Bacterial Vectors for RNAi Delivery

Thu Nguyen and Johannes H. Fruehauf*

* Corresponding Author: Johannes H. Fruehauf—Skip Ackerman Center for Molecular Therapeutics, GI Cancer Laboratory, DANA 607, Beth Israel Deaconess Medical Center, Harvard Medical School, 330 Brookline Ave, Boston, MA 02215, USA. Email: jfruehau@bidmc.harvard.edu

RNA interference (RNAi) is a recently discovered powerful research tool which allows the targeted "silencing" of particular genes. RNAi is also thought to have immense therapeutic potential to treat and prevent a wide range of diseases from inflammation to cancer and to target genes which have formerly been considered "undruggable". However, the advancement of RNAi technology as a means of therapy is hindered by the challenges of delivering RNAi into the cytoplasm of target cells. As a versatile gene vector, bacteria have been employed and shown to be an effective, safe and inexpensive measure for delivering RNAi to mammalian cells. Two systems that exploit the therapeutic benefits of bacteria to deliver RNAi are *transkingdom* RNA interference (*tk*RNAi) and bacteria-mediated RNA interference (*bm*-RNAi). Both systems are effective in eliciting gene silencing both in vitro and in vivo and they suggest an important role for bacteria in future RNAi therapeutics.

RNA interference (RNAi) is a highly conserved, potent gene-silencing mechanism whereby endogenous genes of interest are down-regulated by a targeted enzymatic cleavage of messenger RNA (mRNA) in a highly specific and efficient manner. This phenomenon had been observed in plants in the 1990s[1,2] and its mechanism was elucidated in animals in 1998 by Drs Fire and Mello,[3] who published their findings on RNA induced silencing in the nematode worm C. Elegans, work for which they were awarded the Nobel Prize for Physiology or Medicine in 2006.The RNAi pathway is conserved in many species and has likely evolved as innate immunity against viral infection. This discovery of RNAi gained even more importance after it was demonstrated that RNAi is also active in mammalian cells.[4] Since then, RNAi has been found to be a highly useful tool for basic research as it helps to rapidly elucidate gene function through targeted knockdown studies. Furthermore, RNAi has also spawned a novel field of drug research, RNAi-based therapeutics, which has become a quickly growing area of drug development. RNAi-based therapy attracts a lot of interest, since—at least theoretically- every disease caused by an upregulation of a known gene could be targeted using RNAi-based drugs. Therapeutic applications could range from viral infection to cancer treatment.

The classical RNAi pathway is triggered by the introduction of short interfering RNA (siRNA) to the cytoplasm. siRNA consists of short (19-21 bp) double stranded RNA with two nucleotide overhangs at each 3' end.[5] One of the strands (the sense strand) is homologous to an area in the mRNA of the gene to be silenced. Alternatively, the RNAi pathway can also be triggered by the introduction of siRNA precursors such as double-stranded RNA (dsRNA) or short hairpin RNA (shRNA), which may be transcribed from eukaryotic plasmids inside the target cell or from viral vectors.

Both shRNA and dsRNA can be processed by Dicer, an enzyme with RNAse III

activity, to become siRNA and induce RNAi-mediated gene silencing in the cytoplasm of mammalian cells or live animals.[6,7] shRNA molecules can function as siRNA following the cleavage of the loop by Dicer while long dsRNA can be cleaved by the same cytoplasmic enzyme into short, double-stranded siRNA fragments of 19-21bp with 2-4 nucleotide overhangs at the 3' ends. The siRNA duplexes are then unwound via an ATP-dependent mechanism and the antisense or guide strand is bound to a multi-protein structure called the RNA-induced silencing complex (RISC).[7] This silencing complex catalyzes the cleavage of a homologous mRNA within the cytoplasm to inhibit translation and thereby prevent protein expression of the gene of interest.

