BACTERIAL MEDIATED THF ALPHA GENE SILENCING

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ABSTRACT

Methods are described for the delivery of one or more small interfering RNAs (siRNAs) to a eukaryotic cell using a bacterium. Methods are also described for using this bacterium to regulate gene expression in eukaryotic cells using RNA interference, and methods for treating an inflammatory disease or disorder. The bacterium includes one or more siRNAs or one or more DNA molecules encoding one or more siRNAs. Vectors are also described for use with the bacterium of the invention for causing RNA interference in eukaryotic cells.
Fig. 1B
Fig. 2A

- k-Ras levels at 48 hrs and 72 hrs compared to control.
- β-ACTIN levels at 48 hrs and 72 hrs compared to control.
- β-CATENIN levels at 72 hrs, 96 hrs, 120 hrs, and 144 hrs compared to control.
- β-ACTIN levels at 72 hrs, 96 hrs, 120 hrs, and 144 hrs compared to control.
SL-siRAS TREATMENT: VIABILITY (MTT)

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>SL-7207</th>
<th>SL-siRAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>72</td>
<td>100</td>
<td>71.9</td>
</tr>
<tr>
<td>96</td>
<td>100</td>
<td>53.8</td>
</tr>
<tr>
<td>120</td>
<td>100</td>
<td>62.5</td>
</tr>
</tbody>
</table>

COMBINED TREATMENT SL-siCAT AND SL-siRAS: VIABILITY (MTT)

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>SL-7207</th>
<th>COMBINATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>96</td>
<td>100</td>
<td>40.2</td>
</tr>
<tr>
<td>120</td>
<td>100</td>
<td>29</td>
</tr>
<tr>
<td>144</td>
<td>100</td>
<td>32.1</td>
</tr>
</tbody>
</table>

Fig. 2B
SL-siCAT TREATMENT: VIABILITY (MTT)

- SL-7207
- SL-siCAT

COLONY FORMATION EFFICIENCY (%)

- CONTROL
- SL-siRAS
- SL-siCAT
- COMBINED

Fig. 2C
S-siRAS

SL-siRAS

No decrease of fluorescence seen for splenocyte cell suspensions of treated vs control animals:

SL-siGFP

Fig. 3B
T7 PROMOTER: TAATACGACTCACTATAG (SEQ. ID NO:111)

ENHANCER: GAGACAGG (SEQ ID NO:112)

T7 TERMINATOR:
TAGCATAACCCCTTGGG
CCCTCTACGGGCTCTTGA
GGGGTTTTTG (SEQ ID NO:113)

LOOP:
TTCAAGAGA (SEQ ID NO:114)

shRNA-ENCODING
OLIGONUCLEOTIDES OF HUMAN, MOUSE
β-CATENIN AND HUMAN k-RAS (V12G):

HUMAN β-CATENIN
ggATCCGCTGATATTGATGGACAGTTCAAGAGA
CTGTTCTCAATATTACGGCTTTgTgTgAC
(SEQ ID NO:115)

MOUSE β-CATENIN
ggATCCGCTGAGGTAGTTAAATAAAGCTTTCAAGAGA
AGCTTTATTAACTACCCCTTTTgTgTgAC(SEQ ID NO:116)

HUMAN k-RAS (V12G)
ggATCCGCTGGAGCTTGTGGGCCGTAGTTCAAGAGA
CTACGCCAAGCTCAACTTTTgTgTgAC(SEQ ID NO:117)

Fig. 6A
Fig. 7B

Fig. 7C
<table>
<thead>
<tr>
<th>1Kb PLUS DNA LADDER</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CONTROL</td>
<td>TREATED</td>
<td>CONTROL</td>
</tr>
<tr>
<td>1Kb PLUS DNA LADDER</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-ACTIN (110 nt)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OAS1 (144 nt)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OAS2 (234 nt)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MX1 (402 nt)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFITM1 (128 nt)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISGF3γ (333 nt)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 7D

Fig. 8A
Fig. 8B

Fig. 8C

Fig. 8D

Fig. 8E
Fig. 9D

% $\beta$-CATENIN POSITIVE NUCLEI

CONTROL  TREATMENT

P < 0.01

Fig. 9E

CONTROL  TREATMENT
RELATIVE QUANTITATION: TNFα NORMALIZED TO GAPDH IN siRNA TRANSFECTED THP-1 CELLS (HUMAN)

RAW264.7 CELLS (MOUSE)

Fig. 11
BACTERIAL MEDIATED THF ALPHA GENE SILENCING

RELATED APPLICATIONS


BACKGROUND

[0002] Gene silencing through RNAi (RNA-interference) by use of short interfering RNA (siRNA) has emerged as a powerful tool for molecular biology and holds the potential to be used for therapeutic gene silencing. Short hairpin RNA (shRNA) transcribed from small DNA plasmids within the target cell has also been shown to mediate stable gene silencing and achieve gene knockdown at levels comparable to those obtained by transfection with chemically synthesized siRNA (T. R. Brummelkamp, R. Bernards, R. Agami, Science 296, 550 (2002), P. J. Paddison, A. A. Caudy, G. J. Hannon, PNAS 99, 1443 (2002)).

[0003] Possible applications of RNAi for therapeutic purposes are extensive and include silencing and knockdown of disease genes such as oncogenes or viral genes. One major obstacle for the therapeutic use of RNAi is the delivery of siRNA to the target cell (Zamore P D, Aronin N. Nature Medicine 9, (3):266-8 (2003)). In fact, delivery has been described as the major hurdle now for RNAi (Phillip Sharp, cited by Nature news feature, Vol 425, 2003, 10-12).

[0004] Two methods have been described which can be used in mouse models:


[0006] (2) Direct injection into the target tissue (brain) of an siRNA encoding adenoviral vector (H. Xie, Q. Mao, H. L. Paulson, B. L. Davidson, Nat Biotechnol, 20, 1006 (2002)). This method showed silencing of transgene (GFP) expression in the brain tissues reached by the adenoviral vector. However, the area of silencing could not be predicted reliably. This method might be developed further and might become applicable for local, e.g. intratumoral injection. Viral vectors have been used widely for gene therapy purposes, but one lesson learned from gene therapy experiments is that viral spreading can be unpredictable at times and lead to unwanted side effects (Marshall E. Science 286(5448): 2244-5 (1999)). A new method is needed for the safe and predictable administration of interfering RNAs to mammals.

SUMMARY OF THE INVENTION

[0007] The invention generally pertains to methods of delivering one or more siRNAs to a eukaryotic cell by introducing a bacterium to the cell, wherein the bacterium contains one or more siRNAs or one or more DNA molecules encoding one or more siRNAs.

[0008] In one embodiment of this method, the eukaryotic cell is in vivo. In another embodiment of this invention, the eukaryotic cell is in vitro.

[0009] The invention also pertains to a method of regulating gene expression in a eukaryotic cell, by introducing a bacterium to the cell, wherein the bacterium contains one or more siRNAs or one or more DNA molecules encoding one or more siRNAs, wherein the expressed siRNAs interfere with the mRNA of the gene to be regulated, thereby regulating expression of the gene.

[0010] In one embodiment of this method, the expressed siRNAs direct the multienzyme complex RISC (RNA-induced silencing complex) of the cell to interact with the mRNA to be regulated. This complex degrades the mRNA. This causes the expression of the gene to be decreased or inhibited. In another embodiment of this method, the gene is Tumor Necrosis Factor (TNF), ras or pcatenin. In one aspect of this embodiment, the ras is k-Ras. In another aspect of this embodiment, the TNF is TNFα.

[0011] In one embodiment of the above methods of the invention, the eukaryotic cell is a mammalian cell. In one aspect of this embodiment, the mammalian cell is a human cell.

[0012] The invention also pertains to a method of treating or preventing cancer or a cell proliferation disorder in a mammal, by regulating the expression of a gene or several genes in a cell known to increase cell proliferation by introducing a bacterium to the cell. The bacterium contains one or more siRNAs or one or more DNA molecules encoding one or more siRNAs.

[0013] In one embodiment of this method of the invention, the mammal can be, but it not limited to, human, bovine, ovine, porcine, feline, buffalo, canine, goat, equine, donkey, deer, avian, bird, chicken and primate. The most preferred mammal is a human. In another embodiment of this method the expressed siRNAs interfere with the mRNA of the gene to be regulated. In one aspect of this embodiment, the expressed siRNAs direct the multienzyme complex RISC (RNA-induced silencing complex) of the cell to interact with the mRNA to be regulated. This complex degrades the mRNA. This causes the expression of the gene to be decreased or inhibited.

[0014] In another embodiment of this method, the gene is ras or β-catenin. In one aspect of this embodiment, the ras is k-Ras.

[0015] In another embodiment of this method of the invention, the cell is a colon cancer cell or a pancreatic cancer cell. In one aspect of this embodiment, the colon cancer cell is an SW 480 cell. In another aspect of this embodiment, the pancreatic cancer cell is a CAPAN-1 cell.

[0016] The invention also pertains to a method of treating or preventing an inflammatory disease or disorder in a mamm-
mal, by regulating the expression of a gene or several genes known to cause an inflammatory disease or disorder in a cell, by introducing at least one bacterium to the cell. The bacterium contains one or more siRNAs or one or more DNA molecules encoding one or more siRNAs. In a preferred embodiment of this method, the gene to be regulated can be, but is not limited to, TNFα. Preferably, the regulation of the gene decreases or lessens the expression and/or activity of the gene known to cause an inflammatory disease or disorder.

In one embodiment of this method, the mammal can be, but it not limited to, human, bovine, ovine, porcine, feline, canidae, equine, donkey, deer, avian, bird, chicken and primate. The most preferred mammal is a human. In another embodiment of this method the expressed siRNAs interfere with the mRNA of the gene or genes to be regulated. In one aspect of this embodiment, the expressed siRNAs direct the multienzyme complex RISC (RNA-induced silencing complex) of the cell to interact with the mRNA to be regulated. This complex degrades the mRNA. This causes the expression of the gene to be decreased or inhibited.

In another embodiment of this method, the inflammatory disease or disorder is inflammatory bowel disease. In another embodiment of this method, the inflammatory disease or disorder is Crohn’s disease. In another embodiment of this method, the inflammatory disease or disorder is ulcerative colitis. In another embodiment of this method, the inflammatory disease or disorder is an allergy. In another embodiment of this method, the cell is a gastrointestinal epithelial cell. In another embodiment of this method, the cell is a macrophage cell. The cell can be in vivo, in vitro or ex vivo.

In one embodiment of the above methods of the invention, the bacterium is non-pathogenic or non-virulent. In another aspect of this embodiment, the bacterium is therapeutic. In another aspect of this embodiment, the bacterium is an attenuated strain selected from the group consisting of *Listeria*, *Shigella*, *Salmonella*, *E. coli*, and *Bifidobacterium*. Optionally, the *Salmonella* strain is an attenuated strain of the *Salmonella typhimurium* species. Optionally, the *Salmonella typhimurium* strain is SL 7207 or VNP20009.

In another embodiment of the above methods of the invention, the one or more DNA molecules encoding the one or more siRNAs are transcribed within the eukaryotic cell. In one aspect of this embodiment, the one or more siRNAs are transcribed within the eukaryotic cells as siRNAs. In another aspect of this embodiment, the one or more DNA molecules encoding the one or more siRNAs contains an RNA-polymerase II promoter. Optionally, the RNA polymerase II promoter is a U6 promoter or an H1 promoter.

In another embodiment of the above methods of the invention, the one or more DNA molecules encoding the one or more siRNAs are transcribed within the bacterium. In one aspect of this embodiment, the one or more DNA molecules contain a prokaryotic promoter. Optionally, the prokaryotic promoter is a T7 promoter.

In another embodiment of the above methods of the invention, the one or more DNA molecules are introduced to the eukaryotic cell through type II export or bacterial lysis. In one aspect of this embodiment, the bacterial lysis is triggered by the addition of an intracellular active antibiotic. Optionally, the antibiotic is tetracycline. In another aspect of this embodiment, the bacterial lysis is triggered through bacterial metabolic attenuation. Optionally, the metabolic attenuation is auxotrophy.

The invention also pertains to a bacterium containing one or more siRNAs or one or more DNA molecules encoding one or more siRNAs.

In one embodiment of this invention, the bacterium is a non-pathogenic or a non-virulent bacterium. In another aspect of this embodiment, the bacterium is a therapeutic bacterium.

In another embodiment of this invention, the bacterium is an attenuated strain or derivative thereof selected from, but not limited to *Yersinia*, *Rickettsia*, *Legionella*, *Brucella*, *Mycobacterium*, *Helicobacter*, *Haemophilus*, *Coxiella*, *Chlamydia*, *Neisseria*, *Bordetella*, *Borrelia*, *Listeria*, *Shigella*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Porphyromonas*, *Treponema*, *Vibrio*, *E. coli*, and *Bifidobacterium*. Optionally, the *Yersinia* strain is an attenuated strain of the *Yersinia pseudotuberculosis* species. Optionally, the *Yersinia* strain is an attenuated strain of the *Yersinia enterocolitica* species. Optionally, the *Rickettsia* strain is an attenuated strain of the *Rickettsia conorii* species. Optionally, the *Legionella* strain is an attenuated strain of the *Legionella pneumophila* species. Optionally, the *Mycobacterium* strain is an attenuated strain of the *Mycobacterium tuberculosis* species. Optionally, the *Mycobacterium* strain is an attenuated strain of the *Mycobacterium bovis BCG* species. Optionally, the *Helicobacter* strain is an attenuated strain of the *Helicobacter pylori* species. Optionally, the *Coxiella* strain is an attenuated strain of *Coxiella burnetii*. Optionally, the *Haemophilus* strain is an attenuated strain of the *Haemophilus influenzae* species. Optionally, the *Chlamydia* strain is an attenuated strain of the *Chlamydia trachomatis* species. Optionally, the *Chlamydia* strain is an attenuated strain of the *Chlamydia pneumoniae* species. Optionally, the *Neisseria* strain is an attenuated strain of the *Neisseria gonorrhoeae* species. Optionally, the *Neisseria* strain is an attenuated strain of the *Neisseria meningitides* species. Optionally, the *Bordetella* strain is an attenuated strain of the *Bordetella pertussis* species. Optionally, the *Borrelia* strain is an attenuated strain of the *Borrelia hermsii* species. Optionally, the *Listeria* strain is an attenuated strain of the *Listeria monocytogenes* species. Optionally, the *Listeria* strain is an attenuated strain of the *Listeria ivanovii* species. Optionally, the *Salmonella* strain is an attenuated strain of the *Salmonella enterica* species. Optionally, the *Salmonella* strain is an attenuated strain of the *Salmonella typhimurium* species. Optionally, the *Salmonella typhimurium* strain is SL 7207 or VNP20009. Optionally, the *Staphylococcus* strain is an attenuated strain of the *Staphylococcus aureus* species. Optionally, the *Streptococcus* strain is an attenuated strain of the *Streptococcus mutans* species. Optionally, the *Streptococcus* strain is an attenuated strain of the *Streptococcus salivarus* species. Optionally, the *Streptococcus* strain is an attenuated strain of the *Porphyromonas gingivalis* species. Optionally, the *Pseudomonas aeruginosa* species. Optionally, the *Treponema* strain is...
an attenuated strain of the *Treponema pallidum* species. Optionally, the *Vibrio* strain is an attenuated strain of the *Vibrio cholerae* species.

