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**ORIGINAL ARTICLE**

# *Bifidobacterium breve* as a delivery vector of IL-24 gene therapy for head and neck squamous cell carcinoma *in vivo*

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Beneficial bacteria are becoming ever more popular gene delivery method for hypoxia-tumor targeting *in vivo*. In this study we investigated the therapeutic effect of new recombinant *Bifidobacterium breve* strain expressing interleukin (IL)-24 gene (*B. breve-IL24*) on head and neck tumor xenograft in mice. Briefly, *B. breve* transformants were obtained through electro-transformation. Bacteria-tumor-targeting ability were analyzed *in vivo* over different time points (1, 3 and 7 days post-bacteria injection). Furthermore, the therapeutic effect of bacteria on tumor cells *in vivo* were analyzed as follows: 30 Balb/c nude mice bearing subcutaneous tumor were randomly divided in three groups (Drug group, green fluorescent protein (GFP) group and Saline group). The therapy lasted for 2 weeks and included *B. breve-IL24* administration via tail vein for Drug group, *B. breve-GFP* for GFP group and phosphate buffered saline for Saline group. The tumor growth was monitored using standard caliper technique, while the apoptosis induction *in vivo* was analyzed by Real-time Positron Emission Tomography/Computed Tomography (PET/CT) imaging ([18F]-ML-10 tracer). At the end of the experiment, tumor tissues were collected and analyzed by western blotting. Briefly, our results suggested that our new recombinant bacterium has the capability of targeting tumor tissue *in vivo*. As for the therapeutic effect, our new strain has revealed to be a promising therapeutic approach against tumor growth *in vivo*. Briefly, higher tumor growth inhibition and higher tumor cell apoptosis induction were observed in Drug group compared with the GFP and Saline groups. To conclude, a new recombinant strain *B. breve-IL24* offers a novel, safe and clinically acceptable therapeutic approach for tumor therapy *in vivo*.

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## INTRODUCTION

Currently, gene therapy is a realistic option for all types of cancer treatment. Considering the poor survival rates due to drug resistance and lack of tumor specificity by using conventional methods, gene therapy offers a prospective approach for delivering the transgene to a desired location in human body. The chemical and biological gene delivery techniques are currently considered as model tools for cancer gene therapy *in vivo*. These approaches are less costly and more effective (higher gene transformation and no DNA degradation from serum nucleases) compared with mechanical and physical methodologies. Chemical methods are usually based on coating or incorporating a piece of nucleic acid (small interfering RNA or microRNA) on nanomaterials' surface such as cationic polymers, cationic peptides and cationic liposomes,[1,2] whereas biological methods rely on viruses or bacteria as main delivery vectors. When using viral vectors, the key advantages are the super efficiency in invading and delivering therapeutic genes into the cell. Nevertheless, from a safety point of view, numerous viruses may cause insertional mutagenesis and systemic inflammation responses, thereby limiting clinical efficacy and restricting the range of applicable therapeutic approaches.[3]

The use of bacteria as gene delivery method for cancer therapy has been known for decades. The correlation between the tumor regression and bacterial infection dates back to nineteenth century.[4] To date, different bacteria including both pathogenic strains and non-pathogenic strains have been tested in preclinical works.[5] In short, it has been shown that bacteria organisms have the ability to replicate and target the hypoxic region of the tumor *in vivo*; usually only 3–5% of tumor cells are considered in the growth fraction, whereas the remaining 95% of tumor tissue is hypoxic to some degree.[6] Nevertheless, there are notable differences in using pathogenic and non-pathogenic strains. For example, pathogenic bacteria such as *Salmonella*, *Clostridium and Listeria* have the capability of invading and amplifying gene encoding factors specifically within tumors.[7–9] However, due to the safety reasons, this approach does not provide neither optimal nor prospective clinical approach. On the other hand, the use of beneficial bacteria such as *Bifidobacterium* strains offers a straightforward, safe and clinically acceptable approach for delivering therapeutic proteins locally within the tumor environment, external to tumor cells. Over the last decade, numbers of data have been published on the use of commensal gut bacteria that is, *Bifidobacterium* species such as *breve*, *infantis*, *longum* and *adolescentis* for the liver, lung and melanoma tumor therapy *in vivo*.[10–13]

