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Short hairpin RNA–expressing bacteria elicit RNA interference in mammals

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RNA-interference (RNAi) is a potent mechanism, conserved from plants to humans for specific silencing of genes, which holds promise for functional genomics and gene-targeted therapies. Here we show that bacteria engineered to produce a short hairpin RNA (shRNA) targeting a mammalian gene induce trans-kingdom RNAi *in vitro* and *in vivo*. Nonpathogenic *Escherichia coli* were engineered to transcribe shRNAs from a plasmid containing the invasin gene *Inv* and the listeriolysin O gene *HlyA*, which encode two bacterial factors needed for successful transfer of the shRNAs into mammalian cells. Upon oral or intravenous administration, *E. coli* encoding shRNA against *CTNNB1* (catenin β-1) induce significant gene silencing in the intestinal epithelium and in human colon cancer xenografts in mice. These results provide an example of trans-kingdom RNAi in higher organisms and suggest the potential of bacteria-mediated RNAi for functional genomics, therapeutic target validation and development of clinically compatible RNAi-based therapies.

Several years ago, it was demonstrated that systemic gene silencing could be attained in the nematode *Caenorhabditis elegans* when it ingested *E. coli* engineered to produce interfering RNAs, suggesting that RNAi-mediated information transfer between species or kingdoms might be possible[1,2]. This phenomenon has offered a practical solution for the *in vivo* application of RNAi to high-throughput functional genomic analyses of *C. elegans*[3–6]. In the absence of RNAi spreading[2], however, it is unknown whether and how mammals respond to bacterial interfering RNA under commensal, pathological or other conditions. Given the significance of bacteria in biology and medicine and their versatility as gene vectors[7], we decided to explore the possibility of targeting genes in mammalian cells with engineered bacteria that produce interfering RNAs. Bacteria-mediated RNAi in higher organisms may hold potential for functional genomics in mammalian systems, as well as for other *in vivo* applications.

To investigate this possibility, we first constructed the bacterial plasmid pT7RNAi-*Hly-Inv*, termed TRIP (trans-kingdom RNAi plasmid) (**Supplementary Fig. 1** online). In this plasmid, the expression of shRNA was controlled by the bacteriophage T7 promoter[8,9] rather than by a mammalian promoter or enhancer. shRNA was detected in the bacterial system (**Fig. 1a**). The TRIP vector contains the *Inv* locus that encodes invasin[10], which permits the noninvasive *E. coli* to enter β1-integrin-positive mammalian cells[11]. The TRIP vector also contains *HlyA* whose product, listeriolysin O, permits genetic materials to escape from entry vesicles[12,13]. TRIP constructs were introduced into a competent strain of nonpathogenic *E. coli*, BL21DE3, which contains the T7 RNA polymerase needed for expression of shRNA. As a proof of concept, we constructed a TRIP against the cancer gene *CTNNB1*. Activation of the *CTNNB1* pathway from overexpression or oncogenic mutation of *CTNNB1* is responsible for the initiation of the vast majority of colon cancers and is involved in a variety of other cancer types[14]. Despite the potential of *CTNNB1* as a cancer therapeutic target, the *CTNNB1* pathway has been recalcitrant to inhibition by small molecules. *CTNNB1* is a preferred choice in proof-of-concept experiments for testing the potency of new RNAi approaches because it is commonly stabilized in cancer cells. Theoretically, TRIP could be modified to enable bacteria to express interfering RNA against various genes of interest.

To determine if gene silencing can be achieved through this trans-kingdom system, we cocultured human colon cancer cells (SW480) *in vitro* with *E. coli* for 2 h (**Fig. 1b–e**), then treated them with antibiotics to remove extracellular bacteria. Cells were further cultured for 16–48 h before harvest for analysis of gene silencing. *CTNNB1* was potently and specifically silenced at the mRNA and protein level (**Fig. 1b,c**), whereas β-*actin*, *KRAS* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were not affected (**Fig. 1b–f** and **Supplementary Fig. 2** online). Direct introduction of the TRIP plasmids into SW480 cells by transfection induced no gene silencing, further confirming that active shRNA was made by the bacteria but not by mammalian cells (**Supplementary Fig. 3** online). As an irrelevant shRNA control, *E. coli* containing the TRIP against wild-type *KRAS* exerted no gene-silencing effect on mutated *KRAS* in SW480 cells (**Fig. 1d**). As another control for the specificity of the trans-kingdom RNAi, *E. coli* containing the TRIP against mutant *KRAS* (GGT→GTT at codon 12) silenced *KRAS* expression in SW480 cells containing the same codon 12 mutation, but not in DLD1 cells with a mutation in a different codon of *KRAS* (GGC→GAC at codon 13, **Fig. 1e**). These results suggest that the trans-kingdom RNAi is gene-specific and sufficient to discriminate a point mutation.