[0026] The invention also pertains to a prokaryotic vector containing a DNA encoding one or more siRNAs and an RNA-polymerase III compatible promoter or a prokaryotic promoter.

[0027] In one embodiment of this vector of the invention, the RNA polymerase III promoter is a U6 promoter or an H1 promoter. In another embodiment of this vector of the invention, the prokaryotic promoter is a T7 promoter.

[0028] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be limiting.

[0029] Other features and advantages of the invention will be apparent from the following detailed description and claims.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0030] FIG. 1A shows micrographs of invasion of SL 7207 into SW 480 cells. FIG. 1B shows FACS analysis of a knockdown of green fluorescent protein expression in CRL 2583 cells. FIG. 1C shows micrographs showing loss of fluorescence in CRL 2583 cells.

[0031] FIG. 2A shows Western blots of k-Ras and β-catenin in SW 480 cells.

[0032] FIG. 2B is a series of bar charts showing viability of SW 480 cells under treatment regimes with SL-siRAS and resulting knockdown of k-Ras.

[0033] FIG. 2C is a series of bar charts showing viability of SW 480 cells under treatment regimes with SL-siCAT.

[0034] FIG. 2D shows photographs of tumorigenicity in nude mice injected with SW 480 cells transfected with various siRNAs.

[0035] FIG. 3A shows micrographs of transgenic mouse liver sections. FIG. 3B shows flow cytometry measurements of hepatocyte and splenocyte suspensions.

[0036] FIG. 4A shows Western blots of k-Ras from SW 480 cells transfected with silencer plasmid.

[0037] FIG. 4B shows Western blots of β-catenin from SW 480 cells transfected with silencer plasmid.

[0038] FIG. 5 shows micrographs of histochemical staining of liver sections of mice showing changes in GFP expression levels.

[0039] FIG. 6A is a schematic showing the Transkingdom RNA Interference Plasmid (TRIP). FIG. 6B is a photograph showing an immunoblot of k-Ras from SW 480 cells transfected with TRIP. FIG. 6C is a photograph showing an immunoblot of β-catenin from SW 480 cells transfected with TRIP for exposure time from 30 to 120 minutes. FIG. 6D shows an RT-PCR photograph of β-catenin and k-Ras mRNA from SW 480 cells transfected with TRIP. FIG. 6E is a photograph showing an immunoblot of k-Ras in SW 480 and DLD4 cells following transfection with a TRIP against mutant k-Ras (GGT-GTT at codon 12) mutant k-Ras (GTC-GAC at codon 13). FIG. 6F is a photograph showing an immunoblot of SW 480 cells transfected with a TRIP against wild type k-Ras.

[0040] FIG. 7A is a photograph of RT-PCR showing β-catenin silencing following treatment with *E. coli* expressed shRNAs. FIG. 7B is a schematic showing specific cleavage sites in β-catenin. FIG. 7C is a photograph of a 5’-RACE-PCR showing specific cleavage products. FIG. 7D is a photograph of a blot showing the mRNA expression of various genes.

[0041] FIG. 8A is a photograph of cellular staining showing that both lav and Hly are required for bacterial entry. FIG. 8B is a photograph of an RNA blot showing that TRIP lacking Hly is unable to induce knockdown of a target gene. FIG. 8C is a photograph of an RNA blot showing that both lav and Hly are required to facilitate efficient transkingdom iRNA. FIG. 8D is a photograph of an RNA blot showing the effect of delayed addition of tetracycline on gene silencing. FIG. 8E is a photograph of cellular staining showing lack of significant bacterial replication in the absence of antibiotics beyond 2 h incubation.

[0042] FIG. 9A is a graph showing that oral administration of *E. coli* expressing shRNA against β-catenin in mice leads to significant reduction of β-catenin expression in the intestinal epithelium. FIG. 9B is a photograph of immunohistochemistry staining of intestinal epithelium with or without treatment. FIG. 9C is a graph showing a decrease in β-catenin mRNA expression following treatment. FIG. 9D is a graph showing a decrease in β-catenin protein expression following treatment. FIG. 9E is a photograph of immunohistochemistry staining showing decrease in β-catenin protein expression following treatment.

[0043] FIG. 10 is a photograph of immunohistochemistry staining showing that GAPDH expression is not altered by *E. coli* expressing shRNA against β-catenin after oral dosing in mice.

[0044] FIG. 11 is a graph illustrating reduction of TNF-α expression.

**DETAILED DESCRIPTION OF THE INVENTION**

[0045] The invention pertains to methods of delivering small interfering RNAs (siRNAs) to eukaryotic cells using non-pathogenic or therapeutic strains of bacteria. The bacteria deliver RNA encoding DNA or RNA, itself to effect RNA interference (RNAi). The interfering RNA of the invention regulates gene expression in eukaryotic cells. It silences or knocks down genes of interest inside target cells. The interfering RNA directs the cell-owned multienzyme-complex RISC (RNA-induced silencing complex) to the mRNA of the gene to be silenced. Interaction of RISC and mRNA results in degradation of the mRNA. This leads to effective post-transcriptional silencing of the gene of interest. This method is referred to as Bacteria Mediated Gene Silencing (BMGS).

[0046] Bacterial delivery is more attractive than viral delivery as it can be controlled by use of antibiotics and attenuated bacterial strains which are unable to multiply. Also, bacteria are much more accessible to genetic manipulation which allows the production of vector strains specifically tailored to certain applications. In one embodiment of the invention, the methods of the invention are used to create bacteria which cause RNAi in a tissue specific manner.

[0047] The siRNA is either introduced into the target cell directly or by transfection or can be transcribed within the target cell as hairpin-structured dsRNA (shRNA) from specific plasmids with RNA-polymerase III compatible promoters (U6, H1) (P. J. Paddison, A. A. Caudy, G. J. Hannon,
Liberation of siRNA encoding plasmid from the intracellular bacteria occurs through active mechanisms. One mechanism involves the type III export system in *S. typhimurium*, a specialized multiprotein complex spanning the bacterial cell membrane whose functions include secretion of virulence factors to the outside of the cell to allow signaling towards the target cell, but which can also be used to deliver antigens into target cells. (Rüssmann H. Int J Med Microbiol. 293:107-12 (2003)) or through bacterial lysis and liberation of bacterial contents into the cytoplasm. The lysis of intracellular bacteria is triggered through addition of an intracellularly active antibiotic (tetracycline) or occurs naturally through bacterial metabolic attenuation (auxotrophy). After liberation of the eukaryotic transcription plasmid, siRNA or sRNA are produced within the target cell and trigger the highly specific process of mRNA degradation, which results in silencing of the targeted gene.


BMGS is performed using the naturally invasive pathogen *Salmonella typhimurium*. In one aspect of this embodiment, the strains of *Salmonella typhimurium* include SL 7207 and VNP20009 (S. K. Hoiseth, B. A. D. Stocker, Nature 291, 238 (1981); Pawelcek J M, I Low, K B, Bermedes D. Cancer Res. 57(20):4537-44 (Oct. 15, 1997)). In another embodiment of the invention, BMGS is performed using attenuated *E. coli*. In one aspect of this embodiment, the strain of *E. coli* is BM 2710 (C. Grellot-Courvalin, S. Goussard, F. Huetz, D. M. Ojcius, P. Courvalin, Nat Biotechnol 16, 862 (1998)). In another aspect of this embodiment, the BM 2710 strain is engineered to possess cell-invading properties through an invasion plasmid. In one aspect of the invention, this plasmid is pGB2inv-hly.

A double “trojan horse” technique is also used with an invasive and auxotrophic bacterium carrying a eukaryotic transcription plasmid. This plasmid is, in turn, transcribed by the target cell to form a hairpin RNA structure that triggers the intracellular process of RNAi. This method of the invention induces significant gene silencing of a variety of genes. In certain aspects of this embodiment, the genes include a transgene (GFP), a mutated oncogene (K-Ras) and a cancer related gene (β-catenin) In vitro.

The invention also pertains to a variation of the described method, termed Bacteria Transcribed Gene Silencing (BTGS). In this aspect of the invention, siRNA is directly produced by the invasive bacteria as opposed to the target cell. A transcription plasmid controlled by a prokaryotic promoter (e.g. T7) is inserted into the carrier bacteria through standard transformation protocols. siRNA is produced within the bacteria and is liberated within the mammalian target cell after bacterial lysis triggered either by auxotrophy or by timed addition of antibiotics.

The RNAi methods of the invention, including BMGS and BTGS are used as a cancer therapy or to prevent cancer. This method is affected by silencing or knocking down genes involved with cell proliferation or other cancer phenotypes. Examples of these genes are K-Ras and β-catenin. Specifically, K-Ras and β-catenin are targets for RNAi based therapy of colon cancer. These oncogenes are active and relevant in the majority of clinical cases. BMGS is applied to reach the intestinal tract for colon cancer treatment and prevention. These methods are also used to treat animals carrying xenograft tumors, to treat and prevent cancer in k-Ras V12 model of intestinal tumorigenesis, and to prevent and treat tumors in the adenomatous polyposis coli min mouse model (APC-min model). In this model, the mouse has a defective APC gene resulting in the formation of numerous intestinal and colonic polyps which is used as an animal model for human familial adenomatous polyposis coli (FAP) of intestinal tumorigenesis.

The invention also encompasses a prokaryotic siRNA-encoding transcription plasmid for use with invasive bacteria to perform Bacteria-Transcribed Gene Silencing (BTGS). These plasmids are used to screen different cancer-related targets in transgenic as well as wild type animals for therapeutic experiments.

The RNAi methods of the invention, including BMGS and BTGS are also used to treat or prevent viral diseases (e.g. hepatitis) and genetic disorders.

The RNAi methods of the invention, including BMGS and BTGS are also used to create cancer-preventing “probiotic bacteria” for use, especially with the target of Gl tract or liver.

The RNAi methods of the invention, including BMGS and BTGS are used as therapy against inflammatory conditions, e.g. inflammatory bowel disease (IBD) or colitis. These methods are used to silence or knockdown non-cancer gene targets (viral genes, for treatment and prevention of hepatitis B, C; inflammatory genes, for treatment and prevention of inflammatory bowel disease) and others.

The invention also pertains to a method of treating or preventing an inflammatory disease or disorder in a mammal, by regulating the expression of a gene or several genes known to cause an inflammatory disease or disorder in a cell,
by introducing at least one bacterium to the cell. The bacterium contains one or more siRNAs or one or more DNA molecules encoding one or more siRNAs.

[0059] In one embodiment of this method of the invention, the mammal can be, but it is not limited to, human, bovine, ovine, porcine, feline, buffalo, canine, goat, equine, donkey, deer, avian, bird, chicken and primate. The most preferred mammal is a human. In another embodiment of this method the expressed siRNAs interfere with the mRNA of the gene or genes to be regulated. In one aspect of this embodiment, the expressed siRNAs direct the multi-enzyme complex RISC (RNA-induced silencing complex) of the cell to interact with the mRNA to be regulated. This complex degrades the mRNA. This causes the expression of the gene to be decreased or inhibited.

[0060] In another embodiment of this method, the inflammatory disease or disorder is inflammatory bowel disease. In another embodiment of this method, the inflammatory disease or disorder is Crohn’s disease. In another embodiment of this method, the inflammatory disease or disorder is ulcerative colitis. In another embodiment of this method, the inflammatory disease or disorder is an allergy. In another embodiment of this method, the cell is a gastrointestinal epithelial cell. In another embodiment of this method, the cell is a macrophage cell. The cell can be in vivo, in vitro or ex vivo. In another embodiment of this method, the gene can be, but is not limited to, TNFα.

[0061] In a preferred embodiment of this method, the gene to be regulated can be, but is not limited to, TNFα. Preferably, the regulation of the gene decreases or lessens the expression and/or activity of the gene known to cause an inflammatory disease or disorder.

[0062] In another aspect of this embodiment, the TNFα target gene sequence can be, but is not limited to, those shown in Table 1. The sequences in Table 1 are cross-species target sequences as they are capable of silencing the TNFα in human and mouse.

<table>
<thead>
<tr>
<th>TABLE 1</th>
</tr>
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<tbody>
<tr>
<td>CCCGAGTGGACAAGCCTGTTGCC (SEQ ID NO: 1)</td>
</tr>
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<td>CGTGGAGCAGCCGCTGAAG (SEQ ID NO: 2)</td>
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<tr>
<td>CAGTGACAAAGCGCTGTAAGCC (SEQ ID NO: 3)</td>
</tr>
<tr>
<td>GAGCTGACAGCGCTGAGCGCA (SEQ ID NO: 4)</td>
</tr>
<tr>
<td>AGTGAGAAGCTGAGGCGCA (SEQ ID NO: 5)</td>
</tr>
<tr>
<td>AGTGCCCTGGACCCCAACCA (SEQ ID NO: 6)</td>
</tr>
<tr>
<td>AGTGCACTTCTGTTGTGGA (SEQ ID NO: 7)</td>
</tr>
<tr>
<td>GGTCTTCCGACCCGAAAGG (SEQ ID NO: 8)</td>
</tr>
<tr>
<td>AGATGAACTTTATATTGGG (SEQ ID NO: 9)</td>
</tr>
</tbody>
</table>

[0063] In another aspect of this embodiment, the TNFα target gene sequence can be, but is not limited to, those shown in Table 2. The sequences in Table 2 are target sequences as they are capable of silencing the TNFα in human.

<table>
<thead>
<tr>
<th>TABLE 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTTGATCGCGGCAGCAGGCGGCA (SEQ ID NO: 10)</td>
</tr>
<tr>
<td>GCCGGGCGCCACGCGCTCT (SEQ ID NO: 11)</td>
</tr>
</tbody>
</table>

[0064] In another aspect of this embodiment, the TNFα target gene sequence can be, but is not limited to, those shown in Table 3. The sequences in Table 3 are target sequences as they are capable of silencing the TNFα in mouse.

