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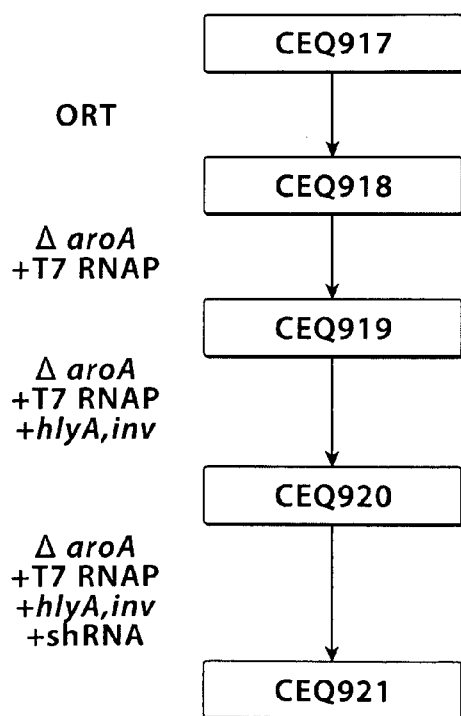


Fig. 2

(57) Abstract: Methods are described for the delivery of one or more small interfering RNAs (siRNAs) to a eukaryotic cell using a bacterium or BTP. Methods are also described for using this bacterium to regulate gene expression in eukaryotic cells using RNA interference, and methods for treating viral diseases and disorders. The bacterium or BTP includes one or more siRNAs or one or more DNA molecules encoding one or more siRNAs. Vectors are also described for use with the bacteria of the invention for causing RNA interference in eukaryotic cells.



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BACTERIA MEDIATED GENE SILENCING

RELATED APPLICATIONS

This application claims priority to, and the benefit of, U.S. Provisional Patent Application No. 60/934,751, filed June 15, 2007 and U.S. Provisional Patent Application No. 61/010,028, filed January 4, 2008. The contents of these applications are incorporated by reference in their entireties.

BACKGROUND

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)).

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 PD, 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).

Therefore, new methods are needed for the safe and predictable administration of interfering RNAs to mammals.

SUMMARY OF THE INVENTION

The present invention provides at least one invasive bacterium, or at least one bacterial therapeutic particle (BTP), including one or more siRNAs or one or more DNA molecules encoding one or more siRNAs. The present invention also provides at least one prokaryotic vector including 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 at least one mRNA of a gene of interest.

The present invention also provides methods of using the various bacterium, BTP and vectors provided in the invention. For example, the present invention provides methods of delivering one or more siRNAs to mammalian cells. The methods include introducing at least one invasive bacterium, or at least one bacterial therapeutic particle (BTP), containing one or more siRNAs or one or more DNA molecules encoding one or more siRNAs to the mammalian cells.

The present invention also provides methods of regulating gene expression in mammalian cells. The method includes introducing at least one invasive bacterium, or at least one bacterial

therapeutic particle (BTP), containing one or more siRNAs or one or more DNA molecules encoding one or more siRNAs to the mammalian cells, where the expressed siRNAs interfere with at least one mRNA of a gene of interest thereby regulating gene expression.

5 The present invention also provides methods of treating or preventing a viral disease or disorder in a mammal. The methods include regulating the expression of at least one gene in a cell known to cause a viral disease or disorder (e.g., known to increase proliferation, growth or dysplasia) by introducing to the cells of the mammal at least one invasive bacterium, or at least one bacterial therapeutic particle (BTP), containing one or more siRNAs or one or more DNA molecules encoding one or more siRNAs, where the expressed siRNAs interfere with the mRNA of the gene known to
10 cause a viral disease or disorder.

Preferably, the viral disease or disorder can be, but is not limited to, infection, epithelial dysplasia and cancer caused by HPV infection

The present invention also provides a composition containing at least one invasive bacterium or BTP and a pharmaceutically acceptable carrier. The present invention also provides a eukaryotic
15 host cell containing at least one invasive bacterium or BTP and a pharmaceutically acceptable carrier.

The invasive bacterium or BTPs of the present invention can be non-pathogenic, non-virulent bacterium or therapeutic bacterium

The mammalian cells can be *ex vivo*, *in vivo* or *in vitro*. The mammalian cells can be, but are not limited to, human, bovine, ovine, porcine, feline, buffalo, canine, goat, equine, donkey, deer,
20 avian, bird, chicken, and primate cells. Preferably, the mammalian cells are human cells. In some preferred embodiments, the mammalian cells can be, but are not limited to, gastrointestinal epithelial cells, macrophages, cervical epithelial cells, rectal epithelial cells and a pharyngeal epithelial cells.

The mammalian cells can be infected with about 10^3 to 10^{11} viable invasive bacterium or BTPs (or any integer within said ranges). Preferably, the mammalian cells can be infected with about
25 10^5 to 10^9 viable invasive bacterium or BTPs (or any integer within said ranges). The mammalian cells can be infected at a multiplicity of infection ranging from about 0.1 to 10^6 (or any integer within said ranges). Preferably, the mammalian cells can be infected at a multiplicity of infection ranging from about 10^2 to 10^4 (or any integer within said ranges).

The mammal can be, but is not limited to, human, bovine, ovine, porcine, feline, buffalo,
30 canine, goat, equine, donkey, deer, avian, bird, chicken, and primate. Preferably, the mammal is a human.

The one or more DNA molecules encoding the one or more siRNAs can be transcribed within the animal cell or transcribed within the bacterium. Preferably, the one or more siRNAs are transcribed within the animal cell as shRNAs.

35 The one or more DNA molecules encoding the one or more siRNAs can include one or more promoter sequences, enhancer sequences, terminator sequences, invasion factor sequences or lysis regulation sequences. The promoter can be a prokaryotic promoter. Preferably, the prokaryotic

promoter is a T7 promoter, a P_{gapA} promoter, a P_{araBAD} promoter, a P_{tac} promoter, a P_{lacUV5} promoter, or a recA promoter.

The expressed siRNAs can direct the multienzyme complex RNA-induced silencing complex of the cell to interact with the mRNA of one or more genes of interest. Preferably, the siRNAs
5 interact with the mRNA of one or more HPV oncogenes. Preferably, the complex can degrade the mRNA. Preferably, the expression of one or more genes of interest is decreased or inhibited. The expression is decreased or inhibited as compared to the expression of the gene prior to administration or treatment with an invasive bacterium or BTP containing one or more siRNA or a DNA encoding for one or more siRNAs. Preferably, the expression of one or more HPV oncogenes is decreased or
10 inhibited.

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. In the specification, the singular forms also include the plural unless the context clearly dictates otherwise. Although methods and materials similar or equivalent to those described herein can be
15 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.

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

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic showing the Transkingdom RNA Interference Plasmid (TRIP) with
25 multiple hairpin express cassettes.

Figure 2 is a schematic showing the TRIP system (bacteria and plasmid) modified with the Operator Repressor Titration (ORT) system.

Figure 3 is a photograph showing cellular staining of the intestinal epithelial layer demonstrating efficient invasion and plasmid delivery by *S. typhimurium*.

30 Figure 4 is a photograph showing that bacteria-mediated RNA interference reduces target gene expression in the gastrointestinal epithelium.

Figure 5 is a schematic showing pNJSZ plasmid construct.

Figure 6 is a schematic showing the use of lambda S and R genes to kill intact bacteria.

Figure 7 is a bar graph showing a reduction in HPV oncogene expression with bacterial
35 delivered siRNA.

Figure 8 is a bar graph showing a reduction in HPV oncogene expression with bacterial delivered siRNA.

Figure 9 is a bar graph showing a reduction in HPV oncogene expression with bacterial delivered siRNA.

Figure 10, Panels A-C, are a series of bar graphs showing real time PCR results following invasion of Hela cells with various siRNAs.

5 Figure 11 is a photograph of an immunoblot showing the effects of downregulation of HPV E6 and E7 genes on tumor suppressor pathways and other downstream targets.

Figure 12 is a photograph of a colony forming assay showing infection at different multiplicities of infection (MOI).

10 Figure 13 is a line graph of a MTT assay showing infection at different multiplicities of infection (MOI).

Figure 14 is a bar graph showing real time PCR results following invasion of Hela cells with various siRNAs

Figure 15, Panels A-C, are a series of bar graphs showing real time PCR results following invasion of Hela cells with various siRNAs.

15 Figure 16 is a photograph of an immunoblot showing the effects of downregulation of HPV E6 and E7 genes on tumor suppressor pathways and other downstream targets.

Figure 17 is a bar graph showing real time PCR results following invasion assay of Hela cells with a frozen aliquot of negative sHRNA control and HPV sHRNA in BL21.

20 Figure 18 is a photograph showing the plating efficiency of frozen aliquots of negative sHRNA control and HPV sHRNA in BL21.

Figure 19 is a photograph of an immunoblot showing the knockdown of HPV E6 gene assessed by western blotting with HPV 18 E7 antibody.

Figure 20 is a bar graph showing the knockdown of CCL20 expression with the various siRNA sequences in CMT93 cells.

25 Figure 21 is a bar graph showing the knockdown of Claudin-2 expression with the various siRNA sequences in CMT93 cells post 24hr transfection.

Figure 22 is a bar graph showing the knockdown of IL6-RA expression with the various siRNA sequences in CMT93 cells post 24hr transfection.

30 Figure 23 is a bar graph showing the knockdown of IL13-RA1 expression with the various siRNA sequences in CMT93 cells post 24hr transfection.

Figure 24 is a bar graph showing the knockdown of IL18 expression with the various siRNA sequences in CMT93 cells post 24hr transfection.

Figure 25 is a bar graph showing the knockdown of IL-7 expression with the various siRNA sequences in CMT93 cells post 24hr transfection.

35 Figure 26 is a bar graph showing the knockdown of CH13L1 expression with the various siRNA sequences in CMT93 cells post 24hr transfection.

Figure 27 is a schematic of the pMBV40 or pMBV43 or pMBV44 plasmids.

Figure 28 is a schematic of the pNJSZ Δ BamH1 plasmid.

Figure 29 is a schematic of the pNJSZ pNJSZc plasmid.

Figure 30 is a schematic showing a lysis regulation system in combination with strain-specific nutritional attenuation.

5 Figure 31 is a schematic showing three cassettes in the combination lysis regulation system/nutritional attenuation system.

Figure 32 is a schematic showing a lysis regulation system in combination with a Tet-on expression system.

10 **DETAILED DESCRIPTION OF THE INVENTION**

The invention pertains to compositions and methods of delivering small interfering RNAs (siRNAs) to eukaryotic cells using non-pathogenic or therapeutic strains of bacteria or bacterial therapeutic particles (BTPs). The bacteria or BTPs deliver DNA encoding siRNA, or siRNA itself, to effect RNA interference (RNAi) by invading into the eukaryotic host cells. Generally, to trigger RNA interference in a target cell, it is required to introduce siRNA into the cell. 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 (e.g., U6, H1) (P. J. Paddison, A. A. Caudiy, G. J. Hannon, *PNAS* 99, 1443 (2002), T. R. Brummelkamp, R. Bernards, R. Agami, *Science* 296, 550 (2002)).

The interfering RNA of the invention regulates gene expression in eukaryotic cells. It silences or knocks down genes of interest inside target cells (*e.g.*, decreases gene activity). 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 or sequestration 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).

In the case of BMGS through delivery of siRNA expressing DNA plasmids, shRNA or siRNA are produced within the target cell after liberation of the eukaryotic transcription plasmid and trigger the highly specific process of mRNA degradation, which results in silencing of the targeted gene. Additionally, one or more cell-specific eukaryotic promoters may be used that limit the expression of siRNA or shRNA to specific target cells or tissues that are in particular metabolic states. In one embodiment of this method, the cell-specific promoter is albumin and the target cell or tissue is the liver. In another embodiment of this method, the cell-specific promoter is keratin and the specific target cell or tissue is the skin.

The non-virulent bacteria and BTPs of the invention have invasive properties (or are modified to have invasive properties) and may enter a mammalian host cell through various mechanisms. In contrast to uptake of bacteria or BTPs by professional phagocytes, which normally results in the destruction of the bacterium or BTP within a specialized lysosome, invasive bacteria or BTP strains

have the ability to invade non-phagocytic host cells. Naturally occurring examples of such bacteria or BTPs are intracellular pathogens such as *Yersinia*, *Rickettsia*, *Legionella*, *Brucella*, *Mycobacterium*, *Helicobacter*, *Coxiella*, *Chlamydia*, *Neisseria*, *Burkholderia*, *Bordetella*, *Borrelia*, *Listeria*, *Shigella*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Porphyromonas*, *Treponema*, and *Vibrio*, but this

5 property can also be transferred to other bacteria or BTPs such as *E. coli*, *Lactobacillus* or *Bifidobacteriae*, including probiotics through transfer of invasion-related genes (P. Courvalin, S. Goussard, C. Grillot-Courvalin, *C.R.Acad.Sci.Paris* 318,1207 (1995)). In other embodiments of the invention, bacteria or BTPs used to deliver interfering RNAs to host cells include *Shigella flexneri* (D. R. Sizemore, A. A. Branstrom, J. C. Sadoff, *Science* 270, 299 (1995)), invasive *E. coli* (P. Courvalin,

10 S. Goussard, C. Grillot-Courvalin, *C.R.Acad.Sci.Paris* 318,1207 (1995), C. Grillot-Courvalin, S. Goussard, F. Huetz, D. M. Ojcius, P. Courvalin, *Nat Biotechnol* 16, 862 (1998)), *Yersinia enterocolitica* (A. Al-Mariri A, A. Tibor, P. Lestrade, P. Mertens, X. De Bolle, J. J. Letesson *Infect Immun* 70, 1915 (2002)) and *Listeria monocytogenes* (M. Hense, E. Domann, S. Krusch, P. Wachholz, K. E. Dittmar, M. Rohde, J. Wehland, T. Chakraborty, S. Weiss, *Cell Microbiol* 3, 599

15 (2001), S. Pilgrim, J. Stritzker, C. Schoen, A. Kolb-Mäurer, G. Geginat, M. J. Loessner, I. Gentschev, W. Goebel, *Gene Therapy* 10, 2036 (2003)). Any invasive bacterium or BTP is useful for DNA transfer into eukaryotic cells (S. Weiss, T. Chakraborty, *Curr Opinion Biotechnol* 12, 467 (2001)).

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.

20 K. Hoiseth, B. A. D. Stocker, *Nature* 291, 238 (1981); Pawelek JM, Low KB, Bermudes D. *Cancer Res.* 57(20): 4537-44 (Oct. 15 1997)). In another embodiment of the invention, BMGS is performed using attenuated *E. coli*. In another aspect of this embodiment, the CEQ201 strain is engineered to possess cell-invading properties through an invasion plasmid. In one aspect of the invention, this plasmid is a TRIP (Transkingdom RNA interference plasmid) plasmid or pNJSZ.

25 A double "trojan horse" technique is also used with an invasive and auxotrophic bacterium or BTP carrying a eukaryotic transcription plasmid. This plasmid is, in turn, transcribed by the target cell to form one or more hairpin RNA structures 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

30 related gene (*β -catenin*) *in vitro*.

Another aspect of BMGS according to this invention is termed Transkingdom RNAi (tkRNAi). In this aspect of the invention, siRNA is directly produced by the invasive bacteria, or accumulated in the BTPs after production in the 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

35 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 tkRNAi 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) to perform BMGS and tkRNAi. Invasiveness as well as eukaryotic or prokaryotic transcription of one or several shRNA is conferred to a bacterium or BTP using plasmids (*e.g.*, TRIP) and vectors as described in greater detail herein.

1. Bacterium and/or Bacterial Therapeutic Particles (BTPs)

The present invention provides at least one invasive bacterium, or at least one bacterial therapeutic particle (BTP), including one or more siRNAs or one or more DNA molecules encoding one or more siRNAs.

According to the invention, any microorganism that is capable of delivering a molecule, *e.g.*, an RNA molecule or an RNA-encoding DNA 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 or BTP. Also within the scope of the invention are microorganisms other than bacteria that can be used for delivering RNA to a cell. For example, the microorganism can be a fungus, *e.g.*, *Cryptococcus neoformans*, protozoan, *e.g.*, *Trypanosoma cruzi*, *Toxoplasma gondii*, *Leishmania donovani*, and *plasmodia*.

In a preferred embodiment, the microorganism is a bacterium or BTP. A preferred invasive bacterium or BTP 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. Preferably, the RNA is siRNA or shRNA and the RNA-encoding DNA molecule encodes for siRNA or shRNA.

BTPs are fragments of bacteria used for therapeutic or preventive purposes. BTPs may include particles known in the art as minicells. Minicells are small cells produced by cell division that is faulty near the pole. They are devoid of nucleoid and, therefore, unable to grow and form colonies (Alder et al., (1967) Proc. Nat. Acad. Sci. U.S.A. 57, 321-326; for reviews see Sullivan and Maddock, (2000) Curr. Biol. 10:R249-R252; Margolin, (2001) Curr. Biol. 11, R395-R398; Howard and Kruse, (2005) J. Cell Biol. 168, 533-536). Minicell formation results due to mutations causing a defect in selection of the site for the septum formation for cell division. Such mutations include null alleles of minC, minD (Davie et al, (1984) J. Bacteriol. 158, 1202-1203; de Boer et al., 1988) J. Bacteriol. 170, 2106-2112) and certain alleles of ftsZ (Bi and Lutkenhaus, (1992) J. Bacteriol. 174, 5414-5423). Overexpression of FtsZ or MinC-MinD proteins has also been reported to cause the formation of minicells (Ward and Lutkenhaus, 1985; de Boer et al., 1988). Although minicells are devoid of

nucleoid, they are capable of transcription and translation (Roozen et al., (1971) J. Bacteriol. 107, 21-33; Shepherd et al., (2001) J. Bacteriol. 183, 2527-34).

BTPs are distinct from bacteria in that they lack the bacterial genome and, therefore, provide a decreased risk of bacterial proliferation in patients. This is of particular value for immune-
5 compromised patients. Furthermore, the inability of BTPs to proliferate allows for their use in sensitive tissues, *e.g.*, the brain, and other areas of the body traditionally considered inaccessible to traditional siRNA. For example, the intraperitoneal delivery of bacteria can include the risk of adhesions and peritonitis, which is eliminated by utilizing BTPs. However, like the bacteria of this invention, BTPs contain the bacterial cell wall, some bacterial plasma contents and subcellular
10 particles, one or more therapeutic components, *e.g.*, one or more siRNAs, one or more invasion factors, one or more phagosome degradation factors, and one or more factors for targeting specific tissues. The BTPs are produced from bacteria that have produced and accumulated siRNAs inside the bacteria, and then segregate the bacterial fragment (BTP) during cell division. In one embodiment of this invention, BTPs are obtained by fermenting the bacteria, during which the BTPs form abundantly,
15 followed by isolation of the BTPs from live bacteria using differential size filtration, which will retain the bacteria but allow passage and collection of BTPs. In another embodiment of this invention, BTPs are separated from bacteria by centrifugation. In another embodiment of this invention, live bacterial cells are lysed through activation of a death signal. Once isolated, the BTPs can be lyophilized and formulated for use.

20 As used herein, the term "invasive" when referring to a microorganism, *e.g.*, a bacterium or BTP, refers to a microorganism that 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 that 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
25 process of delivery of the at least one molecule into the target cell preferably does not significantly modify the invasion apparatus.

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
30 and which have been modified, *e.g.*, genetically modified, to be invasive. In another preferred embodiment, a microorganism that is not naturally invasive can be modified to become invasive by linking the bacterium or BTP 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 or BTP, render the bacterium or BTP invasive.
35 As used herein, an "invasion factor" is encoded by a "cytoplasm-targeting gene".

In one embodiment of this invention, the microorganism is a naturally invasive bacterium or BTP selected from the group that includes, but is not limited to, *Yersinia*, *Rickettsia*, *Legionella*,

Brucella, *Mycobacterium*, *Helicobacter*, *Coxiella*, *Chlamydia*, *Neisseria*, *Burkholderia*, *Bordetella*, *Borrelia*, *Listeria*, *Shigella*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Porphyromonas*, *Treponema*, *Vibrio*, *E. coli*, and *Bifidobacteriae*. Optionally, the naturally invasive bacterium or BTP is *Yersinia* expressing an invasion factor selected from the group including, but not limited to, invasin and YadA (5 *Yersinia enterocolitica* plasmid adhesion factor). Optionally, the naturally invasive bacterium or BTP is *Rickettsia* expressing the invasion factor RickA (actin polymerization protein). Optionally, the naturally invasive bacterium or BTP is *Legionella* expressing the invasion factor RaIF (guanine exchange factor). Optionally, the naturally invasive bacterium or BTP is *Neisseria* expressing an invasion factor selected from the group including, but not limited to, NadA (*Neisseria* 10 adhesion/invasion factor), OpA and OpC (opacity-associated adhesions). Optionally, the naturally invasive bacterium or BTP is *Listeria* expressing an invasion factor selected from the group including, but not limited to, InlA (internalin factor), InlB (internalin factor), Hpt (hexose phosphate transporter), and ActA (actin polymerization protein). Optionally, the naturally invasive bacterium or BTP is *Shigella* expressing an invasion factor selected from the group including, but not limited to, the 15 *Shigella* secreting factors IpaA (invasion plasmid antigen), IpaB, IpaC, IpgD, IpaB-IpaC complex, VirA, and IcsA. Optionally, the naturally invasive bacterium or BTP is *Salmonella* expressing an invasion factor selected from the group including, but not limited to, *Salmonella* secreting/exchange factors SipA, SipC, SpiC, SigD, SopB, SopE, SopE2, and SptP. Optionally, the naturally invasive bacterium or BTP is *Staphylococcus* expressing an invasion factor selected from the group including, 20 but not limited to, the fibronectin binding proteins FnBPA and FnBPB. Optionally, the naturally invasive bacterium or BTP is *Streptococcus* expressing an invasion factor selected from the group including, but not limited to, the fibronectin binding proteins ACP, Fba, F2, Sfb1, Sfb2, SOF, and PFBP. Optionally, the naturally invasive bacterium or BTP is *Porphyromonas gingivalis* expressing the invasion factor FimB (integrin binding protein fibriae).

25 In another embodiment of this invention, the microorganism is a bacterium or BTP that is not naturally invasive but has been modified, e.g., genetically modified, to be invasive. Optionally, the bacterium or BTP that is not naturally invasive has been genetically modified to be invasive by expressing an invasion factor selected from the group including, but not limited to, invasin, YadA, RickA, RaIF, NadA, OpA, OpC, InlA, InlB, Hpt, ActA, IpaA, IpaB, IpaC, IpgD, IpaB-IpaC complex, 30 VirA, IcsA, SipA, SipC, SpiC, SigD, SopB, SopE, SopE2, SptP, FnBPA, FnBPB, ACP, Fba, F2, Sfb1, Sfb2, SOF, PFBP, and FimB.

In another embodiment of this invention, the microorganism is a bacterium or BTP that may be naturally invasive but has been modified, e.g., genetically modified, to express one or more additional invasion factors. Optionally, the invasion factor is selected from the group that includes, 35 but is not limited to, invasin, YadA, RickA, RaIF, NadA, OpA, OpC, InlA, InlB, Hpt, ActA, IpaA, IpaB, IpaC, IpgD, IpaB-IpaC complex, VirA, IcsA, SipA, SipC, SpiC, SigD, SopB, SopE, SopE2, SptP, FnBPA, FnBPB, ACP, Fba, F2, Sfb1, Sfb2, SOF, PFBP, and FimB.

Naturally invasive microorganisms, *e.g.*, bacteria or BTPs, may have a certain tropism, *i.e.*, preferred target cells. Alternatively, microorganisms, *e.g.*, bacteria or BTPs can be modified, *e.g.*, genetically, to mimic the tropism of a second microorganism. Optionally, the bacterium or BTP is *Streptococcus* and the preferred target cells are selected from the group including, but not limited to, pharyngeal epithelial cells, buccal epithelial cells of the tongue, and mucosal epithelial cells. Optionally, the bacterium or BTP is *Porphyromonas* and the preferred target cells are selected from the group including, but not limited to, oral epithelial cells. Optionally, the bacterium or BTP is *Staphylococcus* and the preferred target cells are mucosal epithelial cells. Optionally, the bacterium or BTP is *Neisseria* and the preferred target cells are selected from the group including, but not limited to, urethral epithelial cells and cervical epithelial cells. Optionally, the bacterium or BTP is *E. coli* and the preferred target cells are selected from the group, including but not limited to, intestinal epithelial cells, urethral epithelial cells, and the cells of the upper urinary tract. Optionally, the bacterium or BTP is *Bordetella* and the preferred target cells are respiratory epithelial cells. Optionally, the bacterium or BTP is *Vibrio* and the preferred target cells are intestinal epithelial cells. Optionally, the bacterium or BTP is *Treponema* and the preferred target cells are mucosal epithelial cells. Optionally, the bacterium or BTP is *Mycoplasma* and the preferred target cells are respiratory epithelial cells. Optionally, the bacterium or BTP is *Helicobacter* and the preferred target cells are the endothelial cells of the stomach. Optionally, the bacterium or BTP is *Chlamydia* and the preferred target cells are selected from the group including, but not limited to, conjunctival cells and urethral epithelial cells.

In another embodiment of this invention, the microorganism is a bacterium or BTP that has been modified, *e.g.*, genetically modified, to have a certain tropism. Optionally, the preferred target cells are selected from the group including, but not limited to, pharyngeal epithelial cells, buccal epithelial cells of the tongue, mucosal epithelial cells, oral epithelial cells, epithelial cells of the urethra, cervical epithelial cells, intestinal epithelial cells, respiratory epithelial cells, cells of the upper urinary tract, epithelial cells of the stomach, and conjunctival cells. Optionally, the preferred target cells are dysplastic or cancerous epithelial cells. Optionally, the preferred target cells are activated or resting immune cells.

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 that may include the use of an antibody.

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

Bacterial or BTP entry into cells can be measured by various methods. Intracellular bacteria or BTPs survive treatment by aminoglycoside antibiotics, whereas extracellular bacteria are rapidly killed. A quantitative estimate of bacterial or BTP uptake can be achieved by treating cell monolayers with the antibiotic gentamicin to inactivate extracellular bacteria or BTPs, then by removing said
5 antibiotic before liberating the surviving intracellular organisms with gentle detergent and determining viable counts on standard bacteriological medium. Furthermore, bacterial or BTP 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 bacterium or BTP is capable of
10 invading a specific type of cell or to confirm bacterial invasion following modification of the bacteria or BTP, such modification of the tropism of the bacteria to mimic that of a second bacterium.

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

Without wanting to be limited to a specific mechanism of action, the bacterium or BTP delivering the RNA into the eukaryotic cell can enter various compartments of the cell, depending on
20 the type of bacterium or BTP. For example, the bacterium or BTP can be in a vesicle, *e.g.*, a phagocytic vesicle. Once inside the cell, the bacterium or BTP can be destroyed or lysed and its contents delivered to the eukaryotic cell. A bacterium or BTP can also be engineered to express a phagosome degrading protein to allow leakage of RNA from the phagosome. In one embodiment of this invention, the bacterium or BTP expresses, either naturally or through modification, *e.g.*, genetic
25 modification, a protein that contributes to pore-formation, breakage or degradation of the phagosome. Optionally, the protein is a cholesterol-dependent cytolysin. Optionally, the protein is selected from the group consisting of listeriolysin, ivanolysin, streptolysin, sphingomyelinase, perfringolysin, botulinolysin, leukocidin, anthrax toxin, phospholipase, IpaB (invasion plasmid antigen), IpaH, IcsB (intercellular spread), DOT/Icm (defect in organelle trafficking/intracellular multiplication defective),
30 DOTU (stabilization factor for the DOT/Icm complex), IcmF, and PmrA (multidrug resistance efflux pump).

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
35 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 compositions permitting delivery of RNA or RNA-encoding DNA by a bacterium independently of the mechanism of delivery.

In one embodiment, the bacterium or BTP for use in the present invention is non-pathogenic or non-virulent. In another aspect of this embodiment, the bacterium or BTP is therapeutic. In
 5 another aspect of this embodiment, the bacterium or BTP is an attenuated strain or derivative thereof selected from, but not limited to, *Yersinia*, *Rickettsia*, *Legionella*, *Brucella*, *Mycobacterium*, *Helicobacter*, *Haemophilus*, *Coxiella*, *Chlamydia*, *Neisseria*, *Burkholderia*, *Bordetella*, *Borrelia*, *Listeria*, *Shigella*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Porphyromonas*, *Treponema*, *Vibrio*, *E. coli*, and *Bifidobacteriae*. Optionally, the *Yersinia* strain is an attenuated strain of the *Yersinia*
 10 *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 coronii* 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
 15 *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 burnetti*. Optionally, the *Haemophilus* strain is an attenuated strain of the *Haemophilus influenza* 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*
 20 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 meningitidis* species. Optionally, the *Burkholderia* strain is an attenuated strain of the *Burkholderia cepacia* 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
 25 *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
 30 *Staphylococcus aureus* species. Optionally, the *Streptococcus* strain is an attenuated strain of the *Streptococcus pyogenes* 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 salivarius* species. Optionally, the *Streptococcus* strain is an attenuated strain of the *Streptococcus pneumoniae* species. Optionally, the *Porphyromonas* strain is an attenuated strain of the
 35 *Porphyromonas gingivalis* species. Optionally, the *Pseudomonas* strain is an attenuated strain of 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. Optionally, the *E. coli* strain is MM294.

Set forth below are examples of bacteria that 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).

1.1 Naturally Invasive Bacteria

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

The particular *Shigella* strain employed is not critical to the present invention. Examples of *Shigella* strains that can be employed in the present invention include *Shigella flexneri* 2a (ATCC No. 29903), *Shigella sonnei* (ATCC No. 29930), and *Shigella dysenteriae* (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.*, 62:5664-5668 (1994)), *Shigella flexneri* Y SFL114 aroD mutant (Karnell *et al. Vacc.*, 10:167-174 (1992)), and *Shigella flexneri* aroA aroD mutant (Verma *et al. Vacc.*, 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.

At least one advantage to *Shigella* bacteria as delivery vectors is their tropism for lymphoid tissue in the colonic mucosal surface. In addition, the primary site of *Shigella* replication is believed to be within dendritic cells and macrophages, which are commonly found at the basal lateral surface of M cells in mucosal lymphoid tissues (reviewed by McGhee, J. R. *et al. (1994) Reproduction, Fertility, & Development* 6:369; Pascual, D. W. *et al. (1994) Immunomethods* 5:56). As such, *Shigella* vectors may provide a means to target RNA interference or deliver therapeutic molecules to these professional antigen-presenting cells. Another advantage of *Shigella* vectors is that attenuated *Shigella* strains deliver nucleic acid reporter genes *in vitro* and *in vivo* (Sizemore, D. R. *et al. (1995) Science* 270:299; Courvalin, P. *et al. (1995) Comptes Rendus de l'Academie des Sciences Serie III-Sciences de la Vie-Life Sciences* 318:1207; Powell, R. J. *et al. (1996) In: Molecular approaches to the control of infectious diseases*. F. Brown, E. Norrby, D. Burton and J. Mekalanos, eds. Cold Spring Harbor Laboratory Press, New York. 183; Anderson, R. J. *et al. (1997) Abstracts for the 97th General*

Meeting of the American Society for Microbiology:E.). On the practical side, the tightly restricted host specificity of *Shigella* stands to prevent the spread of *Shigella* vectors into the food chain via intermediate hosts. Furthermore, attenuated strains that are highly attenuated in rodents, primates and volunteers have been developed (Anderson *et al.* (1997) *supra*; Li, A. *et al.* (1992) *Vaccine* 10:395;
 5 Li, A. *et al.* (1993) *Vaccine* 11:180; Karnell, A. *et al.* (1995) *Vaccine* 13:88; Sansonetti, P. J. and J. Arondel (1989) *Vaccine* 7:443; Fontaine, A. *et al.* (1990) *Research in Microbiology* 141:907; Sansonetti, P. J. *et al.* (1991) *Vaccine* 9:416; Noriega, F. R. *et al.* (1994) *Infection & Immunity* 62:5168; Noriega, F. R. *et al.* (1996) *Infection & Immunity* 64:3055; Noriega, F. R. *et al.* (1996) *Infection & Immunity* 64:23; Noriega, F. R. *et al.* (1996) *Infection & Immunity* 64:3055; Kotloff, K.
 10 L. *et al.* (1996) *Infection & Immunity* 64:4542). This latter knowledge will allow the development of well-tolerated *Shigella* vectors for use in humans.

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
 15 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:

- (i) auxotrophic mutations, such as *aro* (Hoiseth *et al.* *Nature*, 291:238-239 (1981)), *gua*
 20 (McFarland *et al.* *Microbiol. Path.*, 3:129-141 (1987)), *nad* (Park *et al.* *J. Bact.*, 170:3725-3730 (1988)), *thy* (Nnalue *et al.* *Infect. Immun.*, 55:955-962 (1987)), and *asd* (Curtiss, *supra*) mutations;
- (ii) mutations that inactivate global regulatory functions, such as *cya* (Curtiss *et al.* *Infect. Immun.*, 55:3035-3043 (1987)), *crp* (Curtiss *et al.* (1987), *supra*), *phoP/phoQ* (Groisman *et al.* *Proc. Natl. Acad. Sci., USA*, 86:7077-7081 (1989); and Miller *et al.* *Proc. Natl. Acad. Sci., USA*, 86:5054-
 25 5058 (1989)), *phop^c* (Miller *et al.* *J. Bact.*, 172:2485-2490 (1990)) or *ompR* (Dorman *et al.* *Infect. Immun.*, 57:2136-2140 (1989)) mutations;
- (iii) mutations that modify the stress response, such as *recA* (Buchmeier *et al.* *Mol. Micro.*, 7:933-936 (1993)), *htrA* (Johnson *et al.* *Mol. Micro.*, 5:401-407 (1991)), *htpR* (Neidhardt *et al.* *Biochem. Biophys. Res. Com.*, 100:894-900 (1981)), *hsp* (Neidhardt *et al.* *Ann. Rev. Genet.*, 18:295-
 30 329 (1984)) and *groEL* (Buchmeier *et al.* *Sci.*, 248:730-732 (1990)) mutations;
- (iv) mutations in specific virulence factors, such as *IsyA* (Libby *et al.* *Proc. Natl. Acad. Sci., USA*, 91:489-493 (1994)), *pag* or *prg* (Miller *et al.* (1990), *supra*; and Miller *et al.* (1989), *supra*), *iscA* or *virG* (d'Hauteville *et al.* *Mol. Micro.*, 6:833-841 (1992)), *plcA* (Mengaud *et al.* *Mol. Microbiol.*, 5:367-72 (1991); Camilli *et al.* *J. Exp. Med.*, 173:751-754 (1991)), and *act* (Brundage *et al.* *Proc. Natl.*
 35 *Acad. Sci., USA*, 90:11890-11894 (1993)) mutations;
- (v) mutations that affect DNA topology, such as *topA* (Galan *et al.* *Infect. Immun.*, 58:1879-1885 (1990));

(vi) mutations that disrupt or modify the cell cycle, such as min (de Boer *et al.* Cell, 56:641-649 (1989)).

(vii) introduction of a gene encoding a suicide system, such as sacB (Recorbet *et al.* App. Environ. Micro., 59:1361-1366 (1993); Quandt *et al.* Gene, 127:15-21 (1993)), nuc (Ahrenholtz *et al.* App. Environ. Micro., 60:3746-3751 (1994)), hok, gef, kil, or phlA (Molin *et al.* Ann. Rev. Microbiol., 47:139-166 (1993));

(viii) mutations that alter the biogenesis of lipopolysaccharide and/or lipid A, such as rFb (Raetz in *Escherichia coli* and *Salmonella typhimurium*, Neidhardt *et al.*, Ed., ASM Press, Washington D.C. pp 1035-1063 (1996)), galE (Hone *et al.* J. Infect. Dis., 156:164-167 (1987)) and htrB (Raetz, *supra*), msbB (Raetz, *supra*)

(ix) introduction of a bacteriophage lysis system, such as lysogens encoded by P22 (Rennell *et al.* Virol, 143:280-289 (1985)), λ murein transglycosylase (Bienkowska-Szewczyk *et al.* Mol. Gen. Genet., 184:111-114 (1981)) or S-gene (Reader *et al.* Virol, 43:623-628 (1971)); and

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 uapA (Gorfinkiel *et al.* J. Biol. Chem., 268:23376-23381 (1993)) or gcv (Stauffer *et al.* J. Bact., 176:6159-6164 (1994)).

The particular *Listeria* strain employed is not critical to the present invention. Examples of *Listeria* strains that 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, new attenuated *Listeria* strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Salmonella* strain employed is not critical to the present invention. Examples of *Salmonella* strains that 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.* Vacc. 9:810 (1991)) and *S. typhimurium*-aroA mutant (Mastroeni *et al.* Micro. Pathol. 13:477 (1992)). Alternatively, new attenuated *Salmonella* strains can be constructed by introducing one or more attenuating mutations as described for *Shigella spp.* above.

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 Rickettsiae* (ATCC Nos. VR149 and VR891), *Rickettsia prowaseckii* (ATCC No. VR233), *Rickettsia tsutsugamuchi* (ATCC Nos. VR312, VR150 and VR609), *Rickettsia mooseri* (ATCC No. VR144), *Rickettsia sibirica* (ATCC No. VR151), and *Rochalimaea quitana* (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.