To investigate the variables that affect the potency of the trans-kingdom RNAi system, cells were incubated for 2 h with *E. coli* at different multiplicities of infection (MOIs). The potency of gene silencing was dependent on MOI, with near complete gene silencing

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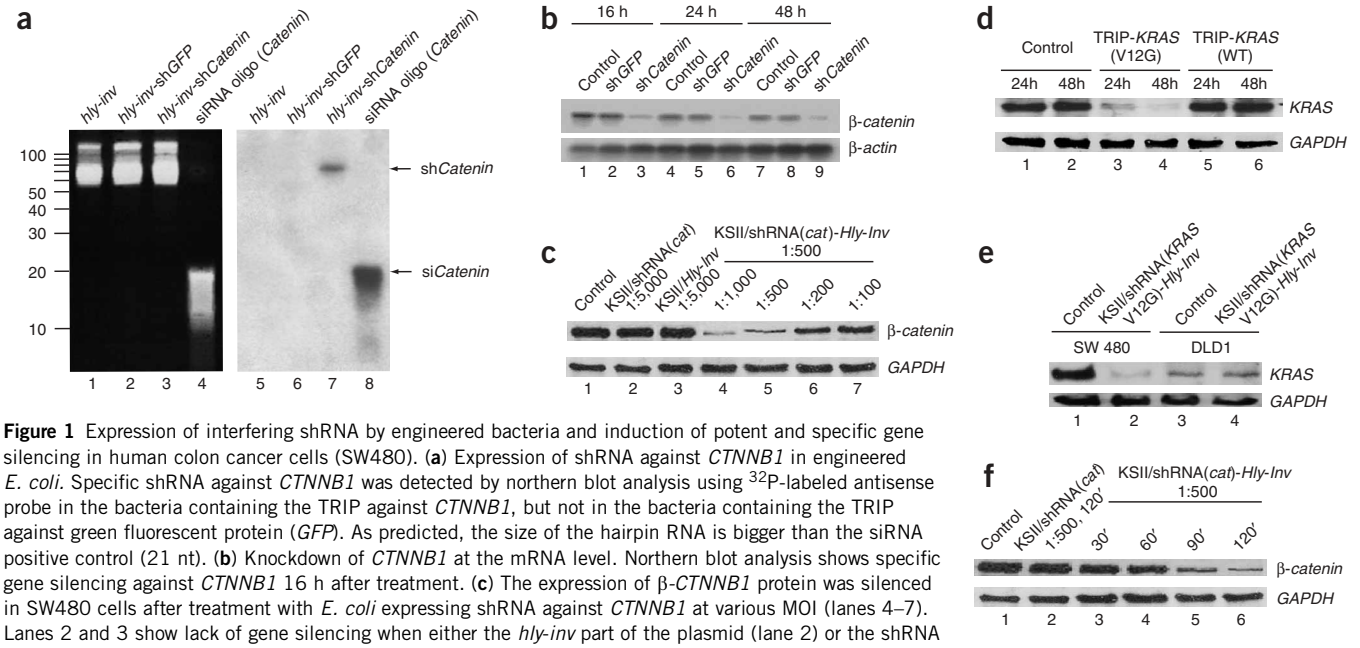

Figure 1 Expression of interfering shRNA by engineered bacteria and induction of potent and specific gene silencing in human colon cancer cells (SW480). (**a**) Expression of shRNA against *CTNNB1* in engineered *E. coli*. Specific shRNA against *CTNNB1* was detected by northern blot analysis using ^{32}P-labeled antisense probe in the bacteria containing the TRIP against *CTNNB1*, but not in the bacteria containing the TRIP against green fluorescent protein (*GFP*). As predicted, the size of the hairpin RNA is bigger than the siRNA positive control (21 nt). (**b**) Knockdown of *CTNNB1* at the mRNA level. Northern blot analysis shows specific gene silencing against *CTNNB1* 16 h after treatment. (**c**) The expression of β-*CTNNB1* protein was silenced in SW480 cells after treatment with *E. coli* expressing shRNA against *CTNNB1* at various MOI (lanes 4–7). Lanes 2 and 3 show lack of gene silencing when either the *hly-inv* part of the plasmid (lane 2) or the shRNA encoding part (lane 3) of the plasmid are missing, even at very high MOI. (**d**) *E. coli* containing the TRIP against wild-type *KRAS* exerted no gene silencing effects against mutant *KRAS* (V12G). (**e**) *E. coli* containing the TRIP against mutant *KRAS* (V12G) silenced codon-matched mutant *KRAS* expression in SW480 cells, but not in DLD1 cells with *KRAS* mutation in a different codon (GGC→GAC at codon 13). (**f**) The gene-silencing effect depends on the coculture time of human cells with the bacteria (lanes 3–6).

at an MOI of 1:1,000 (**Fig. 1c**). To determine the effect of coculture time on gene silencing, we incubated cells with the *E. coli* at an MOI of 1:500 for different times. Gene-silencing potency increased with incubation times up to 2 h (**Fig. 1f**). The dependency of gene silencing on MOI and coculture time provides controllable flexibility for gene silencing in various applications.