<table>
<thead>
<tr>
<th>TABLE 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCCAGTCGCCGCCTCTCTACCG (SEQ ID NO: 12)</td>
</tr>
<tr>
<td>GCTCGGACGGTCGCCCTTGG (SEQ ID NO: 13)</td>
</tr>
<tr>
<td>ACTGGCGGCGGCTGACAATCG (SEQ ID NO: 14)</td>
</tr>
<tr>
<td>CTTGGGCGGCGGCTGACAATCG (SEQ ID NO: 15)</td>
</tr>
<tr>
<td>TGGAGCGGCGGCTGACAATCG (SEQ ID NO: 16)</td>
</tr>
<tr>
<td>CAGCGGCGGCGGCTGACAATCG (SEQ ID NO: 17)</td>
</tr>
<tr>
<td>CTGAGACCTCACTCGGCCGACT (SEQ ID NO: 18)</td>
</tr>
<tr>
<td>TGGATACCTCGGCCGACT (SEQ ID NO: 19)</td>
</tr>
<tr>
<td>GAGATCGAGGCGCGGACT (SEQ ID NO: 20)</td>
</tr>
<tr>
<td>AGATCGAGGCGCGGACT (SEQ ID NO: 21)</td>
</tr>
<tr>
<td>AGATCGAGGCGCGGACT (SEQ ID NO: 22)</td>
</tr>
<tr>
<td>ATCACTCGGCCGACT (SEQ ID NO: 23)</td>
</tr>
<tr>
<td>TGGAGCGGCGGCTGACAATCG (SEQ ID NO: 24)</td>
</tr>
<tr>
<td>CAGCGGCGGCGGCTGACAATCG (SEQ ID NO: 25)</td>
</tr>
<tr>
<td>AATCGAGGCGGCTGACAATCG (SEQ ID NO: 26)</td>
</tr>
<tr>
<td>AATCGAGGCGGCTGACAATCG (SEQ ID NO: 27)</td>
</tr>
<tr>
<td>TGGAGCGGCGGCTGACAATCG (SEQ ID NO: 28)</td>
</tr>
<tr>
<td>CGGAGGGCTGACAATCG (SEQ ID NO: 29)</td>
</tr>
<tr>
<td>GCCGAGTCCTGCGATTTGCG (SEQ ID NO: 30)</td>
</tr>
<tr>
<td>CCCGAGTCCTGCGATTTGCG (SEQ ID NO: 31)</td>
</tr>
<tr>
<td>GACTCTCGATCTTTGCGGAG (SEQ ID NO: 32)</td>
</tr>
<tr>
<td>ACTACTCGACCTTGGCGCG (SEQ ID NO: 33)</td>
</tr>
<tr>
<td>CTACTCGACCTTGGCGCG (SEQ ID NO: 34)</td>
</tr>
<tr>
<td>TATCTCGACCTTGGCGCG (SEQ ID NO: 35)</td>
</tr>
<tr>
<td>GGAGGGCTGACAATCG (SEQ ID NO: 36)</td>
</tr>
<tr>
<td>TAGGTCGAGGCGGACT (SEQ ID NO: 37)</td>
</tr>
<tr>
<td>GGAGGGCTGACAATCG (SEQ ID NO: 38)</td>
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<tr>
<td>GTCCGAGGCGGACT (SEQ ID NO: 39)</td>
</tr>
<tr>
<td>CTGATACCTCACTCGGCCGACT (SEQ ID NO: 40)</td>
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<tr>
<td>TGGATACCTCGGCCGACT (SEQ ID NO: 41)</td>
</tr>
<tr>
<td>GCCGAGTCCTGCGATTTGCG (SEQ ID NO: 42)</td>
</tr>
<tr>
<td>CCGAGTCCTGCGATTTGCG (SEQ ID NO: 43)</td>
</tr>
<tr>
<td>CCTGAGACCTCACTCGGCCGACT (SEQ ID NO: 44)</td>
</tr>
<tr>
<td>GTCCGAGGCGGACT (SEQ ID NO: 45)</td>
</tr>
</tbody>
</table>
**TABLE 3**

<table>
<thead>
<tr>
<th>Sense (19bp)</th>
<th>Loop (19bp)</th>
<th>Antisense (19bp)</th>
<th>SalI</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGAAAGCATGATGCCGACGT</td>
<td>(SEQ ID NO: 46)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAGCATGATGCCGACGTGGGA</td>
<td>(SEQ ID NO: 47)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGCTGATGCCGACGTGGGA</td>
<td>(SEQ ID NO: 48)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CATGATGCCGACGTGGACCT</td>
<td>(SEQ ID NO: 49)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATGATGCCGACGTGGAACTG</td>
<td>(SEQ ID NO: 50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCCGCCGACGTGGACCTGCA</td>
<td>(SEQ ID NO: 51)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AACGCTGATGCCGACGTGTA</td>
<td>(SEQ ID NO: 52)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCGTGATGCCGACGTGTAACGA</td>
<td>(SEQ ID NO: 53)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCCACGTGATGCCGACGTGAA</td>
<td>(SEQ ID NO: 54)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GACCGCAGCGACGTCCCGCT</td>
<td>(SEQ ID NO: 55)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAACTCGTCCGCAGCGACGTGA</td>
<td>(SEQ ID NO: 56)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAATCTGGTGACGCAGCGAGT</td>
<td>(SEQ ID NO: 57)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AACCTGAGCCGACCGATG</td>
<td>(SEQ ID NO: 58)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCACCGAGCGCTGCTCTCTCA</td>
<td>(SEQ ID NO: 59)</td>
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<td></td>
</tr>
<tr>
<td>ACCCACCAGCTAACCGCATT</td>
<td>(SEQ ID NO: 60)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCCACCGAGCTAACCGCATTG</td>
<td>(SEQ ID NO: 61)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCACCGAGCTAACCGCATTGTC</td>
<td>(SEQ ID NO: 62)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CACCACGTGATGCCGACGTGA</td>
<td>(SEQ ID NO: 63)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CACGTGATGCCGACGTGAATTTCTAT</td>
<td>(SEQ ID NO: 64)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACCGTGATGCCGACGTGAATTCTATC</td>
<td>(SEQ ID NO: 65)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAAGTACTAGCTATTTGCCGA</td>
<td>(SEQ ID NO: 66)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAATCATGTTAGCTTGCGGGA</td>
<td>(SEQ ID NO: 67)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AGTACTAGCTATTTGCCGAT</td>
<td>(SEQ ID NO: 68)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GACTTTGCCGAGCTGCGCGAG</td>
<td>(SEQ ID NO: 69)</td>
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<td></td>
</tr>
<tr>
<td>GAGCTCAGGCGAGCTCATTCTT</td>
<td>(SEQ ID NO: 70)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAGGGGCGCTGACTGTAATCGC</td>
<td>(SEQ ID NO: 71)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BamHI</td>
<td>sense (19bp)</td>
<td>loop</td>
<td>antisense (21bp)</td>
</tr>
<tr>
<td>5'-GATCCCGCCTAGGACACCTGGTACG</td>
<td>TTCAARGAGA</td>
<td>GCTACAGGGCTGCCATCGGTGCCTGC</td>
<td>G-3' (SEQ ID NO: 83)</td>
</tr>
<tr>
<td>3'-G</td>
<td>CGTCTGCTTTCCGACAGCTCGGAGC</td>
<td>AAGTCCTCTTCCGACAGCTCGGAGC</td>
<td>CGCCCTACGGGTCATTGAG</td>
</tr>
<tr>
<td>5'-GATCCCGCCTAGGACACCTGGTACG</td>
<td>TTCAARGAGA</td>
<td>GCTACAGGGCTGCCATCGGTGCCTGC</td>
<td>G-3' (SEQ ID NO: 85)</td>
</tr>
<tr>
<td>3'-G</td>
<td>CGTCTGCTTTCCGACAGCTCGGAGC</td>
<td>AAGTCCTCTTCCGACAGCTCGGAGC</td>
<td>CGCCCTACGGGTCATTGAG</td>
</tr>
<tr>
<td>5'-GATCCCGCCTAGGACACCTGGTACG</td>
<td>TTCAARGAGA</td>
<td>GCTACAGGGCTGCCATCGGTGCCTGC</td>
<td>G-3' (SEQ ID NO: 85)</td>
</tr>
<tr>
<td>3'-G</td>
<td>CGTCTGCTTTCCGACAGCTCGGAGC</td>
<td>AAGTCCTCTTCCGACAGCTCGGAGC</td>
<td>CGCCCTACGGGTCATTGAG</td>
</tr>
<tr>
<td>5'-GATCCCGCCTAGGACACCTGGTACG</td>
<td>TTCAARGAGA</td>
<td>GCTACAGGGCTGCCATCGGTGCCTGC</td>
<td>G-3' (SEQ ID NO: 85)</td>
</tr>
<tr>
<td>3'-G</td>
<td>CGTCTGCTTTCCGACAGCTCGGAGC</td>
<td>AAGTCCTCTTCCGACAGCTCGGAGC</td>
<td>CGCCCTACGGGTCATTGAG</td>
</tr>
<tr>
<td>5'-GATCCCGCCTAGGACACCTGGTACG</td>
<td>TTCAARGAGA</td>
<td>GCTACAGGGCTGCCATCGGTGCCTGC</td>
<td>G-3' (SEQ ID NO: 85)</td>
</tr>
<tr>
<td>3'-G</td>
<td>CGTCTGCTTTCCGACAGCTCGGAGC</td>
<td>AAGTCCTCTTCCGACAGCTCGGAGC</td>
<td>CGCCCTACGGGTCATTGAG</td>
</tr>
</tbody>
</table>

[0065] In one embodiment to target these sequences, a Transkingdom RNA Interference Plasmid (TRIP) will incorporate a hairpin RNA expression cassette encoding short hairpin RNA under the control of a T7 RNA polymerase promoter and terminator. In the design of these constructs, an algorithm was utilized to take into account some known difficulties with the development of siRNA, namely: (1) Exclusion of disqualifying properties (SNPs, interferon motifs), (2) Exclusion of the sequence if there was homology in ref seq (19/21, >17 contiguous to any other genes) and (3) Exclusion of the sequence if there were significant miRNA seed type matches. After screening and rejection of all possible siRNA target sequences for human beta catenin according to these criteria, the remaining ones were checked for conservation in the other species as indicated in this list.

[0066] In one aspect of this embodiment, the DNA insert comprises one or more of the following constructs, each of which contains a TNFα target sequence, a hairpin sequence and BamHI and SalI restriction sites to facilitate incorporation into the hairpin RNA expression cassette of the TRIP plasmid:
The RNA methods of the invention, including BMGS and BTGS are used to create transient “knockdown” genetic animal models as opposed to genetically engineered knockout models to discover gene functions. The methods are also used as in vitro transfection tool for research and drug development.

These methods use bacteria with desirable properties (invasiveness attenuation, steerability) for example, *Bifidobacteria* and *Listeria*, are used to perform BMGS and BTGS. Invasiveness as well as eukaryotic or prokaryotic transcription of one or several shRNA is conferred to a bacterium using plasmids.

The RNAi methods of the invention, including BMGS and BTGS are used for delivery of gene silencing to the gut and colon, and for oral application in the treatment of various diseases, namely colon cancer treatment and prevention. In another aspect of this embodiment, delivery of gene silencing is extra-intestinal.

Bacteria Delivering RNA to Eukaryotic Cells

According to the invention, any microorganism which is capable of delivering a molecule, e.g., an RNA molecule, into the cytoplasm of a target cell, such as by traversing the membrane and entering the cytoplasm of a cell, can be used to deliver RNA to such cells. In a preferred embodiment, the microorganism is a prokaryote. In an even more preferred embodiment, the prokaryote is a bacterium. Also within the scope of the invention are microorganisms other than bacteria which can be used for delivering RNA to a cell. For example, the microorganism can be a *Amans*, *Cryptococcus neoformans*, *protozoan*, e.g., *Trypanosoma cruzi*, *Toxoplasma gondii*, *Leishmania donovani*, and *plasmodia*.

As used herein, the term “invasive” when referring to a microorganism, e.g., a bacterium, refers to a microorganism which is capable of delivering at least one molecule, e.g., an RNA or RNA-encoding DNA molecule, to a target cell. An invasive microorganism can be a microorganism which is capable of traversing a cell membrane, thereby entering the cytoplasm of said cell, and delivering at least some of its content, e.g., RNA or RNA-encoding DNA, into the target cell. The process of delivery of the at least one molecule into the target cell preferably does not significantly modify the invasion apparatus.

In a preferred embodiment, the microorganism is a bacterium. A preferred invasive bacterium is a bacterium which is capable of delivering at least one molecule, e.g., an RNA or RNA-encoding DNA molecule, to a target cells, such as by entering the cytoplasm of a eukaryotic cell. Preferred invasive bacteria are live bacteria, e.g., live invasive bacteria.

Invasive microorganisms include microorganisms that are naturally capable of delivering at least one molecule to a target cell, such as by traversing the cell membrane, e.g., a eukaryotic cell membrane, and entering the cytoplasm, as well as microorganisms which are not naturally invasive and which have been modified, e.g., genetically modified, to be invasive. In another preferred embodiment, a microorganism which is not naturally invasive can be modified to become invasive by linking the bacterium to an “invasion factor”, also termed “entry factor” or “cytoplasm-targeting factor”. As used herein, an “invasion factor” is a factor, e.g., a protein or a group of proteins which, when expressed by a non-invasive bacterium, render the bacterium invasive. As used herein, an “invasion factor” is encoded by a “cytoplasm-targeting gene.”

Naturally invasive microorganisms, e.g., bacteria, may have a certain tropism, i.e., preferred target cells. Alternatively, microorganisms, e.g., bacteria can be modified, e.g., genetically, to mimic the tropism of a second microorganism.

Delivery of at least one molecule into a target cell can be determined according to methods known in the art. For example, the presence of the molecule, by the decrease in expression of an RNA or protein silenced thereby, can be detected by hybridization or PCR methods, or by immunological methods which may include the use of an antibody.

Determining whether a microorganism is sufficiently invasive for use in the invention may include determining whether sufficient RNA, was delivered to host cells, relative to the number of microorganisms contacted with the host cells. If the amount of RNA, is low relative to the number of microorganisms used, it may be desirable to further modify the microorganism to increase its invasive potential.

Bacterial entry into cells can be measured by various methods. Intracellular bacteria survive treatment by amnoglycoside antibiotics, whereas extracellular bacteria are rapidly killed. A quantitative estimate of bacterial uptake can be achieved by treating cell monolayers with the antibiotic gentamicin to inactivate extracellular bacteria, then by removing said antibiotic before liberating the surviving intracellular organisms with gentle detergent and determining viable counts on standard bacteriological medium. Furthermore, bacterial entry into cells can be directly observed, e.g., by thin-section-transmission electron microscopy of cell layers or by immunofluorescent techniques (Falkow et al. (1992) Annual Rev. Cell Biol. 8:333). Thus, various techniques can be used to determine whether a specific bacteria is capable of invading a specific type of cell or to confirm bacterial invasion following modification of the bacteria, such modification of the tropism of the bacteria to mimic that of a second bacterium.

Bacteria that can be used for delivering RNA according to the method of the invention are preferably non-pathogenic. However, pathogenic bacteria can also be used, so long as their pathogenicity has been attenuated, to thereby render the bacteria non-harmful to a subject to which it is administered. As used herein, the term “attenuated bacteria” refers to a bacteria that has been modified to significantly reduce or eliminate its harmfulness to a subject. A pathogenic bacterium can be attenuated by various methods, set forth below.

