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RNA干扰
从生物学到临床应用

RNA Interference
From Biology to Clinical Applications

Wei-Ping Min and Thomas Ichim

科学出版社

实验室解决方案

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RNA 干扰

从生物学到临床应用

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RNA 干扰：沉默是理，沉默是金

RNA 干扰从其发现至今不足 15 年的时间，已经成为生命科学领域一种不可或缺的知识结构及研究手段。从生物学角度而言，RNA 干扰是存在于生物体内的一种天然过程，负责限制外源性核酸，包括病毒及其他 DNA 或 RNA 分子，从而稳定体内的核酸。我们传统意义上对 RNA 干扰的理解还局限于外源性的双链 RNA 进入体内后产生的siRNA所诱导产生的基因转录后沉默效应，但是，近些年来，越来越多的文献报道不仅拓宽了原来关于 siRNA 所诱发的 RNA 干扰的理解，发现其不仅局限于对基因的转录后沉默，还参与了基因的转录水平的调节及翻译的抑制过程；而且，证明一些内源性的非编码 RNA，包括 miRNA 事实上属于 RNA 干扰的内源性过程，这一过程尤其在病毒与宿主细胞相互作用过程中得到很好地体现。本书的第 1 章和第 3 章中，针对上述 RNA 干扰概念的理解及其拓展都有系统的介绍，从而为使用 RNA 干扰作为重要的技术进行基因调节治疗奠定了基础。

RNA 干扰可以高效、特异地沉默目标基因，从而不仅可以在功能基因组学中用于研究基因的功能，而且可以用于基因高表达相关的疾病的治疗中，如病毒感染、肿瘤及神经退行性疾病等。但是，这一技术的具体应用却存在很多的障碍。

第一，人们曾经以为 siRNA 足够小，不会诱发机体的先天免疫，但是自 2005 年开始，人们逐渐发现 siRNA 同样可以诱发机体非常强的先天免疫反应，从而一定程度上阻碍了 RNA 干扰的应用。针对这一问题，本书第 2 章不仅概述了哪些 siRNA 分子容易诱发机体的先天免疫，而且给出了快速鉴定可以诱发先天免疫反应的 siRNA 分子的具体方案，从而为筛选可用于体内治疗性研究的 siRNA 提供了有效的筛选手段。

第二，许多疾病中，遗传基因的改变可能仅仅发生在等位基因中的一个，这就要求应用 RNA 干扰手段进行这种疾病治疗时，所使用的 siRNA 必须只能抑制突变后的等位基因，而对野生型等位基因没有影响。这就是本书中第 4 章所提到的等位基因特异性基因沉默。这一点对于疾病的治疗是非常关键的。

第三，RNA 沉默最主要的特点在于其特异性及高效性，但是大量研究已经发现 siRNA在很多情况下会产生“脱靶效应”，即导致非靶基因的沉默；而且，很多情况下 siRNA 的沉默效果也不是很好。针对这些情况，本书第 5 章、第 6 章及第 9 章不仅综述了目前 siRNA 设计的一些主要原则，而且增加了一些新的考虑因素，如在哺乳动物体内最常见的可变性剪接以及 siRNA 针对靶 mRNA 的可接近性问题等，从而为设计具有更高特异性、有效性的 siRNA 分子奠定生物信息学基础。同时，为了达到对基因相对较高的沉默效率，可以选择将多个 shRNA 串联表达，使用 RNA pol III（第 7 章）或 RNA pol II（第 8 章）的启动子，都可以达到相对较高的沉默效率，相比较而言，后者毒副作用更小。另外，siRNA 的稳定性问题也很大程度上影响其沉默效果。第 10 章给出了将 siRNA 进行 2′-氧-烷基修饰的具体过程，这种修饰不仅增强了 siRNA 的稳定性，而且更容易被细胞摄取，从而增强其沉默效率。

