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TransKingdom RNA interference: a bacterial approach to challenges in RNAi therapy and delivery

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Abstract

Since its discovery in 1998 RNA interference (RNAi), a potent and highly selective gene silencing mechanism, has revolutionized the field of biological science. The ability of RNAi to specifically down-regulate the expression of any cellular protein has had a profound impact on the study of gene function *in vitro*. This property of RNAi also holds great promise for *in vivo* functional genomics and interventions against a wide spectrum of diseases, especially those with "undruggable" therapeutic targets. Despite the enormous potential of RNAi for medicine, development of *in vivo* applications has met with significant problems, particularly in terms of delivery. For effective gene silencing to occur, silencing RNA must reach the cytoplasm of the target cell. Consequently, various strategies using chemically modified siRNA, liposomes, nanoparticles and viral vectors are being developed to deliver silencing RNA. These approaches, however, can be expensive and in many cases they lack target cell specificity or clinical compatibility. Recently, we have shown that RNAi can be activated *in vitro* and *in vivo* by non-pathogenic bacteria engineered to manufacture

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Abbreviations: APC, adenatomous polyposis coli; dsRNA, double-stranded RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; mRNA, messenger RNA; PKR, protein kinase R; RISC, RNA-induced silencing complex; RNA, ribonucleic acid; RNAi, RNA interference; miRNA, micro RNA; MOI, multiplicity of infection; RIG-I, retinoic acid inducible gene I; shRNA, short hairpin RNA; siRNA, short interfering RNA; SNALP, stabilized nucleic acid lipid particle; *tk*RNAi, TransKingdom RNA interference; TLR, toll-like receptor; TRIP, TransKingdom RNA interference plasmid.

and deliver silencing shRNA to target cells. This new approach, termed TransKingdom RNAi (*tk*RNAi), has several key advantages. First, *tk*RNAi may provide a viable means to accomplish therapeutic RNAi since non-pathogenic bacteria have a proven safety record in clinical applications. Second, *tk*RNAi eliminates the cost of siRNA manufacture since silencing shRNA are produced inside bacteria. Moreover, the intracellular mechanism of shRNA release inherent to *tk*RNAi may circumvent, or mitigate, the activation of host immune responses. Finally, *tk*RNAi may facilitate high-throughput *in vivo* functional genomics screening since bacteria-based RNAi libraries can be easily constructed, stored, reproduced and amplified, thereby allowing for the creation of a stable gene silencing system.

Introduction

Post-transcriptional gene silencing mediated by short interfering RNA (siRNA) is a naturally occurring biological process that is highly conserved among plants and animals. In mammalian cells, this process is referred to as RNA interference (RNAi) and it is thought to have evolved primarily as an anti-viral defense mechanism (Caplen *et al.,* 2001; Elbashir *et al.,* 2001). The discovery that RNAi-mediated gene silencing can be activated using synthetic siRNA, or siRNA derived from shRNA precursors, has revolutionized our understanding of gene function (Dykxhoorn *et al.,* 2005). In addition, the ability of RNAi to specifically target any cellular protein, including those which are currently considered "undruggable" by small molecules, has created the opportunity to develop an entirely new class of drugs against a wide variety of diseases (Shankar *et al.,* 2005; Kim *et al.,* 2007).

RNAi is triggered when endogenous micro RNA (miRNA), or exogenous double stranded RNA (dsRNA) or short hairpin RNA (shRNA) are processed by the cytoplasmic enzyme Dicer into 21- to 23-nucleotide short interfering RNA (siRNA) duplexes (Provost *et al.,* 2002; Hannon *et al.,* 2004; Tijsterman *et al.,* 2004). The processed siRNA duplexes are then loaded into a large multi-protein complex called RISC (RNA-induced silencing complex) where the siRNA duplex is unwound and the passenger (sense) siRNA strand is discarded (Hammond *et al.,* 2000; Gregory *et al.,* 2005). The RISC complex then locates target mRNA using the incorporated guide (antisense) siRNA strand. For endogenous miRNA, the RISC complex typically binds to partially complementary areas in the 3' untranslated regions of target mRNA and mediates gene silencing by translation repression. For exogenously applied siRNA or shRNA, the RISC complex binds to perfectly complementary regions in target mRNA and cleaves them using the slicer activity of the Argonaute 2 protein, thereby preventing protein production.

As a therapeutic modality, RNAi has several advantages over traditional pharmaceutical approaches (Aagaard *et al.,* 2007). First, RNAi-mediated gene silencing is capable of very high specificity since it relies on the recognition of perfectly complementary nucleotide sequences in target mRNA. Indeed, disease-specific alleles can often be discriminated by RNAi. Second, compared to other antisense strategies RNAi is capable of inducing extremely potent (> 90%) gene silencing lasting several days *in vivo*. Finally, the identification and subsequent optimization of lead candidate molecules is significantly faster for RNAi-based therapeutics than for either small molecules or antibodies/proteins.