Without wanting to be limited to a specific mechanism of action, the bacterium delivering the RNA into the eukaryotic cell can enter various compartments of the cell, depending on the type of bacterium. For example, the bacterium can be in a vesicle, e.g., a phagocytic vesicle. Once inside the cell, the bacterium can be destroyed or lysed and its contents delivered to the eukaryotic cell. A bacterium can also be engineered to express a plasmid degrading enzyme to allow leakage of RNA from the plasmosome. In some embodiments, the bacterium can stay alive for various times in the eukaryotic cell and may continue to produce RNA. The RNA or RNA-encoding DNA can then be released from the bacterium into the cell by, e.g., leakage. In certain embodiments of the invention, the bacterium can also replicate in the eukaryotic cell. In a preferred embodiment, bacterial replication does not kill the host cell. The invention is not limited to delivery of RNA or RNA-encoding DNA by a specific mechanism and is intended to encompass methods and composi-
tions permitting delivery of RNA or RNA-encoding DNA by a bacterium independently of the mechanism of delivery.

[0081] Set forth below are examples of bacteria which have been described in the literature as being naturally invasive (section 1.1), as well as bacteria which have been described in the literature as being naturally non-invasive bacteria (section 1.2), as well as bacteria which are naturally non-pathogenic or which are attenuated. Although some bacteria have been described as being non-invasive (section 1.2), these may still be sufficiently invasive for use according to the invention. Whether traditionally described as naturally invasive or non-invasive, any bacterial strain can be modified to modulate, in particular to increase, its invasive characteristics (e.g., as described in section 1.3).

[0082] Naturally Invasive Bacteria

[0083] The particular naturally invasive bacteria employed in the present invention is not critical thereto. Examples of such naturally-occurring invasive bacteria include, but are not limited to, Shigella spp., Salmonella spp., Listeria spp., Ricketsia spp., and enteroinvasive Escherichia coli.

[0084] The particular Shigella strain employed is not critical to the present invention. Examples of Shigella strains which can be employed in the present invention include Shigella flexneri 2a (ATCC No. 29903), Shigella sonnei (ATCC No. 29930), and Shigella disenteriae (ATCC No. 13313). An attenuated Shigella strain, such as Shigella flexneri 2a 2457T arOa virG mutant CVD 1203 (Noriega et al. supra), Shigella flexneri M90T icsA mutant (Goldberg et al. Infect. Immun., 62:564-568 (1994)), Shigella flexneri Y SFT.14 arOaD mutant (Karnell et al. Vac., 10:167-174 (1992)), and Shigella flexneri arOa arOaD mutant (Verma et al. Vac., 9:6-9 (1991)) are preferably employed in the present invention. Alternatively, new attenuated Shigella spp. strains can be constructed by introducing an attenuating mutation either singularly or in conjunction with one or more additional attenuating mutations.


[0086] Attenuating mutations can be introduced into bacterial pathogens using non-specific mutagenesis either chemically, using agents such as N-methyl-N-nitro-N-nitrosoguanidine, or using recombinant DNA techniques; classic genetic techniques, such as Tn10 mutagenesis, P22-mediated transduction, λ phage mediated crossover, and conjugational transfer; or site-directed mutagenesis using recombinant DNA techniques. Recombinant DNA techniques are preferable since strains constructed by recombinant DNA techniques are far more defined. Examples of such attenuating mutations include, but are not limited to:


[0091] (v) mutations that affect DNA topology, such as topA (Galan et al. Infect. Immun., 58:1879-1885 (1990));

[0092] (vi) mutations that disrupt or modify the cell cycle, such as min (do Boer et al. Cell, 56:641-649 (1989));


[0094] (viii) mutations that alter the biogenesis of lipopolysaccharide and/or lipid A, such as rfb (Raetz in Escherichia coli and Salmonella typhimurium, Neidhart et al., Ed., ASM Press, Washington D.C. pp 1025-1063 (1996)), gell (Hone et al. J. Infect. Dis., 156:164-167 (1987)) and htrB (Raetz, supra), mabB (Raetz, supra)

[0096] The attenuating mutations can be either constitutively expressed or under the control of inducible promoters, such as the temperature sensitive heat shock family of promoters (Neidhardt et al. supra), or the anaerobically induced nirB promoter (Harborne et al. Mol. Micro., 6:2805-2813 (1992)) or repressible promoters, such as upA (Gorfeinik et al. J. Biol. Chem., 268:23376-23381 (1993)) or gcv (Stauffer et al. J. Bact., 176:6159-6164 (1994)).

[0097] The particular Listeria strain employed is not critical to the present invention. Examples of Listeria strains which can be employed in the present invention include Listeria monocytogenes (ATCC No. 15313), Attenuated Listeria strains, such as L. monocytogenes actA mutant (Brundage et al. supra) or L. monocytogenes plcA (Camilli et al J. Exp. Med., 173:751-754 (1991)) are preferably used in the present invention. Alternatively, newly attenuated Listeria strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0098] The particular Salmonella strain employed is not critical to the present invention. Examples of Salmonella strains which can be employed in the present invention include Salmonella typhi (ATCC No. 725.1) and S. typhimurium (ATCC No. 13311). Attenuated Salmonella strains are preferably used in the present invention and include S. typhi A-carD (Hone et al. J. Bacteriol., 9:810 (1991)) and S. typhimurium oroA mutant (Mastromen et al. Micro. Pathol. 13:477 (1992)). Alternatively, newly attenuated Salmonella strains can be constructed by introducing one or more attenuating mutations as described for Shigella spp. above.

[0099] The particular Rickettsia strain employed is not critical to the present invention. Examples of Rickettsia strains which can be employed in the present invention include Rickettsia Rickettisiæ (ATCC Nos. VR149 and VR891), Rickettsia prowasecki (ATCC No. VR233), Rickettsia tsutsugamushi (ATCC Nos. VR312, VR150 and VR609), Rickettsia mooseri (ATCC No. VR144), Rickettsia sibirica (ATCC No. VR151), and Rochalimaea quintana (ATCC No. VR358). Attenuated Rickettsia strains are preferably used in the present invention and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0100] The particular enteroinvasive Escherichia strain employed is not critical to the present invention. Examples of enteroinvasive Escherichia strains which can be employed in the present invention include Escherichia coli strains 4608-58, 1184-68, 53638-C-17, 13-80, and 6-81 (Sansonetti et al. Ann. Microbiol. (Inst. Pasteur), 132A:351-355 (1982)). Attenuated enteroinvasive Escherichia strains are preferably used in the present invention and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0101] Furthermore, since certain microorganisms other than bacteria can also interact with integrin molecules (which are receptors for certain invasion factors) for cellular uptake, such microorganisms can also be used for introducing RNA into target cells. For example, viruses, e.g., foot-and-mouth disease virus, echovirus, and adenovirus, and eukaryotic pathogens, e.g., Histoplasma capsulatum and Leishmania major interact with integrin molecules.

[0102] 1.2 Less Invasive Bacteria

[0103] Examples of bacteria which can be used in the invention and which have been described in the literature as being non-invasive or at least less invasive than the bacteria listed in the previous section (1.1) include, but are not limited to, Yersinia spp., Escherichia spp., Klebsiella spp., Bordetella spp., Neisseria spp., Aeromonas spp., Francisella spp., Corynebacterium spp., Citrobacter spp., Chlamydia spp., Hemophilus spp., Brucella spp., Mycobacterium spp., Legionella spp., Rhodococcus spp., Pseudomonas spp., Helicobacter spp., Vibrio spp., Bacillus spp., and Erysipelothrix spp. It may be necessary to modify these bacteria to increase their invasive potential.

[0104] The particular Yersinia strain employed is not critical to the present invention. Examples of Yersinia strains which can be employed in the present invention include Y. enterocolitica (ATCC No. 9610) or Y. pestis (ATCC No. 19428). Attenuated Yersinia strains, such as Y. enterocolitica Ys93-R2 (al-Hendy et al. Infect. Immun., 60:870-875 (1992)) or Y. enterocolitica or A (O’Gara et al. Micro. Path., 9:105-116 (1990)) are preferably used in the present invention. Alternatively, newly attenuated Yersinia strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0105] The particular Escherichia strain employed is not critical to the present invention. Examples of Escherichia strains which can be employed in the present invention include E. coli N10407 (Elinghorst et al. Infect. Immun., 60:2409-2417 (1992)) and E. coli EFC4, CFT325 and CPZ005 (Donnenberg et al. J. Infect. Dis., 169:831-838 (1994)). Attenuated Escherichia strains, such as the attenuated turkey pathogen E. coli O22a31B mutant (Kwaga et al. Infect. Immun., 62:3766-3772 (1994)) are preferably used in the present invention. Alternatively, newly attenuated Escherichia strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0106] The particular Klebsiella strain employed is not critical to the present invention. Examples of Klebsiella strains which can be employed in the present invention include K. pneumoniae (ATCC No. 13884). Attenuated Klebsiella strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0107] The particular Bordetella strain employed is not critical to the present invention. Examples of Bordetella strains which can be employed in the present invention include B. bronchiseptica (ATCC No. 19395). Attenuated Bordetella strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0108] The particular Neisseria strain employed is not critical to the present invention. Examples of Neisseria strains which can be employed in the present invention include N. meningitidis (ATCC No. 13077) and N. gonorrhoeae (ATCC No. 19424). Attenuated Neisseria strains, such as N. gonorrhoeae MS11 arco mutant (Chamberlain et al. Micro. Path., 15:51-63 (1993)) are preferably used in the present invention. Alternatively, newly attenuated Neisseria strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.
[0109] The particular Aeromonas strain employed is not critical to the present invention. Examples of Aeromonas strains which can be employed in the present invention include A. eucrenophila (ATCC No. 23309). Alternatively, new attenuated Aeromonas strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0110] The particular Francisella strain employed is not critical to the present invention. Examples of Francisella strains which can be employed in the present invention include F. tularensis (ATCC No. 15482). Attenuated Francisella strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0111] The particular Corynebacterium strain employed is not critical to the present invention. Examples of Corynebacterium strains which can be employed in the present invention include C. pseudotuberculosis (ATCC No. 19410). Attenuated Corynebacterium strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0112] The particular Citrobacter strain employed is not critical to the present invention. Examples of Citrobacter strains which can be employed in the present invention include C. freundii (ATCC No. 80090). Attenuated Citrobacter strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0113] The particular Chlamydia strain employed is not critical to the present invention. Examples of Chlamydia strains which can be employed in the present invention include C. pneumontiae (ATCC No. VR1310). Attenuated Chlamydia strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0114] The particular Hemophilus strain employed is not critical to the present invention. Examples of Hemophilus strains which can be employed in the present invention include H. sornus (ATCC No. 43625). Attenuated Hemophilus strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0115] The particular Brucella strain employed is not critical to the present invention. Examples of Brucella strains which can be employed in the present invention include B. abortus (ATCC No. 23448). Attenuated Brucella strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0116] The particular Mycobacterium strain employed is not critical to the present invention. Examples of Mycobacterium strains which can be employed in the present invention include M. intracellulare (ATCC No. 13950) and M. tuberculosis (ATCC No. 27294). Attenuated Mycobacterium strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0117] The particular Legionella strain employed is not critical to the present invention. Examples of Legionella strains which can be employed in the present invention include L. pneumophila (ATCC No. 33156). Attenuated Legionella strains, such as a L. pneumophila mip mutant (Ott, FEMS Micro. Rev., 14:161-176 (1994)) are preferably used in the present invention. Alternatively, new attenuated Legionella strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0118] The particular Rhodococcus strain employed is not critical to the present invention. Examples of Rhodococcus strains which can be employed in the present invention include R. equi (ATCC No. 6939). Attenuated Rhodococcus strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0119] The particular Pseudomonas strain employed is not critical to the present invention. Examples of Pseudomonas strains which can be employed in the present invention include P. aeruginosa (ATCC No. 23267). Attenuated Pseudomonas strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0120] The particular Helicobacter strain employed is not critical to the present invention. Examples of Helicobacter strains which can be employed in the present invention include H. mustelae (ATCC No. 43772). Attenuated Helicobacter strains are preferably used in the present invention, and can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0121] The particular Salmonella strain employed is not critical to the present invention. Examples of Salmonella strains which can be employed in the present invention include Salmonella typhi (ATCC No. 7251) and S. typhimurium (ATCC No. 13311). Attenuated Salmonella strains are preferably used in the present invention, and include S. typhi aroC aroD (Hone et al. Vac., 9:810-816 (1991)) and S. typhimurium aroA mutant (Masiero et al. Micro. Pathol, 13:477-491 (1992)). Alternatively, new attenuated Salmonella strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0122] The particular Vibrio strain employed is not critical to the present invention. Examples of Vibrio strains which can be employed in the present invention include Vibrio cholerae (ATCC No. 14035) and Vibrio cincinnatiensis (ATCC No. 35912). Attenuated Vibrio strains are preferably used in the present invention, and include V. cholerae RSI virulence mutant (Taylor et al. J. Infect Dis., 170:1518-1523 (1994)) and V. cholerae caza, ace, zot, cep mutant (Waldor et al. J. Infect. Dis., 170:278-283 (1994)). Alternatively, new attenuated Vibrio strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0123] The particular Bacillus strain employed is not critical to the present invention. Examples of Bacillus strains which can be employed in the present invention include Bacillus subtilis (ATCC No. 6051). Attenuated Bacillus strains are preferably used in the present invention and include B. anthracis mutant pX01 (Welkos et al. Micro. Pathol, 14:381-388 (1993)) and attenuated BCG strains (Stover et al. Nat., 351:456-460 (1991)). Alternatively, new attenuated Bacillus strains can be constructed by introducing
one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0124] The particular *Erysipelothrix* strain employed is not critical to the present invention. Examples of *Erysipelothrix* strains which can be employed in the present invention include *Erysipelothrix rhusiopathiae* (ATCC No. 19414) and *Erysipelothrix rhusiopathiae* *tummarum* (ATCC No. 45339). Attenuated *Erysipelothrix* strains are preferably used in the present invention and include *E. rhusiopathiae* Kga-1a and Kga-2 (Watarai et al. J. Vet. Med. Sci., 35:505-600 (1993)) and *E. rhusiopathiae* ORVAC mutant (Markowska-Daniel et al. Int. J. Med. Microbiol. Virol. Parisi. Infect. Dis., 277:547-553 (1992)). Alternatively, new attenuated *Erysipelothrix* strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for Shigella spp. above.

[0125] 1.3. Methods for Increasing the Invasive Properties of a Bacterial Strain

[0126] Whether organisms have been traditionally described as invasive or non-invasive, these organisms can be engineered to increase their invasive properties, e.g., by mimicking the invasive properties of *Shigella* spp., *Listeria* spp., *Rickettsia* spp., or enteroinvasive *E. coli* spp. For example, one or more genes that enable the microorganism to access the cytoplasm of a cell, e.g., a cell in the natural host of said non-invasive bacteria, can be introduced into the microorganism.