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In the present work we have established a new recombinant *B. breve* strain expressing a human *MDA-7/IL-24* gene for induction of head and neck squamous cell carcinoma (HNSCC) apoptosis *in vivo*. MDA-7/IL-24 is a member of the interleukin (IL)-10 family and has been strongly correlated with apoptosis induction in a variety of cancer including melanoma, breast, liver, prostate, ovarian and nasopharyngeal cancer.[14–19] In our previous study we have demonstrated that IL-24 could be a good prognostic biomarker and a valuable indicator of second primary malignancies in HNSCC.[20] According to our knowledge, this will be the first time of using *Bifidobacterium* species as delivery strategy of IL-24 for cancer therapy *in vivo*.

## RESULTS

### Construction of *B. breve* recombinant strains

The recombinant *Escherichia coli–Bifidobacterium* shuttle vectors pLW5 and pLW9, which include IL24 (835 bp) and green fluorescent protein (GFP, 930bp) fragments, respectively, were used to obtain *B. breve-IL24* (pLW5-breve) and *B. breve-GFP* (pLW9-breve) transformants. Contrary, recombinant strain *B. breve-Control* was transformed using the pAM4 (naked plasmid). The correct construction patterns were analyzed by digesting plasmids isolated from *B. breve* transformants with *Xba*I and *Xho*I (Figure 1a). Differences in bacteria growth rate between the wild-type strain and the transformants were analyzed. Briefly, the results showed higher growth rate in *B. breve-IL24* and *B. breve-GFP* compared with *B. breve-Control*, but no difference in comparison to *B. breve* wild-type strain, during the exponential phase. Furthermore, during the last growth stage, there was no difference among the four observed groups (Figure 1b). In addition, as shown in Figure 1c, no difference was observed in bacteria shape between transformants and the wild-type bacteria.

### Target gene expression *in vitro*

Real-time PCR was used to investigate the IL-24 mRNA expression level in the *B. breve-IL24* and *B. breve-GFP* strains, respectively; all data were compared with the *B. breve-Control* (naked plasmid) strain. Briefly, the results showed that the mRNA level of IL24 was 9466.72 ± 440.241-fold in *B. breve-IL24* group compared with 2.76 ± 1.059-fold registered in *B. breve-GFP* and 1.06 ± 0.502-fold registered in the *B. breve-Control* strain (Figure 2a). The *P*-value and multiple comparisons were shown in Figure 2b.

### Tumor-targeting property of recombinant *B. breve in vivo*

Tumor-specific targeting of the new recombinant *B. breve* strains were monitored *in vivo*, over 1, 3 and 7 days post injection (Figure 3). Briefly, 24 h post injection, bacteria clones were detected in the heart, liver, spleen, lung, kidney and tumor of the nude mice. Over the next 7 days, growing clones were visible only in the liver and tumor (day 3) and afterwards only in the tumor tissue (day 7). These results suggested that our new recombinant *B. breve* strain has the capability of targeting the tumor tissue *in vivo*.

### *B. breve-IL24* induce tumor apoptosis and tumor growth inhibition *in vivo*

Tumor size was measured to monitor the growth inhibition efficiency *in vivo*. Saline group was used as blank control and *B. breve-GFP* was used as negative control. Our data suggested a higher tumor growth inhibition in *B. breve-IL24* compared with *B. breve-GFP* group and higher tumor growth inhibition in *B. breve-GFP* compared with the Saline group, Figure 4a. The *P*-value among each group was shown in Figure 4b. In general, there were statistical differences in tumor size between Saline group and *B. breve-GFP* group from day 9. The statistical differences between Saline group and *B. breve-IL24* group in tumor size was from day 3, whereas no significance between *B. breve-GFP* and *B. breve-IL24* group were observed. Nevertheless, the average tumor size (including slower tumor growth rate) in *B. breve-IL24* was smaller compared with the *B. breve-GFP* group.