Despite the enormous potential of RNAi-based therapeutics there have, to date, been relatively few clinical studies using this technology (Kim *et al.,* 2007; de Fougerolles 2008). Moreover, these initial trials have focused mainly on direct local application of siRNA rather than systemic administration. Of the five trials currently ongoing, three (Besvasiranib in phase III testing; AGN-745 in phase II testing; and RTP801i-14 in phase I/II testing) are for the treatment of wet age-related macular degeneration, a vascular disorder of the retina. In each case, silencing siRNA is administered by direct intravitreal injection with the goal of inhibiting retinal angiogenesis. RNAi-based therapeutics are also being developed for the treatment of respiratory syncytial virus (RSV), a common cause of bronchiolitis and pneumonia in infants and immunocompromised adults. In a recently completed phase I trials ALN-RSV01, an siRNA against the viral nucleocapsid (N) gene, was administered by intranasal instillation or inhalation and found to be well tolerated. Finally, phase I trials have just commenced for AKI-i5, a systemically delivered siRNA against p53 for the treatment of acute kidney injury.

Although there has been progress with direct local application of therapeutic siRNA for a limited number of indications, the application of RNAi-based therapies *in vivo* has been hampered due to difficulties with delivery, the cost of manufacturing large quantities of siRNA and the activation of non-specific interferon responses (Shankar *et al.,* 2005; Li *et al.,* 2006). Of these, delivery has proven to be the main obstacle. So far, strategies to overcome the delivery problem have focused on various pharmaceutical technologies using chemically modified siRNA, liposomes or nanoparticles to enhance target cell siRNA uptake (Li *et al.,* 2006). These methods tend to be expensive, however, and have a limited ability to effect tissue-specific siRNA delivery. Viral vectors are also being explored for the delivery of shRNA (Li *et al.,* 2006). However, lack of tropism and the potential to cause disease have significantly limited the use of viral vectors for RNAi applications *in vivo*.

Recently, we have developed a bacteria-based RNAi delivery technology called TransKingdom RNAi (*tk*RNAi) for *in vitro* and *in vivo* gene silencing in mammalian cells (Xiang *et al.,* 2006). Our method utilizes genetically engineered, non-pathogenic, *E. coli* to simultaneously manufacture shRNA and deliver them to target cells. This technique offers several advantages over chemically modified siRNA and viral vector-mediated shRNA delivery for biomedical research and development of RNAi-based medical therapies. Foremost is clinical safety; attenuated therapeutic bacteria have been given intravenously to cancer patients with demonstrated safety. This approach also eliminates the siRNA manufacture issue, and may circumvent or mitigate the generation of interferon-like responses since the silencing siRNA are produced intracellularly. Furthermore, the risk of immune system activation, the need for attenuation, and the risk of environmental release can be addressed by technologies already developed for bacteria-based interventions.

The overall goal of this article is to provide an overview of bacteria-mediated RNAi with particular emphasis on the application of the *tk*RNAi approach to study gene function *in vitro* and induce therapeutic gene silencing *in vivo*.

The RNAi Delivery Problem

Development of RNAi-based drugs and therapeutics has been complicated by properties inherent to the chemical nature of siRNA, the molecules that mediate RNAi.

siRNA are double stranded short RNA molecules that are subject to enzymatic degradation when exposed to RNases present in the serum (Elbashir *et al.,* 2001). This results in low siRNA stability and a short half life when injected into the blood for use as a drug (Soutschek *et al.,* 2004).

A second challenge is posed at the level of entry into the target cells. In order to efficiently suppress gene expression, siRNA must gain access to the cytoplasm of target cells and siRNA molecules need to be delivered to each cell in which gene silencing is to be achieved. This is not trivial, however, as siRNA are polar molecules that cannot easily penetrate lipophilic cell membranes, and therefore require some delivery mechanism to gain access to the cytoplasm. This has been achieved *in vitro* through the use of transfection reagents, such as liposomes and lipoplexes, or through physico-chemical manipulation, such as the application of pulsed electric fields that enable the formation of transient membrane pores through which siRNA can gain access to the cell cytoplasm (electroporation). Another approach is the use of viruses for transduction, in which shRNA-encoding DNA sequences are transported into the target cells and the silencing RNA is synthesized by the cell transcription machinery (Brummelkamp *et al.,* 2002).

