replication-incompetent viruses.	- Relatively high transduction efficiency and persistent gene expression. - Can be used with dividing and non-dividing cells. - Highly effective in *in vivo* and *in vitro* trials.	- Strong induction of immune response. - Oncogenesis and insertional mutagenesis. - High cost. - Restrictions on the size of transgene.
Chemical Methods	Transfer of DNA or RNA in complex with cationic lipids or polymers through cellular endocytosis pathway	- Much safer and cheaper than viral vectors. - Amenable for chemical modification for targeted delivery. - Common and effective in *in vitro* experiments.	- Short duration of gene expression. - Low transfection efficiency in *in vivo* systems. - Low efficiency in non-dividing cells.
Physical Methods	Transfer of DNA or RNA through transient pores in plasma membrane created by mechanical forces.	- Can be used effectively in *in vitro* and *in vivo* experiments. - Specific tissue transfection. - Can be used with dividing and non-dividing cells.	- Local tissue damage at the site of application. - Specialized instrument may be required. - Optimized procedure parameters are required for different types of tissues.

been utilized are electrical pulses, ultrasound waves, hydrodynamic pressure, and others (Villemejane & Mir, 2009). Physical methods of gene transfer become increasingly applied in biomedical research due to its safety and simplicity in comparison to the other methods, and importantly, its ability to manipulate procedure parameters toward specific therapeutic needs. However, gene transfer efficiency of most physical methods is inferior to that of viral methods. Moreover, it becomes challenging when the internal organs are the primary tissues to be targeted for gene delivery, because it usually requires an invasive procedure to access target tissue (Kamimura & Liu, 2008). This chapter aims to provide an overview of the implied principles and techniques, the current status of various physical methods of gene transfer that have been developed, and the advantages and limitations of each method. In addition, we provide our perspective on future directions and how to address remaining challenges that restrict their applications in biomedical research and clinical practice.

Most Commonly Used Physical Methods

The barrier function of cell membrane is attributed to the dynamic nature of the membrane bilayer held together primarily by hydrophobic interaction of phospholipids, membrane proteins, and cholesterol. The rationale of physical methods for gene delivery at cellular level is to overcome membrane barrier and facilitate gene transfer into cells by generating transient pores or defects in plasma membrane through which DNA can get into cells. Importantly, these pores are transient in nature because, when membrane is broken open, the hydrophobic boundaries created are not stable in aqueous environment and reseal quickly to limiting leakage of cellular content, while allowing DNA diffuse into cells. The following is a brief description of each of the physical methods developed and **Figure A-1** presents the

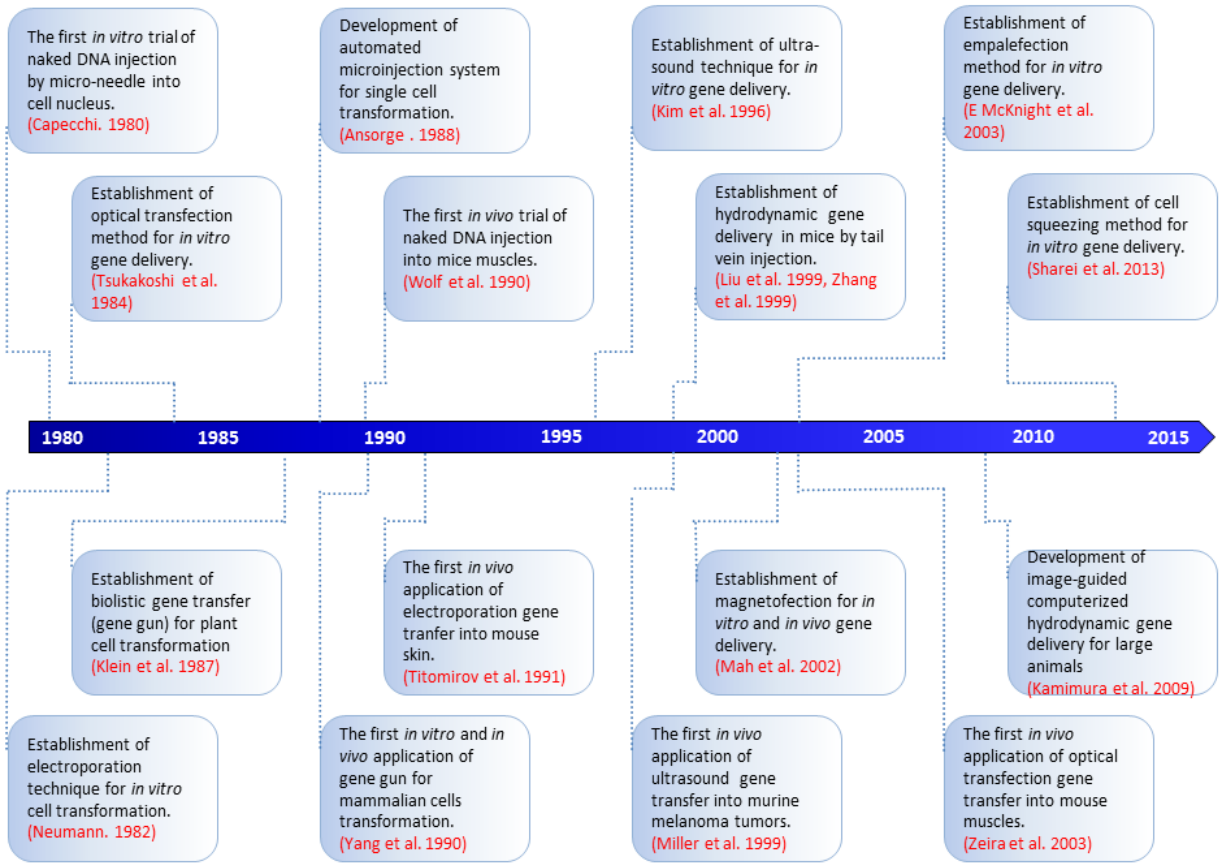

Figure A-1: A timeline of the milestones in the development of physical methods for gene delivery.

milestones that contributed to the successful implementation of physical methods of gene transfer in gene therapy trials.