第四，RNA 干扰成功应用之前需要解决的另一个非常重要的问题就是 siRNA 的导入

问题，如何做到高效、特异、靶向。本书的第11～19章着重描述这一问题的解决方法。根据实验目的的不同，可以选择抗体导向的免疫脂质体将siRNA特异性的靶定目标细胞（第11章），也可以利用流体力学注射的方法将siRNA成功地导向肝脏、肺或脑部（第12章）；如需穿过血脑屏障，则可以选择阳离子纳米技术（第11章）；或者利用可降解的阳离子多聚物将siRNA定向导入癌症模型（第16章）；如果仅需要进行局部基因沉默，则有光诱导的RNA干扰方法可选（第17章）；或者也可以利用聚乙烯亚胺类物质PEI包裹的方法将siRNA导入目标细胞，不仅不容易被核酸酶降解，而且细胞摄入率高，细胞内的释放率也很高（第18章）。对于那些极难转染的细胞，则可以应用链球菌溶血素O可逆性渗透的方法将siRNA导入目标细胞（第19章）。另外，还可以利用固体表面进行siRNA的反式转染（第13章）或结合基因芯片、生物信息学及siRNA转染等方法（第15章），筛选并鉴定功能基因。这不仅解决了siRNA的导入问题，对于功能基因组研究中基因功能的研究也非常重要。

最后，还要考虑到siRNA应用于临床治疗可能遇到的问题。这里列举了目前RNA干扰应用于临床治疗的一些领域，包括神经病学相关疾病治疗（第20章）、癌症免疫治疗（第21章）、组织损伤（第22章）及免疫排斥反应（第23章）、过敏性疾病治疗（第24章）、疼痛（第25章）、流感（第26章）及卵巢癌的治疗（第27章）等。这些领域RNA干扰应用过程中特异性靶mRNA的选择及siRNA的高效靶向性导入问题等都在书中有详细介绍。

总之，RNA干扰引发的基因特异性沉默，不仅是生物进化过程中一种保守的机体防御机制，由此所产生的技术更为生命科学领域的研究开拓了新思路、新方法，并为疾病的治疗带来了新途径、新希望！因此，RNA干扰引发的基因沉默是“理”，也是“金”。

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前　言

在每位研究者的科研之路上，总有一些时刻好像昨天一样清晰并令人记忆深刻。对我们两个而言，在这一刻，我们了解了 RNA 干扰的过程，并实现了它在我们学科的应用。作为免疫学家，我们一直试图研究出如何激活更多的 T 细胞或者可以控制树突细胞。为达到这个目的，我们已经习惯于采用不同的组织培养条件和添加化学抑制剂的方法。但是二者都存在缺点：前者无法定量，后者则不具有特异性。

那是 2001 年加拿大的一个寒冷的夜晚。我们在医院的自助餐厅里享用咖啡，一边等待实验室的数据结果一边畅谈免疫学未来的发展。我们都认为，现在急需一种专门的技术可以调控基因，这样就能少走一些弯路。“如果只用反义寡核苷酸就能将树突状细胞的免疫刺激基因沉默，那该多好?”“肯定已经有人做过了。”“如果是这样，我们为什么不知道?”“这与病毒癌基因或癌基因是不一样的，调节免疫相关的基因一定极易产生治疗效应，而且即使仅抑制 20%的基因，也将产生生物学应答反应。”“肯定已经有人用这种方法试过了。”当时，不像现在人人都有黑莓（Blackberry）或苹果（iPhone）智能手机，我们必须去楼上通过 Pubmed 查询才能知道。但我们的谈话没有终止，“有没有比反义寡核苷酸更好的办法呢？核酸酶怎么样?”就这样我们谈到了 RNA 干扰。

当时，RNA 干扰这个概念还只是局限在分子生物学家的圈子里。还记得当时一个好朋友告诉过我们这种奇怪的现象，当导入一段双链的 RNA 之后，不仅导入的 RNA 本身会被剪切，而且还会使和其相似的任何 RNA 被剪切。他告诉我们，这就是未来的“反义核酸”。因为它是机体内源性的抗病毒机制，因此从理论上来说这种沉默会有更好的效果。“减少一个基因比增加一个基因要简单的多。”“是的，但双链的核酸会引起机体的干扰素反应——朋友给的文献中，实验是在线虫中做的。”“但如果我们可以避免呢？另外，如果是在癌细胞中用它去沉默免疫抑制细胞因子，那么干扰素 α 是有益的”。

我们放下咖啡，急忙去楼上用电脑查找这个领域的研究进展情况。我们将所有有关 RNA 干扰的资料都打印出来。1998 年，Nature 杂志上的文献第一次描述了线虫中的 RNA 干扰现象（这项工作使 Fire 和 Mello 后来获得了诺贝尔奖），Elbashir 的工作则表明在哺乳动物中可以避免干扰素 α 反应，他们是用 siRNA 来研究哺乳动物基因的。那晚，我们都没怎么睡觉，一直在思考特异性的沉默某个免疫基因的可能性。我们已经有一个很好的模型，即处于免疫系统中心地位的树突状细胞，这些细胞很容易进行转染和操作。而且，Wei-Ping Min 已经诱导这些细胞表达多种免疫调控基因，如 FasL。