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 (Sansone *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.

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.

1.2 Less Invasive Bacteria

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.

The particular *Yersinia* strain employed is not critical to the present invention. Examples of *Yersinia* strains that 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* Ye03-R2 (al-Hendy *et al.* Infect. Immun., 60:870-875 (1992)) or *Y. enterocolitica* aroA (O'Gaora *et al.* Micro. Path., 9:105-116 (1990)) are preferably used in the present invention. Alternatively, new attenuated *Yersinia* strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Escherichia* strain employed is not critical to the present invention. Examples of *Escherichia* strains that can be employed in the present invention include *E. coli* Nissle 1917, MM294, H10407 (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* 02 carAB mutant (Kwaga *et al.* Infect. Immun., 62:3766-3772 (1994)) or CEQ201 are preferably used in the present invention. Alternatively, new attenuated *Escherichia* strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Klebsiella* strain employed is not critical to the present invention. Examples of *Klebsiella* strains that 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.

The particular *Bordetella* strain employed is not critical to the present invention. Examples of *Bordetella* strains that 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.

The particular *Neisseria* strain employed is not critical to the present invention. Examples of *Neisseria* strains that 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 aro mutant (Chamberlain *et al.* Micro. Path., 15:51-63 (1993)) are preferably used in the present invention. Alternatively, new attenuated *Neisseria* strains can be constructed by introducing one or more attenuating mutations in groups (i) to (vii) as described for *Shigella spp.* above.

The particular *Aeromonas* strain employed is not critical to the present invention. Examples of *Aeromonas* strains that 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.

The particular *Francisella* strain employed is not critical to the present invention. Examples of *Francisella* strains that 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.

The particular *Corynebacterium* strain employed is not critical to the present invention. Examples of *Corynebacterium* strains that 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.

The particular *Citrobacter* strain employed is not critical to the present invention. Examples of *Citrobacter* strains that can be employed in the present invention include *C. freundii* (ATCC No. 8090). 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.

The particular *Chlamydia* strain employed is not critical to the present invention. Examples of *Chlamydia* strains that can be employed in the present invention include *C. pneumoniae* (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.

5 The particular *Hemophilus* strain employed is not critical to the present invention. Examples of *Hemophilus* strains that can be employed in the present invention include *H. sornnus* (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.

10 The particular *Brucella* strain employed is not critical to the present invention. Examples of *Brucella* strains that 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.

15 The particular *Mycobacterium* strain employed is not critical to the present invention. Examples of *Mycobacterium* strains that 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.

20 The particular *Legionella* strain employed is not critical to the present invention. Examples of *Legionella* strains that 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.

25 The particular *Rhodococcus* strain employed is not critical to the present invention. Examples of *Rhodococcus* strains that 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.

30 The particular *Pseudomonas* strain employed is not critical to the present invention. Examples of *Pseudomonas* strains that 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.

35 The particular *Helicobacter* strain employed is not critical to the present invention. Examples of *Helicobacter* strains that 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.

The particular *Salmonella* strain employed is not critical to the present invention. Examples of *Salmonella* strains that 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.* Vacc., 9:810-816 (1991)) and *S. typhimurium* aroA mutant (Mastroeni *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.

The particular *Vibrio* strain employed is not critical to the present invention. Examples of *Vibrio* strains that 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* ctxA, 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.

The particular *Bacillus* strain employed is not critical to the present invention. Examples of *Bacillus* strains that 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.

The particular *Erysipelothrix* strain employed is not critical to the present invention. Examples of *Erysipelothrix* strains that can be employed in the present invention include *Erysipelothrix rhusiopathiae* (ATCC No. 19414) and *Erysipelothrix tonsillarum* (ATCC No. 43339). Attenuated *Erysipelothrix* strains are preferably used in the present invention and include *E. rhusiopathiae* Kg-1a and Kg-2 (Watarai *et al.* J. Vet. Med. Sci., 55:595-600 (1993)) and *E. rhusiopathiae* ORVAC mutant (Markowska-Daniel *et al.* Int. J. Med. Microb. Virol. Parasit. 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.

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

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.

Examples of such genes referred to herein as "cytoplasm-targeting genes" include genes
5 encoding the proteins that enable invasion by *Shigella* or the analogous invasion genes of entero-
invasive *Escherichia*, or listeriolysin O of *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. Immun., 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,
10 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 that can be introduced into
bacteria to increase their invasive character encodes the invasin protein from *Yersinia*
15 *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
20 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 that promotes endosmosis (Plank *et al.* J. Biol. Chem.,
269:12918-12924 (1994)).

The above cytoplasm-targeting genes can be obtained by, *e.g.*, PCR amplification from DNA
25 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
30 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,
35 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
 5 spliced into the chromosome (Hone *et al. supra*) of the target strain.

In addition to genetically engineering bacteria and BTPs 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
 10 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-
 15 immobilized invasin (reviewed in Isberg and Tran van Nhieu (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.*, $\alpha 5 \beta 1$, known to be the surface molecule with which the bacterial
 20 invasin protein interacts (Isberg and Tran van Nhieu, *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
 25 factor to the surface of a bacterium are known in the art and include cross-linking.

3. Plasmids and Vectors

The present invention also provides at least one vector or plasmid including at least one DNA molecule encoding one or more siRNAs and at least one promoter, wherein the expressed siRNAs
 30 interfere with at least one mRNA of a gene of interest. In one preferred embodiment, the present invention provides at least one prokaryotic vector including 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 at least one mRNA of a gene of interest.

The TRIP (transkingdom RNA interference plasmid) vectors and plasmids of the present
 35 invention include a multiple cloning site, a promoter sequence and a terminator sequence. The TRIP vectors and plasmids also include one or more sequences encoding for an invasion factor to permit the non-invasive bacterium or BTP to enter mammalian cells (*e.g.*, the *Inv* locus that encodes invasion

that permits the bacterium or BTP to enter β 1-integrin-positive mammalian cells) (Young et al., *J. Cell Biol.* 116, 197-207 (1992)) and one or more sequences to permit the genetic material to escape from the entry vesicles (e.g., *Hly A* gene that encodes listeriolysin O) (Mathew et al., *Gene Ther.* 10, 1105-1115 (2003) and Grillot-Courvalin et al., *Nat. Biotechnol.* 16, 862-866 (1998)). TRIP is further described (including a vector/plasmid schematic) in PCT Publication No. WO 06/066048. In preferred embodiments, the TRIP vectors and plasmids will incorporate a hairpin RNA expression cassette encoding short hairpin RNA under the control of an appropriate promoter sequence and terminator sequence.

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.

As described herein, the one or more DNA molecules encoding the one or more siRNAs are transcribed within the eukaryotic target cell or transcribed within the bacterium or BTP.

In embodiments where the DNA is transcribed within the eukaryotic cell, the one or more siRNAs are transcribed within the eukaryotic cells as shRNAs. The eukaryotic cell can be *in vivo*, *in vitro* or *ex vivo*. In one aspect of this embodiment, the one or more DNA molecules encoding the one or more siRNAs contain a eukaryotic promoter. Optionally, the eukaryotic promoter is a RNA-polymerase III promoter. Optionally, the RNA polymerase III promoter is a U6 promoter or an H1 promoter.

In embodiments where the DNA is transcribed within the bacterium or BTP, the one or more DNA molecules contain a prokaryotic promoter. Optionally, the prokaryotic promoter is an *E. coli* promoter. Preferably, the *E. coli* promoter can be a T7 promoter, lacUV5 promoter, RNA polymerase promoter, gapA promoter, pA1 promoter, lac regulated promoter, araC+ P_{araBAD} promoter, T5 promoter, P_{tac} promoter (Estrem et al, 1998, Proc. Natl. Acad. Sci. USA 95, 9761-9766; Meng et al., 2001, Nucleic Acids Res. 29, 4166-417; De Boer et al., 1983, Proc. NatL Acad. Sci. USA 80, 21-25) or recA promoter.

Preferable, promoter sequences are recited in Table 1.

Table 1

Promoter	Sequence	SEQ ID NO:
T7 promoter	TAATACGACTCACTATAG	1
lacUV5 promoter	TAACCAGGCTTTACACTTTATG CTTCCGGCTCGTATAATGTGTG GAAGGATCC	2
RNA polymerase promoter	TAACCAGGCTTTACACTTTATG CTTCCGGCTCGTATAATGTGTG GAA	3
RNA polymerase promoter	TAAAATTCAAAAATTTATTTGC TTTCAGGAAAATTTTCTGTAT AATAGATTC	4

RNA polymerase promoter	TAATTGATACTTTATGCTTTTT TCTGTATAAT	5
gapA promoter	AAGCTTTCAGTCGCGTAATGCT TAGGCACAGGATTGATTGTGCG CAATGATTGACACGATTCCGCT TGACACTGCGTAAGTTTGTGT TATAATGGATCC	6
pA1 promoter	AAGCTTAAGGAGAGACAACTTA AAGAGACTTAAAAGATTAATTT AAAATTTATCAAAAAGAGTATT GACTTAAAGTCTAACCTATAGG ATACTTGGATCC	7
lac regulated promoter	AAGCTTTGTGTGGAATTGTGAG CGGATAACAATTCACACATTG ACACTTTATGCTTCCGGCTCGT ATAATGGATCC	8
lac regulated promoter	AAGCTTGGAAAATTTTTTTAA AAAAGTCATGTGTGGAATTGTG AGCGGATAACAATTCACATAT AATGGATCC	9
araC+ P _{araBAD} promoter	GACTTCATATACCCAAGCTTTA AAAAAAAAATCCTTAGCTTTTCG CTAAGGATCTCCGTCAAGCCGT CAATTGTCTGATTGCTTACCAA TTATGACAACTTGACGGCTACA TCATTCACTTTTCTTCACAAC CGGCACGAACTCGCTCGGGCT GGCCCCGGTGCATTTTTAAAT ACTCGCGAGAAATAGAGTTGAT CGTCAAACCAACATTGCGACC GACGGTGGCGATAGGCATCCGG GTAGTGCTCAAAGCAGCTTCG CCTGACTAATGCGTTGGTCCTC GCGCCAGCTTAAGACGCTAATC CCTAACTGCTGGCGGAAAAGAT GTGACAGACGCGACGGCGACAA GCAAACATGCTGTGCGACGCTG GCGATATCAAAATTGCTGTCTG CCAGGTGATCGCTGATGTACTG ACAAGCCTCGCGTACCCGATTA TCCATCGGTGGATGGAGCGACT CGTTAATCGCTTCCATGCGCCG CAGTAACAATTGCTCAAGCAGA TTTATCGCCAGCAGCTCCGAAT AGCGCCCTTCCCCTTGCCCGGC GTTAATGATTGCCCCAAACAGG TCGCTGAAATGCGGCTGGTGCG CTTCATCCGGGCGAAAGAAACC CGTATTGGCAAATATTGACGGC CAGTTAAGCCATTCATGCCAGT AGGCGCGCGACGAAAGTAAAC CCACTGGTGATAACCATTCGCGA GCCTCCGGATGACGACCGTAGT GATGAATCTCTCTGGCGGGAA CAGCAAAAATATCACCCGGTCGG CAGACAAATTCTCGTCCCTGAT TTTTCAACCCCCCTGACCGCG AATGGTGAGATTGAGAATATAA CCTTTCATTCCCAGCGGTGCGT CGATAAAAAAATCGAGATAACC GTTGGCCTCAATCGGCGTTAA CCCGCCACCAGATGGGCGTTAA	10

	ACGAGTATCCCGGCAGCAGGGG ATCATTTTGCCTTCAGCCATA CTTTTCATACTCCCACCATTCA GAGAAGAAACCAATTGTCCATA TTGCATCAGACATTGCCGTCAC TGCGTCTTTTACTGGCTCTTCT CGCTAACCCAACCGTAACCCC GCTTATTAAAAGCATCTGTAA CAAAGCGGGACCAAAGCCATGA CAAAAACGCGTAACAAAAGTGT CTATAATCACGGCAGAAAAGTC CACATTGATTATTTGCACGGCG TCACACTTTGCTATGCCATAGC ATTTTATCCATAAGATTAGCG GATCCTACCTGACGCTTTTTAT CGCAACTCTCTACTGTAGATCT ATCTGCGAT	
T5 promoter	TAAAATTCAAAAATTTATTTGC TTTCAGGAAAATTTTCTGTAT AATAGATTCGGATCC	11
recA promoter	TAATTGATACTTTATGCTTTTT TCTGTATAATGGATCC	12
P _{tac} promoter	GACTTCATATACCCAAGCTTGG AAAATTTTTTTTAAAAAAGTCT TGACACTTTATGCTTCCGGCTC GTATAATGGATCC	378
P _{atac} promoter	GGAAAATTTTTTTTAAAAAAGT C	379

In embodiments where the DNA is transcribed within the bacterium or BTP, the *E. coli* promoter is associated with a terminator. Preferably, the *E. coli* terminator can be a T7 terminator, lacUV5 terminator, Rho-independent terminator, Rho-dependent terminator, or RNA polymerase terminator.

Preferable, terminator sequences are recited in Table 2.

Table 2

Terminator	Sequence	SEQ ID NO:
T7 terminator	TAGCATAACCCCTTGGGGCCTC TAAACGGGTCTTGAGGGGTTTT TTG	13
lacUV5 terminator	TTGTCACGTGAGCGGATAACAA TTTCACACAGGAAACAGAATTC TTAAT	14
Rho-independent terminator	TTGTCACAAACCCCGCCACCGG CGGGGTTTTTTCTGCTTAAT	15
Rho-dependent terminator	TTGTCACAATTCTATGGTGTAT GCATTATTTGCATACATTCAA TCAATTGGATCCTGCATTAAT	16
RNA polymerase terminator	GTGAGCGGATAACAATTTACA CAGGAAACAGAATTCTTAAT	17
RNA polymerase terminator	AAACCCCGCCACCGGCGGGGT TTTTCTGCTTAAT	18
RNA polymerase terminator	AATTCTATGGTGTATGCATTTA TTTGCATACATTCAATCAATTG GATCCTGCATTAAT	19

In additional embodiments, the vectors and plasmids of the present invention further include one or more enhancer sequences, selection markers, or lysis regulation system sequences.

In one aspect of the invention, the one or more DNA molecules contain a prokaryotic enhancer. Optionally, the prokaryotic enhancer is a T7 enhancer. Optionally, the T7 enhancer has the sequence GAGACAGG (SEQ ID NO: 42). In another aspect of this embodiment, the one or more DNA molecules contain a prokaryotic terminator.

In another aspect of the, the one or more DNA molecules are associated with one or more selection markers. In one aspect of this embodiment, the selection marker is an amber suppressor containing one or more mutations or an diamino pimelic acid (DAP) containing one or more mutations. Optionally, the dap gene is selected from, but not limited to, dapA and dapE.

Preferable, selection marker sequences are recited in Table 3.

Table 3

Selection Marker	Sequence	SEQ ID NO:
amber suppressor gene sequence	AATTCGGGGCTATAGCTCAGCT GGGAGAGCGCTTGCACTAATG CAAGAGGTCAGCGGTTTCGATCC CGCTTAGCTCCACCACTGCA	20
amber suppressor sequence	AATTCGCCCCGATAGCTCAGTC GGTAGAGCAGGGGATTCTAAAT CCCCGTGTCCTTGGTTCGATTTC CGAGTCCGGGCACTGCA	21
Rho- <i>lgt</i> with double amber mutation (<i>lgt am-am</i> allele of <i>lgt</i> gene) sequence	ATGACCAGTAGCTATCTGCATT AGCCGGAGTAGGATCCGGTCAT TTTCTCAATAGGACCGTGGCG CTTCACTGGTACGGCCTGATGT ATCTGGTGGGTTTCATTTTTGC AATGTGGCTGGCAACACGACGG GCGAATCGTCCGGGCAGCGGCT GGACCAAAAATGAAGTTGAAAA CTTACTCTATGCGGGCTTCCTC GGCGTCTTCCTCGGGGGACGTA TTGGTTATGTTCTGTTCTACAA TTTCCCGCAGTTTATGGCCGAT CCGCTGTATCTGTTCCGTGTCT GGGACGGCGGCATGTCTTTCCA CGGCGGCCCTGATTGGCGTTATC GTGGTGATGATTATCTTCGCCC GCCGTACTAAACGTTCCCTTCTT CCAGGTCTCTGATTTTATCGCA CCACTCATTCCGTTTGGTCTTG GTGCCGGGCGTCTGGGCAACTT TATTAACGGTGAATTGTGGGGC CGCGTTGACCGAACTTCCCGT TTGCCATGCTGTTCCCTGGCTC CCGTACAGAAGATATTTTGCTG CTGCAAACCAACCCGAGTGGC AATCCATTTTCGACACTTACGG TGTGCTGCCGCGCCACCCATCA CAGCTTTACGAGCTGCTGCTGG AAGGTGTGGTGCTGTTTATTAT CCTCAACCTGTATATTTCGTAAA CCACGCCCAATGGGAGCTGTCT CAGGTTGTTCCTGATTGGTTA CGGCGCGTTTCGCATCATTGTT	22

	GAGTTTTTCCGCCAGCCCGACG CGCAGTTTACCGGTGCCTGGGT GCAGTACATCAGCATGGGGCAA ATTCTTTCCATCCCGATGATTG TCGCGGGTGTGATCATGATGGT CTGGGCATATCGTCGCAGCCCA CAGCAACACGTTTCCTGA	
<i>murA</i> with double amber mutation (<i>murA</i> <i>am-am</i> allele of <i>murA</i> gene) sequence	ATGGATAAATTTCTGTTCAGG GGCCAACGAAGCTCCAGGGCGA AGTCACAATTTCCGGCGCTAAA AATTAGTAGCTGCCTATCCTTT TTGCCGCACTACTGGCGGAAGA ACCGGTAGAGATCCAGAACGTC CCGAAACTGAAAGACGTCGATA CATCAATGAAGCTGCTAAGCCA GCTGGGTGCGAAAGTAGAACGT AATGGTTCTGTGCATATTGATG CCCGCGACGTTAATGTATTCTG CGCACCTTACGATCTGGTAAAA ACCATGCGTGCTTCTATCTGGG CGCTGGGGCCGCTGGTAGCGCG CTTTGGTCAGGGGCAAGTTTCA CTACCTGGCGGTTGTACGATCG GTGCGCGTCCGGTTGATCTACA CATTTCTGGCCTCGAACAATTA GGCGCGACCATCAAACGGAAG AAGGTTACGTTAAAGCTTCCGT CGATGGTCGTTTGAAAGGTGCA CATATCGTGATGGATAAAGTCA GCGTTGGCGCAACGGTGACCAT CATGTGTGCTGCAACCCTGGCG GAAGGCACCACGATTATTGAAA ACGCAGCGCGTGAACCGGAAAT CGTCGATACCGCGAACTTCCTG ATTACGCTGGGTGCGAAAATTA GCGGTCAGGGCACCGATCGTAT CGTCATCGAAGGTGTGGAACGT TTAGGCGGCGGTGTCTATCGCG TTCTGCCGGATCGTATCGAAAC CGGTACTTTCTGGTGGCGGGCG GCGATTTCTCGCGCAAAATTA TCTGCCGTAACGCGCAGCCAGA TACTCTCGACGCCGTGCTGGCG AAACTGCGTGACGCTGGAGCGG ACATCGAAGTCGGCGAAGACTG GATTAGCCTGGATATGCATGGC AAACGTCCGAAGGCTGTTAACG TACGTACCGCGCCGCATCCGGC ATTCCCGACCGATATGCAGGCC CAGTTCACGCTGTTGAACCTGG TGGCAGAAGGGACCGGGTTAT CACCGAAACGGTCTTTGAAAAC CGCTTTATGCATGTGCCAGAGC TGAGCCGTATGGGCGCGCACGC CGAAATCGAAAGCAATACCGTT ATTTGTCACGGTGTTGAAAAAC TTTCTGGCGCACAGGTTATGGC AACCGATCTGCGTGATCAGCA AGCCTGGTGCTGGCTGGCTGTA TTGCGGAAGGGACGACGGTGGT TGATCGTATTTATCACATCGAT CGTGGCTACGAACGCATTGAAG	23

	ACAAACTGCGCGCTTTAGGTGC AAATATTGAGCGTGTGAAAGGC GAATAA	
dapA sequence	GCCAGGCGACTGTCTTCAATAT TACAGCCGCAACTACTGACATG ACGGGTGATGGTGTTCACAATT CCAGGGCGATCGGCACCCAACG CAGTGATCACCAGATAATGTTG CGATGACAGTGTCAAAGTGGTT ATTCCTTTAAAGGGGTGAGTTGT TCTTAAGGAAAGCATAAAAAAA ACATGCATACAACAATCAGAAC GGTTCTGTCTGCTTGCTTTTAA TGCCATACCAAACGTACCATTG AGACACTTGTGTTGCACAGAGGA TGGCCCATGTTACGGGAAGTA TTGTGCGGATTGTTACTCCGAT GGATGAAAAAGGTAATGTCTGT CGGGCTAGCTTGAAAAAACTGA TTGATTATCATGTCGCCAGCGG TACTTCGGCGATCGTTTCTGTT GGCACCCTGGCGAGTCCGCTA CCTTAAATCATGACGAACATGC TGATGTGGTGATGATGACGCTG GATCTGGCTGATGGGCGCATT CGGTAATTGCCGGGACCGGCGC TAACGCTACTGCGGAAGCCATT AGCCTGACGCAGCGCTTCAATG ACAGTGGTATCGTCGGCTGCCT GACGGTAACCCCTTACTACAAT CGTCCGTGCAAGAAGGTTTGT ATCAGCATTTCAAAGCCATCGC TGAGCATACTGACCTGCCGCAA ATTCTGTATAATGTGCCGTCCC GTACTGGCTGCGATCTGCTCCC GGAAACGGTGGGCCGTCTGGCG AAAGTAAAAAATATTATCGGAA TCAAAGAGGCAACAGGGAAGTT AACGCGTGTAACCAAGATCAAA GAGCTGGTTTCAGATGATTTTG TTCTGCTGAGCGCGCATGATGC GAGCGCGCTGGACTTCATGCAA TTGGGCGGTGATGGGGTTATTT CCGTTACGGCTAACGTGCGAGC GCGTGATATGGCCAGATGTGC AACTGGCAGCAGAAGGGCATT TTGCCGAGGCACGCGTTATTAA TCAGCGTCTGATGCCATTACAC AACAACTATTTGTGGAACCCA ATCCAATCCCGGTGAAATGGGC ATGTAAGGAACTGGGTCTTGTG GCGACCGATACGCTGCGCCTGC CAATGACACCAATCACCGACAG TGGTCGTGAGACGGTCAGAGCG GCGCTTAAGCATGCCGGTTTGC TGTAAGTTTAGGGAGATTGTA TGGCTTACTCTGTTCAAAAGTC GCGCCTGGCAAAGGTTGCGGGT GTTTCGCTTGTTTTATTACTCG CTGCCTGTAGTTCTGACTCAGC CTATAAGCGTCAGGTCAGTGGT GATGAAGCCTACCTGGAAGCG	24

Optionally, the amber suppressor is associated with a promoter or a terminator. Optionally, the promoter is a lipoprotein promoter. Preferable, promoter sequences are recited in Table 4.

Table 4

Amber Suppressor Promoter Sequence	Sequence	SEQ ID NO:
lipoprotein promoter	CATGGCGCCGCTTCTTTGAGCG AACGATCAAAAAATAAGTGGCGC CCCATCAAAAAATATTCTCAA CATAAAAACTTTGTGTAATAC TTGTAACGCTG	25
lipoprotein promoter	CATGGCGCCCCATCAAAAAAAT ATTCTCAACATAAAAAACTTTG TGTAATACTTGTACGCTG	26

Optionally, the terminator is an *rrnC* terminator. Preferable, terminator sequences are recited in Table 5.

Table 5

Amber Suppressor Terminator Sequence	Sequence	SEQ ID NO:
<i>rrnC</i> terminator	GATCCTTAGCGAAAGCTAAGGA TTTTTTTTTAC	27
<i>rrnC</i> terminator	GATCCTTAGCGAAAGCTAAGGA TTTTTTTTTTT	28

Bacterial and BTP delivery is more attractive than viral delivery because they are 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 and BTPs that cause RNAi in a tissue specific manner.

Liberation of the siRNA encoding plasmid or the one or more siRNAs from the intracellular bacteria or BTPs occurs through active mechanisms. One mechanism involves the type III export system in *S. typhimurium*, a specialized multiprotein complex spanning the bacterial or BTP 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 or BTP contents into the cytoplasm. The lysis of intracellular bacteria or BTPs is triggered through various mechanisms, including addition of an intracellularly active antibiotic (tetracycline), naturally through bacterial metabolic attenuation (auxotrophy), or through a lysis regulation system or bacterial suicide system comprising a bacterial regulator, promoter and sensor that is sensitive to the environment, *e.g.*, the pH, magnesium concentration, phosphate concentration, ferric ion concentration, osmolarity, anaerobic conditions, nutritional deficiency and general stress of the target cell or the host phagosome. When the bacteria or BTP lysis regulation system senses one or more of the above environmental conditions, bacterial or BTP lysis is triggered by one or more mechanisms

including but not limited to antimicrobial proteins, bacteriophage lysins and autolysins expressed by the bacteria or BTP, either naturally or through modification, or through pore-forming proteins expressed by the bacteria or BTPs, either naturally or through modification, *e.g.*, genetic modification, which break the phagosomes containing the bacteria or BTPs and liberate the siRNA-encoding plasmid or the one or more siRNAs.

5

The regulator of the lysis regulation system may be selected from the group that includes but is not limited to OmpR, ArcA, PhoP, PhoB, Fur, RstA, EvgA and RpoS. Preferable, lysis regulator sequences are recited in Table 6.

Table 6

Lysis Regulation System Regulator Sequence	Sequence	SEQ ID NO:
OmpR regulator	ATGCAAGAGAACTACAAGATTC TGGTGGTCGATGACGACATGCG CCTGCGTGCGCTGCTGGAACGT TATCTCACCGAACAAGGCTTCC AGGTTCGAAGCGTCGCTAATGC AGAACAGATGGATCGCCTGCTG ACTCGTGAATCTTCCATCTTA TGGTACTGGATTAAATGTTACC TGGTGAAGATGGCTTGTCGATT TGCCGACGTCTTCGTAGTCAGA GCAACCCGATGCCGATCATTAT GGTGACGGCGAAAGGGGAAGAA GTGGACCGTATCGTAGGCCTGG AGATTGGCGCTGACGACTACAT TCCAAAACCGTTAAACCCGCGT GAACTGCTGGCCCGTATCCCGTG CGGTGCTGCGTCGTCAGGCGAA CGAACTGCCAGGCGCACCGTCA CAGGAAGAGGCGGTAATTGCTT TCGGTAAGTTCAAACCTAACCT CGGTACGCGCGAAATGTTCCGC GAAGACGAGCCGATGCCGCTCA CCAGCGGTGAGTTTGCGGTACT GAAGGCACTGGTCAGCCATCCG CGTGAGCCGCTCTCCCGCGATA AGCTGATGAACCTTGCCCGTGG TCGTGAATATTCCGCAATGGAA CGCTCCATCGACGTGCAGATTT CGCGTCTGCGCCGATGGTGGA AGAAGATCCAGCGCATCCGCGT TACATTCAGACCGTCTGGGGTC TGGGCTACGTCTTTGTACCGGA CGGCTCTAAAGCATGA	29
PhoP regulator	ATGCGCGTACTGGTTGTTGAAG ACAATGCGTTGTTACGTACCA CCTTAAAGTTCAGATTCAAGAT GCTGGTCATCAGGTGATGACG CAGAAGATGCCAAAGAAGCCGA TTATTATCTCAATGAACATATA CCGGATATTGCGATTGTCGATC TCGGATTGCCAGACGAGGACGG TCTGTCACTGATTGCGCCGTGG CGTAGCAACGATGTTTCACTGC CGATTCTGGTATTAACCGCCCG TGAAAGCTGGCAGGACAAAGTC	30

	GAAGTATTAAGTGCCGGTGCTG ATGATTATGTGACTAAACCGTT TCATATTGAAGAGGTGATGGCG CGAATGCAGGCATTAATGCGGC GTAATAGCGGTCTGGCTTCACA GGTCATTTTCGCTCCCCCGTTT CAGGTTGATCTCTCTCGCCGTG AATTATCTATTAATGACGAAGT GATCAAACGACCGCGTTCGAA TACACTATTATGGAACGTTGA TACGCAATAATGGCAAAGTGGT CAGCAAAGATTCGTTAATGCTC CAACTCTATCCGGATGCGGAGC TGCGGGAAAGCCATACCATTGA TGTAATGATGGGACGTCTGCGC AAAAAAATTCAGGCACAATATC CCCAAGAAAGTATTACCACCGT TCGCGGCCAGGGCTATCTGTTC GAATTGCGCTGA	
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The promoter of the lysis regulation system may be selected from the group that includes but is not limited to ompF, ompC, fadB, phoPQ, mgtA, mgrB, psiB, phnD, Ptp, sodA, sodB, sltA, sltB, asr, csgD, emrKY, yhiUV, acrAB, mdx and tolC. Preferable, lysis regulation system promoter

5 sequences are recited in Table 7.

Table 7

Lysis Regulation System Promoter Sequence	Sequence	SEQ ID NO:
ompF promoter	GATCATCCTGTTACGGAATATT ACATTGCAACATTTACGCGCAA AACTAATCCGCATTCTTATTG CGGATTAGTTTTTTCTTAGCTA ATAGCACAATTTTCATACTATT TTTTGGCATTCTGGATGTCTGA AAGAAGATTTTGTGCCAGGTCG ATAAAGTTTCCATCAGAAACAA AATTTCCGTTTAGTTAATTTAA ATATAAGGAAATCATATAAATA GATTAAAATTGCTGTAAATATC ATCACGTCTCTATGGAAATATG ACGGTGTTACAAAAGTTCCTTA AATTTTACTTTTGGTTACATAT TTTTTCTTTTGAACCAAATC TTTATCTTTGTAGCACTTTCAC GGTAGCGAAACGTTAGTTTGAA TGGAAAGATGCCTGCA	31
ompC promoter	TTTAAAAAAGTTCCGTAAAATT CATATTTGAAACATCTATGTA GATAACTGTAACATCTTAAAG TTTAGTATCATATTCGTGTTG GATTATTCTGTATTTTGCAGGA GAATGGACTTGCCGACTGGTTA ATGAGGGTTAACCAGTAAGCAG TGGCATAAAAAAGCAATAAAGG CATATAACAGAGGGTTAATAAC	32
fadB promoter	AGTGATTCCATTTTTTACCCTT CTGTTTTTTTGACCTTAAGTCT CCGCATCTTAGCACATCGTTCA TCCAGAGCGTGATTTCTGCCGA	33

	CGGTGATCAGATCGGCATTTCT TTAATCTTTTGTTCATATTT TTAACACAAAATACACACTTCG ACTCATCTGGTACGACCAGATC ACCTTGCGGATTCAGGAGACTG AC	
phoPQ promoter	GAGCTATCACGATGGTTGATGA GCTGAAATAAACCTCGTATCAG TGCCGGATGGCGATGCTGTCCG GCCTGCTTATTAAGATTATCCG CTTTTTATTTTTCACTTTACC TCCCCTCCCCGCTGGTTATTT AATGTTTACCCCCATAACCACA TAATCGCGTTACACTATTTTAA TAATTAAGACAGGGAGAAATAA AA	34
mgtA promoter	GCTTCAACACGCTCGCGGGTGA GCTGGCTCACGCCGCTTTCGTT ATTCAGCACCCGGGAACTGTA GATTTCCCCACGCCGCTTAAGC GCGCGATATCTTGATGGTCAG CCGATTTTGCATCCTGTTGTCC TGTAACGTGTTGTTTAATTATT TGAGCCTAACGTTACCCGTGCA TTCAGCAATGGGTAAAGTCTGG TTTATCGTTGGTTTAGTTGTCA GCAGGTATTATATCGCCA	35
Ptp promoter	GAGCTGTTGACAATTAATCATC GAACTAGTTAACTAGTACGCAA GTTACGTA AAAAGGGTATCTA GAATTCT	36

The sensor of the lysis regulation system may be selected from the group that includes but is not limited to EnvZ, ArcB, PhoQ, PhoR, RstB and EvgS. Preferable, lysis regulation system sensor sequences are recited in Table 8.

5

Table 8

Lysis Regulation System Sensor Sequence	Sequence	SEQ ID NO:
EnvZ sensor	ATGAGGCGATTGCGCTTCTCGC CACGAAGTTCATTTGCCGTAC GTTATTGCTCATCGTCACCTTG CTGTTGCCAGCCTGGTGACGA CTTATCTGGTGGTGCTGAACTT CGCGATTTTGCCGAGCCTCCAG CAGTTTAATAAAGTCCTCGCGT ACGAAGTGCGTATGTTGATGAC CGACAACTGCAACTGGAGGAC GGCACGCAGTTGGTTGTGCCTC CCGCTTCCGTCGGGAGATCTA CCGTGAGCTGGGGATCTCTCTC TACTCCAACGAGGCTGCCGAAG AGGCAGGTCTGCGTTGGGCGCA ACACTATGAATTCTTAAGCCAT CAGATGGCGCAGCAACTGGGCG GCCCCACGGAAGTGCGCGTTGA GGTCAACAAAAGTTGCGCTGTC GTCTGGCTGAAAACCTGGCTGT CGCCCAATATCTGGGTACGCGT	37

	GCCGCTGACCGAAATTCATCAG GGCGATTTCTCTCCGCTGTTCC GCTATACGCTGGCGATTATGCT ATTGGCGATAGGCGGGCGTGG CTGTTTATTCGTATCCAGAACC GACCGTTGGTCGATCTCGAACA CGCAGCCTTGCAGGTTGGTAAA GGGATTATTCCGCCGCCGCTGC GTGAGTATGGCGCTTCGGAGGT GCGTTCCGTTACCCGTGCCTTT AACCATATGGCGGCTGGTGTTA AGCAACTGGCGGATGACCGCAC GCTGCTGATGGCGGGGGTAAGT CACGACTTGCACGCCGCTGA CGCGTATTCGCCTGGCGACTGA GATGATGAGCGAGCAGGATGGC TATCTGGCAGAATCGATCAATA AAGATATCGAAGAGTGCAACGC CATCATTTAGCAGTTTATCGAC TACCTGCGCACCGGGCAGGAGA TGCCGATGGAAATGGCGGATCT TAATGCAGTACTCGGTGAGGTG ATTGCTGCCGAAAGTGGCTATG AGCGGGAAATTGAAACCGCGCT TTACCCCGGCAGCATTGAAGTG AAAATGCACCCGCTGTCGATCA AACGCGCGGTGGCGAATATGGT GGTCAACGCCGCCCGTTATGGC AATGGCTGGATCAAAGTCAGCA GCGGAACGGAGCCGAATCGCGC CTGGTTCCAGGTGGAAGATGAC GGTCCGGGAATTGCGCCGGAAC AACGTAAGCACCTGTTCCAGCC GTTTGTCCGCGGCGACAGTGCG CGCACCATTAGCGGCACGGGAT TAGGGCTGGCAATTGTGCAGCG TATCGTGGATAACCATAACGGG ATGCTGGAGCTTGGCACCAGCG AGCGGGGCGGGCTTTCATTTCG CGCCTGGCTGCCAGTGCCGGTA ACGCGGGCGCAGGGCACGACAA AAGAAGGGTAA	
PhoQ sensor	ATGAAAAAATTACTGCGTCTTT TTTTCCCGCTCTCGCTGCGGGT ACGTTTTCTGTTGGCAACGGCA GCGGTAGTACTGGTGCTTTCGC TTGCCTACGGAATGGTCGCGCT GATCGGTTATAGCGTCAGTTTC GATAAACTACGTTTCGGCTGT TACGTGGCGAGAGCAATCTGTT CTATACCCCTGCGAAGTGGGAA AACATAAGTTGCATGTCGAGT TACCCGAAAATATCGACAAGCA AAGCCCCACCATGACGCTAATT TATGATGAGAACGGGCAGCTTT TATGGGCGCAACGTGACGTGCC CTGGCTGATGAAGATGATCCAG CCTGACTGGCTGAAATCGAATG GTTTTTCATGAAATTGAAGCGGA TGTTAACGATACCAGCCTCTTG CTGAGTGGAGATCATTGATAC AGCAACAGTTGCAGGAAGTGCG	38

	GGAAGATGATGACGACGCGGAG ATGACCCACTCGGTGGCAGTAA ACGTCTACCCGGCAACATCGCG GATGCCAAAATTAACCATTGTG GTGGTGGATACCATTCCGGTGG AGCTAAAAAGTTCCTATATGGT CTGGAGCTGGTTTATCTATGTG CTCTCAGCCAATCTGCTGTTAG TGATCCCGCTGCTGTGGGTCGC CGCCTGGTGGAGTTTACGCCCC ATCGAAGCCCTGGCAAAGAAG TCCGCGAACTGGAAGAACATAA CCGCGAATTGCTCAATCCAGCC ACAACGCGAGAACTGACCAGTC TGGTACGAAACCTGAACCGATT GTTAAAAAGTGAACGCGAACGT TACGACAAATACCGTACGACGC TCACCGACCTGACCCATAGTCT GAAAACGCCACTGGCGGTGCTG CAAAGTACGCTGCGTTCTCTGC GTAGTGAAAAGATGAGCGTCAG TGATGCTGAGCCGGTAATGCTG GAGCAAATCAGCCGCATTTTAC AGCAAATTGGCTACTACCTGCA TCGTGCCAGTATGCGCGGCGGG ACATTGCTCAGCCGCGAGCTGC ATCCGGTCGCCCCACTGCTGGA CAATCTCACCTCAGCGCTGAAC AAAGTGTATCAACGCAAAGGGG TCAATATCTCTCTCGATATTTT GCCAGAGATCAGCTTTGTGCGGT GAGCAGAACGATTTTGTGCGAGG TGATGGGCAACGTGCTGGATAA TGCCTGTAAATATTGCCTCGAG TTTGTGCGAAATTTCTGCAAGGC AAACCGACGAGCATCTCTATAT TGTGGTCGAGGATGATGGCCCC GGTATTCCATTAAGCAAGCGAG AGGTCATTTTCGACCGTGGTCA ACGGGTTGATACTTTACGCCCT GGGCAAGGTGTAGGGCTGGCGG TAGCCCGCGAAATCACCAGCA ATATGAGGGTAAAATCGTCGCC GGAGAGAGCATGCTGGGCGGTG CGCGGATGGAGGTGATTTTTGG TCGCCAGCATTTCTGCGCCGAAA GATGAATAA	
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The lysis regulation system may comprise any combination of one or more of the above regulators, promoters and sensors.