Needle Injection

Practically, the simplest intracellular gene delivery would be a direct injection of DNA into cells. Indeed, direct injection of DNA into the cell cytoplasm or nucleus by means of microneedle has been a common practice since 1980s (Capecchi, 1980). However, minimal progress has been achieved toward in vivo application. This is largely due to the technical difficulties in the procedure, making it extensively laborious because only one single cell can be injected at a time. Moreover, specialized tools are needed to perform injection, such as glass microneedle, micropipette, and precise positioning manipulator to control the movement of the micropipette. Typically, all the work is carried out under a proper microscope. Though, the high efficiency of gene transfer triggers outstanding improvement in the technique to include automated microinjection system controlling movement of micropipette and manipulator with precise cell positioning and injection times (Ansorge & Pepperkok, 1988). Computer-guided microinjection has also been developed to enhance reproducibility and minimize variation associated with manual operation (Pepperkok, Schneider, Philipson, & Ansorge, 1991). Such developments promoted microinjection as a typical procedure for single cell assays involving nuclear DNA transfer (Lamb, Gauthier-Rouviere, & Fernandez, 1996), and cytoplasmic, i.e., mitochondrial DNA transfer (Kagawa, Inoki, & Endo, 2001). To date, production of recombinant cell lines and transgenic animals remains the standard application of microinjection, because it is advantageous in bypassing cytoplasmic nucleases, and delivering precisely defined copy number of transgenes (Auerbach, 2004; Chenuet et al., 2009).

In vivo needle injection was also successfully demonstrated in mouse skeletal muscles and in other tissues, albeit low level of gene expression that was limited to the site of injection (Wolff et al., 1990). Although it has been suggested that the injected DNA is taken by cells through an active receptormediated process (Budker et al., 2000), DNA diffusion into cells through the membrane defects generated by needle insertion is more likely the mechanism as the gene expression was primarily located in the needle track. Although limited, the expression level of the transgene was sufficient in eliciting biological responses such as immune response, justifying the use of intramuscular injection of plasmid DNA as a vaccination strategy to express viral antigens (Danko & Wolff, 1994). Further success was demonstrated in gene therapy of ischemia in rabbits (Vincent et al., 2000), and in patients with limb ischemia (Kalka et al., 2000), both aimed to induce angiogenesis by increasing levels of vascular endothelial growth factor (VEGF) upon intramuscular injection of VEGF-coding plasmid DNA. Importantly, DNA transfer was not solely confined to muscular tissue, as evidenced by several studies involving direct injection of naked DNA into various tissues for different therapeutic purposes, such as liver (Hickman et al., 1994; Zhang et al., 1997), skin (Yu et al., 1999), lungs (Meyer, Thompson, Levy, Barron, & Szoka, 1995), and for intratumoral gene delivery (Yang & Huang, 1996).

A modified version of needle injection is impalefection, the use of nano-fibers or nanowires instead of a needle to deliver genetic material into target cells. This method was established in 2003 using vertically aligned carbon nanofiber (VACNFs) array fixed on chips, and plasmid DNA adsorbed and tethered to VACNFs (McKnight, 2003). DNA-loaded VACNFs are integrated into cells by means of centrifugation of suspended cells into chips. Following centrifugation and integration, chips are transferred into growth medium in culture dish to allow recovery and proliferation of transfected cells. This technique demonstrated promising

transfection efficiency, while maintaining cell viability. Impalefection was later improved to increase the efficiency of transfection and to allow tracking of transgene expression spatially and temporally, along with viability assessment of transfected cells (McKnight et al., 2004). To date, impalefection has been explored for in vitro transfection, and further fabrication is clearly needed toward its in vivo applications. Though, the method holds the potential that was evident in several in vitro impalefection gene transfer studies (Mann et al., 2008; Pearce et al., 2013; Peckys, Melechko, Simpson, & McKnight, 2009). Presumably, in vivo application of impalefection would be utilizing chip arrays of optimized nanofibers with proper height and thickness that are pressed against target tissue or organ, forcing nanofiber penetration into target cells and release of surface-bound genetic material.

Gene Gun or Ballistic Gene Transfer

Gene gun, also called biolistic gene transfer, was first established in 1987 for plant cell transformation (Klein, Wolf, Wu, & Sanford, 1987), and later, it was successfully applied for gene transfer studies in mammalian cells in vitro as well as in vivo (Williams et al., 1991; Yang, Burkholder, Roberts, Martinell, & McCabe, 1990). Gene transfer is accomplished by bombarding target cells with DNA-coated gold particles driven by pressurized inert gas such as helium or by high-voltage electronic discharge. Efficient gene transfer necessitates fine optimization of the procedure to maintain penetration capacity, while minimizing tissue/cell damage. Among the parameters that impact the efficiency of gene transfer are the size and the density of microspheres, bombardment force, gene gun instrumentation, and microspheres to DNA ratio. Typically, these parameters vary with different types of cells and different tissues in animals (Eisenbraun, Fuller, & Haynes, 1993; Sanford, Smith, & Russell, 1993). Biolistic gene

transfer is advantageous in being fast, simple, and highly efficient. Moreover, the technique is permissive to deliver wide range of macromolecules, such as nucleic acids and proteins. To date, DNA vaccination is the most common application of biolistic gene transfer because the technique efficiently delivers small amount of DNA sufficient to induce immune response against gene product, besides the immunogenicity of the technique itself; making the technique superior to other physical methods for DNA vaccination (Wang et al., 2008). Indeed, biolistic gene transfer has demonstrated great promise in preclinical models for DNA vaccination, such as mice, rabbits, nonhuman primates, as well as in human clinical trials (Fuller, Loudon, & Schmaljohn, 2006). Aside from vaccination, the technique has been modestly applied in gene therapy trials in which a little amount of therapeutic protein is enough to elicit therapeutic response for cancer immunotherapy (Lin, Pulkkinen, Uitto, & Yoon, 2000; Seigne et al., 1999; Sun et al., 1995). Most applications of gene gun are limited to exposed tissues including skin and muscles. Though, with the aid of surgical procedures, inner organs were also targeted with gene gun such as liver (Kuriyama et al., 2000), neurons (McAllister, 2000), and brain (Zhang & Selzer, 2001). Despite the promising results in vaccination trials, the progress of biolistic gene transfer in clinics is challenged by limited efficiency to transfect larger and deeper areas, and the cost of the specialized gene gun and preparation of pure gold particles.