[0127] Examples of such genes referred to herein as "cytoplasm-targeting genes" include genes encoding the proteins that enable invasion by *Shigella* or the analogous invasion genes of enteroinvasive *E. coli* or *Listeria*, as such techniques are known to result in rendering a wide array of invasive bacteria capable of invading and entering the cytoplasm of animal cells (Formal et al. Infect. Immum., 46:465 (1984); Bielecke et al. Nature, 345:175-176 (1990); Small et al. In: *Microbiology* 1986, pages 121-124, Levine et al. Eds., *American Society for Microbiology*, Washington, D.C. (1986); Zychlinsky et al. Molec. Micro. 11:619-627 (1994); Gentschev et al. (1995) Infection & Immunity 63:4202; Isberg, R. R. and S. Falkow (1985) Nature 317:262; and Isberg R. R. et al. (1987) Cell 50:769). Methods for transferring the above cytoplasm-targeting genes into a bacterial strain are well known in the art. Another preferred gene which can be introduced into bacteria to increase their invasive character encodes the invasion protein from *Yersinia pseudotuberculosis* (Leong et al. EMBO J., 9:1979 (1990)). Invasin can also be introduced in combination with listeriolysin, thereby further increasing the invasive character of the bacteria relative to the introduction of either of these genes. The above genes have been described for illustrative purposes; however, it will be obvious to those skilled in the art that any gene or combination of genes, from one or more sources, that participates in the delivery of a molecule, in particular an RNA or RNA-encoding DNA molecule, from a microorganism into the cytoplasm of a cell, e.g., an animal cell, will suffice. Thus, such genes are not limited to bacterial genes, and include viral genes, such as influenza virus hemagglutinin HA-2 which promotes endosmosis (Plunk et al. J. Biol. Chem., 269:12918-12924 (1994)).

[0128] The above cytoplasm-targeting genes can be obtained by, e.g., PCR amplification from DNA isolated from an invasive bacterium carrying the desired cytoplasm-targeting gene. Primers for PCR can be designed from the nucleotide sequences available in the art, e.g., in the above-listed references and/or in GenBank, which is publicly available on the internet (www.ncbi.nlm.nih.gov/). The PCR primers can be designed to amplify a cytoplasm-targeting gene, a cytoplasm-targeting operon, a cluster of cytoplasm-targeting genes, or a regulon of cytoplasm-targeting genes. The PCR strategy employed will depend on the genetic organization of the cytoplasm-targeting gene or genes in the target invasive bacteria. The PCR primers are designed to contain a sequence that is homologous to DNA sequences at the beginning and end of the target DNA sequence. The cytoplasm-targeting genes can then be introduced into the target bacterial strain, e.g., by using Hfr transfer or plasmid mobilization (Miller, A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1992); Bothwell et al. supra; and Ausubel et al. supra), bacteriophage-mediated transduction (de Boer, supra; Miller, supra; and Ausubel et al. supra), chemical transformation (Bothwell et al. supra; Ausubel et al. supra), electroporation (Bothwell et al. supra; Ausubel et al. supra; and Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) and physical transformation techniques (Johnston et al. supra; and Bothwell, supra). The cytoplasm-targeting genes can be incorporated into lysogenic bacteriophage (de Boer et al. Cell, 56:641-649 (1989)), plasmids vectors (Curtiss et al. supra) or spliced into the chromosome (Horne et al. supra) of the target strain.

[0129] In addition to genetically engineering bacteria to increase their invasive properties, as set forth above, bacteria can also be modified by linking an invasion factor to the bacteria. Accordingly, in one embodiment, a bacterium is rendered more invasive by coating the bacterium, either covalently or non-covalently, with an invasion factor, e.g., the protein invasin, invasin derivatives, or a fragment thereof sufficient for invasiveness. In fact, it has been shown that non-invasive bacterial cells coated with purified invasin from *Yersinia pseudotuberculosis* or the carboxyl-terminal 192 amino acids of invasin are able to enter mammalian cells (Leong et al. 1990) EMBO J. 9:1979). Furthermore, latex beads coated with the carboxyl terminal region of invasin are efficiently internalized by mammalian cells, as are strains of *Staphylococcus aureus* coated with antibody-immobilized invasin (reviewed in Isberg and Tran van Nhuie (1994) Ann. Rev. Genet. 27:395). Alternatively, a bacterium can also be coated with an antibody, variant thereof, or fragment thereof which binds specifically to a surface molecule recognized by a bacterial entry factor. For example, it has been shown that bacteria are internalized if they are coated with a monoclonal antibody directed against an integrin molecule, e.g., cαβ1, known to be the surface molecule with which the bacterial invasin protein interacts (Isberg and Tran van Nhuie, supra). Such antibodies can be prepared according to methods known in the art. The antibodies can be tested for efficacy in mediating bacterial invasiveness by, e.g., coating bacteria with the antibody, contacting the bacteria with eukaryotic cells having a surface receptor recognized by the antibody, and monitoring the presence of intracellular bacteria, according to the methods described above. Methods for linking an invasion factor to the surface of a bacterium are known in the art and include cross-linking.

[0130] 2. Target Cells

[0131] The invention provides a method for delivering RNA to any type of target cell. As used herein, the term
“target cell” refers to a cell which can be invaded by a bacterium, i.e., a cell which has the necessary surface receptor for recognition by the bacterium. [0132] Preferred target cells are eukaryotic cells. Even more preferred target cells are animal cells. “Animal cells” are defined as nucleated, non-chloroplast containing cells derived from or present in multicellular organisms whose taxonomic position lies within the kingdom animalia. The cells may be present in the intact animal, a primary cell culture, explant culture or a transformed cell line. The particular tissue source of the cells is not critical to the present invention. [0133] The recipient animal cells employed in the present invention are not critical thereto and include cells present in or derived from all organisms within the kingdom animalia, such as those of the families mammalia, piscis, avian, reptilia. [0134] Preferred animal cells are mammalian cells, such as human, bovine, ovine, porcine, feline, buffalo, canine, goat, equine, donkey, deer, avian, bird, chicken and primate cells. The most preferred animal cells are human cells. [0135] In a preferred embodiment, the target cell is in a mucosal surface. Certain enteric pathogens, e.g., E. coli, Shigella, Listeria, and Salmonella, are naturally adapted for this application, as these organisms possess the ability to attach to and invade host mucosal surfaces (Kreig et al. supra). Therefore, in the present invention, such bacteria can deliver RNA molecules or RNA-encoding DNA to the host mucosal compartment. [0136] Although certain types of bacteria may have a certain tropism, i.e., preferred target cells, delivery of RNA or RNA-encoding DNA to a certain type of cell can be achieved by choosing a bacterium which has a tropism for the desired cell type or which is modified such as to be able to invade the desired cell type. Thus, e.g., a bacterium could be genetically engineered to mimic mucosal tissue tropism and invasive properties, as discussed above, to thereby allow said bacteria to invade mucosal tissue, and deliver RNA or RNA-encoding DNA to cells in those sites. [0137] Bacteria can also be targeted to other types of cells. For example, bacteria can be targeted to erythrocytes of humans and primates by modifying bacteria to express on their surface either, or both of, the Plasmodium vivax reticuloocyte binding proteins 1 and 2, which bind specifically to erythrocytes in humans and primates (Galiniski et al. Cell, 69:1213-1226 (1992)). In another embodiment, bacteria are modified to have on their surface asialoorosomucoid, which is a ligand for the asialoglycoprotein receptor on hepatocytes (Wu et al. J. Biol. Chem., 263:14621-14624 (1988)). In yet another embodiment, bacteria are coated with insulin-poly-L-lysine, which has been shown to target plasmid uptake to cells with an insulin receptor (Rosenkranz et al. Exp. Cell Res., 199:323-329 (1992)). Also within the scope of the invention are bacteria modified to have on their surface p60 of Listeria monocytogenes, which allows for tropism for hepatocytes (Hess et al. Infect. Immun., 63:2047-2053 (1995)), or a 60 kD protein from Yersinosa enterocolitica which causes specific binding to the mammalian extra-cellular matrix by binding to heparin, heparin sulfate and collagen (Ortega-Barría et al. Cell, 67:411-421 (1991)). [0138] Yet in another embodiment, a cell can be modified to become a target cell of a bacterium for delivery of RNA. Accordingly, a cell can be modified to express a surface antigen which is recognized by a bacterium for its entry into the cell, i.e., a receptor of an invasion factor. The cell can be modified either by introducing into the cell a nucleic acid encoding a receptor of an invasion factor, such that the surface antigen is expressed in the desired conditions. Alternatively, the cell can be coated with a receptor of an invasion factor. Receptors of invasion factors include proteins belonging to the integrin receptor superfamily. A list of the type of integrin receptors recognized by various bacteria and other microorganisms can be found, e.g., in Isberg and Tran Van Nieuw (1984) Ann. Rev. Genet. 27:395. Nucleotide sequences for the integrin subunits can be found, e.g., in GenBank, publicly available on the internet. [0139] As set forth above, yet other target cells include fish, avian, and reptilian cells. Examples of bacteria which are naturally invasive for fish, avian, and reptilian cells are set forth below. [0140] Examples of bacteria which can naturally access the cytoplasm of fish cells include, but are not limited to Aeromonas salmonicida (ATCC No. 33658) and Aeromonas schuberti (ATCC No. 43700). Attenuated bacteria are preferably used in the invention, and include A. salmonicida vacP (Gustafson et al. J. Mol. Biol., 237:452-463 (1994)) or A. salmonicida aromatic-dependent mutant (Vaughan et al. Infect. Immun., 61:2172-2181 (1993)). [0141] Examples of bacteria which can naturally access the cytoplasm of avian cells include, but are not restricted to, Salmonella gallinarum (ATCC No. 9184), Salmonella enteritidis (ATCC No. 4931) and Salmonella typhimurium (ATCC No. 6994). Attenuated bacteria are preferred to the invention and include attenuated Salmonella strains such as S. gallinarum cya crp mutant (Curtiss et al. (1987) supra) or S. enteritidis aroA aromatic-dependent mutant CVL30 (Cooper et al. Infect. Immun., 62:4739-4746 (1994)). [0142] Examples of bacteria which can naturally access the cytoplasm of reptilian cells include, but are not restricted to, Salmonella typhimurium (ATCC No. 6994). Attenuated bacteria are preferable to the invention and include attenuated strains such as S. typhimurium aromatic-dependent variant (Bernacek et al. supra). [0143] The invention also provides for delivery of RNA to other eukaryotic cells, e.g., plant cells, so long as there are microorganisms which are capable of invading such cells, either naturally or after having been modified to become invasive. Examples of microorganisms which can invade plant cells include Agrobacterium tumefaciens, which uses a plus-like structure which binds to the plant cell via specific receptors, and then through a process that resembles bacterial conjugation, delivers at least some of its content to the plant cell. [0144] Set forth below are examples of cell lines to which RNA can be delivered according to the method of this invention. [0145] Examples of human cell lines include but are not limited to ATCC Nos. CCL 62, CCL 159, HTB 151, HTB 22, CCL 2, CRL 1634, CRL 8155, HTB 61, and HTB104. Examples of bovine cell lines include ATCC Nos. CRL 6021, CRL 1733, CRL 6033, CRL 6023, CRL 44 and CRL 1390. [0146] Examples of ovine cell lines include ATCC Nos. CRL 6540, CRL 6538, CRL 6548 and CRL 6546. [0147] Examples of porcine cell lines include ATCC Nos. CRL 184, CRL 6492, and CRL 1746. [0148] Examples of feline cell lines include CRL 6077, CRL 6113, CRL 6140, CRL 6164, CCL 94, CCL 150, CRL 6075 and CRL 6123.
Examples of buffalo cell lines include CCL 40 and CRL 6072.

Examples of canine cells include ATCC Nos. CRL 6213, CCL 34, CRL 6202, CRL 6225, CRL 6215, CRL 6203 and CRL 6575.

Examples of goat derived cell lines include ATCC No. CCL 73 and ATCC No. CRL 6270.

Examples of horse derived cell lines include ATCC Nos. CCL 57 and CRL 6583.

Examples of deer cell lines include ATCC Nos. CRL 6193-6196.

Examples of primate derived cell lines include those from chimpanzee’s such as ATCC Nos. CRL 6312, CRL 6304, and CRL 1868; monkey cell lines such as ATCC Nos. CRL 1576, CCL 26, and CCL 161; orangustan cell line ATCC No. CRL 1850; and gorilla cell line ATCC No. CRL 1854.

4. Pharmaceutical Compositions

In a preferred embodiment of the invention, the invasive bacteria containing the RNA molecules, and/or DNA encoding such, are introduced into an intravenous, intramuscular, intradermal, intraperitoneally, peroral, intranasal, intraocular, intrarectal, intravaginal, intranasal, oral, immersion and intraurethral inoculation routes.

The amount of the live invasive bacteria of the present invention to be administered to a subject will vary depending on the species of the subject, as well as the disease or condition that is being treated. Generally, the dosage employed will be about $10^5$ to $10^{11}$ viable organisms, preferably about $10^5$ to $10^6$ viable organisms per subject.

The invasive bacteria of the present invention are generally administered along with a pharmaceutically acceptable carrier and/or diluent. The particular pharmaceutically acceptable carrier and/or diluent employed is not critical to the present invention. Examples of diluents include a phosphate buffered saline, buffer for buffering against gastric acid in the stomach, such as citrate buffer (pH 7.0) containing sucrose, bicarbonate buffer (pH 7.0) alone (Levine et al. J. Clin. Invest. 79:888-902 (1987); and Black et al. J. Infect. Dis., 155:1260-1265 (1987)), or bicarbonate buffer (pH 7.0) containing ascorbic acid, lactose, and optionally aspartame (Levine et al. Lancet, II:467-470 (1986)). Examples of carriers include proteins, e.g., as found in skim milk, sugars, e.g., sucrose, or polyvinylpyrrolidone. Typically these carriers would be used at a concentration of about 0.1-30% (w/v) but preferably at a range of 1-10% (w/v).

Set forth below are other pharmaceutically acceptable carriers or diluents which may be used for delivery specific routes. Any such carrier or diluent can be used for administration of the bacteria of the invention, so long as the bacteria are still capable of invading a target cell. In vitro or in vivo tests for invasiveness can be performed to determine appropriate diluents and carriers. The compositions of the invention can be formulated for a variety of types of administration, including systemic and topical or localized administration. Lyophilized forms are also included, so long as the bacteria are invasive upon contact with a target cell or upon administration to the subject. Techniques and formulations generally may be found in Remington’s Pharmaceutical Sciences, Meade Publishing Co., Easton, Pa. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the composition, e.g., bacteria, of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank’s solution or Ringer’s solution.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium laurel sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-tert-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound. For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manners.