When used [18F]-ML-10, a Positron Emission Tomography (PET) tracer for apoptosis, our micro Positron Emission Tomography/Computed Tomography (PET/CT) scan showed that a tumor treated with *B. breve-IL24* has significantly higher apoptotic induction (namely higher radioactivity) compared with the Saline group *in vivo*, Figure 4c. To explore the underlying mechanism of apoptosis, anti- and pro- apoptotic protein expression was tested *ex vivo*, by western blot approach. In general, pro-apoptotic

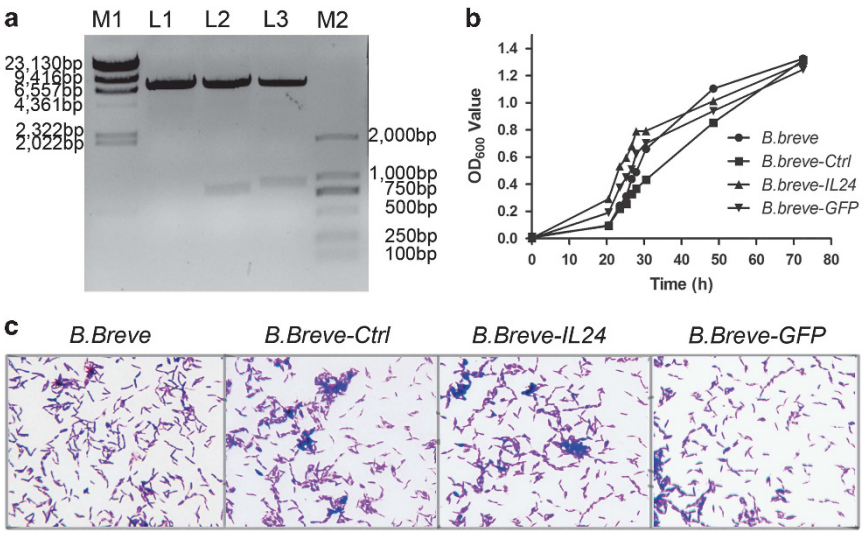
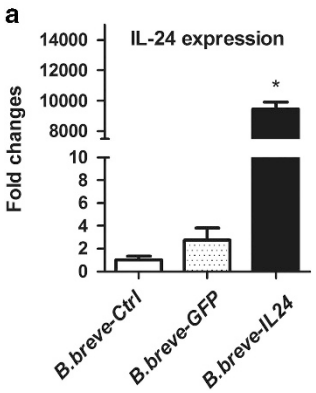



**Figure 1.** New recombinant *B. breve* transformants construction, growth curve and Gram staining difference compared with *B. breve* wild-type strain. (**a**) Enzyme (*Xba*I/*Xho*I) digestion pattern of plasmids isolated from *B. breve-Control* (L1), *B. breve-IL24* (L2) and *B. breve-GFP* (L3). For *Breve-IL24* and *Breve-GFP*, 835 bp and 930 bp fragment were obtained individually as designed. (**b**) Growth curve of *B. breve* wild type compared with the transformants over 72 h. (**c**) A gram staining of *B. breve* wild type and its transformants, magnified by ×100 oil. *B. breve* 1.3001 and its transformants were often branched, Gram-positive bacterium.




**b**

**Statistic of IL-24 expression**

| *P* value | | IL-24 expression |
|---|---|---|
| Levene statistic | | 0.001 |
| ANOVA | | **0.001** * |
| Multiple Comparisons (Games-Howell's correction) | *B.breve-Ctrl* vs *B.breve-GFP* | 0.362 |
| | *B.breve-Ctrl* vs *B.breve-IL24* | **0.031** * |
| | *B.breve-GFP* vs *B.breve-IL24* | **0.031** * |

* *P*<0.05

**Figure 2.** IL-24 gene expression between *B. breve-Control* and other two transformants (*B. breve-IL24* and *B. breve-GFP*). *$P < 0.05$. (**a**) Fold changes of IL-24 expression in mRNA level among *B. breve-Ctrl, B. breve-GFP* and *B. breve-IL24*. (**b**) The *P* value and multiple comparisons among three groups.