Electroporation

Electroporation-mediated gene transfer has been first and successfully established in 1982 by Neumann and collaborators (Neumann, Schaefer-Ridder, Wang, & Hofschneider, 1982), and since then, the technique has evolved as a powerful and widely used method of gene transfer that demonstrated prominent success and versatility in studies involved in vitro and in vivo gene

delivery to various prokaryotic and eukaryotic cells. Historically, the concept of membrane permeation through the application of electrical impulses preceded studies of gene transfer, and was originally explored to understand membrane permeability to biological molecules such as catecholamines (Neumann & Rosenheck, 1972). The mechanism of permeation was revealed later to be transient pores created in the membranes, allowing large and/or ionic macromolecules like DNA, proteins, and even drugs to pass through (Chang & Reese, 1990). Importantly, these pores reseal within a few seconds to minutes, without significant impacts on membrane structure or cell viability (Weaver, 1995). The procedure of electroporation-mediated gene transfer comprises at least two electrodes connected to a power supply and the target cells are in between. In vitro electroporation is applied in a specialized cuvette having a suspension of cells and DNA and connected to a power supply, while the in vivo system involves electrodes inserted into and enclose the target tissue (Gehl, 2003). An electrical pulse is applied to cells, allowing DNA to get into cells. Transfection efficiency and reproducibility are controlled by tight adjustment of procedure parameters, such as the duration of pulse, frequency of electric shock, and the intensity of the electrical field. Gene transfer efficiency varies significantly among different cell types and experimental conditions. For example, the use of dimethyl sulfoxide (DMSO) significantly enhanced the efficiency of electroporation and DNA uptake in mammalian cells (Melkonyan, Sorg, & Klempt, 1996). Moreover, several designs of electrodes have been developed such as plate electrodes, needle pair electrodes, needle array electrodes, and meander electrodes (Gilbert, Jaroszeski, & Heller, 1997; Tjelle, Salte, Mathiesen, & Kjeken, 2006; Zhang, Nolan, Kreitschitz, & Rabussay, 2002), each manipulates and tailors electrical field configuration toward the maximal efficiency of gene transfer and minimal tissue damage. Apart from technical features, in vivo electroporation for gene transfer started in the early 1990s

(Titomirov, Sukharev, & Kistanova, 1991) even though the impressive outcomes came from clinical trials using electroporation to deliver chemotherapy drugs to tumors (Glass et al., 1996; Heller et al., 1996; Mir et al., 1991). The field of electroporation was then expanded to become among the most commonly used methods of gene transfer into various tissues and in different animal models. Skeletal muscles have been extensively utilized in gene transfer experiments using electroporation, as being easily accessible. Highly efficient platform for long duration of gene expression has been established for muscle-gene transfer comparing to other soft organs (Li & Benninger, 2002; McMahon & Wells, 2004). Certainly, different parameters were applied for optimal muscle transfection in murine (Tevz et al., 2008) versus large animals (Khan et al., 2003). The liver has also attained significant attention in gene therapy trials for liver diseases as well as other diseases, and indeed demonstrated great efficiency in transgene expression, largely due to its inherent function of protein synthesis, and highly vascularized structure. Strikingly, systemic administration of plasmid DNA via tail vein is superior to intrahepatic injection, resulting in more efficient gene transfer (Jaichandran et al., 2006). Electroporation-mediated gene transfer has been also assessed in other tissues and shown very encouraging results in pulmonary (Dean, Machado-Aranda, Blair-Parks, Yeldandi, & Young, 2003), renal (Tsujie, Isaka, Nakamura, Imai, & Hori, 2001), dermal (Gothelf & Gehl, 2010), cardiac (Harrison, Byrne, & Tung, 1998), pancreatic (Sato et al., 2013), corneal (Blair-Parks, Weston, & Dean, 2002), and intratumoral gene transfer (Heller & Heller, 2010). This prominent success advanced electroporation into gene therapy trials such as cancer gene therapy (Dolinsek et al., 2013; Sin et al., 2012), DNA vaccination (Ligtenberg, RojasColonelli, Kiessling, & Lladser, 2013), gene therapy for liver cirrhosis (Kiyama et al., 2008) and hepatitis C infection (Weiland et al., 2013), renal failure (Brown, Bodles-Brakhop, Pope, & Draghia-Akli, 2009), and ischemic diseases

(Ouma et al., 2014). Similar to other methods of gene transfer, however, several challenges are yet to overcome, such as the collateral tissue damage, invasiveness, and the limited area of efficacy between electrodes, making it difficult to transfect large number of cells.

Hydrodynamic Gene Transfer "Hydroporation"

Hydrodynamic gene delivery was established in late-1990s (Liu, Song, & Liu, 1999; Zhang, Budker, & Wolff, 1999). It involves a rapid tail-vein injection of plasmid DNA into a mouse using a relatively large volume of DNA solution that induces transient cardiac congestion, resulting in an elevated hydrodynamic pressure in the inferior vena cava that drives DNA solution back to the liver and kidneys through hepatic and renal vein, respectively. Due to fenestrated endothelium in the liver, this pressure widens the pores of the liver fenestration and subsequently impacts hepatocytes surrounding the capillaries. It has been confirmed that hydrodynamic pressure-derived perforation or the so-called "hydroporation" of cell membrane and fluid entry is the underlying mechanism of gene transfer (Crespo et al., 2005; Zhang et al., 2004). Importantly, the pressure impact on the liver is transient and reversible, and hepatocytes recover functionally and structurally within 24–48 h post injection (Suda, Gao, Stolz, & Liu, 2007). The dynamic pressure is a function of the volume injected and the speed of injection, and thus, needs careful adjustment to ensure proper hydrodynamic pressure force that drives efficient gene transfer with minimal side effects. Certainly, these parameters vary with different anatomical features of the target organ, different structures of parenchymal cells, and different capillary types, i.e., fenestrated or continuous (Chen, Liu, & Lin, 2005; Danialou et al., 2005; Maruyama et al., 2002; Yoshino, Hashizume, & Kobayashi, 2006). Hydrodynamic gene transfer has been proven superior to the existing nonviral methods because it is highly efficient, relatively

simple, safe, and versatile (Suda & Liu, 2007). Thus far, the liver is the primary organ targeted by hydrodynamic gene transfer, as being easily and noninvasively accessible via tail vein, as well as demonstrating highest levels of transgene expression among targeted organs (Song, Liu, Zhang, & Liu, 2002). Liver-directed hydrodynamic gene delivery is increasingly used with numerous applications, such as liver gene therapy and gene drug discovery, animal model establishment, and genetic studies of gene expression regulation (Bonamassa, Hai, & Liu, 2011). Gene transfer to other organs using hydrodynamics has also been explored, such as muscles that have shown comparable levels of transgene expression to liver in rodent and large animal models (Hagstrom et al., 2004; Kamimura, Suda, Xu, Zhang, & Liu, 2009). In spite of similar underlying mechanism of gene transfer, technical aspects are quite different. DNA solution is typically applied into afferent or efferent vessels that are transiently occluded around the target tissue, and DNA solution is administered in the enclosed compartment. Hydrodynamic gene transfer has gained increased attention and becomes broadly applied in biomedical research to deliver DNA, RNA, proteins, and synthetic compounds to various tissues for different purposes. Indeed, it has been used in gene therapy studies for treatment of several diseases including growth hormone deficiency (Sondergaard, Dagnaes-Hansen, Flyvbjerg, & Jensen, 2003), hemophilia (Miao, 2005), diabetes (He et al., 2004; Vakili et al., 2013), obesity (Gao et al., 2013; Jiang, Yamato, & Miyazaki, 2003), hypertension (Romero-Vasquez et al., 2012), autoimmune myocarditis (Liu et al., 2005), muscular dystrophy (Zhang et al., 2010), renal ischemia (Hamar et al., 2004), DNA vaccination (Neal, Bates, Albertini, & Herweijer, 2007), and different types of cancers (Barnett et al., 2004; Maruyama et al., 2012; Wang, Chen, Tang, Zhang, & Hua, 2013; Wen, Matsumoto, Taniura, Tomioka, & Nakamura, 2004; Yazawa et al., 2006). In addition, the method was used to establish animal models for different diseases, such as mouse hepatitis B viral infection by

