Preface

There are a few moments, defining the research path of one's career that remain crystal clear and as memorable as yesterday. For both of us, one such moment was our learning of the process of RNA interference and the stunning realization of its implications in our discipline. Being immunologists by training, we have been interested in exploring how to either activate this T cell more toward one direction or manipulate this dendritic cell in another. We have been used to doing this through different tissue culture conditions, or addition of chemical inhibitors: these having the drawbacks of unscalability and unspecificity, respectively.

It was a cold Canadian night in the winter of 2001. We were having coffee at the Hospital Cafeteria waiting for some data coming out of the laboratory, and both of us were talking about the future of immunology. The need for specific ways of modulating genes so that we would be spared of the need for impractical approaches was discussed. "How exciting would it be just to use antisense oligonucleotides to silence immune stimulatory genes in the dendritic cell?" "It must have been performed already." "If it has, then why don't we know about it?" "It's much easier to evoke a therapeutic effect by modulating immunological genes, in that, unlike viral or oncogenes, even a 20% gene inhibition will cause a biological response." "Someone must have done that with antisense already." As this was before the time everyone had a Blackberry and an iPhone, we would have to wait until we got upstairs to check Pubmed. But we continued the conversation: "Is there anything better than antisense? What about ribozymes?" And this led to the discussion regarding RNA interference.

At that time, the concept of RNA interference was still restricted mainly to the world of molecular biologists. We remembered a dear friend telling us about this bizarre phenomena, whereby introduction of a double strand of RNA would induce cleavage not only of the introduced nucleic acids but also any other nucleic acids that resembled it. He told us about this being the "next antisense" since it is part of the body's endogenous defenses against viruses and therefore theoretically should be more potent for silencing. "It's easier to take away a gene than add one." "Yes, but the double strands would activate interferon responses – The paper our friend told us about was in worms." "But imagine if there was a way to get around that? Plus if you use it to suppress immune suppressive cytokines in cancer then the interferon alpha response is actually beneficial."

We left our coffees and hurriedly went to the computers upstairs to see what has and has not been done in this field. We printed out everything that had the words "RNA interference" the 1998 Nature paper that described RNA interference in worms (which subsequently won Fire and Mello the Nobel Prize), the paper by Elbashir et al. showing that the interferon alpha response can be avoided in mammals, the work describing use of siRNA for studying mammalian genes. That night, neither of us had much sleep thinking about the possibility of specifically silencing immunological genes. We had a perfect model, the dendritic cells, which reside at the center of the "immunological universe," and are relatively simple cells to transfect and manipulate, Wei-Ping having already induced them to express various immune regulatory genes such as FasL

Initial silencing of the interleukin-12 p35 gene was performed. The degree of knock-down was phenomenal. These data led us into a journey that continues today, having silenced both immune suppressive and immune stimulatory genes ranging from cytokines, to membrane proteins, to oncogenes, to transcription factors. This journey has taken us personally from ex vivo cell manipulation to current cell-targeting immunoliposomes that deliver siRNA to dendritic cells only, thus alleviating the need for hydrodynamic injection. Disease models treated have included rheumatoid arthritis, allergy, transplant rejection, and cancer.

When we were contacted by Humana with the possibility of being editors for this volume, we gladly accepted it. In the same way that we described our personal journey, we aimed in this book to represent the journey of our field. From those early days where RNA interference was a strange artifact in worms, to the 2006 Noble Prize to Fire and Mello, to the current clinical trials and the $1 billion purchase of a siRNA company by Merck, the field of RNA interference has grown at a breakneck pace.

In this volume, we will overview the science and the Protocols at present that span the biological disciplines from detailed nucleic acid chemistry, to pharmacology, to manipulation of signal transduction pathways. By compiling an overview of the different ongoing areas of scientific investigation of RNAi, we hope to do two things: stimulate new questions and provide you with the tools to start addressing those questions. The book is divided into three main segments. The first deals with the Physiology of RNA Interference, in which we try to overview the biological relevance of this process and provide a context for the next sections. The second section, entitled "RNA interference in the laboratory and siRNA delivery" outlines practical uses of RNAi either as research tools or as components in the development of therapeutics. Finally, the last part of the book deals with actual preclinical and clinical issues associated with the use of RNAi-inducing agents as drugs. Through this clustering of chapters in segments, we hoped to provide a logical context for the current state of the art.