To further confirm that the *CTNNB1* gene silencing is mediated specifically by shRNA, we attempted to identify the specific cleavage fragment of *CTNNB1* mRNA by using the 5′-RACE (rapid amplification of cDNA ends) PCR technique[15]. A specific hallmark of RNAi-mediated gene silencing is the cleavage of target mRNA at the specific sites of the mRNA as predicted from the shRNA sequence. Based on the time course of *CTNNB1* silencing (**Fig. 2a**), total RNA was isolated from SW480 cells 8 h and 16 h after treatment with *E. coli* expressing shRNA against *CTNNB1* to identify the cleaved fragments of mRNA. The cleaved *CTNNB1* mRNA was found as early as 8 h after treatment with *E. coli* expressing shRNA—no fragments were detected in the control (**Fig. 2b,c**). The sequence analysis of the cleaved intermediate of *CTNNB1* mRNA confirms the cleavage site located within the targeted sequence. This result demonstrates that shRNA produced by bacteria trigger specific cleavage of the *CTNNB1* mRNA through RNAi-mediated gene silencing.

Induction of interferon response has been reported as a challenge to the specificity of some RNAi approaches[16,17]. To test whether the gene

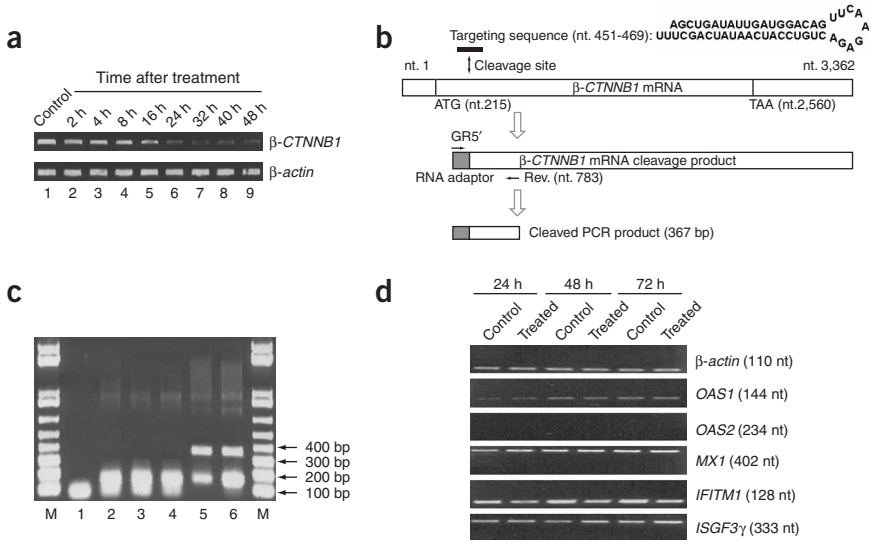

Figure 2 Specific cleavage of *CTNNB1* mRNA in SW480 cells induced by trans-kingdom RNAi. (**a**) The time course of *CTNNB1* silencing as determined by RT-PCR after treatment with *E. coli* expressing shRNAs against *CTNNB1*. (**b**) Specific cleavage site in *CTNNB1* mRNA induced by *E. coli* expressing specific shRNA as determined by 5′-RACE. (**c**) Specific cleavage products were amplified by 5′-RACE-PCR; 1 kb Plus DNA Ladder was used to identify the fragment size. Treatment groups are: 1, reagent control; 2, untreated SW480 cells; 3, KSII(+)/*Hly-Inv*-treated SW480 cells; 4, KSII(+)/*Inv*-shRNA(*cat*)-treated SW480 cells; 5 and 6 represent 8 h and 16 h after TRIP/shRNA against *CTNNB1* treatment, respectively. (**d**) *E. coli*-based trans-kingdom RNAi is not associated with induction of interferon response. The expression levels of *OAS1*, *OAS2*, *MX1*, *ISGF3*γ and *IFITM1* genes were determined by end-point RT-PCR (27 cycles) using ThermoScript RT-PCR System and Expand High-Fidelity PCR System.