The greater difficulty is encountered when one tries to turn RNAi into a drug in order to treat diseases such as cancer, viral infections or inflammatory disease in a living organism. Many of the methods used for *in vitro* treatment of cultured cells are not useful options as drug delivery vehicles (Li *et al.,* 2006). A number of companies are currently developing drugs based on RNAi technology, and a variety of delivery concepts are being evaluated in preclinical and clinical testing. At the moment, it is not clear which delivery method will turn out to be the most useful for a wide range of applications, and it is likely that various delivery techniques will coexist in the area of RNAi-based drugs.

LOCAL APPLICATION OF SiRNA

The earliest clinical trials programs were initiated in areas where direct application of siRNA was feasible because access to the target organ was possible through injection or inhalation. Direct injection of siRNA into the blood as a means of delivering therapeutic RNA interference to more distal organs, or to achieve a systemic effect, is not promising because of the lack of stability of unmodified siRNA. For a few indications where the environment is more favorable to the maintenance of siRNA, such "direct application" may be feasible. This approach is exemplified by RNAi therapeutics in the eye, as well as inhaled siRNA for treatment of respiratory disease. Direct injection into the eye is feasible because the eye has a low concentration of nucleases and siRNA are much more stable after ocular injection compared with injection into the serum (de Fougerolles *et al.,* 2007). Although the concentration of siRNA-degrading nucleases in the nose/lung is higher than in the eye, the ease of access to these tissues has been a major stimulus for developing RNAi-based treatments for various viral infections of the respiratory system (Bitko *et al.,* 2005).

From the experience reported in the literature so far, it seems that the value of direct application of siRNA for therapeutic use will be limited to the few instances where access to the target organ is relatively straight forward. Other than the eye and the respiratory system, the only other tissue currently being explored for local delivery

of siRNA is the nervous system (Thakker *et al.,* 2004). Consequently, alternative delivery strategies for RNAi-based therapeutics are being actively developed.

LIPOSOMAL DELIVERY

In the biology laboratory, liposomes play an important role as transfection reagents, and liposomal formulations are widely used to conduct *in vitro* experiments involving siRNA. For many years liposomes have been used to formulate drugs in order to confer better stability and to facilitate delivery through the cell membrane, and this approach is now being actively pursued by a number of commercial and academic groups for the delivery of RNAi-based drugs (Palliser, 2006). There are a large number of lipid-based delivery methods available some of which are being evaluated for the delivery of siRNA. One approach, developed by Protiva Biotechnology is called SNALP (Stabilized Nucleic Acid Lipid Particle). SNALPs, given intravenously, have been used successfully for *in vivo* delivery of siRNA in a variety of animal models from mice to monkeys (Morrissey *et al.,* 2005; Zimmermann *et al.,* 2006), and may be candidates for future clinical trials targeting liver and tumors.

Lipid particles can also be armed with targeting moieties on their surface to enable them to selectively interact with particular subsets of cells, such as tumor cells or activated inflammatory cells. This has been demonstrated recently for activated leukocytes, where the use of an antibody fragment directed against the activated form of a leukocyte receptor molecule allowed for the targeted delivery of siRNA into the activated subset of leukocytes only. The ability to selectively target activated immune cells will clearly be important for the development of RNAi-based interventions against inflammatory disorders such as rheumatoid arthritis or inflammatory bowel disease (Peer *et al.,* 2008).

However, to make liposomal delivery economically feasible for large-scale clinical trials or even development to a drug product, significant reductions in cost and improvements in efficacy are still required. In a recent trial demonstrating high efficacy siRNA treatment on a metabolic target in non-human primates using liposomal delivery, siRNA doses as high as 2.5mg/kg were used to achieve gene silencing and prolonged metabolic effects (Zimmermann *et al.,* 2006). The expense of synthesizing such large amounts of siRNA and formulating them into complex lipid nanoparticles is enormous, with the anticipated cost of such a treatment being in the order of $100,000 per year.

VIRAL DELIVERY

Viruses have been used to deliver nucleic acids for laboratory and therapeutic applications including gene therapy. Viruses are ideal vectors based on their natural abilities to infect cells and transmit genetic signals. In the area of RNAi research, viruses were used early on to transduce target cells with DNA expression vectors which would use the host cell machinery to produce silencing RNA, mostly in the form of shRNA (Brummelkamp *et al.,* 2002). This approach may have the advantage of allowing for a more persistent silencing effect compared to the delivery of chemically synthesized siRNA since the target cell could be made to express shRNA over a prolonged period of time. Viral delivery vectors are also an economically attrac-

tive alternative to chemically synthesized RNAi therapeutics. However, for use as a vehicle for RNAi-based therapy in humans, viral delivery still faces safety concerns related to serious adverse events observed with viral vectors in the context of gene therapy trials (Hacein-Bey-Abina *et al.,* 2003). The correct dosing of virally-expressed RNAi might be another challenge, as observed in a study reporting lethality in mice after treatment with a virally-delivered shRNA which resulted in overloading of the hepatic micro RNA system and liver failure (Grimm *et al.,* 2006).