In one example of this embodiment, the lysis regulation system comprises OmpR as the regulator, ompF as the promoter and EnvZ as the sensor and the stimulus is reduced osmolarity. In
5 another example of this embodiment, the lysis regulation system comprises OmpR as the regulator, ompC as the promoter and EnvZ as the sensor and the stimulus is reduced osmolarity.

In another example of this embodiment, the lysis regulation system comprises the ArcA as the regulator, fad as the promoter and Arc B as the sensor and the stimulus is anaerobic conditions.

In another example of this embodiment, the lysis regulation system comprises PhoP as the regulator, phoPQ as the promoter and PhoQ as the sensor and the stimulus is reduced magnesium concentration. In another example of this embodiment, the lysis regulation system comprises PhoP as the regulator, mgtA as the promoter and PhoQ as the sensor and the stimulus is reduced magnesium concentration. In another example of this embodiment, the lysis regulation system comprises PhoP as the regulator, mgrB as the promoter and PhoQ as the sensor and the stimulus is reduced magnesium concentration.

In another example of this embodiment, the lysis regulation system comprises PhoB as the regulator, psiB as the promoter and PhoR as the sensor and the stimulus is reduced phosphate concentration. In another example of this embodiment, the lysis regulation system comprises PhoB as the regulator, phnD as the promoter and PhoR as the sensor and the stimulus is reduced phosphate concentration. In another example of this embodiment, the lysis regulation system comprises RstA as the regulator, asr as the promoter and RstB as the sensor. In another example of this embodiment, the lysis regulation system comprises RstA as the regulator, csgD as the promoter and RstB as the sensor.

In another example of this embodiment, the lysis regulation system comprises EvgA as the regulator, emrKY as the promoter and EvgS as the sensor. In another example of this embodiment, the lysis regulation system comprises EvgA as the regulator, yhiUV as the promoter and EvgS as the sensor. In another example of this embodiment, the lysis regulation system comprises EvgA as the regulator, acrAB as the promoter and EvgS as the sensor. In another example of this embodiment, the lysis regulation system comprises EvgA as the regulator, mdfA as the promoter and EvgS as the sensor. In another example of this embodiment, the lysis regulation system comprises EvgA as the regulator, tolC as the promoter and EvgS as the sensor.

In another example of this embodiment, the lysis regulation system comprises Fur as the regulator in combination with a promoter selected from the group comprising sodA, sodB, sltA or sltB.

The antimicrobial protein may be selected from the group that includes but is not limited to α - and β -defensins, protegrins, cathelicidins (e.g., indolicidin and bactenecins), granulysin, lysozyme, lactoferrin, azurocidin, elastase, bactericidal permeability inducing peptide (BPI), adrenomedullin, brevinin, histatins and hepcidin. Additional antimicrobial proteins are disclosed in the following, each of which is incorporated herein by reference in its entirety: Devine, D.A. et al., *Current Pharmaceutical Design*, 8, 703-714 (2002); Jack R.W., et al., *Microbiological Reviews*, 59 (2), 171-200 (June 1995).

Optionally, the antimicrobial protein is an α -defensin, β -defensin, or protegrin. Preferable, antimicrobial protein sequences are recited in Table 9.

Table 9

Antimicrobial Protein Sequence	Sequence	SEQ ID NO:
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α -defensin-1 protein	CTATAGAAGACCTGGGACAGAG GACTGCTGTCTGCCCTCTCTGG TCACCCTGCCTAGCTAGAGGAT CTGTGACCCCAGCCATGAGGAC CCTCGCCATCCTTGCTGCCATT CTCCTGGTGGCCCTGCAGGCCC AGGCTGAGCCACTCCAGGCAAG AGCTGATGAGGTGCTGCAGCC CCGGAGCAGATTGCAGCGGACA TCCCAGAAGTGGTTGTTTCCCT TGCATGGGACGAAAGCTTGGCT CCAAAGCATCCAGGCTCAAGGA AAAACATGGCCTGCTATTGCAG AATACCAGCGTGCATTGCAGGA GAACGTCGCTATGGAACCTGCA TCTACCAGGGAAGACTCTGGGC ATTCTGCTGCTGAGCTTGAGA AAAAGAAAAATGAGCTCAAAAT TTGCTTTGAGAGCTACAGGGAA TTGCTATTACTCCTGTACCTTC TGCTCAATTTCTTTCCTCATC CCAAATAAATGCCTTGGTACAA GAAAAG	39
α -defensin-3 protein	CCTTGCTATAGAAGACCTGGGA CAGAGGACTGCTGTCTGCCCTC TCTGGTCACCCTGCCTAGCTAG AGGATCTGTGACCCCAGCCATG AGGACCCTCGCCATCCTTGCTG CCATTCTCCTGGTGGCCCTGCA GGCCCAGGCTGAGCCACTCCAG GCAAGAGCTGATGAGGTTGCTG CAGCCCCGGAGCAGATTGCAGC GGACATCCCAGAAGTGGTTGTT TCCCTTGCTATGGGACGAAAGCT TGGCTCCAAAGCATCCAGGCTC AAGGAAAAACATGGACTGCTAT TGCAGAATACCAGCGTGCATTG CAGGAGAACGTCGCTATGGAAC CTGCATCTACCAGGGAAGACTC TGGGCATTCTGCTGCTGAGCTT GCAGAAAAAGAAAAATGAGCTC AAAATTTGCTTTGAGAGCTACA GGGAATTGCTATTACTCCTGTA CCTTCTGCTCAATTTCTTTCC TCATCTCAAATAAATGCCTTGT TAC	40
α -defensin-4 protein	GTCTGCCCTCTCTGCTCGCCCT GCCTAGCTTGAGGATCTGTCAC CCCAGCCATGAGGATTATCGCC CTCCTCGCTGCTATTCTCTTGG TAGCCCTCCAGGTCCGGGCAGG CCCACTCCAGGCAAGAGGTGAT GAGGCTCCAGGCCAGGAGCAGC GTGGGCCAGAAGACCAGGACAT ATCTATTTCTTTGCATGGGAT AAAAGCTCTGCTCTTCAGGTTT CAGGCTCAACAAGGGGCATGGT CTGCTCTTGCAATTAGTATTC TGCCGGCGAACAGAACTTCGTG TTGGGAACTGCCTCATTGGTGG TGTGAGTTTCACATACTGCTGC ACGCGTGTGATTACGTTCTG	41

	CTGTCCAAGAGAATGTCATGCT GGGAACGCCATCATCGGTGGTG TTAGCTTCACATGCTTCTGCAG CTGAGCTTGCAGAATAGAGAAA AATGAGCTCATAATTTGCTTTG AGAGCTACAGGAAATGGTTGTT TCTCCTATACTTTGTCTTAAC ATCTTTCTTGATCCTAAATATA TATCTCGTAACAAG	
α -defensin-5 protein	ATATCCACTCCTGCTCTCCCTC CTGCAGGTGACCCAGCCATGA GGACCATCGCCATCCTTGCTGC CATTCTCCTGGTGGCCCTGCAG GCCCAGGCTGAGTCACTCCAGG AAAGAGCTGATGAGGCTACAAC CCAGAAGCAGTCTGGGAAGAC AACCAGGACCTTGCTATCTCCT TTGCAGGAAATGGACTCTCTGC TCTTAGAACCTCAGGTTCTCAG GCAAGAGCCACCTGCTATTGCC GAACCGGCCGTTGTGCTACCCG TGAGTCCCTCTCCGGGGTGTGT GAAATCAGTGGCCGCCTCTACA GACTCTGCTGTCGCTGAGCTTC CTAGATAGAAACCAAAGCAGTG CAAGATTCAGTTCAAGGTCCTG AAAAAAGAAAAACATTTTACTC TGTGTACCTTGTGTCTTTCTAA ATTTCTCTCTCCAAAATAAAGT TCAAGCATT	42
α -defensin-6 protein	ACACATCTGCTCCTGCTCTCTC TCCTCCAGCGACCCTAGCCATG AGAACCCTCACCATCCTCACTG CTGTTCTCCTCGTGGCCCTCCA GGCCAAGGCTGAGCCACTCCAA GCTGAGGATGATCCACTGCAGG CAAAAGCTTATGAGGCTGATGC CCAGGAGCAGCGTGGGGCAAAT GACCAGGACTTTGCCGTCTCCT TTGCAGAGGATGCAAGCTCAAG TCTTAGAGCTTTGGGCTCAACA AGGGCTTTCACTTGCCATTGCA GAAGGTCCTGTTATTCAACAGA ATATTCCCTATGGGACCTGCACT GTCATGGGTATTAACCACAGAT TCTGCTGCCTCTGAGGGATGAG AACAGAGAGAAATATATTCTATA ATTTACTTTATGACCTAGAAGG AAACTGTCGTGTGTCCCATACA TTGCCATCAACTTTGTTTCCTC ATCTCAAATAAAGTCCTTTCAG CAAAAAAAAAAAAA	43
β -defensin-1 protein	TCCCTTCAGTTCGGTCGACGAG GTTGTGCAATCCACCAAGTCTTA TAAATACAGTGACGCTCCAGCC TCTGGAAGCCTCTGTCAGCTCA GCCTCCAAAGGAGCCAGCGTCT CCCCAGTTCCTGAAATCCTGGG TGTTGCCTGCCAGTCGCCATGA GAACTTCCTACCTTCTGCTGTT TACTCTCTGCTTACTTTTGTCT GAGATGGCCTCAGGTGGTAACT	44

	TTCTCACAGGCCTTGGCCACAG ATCTGATCATTACAATTGCGTC AGCAGTGGAGGGCAATGTCTCT ATTCTGCCTGCCCCGATCTTTAC CAAAATTCAAGGCACCTGTTAC AGAGGGAAGGCCAAGTGCTGCA AGTGAGCTGGGAGTGACCAGAA GAAATGACGCAGAAGTGAAATG AACTTTTTATAAGCATTCTTTT AATAAAGGAAAATTGCTTTTGA AGTATACCTCCTTTGGGCCAAA AAAAAAAAAAAAAAAAAAAAA	
β -defensin-3 protein	TGAGTCTCAGCGTGCGGTGAAG CCTAGCAGCTATGAGGATCCAT TATCTTCTGTTTGCTTGTCTCT TCCTGTTTTTTGGTGCCTGTCCC AGGTCATGGAGGAATCATAAAC ACATTACAGAAATATTATTGCA GAGTCAGAGGCGGCCGGTGTGC TGTGCTCAGCTGCCTTCCAAAG GAGGAACAGATCGGCAAGTGCT CGACGCGTGGCCGAAAATGCTG CCGAAGAAAGAAATAAAACCC TGAAACATGACGAGAGTGTGT AAAGTGTGGAATGCCTTCTTA AAGTTTATAAAAGTAAATCAA ATTACATTTTTTTTCAAAAAA AAAAAA	45
β -defensin-4 protein	AGACTCAGCTCCTGGTGAAGCT CCCAGCCATCAGCCATGAGGGT CTTGTATCTCCTCTTCTCGTTC CTCTTCATATTCCTGATGCCTC TTCCAGGTGTTTTTGGTGGTAT AGGCGATCCTGTTACCTGCCTT AAGAGTGGAGCCATATGTCATC CAGTCTTTTGGCCCTAGAAGGTA TAAACAAATTGGCACCTGTGGT CTCCCTGGAACAAAATGCTGCA AAAAGCCATGAGGAGGCCAAGA AGCTGCTGTGGCTGATGCGGAT TCAGAAAGGGCTCCCTCATCAG AGACGTGCGACATGTAAACCAA ATTAACTATGGTGTCCAAAGA TACGCA	46
protegrin-1 protein	ATGGAGACCCAGAGAGCCAGCC TGTGCCTGGGCGCTGGTCACT GTGGCTTCTGCTGCTGGCACTC GTGGTGCCCTCGGCCAGCGCCC AGGCCCTCAGCTACAGGGAGGC CGTGCTTCGTGCTGTGGATCGC CTCAACGAGCAGTCTCGGAAG CTAATCTCTACCGCCTCCTGGA GCTGGACCAGCCGCCCAAGGCC GACGAGGACCCGGGCACCCGA AACCTGTGAGCTTCACGGTGAA GGAGACTGTGTGTCCAGGCCG ACCCGGCAGCCCCGGAGCTGT GTGACTTCAAGGAGAACGGGCG GGTGAAACAGTGTGTGGGGACA GTCACCTGGATCAGATCAAGG ACCCGCTCGACATCACCTGCAA TGAGGTTCAAGGTGTCAAGGGA	47

	GGTCGCCTGTGCTATTGTAGGC GTAGGTTCTGCGTCTGTGTCGG ACGAGGATGACGGTTGCGACGG CAGGCTTTCCCTCCCCCAATTT TCCCGGGGCCAGGTTTCCGTCC CCCAATTTTTCGCTCCACCT TTCCGGCCCGCACCATTGCGTC CACCAAGGTTCCCTGGTAGACG GTGAAGGATTGTCAGGCAACTC ACCCAGAAGGCCTTTCGGTACA TTAAAATCCCAGCAAGGAGACC TAAGCATCTGCTTTGCCAGGC CCGCATCTGTCAAATAAATTCT TGTGAAACC	
protegrin-3 protein	ATGGAGACCCAGAGAGCCAGCC TGTGCCTGGGGCGCTGGTCACT GTGGCTTCTGCTGCTGGCACTC GTGGTGCCCTCGGCCAGCGCCC AGGCCCTCAGCTACAGGGAGGC CGTGCTTCGTGCTGTGGATCGC CTCAACGAGCAGTCTCGGAAG CTAATCTCTACCGCTCCTGGA GCTGGACCAGCCGCCCAAGGCC GACGAGGACCCGGGCACCCGA AACCTGTGAGCTTACGGTGAA GGAGACTGTGTGTCCAGGCCG ACCCGGCAGCCCCGGAGCTGT GTGACTTCAAGGAGAACGGGCG GGTGAACAGTGTGTGGGGACA GTCACCCTGGATCAGATCAAGG ACCCGCTCGACATCACCTGCAA TGAGGTTCAAGGTGTGAGGGGA GGTGGCCTGTGCTATTGTAGGC GTAGGTTCTGCGTCTGTGTCGG ACGAGGATGACGGTTGCGACGG CAGGCTTTCCCTCCCCCAATTT TCCCGGGGCCAGGTTTCCGTCC CCCAATTTTTCGCTCCACCT TTCCGGCCCGCACCATTGCGTC CACCAAGGTTCCCTGGTAGACG GTGAAGGATTGTCAGGCAACTC ACCCAGAAGGCCTTTCGGTACA TTAAAATCCCAGCAAGGAGACC TAAGCATCTGCTTTGCCAGGC CCGCATCTGTCAAATAAATTCT TGTGAAACC	48
protegrin-4 protein	ATGGAGACCCAGAGAGCCAGCC TGTGCCTGGGGCGCTGGTCACT GTGGCTTCTGCTGCTGGCACTC GTGGTGCCCTCGGCCAGCGCCC AGGCCCTCAGCTACAGGGAGGC CGTGCTTCGTGCTGTGGATCGC CTCAACGAGCAGTCTCGGAAG CTAATCTCTACCGCTCCTGGA GCTGGACCAGCCGCCCAAGGCC GACGAGGACCCGGGCACCCGA AACCTGTGAGCTTACGGTGAA GGAGACTGTGTGTCCAGGCCG ACCCGGCAGCCCCGGAGCTGT GTGACTTCAAGGAGAACGGGCG GGTGAACAGTGTGTGGGGACA GTCACCCTGGATCAGATCAAGG	49

	ACCCGCTCGACATCACCTGCAA TGAGGTTCAAGGTGTCAGGGGA GGTCGCCTGTGCTATTGTAGGG GTTGGATCTGCTTCTGTGTCGG ACGAGGATGACGGTTGCGACGG CAGGCTTTCCCTCCCCCAATTT TCCCGGGGCCAGGTTCCGTCC CCCAATTTTCCGCCTCCACCT TTCCGGCCCGCACCATTGCGTC CACCAAGGTTCCCTGGTAGACG GTGAAGGATTGTCAGGCAACTC ACCCAGAAGGCCTTTCGGCACA TTAAAATCCCAGCAAGGAGACC TAAGCATCTGCTTTGCCCAGGC CCGCATCTGTCAAATAAATTCT TGTGAAACC	
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The bacteriophage lysin may be selected from the group that includes but is not limited to holins and endolysins or lysins (e.g., lysozyme, amidase and transglycosylase). Additional lysins are disclosed in the following, each of which is incorporated herein by reference in its entirety: Kloos D.-
5 U., et al., *Journal of Bacteriology*, 176 (23), 7352-7361 (December 1994); Jain V., et al., *Infection and Immunity*, 68 (2), 986-989 (February 2000); Srividhya K.V., et al., *J. Biosci.*, 32, 979-990 (2007); Young R.V., *Microbiological Reviews*, 56 (3), 430-481 (September 1992).

The autolysin may be selected from the group that includes but is not limited to peptidoglycan hydrolases, amidases (e.g., N-acetylmuramyl-L-alanine amidases), transglycosylases, endopeptidases
10 and glucosaminidases. Additional autolysins are disclosed in the following, each of which is incorporated herein by reference in its entirety: Heidrich C., et al., *Molecular Microbiology*, 41 (1), 167-178 (2001); Kitano K., et al., *Journal of Bacteriology*, 167 (3), 759-765 (September 1986); Lommatzsch J., et al., *Journal of Bacteriology*, 179 (17), 5465-5470 (September 1997); Oshida T., et al., *PNAS*, 92, 285-289 (January 1995); Lenz L.L., et al., *PNAS*, 100 (21), 12432-12437 (October 14,
15 2003); Ramadurai L., et al., *Journal of Bacteriology*, 179 (11), 3625-3631 (June 1997); Kraft A.R., et al., *Journal of Bacteriology*, 180 (12), 3441-3447 (July 1998); Dijkstra A.J., et al., *FEBS Letters*, 366, 115-118 (1995); Huard C., et al., *Microbiology*, 149, 695-705 (2003).

In one aspect of the invention, the control exerted by the lysis regulation system may further be enhanced by bacterial or BTP strain-specific regulation. In one aspect of this embodiment, the
20 strain-specific regulation is attenuation caused by deletion of a nutritional gene. The nutritional gene may be selected from the group that includes but is not limited to *dapA*, *aroA* and *guaBA*. In one example of this embodiment, *dapA* attenuation results in deficiency in the biosynthesis of lysine and peptidoglycan. In this particular embodiment, transcription of genes including but not limited to *lysC* may be activated by mechanisms such as transcriptional induction, antitermination and riboswitch. In
25 another example of this embodiment, *aroA* attenuation results in deficiency in aromatic amino acids and derepression of one or more genes including but not limited to *aroF*, *aroG* and *aroH* by regulators such as TrpR and TyrR. In another example of this embodiment, *guaBA* attenuation results in derepression of one or more genes that are repressed by PurR.

In addition to the lysis regulation system and strain-specific regulation, the bacteria or BTP may further contain an inducible system that includes but is not limited to a Tet-on expression system to facilitate bacterial or BTP lysis at a time desired by the clinician. Upon administration of tetracycline, which activates the Tet-on promoter, the bacteria or BTP express a protein that triggers lysis of the bacteria or BTP. In one example of this embodiment, the protein expressed under the Tet-on expression system is selected from the group that includes but is not limited to defensins and protegrins.

The present invention also provides a lysis regulation system in combination with strain-specific attenuation (e.g., nutritional attenuation). As shown in Figure 30, a global regulator can sense an extracellular condition and regulate transcription, starvation for specific nutrient such as an amino acid *in vivo*, in contrast to laboratory growth in the presence of excess of the nutrient and a positive or negative regulator in response to starvation. In the schematic shown in Figure 31 there can be three cassettes, any of which may be placed on either the bacterial chromosome or on a plasmid.

As described, the present invention provides a plasmid containing a lysis regulation system comprising OmpR as the regulator, ompF or ompC as the promoter and protegrin or β -defensin as the antimicrobial protein, in combination with a Tet-on expression system, which provides two levels of control of bacterial lysis. This embodiment is illustrated in Figure 32.

In another aspect of the invention, the DNA insert comprises one or more of the following constructs, each of which contains an HPV target sequence, a hairpin sequence and BamHI and SalI restriction sites to facilitate incorporation into the hairpin RNA expression cassette of the TRIP plasmid as shown in Table 10.

Table 10

HPV Target Sequence Construct				
BamHI	sense (19bp)	loop	antisense (21bp)	SalI
5'-GATCC	TAGGTATTTGAATTGTCAT	TTCAAGAGA	ATGCAAATTCAAATACCTTTT	G-3' (SEQ ID NO:50)
3'-G	ATCCATAAACTTAAACGTA	AAGTTCTCT	TACGTTTAAGTTTATGGAAAA	CAGCT-5' (SEQ ID NO:51)

4. Cell and Gene Targets

The present invention also provides methods of using the various bacterium, BTP and vectors provided in the invention. For example, the present invention provides methods of delivering one or more siRNAs to mammalian cells. The methods include introducing at least one invasive bacterium, or at least one bacterial therapeutic particle (BTP), containing one or more siRNAs or one or more DNA molecules encoding one or more siRNAs to the mammalian cells.

The present invention also provides methods of regulating gene expression in mammalian cells. The method includes introducing at least one invasive bacterium, or at least one bacterial therapeutic particle (BTP), containing one or more siRNAs or one or more DNA molecules encoding one or more siRNAs to the mammalian cells, where the expressed siRNAs interfere with at least one mRNA of a gene of interest thereby regulating gene expression.

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 that can be invaded by a bacterium, *i.e.*, a cell that has the necessary surface receptor for recognition by the bacterium.

Preferred target cells are eukaryotic cells. Even more preferred target cells are animal cells.

5 "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.

The recipient animal cells employed in the present invention are not critical thereto and
10 include cells present in or derived from all organisms within the kingdom *animalia*, such as those of the families *mammalia*, *pisces*, *avian*, *reptilia*.

Preferred animal cells are mammalian cells, such as humans, bovine, ovine, porcine, feline, canine, goat, equine, and primate cells. The most preferred mammalian cells are human cells. The cells can be *in vivo*, *in vitro* or *ex vivo*.

15 In some embodiments of the invention, the cell is a cervical epithelial cell, a rectal epithelial cell or a pharyngeal epithelial cell, macrophage, gastrointestinal epithelial cell, skin cell, melanocyte, keratinocyte, hair follicle, colon cancer cell, an ovarian cancer cell, a bladder cancer cell, a pharyngeal cancer cell, a rectal cancer cell, a prostate cancer cell, a breast cancer cell, a lung cancer cell, a renal cancer cell, a pancreatic cancer cell, or a hematologic cancer cell such as a lymphoma or leukemia
20 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.

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*).

25 Therefore, in the present invention, such bacteria can deliver RNA molecules or RNA-encoding DNA to cells in the host mucosal compartment.

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
30 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.

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
35 of, the *Plasmodium vivax* reticulocyte binding proteins-1 and -2, which bind specifically to erythrocytes in humans and primates (Galinski *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.* Expt. 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 surface protein from *Trypanosoma cruzi* which causes specific binding to the mammalian extra-cellular matrix by binding to heparin, heparin sulfate and collagen (Ortega-Barria *et al.* Cell, 67:411-421 (1991)).

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 that 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 Nhieu (1994) Ann. Rev. Genet. 27:395. Nucleotide sequences for the integrin subunits can be found, *e.g.*, in GenBank, publicly available on the internet.

As set forth above, yet other target cells include fish, avian, and reptilian cells. Examples of bacteria that are naturally invasive for fish, avian, and reptilian cells are set forth below.

Examples of bacteria that can naturally access the cytoplasm of fish cells include, but are not limited to, *Aeromonas salmonicida* (ATCC No. 33658) and *Aeromonas schubertii* (ATCC No. 43700). Attenuated bacteria are preferably used in the invention, and include *A. salmonicida* vapA (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)).

Examples of bacteria that can naturally access the cytoplasm of avian cells include, but are not restricted to, *Salmonella galinarum* (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. galinarum* 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)).

Examples of bacteria that 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 mutant (Hormaeche *et al.* *supra*).

The invention also provides for delivery of RNA to other eukaryotic cells, *e.g.*, plant cells, so long as there are microorganisms that 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 tumerfacium*, which uses a pilus-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.

5 Set forth below are examples of cell lines to which RNA can be delivered according to the method of this invention.

 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
10 6023, CCL 44 and CRL 1390.

 Examples of ovine cells lines include ATCC Nos. CRL 6540, CRL 6538, CRL 6548 and CRL 6546.

 Examples of porcine cell lines include ATCC Nos. CL 184, CRL 6492, and CRL 1746.

 Examples of feline cell lines include CRL 6077, CRL 6113, CRL 6140, CRL 6164, CCL 94,
15 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.

20 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; orangutan cell line ATCC No. CRL 1850; and gorilla cell line ATCC No. CRL 1854.

25 The invention also provides methods of regulating the expression of one or more genes. Preferably, regulating the expression of one or more genes means decreasing or lessening the expression of the gene and/or decreasing or lessening the activity of the gene and its corresponding gene product.

 In one embodiment, the expressed siRNAs direct the multienzyme complex RISC (RNA-
30 induced silencing complex) of the cell to interact with the mRNAs to be regulated. This complex degrades or sequesters the mRNA. This causes the expression of the gene to be decreased or inhibited.

 In some embodiments, the gene is an animal gene. Preferred animal genes are mammalian genes, such as humans, bovine, ovine, porcine, feline, canine, goat, equine, and primate genes. The
35 most preferred mammalian genes are human cells.

The gene to be regulated can be a viral gene, anti-inflammatory gene, obesity gene or autoimmune disease or disorder gene. In some embodiments, more than one gene can be regulated from a single plasmid or vector.

- In preferred embodiments, the gene can be, but is not limited to, ras, *β-catenin*, one or more HPV oncogenes, APC, HER-2, MDR-1, MRP-2, FATP4, SGLUT-1, GLUT-2, GLUT-5, apobec-1, MTP, IL-6, IL-6R, IL-7, IL-12, IL-13, IL-13 Ra-1, IL-18, p38/JNK MAP kinase, p65/NF-κB, CCL20 (MIP-3α), Claudin-2, Chitinase 3-like 1, apoA-IV, MHC class I and MHC class II. In one aspect of this embodiment, the ras is *k-Ras*. In another aspect of this embodiment, the HPV oncogene is E6 or E7.
- 10 Preferable *β-catenin* target gene sequences are recited in Table 11. The sequences in Table 11 are cross-species target sequences as they are capable of silencing the beta-catenin gene (CTNNB1) in human, mouse, rat, dog and monkey.

Table 11.

<i>β-catenin</i> target gene sequences	SEQ ID NO:
AGCCAATGGCTTGGAATGAGA	52
ATCAGCTGGCCTGGTTTGATA	53
CTGTGAACTTGCTCAGGACAA	54
AGCAATCAGCTGGCCTGGTTT	55
CCTCTGTGAACTTGCTCAGGA	56
TTCCGAATGTCTGAGGACAAG	57
CCAATGGCTTGGAATGAGACT	58
GGTGCTGACTATCCAGTTGAT	59
CAATCAGCTGGCCTGGTTTGA	60
CACCCTGGTGCTGACTATCCA	61
CACCACCCTGGTGCTGACTAT	62
TGCTTTATTCTCCCATTGAAA	63
CTGGTGCTGACTATCCAGTTG	64
TCTGTGCTCTTCGTCTCTGA	65
TGCCATCTGTGCTCTTCGTCA	66
TGGTGCTGACTATCCAGTTGA	67
CCTGGTGCTGACTATCCAGTT	68
ACCCTGGTGCTGACTATCCAG	69
GAGCCTGCCATCTGTGCTCTT	70
CTGGTTTGATACTGACCTGTA	71
TGGTTTGATACTGACCTGTAA	72
TCGAGGAGTAACAATACAAAT	73
ACCATGCAGAATACAAATGAT	74
AGGAGTAACAATACAAATGGA	75
GTCGAGGAGTAACAATACAAA	76
TTGTTGTAACCTGCTGTGATA	77
GAGTAATGGTGTAGAACACTA	78
AGTAATGGTGTAGAACACTAA	79
CACACTAACCAAGCTGAGTTT	80
TTTGGTCGAGGAGTAACAATA	81
TACCATTCCATTGTTTGTGCA	82
TAGGGTAAATCAGTAAGAGGT	83
CTAACCAAGCTGAGTTTCCTA	84
TGGTCGAGGAGTAACAATACA	85
CTGGCCTGGTTTGATACTGAC	86

TAACCTCACTTGCAATAATTA	87
ATCCCCTGGCCTCTGATAAA	88
GACCACAAGCAGAGTGCTGAA	89
CACAAGCAGAGTGCTGAAGGT	90
CTAACCTCACTTGCAATAATT	91
AGCTGATATTGATGGACAG	92

Preferable HPV target gene sequences are recited in Table 12. The sequences in Table 12 are target sequences as they are capable of silencing the HPV E6 oncogene.

Table 12

HPV target gene sequences	SEQ ID NO:
CGGTGCCAGAAACCGTTGAATCC	93
CACTGCAAGACATAGAAATAACC	94
AGGTGCCTGCGGTGCCAGAAACC	95
GCGGTGCCAGAAACCGTTGAATC	96
TCCTGCAAGACATAGAAATAAC	97
CCCATGCTGCATGCCATAAATGT	98
ATGCTGCATGCCATAAATGTATA	99
GTGGTGTATAGAGACAGTATACC	100
GCGCGCTTTGAGGATCCAACACG	101
CTGCGGTGCCAGAAACCGTTGAA	102
CCCCATGCTGCATGCCATAAATG	103
ACCCCATGCTGCATGCCATAAAT	104
AACACTGGGTATATACAATTTATT	105
ACGACGCAGAGAAACACAAGTAT	106
AAGGTGCCTGCGGTGCCAGAAAC	107
GGTGCCTGCGGTGCCAGAAACCG	108
CATGCTGCATGCCATAAATGTAT	109
GACGCAGAGAAACACAAGTATAA	110
TTCCTGCAAGACATAGAAATAA	111
GGTGCCAGAAACCGTTGAATCCA	112
TGGCGCGCTTTGAGGATCCAACA	113
TGTGGTGTATAGAGACAGTATAC	114
GTGCCTGCGGTGCCAGAAACCGT	115
CTGCATGCCATAAATGTATAGAT	116
GACTCCAACGACGCAGAGAAACA	117
CTGGGCACTATAGAGGCCAGTGC	118
TGCTGCATGCCATAAATGTATAG	119
GTGCCAGAAACCGTTGAATCCAG	120
TTACAGAGGTATTTGAATTTGCA	121
GAGGCCAGTGCCATTCGTGCTGC	122

Additional preferable HPV target gene sequences are recited in Table 13. The sequences in Table 13 are target sequences as they are capable of silencing the HPV E7 oncogene.

Table 13

HPV target gene sequences	SEQ ID NO:
ATTCCGGTTGACCTTCTATGTCA	123
GATGGAGTTAATCATCAACATTT	124
AAGCCAGAATTGAGCTAGTAGTA	125
CATGGACCTAAGGCAACATTGCA	126
AACCACAACGTCACACAATGTTG	127
ATGGACCTAAGGCAACATTGCAA	128
TAAGCGACTCAGAGGAAGAAAAC	129
GAAGCCAGAATTGAGCTAGTAGT	130
GAGCCGAACCACAACGTCACACA	131
ACGTCACACAATGTTGTGTATGT	132
GAACCACAACGTCACACAATGTT	133
AGGCAACATTGCAAGACATTGTA	134
AAGACATTGTATTGCATTTAGAG	135
TAAGGCAACATTGCAAGACATTG	136
CCAGCCCGACGAGCCGAACCACA	137
AAGCTCAGCAGACGACCTTCGAG	138
GCCCGACGAGCCGAACCACAACG	139
TTCCGGTTGACCTTCTATGTCAC	140
TGCATGGACCTAAGGCAACATTG	141
TTCCAGCAGCTGTTTCTGAACAC	142
AACACCCTGTCCTTTGTGTGTCC	143
CTTCTATGTCACGAGCAATTAAG	144
ACGAGCCGAACCACAACGTCACA	145
TTGAGCTAGTAGTAGAAAGCTCA	146
CAGCAGACGACCTTCGAGCATTTC	147
AGCCAGAATTGAGCTAGTAGTAG	148
GTCACACAATGTTGTGTATGTGT	149
CCGACGAGCCGAACCACAACGTC	150
AATCCGGTTGACCTTCTATGTC	151
ATTCCAGCAGCTGTTTCTGAACA	152

- 5 Additional preferable HPV target gene sequences are recited in Table 14. The sequences in Table 14 are target sequences shared by both HPV E6 and E6.

Table 14

HPV target gene sequences	SEQ ID NO:
TAGGTATTTGAATTTGCAT	153

GAGGTATTTGAATTGCAT	154
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A preferable MDR-1 target gene sequence is recited in Table 15. The sequence in Table 15 is capable of silencing the MDR-1 gene in human.

Table 15

MDR-1 target gene sequences	SEQ ID NO:
ATGTTGTCTGGACAAGCACT	155

5

A preferable *k-Ras* target gene sequence is recited in Table 16. The sequence in Table 16 is capable of silencing the *k-Ras* gene in human.

Table 16

<i>k-Ras</i> target gene sequences	SEQ ID NO:
GTTGGAGCTGTTGGCGTAG	156

10

Preferable IL-6R target gene sequence are recited in Table 17. The sequences in Table 17 are capable of silencing IL-6R in human.

Table 17

IL-6R target gene sequences	SEQ ID NO:
CTCCTGGAACATCATTTCTA	157
GCTCTCCTGCTTCCGGAAGAG	158
CTCCACGACTCTGGAACTAT	159
CAGAAGTTCTCCTGCCAGTTA	160
CCGGAAGACAATGCCACTGTT	161
CTGAACGGTCAAAGACATTCA	162
CACAACATGGATGGTCAAGGA	163
ATGCAGGCACTTACTACTAAT	164
ATCGGGCTGAACGGTCAAAGA	165
AGCTCTCCTGCTTCCGGAAGA	166
CAGCTCTCCTGCTTCCGGAAG	167
CAGGCACTTACTACTAATAAA	168
CACTTGCTGGTGGATGTTCCC	169
AACGGTCAAAGACATTCAAA	170
TGCACAAGCTGCACCCTCAGG	171

15

Additional referable IL-6R target gene sequences are recited in Table 18. The sequences in Table 18 are capable of silencing the IL-6R gene in mouse.

Table 18

IL-6R target gene sequences	SEQ ID NO:
ATCCTGGAGGGTGACAAAGTA	172
TGGGTCTGACAATACCGTAAA	173
AACGAAGCGTTTCACAGCTTA	174
CCGCTGTTTCCTATAACAGAA	175
ACGAAGCGTTTCACAGCTTAA	176
CTGCTGTGAAAGGGAATTAA	177

AACCTTGTGGTATCAGCCATA	178
CACAGTGTGGTGCTTAGATTA	179
CAGCTTCGATACCGACCTGTA	180
CAGTGTGGTGCTTAGATTAAA	181
CCCGGCAGGAATCCTCTGGAA	182
CCCGCTGTTTCCTATAACAGA	183
AACCACGAGGATCAGTACGAA	184
ACCTGCCGTCTTACTGAACTA	185
ACCACGAGGATCAGTACGAAA	186
ACAGCTTGTGATGACTGAATA	187
AGGATCAGTACGAAAGTTCTA	188
AACCCGCTGTTTCCTATAACA	189
CAGTACGAAAGTTCTACAGAA	190
TACGCGAGTGACAATTTCTCA	191
ACGAAAGTTCTACAGAAGCAA	192
CAGGCACTTACTACTAATAAA	193
CACTTGCTGGTGGATGTTCCC	194
AACGGTCAAAGACATTCAAA	195
TGCACAAGCTGCACCCTCAGG	196

Preferable IL-7 target gene sequences are recited in Table 19. The sequences in Table 19 are capable of silencing the IL-7 gene in human.