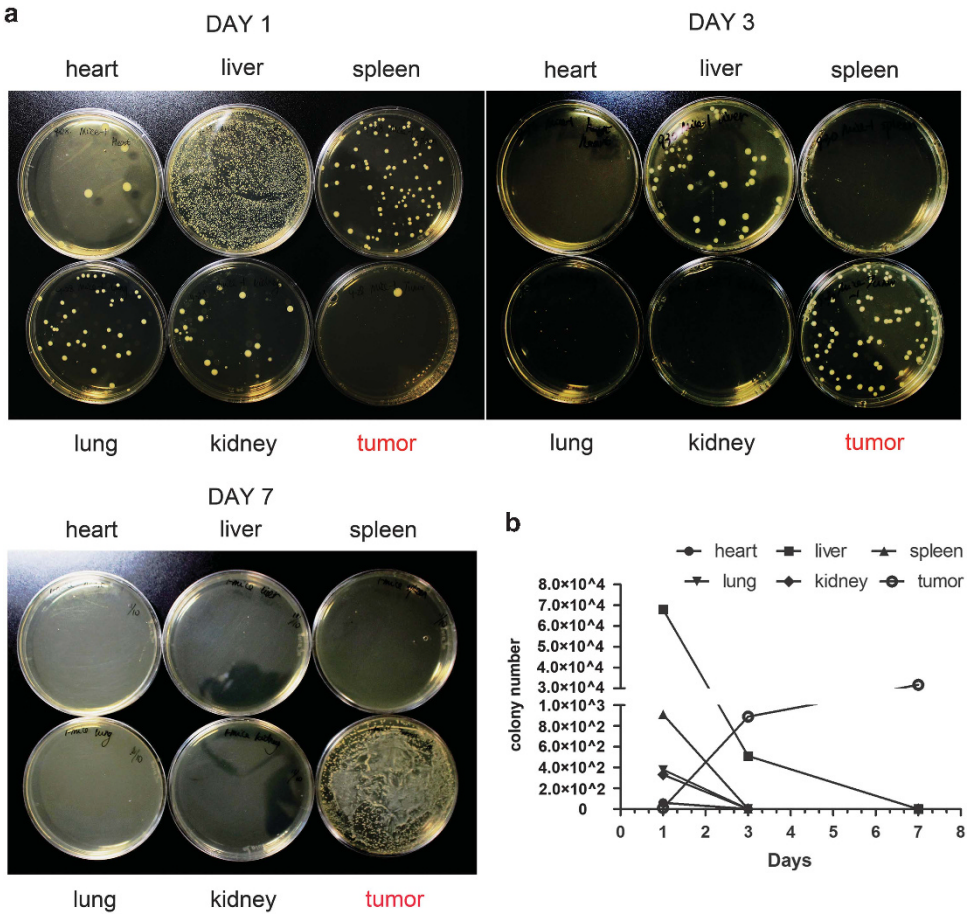
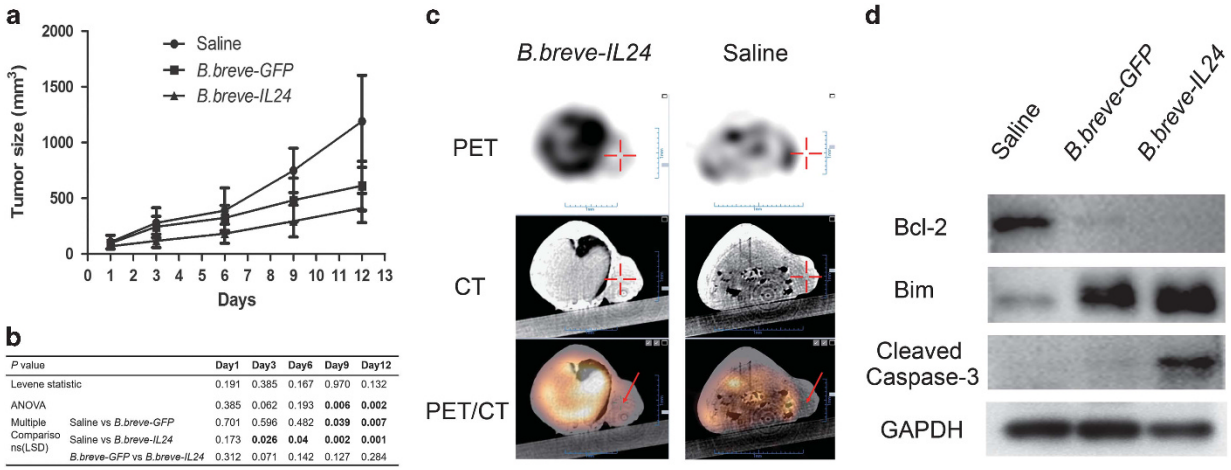