我们首先沉默了白介素-12 p35 基因，其沉默效果很显著。这些数据让我们的探索之旅一直延续到今天，如今，我们已经沉默了包括细胞因子、膜蛋白、癌基因、转录因子在内的免疫抑制和免疫激活基因。此外，我们已经从体外操作发展到通过免疫脂质体靶定细胞，即只将 siRNA 导入树突状细胞中，这样就减少了流体力学注射的使用。已经试验的疾病模型有风湿性关节炎、过敏、移植排斥和癌症。

当 Humana 出版社邀请我们编写这本书时，我们欣然接受了。就像前面所描述的研究之路，我们将通过这本书来展现我们这个领域的研究进展。从最开始 RNA 干扰还只是注

射到线虫里的一个人工合成物，到 2006 年 Fire 和 Mello 获得诺贝尔奖，再到现在临床试验以及 Merck 公司花 10 亿美元购买 siRNA 公司，RNA 干扰领域的发展日新月异。

在这本书中，我们总结了已有的理论知识和实验方法，包括具体的核酸的生化知识、药理学和信号转导通路的调控。通过收集不同领域对 RNA 干扰的探索经验，我们想达到两个目的：启发新的问题，为读者提供解决这些问题的工具。这本书包括三个部分：第一部分是 RNA 干扰的生理机制，主要阐述这个过程的生物学基础并为下一部分做铺垫。第二部分的题目是，“实验室的 RNA 干扰和 siRNA 的导入”，主要介绍 RNA 干扰作为研究手段和治疗方法的实际应用。最后一部分重点讲述 RNA 干扰相关药物使用的临床前期及临床相关的实际问题。通过这样的划分，我们希望可以使本书的编排显得更具逻辑性。

在第一部分的开始，来自加拿大 University of Guelph 的 Abubaker 和 Wilkie 博士从比较生物学的角度分析了 RNA 干扰和病毒防御过程的相关性。他们总结了果蝇、线虫、昆虫和哺乳动物中基因沉默效应机制和宿寄主之间相互关系的异同点。在建立了 RNA 干扰作为机体的一种基因特异性防御机制的相关生物学途径的总体框架后，他们开始讨论先天防御机制，即双链 RNA 分子通过激活 toll 样受体 TLR7/8 和维甲酸诱导基因 RIG-I 所诱导干扰素 α 反应的能力。接下来一章，来自澳大利亚 Clayton 的 Monash University 的 Gantier 和 Williams 博士综述了危险相关的 TLR 途径作为一种免疫激活方法的相关性，并提供了小鼠和人类中评估其免疫激活的方法。miRNA 诱导的 RNAi 也参与机体对病原体的先天防御机制。miRNA 可能提前就存在宿主细胞中也可能由入侵的病毒所转录。加拿大 Laval University 的 Ouellet 和 Provost 博士详细研究了多种病毒，并讨论了宿主和病毒 miRNA 在这场生存斗争中所产生的影响，其中值得注意的是他们介绍了一种可以检测到瞬时表达的 miRNA 的方法。

RNA 干扰诱导特有的灵敏性和选择性使得可以针对一个基因进行等位基因特异性沉默。日本东京 National Institute of Neuroscience 的 Hohjoh 博士提供了在哺乳动物细胞中沉默萤火虫和海肾荧光酶基因的方法。应用 siRNA 进行等位基因特异性沉默时，设计 siRNA 需要非常精心，因为很多的因素都可以影响沉默效率。韩国 Daejeon 的 the University of Science & Technology 的 Kim 博士介绍了一个在线 siRNA 设计程序 AsiDesigner，这个程序在设计最佳 siRNA 时考虑了选择性剪切的因素。爱尔兰 Dublin City University 的 Muhonen 和 Holthöfer 博士继续优化 siRNA 设计，他们考虑了靶 mRNA 的可接近性，并提供不同的生物信息学方法来鉴定待沉默的靶 mRNA 的有效且特异性位点。日本 Kanazawa Medical University 的 Ishigaki 博士小组提供了另一种增强 siRNA 作用的方法。在该章节中，一个质粒可以表达多个 shRNA，通过同时靶定一个转录本的不同区域增强沉默效果。他们给出了表达 2 个 shRNA 的质粒的详细构建方法并从方法学角度描述了其特点。在提到其与治疗的相关性时，作者详细讲述了由于 shRNA 多个启动子可能占用过多的细胞转录机器所产生的负面影响。在实际应用中，单一质粒表达多个 shRNA 的方法已经应用于抑制 HIV。加利福尼亚 City of Hope 的 the Beckman Research Institute 的 Rossi 和 Zhang 博士提出他们的技术是一种新的组合的抗 HIV 基因表达系统，有可能作为治疗手段。这个系统允许从一个 RNA 聚合酶 II 多顺反子转录本上同时转录多个 RNA 干扰的效应子。在这一系统中，通过应用内源性 RNAi 转录本和 miRNA 的方法，从单一一个 RNA 聚合酶 II 的多顺反子转录本表达多个 RNAi 的效应子，