transfecting hepatocytes in vivo with HBV genome expressing viral antigens and replicative intermediates, resulting in production of viral particles (Yang, Althage, Chung, & Chisari, 2002). Numerous modifications have been developed to adapt the technique of hydrodynamics for clinical and experimental needs. Recently, an automated injection device with computerized control has been developed for hydrodynamic gene transfer in large animals (Suda, Suda, & Liu, 2008). The device allows automated adjustment of injection parameters using intravascular pressure as a regulator. Further improvement was made in combining this device with an image-guided catheterization technique, allowing lobe-specific gene transfer to the liver of pigs (Kamimura et al., 2009), and offering great potential as the method of choice for clinical application in human gene therapy. Apart from gene transfer, the principle of hydrodynamics has been utilized for in vivo cell delivery as well. Hydrodynamic cell delivery was recently approved for establishment of the metastatic tumor model in mice, in which tumor cells are simultaneously seeded in liver, kidneys, and lungs (Li, Yao, & Liu, 2011). As being highly efficient and simple, hydrodynamic gene transfer truly boosts research progress in the field of gene therapy.

Ultrasound-Mediated Gene Transfer "Sonoporation"

Since the 1960s, ultrasound technique has been routinely used in clinical practice for various diagnostic and therapeutic purposes, and later in the 1990s, ultrasound was established as a method to enhance transdermal drug delivery (Bommannan, Okuyama, Stauffer, & Guy, 1992; Mitragotri, Edwards, Blankschtein, & Langer, 1995). Sonoporation was then successfully utilized to transfect fibroblast and chondrocytes with plasmid DNA using ultrasound waves applied through the walls of cell-culture plates and flasks (Kim, Greenleaf, Kinnick, Bronk, & Bolander, 1996). Albeit lesser effective than electroporation and hydrodynamic gene transfer,

sonoporation has gained increased attention because it is advantageous for being simple, noninvasive, safe, and more tolerable than the other methods of gene transfer since no tissue damage is associated with application of ultrasound energy (Rychak & Klibanov, 2014). Similar to other physical methods, the underlying mechanism of sonoporation gene transfer is through transient permeation of cell membrane a principle called microbubble and cavitation. Upon application of ultrasound waves to cells in an in vitro or in vivo aqueous media, gas-filled and protein-stabilized bubbles are formed. The size of these bubbles is proportional to the applied energy. These bubbles oscillate in the ultrasound field and eventually collapse and release energy dramatically impacting and permeabilizing nearby cell membranes, and allowing macromolecules to get through transiently created pores (Cool, Geers, Lentacker, De Smedt, & Sanders, 2013; Wells, 2010). The overall efficiency of sonoporation varies in different experimental conditions, including the frequency of the applied ultrasound energy, the duration of treatment, plasmid DNA concentration, and even the ambient temperature. It also varies with different cells or tissue types. Sonoporation efficiency can be further improved by incorporation of echo-contrast agents that act as cavitation nuclei and facilitate energy transfer to increase permeabilization (Greenleaf, Bolander, Sarkar, Goldring, & Greenleaf, 1998; Miller, Pislaru, & Greenleaf, 2002). Therefore, these factors are carefully optimized toward specific tissues, models, and/or therapeutic needs (Pislaru et al., 2003). Sonoporation gene transfer has been investigated to transfect different tissues and demonstrated promising results, such as in muscles (Lu, Liang, Partridge, & Blomley, 2003), liver (Shen, Brayman, Chen, & Miao, 2008), lungs (Xenariou et al., 2007), heart (Fujii et al., 2011), vasculature (Taniyama et al., 2002), and solid tumors (Haag et al., 2006). Promising results were shown in gene therapy trials to treat liver fibrosis (Yang et al., 2013), myocardial ischemia (Korpanty et al., 2005), diabetes (Chen et al.,

2007), and different cancers (Fujii et al., 2013; Liao et al., 2012; Sakakima et al., 2005). Although the method is highly safe, the in vivo application of sonoporation is limited by the modest transfection efficiency in comparison to the other methods. It has been suggested that combined effect of sonoporation and other physical methods would enhance transfection efficiency. Indeed, combined sonoporation and electroporation to muscles showed transfection efficiency superior to either method alone (Yamashita et al., 2002).

Magnetofection

Magnetofection mediates gene transfer using supramagnetic iron oxide nanoparticles coated with DNA in presence of magnetic field. The principle of magnetism in targeted drug delivery has been applied since the early 1980s (Widder & Senyei, 1983), aiming to concentrate drug-loaded magnetic particles at the target site by means of magnetic field application. Magnetic targeting was later implemented in gene delivery (Mah et al., 2002), and thereafter, several systems have been developed where magnetic nanoparticles made of iron oxide are complexed to nonviral or viral vectors (Huth et al., 2004; Scherer et al., 2002), which greatly enhance gene transfer into target tissues. Mechanistically, magnetofection enhances gene delivery by guiding and maintaining DNA-loaded particles in close contact with target cells, and thus increasing cellular uptake of these particles through endocytosis. Further enhancement comes from magnetic field-facilitated extravasation of particles into surrounding tissue (Plank et al., 2003). Moreover, recent studies demonstrated that magnetic field efficiently increases cell membrane permeability by a yet unclear mechanism (Shankayi, Firoozabadi, Mansourian, & Mahna, 2014). Magnetofection has been broadly used in gene transfer for cultured cells, such as cultured endothelial cells (Krotz, Sohn, Gloe, Plank, & Pohl, 2003), and to a lesser extent, for in

vivo gene transfer. The method has been successfully employed for intratumoral delivery of anti-metastatic NM23-H1 gene that results in suppression of pulmonary metastasis in mouse model (Li et al., 2009). It has also been utilized in intradermal gene delivery of VEGF to induce angiogenesis and perfusion in ischemic skin flaps model in rats (Holzbach et al., 2010). Several challenges remain for in vivo magnetofection, such as inferior transfection efficiency, rapid systemic clearance of iron oxide particles, and increased safety concern regarding the accumulation of iron oxide in cells, especially with multidosing experiments.