For administration by inhalation, the pharmaceutical compositions for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the composition, e.g., bacteria, and a suitable powder base such as lactose or starch.

The pharmaceutical compositions may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulation agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The pharmaceutical compositions may also be formulated in rectal, intro vaginal or intraurethral compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example,
transmucosal administration bile salts and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through nasal sprays or by suppositories. For topical administration, the bacteria in the formulation are invented into ointments, salves, gels, or creams as generally known in the art, so long as the bacteria are still invasive upon contact with a target cell.

[0166] The compositions may, if desired, be presented in a pack or dispenser device and/or a kit which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

[0167] The invasive bacteria containing the RNA or DNA encoding DNA to be introduced can be used to infect animal cells that are cultured in vitro, such as cells obtained from a subject. These in vitro-infected cells can then be introduced into animals, e.g., the subject from which the cells were obtained initially, intravenously, intramuscularly, intradermally, or intraperitoneally, or by any inoculation route that allows the cells to enter the host tissue. When delivering RNA to individual cells, the dosage of viable organisms to be administered will be at a multiplicity of infection ranging from about 0.1 to 10⁴, preferably about 10² to 10³ bacteria per cell.

[0168] In yet another embodiment of the present invention, bacteria can also deliver RNA molecules encoding proteins to cells, e.g., animal cells, from which the proteins can later be harvested or purified. For example, a protein can be produced in a tissue culture cell.

[0169] While the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.


EXAMPLES

Methods

[0171] siRNA-Generating Plasmid Construction:

[0172] Oligonucleotides were obtained at 0.2 μmol from QIAgen with PAGE purification. After annealing, oligonucleotides were inserted into the BamHI and HindIII binding sites within psiSilencer 2.0-U6 (Ambion, Inc.) according to the manufacturer’s instructions.

[0173] The following sequences were used:

[0174] The k-Ras-1 and -2 64-mers target the nucleotides encoding for amino acids 9-15 of k-Ras protein which spans the specific mutation of V12.

k-Ras-1 (64-mer):  
5'-gATCCCGTGGAGCTGTTGGCGTCGTAATCCGACAGACTAGCACCACTTTTTTTGGAGAA3'  
k-Ras-2 (64-mer):  
5'-gATCCCGTGGAGCTGTTGGCGTCGTAATCCGACAGACTAGCACCACTTTTTTTGGAGAA3'  

[0175] The β-Catenin-1 and -2 64-mers target the nucleotides encoding for amino acids 79-85 within the catenin protein

β-Catenin-1 (64mer):  
5'-gATCCCGCAGTATTGATGATGGAGATCACCCTTTCCATCATATCAACCCATCGAGTAGCCTCTCTTCTGATCAGACACATCAGCGTGG3'  

β-Catenin-2 (64mer):  
5'-gATCCCGCAGTATTGATGATGGAGATCACCCTTTCCATCATATCAACCCATCGAGTAGCCTCTCTTCTGATCAGACACATCAGCGTGG3'  

[0176] The EGFP-1 and -2 64-mers target the nucleotides encoding for amino acids 22-28 of EGFP.

EGFP-1 (64-mer):  
5'-gATCCCGCAGTATTGATGATGGAGATCACCCTTTCCATCATATCAACCCATCGAGTAGCCTCTCTTCTGATCAGACACATCAGCGTGG3'  

EGFP-2 (64-mer):  
5'-gATCCCGCAGTATTGATGATGGAGATCACCCTTTCCATCATATCAACCCATCGAGTAGCCTCTCTTCTGATCAGACACATCAGCGTGG3'  

Transkingdom RNA Interference Plasmid Construction:

[0177] The engineered plasmid pTTRNAi-Hly-Inv, TRIP was constructed from pGB2inv-hly (Milligan et al., Nucleic Acids Res. 15, 8783 (1987)) and pBlueScript II KS (+). Oligonucleotides containing multiple cloning site (MCS), T7 promoter, enhancer and terminator (synthesized from Qiagen) were ligated into blunt-ended BssHII sites of KS1(+), and β-catenin hairpin oligos were inserted into BamHI and Sall sites of MCS to generate plasmid pTTRNAi. Past fragments containing the inv locus of pGB2inv-hly were inserted into PstI site of KS1(+). Using pGB2 inv-hly as template, N10A
gene was amplified by PCR (Pfx DNA polymerase, Invitrogen Inc.) with primers, hly-1: 5'-CCCTCCTTTGATAGTATAATCTCATCTTTA (SEQ ID NO:101) and hly-2: 5'-AAGCTTATATATGAGCTAGCGCAT (SEQ ID NO:102), and were cloned into EcoRV site of KSII (+) Inv. lly-Irv fragment was excised with BamHI and SalI. After blunting, it was ligated into EcoRV site incorporated within T7 terminator of pT7RNAi.

Bacteria:

[0178] The auxotropic Salmonella typhimurium araA 7207 (S. typhimurium 2337-65 derivative hisG46, DEL407 [aroA544::Tn10(Tc-o-s)]) used as the plasmid carrier in this study was kindly provided by Prof. BAD Stocker, Stanford University, CA. Escherichia coli X1-1 Blue was used to maintain the plasmids (Stratenege).

[0179] Transformation of SL 7207 was achieved using an adapted electroporation protocol (1). Competent SL 7207 and 1 µg plasmid were incubated on ice in a chilled 0.2 cm electroporation cuvette for 5 min. A 2.5 kV, 25 µF, 2000 µs impulse was applied using a BioRad GenePulser. 1 mL of prewarmed SOC medium was added and bacteria were allowed to recover for 1 hr at 37°C. With 225 RPM shaking before plating on selective agar plates. Presence of the plasmids was confirmed using minipreparation after alkaline lysis and separation on 0.7% agarose gel.

[0180] For in vitro experiments, SL 7202 were grown overnight at 37°C in Luria Broth (LB) supplemented with 100 µg/mL Ampicillin (for SL-siRAS, siGFP and SL-siCAT) without shaking. The next morning, bacteria were grown in fresh medium after 1% inoculation from the overnight culture until reaching an OD_{600} of 0.4-0.6. Bacteria were centrifuged (3500 RPM, 4°C) washed once in phosphate-buffered saline (PBS) and resuspended in PBS at the desired concentrations. For all determinations of bacterial number and concentration, the bacterial density was measured spectrometrically and calculated according to the formula cOD_{600} 8*10^{9} µL.

[0181] For animal experiments, SL 7207 were grown in Brain Heart Infusion Broth (BHI) in a stable culture overnight supplemented with the appropriate antibiotics where required. Bacteria were washed and resuspended in PBS at a concentration of 2.5x10^{9} µL/mL. Serial dilutions were done and plated on selective agar at several times during the experiment to verify the actual number of bacteria administered.

[0182] Plasmids were also transformed into BL21DE3 strain (Gene Therapy Systems) according to the manufacturer instructions. Bacteria were grown at 37°C in Brain-Heart-Infusion-broth with addition of 100 µg/mL Ampicillin. Bacteria numbers were calculated using OD_{600} measurement. For cell infection, overnight cultures were inoculated into fresh medium for another 2 h growth.

Cell Culture:

[0183] A human colon cancer cell line (SW 480) was used herein. It carries a mutation of APC protein resulting in increased basal levels of β-catenin. A stably GFP-expressing cell line derived from yolk sac epithelium, CRL 2583 (ATCC, Rockville, Md.) was used for GFP-knockdown experiments. CRL 2583 was maintained in 200 µg/mL G418 until 30 min before bacterial infection. SW 480 were grown in RPMI-1640 supplemented with 10% fetal bovine serum. CRL 2583 were grown in high glucose, high NaHCO₃, DMEM supplemented with 15% FBS as recommended by the supplier. All growth media were routinely supplemented with antibiotics: 100 U/ml penicillin G, 10 µg/mL streptomycin, 2.5 µg/mL amphotericin (all media and additives purchased from Sigma, St. Louis).

[0184] For direct transfection of plasmids, 500,000 cells were seeded into 6 cm petri dishes and allowed to grow overnight before they were transfected using a standard CaP-coprecipitation protocol.

[0185] Briefly, 15 µg plasmid-DNA are mixed in 500 µL reaction mix (2xHEPEs buffer, 60 µL CaP) and dropped to the cells in fresh medium without FBS. Precipitation was allowed to continue for 9 hrs before precipitates were washed away. Cells were harvested at different time points (36, 48, 60, 72, and 96 hrs).

[0186] For standard bacterial infection assays, 500,000 cells were seeded into 6 cm petri dishes and were allowed to attach overnight 30 min prior to addition of the bacteria, the growth medium was replaced with fresh medium without antibiotics and fetal bovine serum. SL 7207-siRAS, siCAT, siGFP were added in 500 µL PBS to reach the designated multiplicity of infection (MOI) of 100, 500 or 1000 and infection was carried out in a standard incubator with 37°C, 5% CO₂. By the end of the indicated infection period, plates were washed once with 4 mL of serum-free RPMI medium and 3 times with 4 mL PBS, then 5 mL of fresh complete RPMI medium containing 100 µg/mL of ampicillin and 150 µg/mL of gentamycin were added. Twenty-four hours later, tetracycline was added to final concentration of 15 µg/mL. At indicated different time points (24-96 h) after bacterial invasion, cells were harvested for western blot or flow cytometry.

[0187] For staining of intracellular bacteria, cells were grown on Lab-Tek II Chamber Slides (Nalgene Nunc, USA). After bacterial invasion as described above, cells were washed with PBS and fixed in 1% paraformaldehyde for 10 min. Acidrin Orange (Siagene solution) 0.01% was added for 45 sec, then washed with PBS. Crystal Violet stain (Siagene) was applied for 45 sec, then washed with PBS. Coverslips were mounted using PERMOUNT™ mounting medium and invasion was assessed using confocal microscopy.

MITT Assay:

[0188] After treatment with SL7207-siRAS and/or SL7207-siCAT, cells were trypsinized (24 h or 48 h later), diluted and seeded into 96-well plate at a concentration of 5000 cells/well. Cells were then allowed to grow for up to 4 days. At the desired incubation time point, medium was removed and 100 µL of MITT solution (5 mg/mL) was added to each well. After an incubation period of 4 h, MITT solution was drained away and cells were lysed by adding 100 µL of solubilization reagent (isopropanol:1HCl:10% SDS 43:2: 5) to each well. The resulting signal of the dark blue formazan-product was photometrically determined at 570 nm wavelength. The amount of color formation is dependent on the number of surviving cells per well.

Colony Formation Test:

[0189] After treatment with SL7207-siRAS and/or SL7207-siCAT, cells were trypsinized (24 h post-transfection), diluted and seeded into 6-well MTP at a concentration of 750 cells/well. Cells were kept growing for two more weeks to let them form visible colonies. Two weeks later, medium was removed and 1 mL of Giemsa stain (7415 g/L) were added to each well. After 10-min incubation at 37°C,
Giems stain was drained away and cells washed with PBS. Groups of more than eight cells were counted as positive colonies.

Western Blot:

Cells were washed with chilled PBS, scraped off the plate, lysed in lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1% NP-40, 1 mM DTT) containing 0.1% protease inhibitor mix (Sigma). 20 µg of protein were separated using 11% SDS-PAGE Gel and transferred to a 0.4 m PVDF membrane (Schleicher and Schuell). The membrane was blocked using 5% milk and incubated for 1 hr with specific antibodies at the indicated concentrations: Living Colors® antibody (Clontech)-1:500, β-catenin antibody (Santa Cruz)-1:500, k-Ras antibody (Santa Cruz)-1:300 and β-actin (Santa Cruz) 1:500. Each was followed by incubation with horseradish-peroxidase conjugated anti-rabbit or anti-goat secondary antibodies (Santa Cruz)-1:1000-1:2000. Bands were detected using ECL® chemoluminescence detection (Amersham).

Flow Cytometry:

For flow cytometry, cells were trypsinized for 3 min, resuspended in fresh medium and washed in PBS. After centrifugation, cells were fixed for 10 min in 1% paraformaldehyde/PBS at 4°C. Flow cytometry was performed using FACScan (Becton Dickinson), data analysis was done using CellQuest® software.

Animal Techniques:

Six to eight week old female GFP+ transgenic mice (Cg(tg2Nagy) were obtained from Jackson Laboratories. They were housed in the INDAM animal research facility with ad libitum access to standard rodent diet and drinking water. Treatment was initiated at ten weeks of age. For the iv treatment protocol, four doses of 10⁸ cfu SL-siRAS or SL-siGFP dissolved in 50 µL PBS were injected into the tail vein on alternating days. Mice were weighed daily and monitored for signs of disease.

Mice were sacrificed one day after the final treatment at which time tissue samples were taken for histology and flow cytometric analysis. Tissues were paraffin embedded and sectioned in 6 µm steps for histology and fluorescence microscopy. For flow cytometry, heparin and spleenocytes suspensions were prepared through the use of cell strainers (Falcon). Organ suspensions were fixed in 4% formalin and flow cytometry was performed using FACScan (Becton Dickinson), data analysis was done using CellQuest® software.

For the Xenograft cancer model, female BALB/c nude mice (Charles River Laboratories) were randomized into two groups (n=6). Three weeks before treatment, 1x10⁷ of SW480 cells were implanted subcutaneously. Treatments were initiated when the tumors reached about 10 mm in diameter. The treatment group was injected through tail vein with 1x10⁸ cfu of E. coli expressing shRNA against β-catenin in PBS. The control group was similarly treated except that the E. coli contains the TRIP vector without shRNA insert. The treatment was carried out every 5 days for a total of three treatments. Mice were sacrificed 5 days after the last treatment. Tissues were frozen and fixed for analysis of β-catenin mRNA level by real-time PCR and β-catenin protein level by immunohistochemistry.

For in vivo silencing experiments, female C57BL6 mice (Charles River Laboratories) were randomly divided into two groups. The treatment group was administered orally with 5x10¹⁰ cfu E. coli expressing shRNA against β-catenin in 200 µL phosphate-buffered saline (PBS). The control group was similarly treated except that the E. coli contains the TRIP vector without the shRNA insert. Two independent experiments were performed with 6 and 5 mice per group used, respectively. The treatment was carried out daily for 5 days per week for a total of 4 weeks. Mice were sacrificed 2 days after the last treatment, and tissues were paraffin-embedded.

Immunohistochemistry

Immunostaining was performed on 6 µm tissue sections using Vectastain Elite ABC avidin-biotin staining kit (Vector laboratories, Burlingame, Calif.) according to the instructions by the manufacturer. Slides were deparaffinized and rehydrated using a standard protocol. For antigen retrieval, slides were heated by microwave in 5% urea for 5 min. Unspecific binding sites were blocked with 1% bovine serum albumin for 10 min and endogenous peroxidase activity was suppressed by treatment with 3% H₂O₂ in methanol for 10 min. Sections were exposed to primary antibody LIV-ING COLORSTM rabbit polyclonal antibody (Clontech) at 1:500 dilution overnight at 4°C. The chromagen used was 3,3’Diamino-enzidine (DAB) (Vector), counterstaining was done with hematoxyline.