Transkingdom RNAi (*tk*RNAi)

As outlined above, the delivery strategies developed thus far have focused mainly on developing chemically modified siRNA to increase stability, and on complexing siRNA with liposomes, nanoparticles and polymers to prevent degradation and promote cell uptake. The main disadvantage of these approaches, however, is that they tend to have a limited ability to target specific cell types or tissues. Moreover, they typically require large quantities of siRNA that is expensive to manufacture. Viral vectors have also been explored as a means of delivering RNAi *in vivo*. While this approach has important research applications, problems associated with insertional mutagenesis, safety, lack of tropism, and the generation of host immune responses have significantly limited the utility of viral vectors for gene therapy.

Recently, in an effort to overcome the "delivery problem", we have developed TransKingdom RNAi (*tk*RNAi) for *in vitro* and *in vivo* gene silencing in mammalian cells (*Figure 1*) (Xiang *et al.,* 2006). In this system, silencing shRNA are transcribed from a TransKingdom RNAi plasmid (TRIP) by T7 RNA polymerase inside non-pathogenic bacteria that have been engineered to invade target cells. Following uptake into phagosomes, the bacteria are lyzed releasing their silencing shRNA into the host cell cytoplasm. RNAi-mediated gene-silencing is then achieved through the canonical Dicer/RISC pathway.

This novel approach has several advantages over delivery mediated by complexed siRNA and viral vectors. First, the TransKingdom system may provide a practical and clinically compatible way to achieve RNAi for medical indications. In contrast to viral vectors, non-pathogenic bacteria have been used clinically for decades with a proven track record of safety in the treatment of gastrointestinal diseases such as diarrhea, irritable bowel syndrome and inflammatory bowel disease. For example, a strain of *Lactococcus lactis* engineered to secrete interleukin-10 has recently been investigated for the treatment of Crohn's disease (Braat *et al.,* 2006). There has also been renewed interest in the use of bacteria to treat human solid tumors (Pawelek *et al.,* 2003; Ryan *et al.,* 2006; Wei *et al.,* 2007). This is based on the observation that various non-pathogenic anaerobic bacteria can infiltrate and replicate within solid tumors when given intravenously. Indeed, attenuated *S. typhimurium* expressing *E. coli* cytosine deaminase has proven effective for the selective conversion of the pro-drug 5-fluorocytosine to 5-fluorouracil in tumors of tumor-bearing mice, and has been tested in 3 phase I clinical trials with demonstrated safety in patients with late stage cancer (Cunningham *et al.,* 2001; King *et al.,* 2002). Thus, this RNAi approach can potentially be exploited to silence genes of interest at various sites colonized by non-pathogenic and/or commensal bacteria.

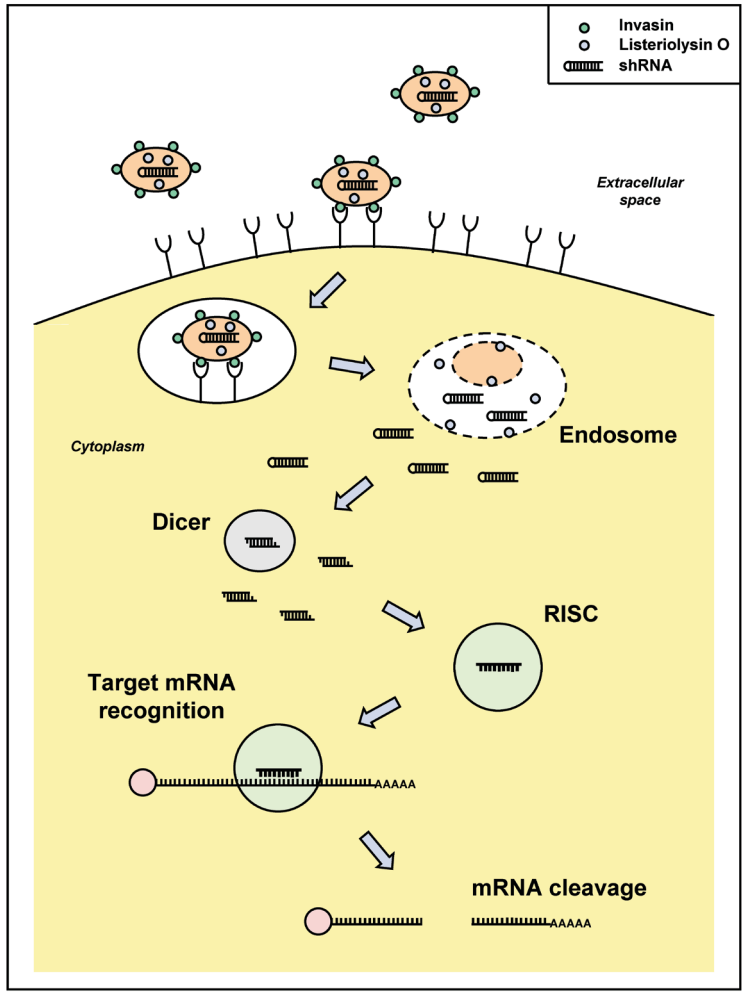

Figure 1. Schematic representation of TransKingdom RNAi.