Laser-Mediated Gene Transfer "Optical Transfection"

Laser irradiation is another form of physical force that has been explored to permeabilize cell membrane in order to facilitate gene transfer. Laser-mediated gene transfer, also called optoporation, has been established in 1980s (Kurata, Tsukakoshi, Kasuya, & Ikawa, 1986; Tsukakoshi, Kurata, Nomiya, Ikawa, & Kasuya, 1984), allowing genetic material in culture media to get into cells. Several advancements have been achieved in the field of optical transfection, allowing selective targeting of single cell, or even a particular subcellular structure, such as nuclei and mitochondria, using various wavelengths and power densities (Yao, Zhang, Rahmanzadeh, & Huettmann, 2008). While Neodymium-doped yttrium aluminum garnet (Nd:YAG) was used originally as a laser source, several sources have later been developed such as argon ion (Palumbo et al., 1996), holmium YAG (Ho:YAG) (Sagi et al., 2003), and titanium sapphire (Zeira et al., 2003). Laser-beam intensity is controlled by pulse generator and focused on the target area by a lens. Optical transfection is advantageous in being safe and noninvasive. Optical transfection has demonstrated promising success in various in vitro studies (Stevenson, Gunn-Moore, Campbell, & Dholakia, 2010), but it is still a subject of research to be applied in

vivo. This is largely due to the relatively high cost and the limited efficiency because of limited penetrating capacity (approximately 2 mm) to deeper tissues as well as limited impacted area. Therefore, in vivo application was limited to exposed regions such as muscles (Zeira et al., 2003), skin (Zeira et al., 2007), and for intratumor gene transfer (Tsen et al., 2009).

Cell Squeezing "Microfluidics Gene Transfer"

Microfluidic gene transfer has been newly developed for in vitro delivery of proteins and nucleic acids into cells using the principle of cell deformation (Sharei et al., 2013). The original concept emerged in early 1990s in studies of macromolecules loading into cells using a method that was developed back then called syringe loading (Clarke & McNeil, 1992). It was shown that the mechanism of transfer is the transient defects in cell membrane that are created as cells are passed back and forth through a standard syringe needle or similar narrow orifice. The principle, also called shear-induced intracellular uptake, was later refined and special devices were developed in which cells are deformed and the membrane is perforated upon flow through microchannels with proper velocity. The method indeed successfully mediated uptake of large molecules such as dextran and bovine serum albumin into suspended prostate cancer cells, without compromising cell viability (Hallow et al., 2008). Recently, this method was implemented in gene transfer and demonstrated promising success for in vitro siRNA delivery (Sharei et al., 2013). It has been shown that the size and the frequency of the transient holes, and hence transfection efficiency are functions of the shear and compressive forces that the cells experience when passing through the constriction. In turn, these forces are determined by the flow rate of cells, dimensions, and the number of constrictions. More recently, kinetic studies of membrane recovery after cell squeezing technique have shown that calcium content in cell

medium is another major determinant of transfection efficiency, and that calcium depletion significantly increased macromolecule uptake by squeezed cells (Sharei et al., 2014). This is because the active membrane recovery is mediated by calcium-dependent signaling (McNeil & Steinhardt, 2003).

Future Perspectives

Revolutionary advances have taken place in the field of gene delivery over the past decades. Physical methods, in particular, have gained increased attention in attempt to overcome barriers of chemical and viral methods. However, clinical application of physical methods is rather limited, largely imposed by some challenges that necessitate additional research and improvements to the current systems. The hallmark for efficient gene transfer is the application of mechanical forces to disrupt cell membranes for the DNA to get in. These forces, however, are not risk free, as in many cases efficient gene transfer is accompanied with local tissue damage, which is often considered as intolerable in clinical practice. In addition, while skin is easily accessible, gene transfer to inner organs, such as liver and kidneys, with physical methods requires invasive, usually surgical procedures to insert the applicators close to the target tissues, presenting an additional medical concern for disease management. Moreover, the tissue fraction that is successfully transfected with most physical methods is often limited. This might explain the bias in exploring physical methods in gene therapy using secreted proteins more than intracellular proteins, where the locally expressed protein is sufficient to drive global effects, such as vaccination and growth factor replacement therapies. Nonetheless, the technology of physics-based gene delivery has now advanced to a point from where it seems feasible to implement minimally invasive strategies for gene transfer into various target tissues, with

adequatelevels of transfection and minimal side effects. This is clearly illustrated in the development of image-guided computerized hydrodynamic gene transfer into large animals. The method combines tunable parameters with facile application into a specific organ, and specific area within the organ, and thus, it truly holds the promise for clinical applications in human gene therapy. Moreover, these computerized methods will also enhance the reproducibility and reduce variations commonly associated with manual procedures. Future research will focus on the amenability to physically manipulate biological and physiological features of tissues aiming to facilitate gene transfer. Joint application of different kinds of physical forces will also be considered sincerely to improve the overall efficiency, and to minimize the magnitude of the applied forces. Innovative integration of physics, chemistry, biology, and computer engineering will pave the way for the urgently needed breakthroughs in the field of nonviral gene delivery and for human gene therapy.

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APPENDIX B

ALTERNATIVE STATISTICAL ANALYSIS FOR CHAPTER 4 FIGURES

The data presented in chapter 4 of this dissertation have been statistically analyzed using the student t-test. The first comparison was made between the two control groups to denote the establishment of obesity phenotype, i.e. statistically significant induction of weight gain, insulin resistance, adipose inflammation and others. The second comparison was made between the HFD-fed control group and the HFD-fed, IFNβ-treated group to denote the therapeutic outcomes of IFNβ overexpression in blockade of obesity development. As per committee members' request, data figures in chapter 4 are represented here with statistical comparison made between the three groups using one-way analysis of variance method (ANOVA), to denote the statistical difference between the three groups together. See chapter 4 for figure legends.

In each subfigure, the shown *P* value is calculated by One-Way ANOVA to reflect statistical difference between the three groups. The asterisks represent the post-hoc statistical analysis between individual groups. (*: $P < 0.05$, **: $P < 0.01$).