从而避免了从 RNA 聚合酶 III 启动转录多个 shRNA 所产生的细胞毒性。接下来，University of Vienna 的 Hofacker 博士，讨论了仅考虑特异性 siRNA 设计标准及整合 mRNA 结构特征和 siRNA 的基本特征，用于筛选 shRNA 及 siRNA 的芯片技术。第一部分的最后一章是由 J. W. Goethe-Universität 的 Engels 博士编写的关于合成各种 siRNA 的具体步骤。

在第二部分中，我们从 RNA 干扰的生物学转移到了其相关应用问题，包括作为实验室的基础研究试剂和治疗疾病的潜在有效工具。加拿大 University of Western Ontario 的 Zheng 博士首先描述了可以靶定特定细胞的含 siRNA 的免疫脂质体的产生方法。由于免疫脂质体具备通过其自身携带的抗体选择性结合表达相应抗原的细胞的能力，因此，为研究者提供了一个很好的递送平台，既容易实现而且已经广泛应用。在接下来一章中，来自 the University of Texas 的 Evers 和 Rychahou 博士回顾了最初在体内导入 siRNA 的流体力学注射方法。这个方法通过快速从静脉注射大量的 siRNA，在内皮引起瞬时的微孔并导致紧密连接的松散，从而使 siRNA 穿过细胞膜进入细胞内部。到目前为止，已经用这种方法向肝、肺和脑中成功导入 siRNA。

DNA 芯片技术可以高通量地检测不同细胞不同生物学条件下基因表达的情况，同样，使用高通量 siRNA 技术沉默基因也可以用来研究基因被抑制后的细胞表型。来自日本东京 the Research Institute for Cell Engineering（RICE），the National Institute of Advanced Industrial Science and Technology（AIST）的 Fujita 等介绍了 2 种在固体表面进行 siRNA 反式转染的方案，一种是在芯片上，另一种是在微量滴定板上。

从一般到特异，在特定的病理条件下使用 siRNA 也备受关注。来自加拿大 McGill University 的 Prakash 等致力于研究神经退行性疾病和穿过血脑屏障的方法。他们概述了神经生物学领域应用 siRNA 的现状，并阐明了如何合成最优 siRNA 分子，以及应用阳离子纳米技术体内定向导入 siRNA 的方法。来自台湾 Chang Gung Memorial Hospital-Kaohsiung Medical Center 的 Huang 等用生物信息学方法通过随机沉默和表型评估来选择性地鉴定肺癌中的基因。他们成功鉴定出一个和癌症侵袭迁移相关的基因 FLJ10540。在这一章中，他们讲述了该基因受到的上下游的调控。Jere 和 Cho 博士（来自韩国 Seoul National University）编写的章节介绍了将感兴趣的 siRNA 和 shRNA 导入癌症模型，并提供了合成生物可降解的阳离子多聚物的具体步骤。此外，还介绍了追踪细胞更新和胞内运输情况的方法，以及评估 siRNA 对癌细胞所产生影响的具体方案。前面已经介绍了用免疫脂质体或具有靶向亲和能力的试剂将引起 RNA 干扰的分子定向导入体内，来自日本 Okayama University 的 Ohtsuki 小组介绍了一种新方法。他们将 siRNA 和 HIV-tat 连接然后导入细胞内，则导入的 siRNA 可以通过光子激活沉默效应。这种方法被称为 CLIP-RNAi（CPP-linked RBP-mediated RNA internalization and photoinduced RNAi），可以用于需要局部基因沉默的治疗方案。