Second, the production of silencing shRNA by engineered non-pathogenic bacteria eliminates the siRNA manufacture issue, and significantly reduces the cost compared to other delivery technologies. Moreover, the bacterial RNAi approach may circumvent, or mitigate, host interferon-like responses since direct cytoplasmic release of shRNA by the engineered bacteria will likely avoid activation of TLR, PKR and RIG-I in target cells.

Third, *tk*RNAi may have significant implications for high throughput functional genomics in mammalian systems. Bacteria, especially *E. coli*, have served as a well-validated and versatile vector system for the revolution in molecular biology and biotechnology that has occurred over the last few decades. Using the *tk*RNAi approach, a laboratory can easily establish *E. coli*-based RNAi against any gene of interest. A major advantage of this system for functional genomics studies is that it can be eas-

ily reproduced and stored for long term use. Thus, *tk*RNAi should provide a stable and consistent gene silencing tool. Another benefit of the *tk*RNAi approach is that it can be easily translated to *in vivo* systems in order to verify *in vitro* observations in live animals. Furthermore, non-pathogenic bacteria can be used in a routine biological laboratory, rather than a BL2 laboratory which is required for viral vector-based systems. Other advantages of using bacteria as a delivery vector for siRNA include the ability to control the vector using antibiotics and/or auxotrophy, and the ease of engineering specific vectors for particular applications.

Application of TransKingsdom RNAi

Colon cancer is one of the most common solid malignancies (Wei *et al.,* 2007). However, despite of the recent introduction of new drugs such as Avastin (a VEGF inhibitor) and Cetuximab (an EGF receptor inhibitor) the treatment of colon cancer remains largely palliative. Over the last 20 years or so the molecular mechanisms that underlie the pathogenesis colon cancer have been extensively studied and many of the key defects have been identified (Vogelstein *et al.,* 2004). In particular, studies have shown that inappropriate activation of the beta-catenin, resulting from mutation of the APC gene, is causally linked with the pathogenesis of almost all colon cancers (Vogelstein *et al.,* 2004). Recent evidence suggests that beta-catenin stabilization may also be linked to the renewal of cancer stem cells. The development of specific drugs against beta-catenin, however, has proven difficult since it is neither an enzyme nor a receptor. Because of this, beta-catenin is currently considered to be a "non-druggable" target for designing direct small molecule antagonists. Blockade of beta-catenin using RNAi, therefore, represents a potential approach to treat colon cancer. The oncogene k-Ras, which mutates in over 80% of colon cancers, represents another attractive target for treating this disease (Vogelstein *et al.,* 2004). Like beta-catenin, targeted therapy against mutant k-Ras has not been successful and RNAi may provide a potential solution.

In the following sections we demonstrate the feasibility of using *tk*RNAi to potently and specifically silence colon cancer oncogenes *in vitro* and *in vivo*.

*IN VITRO tk*RNAi

The application of *tk*RNAi to silence genes *in vitro* is relatively straight forward only requiring the completion of two sequential experimental phases. The first stage consists of generating TRIP silencing plasmids against each target gene (as well as relevant controls e.g. GFP), and transforming these constructs into competent bacteria capable of expressing high levels of silencing shRNA. The second stage consists of infecting tissue culture cells with TRIP-bearing bacteria, and then assessing target gene silencing.

To investigate *tk*RNAi for *in vitro* silencing of colon cancer oncogenes, we synthesized TRIP plasmids against beta-catenin and mutant k-Ras. As noted above, both molecules have previously been recalcitrant to small-molecule inhibition, making them ideal choices for our initial "proof of concept" studies. In order to facilitate uptake and efficient gene silencing in gastrointestinal cells, these constructs were also engineered to express the invasin gene (*Inv*) from *Yersinia pseudotuberculosis*, and

the listeriolysin O gene (*HlyA*) from *Listeria monocytogenes*. The *Inv* gene product permits non-invasive *E. coli* to enter β1-integrin positive colonic epithelial cells (Isberg *et al.,* 1987; Young *et al.,* 1992), whereas the *Hly* gene product allows the release of genetic material from entry vesicles (Grillot-Courvalin *et al.,* 1998; Mathew *et al.,* 2003). TRIP constructs were then transformed into BL21 DE3 *E. coli* that express the T7 RNA polymerase necessary for shRNA expression. Using this approach the engineered bacteria were found to be capable of expressing high levels of target gene silencing shRNA (*Figure 2a*).