Figure 4.1:

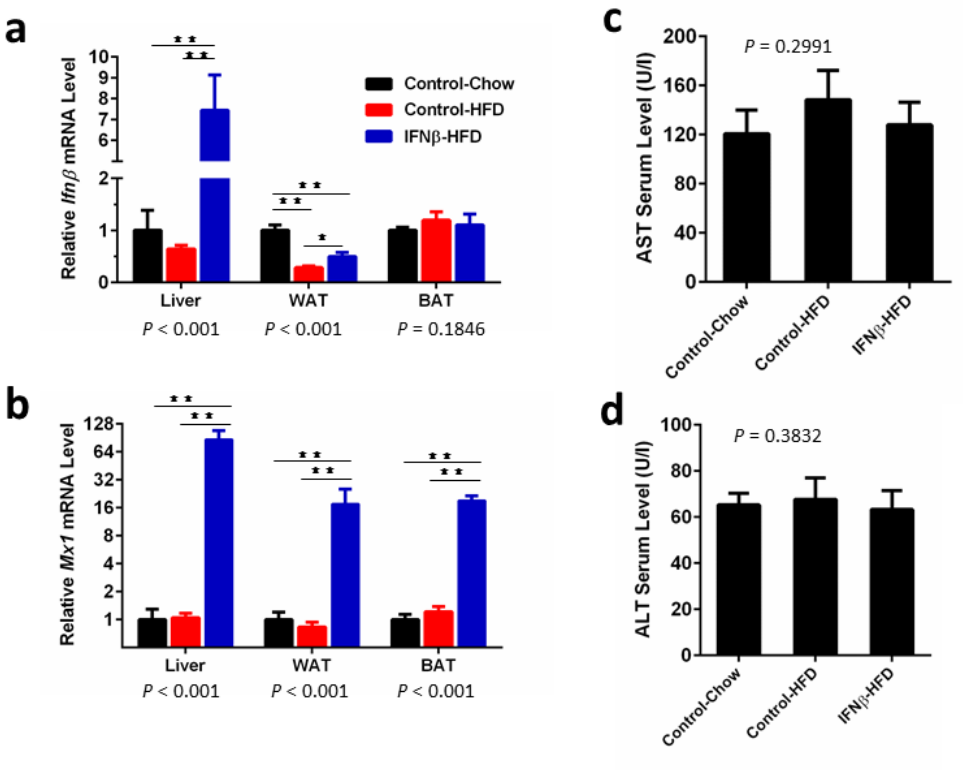

a
Relative Ifnβ mRNA Level
Control-Chow
Control-HFD
IFNβ-HFD
Liver
WAT
BAT
P < 0.001
P < 0.001
P = 0.1846
b
Relative Mx1 mRNA Level
Liver
WAT
BAT
P < 0.001
P < 0.001
P < 0.001
c
AST Serum Level (U/l)
P = 0.2991
Control-Chow
Control-HFD
IFNβ-HFD
d
ALT Serum Level (U/l)
P = 0.3832
Control-Chow
Control-HFD
IFNβ-HFD

Figure 4.2:

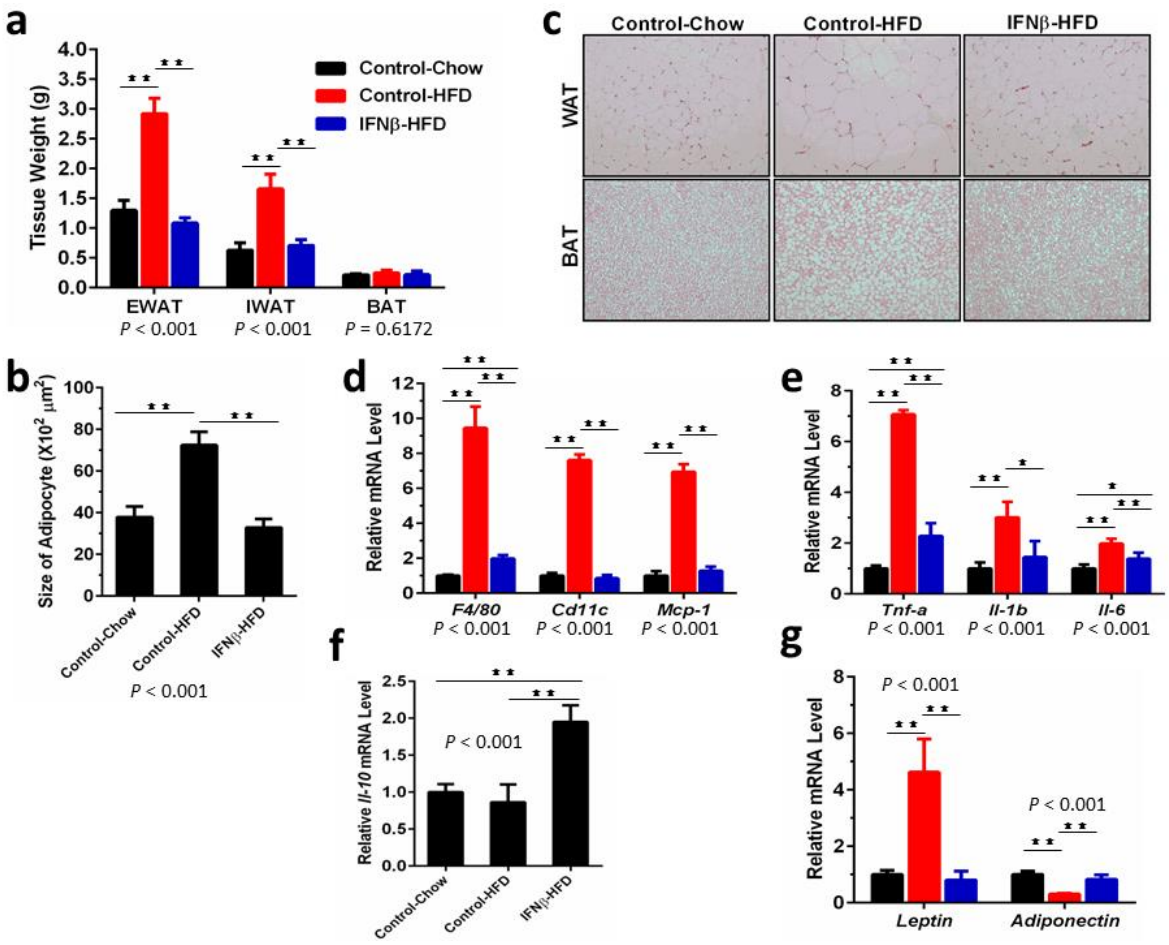

a
Tissue Weight (g)
Control-Chow
Control-HFD
IFNβ-HFD
EWAT
IWAT
BAT
P < 0.001
P < 0.001
P = 0.6172
c
Control-Chow
Control-HFD
IFNβ-HFD
WAT
BAT
b
Size of Adipocyte (X10² μm²)
Control-Chow
Control-HFD
IFNβ-HFD
P < 0.001
d
Relative mRNA Level
F4/80
Cd11c
Mcp-1
P < 0.001
P < 0.001
P < 0.001
e
Relative mRNA Level
Tnf-a
Il-1b
Il-6
P < 0.001
P < 0.001
P < 0.001
f
Relative Il-10 mRNA Level
P < 0.001
Control-Chow
Control-HFD
IFNβ-HFD
g
Relative mRNA Level
P < 0.001
P < 0.001
Leptin
Adiponectin