Interferon Response Detection:

SW480 cells were treated for 2 h with E. coli transformed with the TRIP encoding shRNA against human β-catenin or mutant k-Ras at MOI of 1:1000. Untreated cells were used as control. Cells were harvested at 24, 48 and 72 h. The expression levels of OAS1, OAS2, MX1, ISGF3y and IFITM1 genes were determined by RT-PCR using the Interferon Response Detection Kit (SIB System Biosciences, CA).

Example 1

Knock Down of Green Fluorescent Protein Using Bacteria Mediated Gene Silencing In Vitro and In Vivo

In the following experiments, an attenuated strain of Salmonella typhimurium (SL 7207, obtained from BARD Stecker, Stanford University) was used. To prove that the concept is useful as a general approach, we did confirmation experiments also with another attenuated strain of Salmonella typhimurium (VNP 70009, obtained from VION Pharmaceuticals, New Haven) and an invasive and attenuated strain of E. coli (JM 2710, obtained from P. Courvalin, Institut Pasteur, Paris).

Silencing plasmids were designed based on a commercially available plasmid (pSilencer, Ambion) to knock down the target genes GFP, β-catenin and oncogenic k-Ras (V12G). These plasmids were transformed into SL 7207 by electroporation and positive clones were verified by growth on selective agar and DNA preparation.

For in vitro use, knockdown of GFP expression was demonstrated using the stable GFP+ cell line CRL 2598 (ATCC, Rockland, Va.). Knockdown of oncogenic k-Ras
A system of bacterial delivery using an invasive bacterial strain, *S. typhimurium*, was developed with a commercially available eukaryotic transcription plasmid, pSilencer (Ambion). The *S. typhimurium* strain SL 7207 ( kindly provided by B. Stocker, Stanford University) is attenuated through an auxotrophy in the synthesis pathway for aromatic amino acids, and dies quickly after invasion into a target cell due to lack of nutrients. This strain has been used successfully for delivery of DNA in vitro and in mouse models, mainly with the purpose of DNA vaccination.

To verify bacterial entry into epithelial cells, an invasion assay was performed. SW 480 cells were infected for 2 hrs with SL-siRAS followed by 2 hrs of treatment with gentamycin. Acidine orange/crystal violet staining revealed good invasion efficiency. 90% of the SW 480 cells harbored viable SL 7207 bacteria. The average number of intracellular bacteria was 6 (range, 2-8) (FIG. 1). (Micrograph A1 is the transmission image. Micrograph A2 is the fluorescent image. Micrograph A3 is the merged image.) The number of viable intracellular bacteria reduced quickly over time. After 24 hrs and 48 hrs, only 10% and 3% of cells were found to still contain bacteria.

In the next experiment, the effective reduction of GFP expression in the GFP+ cell line was demonstrated. Successful knockdown of oncogenes k-Ras and β-catenin was confirmed using Western blot and RT-PCR. Oncogene knockdown resulted in growth retardation and decreased tumor formation in a xenograft animal model.

Cells stably expressing GFP (CRL 2583) were infected with SL 7207 carrying pSilencer2.0 including a sequence to silence GFP mRNA (SL-siGFP). (See above). After 48 hrs, cells treated with SL-siGFP showed a marked decrease in GFP expression as compared to cells treated with SL-siRAS and untreated control (FIGS. 1B and 1C). Treatment with SL-siGFP led to loss of GFP signal in a manner dependent on the multiplicity of infection (MOI) applied. In untreated cells, only 4% display low or absent fluorescence. In SL-siGFP treated cells, this fraction increased to 78.1% (treated with MOI 1:500) and 92.3% (MOI 1:1000). Control treatment with SL-siRAS lead to a slight loss of fluorescence (7.5% at MOI 1:500 and 8.4% at MOI 1:1000). This is also shown in the fluorescence microscope photograph in FIG. 1C. The top micrograph is (200×) of SL-siRAS and the bottom of SL-siGFP (below) treated CRL 2583 cells. This finding was confirmed using flow cytometry.

In a series of animal experiments with stably GFP-expressing mice, we were able to demonstrate knockdown of GFP expression in the liver and in the colon (in both organs approx. 50% reduction of GFP expression) after oral and intravenous application of SL 7207 carrying the GFP-silencing plasmid.

In animal experiments, *S. typhimurium* was used to achieve gene silencing in a transgenic mouse model (GFP+). Using this method, silencing of the transgene in the animal experiment is demonstrated at mRNA level as well as on protein levels and tissue sections of various organs (liver, gastrointestinal tract) with limited toxicity.

Next, BMGS was applied to knock down a specific disease-related gene. The specific oncogenic point mutation in the k-Ras gene, k-Ras<sup>G12C</sup>, is present in the human colon carcinoma cell line, SW 480 was targeted. After construction of the silencing plasmids and before they were electroporated into the attenuated SL 7207, their activity was tested by transfecting them into SW 480 cells using CaP coprecipitation.

Western blot (FIG. 4A) shows efficient knockdown of k-Ras using the pSilencer-kras (V12G) insert at 36 h and 48 h posttransfection. At later time points, the protein expression recovers, which is due to outgrowth of transfected clones which have a growth disadvantage versus non transfected clones in which the oncogenic k-Ras would still drive replication. When BMGS with SL 7207 as a carrier was used to mediate the knockdown, k-Ras levels were decreased at MOI of 1:500 and 1:1000. Using BMGS, knockdown of the k-Ras protein was observed with similar efficiency compared to direct transfection of the silencer plasmid using calciphasphate coprecipitation, although the onset of knockdown was slightly delayed by 12 hrs. (FIG. 4A). With an MOI of 1:1000, the result can be observed for a longer time (up to 72 hrs) (FIG. 2A).

The Western blot for β-catenin (FIG. 4B) shows delayed knockdown after transfection, with a maximum effect seen at 96 hrs post transfection. It is assumed that this delay is caused by the survival time of SL 7207 intracellularly before the plasmid is liberated (FIG. 2A).

After treatment with SL-siRAS and resulting knockdown of the oncogenic k-Ras (V12G), SW 480 cells displayed significantly reduced viability and colony formation ability (FIG. 2B). Cells were cocultured with equal amounts (2.5x10<sup>6</sup>) of SL-siRAS and SL-siCAT bacteria. Control cells were treated with untransformed SL 207.48 hrs after transfection, cells were seeded in 96 well plates for MTT test and 6 well plates for colony formation tests. At 120 hrs after treatment, viability, as assessed by MTT assay, was reduced to 62.5% after SL-siRAS treatment and 51% after SL-siCAT treatment. Combined treatment further reduced viability to 29% of control treated cells. SL-siRAS treatment and SL-siCAT treatment reduced the ability of SW 480 to form colonies by 37.7% and 50%, respectively. Combined treatment led to 63.3% reduction.

Further, treatment with SL-RAS completely inhibited the tumor formation ability of SW 480 cells when injected subcutaneously into nude mice, while treatment with empty SL 7207 did not influence their ability to form tumors (FIG. 2C). SW 480 cells (untreated, treated with SL 7207 or SL-siRAS) were subcutaneously injected into nude mice (4x10<sup>6</sup> cells, n=4 animals per group). Pretreatment with SL-siRAS completely abolished the ability to form tumors (no tumors visible in any of the four animals, day 40) (FIG. 2C).

To test whether this approach can be employed universally, another cancer-related gene, β-catenin was targeted (FIG. 2A). Basal levels of β-catenin are high in SW 480 cells, due to a mutated APC-gene, but can be reduced through treatment with hairpin siRNA after pSilencer-siCAT transfection (FIG. 2A). After treatment with SL 7207 carrying pSilencer with the β-catenin construct (SL-siCAT), significant knockdown of β-catenin expression was achieved which
resulted in decreased viability and colony formation ability (FIG. 2A). β-catenin was knocked down from 96 hr, but recovered from 144 hr.

Example 3

In Vivo Bacterial Mediated Gene Silencing

The method of bacterial mediation of RNAi offers the possibility of selectively targeting more than one gene at a time which might allow for increased efficiency for future applications, e.g., anticancer treatment through interference with multiple oncogenic pathways. To test the feasibility of such an approach, both the mutated k-Ras oncogene and β-catenin were targeted simultaneously. After simultaneous treatment with SL-siRAS and SL-siCAT, knockdown of both genes was observed at the protein level and resulted in further decreased viability and colony formation ability (FIG. 2). These findings demonstrate that the proposed concept of bacterial mediated gene silencing can be successfully used in vitro for different target genes and in different cell lines.

A mouse model was chosen to test whether this approach could be used to silence target genes in vivo. C57BL/6-Nagy mice express high levels of GFP in all tissues. 14 mice were randomly assigned to receive four doses of 10^8 cfu of either SL-siGFp or SL-siRAS i.v. on alternating days (seven animals per group). This treatment was well tolerated with no weight loss or clinically apparent signs of disease. All mice were sacrificed one day after the last treatment.

Liver tissue slides were assessed by fluorescence microscopy and immunohistochemistry with GFP antibody. (FIG. 3A). Intravenous treatment with SL-siGFp led to a decrease of fluorescence in the liver sections of the treated animals compared with SL-siRAS treated control animals. Histology staining with anti-GFP antibody, verified that changes in fluorescence were caused by a reduction in GFP and not caused by changes in background fluorescence levels. (FIG. 5) To verify that reductions in fluorescence in the liver sections of treated mice are really caused by changes in GFP expression levels and not due to changes in background fluorescence, tissue slides were stained with GFP specific antibody.

Immunohistochemical staining patterns correlate well with fluorescence microscopy images and confirm that changes in fluorescence are caused by decreased GFP expression. Fluorescence microscopy (50x) and corresponding immunohistochemistry image (50x) of liver section from control (top row) and iv treated (lower row) animal.

Staining patterns correlated well with fluorescence images. Subsequent image analysis revealed reductions in the number of GFP expressing cells between 9-25% after SL-siGFp treatment. These findings were confirmed by flow cytometric analysis of single cell suspensions of hepatocytes which showed a significant decrease in the number of GFP positive hepatocytes in SL-siGFp treated vs SL-siRAS treated animals (FIG. 3B). Flow cytometry measurements of hepatocyte and splenocyte suspensions were performed. After intravenous treatment with SL-siGFp, the number of GFP positive hepatocytes was significantly reduced compared to control treated (SL-siRAS) animals (SL-siRAS: 50.0% [45.4-53.2%;] SL-siGFp: 39.9% [26.1-53.2%], p<0.05).

These results indicate that significant gene silencing can be achieved in vivo using this approach. Using iv application of attenuated S. typhimurium we were able to extend the in vitro findings into a mouse model and achieve significant gene silencing in the liver. Other organs might become accessible through use of different invasive bacterial strains or different routes of application. Professional phagocytes will be a promising target for bacteria-mediated gene silencing, as transfection efficiencies have been reported to be higher for these cells compared to cells of epithelial lineage.

Example 4

Transkingdom RNA Interference

The use of bacteria-mediated RNAi in higher organisms holds the potential for functional genomics in mammalian systems, as previously demonstrated in C. elegans, and for other in vivo applications of RNAi. To investigate this possibility, the bacterial plasmid p17RNAi-Hly-Inv, termed TRIP (transkingdom RNA interference plasmid) was constructed (FIG. 6A). In this novel plasmid construct, the expression of shRNA was directed under the bacteriophage T7 promoter (Milligan and Uhlenbeck, Methods Enzymol. 180, 51-62 (1989) and Milligan et al., Nucleic Acids Res. 15, 8783-8798 (1987), rather than a mammalian promoter or enhancer. The shRNA can only be produced by the bacterial system. TRIP vector contains the Env locus that encodes invasion (Isberg et al., Cell 50, 769-778 (1987)), which permits the non-invasive E. coli to enter β1-integrin-positive mammalian cells (Young et al., J. Cell Biol. 116, 197-207 (1992)). The TRIP vector also contains the Hly A gene that encodes listeriolysin O to permit genetic materials to escape from entry vesicles (Mathew et al., Gene Ther. 10, 1105-1115 (2003) and Grillot-Courvalin et al., Nat. Biotechnol. 16, 862-866 (1998)). TRIP constructs were introduced into a competent strain of non-pathogenic E. coli, BL21DE3, which contains T7 RNA polymerase to express shRNA. A TRIP against the cancer gene β-catenin was constructed as an example. Activation of the β-catenin pathway from over-expression or oncogenic mutation of β-catenin is responsible for the initiation of the vast majority of colon cancers and is involved in a variety of other cancer types (Kim et al., Oncogene 24, 597-604 (2005)). Despite the potential of β-catenin as a cancer therapeutic target, the β-catenin pathway has been recalcitrant to inhibition by small molecules. β-catenin is a preferred target in proof of concept experiments for testing the potential of new RNAi approaches because it is commonly stabilized in cancer cells. TRIP can be modified to enable bacteria to express interfering RNA against various genes of interest.

To determine if gene silencing can be achieved through this transkingdom system, human colon cancer cells (SW 480) were co-cultured in vitro with E. coli for 2 h (FIGS. 6B and 6D) or different time (FIG. 6C), then treated with antibiotics to remove extracellular bacteria. Cells were further cultured for 48 h before harvest for analysis of gene silencing. As shown in FIG. 6D-6I), β-catenin was potently and specifically silenced at protein and mRNA level, while β-actin, k-Ras, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were not affected. To further test the specificity of the transkingdom RNAi, E. coli containing the TRIP against mutant k-Ras (GGT→GT at codon 12) silenced k-Ras expression in SW 480 cells with the same codon 12 mutation, but not in DLD1 cells with mutation in a different codon of k-Ras (GCC→GAC at codon 13, FIG. 6E). As an shRNA control, E. coli containing the TRIP against wild type k-Ras exerted no gene-silencing effect on mutated k-Ras in SW 480 cells (FIG. 6F). These results show that the transk-
ingdom RNA interference is highly gene-specific, sufficient to discriminate a point mutation.

[0222] To investigate the variables that affect the potency of gene silencing by the transkingdom system, cells were incubated for 2 h with the E. coli at different multiplicity of infection (MOI). As shown in Fig. 6B, the potency of gene silencing was dependent on MOI, with near complete gene silencing at a MOI of 1:1,000. To determine the effect of co-culture time on gene silencing, cells were incubated with the E. coli at a MOI of 1:500 for different times. As shown in Fig. 6C, gene-silencing potency increased with incubation time up to 2 h. The dependency of gene silencing on MOI and co-culture time provides controllable flexibility for gene silencing in various applications.