Although RNAi technology holds great promise for the advancement of new gene directed therapies, the difficulty of delivering siRNA to target cells has impeded the rapid expansion of RNAi-based therapies. The use of viral vectors, nanoparticles, liposomes and chemical modifications to siRNA have been exploited to deliver RNAi[6,8] and delivery of therapeutic RNA interference has become a field of its own right in the biotechnology industry.

Currently (Fall 2007), few RNAi-therapeutic compounds are in clinical trials for target organs such as the eye (direct injection of modified siRNA), the pulmonary epithelium (inhalation of unmodified siRNA) or the liver (systemic injection of liposomes or nanoparticles carrying siRNA). However, these delivery methods may not be suitable for a number of other organs or cell types, particularly in the gastrointestinal or genitourinary tract, where numerous potential medical indications exist which could be treated with RNAi based therapeutics, but for which targeting and delivery is more difficult.

Bacteria are highly versatile and useful tools for biotechnology and have helped drive most of the recent rapid growth in our understanding of genes and gene function. Bacteria are increasingly recognized for their vast potential as therapeutics and efforts are being made in various areas of therapeutic drug development based on live attenuated or otherwise modified bacteria. Other chapters in this book cover the use of bacteria as delivery agents for vaccines, chemicals, therapeutic proteins, or even as gene therapy vectors.

Bacteria have also been found to be useful for the delivery of short interfering RNA. Unlike some viral vectors, bacteria do not integrate genetic material into the host genome and they can be controlled with antibiotics or engineered to increase safety using nutrient auxotrophy. Bacteria are versatile gene vectors and have been shown to be an effective, safe and inexpensive measure for delivering RNAi to target cells with high selectivity and high specificity.[6] In a concept called *transkingdom* RNA interference (*tk*RNAi), it was shown that laboratory strains of E.Coli can be harnessed to produce and deliver therapeutic short interfering RNA.[9]

Transkingdom Rna Interference

Transkingdom RNA Interference (*tk*RNAi) is an example of a bacteria-mediated RNAi delivery system that is successful in eliciting efficient gene silencing both in vitro and in vivo, primarily targeting epithelial cells. *tk*RNAi was developed at the Beth Israel Deaconess Medical Center in Boston, MA and uses genetically modified bacteria, e.g., *Escherichia coli*, to produce and deliver short hairpin RNA (shRNA). In the prototype concept, this is achieved through the use of a *transkingdom* RNA interference plasmid (TRIP). The TRIP plasmid enables the bacteria to produce shRNA, invade target cells, release shRNA into the cells' cytoplasm and activate the RNAi pathway to induce gene silencing.

The TRIP prototype plasmid consists of three main components:

(1) A shRNA expression cassette, which drives the expression of a short hairpin RNA under the control of the T7RNA polymerase promoter and terminator. The cassette is flanked by two characteristic restriction enzyme sites (Sal 1 and BamH1) which allow for a rapid exchange of the shRNA sequence to develop TRIP vectors against different targets. shRNA is produced after induction of the T7RNA polymerase which causes shRNA to accumulate inside the bacteria and becomes released after the bacteria dies inside the host cell.

(2) The *inv* gene on the TRIP encodes for the expression of invasin protein on the bacterial surface (derived from *Yersinia pseudotuberculosis*),[10] which enables bacterial entry into the target (host) cell. Invasin interacts in a highly efficient manner with beta(1)integrins which are expressed on the surface of epithelial cells. This leads to the rearrangement of host cell cytoskeleton, formation of a groove and uptake of the invasin-expressing bacteria into an endosome.

(3) The *hly* gene on the TRIP encodes for listeriolysin O.[11,12] This protein is derived from *Listeria Monocytogenes*, a pore-forming toxin which is released upon bacterial lysis inside the endosome and ruptures the endosomal membrane. This results in the release of the accumulated shRNA into the cytoplasm of the host cell.