Aigner 等研究了 siRNA 的降解问题，他们利用多种聚乙烯亚胺类物质保护 siRNA 免受细胞内外核酸酶的降解。在该章节中，作者比较了带有不同聚乙烯亚胺类物质的阳离子电荷、与 siRNA 形成非共价连接的能力以及 siRNA 复合物通过内吞作用进入细胞的能力。在如何穿过膜的问题上，来自英国伦敦 King's College 的 Brito 等介绍了一种很新奇的转染方法：即用链球菌溶血素-O 进行短暂的透析。此外，他们还提供了在多种骨髓瘤

细胞系中进行基因沉默操作的优化方案，这对治疗学的发展有重要作用。

本书的第三部分介绍了 RNAi 临床应用的相关问题。登陆美国国家医学图书馆 NIH 记录正在进行的临床试验的网站 www. clinicaltrials. gov，就会发现有 7 个正在使用 RNA 干扰技术的临床试验，涉及湿黄斑变性、传染病和癌症。这章还强调了在应用这项新技术时应该注意的一些问题。来自日本 Osaka University Graduate School of Medicine 的 Akaneya 博士，介绍了在神经学领域使用 RNA 干扰方法的一些优缺点，重点谈到 ALS 和炎症性疾病。关于免疫原性、干扰素反应以及定位的相关问题也都有涉及。来自巴尔的摩 Johns Hopkins School of Medicine 的 Mao 和 Wu 博士介绍了在癌症免疫疗法中 RNA 干扰技术的使用。他们研究了多种重要的免疫相关靶标，从特异的效应分子到一般的上游转录因子，如 STAT，以及多个免疫应答基因的其他调控因子。此外还概述了一些调控先天及后天免疫反应的内源性 miRNA 分子。最后作者评价了在最快速进行临床转化的癌症免疫治疗中各种诱导 RNA 干扰方法。

来自加拿大 University of Western Ontario 的 Zhang 等介绍了 RNA 干扰在保护组织免受损伤方面的作用。他们提供了用于分析缺血/再灌注的肾损伤模型中通过抑制 caspase 转录所起到的保护作用的具体实验。同样是这所大学的 Zhang 和 Li 博士，介绍了在体外通过 siRNA 沉默树突状细胞的方法并且用这些细胞调节和/或抑制了移植排斥反应。这个方法的优点是树突状细胞的免疫激活或抑制潜能取决于共刺激分子的表达。书中描述了对树突状细胞中 NF-kB 家族成员之一 RelB 的靶向抑制作用，导致了多种细胞因子和共刺激分子的下调，而这种下调和抑制的免疫原性相关。

下面的章节继续探讨了免疫调控的问题，来自日本东京的 Tokyo Medical and Dental University 的 Ritprajak 等使用 siRNA 穿过角质层进入真皮层中的树突状细胞。通过调控这些细胞，作者发现共刺激分子受到抑制，从而有可能用于过敏性疾病治疗。来自加拿大 University of Sherbrooke 的 Sarret 等应用 RNA 干扰技术以非药物的方式解决疼痛问题。他们讨论了关于 siRNA 给药的流程、靶基因以及这种方法在疼痛管理研究中使用的行为系统，并以 G-蛋白偶联受体为例进行特别说明。来自美国 Bothell MDRNA 公司的 Seth 等，介绍了呼吸道病毒治疗，尤其是流感病毒治疗中 RNA 干扰的使用情况。他们提到了多种病毒的靶标、动物模型以及能达到最大抗病毒效果的导入方法。一个很有意思的问题是可以激活干扰素-α 反应的 siRNA 和这些分子的基本的抗病毒效应之间的相互作用。来自德国柏林 Charité-Universitätsmedizin 病理研究所的 Malek 博士和 Oncology Institute of Southern Switzerland 的 Tchernitsa 博士，详细介绍了在体内和体外沉默卵巢癌细胞的具体步骤，尤其是他们用到了临床相关的异种移植腹水模型。

正如你所看到的，RNA 干扰的研究进展是引人注目的。虽然不知道它是否可以像人们最初设想的那样发挥作用，但我们希望这本书可以让读者也感受到这项技术发展到今天所带给我们的惊喜。

Wei-Ping Min 和 Thomas Ichim

（王芳 译）

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.

2009 ***Wei-Ping Min and Thomas Ichim***

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