Figure 4.3:

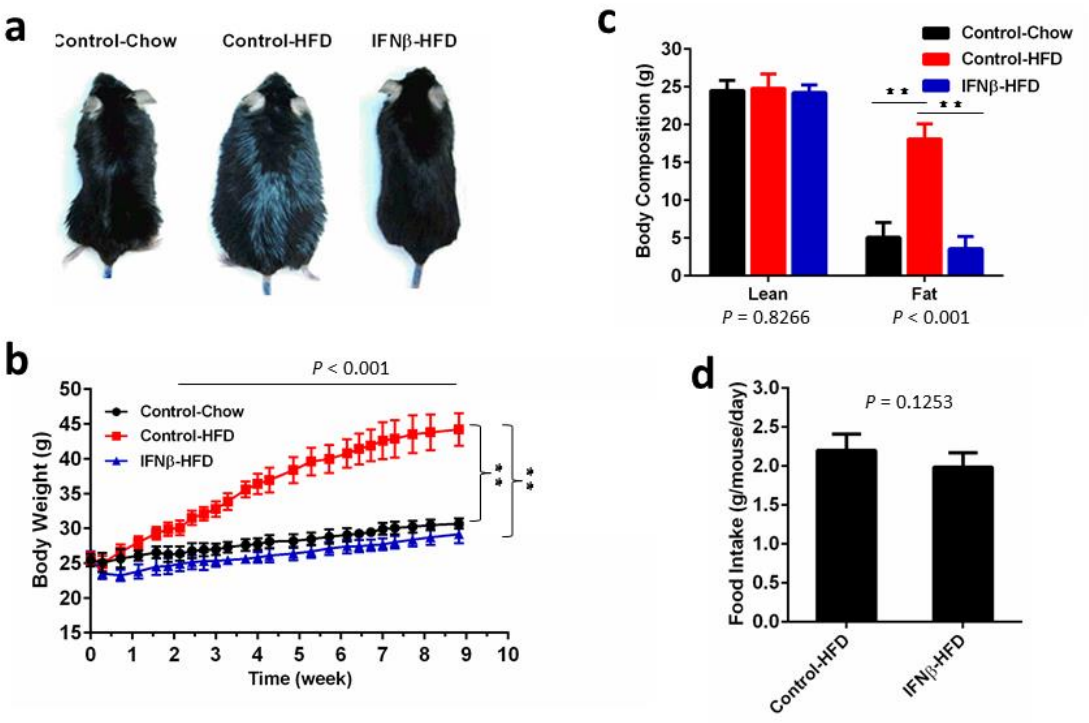

a
Control-Chow
Control-HFD
IFNβ-HFD
b
P < 0.001
Control-Chow
Control-HFD
IFNβ-HFD
Body Weight (g)
Time (week)
c
Control-Chow
Control-HFD
IFNβ-HFD
Body Composition (g)
Lean
P = 0.8266
Fat
P < 0.001
d
P = 0.1253
Food Intake (g/mouse/day)
Control-HFD
IFNβ-HFD

Figure 4.4:

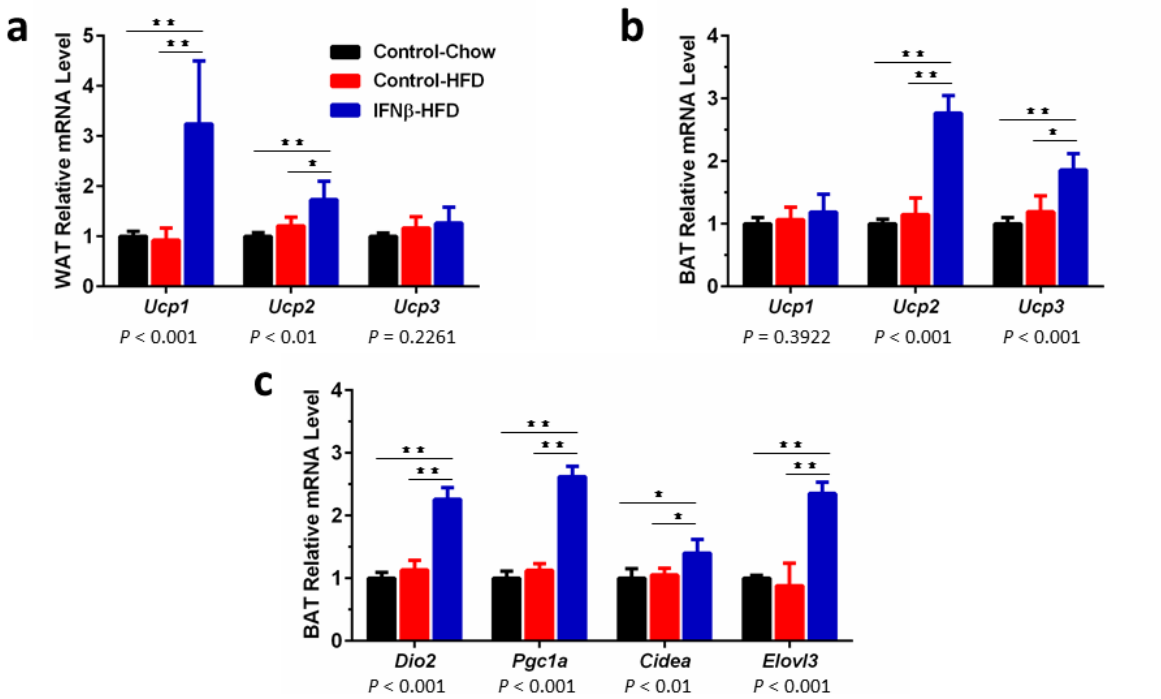

a
WAT Relative mRNA Level
Control-Chow
Control-HFD
IFNβ-HFD
Ucp1
P < 0.001
Ucp2
P < 0.01
Ucp3
P = 0.2261
b
BAT Relative mRNA Level
Ucp1
P = 0.3922
Ucp2
P < 0.001
Ucp3
P < 0.001
c
BAT Relative mRNA Level
Dio2
P < 0.001
Pgc1a
P < 0.001
Cidea
P < 0.01
Elovl3
P < 0.001

Figure 4.5:

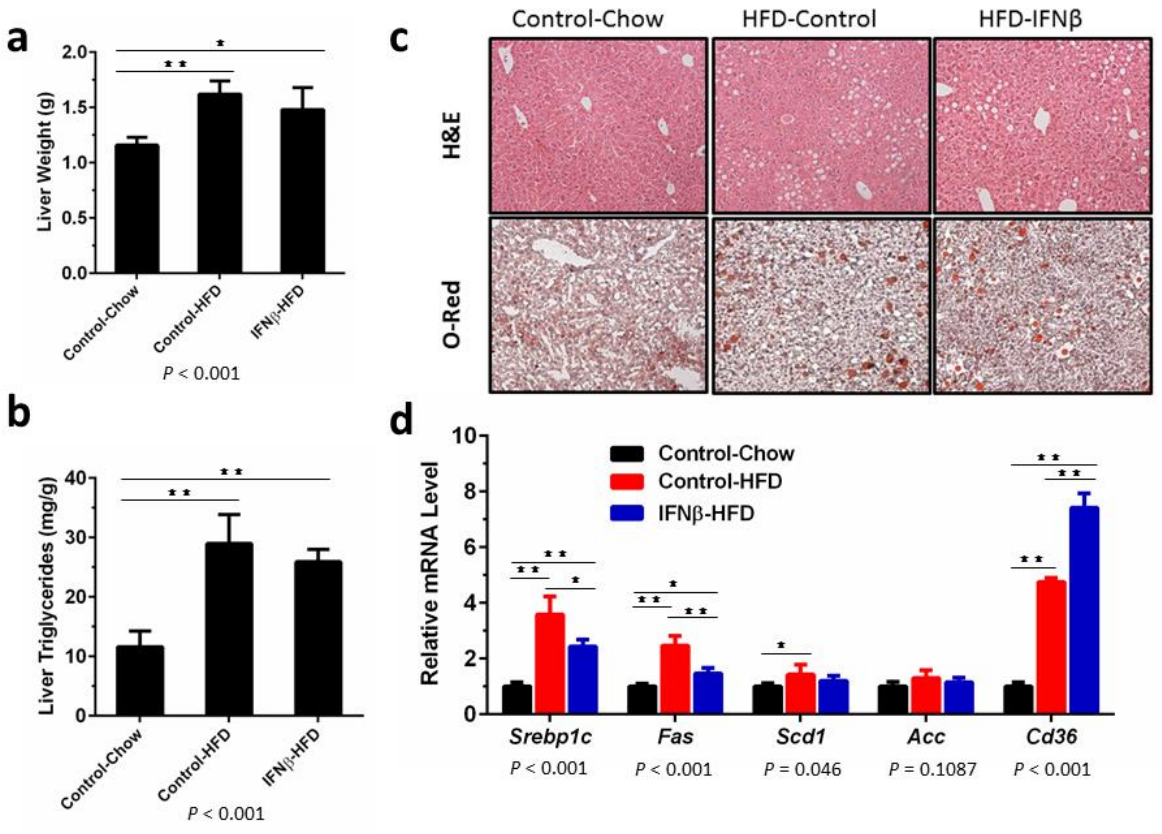
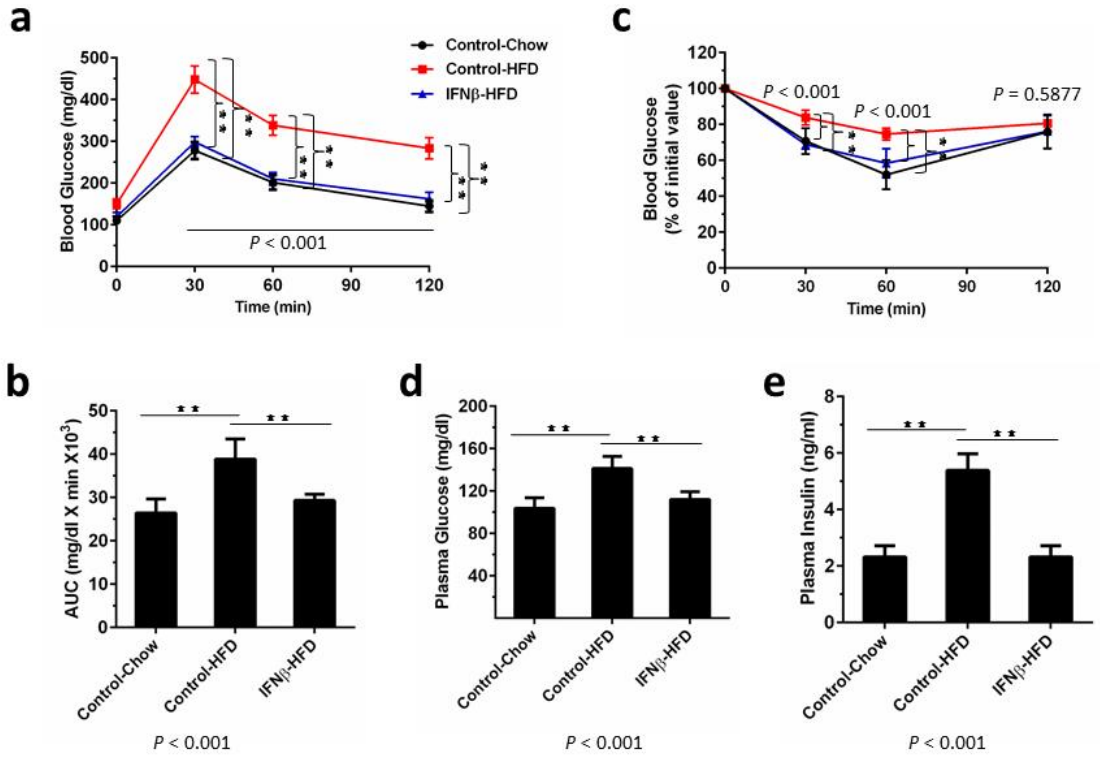

a
Liver Weight (g)
2.0
1.5
1.0
0.5
0.0
Control-Chow
Control-HFD
IFNβ-HFD
P < 0.001
b
Liver Triglycerides (mg/g)
40
30
20
10
0
Control-Chow
Control-HFD
IFNβ-HFD
P < 0.001
c
Control-Chow
HFD-Control
HFD-IFNβ
H&E
O-Red
d
Relative mRNA Level
10
8
6
4
2
0
Control-Chow
Control-HFD
IFNβ-HFD
Srebp1c
Fas
Scd1
Acc
Cd36
P < 0.001
P < 0.001
P = 0.046
P = 0.1087
P < 0.001

Figure 4.6:


a
Control-Chow
Control-HFD
IFNβ-HFD
Blood Glucose (mg/dl)
Time (min)
P < 0.001
c
Blood Glucose (% of initial value)
P < 0.001
P < 0.001
P = 0.5877
Time (min)
b
AUC (mg/dl X min X10³)
Control-Chow
Control-HFD
IFNβ-HFD
P < 0.001
d
Plasma Glucose (mg/dl)
P < 0.001
e
Plasma Insulin (ng/ml)
P < 0.001