Table 19

IL-7 target gene sequences	SEQ ID NO:
TAAGAGAGTCATAAACCTTAA	197
AACAAGGTCCAAGATACCTAA	198
AAGATTGAACCTGCAGACCAA	199
AAGAGATTTCAAGAGATTTAA	200
AAGCGCAAAGTAGAAACTGAA	201
TAGCATCATCTGATTGTGATA	202
TAAGATAATAATATATGTTTA	203
ATGGTCAGCATCGATCAATTA	204
TGCTTGAATAATGAATTTAA	205
ATCTGTGATGCTAATAAGGAA	206
AACAACTATTTCTTATATAT	207
AACATTTATCAATCAGTATAA	208
ATCAATCAGTATAATTCTGTA	209
AAGGTATCAGTTGCAATAATA	210

5

Additional preferable IL-7 target gene sequences are recited in Table 20. The sequences in Table 20 are capable of silencing the IL-7 gene in mouse.

Table 20

IL-7 target gene sequences	SEQ ID NO:
CGGATCCTACGGAAGTTATGG	211
GACCATGTTCCATGTTTCTTT	212
AACCTAAATGACCTTTATTAA	213
CAGGAGACTAGGACCCTATAA	214
TAGGGTCTTATTCGTATCTAA	215
ATGAGCCAATATGCTTAATTA	216
GCCAATATGCTTAATTAGAAA	217

CAGCATCGATGAATTGGACAA	218
TTGCCTGAATAATGAATTTAA	219
CTGATAGTAATTGCCCCAATA	220
AAGGGTTTGCTTGACTGAAT	221
AACATGTATGTGATGATACAA	222
TTGCAACATGTAATAATTTAA	223
AAGAGACTACTGAGAGAAATA	224
AAGAATCTACTGGTTCATATA	225
TGCCGTCAGCATATACATATA	226
AGGGCTCACGGTGATGGATAA	227

Additional preferable IL-7 target gene sequences are recited in Table 21. The sequences in Table 21 are cross species sequences as they are capable of silencing the IL-7 gene in human and mouse.

5 Table 21

IL-7 target gene sequences	SEQ ID NO:
CGCCTCCCGCAGACCATGTTTC	228
TCCGTGCTGCTCGCAAGTTGA	229
GCCTCCCGCAGACCATGTTCC	230
CCTCCCGCAGACCATGTTCCA	231
CTCCCGCAGACCATGTTCCAT	232
TCCCGCAGACCATGTTCCATG	233
CCCGCAGACCATGTTCCATGT	234
CCGCAGACCATGTTCCATGTT	235
CGCAGACCATGTTCCATGTTT	236
GCAGACCATGTTCCATGTTTC	237
CAGACCATGTTCCATGTTTCT	238
AGACCATGTTCCATGTTTCTT	239

Preferable IL-13Ra-1 target gene sequences are recited in Table 22. The sequences in Table 22 are capable of silencing the IL-13Ra-1 gene in human.

Table 22

IL-13Ra-1 target gene sequences	SEQ ID NO:
AACCTGATCCTCCACATATTA	240
CCTGATCCTCCACATATTAAA	241
AGAAATGTTTGGAGACCAGAA	242
CAAATAATGGTCAAGGATAAT	243
TTCTGATCCTGGCAAGATTT	244
TAAAGAAATGTTTGGAGACCA	245
ATGTTTGGAGACCAGAATGAT	246
CTCCAATTCCTGATCCTGGCA	247

10

Additional preferable IL-13Ra-1 target gene sequences are recited in Table 23. The sequences in Table 23 are capable of silencing the IL-13Ra-1 gene in mouse.

Table 23

IL-13Ra-1 target gene sequences	SEQ ID NO:
CAAGAAGACTCTAATGATGTA	248
CACAGTCAGAGTAAGAGTCAA	249

ACCCAGGGTATCATAGTTCTA	250
CTGCTTTGAAATTTCCAGAAA	251
ATCATAGTTCTAAGAATGAAA	252
AAGGCTTAAGATCATTATATT	253
AACTACTTATAAGAAAGTAAA	254
CACAGAACATCTAGCAAACAA	255
CTCGTTCTTGTTCAATCCTAA	256
AACTTGTAGGTTACATATTA	257
AACCATTTCTGCAAATTTAAA	258
CTCAGTGTAGTGCCAATGAAA	259
CAGGCCTTAGGGACTCATAAA	260
AAGTATGACATCTATGAGAAA	261
GTGGAGGTCAATAACTCAA	262
CAGAGTATAGGTAAGGAGCAA	263

A preferable IL-18 target gene sequence is recited in Table 24. The sequence in Table 24 is capable of silencing the IL-18 gene in human.

Table 24

IL-18 target gene sequences	SEQ ID NO:
TTGAATGACCAAGTTCTCTTC	264

5

Additional preferable IL-18 target gene sequences are recited in Table 25. The sequences in Table 25 are capable of silencing the IL-18 gene in mouse.

Table 25

IL-18 target gene sequences	SEQ ID NO:
CTCTCTGTGAAGGATAGTAAA	265
CCGCAGTAATACGGAATATAA	266
CAAGGAAATGATGTTTATTGA	267
CAGACTGATAATATACATGTA	268
TTGGCCGACTTCACTGTACAA	269
CCAGACCAGACTGATAATATA	270
AAGATGGAGTTTGAATCTTCA	271
ACGCTTTACTTTATACCTGAA	272
TACAACCGCAGTAATACGGAA	273
CTGCATGATTTATAGAGTAAA	274
CCCGAGGCTGCATGATTTATA	275
CACGCTTTACTTTATACCTGA	276
CGCCTGTATTTCCATAACAGA	277
CGCAGTAATACGGAATATAAA	278
TACATGTACAAAGACAGTGAA	279
CAGGCCTGACATCTTCTGCAA	280
TTCGAGGATATGACTGATATT	281
CTGTATTTCCATAACAGAATA	282
GAGGATATGACTGATATTGAT	283
CAAGTTCTCTTCGTTGACAAA	284
CACTAACTTACATCAAAGTTA	285
ACCGCAGTAATACGGAATATA	286
CTCTCACTAACTTACATCAAA	287

Preferable CCL20 target gene sequences are recited in Table 26. The sequences in Table 26 are capable of silencing the CCL20 gene in human.

Table 26

CCL20 target gene sequences	SEQ ID NO:
ATCATCTTTCACACAAAGAAA	288
AACAGACTTGGGTGAAATATA	289
ATGGAATTGGACATAGCCCAA	290
GAGGGTTTAGTGCTTATCTAA	291
CTCACTGGACTTGTCCAATTA	292
ATCATAGTTTGCTTTGTTTAA	293
TTGTTTAAGCATCACATTAAA	294
AAGCATCACATTAAAGTTAAA	295
CCCAAAGAACTGGGTACTCAA	296
CACATTAAAGTTAACTGTAT	297
CAGATCTGTTCTTTGAGCTAA	298
TTGGTTTAGTGCAAAGTATAA	299
CAGACCGTATTCTTCATCCTA	300
AACATTAATAAGACAAATATT	301
GACCGTATTCTTCATCCTAAA	302

- 5 Additional referable CCL20 target gene sequences are recited in Table 27. The sequences in Table 27 are capable of silencing the CCL20 gene in mouse.

Table 27

CCL20 target gene sequences	SEQ ID NO:
AAGCTTGTGACATTAATGCTA	303
CAATAAGCTATTGTAAAGATA	304
ATCATCTTTCACACGAAGAAA	305
AGCTATTGTAAAGATATTTAA	306
CAGCCTAAGAGTCAAGAAGAT	307
CCCAGTGGACTTGTCAATGGA	308
ATGAAGTTGATTCATATTGCA	309
AAGTTGATTCATATTGCATCA	310
TCACATTAGAGTTAAGTTGTA	311
CACATTAGAGTTAAGTTGTAT	312
TATGTTATTTATAGATCTGAA	313
ATGTTTAGCTATTTAATGTTA	314
TTAGTGAAGGATTAATATTA	315
ACCCAGCACTGAGTACATCAA	316
TATGTTTAAGGGAATAGTTTA	317

- 10 Additional preferable CCL20 target gene sequences are recited in Table 28. The sequences in Table 28 are cross-species target sequences as they are capable of silencing the CCL20 gene in human and mouse.

Table 28.

CCL20 target gene sequences	SEQ ID NO:
ATGAAGTTGATTCATATTGCA	318
TGAAGTTGATTCATATTGCAT	319
GAAGTTGATTCATATTGCATC	320

AAGTTGATTCATATTGCATCA	321
AGTTGATTCATATTGCATCAT	322
GTTGATTCATATTGCATCATA	323
TTGATTCATATTGCATCATAG	324
TGATTCATATTGCATCATAGT	325
TCAATGCTATCATCTTTCACA	326
CAATGCTATCATCTTTCACAC	327
TAATGAAGTTGATTCATATTG	328
AATGAAGTTGATTCATATTGC	329

Preferable CCL20 target gene sequences are recited in Table 29. The sequences in Table 29 are capable of silencing the CCL20 gene in human.

Table 29

Claudin-2 target gene sequences	SEQ ID NO:
AGCATGAAATTTGAGATTGGA	330
TACAGAGCCTCTGAAAGACCA	331
CACTACAGAGCCTCTGAAAGA	332
CTGACAGCATGAAATTTGAGA	333
ATCTCTGTGGTGGGCATGAGA	334
CATGAAATTTGAGATTGGAGA	335
TCTGGCTGAGGTTGGCTCTTA	336
GTGGGCTACATCCTAGGCCTT	337

5

Additional preferable CCL20 target gene sequences are recited in Table 30. The sequences in Table 30 are capable of silencing the CCL20 gene in mouse.

Table 30

Claudin-2 target gene sequences	SEQ ID NO:
CAGCTTCCTGCTAAACCACAA	338
CAAGAGTGAGTTCAACTCATA	339
CTGGTTCCTGACAGCATGAAA	340
TGGCTGGGACTATATATATAA	341
GAGGGCAATTGCTATATCTTA	342
CAGCAGCCAAACGACAAGCAA	343
CAAGGGTTTCCTTAAGGACAA	344
CAGATACTTGTAAGGAGGAAA	345
AAGAAATGGATTAGTCAGTAA	346
AAGGAAAGCACAAGAAGCCAA	347
CTGGCTGAGGTTGGCTCTTAA	348
AACCTGGGATCTAAAGAAACA	349
AAGGGCTTGGGTATCAAAGAA	350
CAGGCTCCGAAGATACTTCTA	351
CCCAATATATAAATTGCCTAA	352
CTGACCCAGCTTCCTGCTAAA	353

10

Preferable Chitinase-3 target gene sequences are recited in Table 31. The sequences in Table 31 are capable of silencing the Chitinase-3 gene in human.

Table 31

Chitinase-3 target gene sequences	SEQ ID NO:
--	-------------------

ACCCACATCATCTACAGCTTT	354
CATCATCTACAGCTTTGCCAA	355
CAGCTGGTCCCAGTACCGGGA	356
CACCAAGGAGGCAGGGACCCT	357
CCGGTTCACCAAGGAGGCAGG	358
AGCTGGTCCCAGTACCGGGAA	359
CAGGCCGGTTCACCAAGGAGG	360
GGCCGGTTCACCAAGGAGGCA	361

Additional preferable Chitinase-3 target gene sequences are recited in Table 32. The sequences in Table 32 are capable of silencing the Chitinase-3 gene in mouse.

Table 32

Chitinase-3 target gene sequences	SEQ ID NO:
TAGGTTTGACAGATACAGCAA	362
AACCCTGTTAAGGAATGCAAA	363
ATCAAGTAGGCAAATATCTTA	364
CGCAGCTTTGTGAGCAGGAAA	365
TTGGATCAAGTAGGCAAATAT	366
TTGAGGGACCATACTAATTAT	367
GAGGACAAGGAGAGTGTCAA	368
TGCGTACAAGCTGGTCTGCTA	369
CAGGAGTTTAATCTCTTGCAA	370
ATCAAGGAACTGAATGCGGAA	371
CACCCTGATCAAGGAACTGAA	372
CACCTGGATCAAGTAGGCAAA	373
CAGGATTGAGGGACCATACTA	374
AACATATGACAAGCTGAATAAA	375
ATGCAAATTCTCAGACTCTAA	376
ATCCTTCCCTTAGGAACTTAA	377

5

5. Treatment of Diseases and Disorders

The present invention also provides methods of treating or preventing a disease or disorder in a mammal. The methods include regulating the expression of at least one gene in a cell known to cause a disease or disorder by introducing to the cells of the mammal at least one invasive bacterium, or at least one bacterial therapeutic particle (BTP), containing one or more siRNAs or one or more DNA molecules encoding one or more siRNAs, where the expressed siRNAs interfere with the mRNA of the gene known to cause the disease or disorder of interest.

The RNAi methods of the invention, including BMGS and tkRNAi are used to treat any disease or disorder for which gene expression regulation would be beneficial. This method is effected by silencing or knocking down (decreasing) genes involved with one or more diseases and disorders.

The gene to be regulated to treat or prevent a disease or disorder of interest, can be, but is not limited to, ras, β -catenin, one or more HPV oncogenes, APC, HER-2, MDR-1, MRP-2, FATP4, SGLUT-1, GLUT-2, GLUT-5, apobec-1, MTP, IL-6, IL-6R, IL-7, IL-12, IL-13, IL-13 Ra-1, IL-18, p38/JNK MAP kinase, p65/NF- κ B, CCL20 (MIP-3 α), Claudin-2, Chitinase 3-like 1, apoA-IV, MHC

class I and MHC class II. In one aspect of this embodiment, the ras is *k-Ras*. In another aspect of this embodiment, the HPV oncogene is E6 or E7.

Preferably, the present invention provides methods of treating or preventing cancer or a cell proliferation disorder, viral disease, an inflammatory disease or disorder, a metabolic disease or disorder, an autoimmune disease or disorder, or a disease, disorder or cosmetic concern in the skin or hair in a mammal by regulating the expression of a gene or several genes known to be associated with the onset, propagation or prolongation of the disease or disorder by introducing a bacterium or BTP to the cell. The bacterium or BTP contain one or more siRNAs or one or more DNA molecules encoding one or more siRNAs, where the expressed siRNAs interfere with the mRNA of the gene known to cause, propagate or prolong the disease or disorder of interest.

In some preferred embodiments, the viral disease can be, but is not limited to, hepatitis B, hepatitis C, Human Papilloma Virus (HPV) infection or epithelial dysplasia or cancer caused by HPV infection or HPV induced transformation, including cervical cancer, rectal cancer and pharyngeal cancer.

In some preferred embodiments, the inflammatory disease or disorder can be, but is not limited to, inflammatory bowel disease, Crohn's disease, ulcerative colitis, an allergy, rheumatoid arthritis.

In some preferred embodiments, the autoimmune disease or disorder can be, but is not limited to, celiac disease.

In some preferred embodiments, the disease, disorder or cosmetic concern can be, but is not limited to, psoriasis, eczema, albinism, balding or gray hair.

The mammal can be any mammal including, but not limited to, human, bovine, ovine, porcine, feline, canine, goat, equine, or primate. Preferably, the mammal is a human.

The terms "treating" and "treatment" as used herein refer to the administration of an agent or formulation (e.g., bacterium and/or BTP containing an siRNA or a DNA encoding for an siRNA) to a clinically symptomatic individual afflicted with an adverse condition, disorder, or disease, so as to effect a reduction in severity and/or frequency of symptoms, eliminate the symptoms and/or their underlying cause, and/or facilitate improvement or remediation of damage.

The terms "preventing" and "prevention" refer to the administration of an agent or composition to a clinically asymptomatic individual who is susceptible to a particular adverse condition, disorder, or disease, and thus relates to the prevention of the occurrence of symptoms and/or their underlying cause.

6. Pharmaceutical Compositions and Modes of Administration

In a preferred embodiment of the invention, the invasive bacteria or BTPs containing the RNA molecules, and/or DNA encoding such, are introduced into an animal by intravenous,

intramuscular, intradermal, intraperitoneally, peroral, intranasal, intraocular, intrarectal, intravaginal, intraosseous, oral, immersion and intraurethral inoculation routes.

The amount of the invasive bacteria or BTPs 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 10^3 to 10^{11} viable organisms, preferably about 10^5 to 10^9 viable organisms per subject.

The invasive bacteria or BTPs 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 (1988)). 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 or BTPs 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 or BTPs, 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.*, pregelatinised 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 lauryl 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-*p*-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 manner.

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 nebuliser, 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 formulatory 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, intravaginal 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, for 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 using suppositories. For topical administration, the bacteria of the invention are formulated 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.

The compositions may, if desired, be presented in a pack or dispenser device and/or a kit that 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.

The invasive bacteria or BTPs containing the RNA or RNA-encoding DNA to be introduced can be used to infect animal cells that are cultured *in vitro*, such as cells obtained from a subject.

5 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 administered will be at a multiplicity of infection ranging from about 0.1 to 10^6 , preferably about 10^2 to 10^4 bacteria per cell.

10 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.

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
15 defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

The present invention is further illustrated by the following examples that should not be construed as limiting in any way. The contents of all cited references including literature references, issued patents, published patent applications as cited throughout this application are hereby expressly
20 incorporated by reference. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press:
25 1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis *et al.* U.S. Pat. No: 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods
30 In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu *et al.* eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Manipulating the Mouse Embryo, (Cold Spring
35 Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

The following non-limiting examples are merely illustrative of the preferred embodiments of the present invention, and are not to be construed as limiting the invention.

EXAMPLES

Example 1: Knockdown of β -catenin and *k-Ras*

5 Previous studies have demonstrated the powerful nature of the siRNA knockdown technology disclosed herein. For example, *in vitro* and *in vivo* knockdown of beta catenin and k-ras utilizing bacterial delivery is described in PCT Publication No. WO 06/066048, which is incorporated herein by reference in its entirety.

10 Example 2: TRIP with Multiple shRNA Expression Cassettes

The TRIP described herein, and described in further detail in PCT Publication No. WO 06/066048, can be modified to produce a plasmid which allows targeting of multiple genes simultaneously or multiple sequences within one gene simultaneously. For example, TRIP with multiple hairpin expression cassettes to produce shRNA can target different sequences in a given
15 gene, or target multiple genes through a simultaneous bacterial treatment.

The TRIP plasmid can incorporate multiple (up to ten) cloning sites to express different shRNA constructs (Figure 1). The purpose of such a plasmid will be to allow silencing of various genes through a single therapeutic bacterium which will be empowered by the Multiple-expression cassette-TRIP (mec-TRIP) to synthesize short hairpin RNA against a variety of targets
20 simultaneously.

These different hairpins can either be expressed competitively at high levels through the use of an identical high level promoter (such as T7 promoter or a different high level bacterial promoter), or they can be expressed at different levels through the use of promoters with different levels of activity; this will depend on the intended use of the plasmid and the desired relative silencing levels of
25 the target gene.

This mec-TRIP could be useful to treat complex diseases as described herein (e.g. inflammatory diseases, or cancer), through the simultaneous silencing(targeting) of multiple targets as described herein (e.g. multiple oncogenes, such as k-ras and beta-catenin in the case of colon cancer, or HER-2 and MDR-1 in breast cancer, or other combinations).

30 Example 3: Operator Repressor Titration System

The TRIP system (bacteria and plasmid) have been modified to include the ORT (Operator Repressor Titration) system from Cobra Biomanufacturing (Keele, UK). This adaptation helps to maintain the plasmid in suitable strains in the absence of selective antibiotics. The bacterial carrier
35 strain has been modified accordingly to allow for the ORT system to function (deletion of the DAP gene and replacement with an ORT-controlled DAP gene expression system). The plasmid has been modified to remove the antibiotic selection sequences to support the ORT system. Further changes

have been introduced to the bacterial genome, including for example, (a) deletion of the *aroA* gene (in some CEQ strains) to make the bacteria more susceptible to nutrient shortage, particularly in the intracellular compartment where they will die due to lack of nutrients; (b) insertion of T7RNAPolymerase gene into the chromosome and or (c) integration of a shRNA expression cassette under T7 promoter into the chromosome.

Figure 2 shows development examples of bacterial strains. Further strains developed include, but are not limited to, CEQ922 (CEQ919 without *aroA* deletion), CEQ923 (CEQ920 without *aroA* deletion), CEQ924 (CEQ921 without *aroA* deletion).

10 **Example 4: Intestinal Tract Gene Delivery**

S. typhimurium was investigated to determine if it could be used as a vector for RNAi delivery into the epithelial cells lining the intestinal tract. Mice were treated orally with a single dose of 10^8 SL 7207 and sacrificed at various time points after administration. SL7207 were then stained using the Salmonella specific antibody. 2h after treatment, numerous SL7207 could be seen invading the intestinal epithelial layer (Salmonella stained red), suggesting that oral administration of SL7207 may be a useful tool to deliver payloads to the intestinal and colonic mucosa. In a follow up experiment, mice were treated with SL7207 harboring a GFP expression plasmid (pEGFPC1, Invitrogen). At 24h after a single treatment, a small percentage (approximately 1%) of cells was clearly found to express GFP.

Figure 3 shows the efficient invasion and plasmid delivery into the intestinal mucosa by *S. typhimurium*. SL7207 were stained using red fluorescent antibody 6h after oral administration. Intact SL7207 and fragments of SL7207 were seen in epithelial cells as well as underlying cells of the lamina propria (top left/right). SL7207 successfully deliver expressed DNA into the intestinal mucosa: intestinal mucosal cells expressing GFP after treatment with SL7207 carrying a eukaryotic expression plasmid for GFP (pEGFP-C1)(lower left). For fluorescence microscopy, SL7207 were stained with red fluorescent antibody and nuclei were counterstained with Hoechst 37111.

To test whether SL7207 could be used for the delivery of RNAi to target genes in the intestinal tract, GFP transgenic mice (4 per group) were treated with *S. typhimurium* harboring a shRNA expression plasmid directed against GFP (SL-siGFP) or a shRNA expression plasmid directed against k-RAS (SL-siRAS). 10^8 c.f.u. was given three times weekly for two weeks by oral gavage. Colonic tissues were subsequently reviewed with fluorescent microscopy (data not shown) and stained analyzed after immunohistochemistry staining for GFP expression using a specific antibody (Living Colors®, Invitrogen). There was a significant reduction in the overall GFP expression level and significant reduction in the number of GFP expressing crypts in the SL-siGFP treated animals compared with the SL-siRAS treated animals (33.9% vs 50%, $p < 0.05$), suggesting that this method could be useful to deliver therapeutic RNAi into the colonic epithelium.

Figure 4 shows that bacteria-mediated RNA interference reduces target gene expression in the gastrointestinal epithelium. After treatment with SL7207 carrying expression plasmids targeting GFP (SL-siGFP, right bottom panel), colon tissues showed lower levels of GFP expression, and fewer colonic crypts were stained positive for GFP compared with animals treated with SL-siRAS (left bottom panel). Slides were stained with GFP-specific antibody.

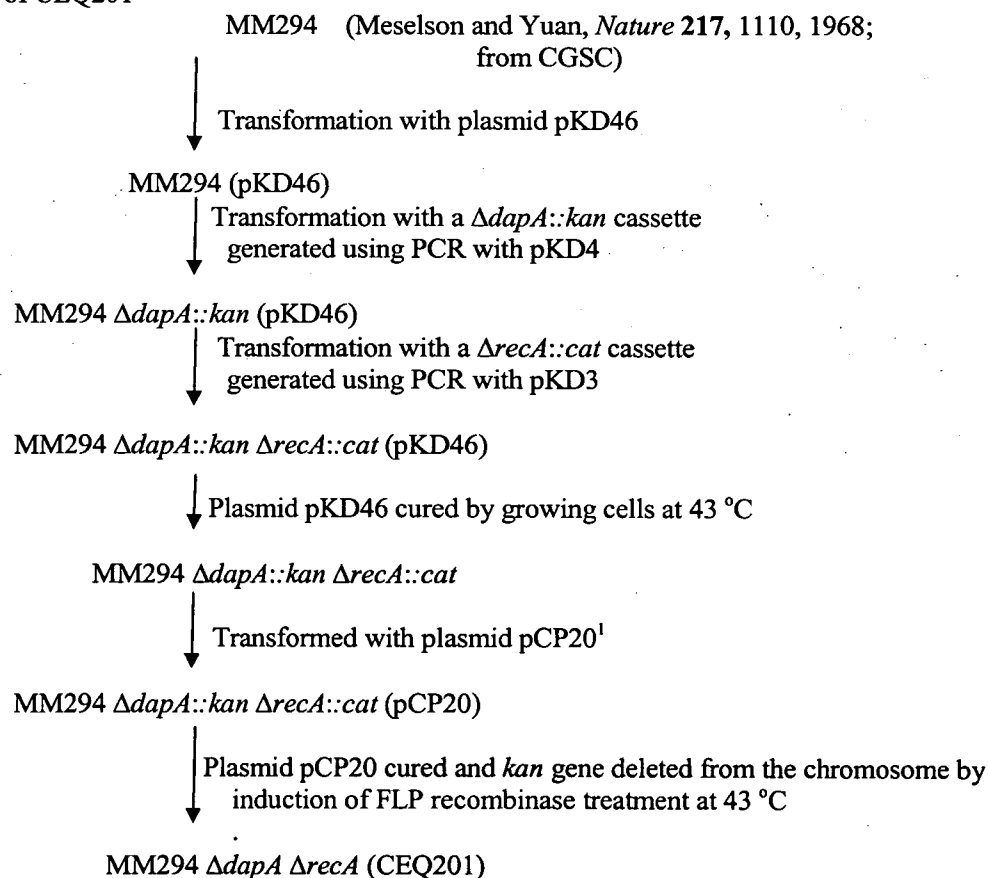
Example 5: Construction of CEQ503 Bacterial Strain

Derivation and Description of CEQ 503 (Strain CEQ201 (pNJSZ))

CEQ503 consists of a combination of an attenuated *E. coli* strain (CEQ201) with a specially engineered TRIP plasmid (pNJSZ). The plasmid confers the abilities required to induce tkRNAi (in this case: invasiveness, escape from the entry vesicle, expression of short hairpin RNA). Strain Description of CEQ503 (pNJSZ):

1. Genotype: *Escherichia coli* CEQ201 [*glnV44(AS)*, *LAM*, *rfbC1*, *endA1*, *spoT1*, *thi-1*, *hsdR17*, (*r_k⁺*), *creC510* Δ *dapA*, Δ *recA*].

2. Derivation of CEQ201



3. Plasmid: pNJSZ, shown schematically in Figure 5, is a 10.4 kb plasmid that confers kanamycin resistance to our bacterial strain (CEQ503). This plasmid contains two genes, *hly* and *inv*, and the

H3 hairpin sequence:

ggatccAGGAGTAACAATACAAATGGATTCAAGAGATCCATTTGTATTGTTACTCCTTTgt
cgac (SEQ ID NO:380), which includes BamHI and SalI restriction sites. To verify the presence
of this plasmid, PCRs are performed to verify chromosomal deletion of *dapA*, and minipreps
and/or PCR are performed to confirm *inv*, *hly* and 341-H3 on the plasmid.

4. Nutritional Requirements: Althea Media Broth or LB, Miller (Luria-Bertani) broth (Amresco; cat. no.: J106-2KG) and 50 µg/ml of DL-Δ;ε-Diaminopemilic acid (DAP) (SIGMA; cat. no.: D1377-10G).

5. Growth Conditions: 37 °C

Example 6: BTP Production

BTPs or minicells containing a suitable plasmid such as TRIP have been engineered for
delivery of tkRNAi. These cells will express invasin or Opa to enable entry into mammalian cells and
listeriolysin will allow lysis of phagosome following minicell degradation/ lysis. Additionally, a
method for manufacturing minicells has been developed that utilizes a suicide construct for killing
intact cells to aid in the purification of minicells. Such suicide plasmids have been described in the
literature (Kloos et al., (1994) J. Bacteriol. 176, 7352-61; Jain and Mekalanos, (2000) Infect. Immun.
68, 986-989). Summarily, the lambda S and R genes that code for holing and lysozyme are placed
under regulation of an inducible promoter on the bacterial chromosome. When induced, they will lyse
intact cells but not minicells since minicells lack chromosomes. A number of different types of
regulators such as *lacI*, *araC*, lambda *cI857* and *rhaS-rhaR* can be used for development of an
inducible suicide gene construct.. Similarly, a number of different types of suicide genes, including *E.*
coli autolysis genes and antimicrobial small peptides, can be used in a similar scheme. Purification is
enhanced by treatments or mutations that induce filamentation (see, for example, Ward and
Lutkenhaus, (1985) Cell 42, 941-949; Bi and Lutkenhaus, 1992). Initial purification involves low
speed centrifugation to separate intact cells and retain minicells in the supernatant. This can be
followed by density gradient purification or filtration (for example, Shull et al., (1971) J. Bacteriol.
106, 626-633).

Any cell death-triggering gene, also known as a suicide gene, including but not limited to
genes encoding antimicrobial proteins, bacteriophage lysins or autolysins can be used in this method
for obtaining BTPs from a mixture containing BTPs and bacteria. Suicide genes can kill live bacteria
by mechanisms that include but are not limited to cell lysis, or by the destruction, degradation or
poisoning of cellular components such as chromosomal DNA or filament components. Any inducible
promoter may be used in conjunction with this system. In one embodiment of this invention, the
suicide genes are integrated within the chromosome, thereby limiting their presence only in intact

bacterial cells as BTPs or minicells will not incorporate these genes because they do not harbor chromosomal DNA.

As shown in Figure 6, induction of suicide genes will lyse intact bacterial cells. The lambda S and R genes (suicide genes) are put under the control of P_{lacUV5} (inducible promoter). The leaky basal activity is repressed by a "super-repressor" coded by *lacI^s* gene on a P_{gapA} (strong promoter).
 5 This cassette is put at the *minCD* locus.

Example 7: siRNA Inhibition of Human Papillomavirus (HPV) Oncogenes

10 Experimental Procedures

Cell Culture: HeLa cells were cultured in Minimum Essential Medium (MEM, ATCC No. 30-2003) with 10% FBS supplemented with antibiotics: 100 U/ml penicillin G, 10 µg/ml streptomycin (Sigma).

Bacterial Culture: Plasmids were transformed into BL21(DE3) strain (Invitrogen). Bacteria
 15 were grown at 37°C in LB Broth containing 100 µg/ml ampicillin. Bacterial cell density (in CFU/ml) was calculated using OD₆₀₀ measurement. For cell infection, overnight cultures were inoculated into fresh medium for another 2-3 h growth until the optical density at 600 nm [OD₆₀₀] reached 0.6.

Invasion Assay: For bacterial invasion, HeLa cells were plated in 6-well dishes at 200,000 cells/well and allowed to incubate overnight in 2 ml complete growth medium. The bacterial cells
 20 were grown to mid-exponential phase with optical density at 600 nm [OD₆₀₀] 0.6 in LB Broth with Ampicillin, and then centrifuged at 3,400 rpm for 10 minutes at 4°C. Bacterial pellets were resuspended in MEM without serum or the antibiotics and the bacteria were added to the cells at an MOI of 1:1000, 1:500, 1:250, 1:125, or 1:62.5 and allowed to invade the HeLa cells for 2 hours at 37°C in 5% CO₂. The cells were washed 4 times with MEM containing 10% FBS and penicillin-
 25 streptomycin (100 IU of penicillin and 100 µg of streptomycin per ml). Cells were incubated in fresh complete medium for further 48 hours at 37°C in 5% CO₂ and total RNA was then isolated by the Qiagen RNeasy system with on-column DNase digestion or by TRIZOL extraction method.

siRNA Transfection: One day before the transfection, cells were plated in complete growth medium without antibiotics so that the cells will be 30-50% confluent at the time of transfection.
 30 Diluted various concentrations of siRNA from a stock of 20µM in 175 µl of Opti-MEM. Mixed 4 µl of Oligofectamine separately in 15 µl of Opti-MEM. Mixed gently and incubated for 5-10 min at room temperature. Combined the diluted siRNA with diluted oligofectamine and incubated for 15-20 min at room temperature. While the complexes were being formed, removed the growth medium from the cells and added 800 µl of medium without serum to each well containing cells. Added the 200 µl
 35 of siRNA/oligofectamine complexes to the cells and incubated at 37°C for 4h. Added 1ml of growth medium containing 3X the normal concentration of serum without removing the transfection mixture. Gene silencing was assayed at 48 h.

RT-PCR: Quantitative real-time reverse transcription PCR (RT-PCR) was performed with the TaqMan RT-PCR master Mix Reagents Kit (Applied Biosystems) using the following primers and a probe set for detection of HPV18E6E7 transcripts:

Forward Primer: 5'-CTGATCTGTGCACGGAAGTGA-3' (148-168) (SEQ ID NO:381)

5 Reverse Primer: 5'-TGTCTAAGTTTTTCTGCTGGATTCA-3'(439-463) (SEQ ID NO:382)

Probe: 5'-TTGGAAGTTACAGAGGTGCCTGCGC-3' (219-233 and 416-425) (SEQ ID NO:383)

The probe was labeled at the 5' end with a reporter fluorescent dye, FAM and at the 3' end with fluorescent dye quencher TAMRA. GAPDH was used to detect human GAPDH transcripts for the normalization.

10 HPVshRNA sequences:

H1 (working sequence)

5' - **ggATCCTAGGTATTTGAATTTGCATTTCAGAGAATGCAAATTCAAATACCTTTTgTCgAC** (SEQ ID NO:384)

5' - **GTCGACAAAGGTATTTGAATTTGCATTCTCTTGAATGCAAATTCAAATACCTAGGATCC** (SEQ ID NO:385)

H2 (ineffective sequence)

15 5' -**ggATCCTCAGAAAACTTAGACACCTTCAAGAGAGGTGTCTAAGTTTTTCTGTTTgTCgAC** (SEQ ID NO:386)

5' - **GTCGACAAACAGAAAACTTAGACACCTCTCTGAAGGTGTCTAAGTTTTTCTGAGGATCC** (SEQ ID NO:387)

Western Blot: Hela cells were lysed using 1X Cell lysis Buffer (Cell Signaling Technology, Cat No. 9803). For electrophoresis, 50 µg of total protein in 2X loading buffer was loaded to each well of a 12% SDS-PAGE gel. After transferring the blot was blocked and probed with primary antibody at 2h followed by incubation with HRP-conjugated secondary antibody before detection by ECL. All primary antibodies were used at 1/1000 dilution except HPV18E7 antibody at 1/250.

Anti-Human pRb antibody: BD Pharmingen (Cat No. 554136), Sec Ab: HRP-anti Mouse
 HPV18E7: Santa Cruz (Cat No. sc-1590), Sec Ab: donkey anti-goat IgG-HRP Cat no. sc 2020
 25 p53: Santa Cruz (Cat No. sc-126), Sec Ab: HRP-anti Mouse
 p21: Santa Cruz (Cat No. sc-397), Sec Ab: HRP-anti Rabbit
 c-Myc: Cell Signaling Technology (Cat No. 9402), Sec Ab: HRP-anti Rabbit

Colony Formation Assay: Hela cells were harvested after bacterial invasion for 2h. The cells in either control treated or HPV shRNA treated cells were washed 3x times with complete MEM and one time with PBS. The cells were then trypsinized and counted. 500 cells from each treatment were added to a single well of a six well plate containing 2 ml of complete growth medium. The cells were allowed to grow for 10 days following which the colonies were fixed with GEIMSA stain.

MTT Assay: Hela cells were harvested after bacterial invasion for 2h. The cells in either control treated or HPV shRNA treated cells were washed 3x times with complete MEM and one time with PBS. The cells were then trypsinized and counted. 5000 cells from each treatment were added to a single well of a 96 well plate in 100 µl of complete growth medium in triplicates. The cells were incubated at 37°C for 48-72h following which 10 µl of 0.5 mg/ml MTT was added to each well. The plate was further incubated at 37°C for 3h, the medium was aspirated off from the wells and after

incubation, 100 µl of MTT solubilization solution [10% Triton X-100 in acidic isopropanol (0.1 N HCl)] was added to each well to stop the reaction. The absorbance was read at 570 nm on the plate reader.

Results

5 In this example, the suppressive effect of a short hairpin RNA directed towards HPV 18 E6 and E7 oncogenes was investigated. The short hairpin RNA was delivered by infecting human cervical cancer cells (Hela) with bacterial strains that produce the short hairpin RNA. The shRNA expression cassette contained 19 nucleotide (nt) of the target sequence followed by the loop sequence (TTCAAGAGA) (SEQ ID NO:388) and the reverse complement to the 19nt. For the 19 nt, two
10 shRNA sequences published in Cancer Gene Therapy (2006) 13, 1023-1032, were used to measure siRNA delivery and gene silencing efficiency, oligofectamine reagent in a 6 well format was used. Briefly, Hela cells were plated at a cell density of about 40% confluence in antibiotic free medium. On the next day, siRNA was added to 6 well plates at varying concentrations of 50, 100, 200 nM. The control siRNA was added at a single concentration of 100 nM.

15 As shown in Figure 7, the oligofectamine transfection method resulted in a decrease in E6 mRNA in Hela cells with respect to the control siRNA. The siRNA (H1) showed up to about 40% of reduction in E6 mRNA. The knockdown response was not dose dependent.