Starting the first section, Drs. Abubaker and Wilkie from University of Guelph, Canada provide a comparative biology examination of the relevance of RNAi processes to viral defense. They overview commonalities and differences between gene silencing effector mechanisms and host–parasite interactions in forms of life ranging from fungi, to worms, to insects, to mammals. Subsequent to establishing an overall framework for understanding the various biological pathways associated with RNA interference as a gene-specific mechanism of defense, they move into a discussion on innate defense mechanisms, namely the ability of double-stranded RNA molecules to activate the interferon alpha response through activation of toll like receptors (TLR) 7/8 and the acid inducible gene I (RIG-I). In the subsequent chapter, Drs. Gantier and Williams from Monash University in Clayton, Australia review the relevance of this "danger-associated" TLR pathway as a method of immune activation and provide methodology for assessment, in both mouse and man, of its activation. RNAi-induction by microRNA (miRNA) also plays a role of fundamental innate protection mechanisms against pathogens. The miRNA can be pre-existing in the host cell or can be transcribed by the invading virus. Drs. Ouellet and Provost from Laval University in Canada, go into considerable detail across the major viruses to discuss the impact of host and viral miRNA in the battle for survival. Of particular interest are the analytical methods for detection of even transiently expressed miRNAs.

The exquisite sensitivity and selectivity of RNAi induction allows for knock-down of specific alleles of a gene. Dr. Hohjoh from the National Institute of Neuroscience in Tokyo, Japan, provides protocols for silencing of the Photinus and *Renilla luciferase* genes

in mammalian cells. The same selectivity that allows for allele-specific silencing by siRNA also requires great care in designing siRNAs, in that numerous factors contribute to silencing efficacy. The issue of siRNA-designing algorithms is reviewed by Dr. Kim from the University of Science & Technology in Daejeon, Korea who presents the AsiDesigner, a web-based siRNA design program that takes into consideration alternative splicing in designing optimum siRNAs. Drs. Muhonen and Holthöfer from Dublin City University, Dublin, Ireland, continue on the theme of optimizing siRNA design by discussing issues of target messenger accessibility and provide various bioinformatics approaches for identifying active and specific sites on the mRNA for silencing. Dr. Ishigaki's group from the Kanazawa Medical University, Kanazawa, Japan, describes another method of increasing potency of siRNA. In their chapter, shRNAs are expressed on a single plasmid, so that by concurrently targeting different areas of the same transcript, increased silencing may be achieved. They proved a detailed protocol for generating dual shRNA expressing plasmids and describe various methodological peculiarities of this approach. Of particular relevance to therapeutic development, the authors detail possible adverse effects by overconsumption of cellular transcription machinery when various promoters of shRNA transcription are used. Practical application of multi-shRNA derived from a single plasmid could include suppression of HIV. Drs. Rossi and Zhang from the Beckman Research Institute, City of Hope, CA, address this possible therapeutic approach through disclosing their technique involving a new combinatorial anti-HIV gene expression system that allows for simultaneous expression of multiple RNAi effector units from a single Pol II polycistronic transcript. In their system, they avoid the cell toxicity associated with expressing numerous shRNAs from Pol III promoters by using endogenous RNAi transcripts and miRNAs for expression of multiple RNAi effector units off a single Pol II polycistronic transcript. University of Vienna's Dr. Hofacker, subsequently discusses in silico tools that consider only siRNA-specific design criteria and those that integrate mRNA structure features as well as basic siRNA features for selection of shRNA and siRNAs. The final chapter of the First Section is by Dr. Engels et al. from J.W. Goethe-Universität in which protocols for synthesis of various siRNAs are provided.

In the Second Section, we transition from the biology of RNA interference to issues related to implementation, both in the laboratory setting as a basic research reagent and as a potent tool useful for the development of therapeutics for diseases. Dr. Zheng et al. from University of Western Ontario, Canada, begin the section by describing methodology for producing cell-targeting siRNA-bearing immunoliposomes. Through the ability of immunoliposomes to selectively bind to antigen-expressing cells corresponding to the antibody on the immunoliposome, the investigators provide a delivery platform that is relatively simple to generate and has widespread applications. The original method of in vivo siRNA delivery, hydrodynamic injection, is reviewed in the next chapter by Drs. Evers and Rychahou from the University of Texas. This method involves a rapid administration of high volume siRNA intravenously, which temporarily causes micropores and loosening of tight junctions in the endothelium, causing siRNA entry across the plasma membrane into intracellular compartments. To date, this method has been used to deliver siRNA to the liver, lungs, and brain.

In the same way that DNA array technologies have allowed for en masse identification of gene expression patterns in various cells and biological conditions, the knock-down of genes using high throughput siRNA technologies has allowed for the understanding of cellular phenotypes after a gene is suppressed. Fujita et al. from the Research Institute for Cell Engineering (RICE) and the National Institute of Advanced Industrial Science and

Technology (AIST), Tokyo, Japan, describe two protocols for reserve transfection of siRNA molecules on solid surfaces, the first for microarrays and the second for microtiter plates.