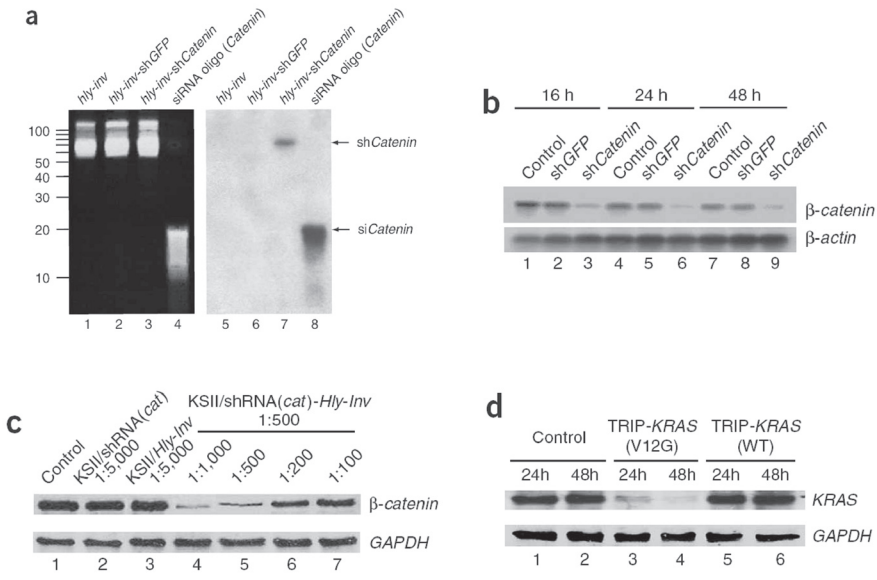

Figure 2. *In vitro* **TransKingdom RNAi. (a)** Expression of shRNA against beta-catenin in engineered *E. coli*. Specific shRNA against beta-catenin was detected by Northern blot analysis in bacteria containing beta-catenin-TRIP, but not GFP-TRIP (green fluorescent protein). As expected, the shRNA is longer than positive control siRNA (21 nt). **(b)** Northern blot analysis shows specific beta-catenin silencing in SW480 colon cancer cells 16 h after infection by *E. coli* containing a beta-catenin TRIP plasmid. **(c)** Beta-catenin expression was silenced in SW480 colon cancer cells after treatment with beta-catenin TRIP-containing *E. coli* at various MOI (lanes 4-7). Lanes 2 and 3 show lack of gene silencing when either the *hly/inv* (lane 2) or shRNA (lane 3) cassettes were deleted, even at very high MOI. **(d)** *E. coli* containing a TRIP against wild-type k-Ras are unable to exert a gene silencing effect in SW480 colon cancer cells containing mutant k-Ras (V12G). Reproduced from Xiang *et al*, 2006 with permission.

The ability of non-pathogenic bacteria expressing shRNA to mediate potent and long-lasting gene silencing *in vitro* is shown in *Figure 2*. In these studies, SW480 human colon cancer cells (at 20% confluency) were co-cultured with beta-catenin TRIP-containing *E. coli* or GFP TRIP-containing *E. coli* (as a plasmid control) at the desired multiplicity of infection (MOI) for 2 h. The SW480 cells were then treated with antibiotics for 2h to remove kill any extracellular bacteria, and *tk*RNAi-mediated target gene silencing was assessed after a period of 16-48 h. As shown in *Figure 2b*, beta-catenin mRNA levels in SW480 cells were potently and specifically silenced after infection by beta-catenin TRIP-bearing *E. coli*. This effect was detectable 16h after bacterial treatment and persisted for least 48h. In contrast, expression of beta-actin mRNA in SW480 cells was unaffected by *tk*RNAi against beta-catenin. In keeping

with these findings, a corresponding reduction in beta-catenin protein levels in SW480 cells was observed 48h following bacterial RNAi against beta-catenin (*Figure 2c*). The potency of gene silencing effect was dependent on MOI, with near complete gene silencing at an MOI of 1:1000. Once again, *tk*RNAi against beta-catenin had no effect on GAPDH levels indicating that the gene silencing effect was specific.