 Next, the hairpin of the siRNA (H1) was cloned into the TRIP vector. In order to determine if gene silencing could be achieved through the transkingdom system, the shRNA in human cervical
20 cancer cells (Hela) was tested in an invasion assay. Briefly, Hela cells were plated in a six-well plate at 2×10^5 cells/well, allowed to grow overnight and incubated the next day for 2h at different MOIs with bacteria (*E.coli*) engineered to produce the hairpin RNA. The bacteria were washed off with medium containing 10% FBS and Pen Strep four times and the mammalian cells were further incubated for an additional 48h in the complete medium. RNA or protein was isolated from the
25 bacteria.

 Figure 8 and Figure 9 demonstrate that siRNA downregulates HPV E6 expression in Hela cells. Cells were plated in six well plates and allowed to grow to a confluence of 40% (about 40,000 cells). Oligofectamine/siRNA transfection complexes were prepared in Opti-MEM serum-free medium by mixing 4 µl of oligofectamine with siRNAs (final concentration in 185 µl of medium is
30 50, 100, 200 nM). 48 hours post-transfection cells were harvested and analyzed by real-time RT-PCR for both target and GAPDH mRNA levels. Data were normalized against the GAPDH signal. Two different negative control siRNAs were used at a single concentration of 200 nM.

 Figure 10, Panels A - C show real time PCR results following invasion assay of Hela cells. Hela cells were incubated for 2h with shRNA-expressing BL21(DE3) at different multiplicities of
35 infection (MOI). Forty-eight hours post-infection the cells were harvested and analyzed by real-time RT-PCR for both target and GAPDH mRNA levels. Data were normalized against the GAPDH signal. These data were then further normalized to untreated control cells.

Figure 11 shows the effects of downregulation of HPV E6 and E7 genes on tumor suppressor pathways and other downstream targets. HeLa cells were incubated for 2h with shRNA-expressing BL21(DE3) at different multiplicities of infection (MOI). Forty-eight hours post-infection cells were harvested and analyzed by western blotting. 50 µg of protein was loaded in each lane and resolved by gel electrophoresis, transferred to a membrane and probed with antibodies specific for HPV 18 E7, p53, actin, p110Rb, p21 and c-myc as indicated.

Figures 12 and 13 show a colony formation and MTT assay, respectively. HeLa cells were incubated for 2h with shRNA-expressing BL21(DE3) at different multiplicities of infection (MOI). 2h post-infection cells were washed trypsinized and counted and an equal number of cells for each MOI was added to a well of a six well plate (For CFA: added 500 cells to each well of a 6 well plate, for MTT added 5000 cells in each well of a 96 well plate). For colony formation, the cells were allowed to grow for 10 days and stained with Geimsa, MTT assay was analyzed at 72h post plating.

Figures 14 and 15 show real time PCR results following invasion assay of HeLa cells. HeLa cells were incubated for 2h with shRNA-expressing BL21(DE3) at different multiplicities of infection (MOI). Forty-eight hours post-infection cells were harvested and analyzed by real-time RT-PCR for both target and GAPDH mRNA levels. Data were normalized against the GAPDH signal. These data were then further normalized to untreated control cells.

Figure 16 shows the effects of downregulation of HPV E6 and E7 genes on tumor suppressor pathways and other downstream targets. HeLa cells were incubated for 2h with shRNA-expressing BL21(DE3) at different multiplicities of infection (MOI). Forty-eight hours post-infection cells were harvested and analyzed by western blotting. 50 µg of protein was loaded in each lane and resolved by gel electrophoresis, transferred to a membrane and probed with antibodies specific for HPV 18 E7, p53, actin, p110Rb as indicated.

Figure 17 shows real time PCR results following invasion assay of HeLa cells with a frozen aliquot of negative shRNA control and HPV shRNA in BL21 (DE3). HeLa cells were incubated for 2h with shRNA-expressing BL21(DE3) at different multiplicities of infection (MOI). Forty-eight hours post-infection cells were harvested and analyzed by real-time RT-PCR for both target and GAPDH mRNA levels. Data were normalized against the GAPDH signal. These data were then further normalized to untreated control cells.

Figure 18 shows the plating efficiency of frozen aliquots of negative shRNA control and HPV shRNA in BL21 (DE3). The frozen bacteria were thawed and resuspended to a final concentration of 3.38×10^8 cells/ml. Invasion assay was performed with this concentration taking 2 ml of 3.38×10^8 cells/ml as an MOI of 1000. Some stock control bacteria or HPV bacteria were serially diluted (1:100) and plated on LB plates to assess for the number and viability of bacteria treated cells at 48h. Gene silencing was analyzed either by quantitative real-time PCR using the $\Delta\Delta C_t$ relative quantitation method or by western blot analysis. HPVE6 mRNA levels were normalized to an endogenous control, GAPDH. The final data were further normalized to the RNA from the untreated

cells. For Protein analysis, cell lysates were prepared in Cell Lysis Buffer (Cell Signaling Technology) and the protein concentration was determined using a BCA kit from BioRad. For electrophoresis, the protein expression was normalized to Actin loading control.

5 Example 8: Knockdown of HPV E6 gene assessed by western blotting with HPV 18 E7 antibody:

Hela cells were incubated for 2h with shRNA-expressing BL21(DE3) (HPVH1 construct below) at different multiplicities of infection (MOI). Forty-eight hours post-infection cells were harvested and analyzed by western blotting. The HPV E6 specific knockdown was compared with a negative shRNA control. Briefly, 50 µg of protein was loaded in each lane and resolved by gel electrophoresis, transferred to a membrane and probed with antibodies specific for HPV 18 E7, and actin as indicated.

HPVH1

15 5'-GATCC TAGGTATTTGAATTTCAT TTCAAGAGA ATGCAAATCAAATACCTTTT G-3' (SEQ ID NO:389)
3'-G ATCCATAAACTTAAACGTA AAGTTCTCT TACGTTTAAGTTTATGGAAAA CAGCT-5' (SEQ ID NO:390)

Figure 19 shows the knockdown of HPV E6 gene assessed by western blotting with HPV 18 E7 antibody. Hela cells were incubated for 2h with shRNA-expressing BL21(DE3) at different multiplicities of infection (MOI). Forty-eight hours post-infection cells were harvested and analyzed by western blotting. The HPV E6 specific knockdown was compared with a negative shRNA control. Briefly, 50 µg of protein was loaded in each lane and resolved by gel electrophoresis, transferred to a membrane and probed with antibodies specific for HPV 18 E7 and actin as indicated.

Example 9: Inhibition of CCL20 Expression in CMT93 Cells

25 One confluent T-175 flask of CMT93 cells was trypsinized in 10mls until the cells detached. Trypsin was inactivated by addition of 30mls of DMEM (10% FCS, pen/strep) and the cells thoroughly mixed by pipetting. From this solution, 8mls was transferred into a sterile 50ml tube and 32mLs of DMEM 10% added. Cells were well mixed and 250uLs added to each well of a 48 well plate and incubated overnight at 37C resulting in adherent cells that were approximately 70% confluent the following morning. The next day, siRNA transfection complexes were created by the following method.

Sequences were ordered from Qiagen as pre-annealed siRNA duplexes. Each well was resuspended in 250ul of siRNA buffer (from Qiagen) to give a stock concentration of 20uM. The plate was then placed in a water bath at 95C for 5 minutes and then allowed to slowly cool to resuspend the duplexes and break apart aggregates. The suspended duplexes were then used in transfection experiments described in standard protocols. The formulation is per well of a 48 well plate containing 250uL of media; each screen was performed in biological triplicate so the solution was made for 4 wells; 3 for transfection and 1 extra.

0.3uL of the appropriate siRNA (from a 20uM stock solution) were diluted to 47uL with serum/antibiotic free media and mixed. To this solution was added 3uL of HiPerfect transfection reagent (Qiagen) followed by brief vortexing and incubation at room temperature for 20 minutes. 50uLs of the complex containing mixture was added to each of 3 wells in a 48 well plate containing CMT93 cells. Transfection was for 24 hours at 37C at which time the media was removed and replaced with 400uLs of DMEM/10% FCS containing 100ng/mL of LPS for 2 hours. Following stimulation, the cells were washed and RNA isolated for qRT-PCR according the Qiagen Quantitech method (see manufacturer's protocol) for 50 cycles.

Figure 20 shows the knockdown of CCL20 expression with the various siRNA sequences in CMT93 cells. The siRNA sequences tested are listed below:

Well	siRNA sense 5'→3'	SEQ ID NO:	siRNA antisense 5'→3'	SEQ ID NO:
G1	GCUUGUGACAUUAAUGCUAtt	391	UAGCAUUAAGUCACAAGCtt	392
H1	AUAAGCUAUUGUAAAGAUAtt	393	UAUCUUUACAAUAGCUUAUtg	394
G2	CAUCUUUCACACGAAGAAAtt	395	UUUCUUCGUGUGAAAGAUGat	396
H2	CUAUUGUAAAGAUUUUAAtt	397	UUAUAUUCUUUACAAUAGct	398
G3	GCCUAAGAGUCAAGAAGAUtt	399	AUCUUCUUGACUCUUAGGctg	400
G4	CAGUGGACUUGUCAUGGAtt	401	UCCAUUGACAAGUCCACUGgg	402
G5	GAAGUUGAUUCAUUGCAAtt	403	UGCAAUAUGAAUCAACUUCat	404
G6	GUUGAUUCAUUGCAUCAAtt	405	UGAUGCAAUAUGAAUCAACtt	406
G7	ACAUUAGAGUUAAGUUGUAAtt	407	UACAACUUAACUCUAAUGUga	408
G8	CAUUAGAGUUAAGUUGUAUtt	409	AUACAACUUAACUCUAAUGtg	410
G9	UGUUAUUUAUAGAUUGAAtt	411	UUCAGAUCUAUAAUAAACata	412
G10	GUUUAGCUAUUAAUGUUAAtt	413	UAACAUAUAAUAGCUAAACat	414
G11	AGUGGAAGGAUUAUUAUUAAtt	415	UAAUAUUAUCCUCCACUaa	416
F12	CCAGCACUGAGUACAUCAAtt	417	UUGAUGUACUCAGUGCUGGgt	418
G12	UGUUUAAGGGAUAGUUUAAtt	419	UAAACUAUCCCUUAAACata	420

Example 10: Inhibition of Expression of Claudin-2 in CMT93 cells

One confluent T-175 flask of CMT93 cells was trypsinized in 10mls until the cells detached. Trypsin was inactivated by addition of 30mls of DMEM (10% FCS, pen/strep) and the cells thoroughly mixed by pipetting. From this solution, 8mls was transferred into a sterile 50ml tube and 32mLs of DMEM 10% added. Cells were well mixed and 250uL added to each well of a 48 well plate and incubated overnight at 37C resulting in adherent cells that were approximately 70% confluent the following morning. The next day, siRNA transfection complexes were created by the following method:

Sequences were ordered from Qiagen as pre-annealed siRNA duplexes. Each well was resuspended in 250ul of siRNA buffer (from Qiagen) to give a stock concentration of 20uM. The plate was then placed in a water bath at 95C for 5 minutes and then allowed to slowly cool to resuspend the duplexes and break apart aggregates. The suspended duplexes were then used in

transfection experiments described in standard protocols. The formulation is per well of a 48 well plate containing 250uL of media; each screen was performed in biological triplicate so the solution was made for 4 wells; 3 for transfection and 1 extra.

- 0.3uL of the appropriate siRNA (from a 20uM stock solution) to 47uL of serum/antibiotic free media and mixed. To this solution was added 3uL of HiPerfect transfection reagent (Qiagen) followed by brief vortexing and incubation at room temperature for 20 minutes. 50uLs of the complex containing mixture was added to each of 3 wells in a 48 well plate containing CMT93 cells. Transfection was for 24 or 48 hours at 37C at which time the cells were washed and RNA isolated for qRT-PCR according the Qiagen Quantitech method (see manufacturer's protocol) for 50 cycles.
- Figure 21 shows the knockdown of Claudin-2 expression with the various siRNA sequences in CMT93 cells post 24hr transfection. The siRNA sequences tested are listed below:

Well	siRNA sense 5'→3'	SEQ ID NO:	siRNA antisense 5'→3'	SEQ ID NO:
D4	GCUGGGACUAUAUAUAUAAt t	421	UUAUAUAUAUAAGUCCCAGCca	422
D5	GGGCAAUUGCUAUAUCUUAAt t	423	UAAGAUUAAGCAAUUGCCCtc	424
D6	GCAGCCAAACGACAAGCAAt t	425	UUGCUGUCGUUUGGCUGCtg	426
D7	AGGGUUUCCUUAAGGACAAt t	427	UUGUCCUUAAGGAAACCCUtg	428
C9	GAAAUUGGAUUAGUCAGUAAt t	429	UUACUGACUAAUCCAUUUCtt	430
D11	GGCUCCGAAGAUACUUCUAAt t	431	UAGAAGUAUCUUCGAGCCTg	432

15

Example 11: Inhibition of Expression of IL6-Ra in CMT93 Cells

- One confluent T-175 flask of CMT93 cells was trypsinized in 10mls until the cells detached. Trypsin was inactivated by addition of 30mls of DMEM (10% FCS, pen/strep) and the cells thoroughly mixed by pipetting. From this solution, 8mls was transferred into a sterile 50ml tube and 32mLs of DMEM 10% added. Cells were well mixed and 250uLs added to each well of a 48 well plate and incubated overnight at 37C resulting in adherent cells that were approximately 70% confluent the following morning. The next day, siRNA transfection complexes were created by the following method:

- Sequences were ordered from Qiagen as pre-annealed siRNA duplexes. Each well was resuspended in 250ul of siRNA buffer (from Qiagen) to give a stock concentration of 20uM. The plate was then placed in a water bath at 95C for 5 minutes and then allowed to slowly cool to resuspend the duplexes and break apart aggregates. The suspended duplexes were then used in transfection experiments described in standard protocols. The formulation is per well of a 48 well plate containing 250uL of media; each screen was performed in biological triplicate so the solution was made for 4 wells; 3 for transfection and 1 extra.

0.3uL of the appropriate siRNA (from a 20uM stock solution) to 47uL of serum/antibiotic free media and mixed. To this solution was added 3uL of HiPerfect transfection reagent (Qiagen)

followed by brief vortexing and incubation at room temperature for 20 minutes. 50uLs of the complex containing mixture was added to each of 3 wells in a 48 well plate containing CMT93 cells. Transfection was for 24, 48 or 72 hours at 37C at which time the cells were washed and RNA isolated for qRT-PCR according the Qiagen Quantitech method (see manufacturer's protocol) for 40 cycles.

- 5 Figure 22 shows the knockdown of IL6-RA expression with the various siRNA sequences in CMT93 cells post 24hr transfection. The siRNA sequences tested are listed below:

Well	siRNA sense 5'→3'	SEQ ID NO:	siRNA antisense 5'→3'	SEQ ID NO:
E1	CCUGGAGGGUGACAAAGUAAt t	433	UACUUUGUCACCCUCCAGGAt	434
F1	GGUCUGACAAUACCGUAAAt t	435	UUUACGGUAUUGUCAGACCca	436
F2	GCUGUUUCCUAUAACAGAAAt t	437	UUCUGUUUAUAGGAAACAGCgg	438
F3	GCUGUGAAAGGGAAAUUUAAt t	439	UAAAUUUCCCUUUCACAGCag	440
E4	CCUUGUGGUAUCAGCCAUAt t	441	UAUGGCUGAUACCACAAGGt t	442
E5	GCUUCGAUACCGACCUGUAAt t	443	UACAGGUCGGUAUCGAAGCt g	444
E6	CGGCAGGAUCCUCUGGAAt t	445	UUCCAGAGGAUUCUCCGCGgg	446
E7	CCACGAGGAUCAGUACGAAt t	447	UUCGUACUGAUCCUCGUGGt t	448
E8	CACGAGGAUCAGUACGAAAt t	449	UUUCGUACUGAUCCUCGUGgt	450
E9	GAUCAGUACGAAAGUUCUAAt t	451	UAGAACUUUCGUACUGAUCCt	452
E10	GUACGAAAGUUCUACAGAAAt t	453	UUCUGUAGAACUUUCGUACt g	454
E11	GAAAGUUCUACAGAAAGCAAAt t	455	UUGCUUCUGUAGAACUUUCgt	456
E12	GGGUCUGACAAUACCGUAAt t	457	UUACGGUAUUGUCAGACCCag	458

Example 12: Inhibition of Expression of IL13-Ra1 in CMT93 Cells

10

One confluent T-175 flask of CMT93 cells was trypsinized in 10mls until the cells detached. Trypsin was inactivated by addition of 30mls of DMEM (10% FCS, pen/strep) and the cells thoroughly mixed by pipetting. From this solution, 8mls was transferred into a sterile 50ml tube and 32mLs of DMEM 10% added. Cells were well mixed and 250uLs added to each well of a 48 well plate and incubated overnight at 37C resulting in adherent cells that were approximately 70% confluent the following morning. The next day, siRNA transfection complexes were created by the following method:

15

Sequences were ordered from Qiagen as pre-annealed siRNA duplexes. Each well was resuspended in 250ul of siRNA buffer (from Qiagen) to give a stock concentration of 20uM. The plate was then placed in a water bath at 95C for 5 minutes and then allowed to slowly cool to resuspend the duplexes and break apart aggregates. The suspended duplexes were then used in transfection experiments described in standard protocols. The formulation is per well of a 48 well plate containing 250uL of media; each screen was performed in biological triplicate so the solution was made for 4 wells; 3 for transfection and 1 extra.

20

- 25 0.3uL of the appropriate siRNA (from a 20uM stock solution) to 47uL of serum/antibiotic free media and mixed. To this solution was added 3uL of HiPerfect transfection reagent (Qiagen)

followed by brief vortexing and incubation at room temperature for 20 minutes. 50uLs of the complex containing mixture was added to each of 3 wells in a 48 well plate containing CMT93 cells. Transfection was for 24 or 72 hours at 37C at which time the cells were washed and RNA isolated for qRT-PCR according the Qiagen Quantitech method (see manufacturer's protocol) for 40 cycles.

5 Figure 23 shows the knockdown of IL13-RA1 expression with the various siRNA sequences in CMT93 cells post 24hr transfection. The siRNA sequences tested are listed below:

Well	siRNA sense 5'→3'	SEQ ID NO:	siRNA antisense 5'→3'	SEQ ID NO:
F1	AGAAGACUCUAAUGAUGUAAt t	459	UACAUCAUUAGAGUCUUCUt g	460
F2	CAGUCAGAGUAAGAGUCAAt t	461	UUGACUCUUACUCUGACUGt g	462
F8	CAGAACAUUCUAGCAAACAAt t	463	UUGUUUGCUAGAUGUUCUGt g	464
F9	CUUGUAGGUUCACAUUUUAAt t	465	UAAUAUGUGAACCUACAAGt t	466
F10	CAGUGUAGUGCCAAUGAAAt t	467	UUUCAUUGGCACUACACUGag	468
F11	GUAUGACAUCUAUGAGAAAt t	469	UUUCUCAUAGAUGUCAUAAct t	470

10

Example 13: Inhibition of Expression of IL-18 in CMT93 Cells

One confluent T-175 flask of CMT93 cells was trypsinized in 10mls until the cells detached. Trypsin was inactivated by addition of 30mls of DMEM (10% FCS, pen/strep) and the cells thoroughly mixed by pipetting. From this solution, 8mls was transferred into a sterile 50ml tube and 15 32mLs of DMEM 10% added. Cells were well mixed and 250uLs added to each well of a 48 well plate and incubated overnight at 37C resulting in adherent cells that were approximately 70% confluent the following morning. The next day, siRNA transfection complexes were created by the following method:

Sequences were ordered from Qiagen as pre-annealed siRNA duplexes. Each well was 20 resuspended in 250ul of siRNA buffer (from Qiagen) to give a stock concentration of 20uM. The plate was then placed in a water bath at 95C for 5 minutes and then allowed to slowly cool to resuspend the duplexes and break apart aggregates. The suspended duplexes were then used in transfection experiments described in standard protocols. The formulation is per well of a 48 well plate containing 250uL of media; each screen was performed in biological triplicate so the solution 25 was made for 4 wells; 3 for transfection and 1 extra.

0.3uL of the appropriate siRNA (from a 20uM stock solution) to 47uL of serum/antibiotic free media and mixed. To this solution was added 3uL of Lipofectamine RNAiMAX transfection reagent (Invitrogen) followed by brief vortexing and incubation at room temperature for 20 minutes. 50uLs of the complex containing mixture was added to each of 3 wells in a 48 well plate containing 30 CMT93 cells. Transfection was for 24 hours at 37C at which time the cells were washed and RNA isolated for qRT-PCR according the Qiagen Quantitech method (see manufacturer's protocol) for 40 cycles.

Figure 24 shows the knockdown of IL18 expression with the various siRNA sequences in CMT93 cells post 24hr transfection. The siRNA sequences tested are listed below:

Well	siRNA sense 5'→3'	SEQ ID NO:	siRNA antisense 5'→3'	SEQ ID NO:
B2	AGGAAAUGAUGUUUAUUGAtt	471	UCAAUAAACAUCAUUUCCUtg	472
B3	GGCCGACUUCACUGUACAAtt	473	UUGUACAGUGAAGUCGGCCaa	474
B4	GAUGGAGUUUGAAUCUUCAtt	475	UGAAGAUUCAAACUCCAUCtt	476
B5	CAACCGCAGUAAUACGGAAtt	477	UUCCGUAAUACUGCGGUUGta	478
B6	CGAGGCUGCAUGAUUUUAUAtt	479	UAUAAAUCAUGCAGCCUCGgg	480
B7	CCUGUAUUUCCAUAAACAGAtt	481	UCUGUUAUGGAAAUACAGGcg	482
B8	CAUGUACAAAGACAGUGAAAtt	483	UUCACUGUCUUUGUACAUGta	484
B9	CGAGGAUAUGACUGAUUAUUt	485	AAUAUCAGUCAUAUCCUCGaa	486
B10	GGAUAUGACUGAUUAUGAUtt	487	AUCAUAUCAGUCAUAUCCtc	488
B11	CUAACUUACAUCAAAGUUAtt	489	UAACUUUGAUGUAAGUUAGtg	490
B12	CUCACUAACUUACAUCAAAtt	491	UUUGAUGUAAGUUAGUGAGag	492

5 Example 14: Inhibition of Expression of IL-7 in CMT93 Cells

One confluent T-175 flask of CMT93 cells was trypsinized in 10mls until the cells detached. Trypsin was inactivated by addition of 30mls of DMEM (10% FCS, pen/strep) and the cells thoroughly mixed by pipetting. From this solution, 8mls was transferred into a sterile 50ml tube and 32mLs of DMEM 10% added. Cells were well mixed and 250uLs added to each well of a 48 well plate and incubated overnight at 37C resulting in adherent cells that were approximately 70% confluent the following morning. The next day, siRNA transfection complexes were created by the following method:

Sequences were ordered from Qiagen as pre-annealed siRNA duplexes. Each well was resuspended in 250ul of siRNA buffer (from Qiagen) to give a stock concentration of 20uM. The plate was then placed in a water bath at 95C for 5 minutes and then allowed to slowly cool to resuspend the duplexes and break apart aggregates. The suspended duplexes were then used in transfection experiments described in standard protocols. The formulation is per well of a 48 well plate containing 250uL of media; each screen was performed in biological triplicate so the solution was made for 4 wells; 3 for transfection and 1 extra.

0.3uL of the appropriate siRNA (from a 20uM stock solution) to 47uL of serum/antibiotic free media and mixed. To this solution was added 3uL of Lipofectamine RNAiMAX transfection reagent (Invitrogen) followed by brief vortexing and incubation at room temperature for 20 minutes. 50uLs of the complex containing mixture was added to each of 3 wells in a 48 well plate containing CMT93 cells. Transfection was for 24 hours at 37C at which time the cells were washed and RNA isolated for qRT-PCR according the Qiagen Quantitech method (see manufacturer's protocol) for 40 cycles.

Figure 25 shows the knockdown of IL-7 expression with the various siRNA sequences in CMT93 cells post 24hr transfection. The siRNA sequences tested are listed below:

Well	siRNA sense 5'→3'	SEQ ID NO:	siRNA antisense 5'→3'	SEQ ID NO:
A1	GAUCCUACGGAAGUUAUGGtt	493	CCAUAACUCCGUAGGAUCcg	494
B1	CCAUGUCCAUGUUUCUUtt	495	AAAGAAACAUGGAACAUGGtc	496
A2	CCUCCCGCAGACCAUGUUCtt	497	GAACAUGGUCUGCGGGAGcg	498
A3	CUCCCGCAGACCAUGUUCtt	499	GGAACAUGGUCUGCGGGAGgc	500
A4	UCCCGCAGACCAUGUCCAtt	501	UGGAACAUGGUCUGCGGGAgg	502
A5	CCCGCAGACCAUGUCCAUtt	503	AUGGAACAUGGUCUGCGGAgg	504
A6	CCGCAGACCAUGUCCAUGtt	505	CAUGGAACAUGGUCUGCGGga	506
A7	CGCAGACCAUGUCCAUGUtt	507	ACAUGGAACAUGGUCUGCGgg	508
A10	AGACCAUGUCCAUGUUUCtt	509	GAAACAUGGAACAUGGUCUgc	510
A12	ACCAUGUCCAUGUUUCUUtt	511	AAGAAACAUGGAACAUGGUct	512

Example 15: Inhibition of Expression of Chitinase3-like-1 (CH13L1) Expression in CMT93

5 Cells

In a 1.7ml microcentrifuge tube, 2.4 µl of 20 µM double-stranded RNA solution (from Qiagen) was diluted into 394 µl Opti-MEM serum-free medium (Invitrogen) containing 1 µl Lipofectamine RNAiMAX (Invitrogen), mixed, and incubated 10 min at room temperature to enable the formation of transfection complexes. 100 µl of this mixture was added to each of three wells of a 24-well tissue culture dish, on top of which CMT-93 cells were plated in a 500 µl volume, resulting in a final volume of 600 µl per well and a final RNA concentration of 20 nM. After 24 h transfection, 0.1 µg/ml lipopolysaccharide (LPS) (Sigma) was added to each well and cells were incubated for a further 24 h to stimulate CHI3L1 production, after which cells were washed in PBS and harvested for RNA extraction. CMT-93 cells were prepared for transfection as follows. 1 confluent T-175 flask of CMT93 cells was trypsinized in 10mls until the cells detached. Trypsin was inactivated by addition of 30mls of DMEM (10% FBS) and the cells thoroughly mixed by pipetting. From this solution, 10mls was transferred into a sterile 50ml tube and 40mLs of DMEM 10% FBS added. Cells were well mixed and 500 µLs added to each well of a 24-well plate. This concentration of cells resulted in approximately 70% confluency after 24 h of growth.

Figure 26 shows the knockdown of CH13L1 expression with the various siRNA sequences in CMT93 cells post 24hr transfection. The siRNA sequences tested are listed below:

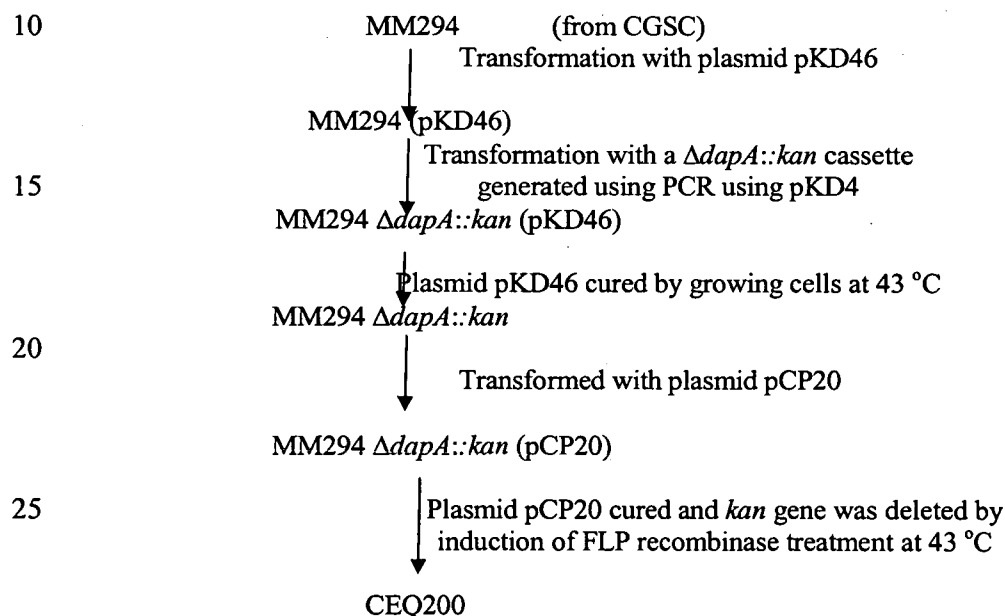
Well	siRNA sense 5'→3'	SEQ ID NO:	siRNA antisense 5'→3'	SEQ ID NO:
G1	CCACAUCAUCUACAGCUUUtt	513	AAAGCUGUAGAUGAUGUGGgt	514
H1	GGUUUGACAGAUACAGCAAtt	515	UUGCUGUAUCUGUCAAACct	516
G2	UCAUCUACAGCUUUGCCAAtt	517	UUGGCAAAGCUGUAGAUGAtg	518
H2	CCCUGUUAAGGAAUGCAAAtt	519	UUUGCAUUCUUAACAGGGtt	520
H3	CAAGUAGGCAAAUAUCUUAAtt	521	UAAGAUUUUGCCUACUUGat	522
H4	CAGCUUUGUCAGCAGGAAAtt	523	UUUCCUGCUGACAAAGCUGcg	524
G5	GGUUCACCAAGGAGGCAGGtt	525	CCUGCCUCCUUGGUGAACCGg	526

H5	GGAUCAAGUAGGCAAUAUtt	527	AUAUUUGCCUACUUGAUCCaa	528
H6	GAGGGACCAUACUAAUUAUtt	529	AUAAUUAGUAUGGUCCCUca	530
G7	GGCCGGUUCACCAAGGAGGtt	531	CCUCCUUGGUGAACCGGCctg	532
H7	GGACAAGGAGAGUGUCAAAtt	533	UUUGACACUCUCCUUGUCctc	534
G8	CCGGUUCACCAAGGAGGCAtt	535	UGCCUCCUUGGUGAACCGGcc	536
H8	CGUACAAGCUGGUCUGCUAtt	537	UAGCAGACCAGCUUGUACGca	538
G9	GGAGUUUAUCUCUUGCAAtt	539	UUGCAAGAGAUUAAACUCctg	540
H9	CAAGGAACUGAAUGCGGAAtt	541	UUCCGCAUUCAGUUCUUGat	542
G10	CCCUGAUCAAGGAACUGAAtt	543	UUCAGUCCUUGAUCAGGGtg	544
H10	CUUGGAUCAAGUAGGCAAAtt	545	UUUGCCUACUUGAUCCAAGtg	546
G11	GGAUUGAGGGACCAUACUAAtt	547	UAGUAUGGUCCCUCAAUCctg	548
G12	GCAAUUCUCAGACUCUAAtt	549	UUAGAGUCUGAGAAUUGCat	550
H12	CCUCCCCUAGGAACUUAAtt	551	UUAAGUCCUAAGGGAAGGat	552

Example 16: Construction of CEQ200

CEQ200 has the following genotype: *glnV44(AS)*, *LAM^r*, *rfbC1*, *endA1*, *spoT1*, *thi-1*, *hsdR17*, (*r_k⁻m_k⁺*), *creC510 ΔdapA*. The MM294 has the following genotype: *glnV44(AS)*, *LAM^r*, *rfbC1*, *endA1*, *spoT1*, *thi-1*, *hsdR17*, (*r_k⁻m_k⁺*), *creC510*. We purchased the plasmids from CGSC (see Datsenko et al., (2000) Proc. Natl. Acad. Sci. USA 97,6640-6645).

Derivation of CEQ200



Example 17: Construction of CEQ201

CEQ201 has the following genotype: CEQ200 [*glnV44(AS)*, *LAM^r*, *rfbC1*, *endA1*, *spoT1*, *thi-1*, *hsdR17*, (*r_k⁻m_k⁺*), *creC510 ΔdapA ΔrecA*]. The MM294 has the following genotype: *glnV44(AS)*, *LAM^r*, *rfbC1*, *endA1*, *spoT1*, *thi-1*, *hsdR17*, (*r_k⁻m_k⁺*), *creC510*. We purchased the plasmids from CGSC (see Datsenko et al., (2000) Proc. Natl. Acad. Sci. USA 97,6640-6645).

5



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Example 19: Illustration of the pMBV40, pMBV43 and pMBV44 plasmids

The pMBV40, pMBV43 and pMBV44 plasmids may be used as final or intermediary plasmid in the tkRNA system and may be constructed as follows: pUC19 digested with restriction enzyme *Pvu*II. Resultant ~ 2.4 kb fragment was ligated with a ~ 200 bp DNA fragment generated by annealing 5 oligonucleotides with each other. The oligonucleotides have the following names and sequences:

10 **OHTOP1**: GACTTCATATACCCAAGCTTGGAAAATTTTTTTTAAAAAGCTTGACACTTTATGCTTCCGGCTCGTATAATGG
ATCCAGGAGTAACAATACAAATGGA (SEQ ID NO:553)

OHTOP2: TTCAAGAGATCCATTTGTATTGTTACTCCTTTTTTTTTTTTGTGACGATCCTTAGCGAAAGCTAAGGATTTTTTT
TTTACTCGAGCGGATTACTACATAC (SEQ ID NO:554)

15 **OHBOT1**: GTATGTAGTAATCCGCTCGAGTAAAAAATCCTTAGCTTTCGCTAAGGATCGTCGACAAAAA
(SEQ ID NO:555)

OHBOT2: AGGAGTAACAATACAAATGGATCTCTGAATCCATTTGTATTGTTACTCCTGGATCCATT (SEQ ID
NO:556)

20 **OHBOT3**: ATACGAGCCGGAAGCATAAAGTGTCAGACTTTTTTAAAAAATTTTCCAAGCTTGGGTATATGAAGTC
(SEQ ID NO:557)

↓

25 Ligation mix was transformed in *E. coli* and Ampicillin
resistant transformants were selected. Plasmid DNA from a
transformant that had the expected DNA sequence of the insert
and restriction map was named pMBV38.