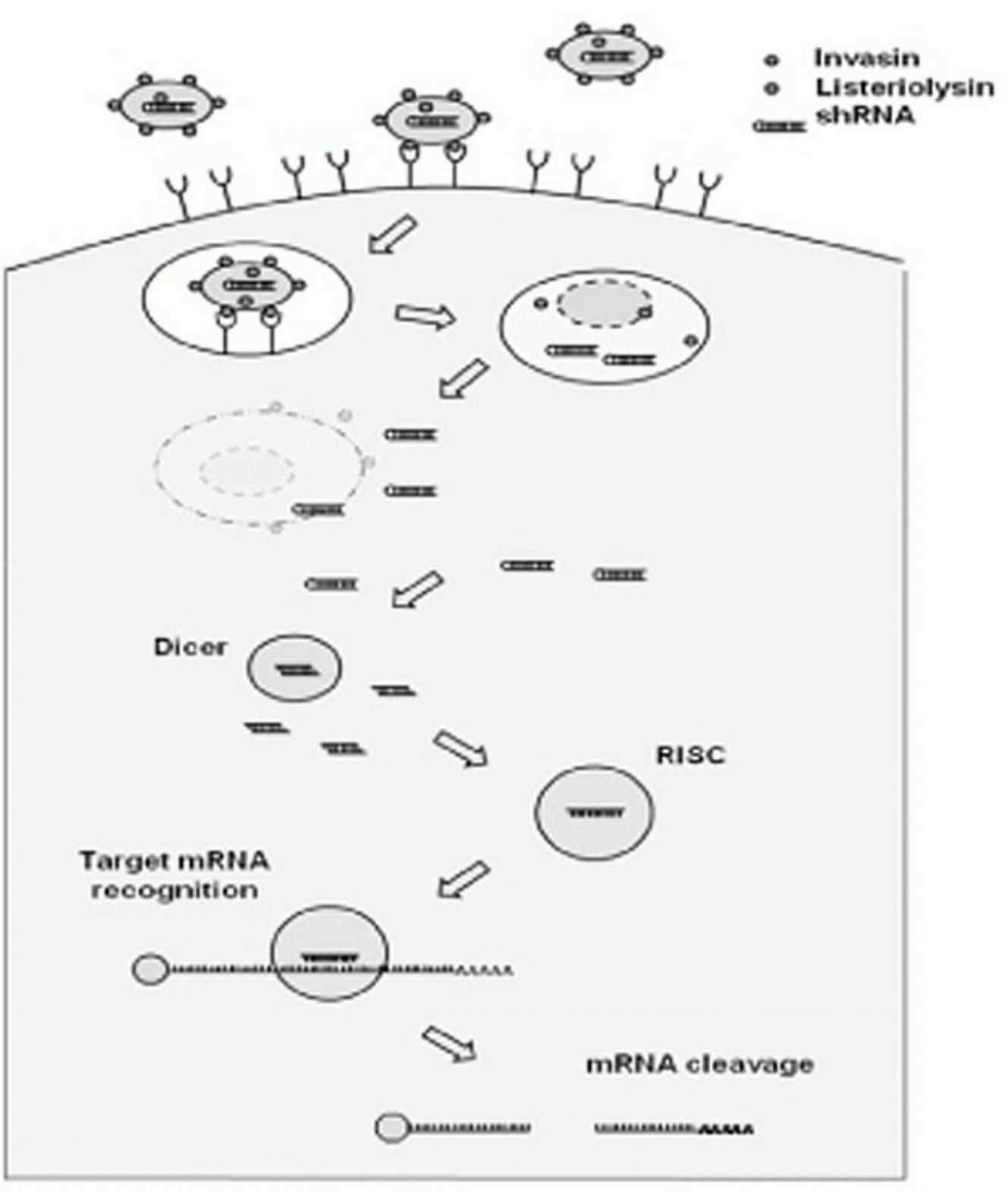

Figure 1.

Transkingdom RNA interference (tkRNAi): mechanism of action.