The specificity of the *tk*RNAi approach is further illustrated in *Figure 2d*. In this experiment, SW480 human colon cancer cells were co-cultured with *E. coli* bearing TRIP plasmids against wild-type k-Ras or mutant k-Ras (V12G) as described above. As shown in *Figure 2d*, *E. coli* bearing the wild-type k-Ras TRIP were unable to silence mutant k-Ras in SW480 cells. In contrast, bacterial RNAi directed against the mutant form of k-Ras (GGT-GTT at codon 12) was able to silence k-Ras expression in SW480 cells containing the same mutation. These data demonstrate that *tk*RNAi can mediate allele-specific gene silencing in colon cancer cells with sufficient precision to discriminate a point mutation.

*IN VIVO tk*RNAi

The application of bacteria-mediated RNAi to silence genes in whole animals is also relatively simple to implement. Similar to *in vitro tk*RNAi, the first step consists of generating *E. coli* that harbor TRIP plasmids directed against the target gene of interest. The second step consists of treating animals, either orally or intravenously, with the TRIP-bearing bacteria and then assessing target gene silencing in selected tissues.

The ability of *tk*RNAi to mediate beta-catenin silencing in intestinal epithelium after oral dosing is shown in *Figure 3*. For these experiments, 5 x 10^{10} BL21 DE3 *E. coli* containing TRIP plasmids directed against murine beta-catenin or *E. coli* bearing an empty TRIP vector (as a control) was fed to C57/BL6 mice by gavage five times per week. Both TRIPs were engineered to express the *Inv* and *HlyA* proteins in order to promote bacterial uptake in the GI tract uptake and efficient gene silencing. Treatment was continued for four weeks before analysis of gene silencing by immunohistochemistry. As shown in *Figure 3a*, oral administration of BL21 DE3 *E. coli* containing the beta-catenin TRIP significantly reduced intestinal beta-catenin expression in C57/BL6 mice compared to animals treated with bacteria bearing the empty TRIP vector. The bacterial treatment appeared to be well tolerated with no gross or microscopic signs of epithelial damage or ulcerations **(***Figure 3b***)**. Interestingly, the gene silencing effect appeared to be more pronounced in regions of, or adjacent to, Peyer's patches **(***Figure 3b***)**. In contrast, GAPDH expression was not reduced following treatment with *E. coli* carrying the beta-catenin TRIP, and *E. coli* bearing a GFP TRIP plasmid did not induce beta-catenin silencing (data not shown). These results demonstrate that *tk*RNAi is capable of mediating powerful regional RNAi effects in whole animals.

Previous studies have suggested that bacteria can be employed to selectively target solid tumors (Pawelek *et al.,* 2003; Ryan *et al.,* 2006; Wei *et al.,* 2007). This is based on the observation that various non-pathogenic facultative anaerobes and obligate anaerobes can infiltrate and replicate within the hypoxic regions of solid tumors when delivered systemically. Indeed, intravenous administration of therapeutic bacteria has been tested in clinical trials with demonstrated safety in late stage cancer patients (Cunningham *et al.,* 2001; King *et al.,* 2002). The ability of *tk*RNAi to mediate

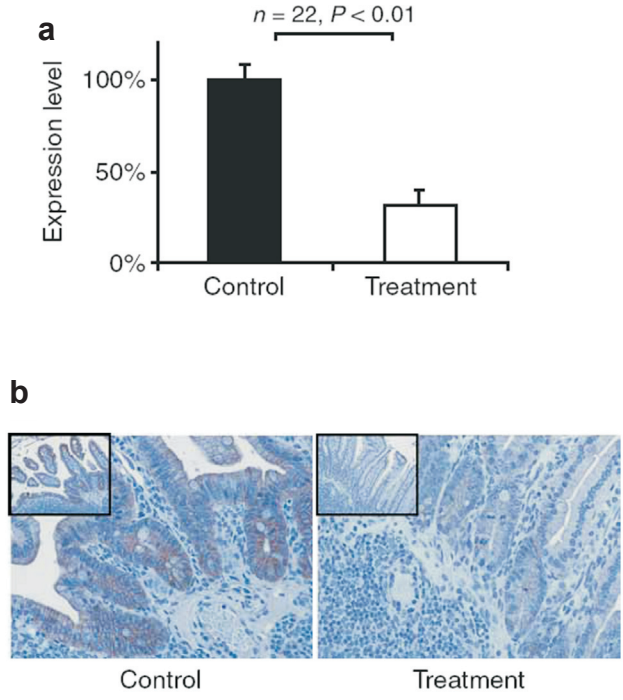

Figure 3. *In vivo* TransKingdom RNAi following oral administration of bacteria. **(a)** Oral administration of *E. coli* expressing shRNA against beta-catenin in C57/BL6 mice leads to significant reduction in beta-catenin protein levels in normal intestinal epithelium, especially in the regions of, or adjacent to, Peyer's patches. **(b)** Representative view of intestinal epithelium from animals treated with *E. coli* bearing an empty TRIP vector (left) or the beta-catenin TRIP plasmid (right). Reproduced from Xiang *et al*, 2006 with permission.