↓

30 pMBV38 was digested with *Nde*I and blunt end ligated with a
~ 6 kb fragment generated by *Bam*HI-*Sal*I digestion of the
plasmid pKSII-inv-hly

The predicted sequence of pKSII-inv-hly is as follows:

35 CTAATTGTAAGCGTTAATATTTTGTAAAATTCGCGTTAAATTTTGTAAATCAGCTCATTTTTTTAACCAA
TAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAGAATAGACCGAGATAGGGTTGAGTGTGTTCCAGTTT
GGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATGG
CCCACTACGTGAACCATCACCTAATCAAGTTTTTTGGGGTTCGAGGTGCCGTAAAGCACTAAATCGGAACCTT
AAAGGGAGCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGA
40 AAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTACGCTGCGCGTAACCACCACACCCGCCGCGCTTAA
TGCGCCGCTACAGGGCGCGTCCCATTCGCCATTAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCC
TCTTCGCTATTACGCCAGCTGGCGAAAGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAAACGCCAGGGTTTT
CCCACTCACGACGTTGTAAAACGACGGCCAGTGAGCGCGGTAATACGACTCACTATAGGGCGAATTGGAGCT
CCACCGCGTGGCGCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGCTGGGCGGTAAGATCGGCATTAA
45 TCGCGACAATCCTTTTTAAAAAACAGCGCGCTCAATTAACCTGAGCGGCGTTGTTCTTCTGGACGTTTGCTA
CTTATGGGGCGAGTCTAGGATTGCCGGAATCCCATTCGCGCCCCAAATAATCAGCTCATTAACTGTTCTTAT
TGCTATCTGTTATCTGGTTATATTGACAGCGCACAGAGCGGGAACGCCAAGTATGCAGGCCCTGGTTGCAGTG
CGCCTGTGTCCATATTCATGGTTTTCAAATCCGTGCTGGTCTTTTTGACCCAATATTCACCAGATTGCCAATC
AGAATATACCGGTCAAGCTCCCCCACTCGCCCCAATGTCCCGTCAGGCGCACGCGTCCGTTGGTTGCA
50 CGTGAGGATTCAAGAACCGCAGACATATCTGAACCTTGGCATTGTCTGCTGGCCTCGAGACTGGATAACGAGC
ATCTGCCGCCATCGTATATCCACCGATTGGGTAGAACCGATAACTACCGAATAACTTGGGAATTTTTTACT
TTTCGCCGTCACAGCCACTTCGCTATAGGTTTGGTAGGTAATCGTCACCTGACCTGATCGTTAACCGATACA
TTGGGTGTGAATGACGACGACCACTCATACTGAGTATTATTAGCAACATCGTTATCCATCTGTAACCTGGAATG
TGGCGTTTTTAAAGATCGTTTTTCGGGAACCTTTATCCGTCAGCGAAATTTTGCCCGTTAACCGAATACCGGT
55 CAGCGTAGGTACCGGAATAGGGATATTTTTTTCTGCAATGTACTCAGTATCAGGGTATCAACCTGCGGCGTG

ATTGTGACATCACCGACACTATTCCCAACCACCGTCGCGGTATAGCTATCTGGCTGCTCGGTAATGGGGCTAA
TACTCACCGGCACACCGTTTTGAGTAAAACTCAAGCCCTGCATCCCACTGATAAAATGGCCATTCTTATCGAC
AGGGACAAAGGATAATGTGGAACATCATCGTGCCATCAGCCAAGATATCCGGTGTGGAGACCGTGAAACTGGAG
CGGCCAGCATCTGGAATAGGATCTGCCGTGAAATTAACCGTCACACTCGGCACACTGAACGCAGCCCCATCCA
5 CTTTCACCGTTACTGTTGCTACCCCCAACGTGGTACTGGTCAATGGTGCGCTATAAGTGCCGTCAATTGTGATC
CGTGATAACGCCCATATTGCCTAAGGTTGTGTCAAAAGCCACATTTCGCGCCAGCCTGCGGGTCCCCATAGGTA
TCCTTCAACTCCAACGTGATGGTTGAAGCCATTAGACCATCAGCGATGATAGATGTCGGTACCGCAGCCAGAG
TGGATTATCCGCCGCGATAGTACCCTTAACAAAGTGGGTATCAACACTTTGCCGTTGCCCTCCACTTCTGC
TGTGACTACCGTCACGCCATCTGTCGTATTGGTTAATGCAATGCGCGGACGCCATTTGCATCTGTCTTTTCC
10 GTGATTTTATTCCGTAGCGCACCATTATTGGTGGTTATCACCACCTCCTGCCCGGCTAAGGGTTTCCCCTCAA
AATCAGCAACGGTGAACCTCAACGGTGATTGCAGTTTCCCATTAGCCGGTGCGCCATCACCATGACGGCCGC
CGTTAATGTCAACTGAGGCTGCTGAACGGTGACGCTCAATGTGAATGAGTTAGATCGGTTTCTTGGTGATCA
ACCGCGAGCGCACTAAGCGAATAAAAGTTGGCTGTGAGGTGCTCCGTTACCCGACTCACTTGTGCTGTGCGTT
TATAAGGCGGTAAAACCAAGTTGAATTGTGTGGTACTCAGTGGTGTAAATGTGCCGCCAGCGGCAATCAGTTC
15 GGCATCACTCCAGACAATTTCCCTTACAGCAGATGCCCTTGTACTTGTGCGTTACCTGATAAACCTGACCC
GGCAGGCCGGAGATAGTTGCTGGCGATAATGTCTAGTTTAAACACCTGCTGTTTCTGATACTCCAACACGATAT
TATTGTTACGATCGACAAGGTTATAGCGGCTCTCCGCCAGTAGACGTGTTCTGCCACCGCTGAAGGGCTAAG
TTGCGACTGAAAACCTCTCGCCAGCGGATAGTTCAATTGGAGGTTCCACTGTGTTTTCATGCTTACTGCTTTTC
CCCATACGCTGATCTACCCCGACAGTGAGTAGAGGCACGGGGGTGTAATTGATCCCGGCAGTCACGGCATAAG
20 GGTGCGTGTGAGATTATCTTTACCAAATAAAGCAACACGCTCACCGGTGTATTGCTCATACATCAACTTCCC
CCCCAGTTGTGGGAGTGACAGGTAATAAGCATTCGCGCGCAATCCCCCCAGTGGCTGGGCGCTCTTTATAG
TCGGAGAAATCACGCGACGAGTGCCATCCATTGAGGCGAAAATACCCATTGGCAGCCAACTGTAAATAATCGG
TCCAGGCTCGGCACCAAGACCGATACGGTGGTTGTGGCCGGTCAAATCATTATCATAAAAAGTATTAAGTCC
GTACAGCCAACCGTTCTCCAATGTACGTATCCCGACGCCAAGGTTAAGTGTGTTGCCGCTGTCTTTATTGCGA
25 ATACCTAAGTACTGATAAAAAGAGGAATGAAGCAGAGTCATACCAAGGAGCCAGCCAATCAAGAGAGCTTTCTT
TTAGCGAAAAATTTTTGTCAAATTCAGATTAACCTTGAGCCGTACCGAATCGATTTAACCACTGTTTGTATTC
TTGATTAACCGCATCGCCACCATTGAGTGAGCAACATCAGATGCCCTGCCTGATGCAGCTAACCTGGCCCCG
GTGCTTATCATCTTATTACCGCTTCAGTCTCCTGCTCCTTATTGGCGCGATCTATTATTGCAGCATTTCTTT
CTGTATCCGATGCGGAAAAGGGATTGATTGAATCTCCATTTTATTATTAGGATGGAGATTTCAAATGCAGA
30 TGAAGAGACAGAATAAGGCTGGACCTGTTGCGGTGCGTTAGCATCATATTTTTCTGAAGCCCCAGCCATGAAC
ATTCCACATATCAAAAAGATACAAATAACTATTTCGTGAAATAATATTAATGAAATTTATTTATTAAATACA
TAGACATTTCCCGCATTCCTTATCAAGAGAACTCACTGATTGGCTGGAAGAACCATCATAATTTAAATGAAATA
AAGCATACCTGTCTACGTCAAACCTGCATGTGCGTTGGCTGTGCTCAACAACCTGAGTTATTTGAGGTATAAC
TGGCCACAAACGAGCATTTGAAATCACCTTGACCATTAATTAAGATGCAATAGTTGAAAGTGAACCTTGTTT
35 TCTAATTTAGTAAAGACATTAAAGAGGATAGCACTTTTTTAAAAAACAGACTGGGCAGATTAAAAATATTCAA
AATATATAATAAAACAGTCTATACCATACAGCGATAGAATTGATTTATTGTAACCTAAGCAGGTGAGAATATCA
AAAAAACAAAAATACAAATGAATATTATCATATAAATAATATCAATTAGAATAAGCCCCCTTCATTTGAT
GTTGTGAGTTGTCTGCTGCGGTTTTTATTCTACTTTAGTCTGAAGTGTACTCCGCAATATCCGCATTAAT
CCTGATGGTTGCCTTGATGACTGCAGGAATTCGATCCCTCCTTTGATTAGTATATCTCTATCTTAAAGTGACT
40 TTTATGTTGAGGCATTAAACATTTGTTAACGACGATAAAGGGACAGCAGGACTAGAAATAAGCTATAAAGCAAG
CATATAATATTGCGTTTCATCTTTAGAAGCGAATTTCCGCAATATTATAATTATCAAAAGAGAGGGGTGGCAA
ACGGTATTTGGCATTATTAGGTTAAAAATGTAGAAGGAGAGTGAAACCCATGAAAAAATAATGCTAGTTTT
TATTACACTATATTAGTTAGTCTACCAATTGCGCAACAACTGAAGCAAGGATGCATCTGCATCTCAATAAA
GAAATTTCAATTTTATCATCCATGGCACCACCGCATCTCCGCTGCAAGTCTTAAGACCGCAATCGAAAAAGAAC
45 ACGCGGATGAAATCGATAAGTATATACAAGGATTGGATTACAATAAAAAACAATGTATTAGTATACCACGGAGA
TGCACTGACAAATGTGCCGCCAAGAAAAGGTTACAAAGATGGAATGAATATATTGTTGTGGAGAAAAAGAAG
AAATCCATCAATCAAAATAATGCAGACATTCAAGTTGTGAATGCAATTTTCGAGCCTAACCTATCCAGGTGCTC
TCGTAAAAGCGAATTCCGAATTAGTAGAAAAATCAACCAGATGTTCTCCCTGTAAAACGTGATTCAATAACCT
CAGCATTTGATTGCCAGGTATGACTAATCAAGACAATAAAATCGTTGTAAAAAATGCCACTAAATCAAACGTT
50 AACAACGCAGTAAATACATTAGTGGAAGATGGAATGAAAAATATGCTCAAGCTTATCCAAATGTAAGTGCAA
AAATTGATTATGATGACGAAATGGCTTACAGTGAATCACAATTAATTGCGAAATTTGGTACAGCATTTAAAGC
TGTAATAATAGATTGAATGTAACCTTCGGCGCAATCAGTGAAGGGAAAAATGCAAGAAGAAGTCATTAGTTTT
AAACAAATTTACTATAACGTGAATGTTAATGAACCTACAAGACCTTCCAGATTTTTCGGCAAAGCTGTTACTA
AAGAGCAGTTGCAAGCGCTTGGAGTGAATGCAGAAAAATCCTCCTGCATATATCTCAAGTGTGGCGTATGGCCG
55 TCAAGTTTATTTGAAATTATCAACTAATTCCTATAGTACTAAAGTAAAAGCTGCTTTTGTGCTGCCGTAAGC
GGAAAACTGTCTCAGGTGATGTAGAATAACAAATATCATCAAAAATTTCTTCTTCAAAGCCGTAATTTACG
GAGGTTCCGCAAAAGATGAAGTTCAAATCATCGACGGCAACCTCGGAGACTTACGCGATATTTTGAAGAAAGG
CGCTACTTTTAATCGAGAAACACCAGGAGTCCCATTTGCTTATACAACAACTTCTTAAAAGACAATGAATTA
GCTGTTATTAACAACTCAGAATATATTGAAACAACCTTCAAAGCTTATACAGATGGAAGAAATTAACATCG
60 ATCACTCTGGAGGATACGTTGCTCAATTCAACATTTCTTGGGATGAAGTAAATTAATGATCCTGAAGGTAACGA
AATTGTTCAACATAAAAACTGGAGCGAAAAACAATAAAAGCAAGCTAGCTCATTTACATCGTCCATCTATTTG
CCAGGTAACGCGAGAAATATTAATGTTTACGCTAAAGAATGCACTGGTTTAGCTTGGGAATGGTGGAGAACGG

TAATTGATGACCGGAACTTACCACTTGTGAAAAATAGAAATATCTCCATCTGGGGCACCACGCTTTATCCGAA
 ATATAGTAATAAAGTAGATAATCCAATCGAATAATTGTAAAAGTAATAAAAAATTAAGAATAAAACCGCTTAA
 CACACACGAAAAATAAGCTTGTGTTTTGCACTCTTCGTAAATTATTTTGTGAAGAATGTAGAAACAGGCTTATT
 5 TTTTAATTTTTTTAGAAGAATTAACAAATGTAAAAGAATATCTGACTGTTTATCCATATAATATAAGCATATC
 CCAAAGTTTAAGCCACCTATAGTTTCTACTGCAAAACGTATAATTTAGTTCCCACATATACTAAAAACGTGT
 CCTTAACCTCTCTGTGTCAGATTAGTTGTAGGTGGCTTAACTTAGTTTACGAATTAAGGAGCGGTGAAA
 TGAAAAGTAACTTATTTGTATCATCATGGTAATAGCTTTTCAGGCTCATTTCACATGACGGTAAAAGCAGA
 TTCTGTGCGGGGAAGAAAACTTCAAAATAATACACAAGCCAAAAAGACCCCTGCTGATTTAAAAGCTTATCAA
 GCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAGCTTTTGTTCCTTTAGTGAGGGTTAATTGC
 10 GCGCTTGGCGTAATCATGGTCATAGCTGTTTCTGTGTGAAATTGTTATCCGCTCACAATTCACACAACATA
 CGAGCCGGAAGCATAAAGTGTAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTCGCGT
 CACTGCCCCGCTTCCAGTCGGGAAACCTGTCTGCGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGG
 CGGTTTGCCTATTGGGCGCTCTTCCGCTTCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCCGGTTCGCGCGA
 GCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGT
 15 GAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCTTGCTGGCGTTTTTCCATAGGCTCCGCCC
 CCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACAG
 GCGTTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCCTGCCGCTTACCGGATACCTGTCCGCT
 TTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCAGCTGTAGGTATCTCAGTTCGGTGTAGGTGCGTTCCG
 CTCCAAGCTGGGCTGTGTGCACGAACCCCGCTTACGCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTT
 20 GAGTCCAACCCGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGT
 ATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAATACGGCTACACTAGAAGGACAGTATTTGGTAT
 CTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCCCGCT
 GGTAGCGGTGGTTTTTTTTGTTTGAAGCAGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGA
 TCTTTTCTACGGGTCTGACGCTCAGTGGAACGAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAA
 25 AAGGATCTTCACCTAGATCCTTTTAAATTAATAAATGAAGTTTAAATCAATCTAAAGTATATATAGTAAACT
 TGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTTCATCCATAG
 TTGCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGTGCAATGAT
 ACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACAGCCAGCCGGAAGGGCCGAGCGCAGA
 AGTGGTCTGCAACTTTATCCGCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGC
 30 CAGTTAATAGTTTGCAGAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTACGCTCGTCTGTTGGTATGGC
 TTCATTACAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGTTAGC
 TCCTTCGGTCTCCGATCGTTGTGAGAAGTAAGTTGGCCGAGTGTATCACTCATGGTTATGGCAGCACTGC
 ATAATTCTCTTACTGTGATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTG
 AGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGA
 35 ACTTTAAAGTGCTCATCATTGGAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGAT
 CCAGTTCGATGTAACCCACTCGTGACCCAACTGATCTTCAGCATCTTTTACTTTTACCAGCGTTTCTGGGTG
 AGCAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAATGTTGAATACTCATACTC
 TTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTGAATGTATTT
 AGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCAC (SEQ ID NO:558)

40

↓
 Ligation mix was transformed in *E. coli* and Ampicillin
 resistant transformants were selected. Plasmid DNA from a
 transformant that had insertion of *inv* and *hly* genes was
 named **pMBV40**.

45

↓
 pMBV40 was digested with *Bsp*HI and the resultant 7.4 kb
 DNA fragment was ligated with a PCR fragment containing
kan gene generated using plasmid pKD4 (purchased from
 CGSC (see Datsenko et al., (2000) Proc. Natl. Acad. Sci. USA
 97,6640-6645) as the template.

50

↓
 Ligation mix was transformed in *E. coli* and Kanamycin
 resistant transformants were selected. They were screened
 restriction mapping. They two different orientation of *kan*
 gene. The plasmids having clockwise and anticlockwise
 orientation of open reading frame of *kan* gene were called
 pMBV43 and pMBV44, respectively

55

As shown in Figure 27, the pMBV40 (*amp* selected having H3 hairpin) or pMBV43 and pMBV44 (*kan* selected having H3 hairpin) plasmids, are followed by the respective sequences.

5 pMBV40
 TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAG
 CGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTGGCGGGTGTGGGGCTGGCTTAACATATG
 CGGCATCAGAGCAGATTGTAAGAGTGCACCATATCGACGGTATCGATAAGCTTGATAAGCTTTTAAATCAGC
 AGGGGTCTTTTGGCTTGTGTAATTTTGAAGTTTTCTTCCCGACAGAATCTGCTTTTACCGTCATAGTGAA
 10 ATGAGCCTGAAAAGCTATTACCATGATGATACAAATAAGTTTACTTTTCATTTACCGCTCCTTTTAAATTCGTA
 AAATAAGTTTAAAGCCACTACAATAATCTGACAGAGAGAGTTAAGGACAGCTTTTGTATATGTGGGAACCT
 AAATTATACGTTTTTGCAGTAGAACTATAGGTGGCTTAAACCTTTGGGATATGCTTATATTATATGGATAAACAGT
 CAGATATTCTTTTACATTTGTTAATTCTTCTAAAAAATTAAGCCTGTTTCTACATTCTTCACAAAAT
 AATTTACGAAGAGTGCAAAACAAGCTTATTTTTCTGTGTGTGTTAAGCGGTTTTATTCTTAATTTTTTATTACTT
 15 TTACAATTATTCGATTGGATTATCTACTTTATTACTATATTTTCGGATAAAGCGTGGTGCCCGAGATGGAGATATT
 TCTATTTTTTACAAAGTGGTAAGTTCCGGTCATCAATTACCGTTCTCCACCATTCCCAAGCTAAACCAGTGCATTC
 TTTAGCGTAACATTAAATTTCTCGCGTTACCTGGCAAATAGATGGACGATGTGAAATGAGCTAGCTTGCCTTTT
 ATTGTTTTCTCGCTCCAGTTTATGTTGAACAATTTCTGTTACCTTCAGGATCATAATTTACTTCATCCCAAGAAAT
 GTTGAATTGAGCAACGTATCCTCCAGAGTGATCGATGTTAATTTTTCCATCTGTATAAGCTTTTGAAGTTGTTTC
 20 AATATATTCTGAGTTGTTTTAATAACAGCTAATTCATTGTCCTTTAGGAAGTTTGTGTATAAGCAATGGGAAC
 TCCTGGTGTTCCTCGATTAAAGTAGCGCCTTTTTTCAAAATATCGCGTAAGTCTCCGAGGTTGCCGTCGATGAT
 TTGAACCTTCATCTTTTGCAGAACCTCCGTAAATTACGGCTTTGAAGGAAGAATTTTTGATGATATTGTTAGTTT
 TACATCACCTGAGACAGATTTTCCGCTTACGGCAGCATCAAAAGCAGCTTTTACTTTAGTACTATGGGAATTAGT
 TGATAATTTCAAATAAACTTGACGGCCATACGCCACACTTGAGATATATGCAGGAGGATTTTCTGCATTCACTCC
 25 AAGCGCTTGCAACTGCTCTTTAGTAACAGCTTTGCCGAAAAATCTGGAAGGTCTGTAGGTTTATTAACATTAC
 GTTATAGTAATTTGTTTTAAACTAATGACTTCTTCTGCAATTTCCCTTCACTGATTGCGCCGAAGTTTACATT
 CAAGCTATTATTTACAGCTTTAAATGCTGTACCAAATTTTCGCAATTAATTGTGATTCACTGTAAGCCATTTCTGTC
 ATCATAATCAATTTTGCACCTTACATTTGGATAAGCTTGAGCATATTTTTTCACTCCATCTTTCCACTAATGTATT
 TACTGCGTTGTTAACGTTTGATTAGTGGCATTTTTTACAACGATTTTATTGTCTTGATTAGTCATACCTGGCAA
 30 ATCAATGCTGAGTGTAAATGAATCAGTTTTTACAGGGAGAACATCTGGTTGATTTTCTACTAATTCGAATTCCGC
 TTTTACGAGAGCACCTGGATAGGTTAGGCTCGAAATTGCATTCAACAATGAAATGTCTGCATTATTTTGATTGAT
 GGATTTCTTCTTTTCTCCACAACAATATATTCATTTCCATCTTTGTAACCTTTTCTTGGCGGCACATTTGTCAC
 TGCATCTCCGTGGTATACTAATACATTGTTTTTATTGTAATCCAATCCTTGTATATACTTATCGATTTCATCCGC
 GTGTTTCTTTTTCGATTGGCGTCTTAGGACTTGCAGGCGGAGATGCTGGTGGTGCCATGGATGAAATTGAATTTTC
 35 TTTATTGAATGCAGATGCATCCTTTGCTTCAGTTTGTGCGCAATTGGTAGACTAACTAATAAGTGAATAAAA
 AACTAGCATTATTTTTTTCATGGGTTTCACTCTCTTCTACATTTTTTAACTAATAATGCCAAATACCGTTTGC
 CACCCCTCTCTTTTGATAATTATAATATTGGCGAAATTCGCTTCTAAAGATGAAACGCAATATTATATGCTTGCT
 TTATAGCTTTATTTAGTCTGCTGCTCCCTTTATCGTCGTTTAAACAAATGTTAATGCCTCAACATAAAAGTCACTT
 TAAGATAGGAATATACTAATCAAAAGGAGGATCGAATTCCTGCATCATCAAGGCAACCATCAGGATTAATGGCG
 40 ATATTGCGGAGTAACACTTCAGACTGAAAGTAGAATAAAAAACCGCAGCAGACAACAGCAACATGAAAGG
 GGGCTTATTCTAATTGATATTATTTATATGATAATAGTTTCATTTGTATTTTTGTTTTTTTGTATTTCTCACCT
 GCTTAGTTACAATAAATCAATTCTATCGCTGTATGGTATAGACTGTTTTATTATATATTTTGAATATTTTAAATC
 TGCCAGTCTGGTTTTTTAAAAAGTGCTATCCTCTTAATGTCTTTACTAAATTAGAAAACAAGTTTCACTTTCA
 ACTATTGCATCTTTAATTAATGGTCAAGGTGATTTCAAATGCTCGTTTGTGGCCAGTTATACCTCAAATAACTCA
 45 AGTTGTTGAGCACAGCCAACGCACATGCAGTTTGACGTATGACAGGTATGCTTTATTTTCAATTTAAATTATGATGG
 TTTTCCAGCCAATCAGTGAGTTTCTCTTGATAAGGAATGCGGGAATGTCTATGTATTTTAAATAAAATAATTTAT
 TTAATATTATTTACGAATAGTTATTTGTATCTTTTTGATATGTGGAATGTTTATGCTGGGCTTCCAGAAAAAT
 ATGATGCTAACGCACCGCAACAGGTCAGCCTTATCTGCTCTTCACTGCAATTTGAAAATCTCCATCTCAATA
 ATGAAATGGAGAGTTCAATCAATCCCTTTTCCGATCGGATACAGAAAAGAAATGCTGCAATAATAGATCGCGCCA
 50 ATAAGGAGCAGGAGACTGAAGCGGTGAATAAGATGATAAGCACCGGGGCCAGGTTAGCTGCATCAGGCAGGGCAT
 CTGATGTTGCTCACTCAATGGTGGGCGATGCGGTTAATCAAGAAATCAAACAGTGGTTAAATCGATTCCGGTACGG
 CTCAAGTTAATCTGAATTTTGACAAAAATTTTTCGCTAAAAGAAAGCTCTCTTGATTGGCTGGCTCCTTGGTATG
 ACTCTGCTTCATTCCTCTTTTTTAGTCAGTTAGGTATTCGCAATAAAGACAGCCGCAACACACTTAACCTTGGCG
 TCGGGATACGTACATTGGAGAACGGTTGGCTGTACGGACTTAATACTTTTTATGATAATGATTTGACCGGCCACA
 55 ACCACCGTATCGGTCTTGGTGCCGAGGCCTGGACCGATTATTTACAGTTGGCTGCCAATGGGTATTTTCGCCTCA
 ATGGATGGCACTCGTCGCGTGATTTCTCCGACTATAAAGAGCGCCAGCCACTGGGGGGGATTTGCGCGCGAATG
 CTTATTTACGTCACTCCCACAACCTGGGGGGGAAGTTGATGTATGAGCAATACACCGGTGAGCGGTGTGCTTTAT
 TTGGTAAAGATAATCTGCAACGCAACCCTTATGCGGTGACTGCCGGGATCAATTACACCCCGTGCCCTACTCA
 CTGTCCGGGTAGATCAGCGTATGGGGAAAAGCAGTAAGCATGAAACACAGTGAACCTCCAAATGAACCTATCGCC
 60 TGGGCGAGAGTTTTTCACTCGCAACTTAGCCCTTACGCGGTGGCAGGAACACGTCTACTGGCGGAGAGCCGCTATA

ACCTTGTGATCGTAACAATAATATCGTGTTGGAGTATCAGAAACAGCAGGTGGTTAAACTGACATTATCGCCAG
CAACTATCTCCGGCCTGCCGGTCAGGTTTATCAGGTGAACGCACAAGTACAAGGGGCATCTGCTGTAAGGGAAA
TTGTCTGGAGTGATGCCGAACGATTGCGGCTGGCGGCACATTAACACCACTGAGTACCACACAATTCAACTTGG
TTTTACCGCCTTATAAACGCACAGCACAAGTGAGTCGGGTAACGGACGACCTGACAGCCAACCTTTTATTCGCTTA
5 GTGCGCTCGCGGTTGATCACCAAGGAAACCGATCTAACTCATTACATTGAGCGTCACCGTTCAGCAGCCTCAGT
TGACATTAAACGGCGGCCGTCATTGGTGATGGCGCACCGGCTAATGGGAAAACCTGCAATCACCGTTGAGTTCACCG
TTGCTGATTTTGGGGGAAACCCCTTAGCCGGGCAGGAGGTGGTGATAACCACCAATAATGGTGCGCTACCGAATA
AAATCACGGAAGACAGATGCAAAATGGCGTCGCGCGCATTGCATTAACCAATACGACAGATGGCGTGACGGTAG
TCACAGCAGAAGTGGAGGGCAACGGCAAAGTGTTGATACCCACTTTGTTAAGGGTACTATCGCGGCGGATAAAT
10 CCACTCTGGCTGCGGTACCGACATCTATCATCGCTGATGGTCTAATGGCTTCAACCATCACGTTGGAGTTGAAGG
ATACCTATGGGGACCCGACGGCTGGCGCGAATGTGGCTTTTGACACAACCTTAGGCAATATGGGCGTTATCACGG
ATCACAATGACGGCACTTATAGCGCACCATTGACCAGTACCACGTTGGGGGTAGCAACAGTAACGGTGAAAGTGG
ATGGGGCTGCGTTCAGTGTGCCGAGTGTGACGGTTAATTTACCGGCAGATCCTATTCCAGATGCTGGCCGCTCCA
GTTTCACCGTCTCCACACCGGATATCTTGGCTGATGGCAGATGAGTTCACATTATCCTTTGTCCCTGTCGATA
15 AGAATGGCCATTTTATCAGTGGGATGCAGGGCTTGAGTTTACTCAAAACGGTGTGCCGGTGAGTATTAGCCCCA
TTACCGAGCAGCCAGATAGCTATACCGCGACGGTGGTGGGAATAGTGTCCGTGATGTACAATCACGCCGACG
TTGATACCCTGATAGTACATTGCAGAAAAAATATCCCTATTCCCGGTACCTACGCTGACCGGTATTCTGG
TTAACGGGCAAAATTTGCTACGGATAAAGGGTTCCCGAAAACGATCTTTAAAAACGCCACATTCCAGTTACAGA
TGGATAACGATGTTGCTAATAATACTCAGTATGAGTGGTCGTCGTCATTACACCCAATGTATCGGTTAACGATC
20 AGGGTCAGGTGACGATTACCTACCAAACCTATAGCGAAGTGGCTGTGACGGCGAAAAGTAAAAAATCCCAAGTT
ATTCCGTGAGTTATCGGTTCTACCCAAATCGGTGGATATACGATGGCGGCAGATCGCTGGTATCCAGTCTCGAGG
CCAGCAGACAATGCCAAGGTTGAGATATGTCTGCGGTTCTTGAATCCTCACGTGCAACCAACGGAACGCGTGCGC
CTGACGGGACATTGTGGGGCAGTGGGGGAGCTTGACCGGTATAGTTCTGATTGGCAATCTGGTGAATATTGGG
TCAAAAAGACCAGCACGGATTTTGAACCATGAATATGGACACAGGCGCACTGCAACACAGGGCCTGCATACCTGG
25 CGTTCGCGCTCTGTGCGCTGTCAATATAACAGATACAGATAGCAATAAGAACAGTTTAAATGAGCTGATTATTT
GGGGCGCAATGGGAGTCCGCAATCTAGACTCGCCCATTAAGTAGCAAAACGTCCCAACGCAACGCGCTCA
GGTTAATTGAGCGGCGCTGTTTTTTTTAAAGGATTGTGCGGATTAAATGCCGATCTTACGGCCCAGCTGCAGCCC
GGGGGATCTATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAAATACCGCATCAGGCGCCATTGCGCCATTC
AGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGGACTTCATATACCCAAGC
30 TTGGAAAATTTTTTTTTTAAAAAAGTCTTGACACTTTATGCTTCCGGCTCGTATAATGGATCCAGGAGTAACAATAC
AAATGGATTCAAGAGATCCATTTGTATTGTTACTCCTTTTTTTTTTTGTGCGACGATCCTTAGCGAAAGCTAAGGA
TTTTTTTTTTACTCGAGCGGATTACTACATACCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGC
GTATTGGGCGCTCTTCCGCTTCTCTGCTCACTGACTCGCTGCGCTCGGTGCTTCCGGCTGCGGCGAGCGGTATCAG
CTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCC
35 AGCAAAAGGCCAGGAACCGTAAAAAGGCCGCTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATC
ACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCGACAGGACTATAAAGATAACAGGCGTTTCCCCCTGGAA
GCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCTTTCTCCCTTCGGGAAGCG
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60 NO: 559)

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 CTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCC
 20 AGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATC
 ACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAA
 GCTCCCTCGTGCGCTCTCCTGTTCCGACCCCTGCCGCTTACCGGATACCTGTCCGCTTTTCTCCCTTCGGGAAGCG
 TGGCGCTTTCTCATAGCTCAGCTGTAGGTATCTCAGTTCGGTGTAGGTGCTTCCGCTCCAAGCTGGGCTGTGTGC
 ACGAATCCCCCGTTTCAGCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACG
 25 ACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCT
 TGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTGTTGTTATCTGCGCTCTGCTGAAGCCAGTTACCT
 TCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTTTGTGTTGCAAGC
 AGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGGA
 ACGAAACTCACGTTAAGGGATTTTGGTCATGATTTTCATAGAAGGCGGCGGTGGAATCGAAATCTCGTGATGGCA
 30 GGTGGGCGTCGCTTGGTTCGGTCATTTTCGAACCCAGAGTCCCGCTCAGAAGAACTCGTCAAGAAGCGGATAGAA
 GGCGATGCGCTGCGAATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTTCAGCCATTTCGCCGCCAAGCTC
 TTCAGCAATATCACGGGTAGCCAACGCTATGTCTGATAGCGGTCCGCCACACCCAGCCGGCCACAGTCGATGAA
 TCCAGAAAAGCGGCCATTTTCACCATGATATTCGGCAAGCAGGCATCGCCATGGGTTCAGACGAGATCCTCGCC
 GTCGGGCATGCGCGCCTTGAGCCTGGCGAACAGTTCCGGTGGCGGAGCCCCCTGATGCTCTTCGTCAGATCATC
 35 CTGATCGACAAGACCGCTTCCATCCGAGTACGTGCTCGCTCGATGCGATGTTTCGTTGGTGGTTCGAATGGGCA
 GGTAGCCGGATCAAGCGTATGCAGCCGCGCATTGCATCAGCCATGATGGATACTTTCTCGGCAGGAGCAAGGTG
 AGATGACAGGAGATCCTGCCCCGGCACTTCGCCAATAGCAGCCAGTCCCTTCCCGCTTCAGTGACAACGTCGAG
 CACAGCTGCGCAAGGAACGCCGCTCGTGGCCAGCCAGATAGCCGCGCTGCCTCGTCTGCAAGTTTCATTAGGGC
 ACCGGACAGGTGGTCTTGACAAAAAGAACGGGGCGCCCCCTGCGCTGACAGCCGGAACACGGCGGCATCAGAGCA
 40 GCCGATTGTCTGTTGTGCCAGTCATAGCCGAATAGCCTCTCCACCAAGCGGCCGAGAACCTGCGTGCAATCC
 ATCTTGTTCAATCATGCGAAACGATCCTCATCTGTCTCTTGATCAGATCTTGATCCCCTGCGCCATCAGATCCT
 TGGCGGCAAGAAAGCCATCCAGTTTACTTTGCAGGGCTTCCCAACCTTACCAGATCATGACATTAACCTATAAAA
 ATAGGCGTATCACGAGGCCCTTTCGTC (SEQ ID NO:561)

45 Example 23: Construction of pNJSZc Plasmid

pNJSZ is a 10.4 kb plasmid that confers the abilities required to induce tkRNAi. It contains
 two genes, *inv* and *hly*, that allows bacteria to invade mammalian cells and to escape from the entry
 vacuole. Expression of the short hairpin RNA is different between the original Trip plasmid and
 pNJSZ. In pNJSZ, expression of shRNA is under the control of a constitutive bacterial promoter
 50 which allows for continuous expression. This is different from the original Trip plasmid, which has an
 ITPG inducible promoter, which controls the expression of the shRNA. Moreover, pNJSZ and the
 original Trip plasmid contain different antibiotic resistant genes. pNJSZ has the kanamycin resistance
 gene, whereas the original Trip plasmid has the ampicillin resistance gene. pNJSZc was constructed
 from pNJSZ by removing any regions of pNJSZ that were not required for its maintenance or abilities
 55 to induce tkRNAi.

Step 1 as shown in Figure 28: Removed an extra BamH1 site at 9778 by digesting pNJSZ with both SpeI (9784) and XmaI (9772), T4 DNA polymerase filled-in these two sites and then allowed the plasmid to self ligate, creating pNJSZ ΔBamH1.

Step 2 as shown in Figure 29: Removed both an extra SalI site at 972 and the fl origin of replication by digesting pNJSZ ΔBamH1 with BglI (208) and PmeI (982), T4 DNA polymerase filled-in these two sites and allowed the plasmid to self ligate, creating pNJSZc.