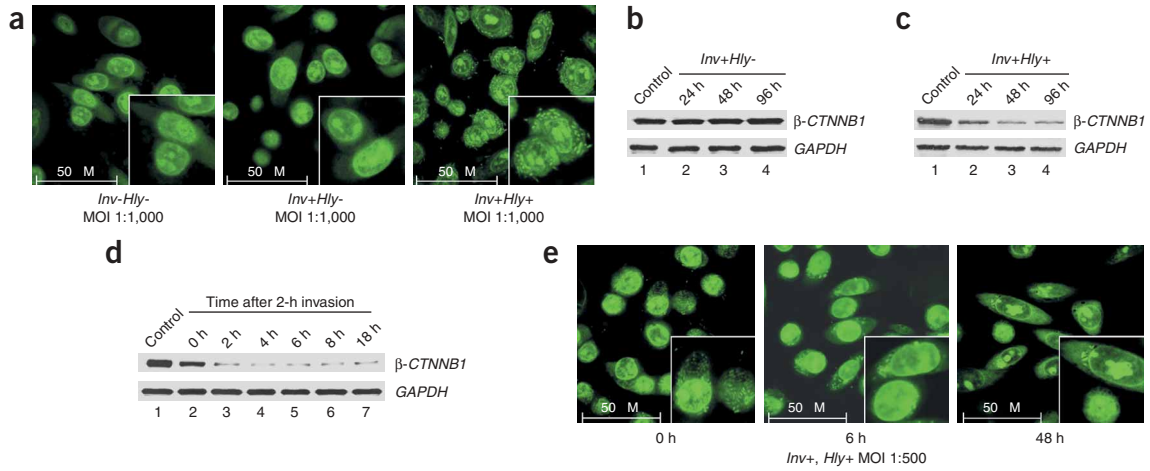

Figure 3 Trans-kingdom gene silencing requires bacterial entry of bacteria into mammalian cells in the presence of both bacterial *Inv* and *Hly* genes. (**a**) Both *Inv* and *Hly* are required for bacterial entry into SW480 cells. (**b**) TRIP lacking the *Hly* component is unable to induce knockdown of the target gene. (**c**) Both *Inv* and *Hly* are needed to facilitate efficient trans-kingdom RNAi. (**d**) Effect of delayed addition of tetracycline on gene silencing. After 2 h invasion, 15 μg/ml tetracycline was added at different time points ($t = 0$–18 h). Cells were harvested at 48 h after invasion. (**e**) Lack of significant bacterial replication in the absence of antibiotics beyond the initial 2-h incubation time.

silencing induced by the trans-kingdom RNAi is associated with interferon-response induction, we measured some key interferon-response genes. The genes encoding 2′,5′-oligoadenylate synthetases (*OAS1* and *OAS2*) are important interferon-induced genes for the inhibition of cellular protein synthesis after viral infection. *MX1*, whose product is a member of the interferon-induced myxovirus resistance protein family (MX proteins), participates in the innate host defense against RNA viruses. *IFITM1*, whose product is a member of the interferon-inducible transmembrane proteins, mediates the anti-proliferation activity of interferon. *ISGF3γ* is part of a cellular interferon receptor involved in interferon-induced transcription regulation and stimulation. We tested the levels of these genes to determine possible induction of the interferon response by RNAi. The mRNAs of the five interferon-response genes were analyzed with semiquantitative RT-PCR. No induction of *OAS1*, *OAS2*, *MX1*, *ISGF3γ* or *IFITM1* was detected after treatment with *E. coli* encoding shRNA against *CTNNB1* (**Fig. 2d** and **Supplementary Table 1** online). These data suggest that gene silencing induced by trans-kingdom RNAi is not associated with nonspecific interferon-response induction.

Next, we investigated the mechanism of the trans-kingdom RNAi transfer. To determine whether cellular entry of *E. coli* is required to induce RNAi, we compared the gene-silencing activity of *E. coli* with or without the *Inv* locus. The *Inv* encodes invasin that interacts with β1-integrin to facilitate the entry of *E. coli* into the cells. As expected, *E. coli* without *Inv* failed to enter cells (**Fig. 3a**). Surprisingly, *Inv* alone is not sufficient to enable *E. coli* to enter colon cancer cells (**Fig. 3a**), and no detectable gene silencing was observed in the absence of intracellular bacteria (**Fig. 3b**). We introduced the *HlyA* gene, which is thought to facilitate delivered genetic materials to escape from the entry vesicles[13]. As expected, *Hly* alone failed to enable cell invasion (data not shown), but commensal *E. coli* with both *Inv* and *Hly* entered colon cancer cells with high efficiency (**Fig. 3a**). Under these conditions *CTNNB1* was potently silenced up to 96 h (**Fig. 3c**). These results suggest that *E. coli* need both *Inv* and *Hly* to enter cells to induce trans-kingdom RNAi.

To determine whether gene silencing requires continued bacterial replication inside target cells, we used tetracycline to block intracellular bacterial replication and gentamycin to remove extracellular bacteria. SW480 cells were incubated with *E. coli* for 2 h,