silencing of beta-catenin in human colon cancer xenografts after systemic bacterial administration is shown in *Figure 4*. For these studies, nude mice were implanted subcutaneously with $1x10^7$ SW480 human colon cancer cells. Three weeks later, the mice were given $1x10^8$ BL21 DE3 *E. coli* containing either the beta-catenin TRIP plasmid, or an empty TRIP vector (as a control), by tail vein injection. A total of three doses were administered at 5-day intervals. Intravenous bacterial treatment was well-tolerated without adverse effects. As shown in *Figure 4*, administration of *E. coli* containing the beta-catenin TRIP resulted in significant reduction in xenograft beta-catenin mRNA (*Figure 4a*) and protein *levels (Figures 4b & 4c*) compared to animals treated with control bacteria containing the empty TRIP vector. These data show that *tk*RNAi can effectively silence a disease gene in a distant part of the body after systemic administration.

Conclusion

Since its initial discovery in plants, RNAi has been quickly developed by the research community into a powerful tool for analyzing gene function. RNAi is also poised to catalyze a medical revolution due to its unlimited therapeutic potential for genetic,

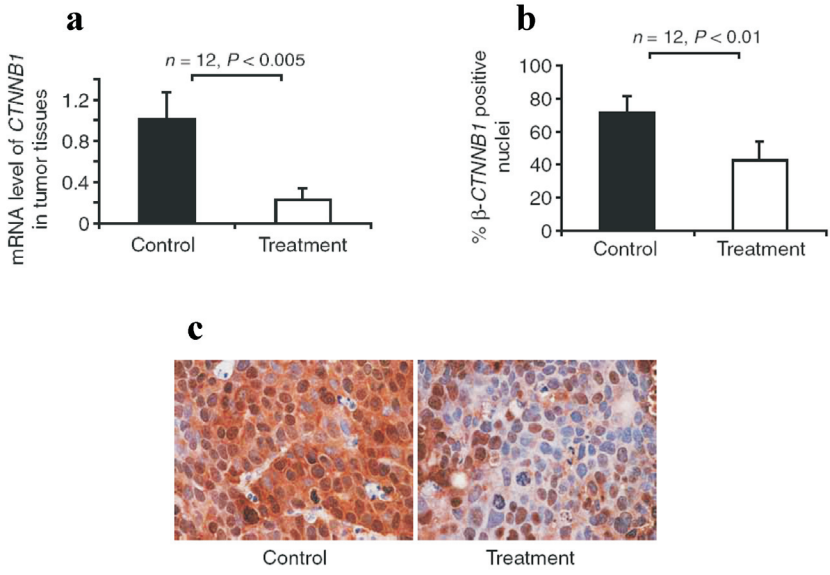

Figure 4: *In vivo* TransKingdom RNAi following intravenous administration of bacteria. Intravenous administration of *E. coli* containing a beta-catenin TRIP to nude mice containing human colon cancer xenograft tumors resulted in decreased in beta-catenin mRNA expression **(a)** and protein expression **(b)** in colon cancer xenograft tissue. **(c)** Representative view of colon cancer xenograft tissue from animals treated with *E. coli* bearing an empty TRIP vector (left) or the beta-catenin TRIP plasmid (right). Reproduced from Xiang *et al*, 2006 with permission.

epigenetic and infectious disease. The development of RNAi-based therapies, however, has been slow mainly due to the difficulty of delivering silencing RNA to specific cells types and/or tissues *in vivo*. The finding that non-pathogenic bacteria can be engineered to simultaneously produce and deliver silencing shRNA into mammalian target cells represents an important step forward in overcoming the delivery problem. The potent and specific intestinal gene silencing seen following oral *tk*RNAi suggests that this approach may be clinically useful for silencing genes of interest in the gastrointestinal mucosa, and possibly other organs that can be colonized by commensal or non-pathogenic bacteria such as the oral cavity, urinary bladder and female genital tract. The therapeutic potential of bacteria-mediated RNAi is further illustrated by its ability to induce gene silencing without toxicity at a distant site following systemic administration, suggesting that the *tk*RNAi approach may also have important applications as a clinically compatible, targeted therapy for cancer.

The *tk*RNAi approach may also be useful for *in vivo* functional genomics studies. Bacteria are particularly useful as an RNAi vector since they can be manipulated with relative ease. Moreover, by using different bacterial strains and routes of application gene silencing can be induced in wide variety of tissues. Thus, *tk*RNAi may provide a convenient and economical system for gene function studies and the validation of therapeutics targets in mammalian systems.

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