The theoretic steps required for *tk*RNAi are described in Figure 1 The successful entry of the bacteria into epithelial cells is dependent upon the expression of invasin on the bacterial surface, encoded by the *inv* gene from *Yersinia pseudotuberculosis*. Invasin endows the bacteria with the capability to invade mammalian cells containing beta-1 integrin receptors. Meanwhile, the *hly* gene encodes for the production of listeriolysin O, a pore-forming toxin, which accumulates in the bacterial cytoplasm and periplasma. Listeriolysin is released into the endosome of the host cell after invasion when the bacterial cell wall starts to disintegrate due to nutrient deficiency and host cell lysosomal action. Listeriolysin O will result in the formation of pores and rupture of the endosomal membrane, allowing the bacterial content (which includes the shRNA) to reach the cytoplasm of the host cell. Once shRNA has escaped from the lysed bacteria into the cytoplasm, it is processed by the host cell machinery. The protein Dicer cleaves off the loop structure of the hairpin RNA which results in the formation of a short double stranded RNA of 21 paired nucleotides (siRNA). The guide strand of the siRNA is then integrated into a multi protein complex called RISC (RNA induced silencing complex) which retrieves mRNA with perfectly homologous regions. In the presence of a perfect match between the guide strand and the mRNA, cleavage of the mRNA is induced by the ago-2, a component of the RISC complex. This results in the rapid degradation of the remaining mRNA fragments. Thereby, the production of the protein encoded by this particular mRNA will not take place (prevention of translation). The RISC complex containing the guide strand of the siRNA continues to target other copies of the mRNA resulting in a very efficient process of degradation of the specific mRNA of the targeted gene.

To demonstrate the effects of *tk*RNAi technology, a TRIP plasmid was constructed against the human colon cancer oncogene *CTNNB1* (catenin β-1) and cloned into a competent strain of nonpathogenic *E. coli*, BL21DE3. The transformed strain of BL21DE3 was shown to successfully down-regulate the beta-catenin level in human colon cancer cells (SW480) in vitro with a clear dose-dependent effect.[9] *tk*RNAi was also shown to be effective in inducing local gene silencing in the intestinal epithelium of mice that were treated orally with *E. coli* expressing shRNA against mouse *Ctnnb1*. In addition, *tk*RNAi is also successful in eliciting systemic gene silencing in mice with xenografts of human colon cancer that were treated intravenously with *E. coli* encoding shRNA against human *CTNNB1*. *tk*RNAi is currently being developed as a novel method for the delivery of RNAi-based therapeutics with applications ranging from colon cancer prevention to treatment of Human Papilloma Virus infection and inflammatory bowel disease (IBD) by Cequent Pharmaceuticals of Cambridge, MA (more information at www.cequentpharma.com).

Bacteria-Mediated Rna Delivery

Bacteria-mediated RNA interference (*bm*RNAi) delivery through the use of invasive bacteria such as Salmonella typhimurium is another approach that employs naturally invasive bacteria to deliver RNA interference. Unlike the *tk*RNAi delivery system, carrier bacteria in the *bm*RNAi system do not produce shRNA, but rather they transfer shRNA expression plasmids to the host cell which then utilizes its own transcriptional machinery to produce shRNA in the nucleus. Some speculate that this approach may induce more sustained silencing since the siRNA is constantly produced by the host cell and may be more stable than that produced by the bacteria and released into the host cell cytoplasm.

Attenuated S. typhimurium has successfully been used in the past as a means of delivery for a wide variety of therapeutic payloads from proteins to DNA for vaccine or gene therapy applications.[13-16] One widely used version of attenuated S. typhimurium is SL7207[17] in which the *aroA* gene is inactivated. It requires aromatic amino acids for survival, which it cannot find after invasion into a host cell. This results in rapid lysis of the bacteria after host cell invasion, liberation of their payload and a lowered toxicity compared with wildtype S. typhimurium.