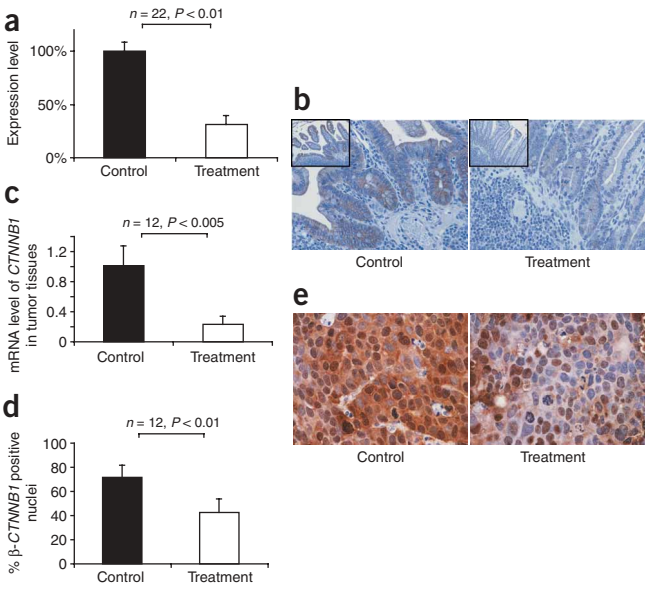

Figure 4 Trans-kingdom RNAi *in vivo*. (**a**) Oral administration of *E. coli* expressing shRNA against *CTNNB1* in mice leads to significant reduction of *CTNNB1* expression in the intestinal epithelium. The data represent the combination of two independent experiments. Quantification was done using Northern Eclipse (Empix Imaging) image analysis software. (**b**) Representative view of intestinal epithelium from treated (right) and control (left) groups. (**c**–**e**) Intravenous administration of *E. coli* transformed with TRIP against human β-*catenin* in mice with xenografted human tumors resulted in a decrease in the mRNA levels (**c**) and in protein levels (**d**,**e**) of *CTNNB1* in tumor tissues. All data are means ± s.e.m. Student's *t*-test (two-tailed) was used.

followed by tetracycline treatment initiated at different times. After the initial 2-h infection time, an additional 2-h incubation time without tetracycline induced near maximum gene silencing (**Fig. 3d**); further delay in tetracycline treatment had no further enhancing effect on the degree of gene silencing. Surprisingly, there was no evidence of substantial intracellular bacterial replication in the absence of tetracycline at 6 h and 48 h (**Fig. 3e**); replication is probably prevented by lysosomes and other intracellular anti-bacterial mechanisms[18,19]. These results suggest that trans-kingdom RNAi is not dependent on persistent bacterial replication inside target cells after the initial infection (2 h) and incubation time (2 h).

We next investigated whether trans-kingdom RNAi also functions *in vivo*. *E. coli* expressing shRNA against *Ctnnb1* were administered to mice orally. An inoculum of 5×10^{10} colony-forming units (c.f.u.) was administered orally five times per week, which is comparable to a human dosage[20] of the probiotic *E. coli Nissle 1917*. Most of the inoculum is eliminated during passage through the bactericidal environment in the upper gastrointestinal tract. Mice were treated with *E. coli* expressing shRNA against mouse *Ctnnb1* or with *E. coli* containing the corresponding TRIP vector. Treatment was continued for four weeks before the analysis of gene silencing by immunohistochemistry. *Ctnnb1* expression was silenced in the intestinal epithelium by *E. coli* expressing *Ctnnb1* shRNA ($P < 0.01$), not by the control *E. coli* containing the TRIP vector (**Fig. 4a–b**). In independent experiments, *E. coli* encoding shRNA against *GFP* did not induce *Ctnnb1* silencing. As a control for gene silencing specificity, *GAPDH* expression was not reduced (**Supplementary Fig. 4** online). The gene silencing effect was more pronounced in the regions of or adjacent to the Peyer's patches (**Fig. 4b**). Treatment was well tolerated with no gross or microscopic signs of epithelial damage or ulcerations (**Fig. 4b**, and data not shown). These results suggest that mammals respond to *E. coli* expressing specific shRNA with powerful regional RNAi *in vivo*.

We investigated whether the trans-kingdom RNAi approach can be used to silence a disease gene after systemic dosing. Intravenous administration of therapeutic bacteria has been tested in clinical trials with demonstrated safety in cancer patients[21]. Nude mice with xenografted human colon cancer cells were treated intravenously with 10^8 c.f.u. of *E. coli* encoding shRNA against human *CTNNB1*. Three doses were given at 5-day intervals. The treatments were well tolerated without adverse effects. Treatment with *E. coli* encoding shRNA against *CTNNB1* resulted in a significant decrease in *CTNNB1* mRNA ($P < 0.005$, **Fig. 4c**) and protein ($P < 0.01$, **Fig. 4d**,**e**) in the tumor tissues. These data suggest that bacteria-mediated trans-kingdom RNAi can silence a disease gene in a distant part of the body after systemic administration.

Our results suggest that bacteria-mediated trans-kingdom RNAi may offer a clinically feasible approach to the *in vivo* application of RNAi technology. Despite advances in chemical modifications and formulations, the synthetic short interfering (si)RNA approach is still not clinically feasible for systemic applications. It was estimated that even using one of the most advanced approaches for RNAi delivery[15], impractical amounts (grams) of siRNA would need to be administered to achieve a modest gene silencing effect[15]. The topical use of synthetic siRNA is limited to some local diseases[22]. The nonsynthetic siRNA–based *in vivo* approaches that use a viral vector, such as retrovirus or adenovirus, are limited to research use in animal models owing to safety concerns. In contrast, nonpathogenic bacteria, such as commensal *E. coli*, have been used clinically as probiotics with demonstrated safety after oral dosing[20]. Intravenous bacterial treatment regimens have been tested in clinical trials for the treatment of cancer[21]. Engineered bacteria may help to achieve potent RNAi for desired therapeutic effects with versatility and less cost. For example, this trans-kingdom RNAi approach can be exploited clinically to silence genes of interest in the colonic mucosa, and possibly also in other organs which can be colonized by commensal or nonpathogenic bacteria, including the oral cavity, urinary bladder and female genital tract. Because the shRNA is released inside target cells by the engineered bacteria, this RNAi approach may have the advantage of mitigating the Toll-like receptor–mediated immunostimulatory effect of siRNA[17].