[0223] To further confirm that the β-catenin gene silencing is mediated specifically by shRNA, identification of the specific cleavage fragment of β-catenin mRNA was attempted by using 5’-RACE (rapid amplification of cDNA ends) PCR technique. A specific hallmark of RNAi-mediated gene silencing is the cleavage of β-catenin mRNA at the specific sites of the mRNA as predicted from the shRNA sequence. Based on the time course of catenin silencing (Fig. 7A), total RNA was isolated from SW 480 cells 8 h and 16 h after treatment with E. coli expressing shRNA against β-catenin to identify the cleaved fragments of mRNA. The cleaved β-catenin mRNA was found as early as 8 h after treatment with E. coli expressing shRNA: no fragments were detected in the control (Fig. 7B and 7C). The sequence analysis of the cleaved intermediate of β-catenin mRNA confirms the cleavage site located within the targeting sequence. This result shows that shRNA produced by bacteria trigger specific cleavage of the β-catenin mRNA through the RNAi-mediated gene silencing.

[0224] Induction of interferon response has been reported as a potential challenge to the specificity of some RNAi approaches (Bridge et al., Nat. Genet. 34, 263-264 (2003) and Hornung et al., Nat. Med. 11, 263-270 (2005)). To test if the gene silencing induced by the transkingdom RNAi is associated with interferon response induction, key interferon response genes were measured. The 2’,5’-oligoadenylate synthetases (OAS1 and OAS2) are important interferon-induced genes for the inhibition of cellular protein synthesis after viral infection. MX1 gene, a member of the interferon-inducible transmembrane proteins, mediates the anti-proliferation activity of interferon. ISGF3γ is part of a cellular interferon receptor involved in interferon-induced transcription regulation and stimulation. These genes have been used as a standard panel for analyzing interferon response induction by interfering RNA (Interferon Response Detection Kit, SBI Systems Biosciences, CA). The mRNA of the five interferon response genes were analyzed with semiquantitative RT-PCR. As shown in Fig. 7D, no induction of OAS1, OAS2, MX1, ISGF3γ and IFITM1 was detected following treatment with E. coli encoding shRNA against β-catenin. These data show that gene silencing induced by transkingdom RNAi is not associated with non-specific interferon response induction.

[0225] The mechanism of the transkingdom RNAi transfer was investigated. To determine if cellular entry of E. coli is required to induce RNAi, the gene-silencing activity of E. coli was compared with or without the Inv locus. The Inv encodes invasin that interacts with β1-integrin to facilitate the entry of E. coli into the cells. As expected, E. coli without Inv failed to enter cells (Fig. 8A). Surprisingly, Inv alone is not sufficient to enable E. coli to enter colon cancer cells (Fig. 8A), and no detectable gene silencing was observed in the absence of intracellular bacteria (Fig. 8B). The Hly A gene was introduced, which is thought to facilitate delivery of genetic materials to escape from the entry vesicles (Grillot-Courvalin et al., Nat. Biotechnol. 16, 862-866 (1998)). As expected, Hly alone failed to enable cell entrance of E. coli, but commensal E. coli with both Inv and Hly entered colon cancer cells with high efficiency (Fig. 8A). Under these conditions β-catenin was potently silenced up to 96 h (Fig. 8C). These results show that E. coli require both Inv and Hly to enter cells to induce transkingdom RNAi.

[0226] To determine whether gene silencing requires continued bacterial replication inside target cells, tetracycline was employed to block intracellular bacterial replication and gentamycin to remove extracellular bacteria. SW 480 cells were incubated with E. coli for 2 h followed by tetracycline treatment initiated at different times. As shown in Fig. 8D, following the initial 2 h infection time, an additional 2 h incubation time without tetracycline induced near maximum gene silencing; further delay in tetracycline treatment had no further enhancing effect on the degree of gene silencing. Surprisingly, there was no evidence of significant intracellular bacterial replication in the absence of tetracycline at 6 h and 48 h (Fig. 8E), which is likely due to the function of lysosomes and other intracellular anti-bacterial mechanisms (Roy et al., Science 304, 1515-1518 (2004) and Battistoni et al., Infect. Immun. 68, 30-37 (2000)). These results show that transkingdom RNAi is not dependent on persistent bacterial replication inside target cells after the initial infection (2 h) and incubation time (2 h).

[0227] It was next determined if the transkingdom RNAi approach works in vivo. E. coli expressing shRNA against β-catenin were administered to mice orally. An inoculum of 5x10^6 was administered orally five times per week, which is comparable to a human dosage of the probiotic E. coli Nissle 1917. Most of the inoculum is eliminated during passage through the bactericidal environment in the upper GI tract. Mice were treated with E. coli expressing shRNA against mouse β-catenin or with E. coli containing the corresponding plasmid vector. Treatment was continued for four weeks before the analysis of gene silencing by immunohistochemistry. As shown in Figs. 9A and 9B, β-catenin expression was silenced in the intestinal epithelium by E. coli expressing β-catenin shRNA (P<0.01), but not by the control E. coli. As a control, GAPDH expression was not reduced (Fig. 10). The gene silencing effect was more pronounced in the regions of or adjacent to the Peyer’s patches (Fig. 9B). Treatment was well tolerated with no gross or microscopic signs of epithelial damage or ulcerations (Fig. 9B). These results show that mammals respond to E. coli expressing specific shRNA with powerful local RNAi in vivo.

[0228] The transkingdom RNAi approach was investigated to determine if it can be used to silence a disease gene after systemic dosing. Intravenous administration of therapeutic bacteria has been tested in clinical trials with demonstrated safety in cancer patients (Toso et al., J. Clin. Oncol. 20, 142-52 (2002)). Nude mice with xenografted human colon cancer were treated intravenously with 10^8 cfu of E. coli encoding shRNA against human β-catenin. Three doses were given at a 5-day interval. The treatments were well tolerated without adverse effects. As shown in Fig. 9, treatment with E.
coli encoding shRNA against β-catenin resulted in significant decrease in β-catenin mRNA (p<0.005, FIG. 9C) and protein (p<0.01, FIGS. 9D and 9E) in the tumor tissues. These data show that bacteria-mediated transkingdom RNAi can silence a disease gene in a distant part of the body after systemic administration. These results show that gene silencing can be achieved through a transkingdom system. Importantly, the potency and specificity of RNAi is preserved. Non-pathogenic E. coli has been used clinically as probiotics with demonstrated safety (Rembergen et al., Lancet 354, 635 (1999)). Therefore, this transkingdom system provides a practical and clinically compatible way to deliver RNA interference for medical indications. This E. coli-based RNAi technology also provides a convenient vector system for conducting RNAi-based functional studies of genes. Finally, the results invite an intriguing possibility that such exchange of interfering RNA may occur in nature under cohabitive, infectious, or symbiotic conditions.

Example 5

In Vitro Assay of siRNAs Directed to TNFα Target Sequences

siRNAs were designed according to algorithm (Huesken D., et al, Design of a Genome-Wide siRNA Library Using an Artificial Neural Network. Nature Biotechnology 23 (8): 995-1001 (August 2005)) and cross-species reactivity, and screened in stimulated mouse macrophage RAW 264.7 and human macrophage THP-1 to determine if they are capable of silencing the TNFα target sequences illustrated in Tables 1-3. Nine mouse and human cross-specific siRNAs were tested in both the mouse and human cells, 36 human-specific siRNAs were tested in the human cells and 37 mouse-specific siRNAs were tested in the mouse cells.

Material and Methods:

Cell Culture and Transfection

The human monocytic cell line THP-1 was cultured in RPMI 1640 medium containing 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate, 10% heat-inactivated fetal bovine serum (FBS), with 100 U penicillin (pen) and 100 µg streptomycin (streptomycin) per ml, and grown at 37°C in the presence of 5% CO2. Prior to transfection, cells were plated in complete medium into 96-well culture dishes (30,000 cells per well) and treated overnight with 162 nM phorbol 12-myristate 13-acetate (PMA) for differentiation into an adherent, macrophage-like form. To remove PMA and pen/streptomycin, cells were washed once in 100 µl complete medium lacking pen/streptomycin.

The murine macrophage cell line RAW264.7 was cultured in DMEM medium containing 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10% heat-inactivated fetal bovine serum (FBS), with 100 U penicillin and 100 µg streptomycin per ml, and grown at 37°C in the presence of 5% CO2. Prior to transfection, cells were plated into 96-well culture dishes (30,000 cells per well) and allowed to adhere overnight. Cells were washed as above to remove penicillin and streptomycin.

In a separate 96-well dish, double-stranded RNAs for transfection were diluted into 50 µl Opti-MEM serum-free medium (Invitrogen) containing 0.3 µl Lipofectamine RNAiMAX (Invitrogen) and incubated 20 min at room temperature to enable the formation of transfection complexes. This mixture was then added to THP-1 or RAW cells (30-50% confluence), resulting in a final volume of 150 µl medium per well and a final RNA concentration of 50 nM. After 48 h, cells were treated with 0.1 µg/ml lipopolysaccharide (LPS) (Sigma) for 2 h to stimulate TNF-alpha production, and then harvested for RNA extraction.

RNA isolation, cDNA synthesis and quantitative real-time RT-PCR RNA was prepared with the SV 96 Total RNA Isolation System (Promega) according to the manufacturer’s protocol. cDNA was synthesized via the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a 20 µl reaction volume with 13.8 µl RNA, and used as template (8.8 µl per 25 µl reaction) in quantitative PCR performed with the Applied Biosystems (ABI) 7500 Fast Real-Time PCR System. Reactions contained 1× GeneAmp Fast PCR Master Mix (Applied Biosystems), custom mouse or human TNF-α primers and TaqMan probes (400 nM each primer and 200 nM probe), and 1× commercial mouse or human GAPDH primers and probes (AB). Relative TNF-α abundance was calculated via the delta Ct method using GAPDH as an endogenous control.

Each siRNA was screened twice and the following lead sequences were identified based upon the level of reduction of TNFα mRNA as determined by qRT-PCR. The data in Table 4 for mouse and Table 5 for human shows the average of both experiments. FIG. 11 illustrates the data in graphical format.

<table>
<thead>
<tr>
<th>TABLE 4</th>
<th>Decrease of TNFα mRNA (%)</th>
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<table>
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US 2015/0030573 A1

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<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 100

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tggg 64
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<210> SEQ ID NO 101
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 101

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<210> SEQ ID NO 102
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 102

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<210> SEQ ID NO 103
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<212> TYPE: DNA
<213> ORGANISM: Mus sp.

<400> SEQUENCE: 103

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<210> SEQ ID NO 104
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Mus sp.

<400> SEQUENCE: 104

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<210> SEQ ID NO 105
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Mus sp.

<400> SEQUENCE: 105
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atgcgcctac gggctattga g 21

<210> SEQ ID NO 106
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Mus sp.
<400> SEQUENCE: 106

aatgcgccta gggctattg a 21

<210> SEQ ID NO 107
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 107

gagtgacagt cctgtagcct a 21

<210> SEQ ID NO 108
<211> LENGTH: 21
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<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 108

agtgacagc cttgagcct c 21

<210> SEQ ID NO 109
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 109

aggtcaact cccctctgca a 21

<210> SEQ ID NO 110
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 110

ccccagtgac aagctgtgag c 21

<210> SEQ ID NO 111
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE: T7 promoter oligonucleotide
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic T7 promoter oligonucleotide
<400> SEQUENCE: 111

taatagact cactatag 18

<210> SEQ ID NO 112
<211> LENGTH: 8
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE: enhancer oligonucleotide
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic enhancer oligonucleotide
<400> SEQUENCE: 112
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gagacagg

<210> SEQ ID NO 113
<211> LENGTH: 47
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic T7 terminator oligonucleotide

<400> SEQUENCE: 113
tagcataac ccotgggggct cttaacggg tcttgagggg ttttttg

<210> SEQ ID NO 114
<211> LENGTH: 9
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic loop oligonucleotide

<400> SEQUENCE: 114
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<210> SEQ ID NO 115
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<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 115
gatatccagt gatattgatg gacagttcag attgatg gacagttcag ttgttgcag

<210> SEQ ID NO 116
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<212> TYPE: DNA
<213> ORGANISM: Mus musculus
<400> SEQUENCE: 116
gatatccggtg gtagtttaata aagcttcgta gaagcttcgta tttttgcag

<210> SEQ ID NO 117
<211> LENGTH: 61
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 117
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<210> SEQ ID NO 118
<211> LENGTH: 49
<212> TYPE: RNA
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 118
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What is claimed is:

1. A method of delivering one or more siRNAs to mammalian cells, the method comprising introducing to said mammalian cells at least one invasive bacterium containing one or more siRNAs or one or more DNA molecules encoding one or more siRNAs, wherein the expressed siRNAs interfere with the mRNA of TNFα.

2. An invasive bacterium comprising one or more siRNAs or one or more DNA molecules encoding one or more siRNAs, wherein the expressed siRNAs interfere with the mRNA of TNFα.

3. A prokaryotic vector comprising at least one DNA molecule encoding one or more siRNAs and at least one RNA-polymerase III compatible promoter or at least one prokaryotic promoter, wherein the expressed siRNAs interfere with the mRNA of TNFα.

4. The method of claim 1, wherein said invasive bacterium is a non-pathogenic or non-virulent bacterium.

5. The method of claim 1, wherein said invasive bacterium is a therapeutic bacterium.

6. The method of claim 1, wherein said mammalian cell is in vivo or in vitro.

7. The method of claim 1, wherein said mammalian cell is selected from the group consisting of human, bovine, ovine, porcine, feline, bufalo, canine, goat, equine, donkey, deer, avian, bird, chicken and primate cells.

8. The method of claim 1, wherein the mammalian cell is selected from the group consisting of a gastrointestinal epithelial cell and a macrophage.

9. The method of claim 1, wherein said one or more DNA molecules encoding said one or more siRNAs are transcribed within the animal cell.

10. The method of claim 1, wherein said one or more DNA molecules encoding said one or more siRNAs are transcribed within the bacterium.

11. The method of claim 1, wherein said one or more DNA molecules encoding one or more siRNAs comprise a prokaryotic promoter.

12. The method of claim 11, wherein said prokaryotic promoter is a T7 promoter.

13. The method of claim 1, wherein said animal cells are infected with about 10^7 to 10^11 viable invasive bacteria.

14. The method of claim 1, wherein said animal cells are infected at a multiplicity of infection ranging from about 0.1 to 10^6.

15. The method of claim 1, wherein the expressed siRNAs direct the multienzyme complex RNA-induced silencing complex of the cell to interact with the mRNA of TNFα.

16. The method of claim 1, wherein expression of TNFα is decreased or inhibited.

17. The invasive bacterium of claim 2, wherein said invasive bacterium is a non-pathogenic or non-virulent bacterium.

18. The invasive bacterium of claim 2, wherein said invasive bacterium is a therapeutic bacterium.

19. A composition comprising the bacterium of claim 2 and a pharmaceutically acceptable carrier.

20. A eukaryotic host cell comprising the bacterium of claim 2, and a pharmaceutically acceptable carrier.

* * * * *