The pNJSZc DNA sequence is as follows:

```

10  GGCCGCTCGAGCATGCATCTAGAGGGCCCAATTGCGCCCTATAGTGAGTCGTATTACAATTCAGTGGCCGTCGTTT
    TACAACGTCGTGACTGGGAAAACCCCTGGCGTTACCCAACCTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCT
    GGCCTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAAAAACCGCGCCATGGTGT
    GTAGGCTGGAGCTGCTTCGAAGTTCCTATACTTTCTAGAGAAATAGGAACCTTCGGAATAGGAACCTCAAGATCCCC
    CACGCTGCCGCAAGCACTCAGGGCGCAAGGGCTGCTAAAGGAAACGGAACACGTAGAAAGCCAGTCCGCAGAAAC
    GGTGCTGACCCCGGATGAATGTCAGCTACTGGGCTATCTGGACAAGGGAAAACGCAAGCGCAAAGAGAAAGCAGG
15  TAGCTTGCACTGGGCTTACATGGCGATAGCTAGACTGGGCGGTTTATGGACAGCAAGCGAACCGGAATTGCCAG
    CTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAGTAACTGGATGGCTTCTTGCCGCCAAGGATCTGAT
    GGCGCAGGGGATCAAGATCTGATCAAGAGACAGGATGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACG
    CAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATG
    CCGCCGTGTTCCGGCTGTGAGCGCAGGGGCGCCGCTTCTTTTGTCAAGACCGACCTGTCCGGTGCCTTGAATG
20  AACTGCAGGACGAGGACGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTG
    TCACTGAAGCGGGAAGGACTGGCTGCTATTGGGCGAAGTGCCGGGCAGGATCTCCTGTCTATCTACCTTGCTC
    CTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCATTGCG
    ACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGG
    ACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACCTGTTCCGCAAGGCTCAAGGCGCGCATGCCGACGGCGAGGATC
25  TCGTCAGTACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTTCATCGACT
    GTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCG
    GCGAGTGGGCTGACCGCTTCTCGTGCTTTACGGTATCGCCGCTCCCGATTTCGACGCGCATCGCCTTCTATCGCC
    TTCTTGACGAGTTCTTCTGAGCGGACTCTGGGTTTCGAAATGACCGACCAAGCGACGCCAACCTGCCATCAGG
    AGATTTTCGATTCCACCGCCGCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCGGGACGCCGGCTGGATGAT
30  CCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCACCCAGCTTCAAAAGCGCTCTGAAGTTCCTATACTT
    TCTAGAGAATAGGAACCTTCGGAATAGGAACCTAAGGAGGATATTCATATGGACCATGGCGCGGCATGCAAGCTCGG
    TATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGAGTCAGGCAAC
    TATGGATGAACGAAATAGACAGATCGCTGAGATAGTGCCCTCACTGATTAAGCATTGGTAACGTGCAGACCAAGT
    TTACTCATATATATTTAGATTGATTTAAACTTCAATTTTAAATTTAAAGGATCTAGGTGAAGATCCTTTTGA
35  TAATCTCATGACCAAAATCCCTTAACGTGAGTTTCGTTCCACTGAGCGTCAGACCCGTAGAAAAGATCAAAGG
    ATCTTCTTGAGATCCTTTTCTGCGCGTAATCTGCTGCTTGCAAAACAAAAAACACCGCTACCAGCGGTGGT
    TTGTTTTCGGGATCAAGAGCTACCAACTCTTTTTCGAAGGTAAGTGGCTTCAGCAGAGCGCAGATACCAAAATAC
    TGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCT
    AATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCTGTCTTACCGGGTTGGACTCAAGACGATAGTTACC
40  GGATAAGGCGCAGCGGTCCGGCTGAACGGGGGGTTCGTGCACACAGCCAGCTTGGAGCGAACGACCTACACCGA
    ACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGT
    AAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAACGCCTGGTATCTTTATAGTCTGT
    CGGGTTTCGCCACCTTGACTTGAGCGTCAATTTTGTGATGCTCGTCAGGGGGCGGAGCCTATGAAAAACGC
    CAGCAACGCGGCCTTTTACGGTTCTTGGCCTTTTGTGCTGCTTTCCTGCTCACATGTTCTTTCCTGCTTATCCCC
45  TGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGACGCCGAACGACCGAGCGCAG
    CGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAAACCGCTCTCCCGCGCGTTGGCCGATTCAATTA
    ATGCAGCTGGCAGCAGTATCGATAAGCTTGATAAGCTTTTAAATCAGCAGGGGTCTTTTGGCTTGTGTATTA
    TTTTGAAGTTTTTCTTCCCGACAGAATCTGCTTTTACCGTCATAGTGAAATGAGCCTGAAAAGCTATTACCATG
    ATGATACAAATAAGTTTACTTTTCAATTCACCGCTCCTTTTAAATTCGTAAAACTAAGTTTAAAGCCCTACAAC
50  TAATCTGACAGAGAGAGTTAAGGACACGTTTTTTAGTATATGTGGGAACTAAATTATACGTTTTGACGTAGAAAC
    TATAGGTGGCTTAACTTTGGGATATGCTTATATTATATGGATAAACAGTCAGATATTCTTTTACATTTGTTAAT
    TCTTCTAAAAAATTAATAAAGCCTGTTTCTACATTCTTCAAAAATAATTTACGAAGAGTGCAAAACAAGC
    TTATTTTTTTCGTGTGTGTTAAGCGGTTTTATTCTTAATTTTTTATTACTTTTACAATTTATCGATTGGATTATCT
    ACTTTATTACTATATTTCCGATAAAGCGTGGTGCCCGAGATGGAGATATTTCTATTTTACAAAGTGGAATGATCT
55  CGGTCAATTAACCGTTCTCCACCATTCCCAAGCTAAACCAGTGCATTCTTTAGCGTAACATTAATATTTCTC
    GCGTTACCTGGCAAATAGATGGACGATGTGAAATGAGCTAGCTTGTCTTTTATTGTTTTCGCTCCAGTTTTTATGT
    TGAACAATTTCTGTTACCTTCAGGATCATAATTTACTTCATCCCAAGAAATGTTGAATTGAGCAACGTATCCTCCA
    GAGTGATCGATGTTAATTTTTCCATCTGTATAAGCTTTTGAAGTTGTTTCAATATATTCTGAGTTGTTTTAATA
  
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5 CTTACGGCAGCATCAAAAGCAGCTTTTACTTTAGTACTATGGGAATTAGTTGATAATTTCAAATAAACTTGACGG
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10 ATGACTTCTTCTTGCAATTTCCCTTCACTGATTGCGCCGAAGTTTACATTCAAGCTATTATTTACAGCTTTAAAT
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TTTGATAAGCTTGAGCATATTTTTCATTCCATCTTTCCACTAATGTATTTACTGCGTTGTTAACGTTTGATTTA
15 GTGGCATTTTTTTACAACGATTTTATTGTCTTGATTAGTCATACCTGGCAAATCAATGCTGAGTGTTAATGAATCA
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20 TTGTTTTTATTGTAATCCAATCCTTGTATATACTTATCGATTTTCATCCGCGTGTCTTTTTCGATTGGCGTCTTA
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15 GCTTCAGTTTGTGCGCAATTGGTAGACTAATAATAGTGAATAAAAACTAGCATTATTTTTTTCATGGGT
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30 CTATCCTCTTAATGTCTTTACTAAATTAGAAAACAAGTTTCACTTTCACTATTGCATCTTTAATTAATGGTCAA
25 GGTGATTTCAAATGCTCGTTTGTGGCCAGTTATACCTCAAATAACTCAAGTTGTTGAGCACAGCCAACGCACATG
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45 CTTATGCCGTGACTGCCGGGATCAATTACACCCCGTGCCTTACTCACTGTCGGGGTAGATCAGCGTATGGGGA
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50 CCGCTGGCGGCACATTAACACCACTGAGTACCACACAATTCACCTTGGTTTTACCGCTTATAAACGACAGCAC
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55 GCGTCGCGCGCATTCGATTAACCAATACGACAGATGGCGTGACGGTAGTCACAGCAGAAGTGGAGGGGCAACGGC
50 AAAGTGTGATACCCACTTTGTAAAGGGTACTATCGCGGCGGATAAATCCACTCTGGCTGCGGTACCGACATCTA
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55 TGGCTGATGGCAGATGAGTTCCACATTATCCTTTGTCCCTGTGCGATAAGAATGGCCATTTTATCAGTGGGATGC
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5 AACCAGATAACAGATAGCAATAAGAACAGTTTAATGAGCTGATTATTTGGGGCGGAATGGGAGTCCGGCAATCC
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AAGGATTTGTGCGGATAAGCGTGAGCTGGCGTTAAATGCCGATCTTACGGCCAGCTGCAGCCCGGCTAGTAACG
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10 GATAATGTTGCGATGACAGTGTCAAACCTGGTTATTCTTCAAGGGGTGAGTTGTTCTTAAGCATGCCGGTTTGTCT
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15 TACTCCTTTGTGCTGACGGACAGTTCAAGAGACTGTCCATCAATATCAGCTTTGTCAAAACCCCGCCACCGGCGG
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20 AAGCCAGTCCGCAGAAACGGTGTGACCCCGGATGAATGTCAGCTACTGGGCTATCTGGACAAGGAAAACGCAA
GCGCAAAGAGAAAGCAGGTAGCTTGCAGTGGGCTTACATGGCGATAGCTAGACTGGGCGGTTTTATGGACAGCAA
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TGCCGCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATCAAGAGACAGGATGAGGATCGTTTCGCATGATTG
AACAAGATGGATTGCACGAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTTCGGCTATGACTGGGCACAACAGA
25 CAATCGGCTGCTCTGATGCCGCGTGTTCGGCTGTGAGCGAGGGGCGCCCGGTTCTTTTGTCAAGACCGACC
TGTCCGGTGCCCTGAATGAACCTGCAGGACGAGGCGAGCGCGCTATCGTGCTGGCCACGACCGGGGTCCTTTCG
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CGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTAAGGATGGAAGCCGGTCTTG
30 TCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAAGTTCGCCAGGCTCAAGCGCGCA
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35 GCCCAACCTGCCATCACGAGATTTGATTCACCGCCGCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCG
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TCTGAAGTTCCTATACTTTCTAGAGAATAGGAACCTCGGAATAGGAACCTAAGGAGGATATTATATGGACCATGG
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40 GTAAGTGTGACACCAAGTTTACTCATATATACTTTAGATTGATTAAAACTTCATTTTTAATTTAAAGGATCTA
GGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCTGTTCCACTGAGCGTCAGACCC
CGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAACCC
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45 TACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTCTTACCGGTTTGA
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GCGAACGACCTACACCGAAGTGAATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAA
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50 GAGCCTATGGAACCAAGCCAGCAACGCGGCCTTTTTACGGTTCTCGGCCTTTTGCTGGCCTTTTGCTCACATGTT