Our finding of trans-kingdom RNAi in higher organisms may have important implications for functional genomics in mammalian systems. Bacteria, *E. coli* in particular, have served as a well-validated and versatile vector system for the revolution in molecular biology and biotechnology[23–26], and may be versatile RNAi vectors for functional genomics in mammalian systems. Compared to synthetic siRNA, the bacteria-based approach is less costly, and can be constructed, stored, reproduced and amplified, as is done for cDNA libraries and genomic libraries. Further, *in vitro* data with an shRNA construct can be further tested *in vivo*, offering a convenient system for the study of gene function and therapeutic target validation. Compared to viral vector–based approaches, the bacteria-based approach demands a lower biohazard level of the laboratory facility, is more convenient in the handling and does not require receptors on the target cells.

Our results provide a synthetic biological system for RNA-mediated trans-kingdom crosstalk in higher organisms, which may add to the complex relationship between mammals and prokaryotes. This transference of interfering RNA is different from 'transduction' since the exchanged genetic material is not a gene, but rather interfering RNAs. Our data invite the intriguing, albeit speculative, possibility that such transfer of interfering RNA between species or kingdoms may occur in natural interactions, such as infection, commensal interaction and symbiosis.

METHODS

TRIP plasmid construction. The TRIP was constructed in our laboratory as described in the followings. Briefly, oligonucleotides containing multiple cloning site (MCS), T7 promoter, enhancer and terminator (synthesized by Qiagen) were ligated into blunted *Bss*HII sites of KSII(+), and an shRNA encoding sequence (*CTNNB1* hairpin oligonucleotides) was inserted into the *Bam*HI and *Sal*I sites of MCS to generate plasmid pT7RNAi. The *HlyA* gene was amplified from pGB2Ω*inv-hly* (kindly provided by C. Grillot-Courvalin) by PCR (Pfx DNA polymerase, Invitrogen) with primers, hly-1: 5′-CCCTCCTTTGATTAGTATATTCCTATCTTA-3′ and hly-2: 5′-AAGCTTT TAAATCAGCAGGGGTCTTTTTGG-3′, and were cloned into the *Eco*RV site of KSII(+). *Pst*I fragments containing the *inv* locus of pGB2Ω*inv-hly* were inserted into the *Pst*I site of KSII(+)/*Hly*. The *Hly-Inv* fragment was excised with *Bam*HI and *Sal*I. After blunting, it was ligated into the *Eco*RV site incorporated within the T7 terminator of pT7RNAi.

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

Cell culture. SW480 cells (human colon cancer cells) were cultured in RPMI1640 medium with 10% FBS supplemented with antibiotics: 100 U/ml penicillin G, 10 μg/ml streptomycin and 2.5 μg/ml amphotericin (Sigma). For bacterial invasion, cells were cultured in the 6-cm dishes at 20% confluency one day before treatment. Medium was replaced with fresh medium without serum and antibiotics 30 min before addition of bacteria. Bacteria in early log phase were washed, diluted in RPMI and added at the desired MOI. After exposure, cells were washed twice and fresh complete medium containing 100 μg/ml of

ampicillin and 150 μg/ml of gentamycin were added. For staining of intracellular bacteria, cells were grown on Lab-Tek II Chamber Slides (Nalgene). A 2-h bacterial invasion was followed by a 30-min gentamycin treatment; cells were stained with Acridin Orange[27].

Northern blot analysis. Total RNA was extracted by TRIZOL (Invitrogen) from overnight bacteria culture (1×10^9 c.f.u.) and SW480 cells ($\sim 3 \times 10^6$) at different time points after treatment using TRIP system at an MOI of 1:1,000. To analyze bacterial shRNA by northern blot analysis, 10 μg of total RNA was loaded to each lane on a 15% TBE-Urea PAGE gel. 10 μl of 20 μM synthetic siRNA against *CTNNB1* (Ambion) was used as positive control. To determine gene silencing of *CTNNB1* by northern blot analysis, 20 μg of total cellular RNA was loaded to each lane on 1% denaturing agarose gel. After electrophoresis, RNA was transferred to Hybond-XL Nylon membrane (Amersham Biosciences) and crosslinked using 1,200 μJ UV irradiation followed by baking at 80 °C for 30 min. Antisense strand (5′-GUAGCUG AUAUUGAUGGACAG-3′) probe labeled with ^{32}P according to standard transcription protocol was used to detect the specific shRNA and siRNA against *CTNNB1*. Probe detecting *CTNNB1* and β-*actin* mRNA was prepared using Prime-It II Random Primer Labeling Kit (Stratagene) from *CTNNB1* template (1–568 nt). Film was exposed at –80 °C to detect shRNA and the *CTNNB1* mRNA.