S. typhimurium have been used successfully to deliver shRNA expression plasmids for the treatment of a mouse prostate cancer model.[18] In our own work, we have seen consistent

and robust gene silencing in vitro and in vivo using attenuated S. typhimurium as carriers for shRNA expression plasmids for various indications (Fruehauf et al, unpublished data).

Bacteria have evolved as an amazingly versatile tool in the last 20 years which drives the genomic revolution and the development of biotechnology. The recent discovery that bacteria can be useful carriers for RNAi is just one more step in our understanding of this kingdom of life. While there are still a host of obstacles to overcome, such as the avoidance of the innate immune system, activation of cellular and humoral immune responses to therapeutically administered bacteria, the remaining risk of bacterially-induced disease and others, we believe that bacteria will soon become much more acceptable as carriers of therapeutic payloads and as little bio-robots to fulfill useful tasks inside a host organism.

References

1. Napoli C, Lemieux C, Jorgensen R. Introduction of a chimeric chalcone synthase gene into petunia results in reversible cosuppression of homologous genes in trans. *Plant Cell.* (1990); 2: 279–289. [PubMed] [Free Full text in PMC]

2. Romano N, Macino G. Q uelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences. *Mol Microbiol.* (1992); 6: 3343–3353. [PubMed]

3. Fire A. et al. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature.* (1998); 391: 806–811. [PubMed]

4. Elbashir SM. et al. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature.* (2001); 411: 494–498. [PubMed]

5. Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* (2001); 15: 188–200. [PubMed] [Free Full text in PMC]

6. Li CX. et al. Delivery of RNA Interference. *Cell Cycle.* (2006); 5

7. Zamore PD, Tuschl T, Sharp PA. et al. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell.* (2000); 101: 25–33. [PubMed]

8. de Fougerolles A, Vornlocher HP, Maraganore J. et al. Interfering with disease: a progress report on siRNA-based therapeutics. *Nat Rev Drug Discov.* (2007); 6: 443–453. [PubMed]

9. Xiang S, Fruehauf J, Li CJ. Short hairpin RNA-expressing bacteria elicit RNA interference in mammals. *Nat Biotechnol.* (2006); 24: 697–702. [PubMed]

10. Isberg RR, Voorhis DL, Falkow S. Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. *Cell.* (1987); 50: 769–778. [PubMed]

11. Courvalin P, Goussard S, Grillot-Courvalin C. Gene transfer from bacteria to mammalian cells. *C R Acad Sci III.* (1995); 318: 1207–1212. [PubMed]

12. Grillot-Courvalin C, Goussard S, Huetz F. et al. Functional gene transfer from intracellular bacteria to mammalian cells. *Nat Biotechnol.* (1998); 16: 862–866. [PubMed]

13. Hense M. et al. Eukaryotic expression plasmid transfer from the intracellular bacterium Listeria monocytogenes to host cells. *Cell Microbiol.* (2001); 3: 599–609. [PubMed]

14. Weiss S, Chakraborty T. Transfer of eukaryotic expression plasmids to mammalian host cells by bacterial carriers. *Curr Opin Biotechnol.* (2001); 12: 467–472. [PubMed]

15. Bermudes D, Zheng LM, King IC. Live bacteria as anticancer agents and tumor-selective protein delivery vectors.
Current opinion in drug discovery & development. (2002); 5: 194–199.

16. Paglia P, Terrazzini N, Schulze K. et al. In vivo correction of genetic defects of monocyte/macrophages using attenuated Salmonella as oral vectors for targeted gene delivery. *Gene Ther.* (2000); 7: 1725–1730. [PubMed]

17. Hoiseth SK, Stocker BA. Aromatic-dependent Salmonella typhimurium are nonvirulent and effective as live vaccines. *Nature.* (1981); 291: 238–239. [PubMed]

18. Zhang L. et al. Intratumoral delivery and suppression of prostate tumor growth by attenuated Salmonella enterica serovar typhimurium carrying plasmid-based small interfering RNAs. *Cancer Res.* (2007); 67: 5859–5864. [PubMed]