Moving from general to specific, the use of siRNA in specific pathologies is examined in greater detail. Prakash et al. from McGill University, Canada, are focused on neurodegenerative diseases and the means of traversing the blood brain barrier. They provide a detailed review of the state of the art regarding neurological uses of siRNA and subsequently describe the generation of optimized siRNA sequences and delivery methods for in vivo targeting using cationic nanoparticles. Huang et al. from the Chang Gung Memorial Hospital-Kaohsiung Medical Center in Taiwan used a bioinformatics approach to selectively identify genes in lung cancer through random knock-down and assessment of phenotype. Using this approach, they identified FLJ10540, a target associated with cancer invasion and migration. In their chapter, they describe upstream and downstream control of this tumor-associated factor. Delivery of siRNA and shRNA, of particular interest to cancer models, is described in the Chapter of Drs. Jere and Cho (Seoul National University, Korea) who provide protocols for generation of biodegradable cationic polymers. Methods of tracking cellular update and intracellular trafficking as well as protocols for the evaluation of the impact on cancer cells are provided. While selective delivery of RNAi-inducing molecules has been performed with immunoliposomes or affinity-targeting agents, an interesting approach is described by Ohtsuki's group from Okayama University in Okayama, Japan, who used HIV-tat conjugation of siRNA to allow intracellular delivery and could activate the gene silencing process using photons. This novel method, termed CPP-linked RBP-mediated RNA internalization and photoinduced RNAi (CLIP-RNAi), could have many applications in therapeutic scenarios where localized silencing is desirable.

The issue of siRNA degradation is examined by Aigner et al. who utilize various polyethylenimines to increase protection from nucleases, both extracellular and intracellular. In their chapter, the authors provide a comparison of the different polyethylenimines in respect to cationic charge, ability to form noncovalent interactions with siRNA, and compaction of the siRNA into complexes that allow for internalization by endocytosis. On the same topic of crossing the plasma membrane, Brito et al. from King's College, London, England provide a rather interesting transfection methodology: temporary permeabilization with streptolysin-O. They provide protocols that have been optimized for gene silencing of multiple myeloma cell lines, which have great importance for therapeutics development.

The third section of the book covers the issue of clinical implementation of RNAi. A look at www.clinicaltrials.gov , the NIH registry for ongoing clinical trials, reveals seven ongoing clinical investigations using RNAi induction for conditions such as wet macular degeneration, infectious diseases, and cancer. The current chapter will address some of the issues that need to be addressed in the translation of this new class of therapeutic approaches. Dr. Akaneya from the Osaka University Graduate School of Medicine, Japan, begins by describing the advantages and disadvantages of using RNAi-inducing approaches for neurological conditions. Specific diseases discussed include ALS and inflammatory conditions. Issues such as immunogenicity, interferon response, and localization are discussed. Drs. Mao and Wu from Johns Hopkins School of Medicine, Baltimore, describe specifics of using RNAi-based approaches in cancer immunotherapy. They discuss various important immunological targets starting with specific effector molecules, and then moving on to more general upstream transcription factors such as STATs and other global regulators of numerous immune response genes. The issue of endogenous miRNA controlling of the immune response, both natural and stimulated, is also overviewed.

The authors conclude by evaluating various RNAi-inducing approaches for the most rapid clinical translation in immunotherapy of cancer.

Tissue injury prevention by RNAi strategies is discussed by Zhang et al. from University of Western Ontario, Canada. They provide details of assays used to assess renal injury in an ischemia/reperfusion model and prevention by suppression of caspase transcription. From the same Institute, Drs. Zhang and Li present protocols for the in vitro silencing of dendritic cells with siRNA and subsequent use of these cells to modulate and/or suppress transplant rejection. The advantage of this approach is the potent immune stimulatory/immune suppressive ability of DC dependent on expression of costimulatory molecules. Targeting of RelB, an NF-kB family member, is demonstrated in the protocols, which causes suppression of various cytokine and costimulatory molecules on the dendritic cell, this suppression associated with inhibited immunogenicity.

Continuing on the theme of immune modulation, Ritprajak et al. from Tokyo Medical and Dental University, Tokyo, Japan utilize siRNA to enter across the stratum corneum and into dermal dendritic cells. By modulating these cells, the authors describe suppression of costimulatory molecules and possible use for treatment of allergic disease. Sarret et al. from University of Sherbrooke, Canada, use RNAi to tackle the problem of pain in a nonpharmacological manner. They discuss protocols for siRNA administration, targets, and behavioral systems used in researching this unique approach to pain management, with particular reference to G protein-coupled receptors. Seth et al. from MDRNA Inc, Bothell, USA, describe the use of RNAi in treatment of respiratory viruses, with emphasis on influenza. They describe various viral targets, animal models, and methods of delivery for maximum antiviral activity. An interesting subject is the interaction between siRNA that stimulates interferon alpha responses and the overall antiviral activity of these molecules. Drs. Malek and Tchernitsa from the Institute of Pathology, Charité – Universitätsmedizin Berlin, Germany and Oncology Institute of Southern Switzerland provide detailed protocols for silencing of ovarian cancer cells in vitro and in vivo. Of particular interest is the clinically relevant human xenograft ascites model that is described.

As you may see, the progress of RNA interference research has been significant. The question of whether it will deliver on its promise is still open; however, we hope this volume will provide to you, our reader, the same amount of excitement we've had in seeing the field progress to where it is today.

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