Western blot. Cells were scraped off, washed and lysed (50 mM pH 7.5 HEPES, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1% NP-40, 1 mM DTT, 1 mM PMSF and 1% Sigma protease inhibitor cocktail). We applied 40 μg of protein to 10% SDS-PAGE and transferred to 0.2 μm nitrocellulose membrane (Schleicher & Schuell). Antibodies (Santa Cruz) against *CTNNB1* (SC-1496, 1:500), GAPDH (SC-20357, 1:400), KRAS (SC-30, 1:100), anti-goat (SC-2350, 1:4000) and anti-mouse (SC-2375, 1:2000) were used. Protein bands were detected using ECL (Amersham).

RT-PCR. Total RNA was prepared using the RNeasy Mini Kit (Qiagen), and cDNA was synthesized using random primers with Thermoscript RT-PCR System (Invitrogen). PCR was run for 20 cycles using Pfx polymerase. Primers: ACTIN-1, 5′-CCATGGATGATGATATCGCC-3′; ACTIN-2, 5′-TAGAAGCATT TGCGGTGGAC-3′; KRAS-1, 5′-GCCTGCTGAAAATGACTGAA- 3′; KRAS-2, 5′-GGCATCATCAACACCCAGAT-3′; CATB-1, 5′-GACAATGGCTACTCAAGC TG-3′; CATB-2, 5′-CAGGTCAGTATCAAACCAGG-3′.

5′-RACE analysis. Total RNA (5 μg) from SW480 cells treated with *E. coli* containing the TRIP encoding shRNA against *CTNNB1* or the TRIP vector was ligated to GeneRacer Oligo (Invitrogen) without any prior processing. Ligated RNA was reverse transcribed into cDNA using a random primer. To detect cleavage product, PCR was performed using Pfx polymerase (Invitrogen) with GeneRacer 5′ primer (Invitrogen) and *CTNNB1* specific primer: 5′-CGCAT GATAGCGTGTCTGGAAGCTT-3′. Amplification fragments were resolved on 1% agarose gel and sized using a 1-kb Plus DNA Ladder (Invitrogen). Specific cleavage site was further confirmed by sequencing.

Interferon-response detection. SW480 cells were treated for 2 h with *E. coli* transformed with the TRIP encoding shRNA against human *CTNNB1* or mutant *KRAS* at an MOI of 1:1,000. Untreated cells were used as control. Cells were harvested at 24, 48 and 72 h. The expression levels of *OAS1*, *OAS2*, *MX1*, *ISGF3γ* and *IFITM1* genes were determined by RT-PCR using the Interferon Response Detection Kit (SBI System Biosciences).

***In vivo* silencing experiments.** Female C57/BL6 mice (Charles River Laboratories) were randomly divided into two groups. The treatment group was administered orally with 5×10^{10} c.f.u. *E. coli* expressing shRNA against *XTNNB1* in 200 μl PBS. The control group was similarly treated except that the *E. coli* contains the TRIP vector without the shRNA insert. Two independent experiments were performed with 6 and 5 mice per group used, respectively. The treatment was carried out daily for 5 d per week for a total of 4 weeks. Mice were killed 2 d after the last treatment, and tissues were paraffin-embedded.

Xenograft cancer model. Female BALB/c nude mice (Charles River Laboratories) were randomized into two groups ($n = 6$). Three weeks before treatment, 1×10^7 of SW480 cells were implanted subcutaneously. Treatments were initiated when the tumors reached about 10 mm in diameter. The treatment group was injected through tail vein with 1×10^8 c.f.u. of *E. coli* expressing shRNA against *CTNNB1* in PBS. The control group was similarly treated except that the *E. coli* contains the TRIP vector without shRNA insert. The treatment was carried out every 5 d for a total of three treatments. Mice were killed 5 d after the last treatment. Tissues were frozen and fixed for analysis of *CTNNB1* mRNA level by real-time PCR and *CTNNB1* protein level by immunohistochemistry.