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CCGAACGACGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGC
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55 TGAAGGCTATTACCATGATGATACAAATAAGTTTTACTTTTCAATTTACCGCTCCTTTTTTAATTCGTAAAACTAA
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60 TATTCGATTGGATTATCTACTTTATTACTATATTTTCGGATAAAGCGTGGTGCCCCAGATGGAGATATTTCTATTT
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TGACGATTACCTACCAAACCTATAGCGAAGTGGCTGTGACGGCGAAAAGTAAAAAATTCCCAAGTTATTTCGGTGA
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5 CATTGTGGGGCGAGTGGGGGAGCTTGACCGCGTATAGTTCTGATTGGCAATCTGGTGAATATTGGGTCAAAAAGA
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10 GCGGCGTTGTTTTTTTAAAAGGATTTGTGCGGATAAGCGTGAGCTGGCGTTAAATGCCGATCTTACGGCCCAGCT
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15 TTGCGGGTGTTTCGCTTGTTTTATTACTCGCTGCCTGTAGTTCTGACTCACGCTATAAGCGTCAGGTCAGTGGTG
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What is claimed:

1. 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 one or more HPV oncogenes.
2. 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 one or more HPV oncogenes.
3. 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 one or more HPV oncogenes.
4. A method of regulating gene expression in 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 one or more HPV oncogenes.
5. A method of treating or preventing a viral disease or disorder in a mammal, the method comprising regulating the expression of at least one gene in a cell known to increase proliferation, growth or dysplasia, by introducing to the cells of the mammal 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 one or more HPV oncogenes.
6. The invasive bacterium of claim 1, wherein said invasive bacterium is a non-pathogenic or non-virulent bacterium.
7. The invasive bacterium of claim 1, wherein said invasive bacterium is a therapeutic bacterium.
8. A composition comprising the invasive bacterium of claim 1 and a pharmaceutically acceptable carrier.
9. A eukaryotic host cell comprising the invasive bacterium of claim 1, and a pharmaceutically acceptable carrier.

10. The method of claims 3 and 4, wherein said mammalian cells are *in vivo* or *in vitro*.
11. The method of claims 3 and 4, wherein said mammalian cells are selected from the group consisting of human, bovine, ovine, porcine, feline, buffalo, canine, goat, equine, donkey, deer, avian, bird, chicken, and primate cells.
12. The method of claims 3 to 5, wherein said invasive bacterium is a non-pathogenic or non-virulent bacterium.
13. The method of claims 3 to 5, wherein said invasive bacterium is a therapeutic bacterium.
14. The method of claims 3 to 5, wherein said one or more DNA molecules encoding said one or more siRNAs are transcribed within the mammalian cell.
15. The method of claim 14, wherein said one or more siRNAs are transcribed within the mammalian cell as shRNAs.
16. The method of claims 3 to 5, wherein said one or more DNA molecules encoding said one or more siRNAs are transcribed within the bacterium.
17. The method of claims 3 to 5, wherein said one or more DNA molecules encoding one or more siRNAs comprise a prokaryotic promoter.
18. The method of claim 17, wherein said prokaryotic promoter is selected from the group consisting of a T7 promoter, a P_{gapA} promoter, a P_{araBAD} promoter, a P_{tac} promoter, a P_{lacUV5} promoter and a *recA* promoter.
19. The method of claims 3 to 5, wherein said one or more DNA molecules encoding one or more siRNAs further comprise at least one of enhancer sequence, terminator sequence, invasion factor sequence or lysis regulation sequence.
20. The method of claims 3 and 4, wherein said mammalian cells are infected with about 10^3 to 10^{11} viable invasive bacteria.
21. The method of claim 20, wherein said mammalian cells are infected with about 10^5 to 10^9 viable invasive bacteria.

22. The method of claims 3 and 4, wherein said mammalian cells are infected at a multiplicity of infection ranging from about 0.1 to 10^6 .
23. The method of claim 22, wherein said mammalian cells are infected at a multiplicity of infection ranging from about 10^2 to 10^4 .
24. The method of claims 3 to 5, wherein the expressed siRNAs direct the multienzyme complex RNA-induced silencing complex of the cell to interact with the mRNA of one or more HPV oncogenes.
25. The method of claim 24, wherein said complex degrades said mRNA.
26. The method of claims 3 to 5, wherein expression of one or more HPV oncogenes is decreased or inhibited.
27. The method of claim 5, wherein the viral disease or disorder is selected from the group consisting of infection, epithelial dysplasia and cancer caused by HPV infection.
28. The method of claim 5, wherein said mammal is selected from the group consisting of human, bovine, ovine, porcine, feline, buffalo, canine, goat, equine, donkey, deer, avian, bird, chicken, and primate.
29. The method of claim 5, wherein the mammalian cells are selected from the group consisting of a gastrointestinal epithelial cell, a macrophage, a cervical epithelial cell, a rectal epithelial cell and a pharyngeal epithelial cell.
30. The invasive bacterium of claim 1, wherein wherein said invasive bacterium contains the pNJSZc plasmid.

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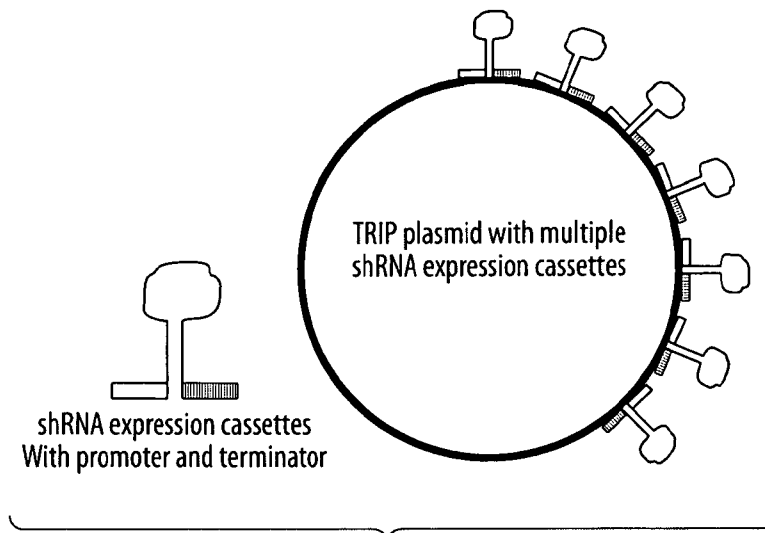


Fig. 1

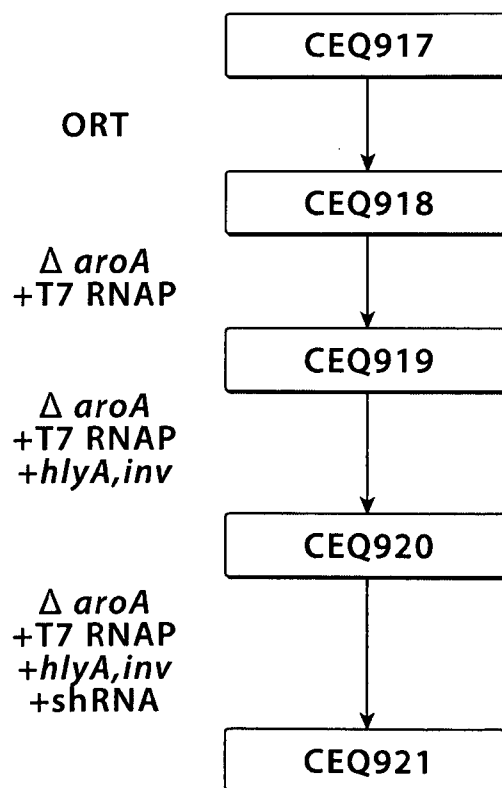


Fig. 2

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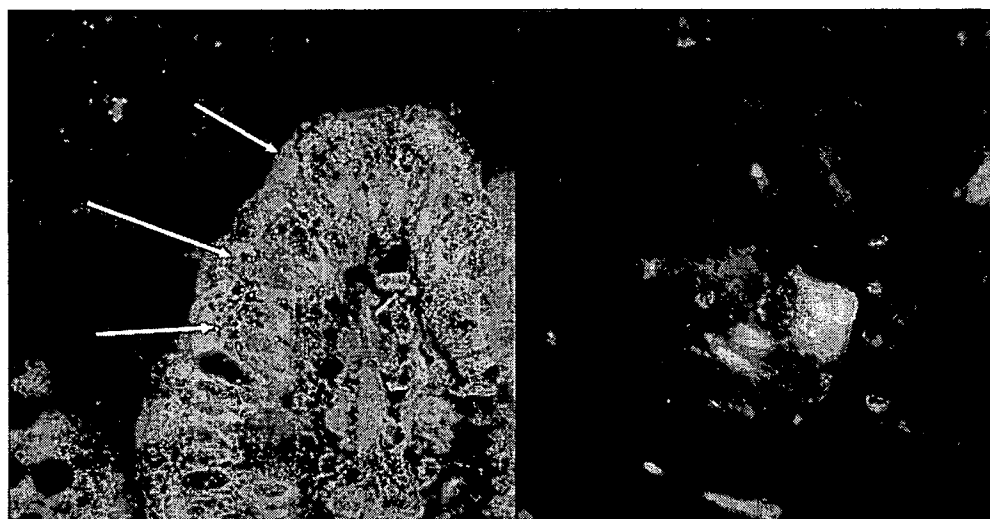


Fig. 3

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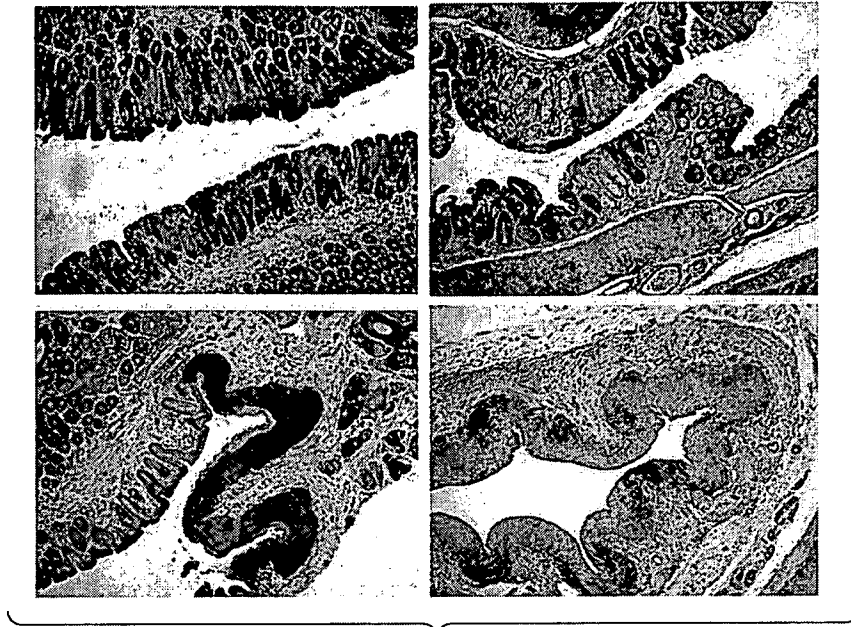


Fig. 4

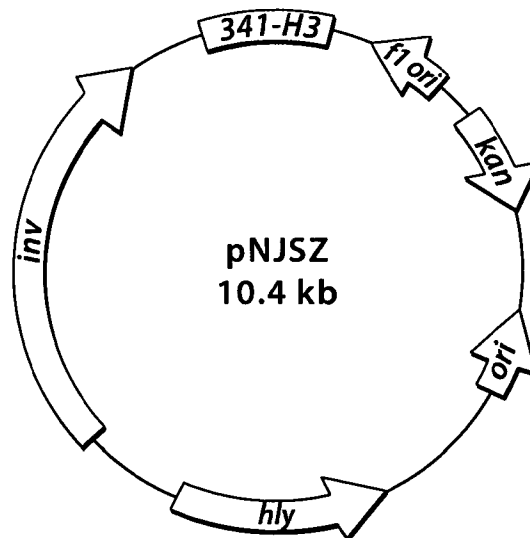


Fig. 5

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Use of lambda S and R genes to kill intact cells

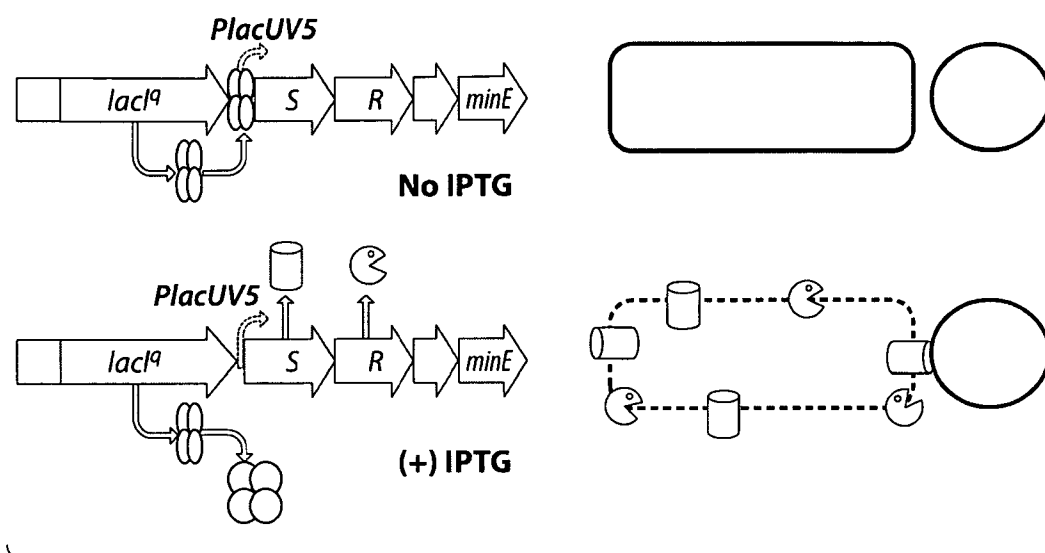


Fig. 6

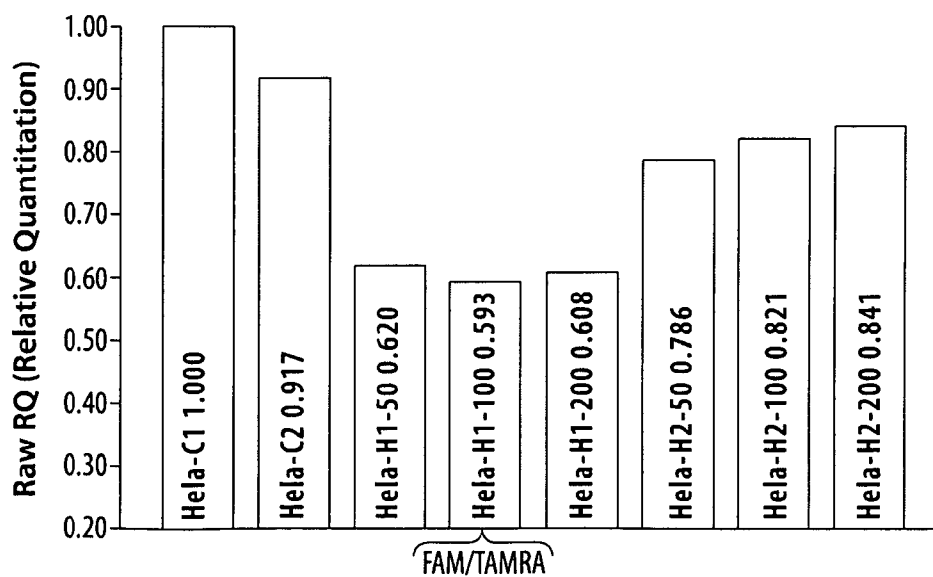


Fig. 7

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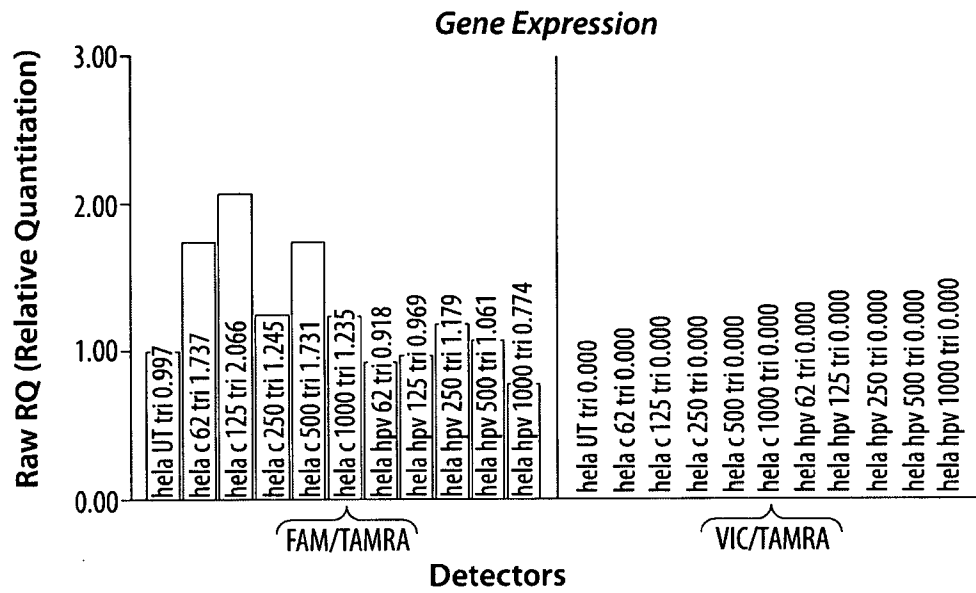


Fig. 8

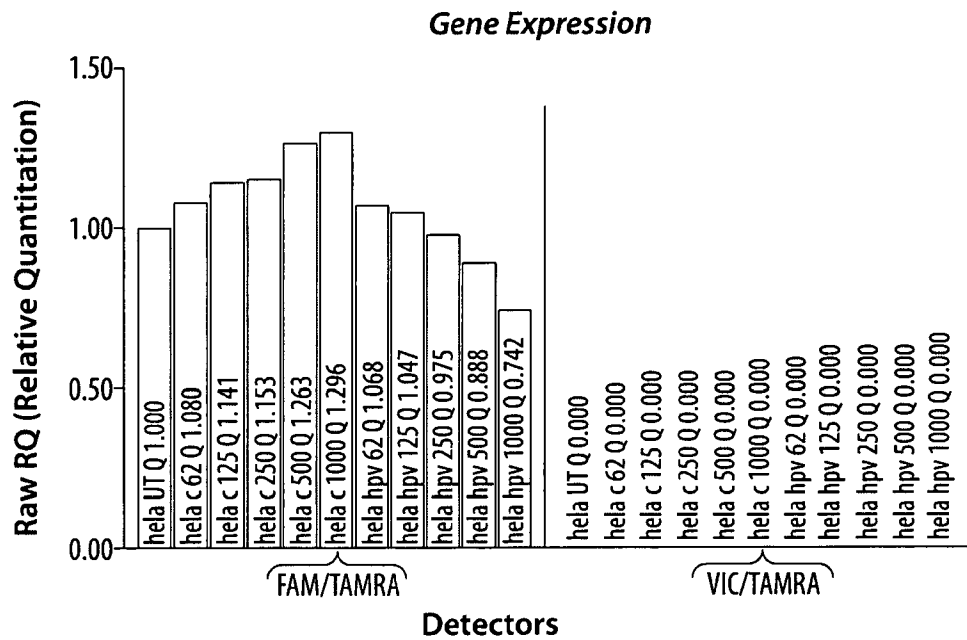


Fig. 9

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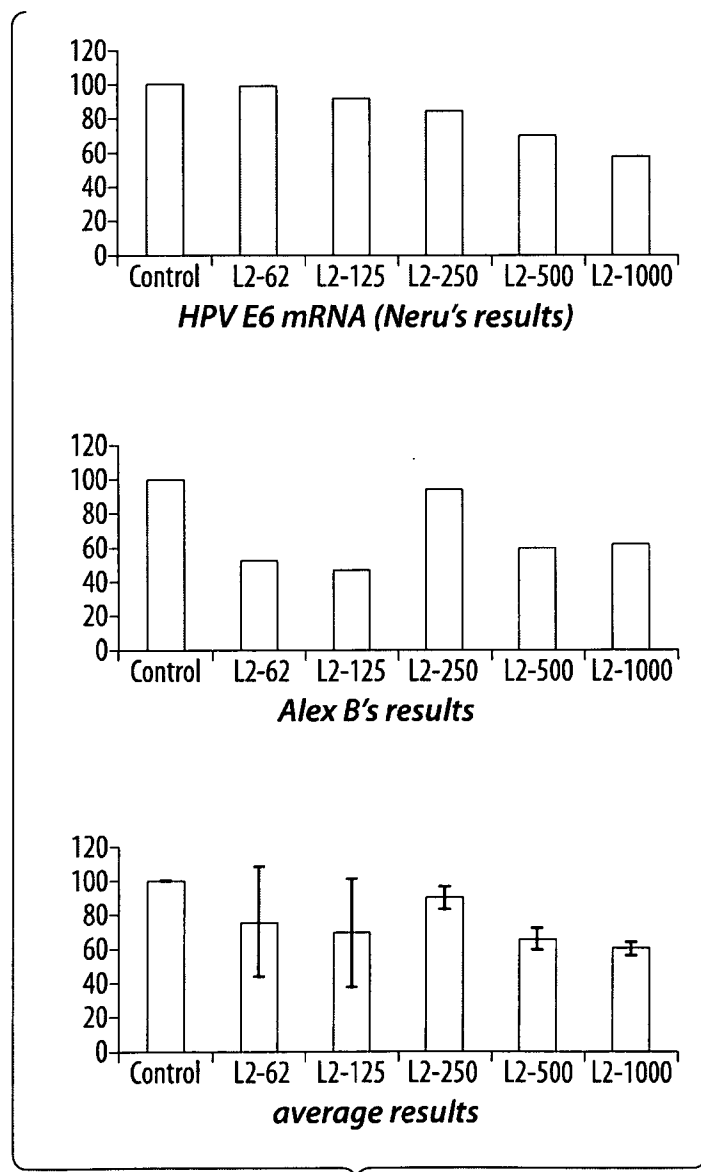


Fig. 10

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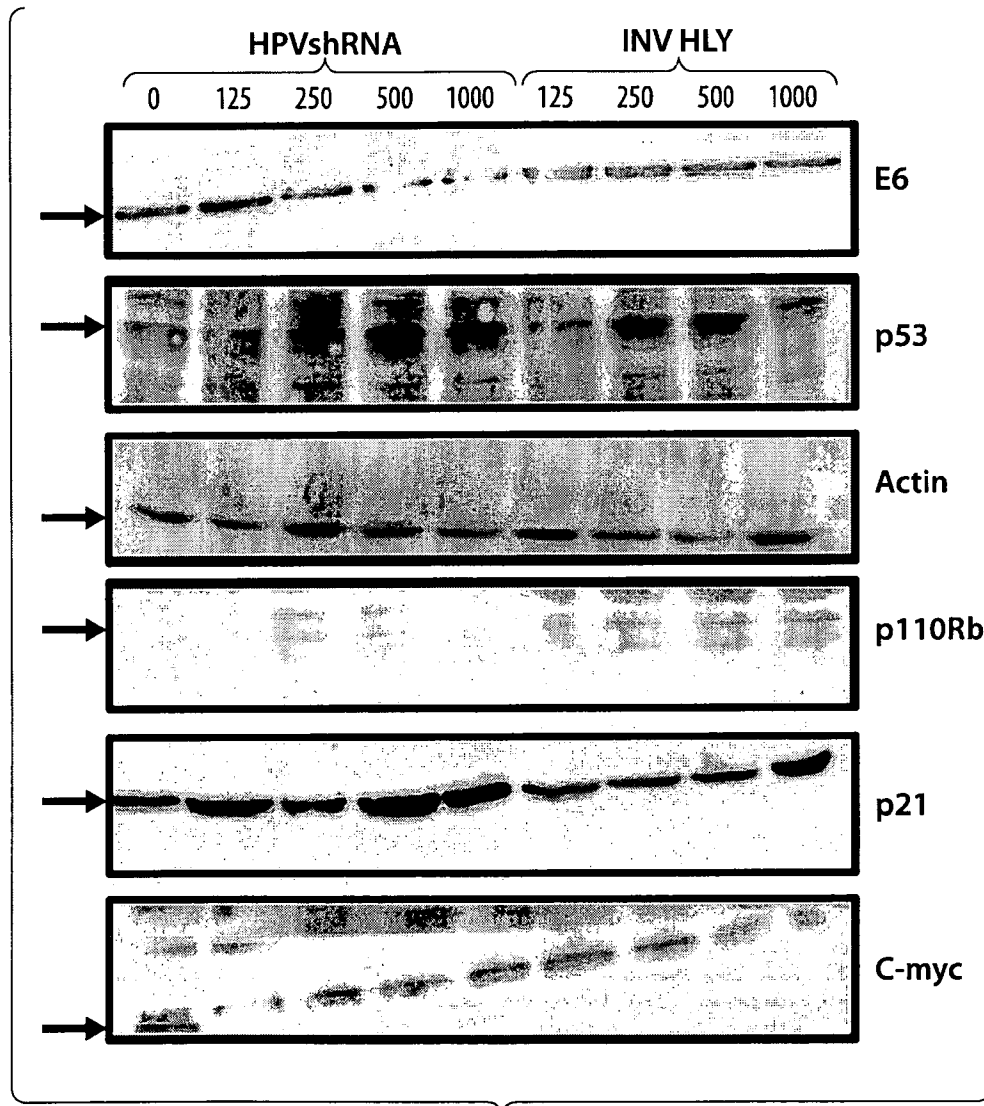


Fig. 11

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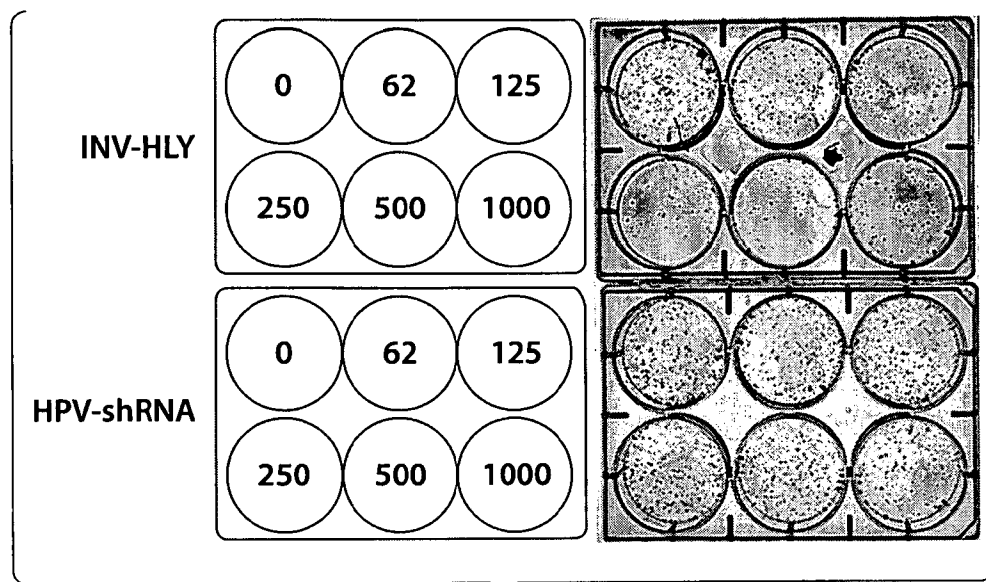


Fig. 12

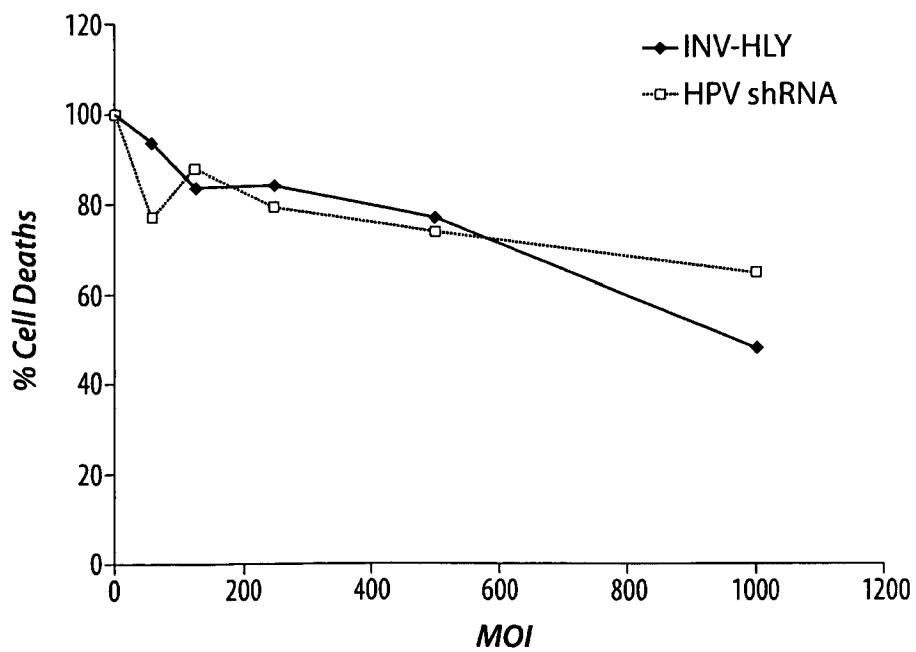
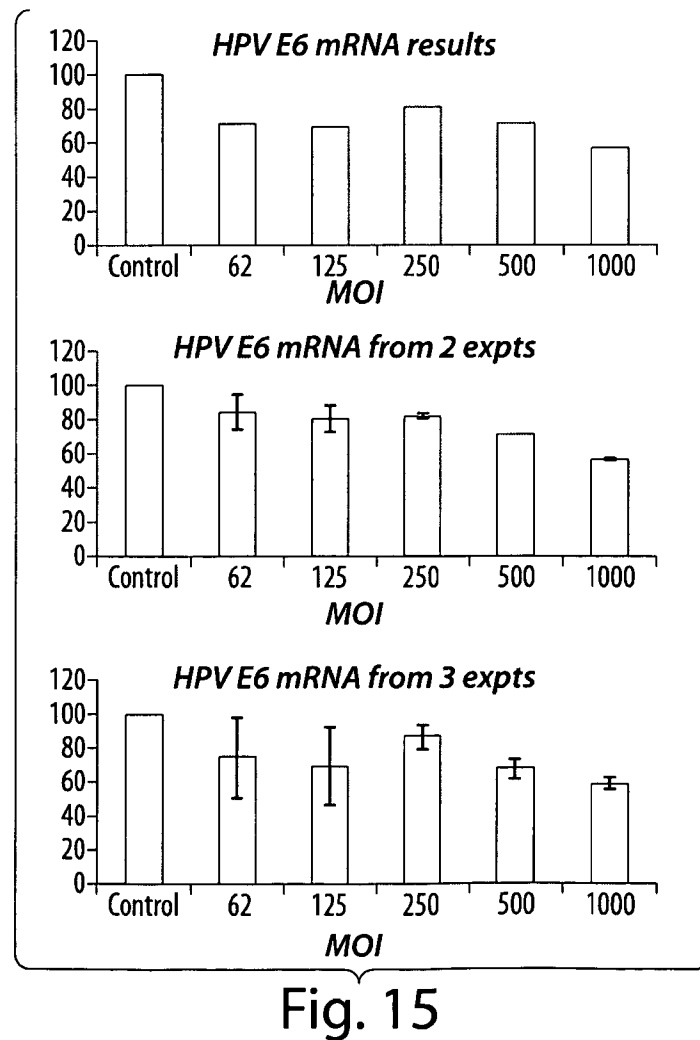
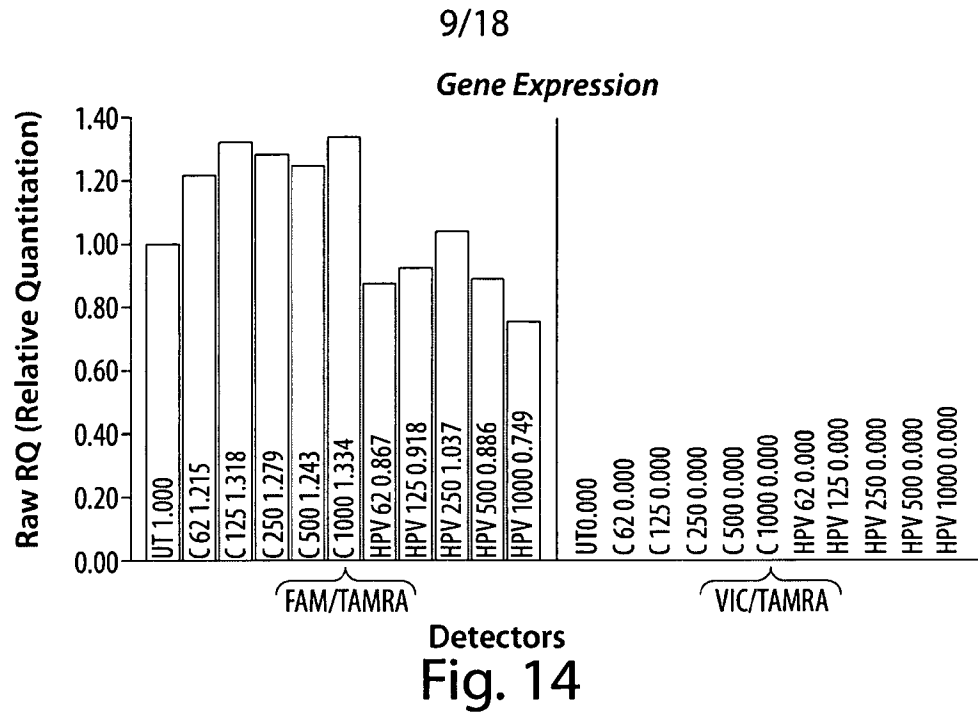


Fig. 13



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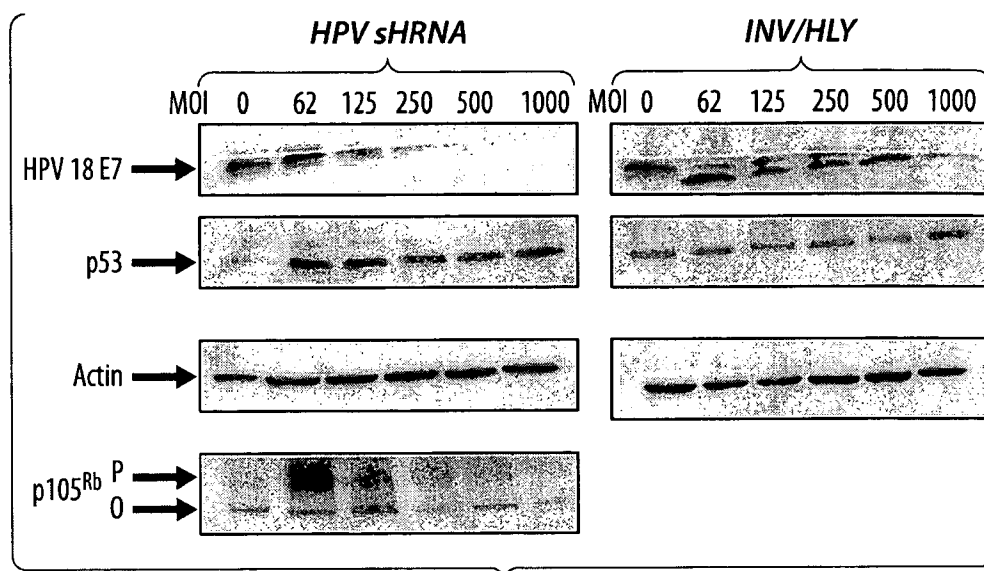


Fig. 16

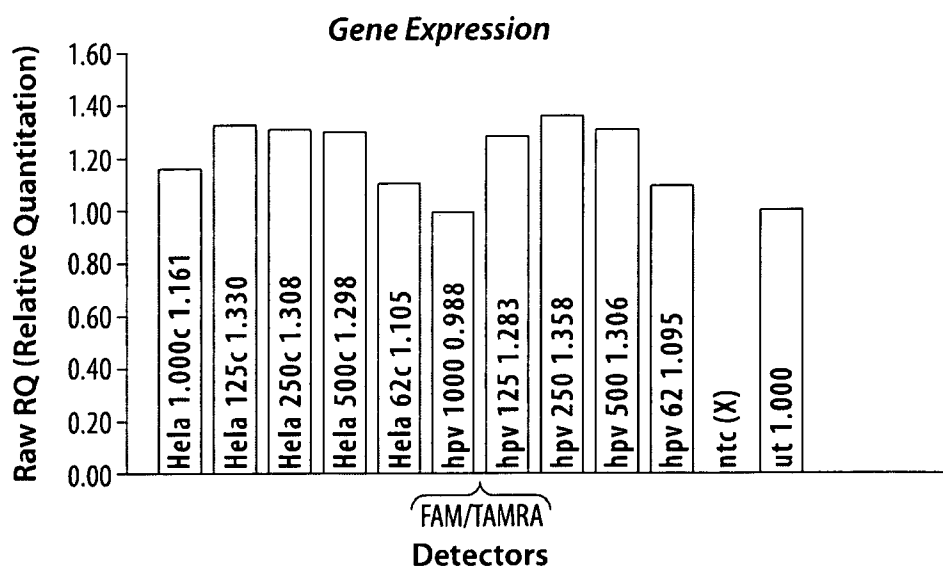


Fig. 17

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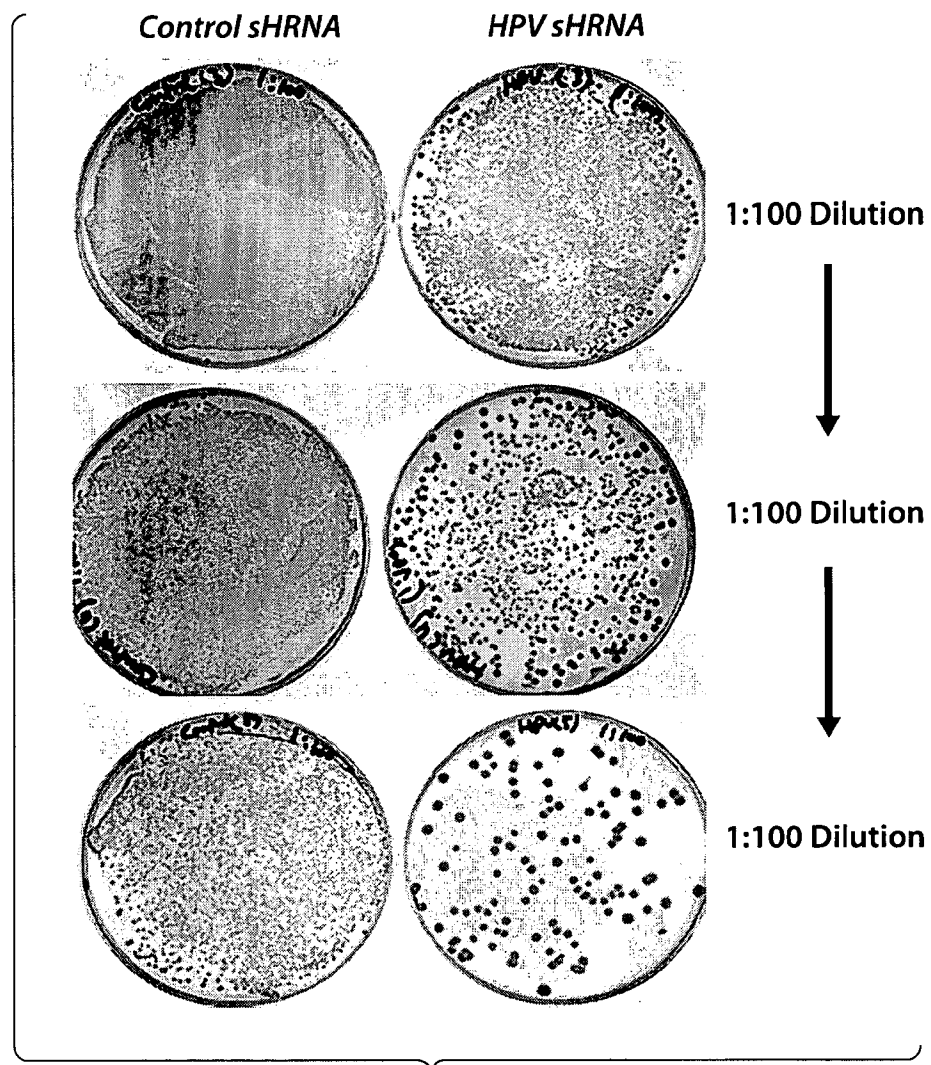


Fig. 18

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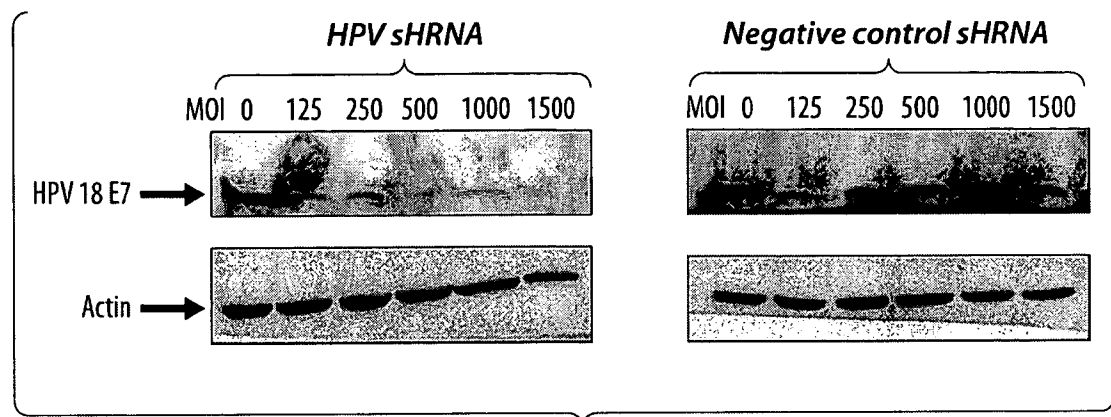


Fig. 19

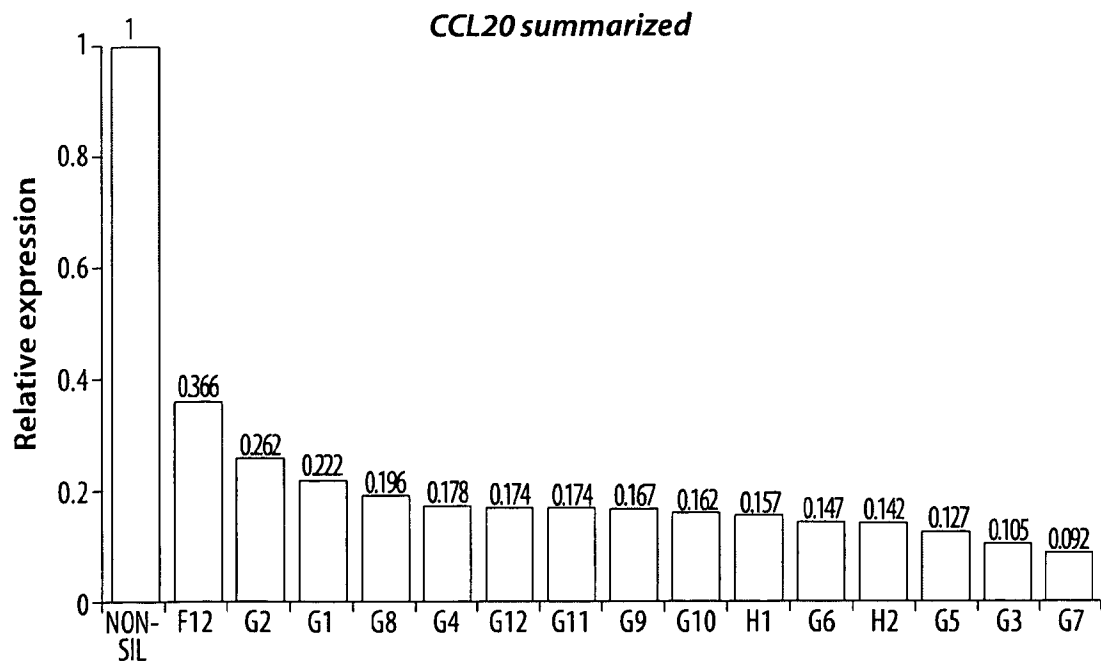


Fig. 20

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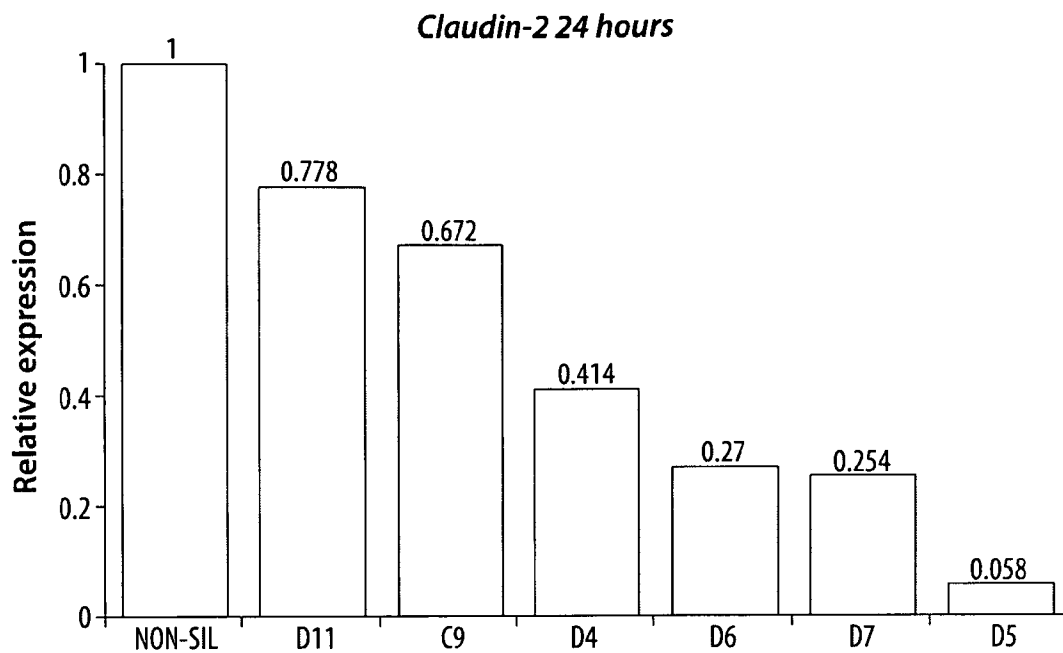


Fig. 21

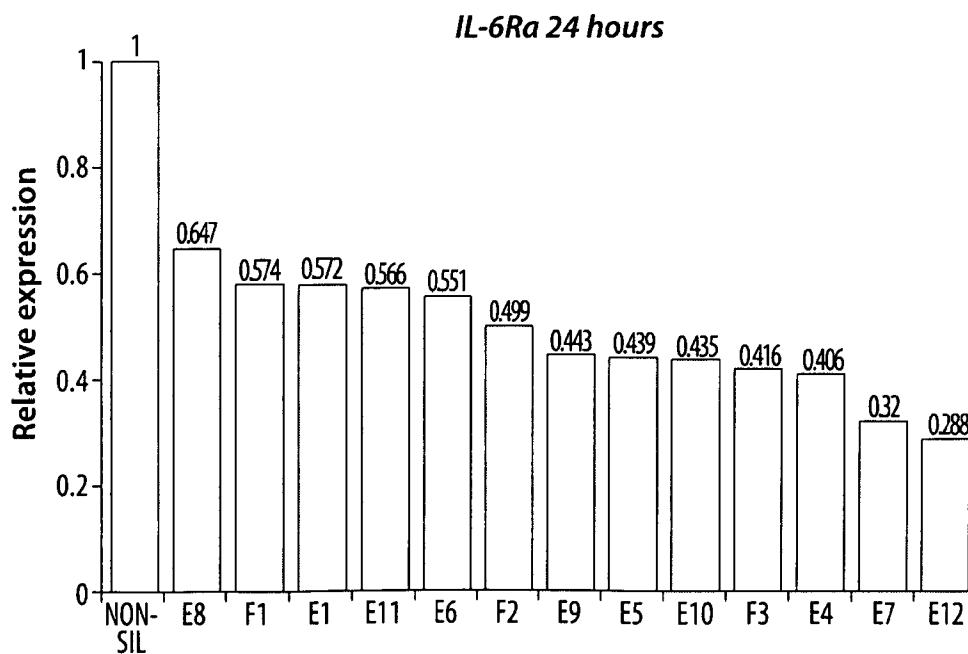


Fig. 22

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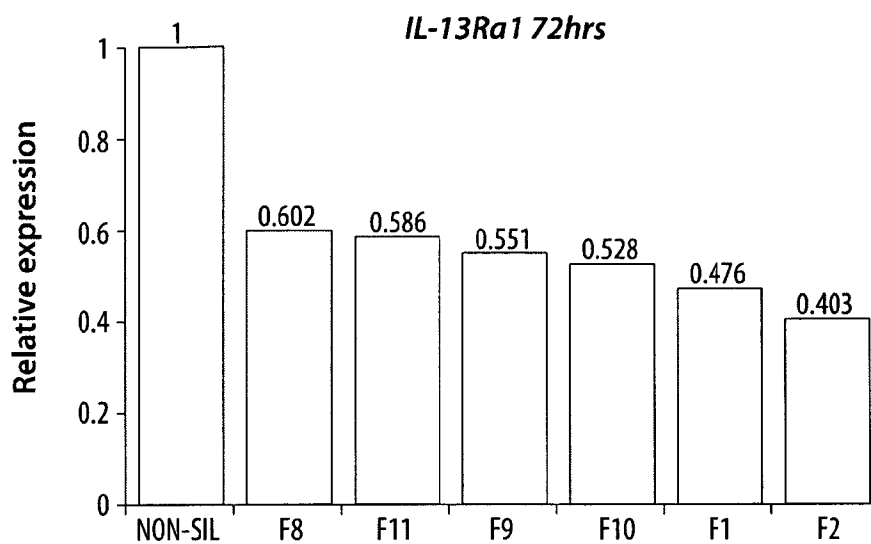


Fig. 23

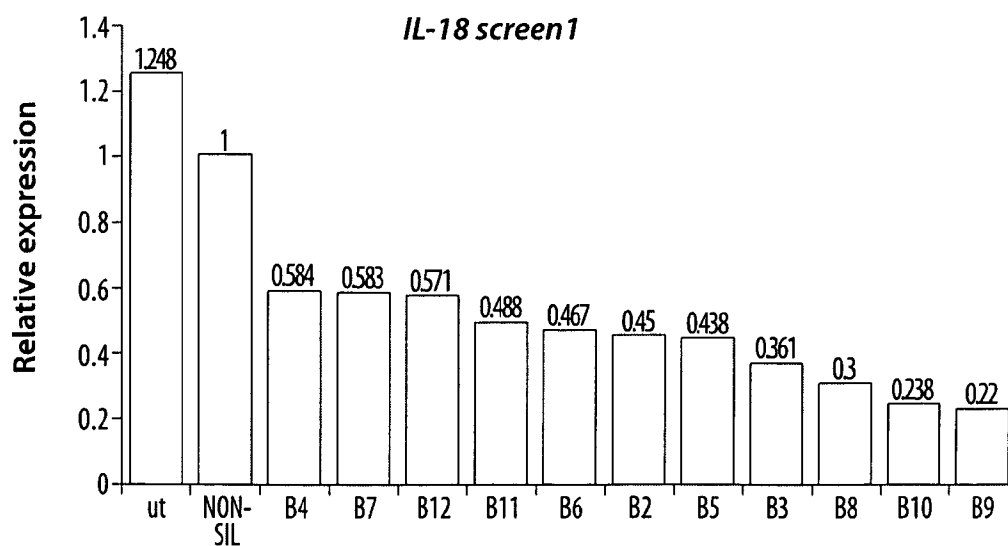


Fig. 24

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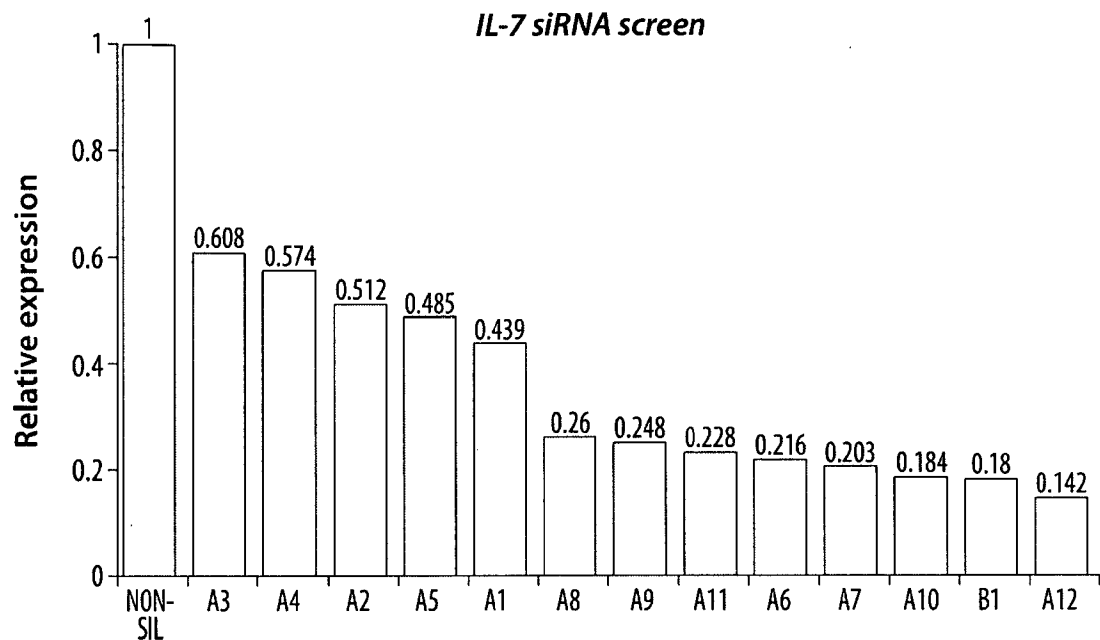


Fig. 25

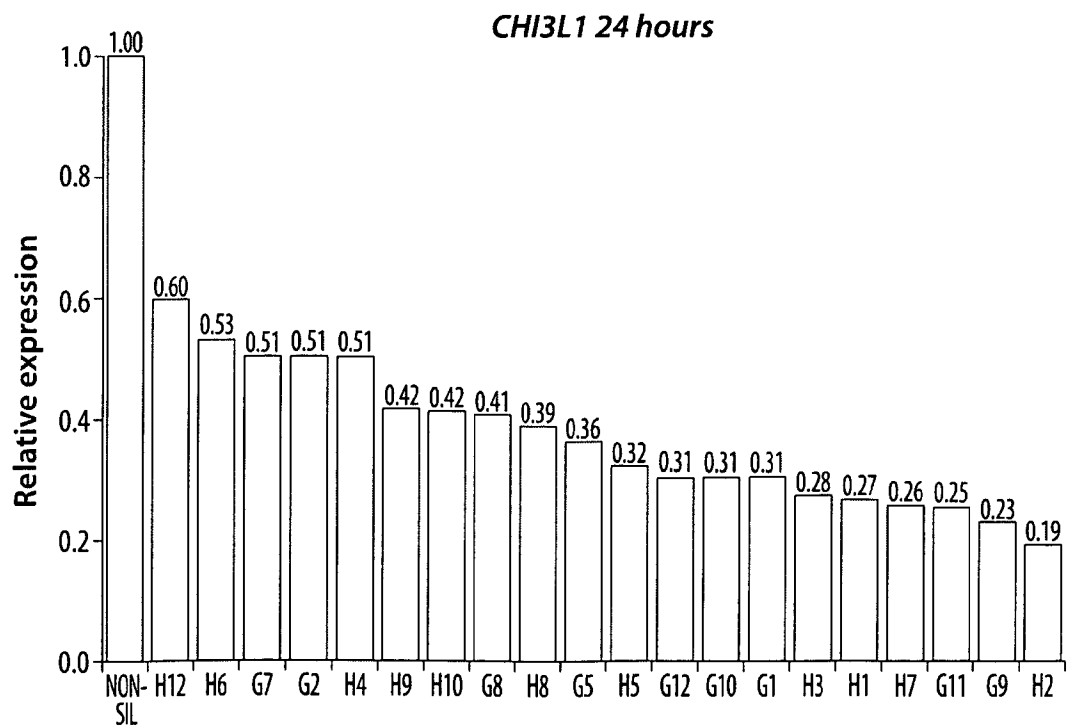


Fig. 26

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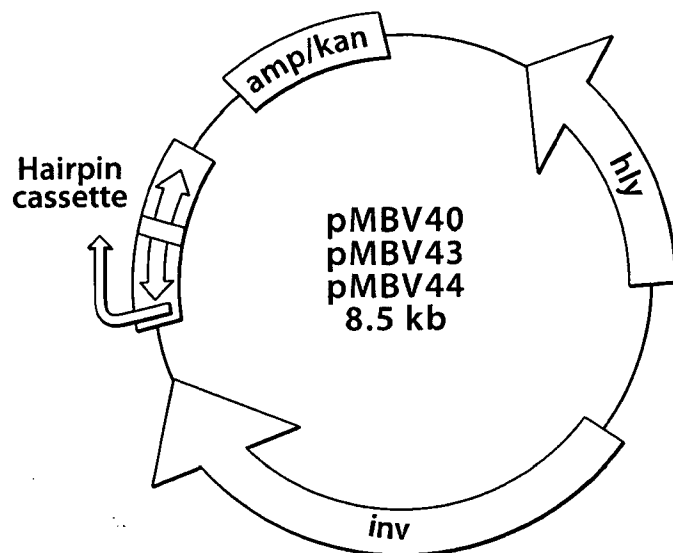


Fig. 27

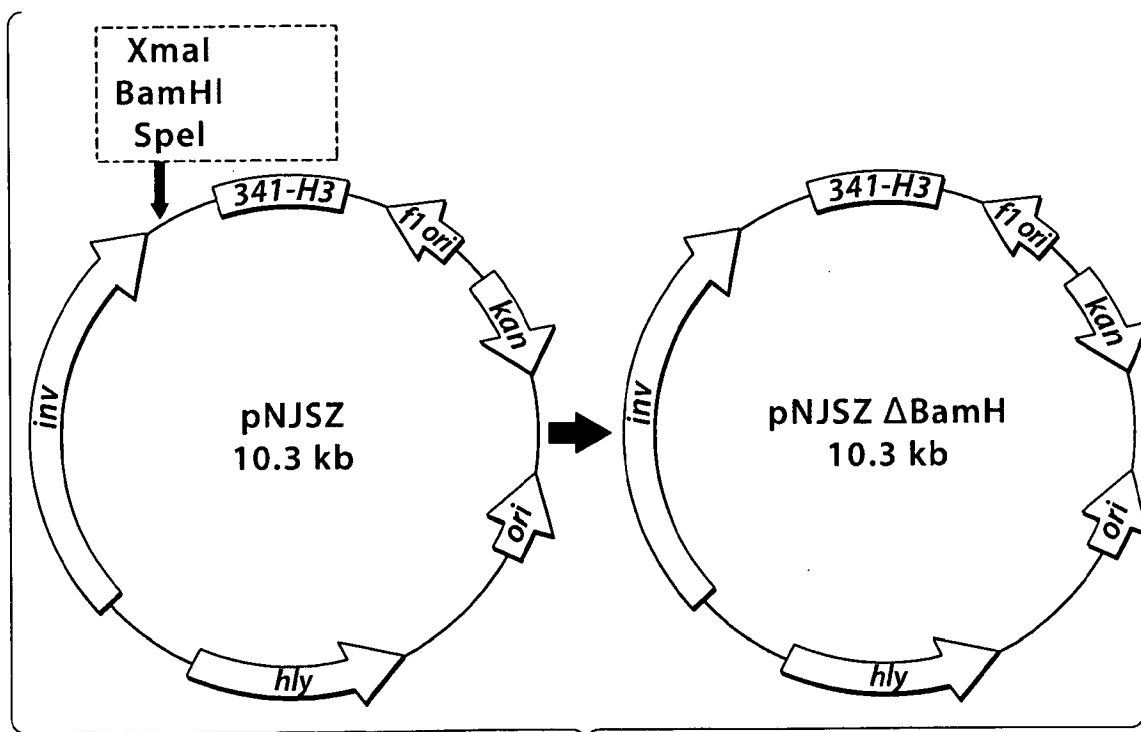


Fig. 28

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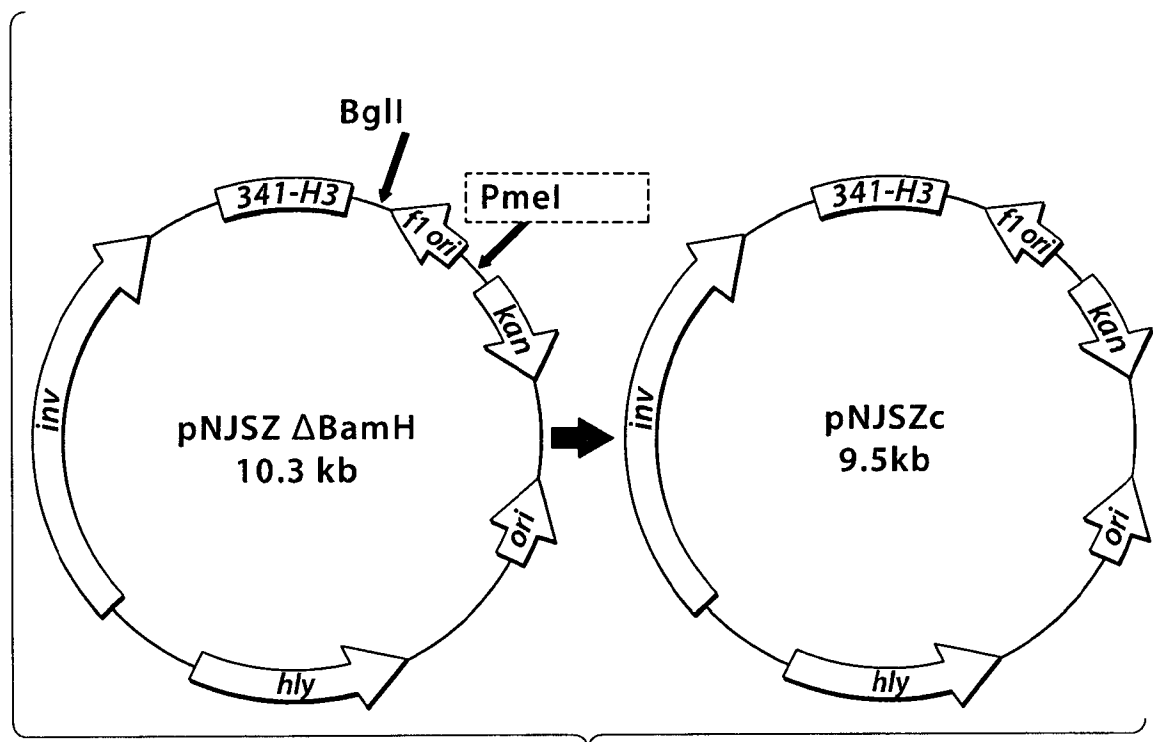


Fig. 29

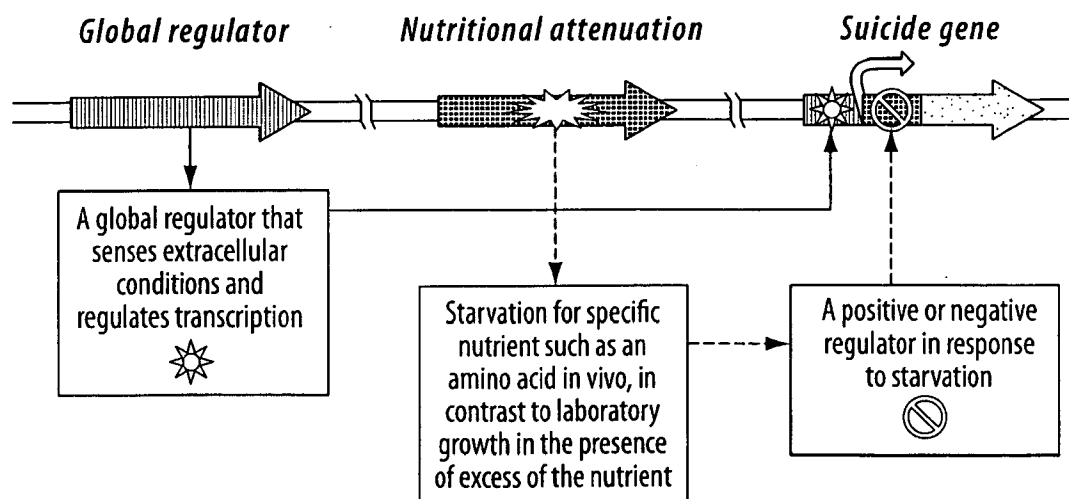


Fig. 30

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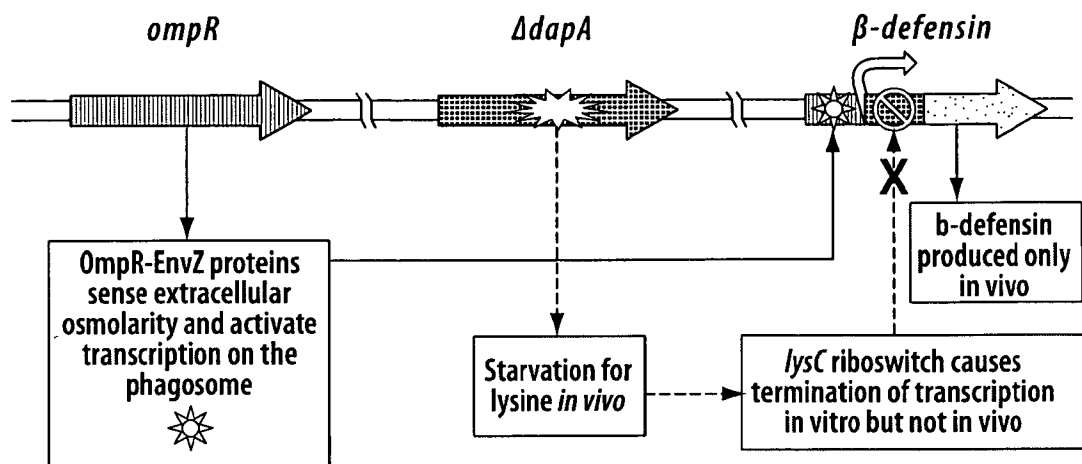


Fig. 31

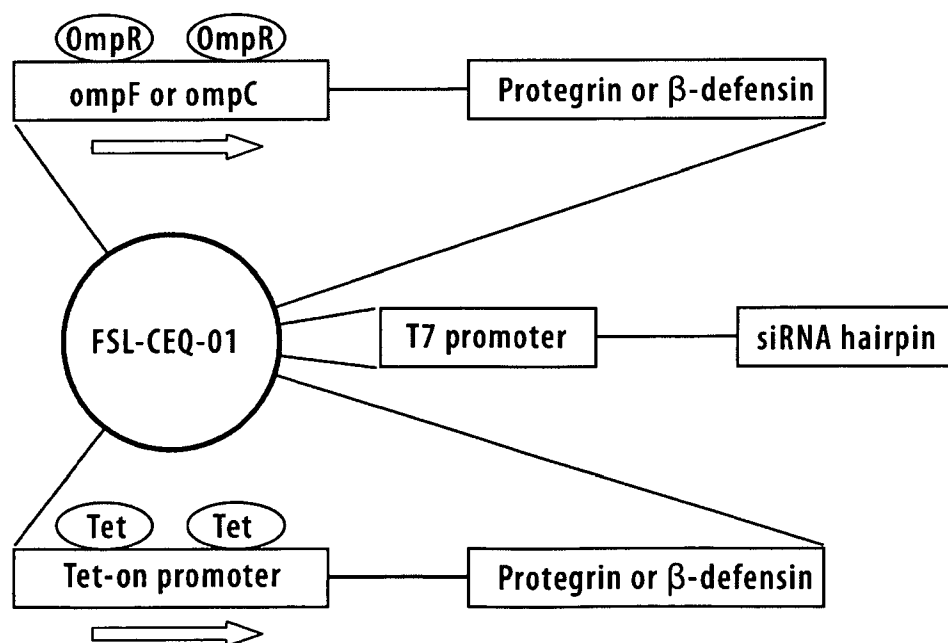


Fig. 32