Immunohistochemistry. Immunostaining was performed on 6-μm tissue sections using Vectastain Elite ABC avidin-biotin staining kit (Vector). For antigen retrieval, slides were heated by microwave in 5% urea. Nonspecific binding sites were blocked with 0.5% BSA for 10 min and endogenous peroxidase activity was suppressed by treatment with 3% H_2O_2 in methanol for 10 min. Sections were exposed to primary antibody *CTNNB1* (SC-1496, 1:250) and GAPDH (SC-20357, 1:400) overnight at 4 °C. The chromogen was 3,3′-diamino-enzidine (Vector); counterstaining was done with hematoxylin. A total of ten images were randomly taken from each animal in the small and large intestine regions. Expression of *CTNNB1* was quantitatively analyzed using Northern Eclipse (Empix Imaging) software and Student's *t*-test to calculate significance.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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1. Timmons, L. & Fire, A. Specific interference by ingested dsRNA. *Nature* **395**, 854 (1998).
2. May, R.C. & Plasterk, R.H. RNA interference spreading in *C. elegans*. *Methods Enzymol.* **392**, 308–315 (2005).
3. Fraser, A.G. *et al.* Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature* **408**, 325–330 (2000).
4. Kamath, R.S. *et al.* Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* **421**, 231–237 (2003).
5. Ashrafi, K. *et al.* Genome-wide RNAi analysis of *Caenorhabditis elegans* fat regulatory genes. *Nature* **421**, 268–272 (2003).
6. Feinberg, E.H. & Hunter, C.P. Transport of dsRNA into cells by the transmembrane protein SID-1. *Science* **301**, 1545–1547 (2003).
7. Xiang, R. *et al.* A DNA vaccine targeting survivin combines apoptosis with suppression of angiogenesis in lung tumor eradication. *Cancer Res.* **65**, 553–561 (2005).
8. Milligan, J.F. & Uhlenbeck, O.C. Synthesis of small RNAs using T7 RNA polymerase. *Methods Enzymol.* **180**, 51–62 (1989).
9. Milligan, J.F., Groebe, D.R., Witherell, G.W. & Uhlenbeck, O.C. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res.* **15**, 8783–8798 (1987).
10. Isberg, R.R., Voorhis, D.L. & Falkow, S. Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. *Cell* **50**, 769–778 (1987).
11. Young, V.B., Falkow, S. & Schoolnik, G.K. The invasin protein of Yersinia enterocolitica: internalization of invasin-bearing bacteria by eukaryotic cells is associated with reorganization of the cytoskeleton. *J. Cell Biol.* **116**, 197–207 (1992).
12. Mathew, E., Hardee, G.E., Bennett, C.F. & Lee, K.D. Cytosolic delivery of antisense oligonucleotides by listeriolysin O-containing liposomes. *Gene Ther.* **10**, 1105–1115 (2003).
13. Grillot-Courvalin, C., Goussard, S., Huetz, F., Ojcius, D.M. & Courvalin, P. Functional gene transfer from intracellular bacteria to mammalian cells. *Nat. Biotechnol.* **16**, 862–866 (1998).
14. Kim, T.H., Xiong, H., Zhang, Z. & Ren, B. β-Catenin activates the growth factor endothelin-1 in colon cancer cells. *Oncogene* **24**, 597–604 (2005).
15. Soutschek, J. *et al.* Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* **432**, 173–178 (2004).
16. Bridge, A.J., Pebernard, S., Ducraux, A., Nicoulaz, A.L. & Iggo, R. Induction of an interferon response by RNAi vectors in mammalian cells. *Nat. Genet.* **34**, 263–264 (2003).

17. Hornung, V. *et al.* Sequence-specific potent induction o f IFN-α by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat. Med.* **11**, 263–270 (2005).
18. Roy, D. *et al.* A process for controlling intracellular bacterial infections induced by membrane injury. *Science* **304**, 1515–1518 (2004).
19. Battistoni, A. *et al.* Increased expression of periplasmic Cu,Zn superoxide dismutase enhances survival of *Escherichia coli* invasive strains within nonphagocytic cells. *Infect. Immun.* **68**, 30–37 (2000).
20. Rembacken, B.J., Snelling, A.M., Hawkey, P.M., Chalmers, D.M. & Axon, A.T. Non-pathogenic *Escherichia coli* versus mesalazine for the treatment of ulcerative colitis: a randomised trial. *Lancet* **354**, 635–639 (1999).
21. Toso, J.F. *et al.* Phase I study of the intravenous administration of attenuated Salmonella typhimurium to patients with metastatic melanoma. *J. Clin. Oncol.* **20**, 142–152 (2002).
22. Bitko, V., Musiyenko, A., Shulyayeva, O. & Barik, S. Inhibition of respiratory viruses by nasally administered siRNA. *Nat. Med.* **11**, 50–55 (2005).
23. Zhao, M. *et al.* Tumor-targeting bacterial therapy with amino acid auxotrophs of GFP-expressing Salmonella typhimurium. *Proc. Natl. Acad. Sci. USA* **102**, 755–760 (2005).
24. Narayanan, K. & Warburton, P.E. DNA modification and functional delivery into human cells using *Escherichia coli* DH10B. *Nucleic Acids Res.* **31**, e51 (2003).
25. Darji, A. *et al.* Oral somatic transgene vaccination using attenuated *S. typhimurium*. *Cell* **91**, 765–775 (1997).
26. Pawelek, J.M., Low, K.B. & Bermudes, D. Bacteria as tumour-targeting vectors. *Lancet Oncol.* **4**, 548–556 (2003).
27. Miliotis, M.D. Acridine orange stain for determining intracellular enteropathogens in HeLa cells. *J. Clin. Microbiol.* **29**, 830–831 (1991).