**Figure 3.** Analysis of targeting property of *B. breve* transformants to tumor tissue *in vivo*. (**a**) Day 1: bacteria visible in all the main organs including tumor tissue, ×10 dilution. Day 3: bacteria visible only in the liver and tumor tissue, ×10 dilution. Day 7: bacteria visible only in the tumor tissue, ×10 dilution. (**b**) The number of bacteria clones in different organs over Day 1, 3 and 7.

protein Bim was highly expressed in *B. breve-IL24*, whereas little Bcl-2 expression was detected in this strain compared with *B. breve-GFP* and Saline groups, Figure 4d. In addition, the expression of caspase 3 and cleaved caspase-3 was elevated in the *B. breve-IL24* group. Nevertheless, the expression of cleaved caspase-3, which is the active component of caspase-3, was difficult to test when using the total caspase-3 antibody analysis (Supplementary Figure S1 in Supplementary Information). To conclude, this data suggested that the IL-24 expressing new recombinant *B. breve-IL24* strain may be a suitable approach for cancer therapy *in vivo*.

In addition, compared with Saline group, higher Bim and lower Bcl-2 expression (but no cleaved caspase-3) was observed in *B. breve-GFP* group. This result was consistent with the tumor growth inhibition rate, thus suggesting the ability of bacteria itself to induce tumor inhibition *in vivo*.




**b**

| *P* value | | Day1 | Day3 | Day6 | Day9 | Day12 |
|---|---|---|---|---|---|---|
| Levene statistic | | 0.191 | 0.385 | 0.167 | 0.970 | 0.132 |
| ANOVA | | 0.385 | 0.062 | 0.193 | **0.006** | **0.002** |
| Multiple Comparisons(LSD) | Saline vs *B.breve-GFP* | 0.701 | 0.596 | 0.482 | **0.039** | **0.007** |
| | Saline vs *B.breve-IL24* | 0.173 | **0.026** | **0.04** | **0.002** | **0.001** |
| | *B.breve-GFP* vs *B.breve-IL24* | 0.312 | 0.071 | 0.142 | 0.127 | 0.284 |

**Figure 4.** Growth curve and apoptosis induction in mice bearing a WSU-HN6 tumor, after treatment with *B. breve-IL24*, *B. breve-GFP* and the Saline *in vivo*. (**a**) Tumor growth curve over 2 weeks of therapy with different therapeutic approach ($n = 10$). (**b**) *P*-value among each group. (**c**) Tumor uptake of [18F]-ML-10 tracer *in vivo*, by PET/CT scanning; the detected radioactivity was 135.35 for *B. breve-IL24* group (volume 169.13 $mm^3$) and 20.09 for the Saline group (volume 129.96 $mm^3$). (**d**) Anti- and Pro- apoptotic protein expression post therapy, in *ex vivo*.

## DISCUSSION

Mda-7/IL-24, a member of the IL-10 family, has been shown to function as a cytokine at physiological level and to exhibit anti-cancer effects at supra-physiological level. During physiological conditions, IL-24 protein can interact in a paracrine manner with IL-22R1/IL-20R2 and IL-20R1/IL-20R2 receptors expressed in immune system cells, resulting in the release of secondary cytokines.[21] These cytokines might then produce additional changes in cellular physiology, including cell proliferation in specific immune cell subsets, antitumor immune responses, and/or effects on immune cell growth. At supra-physiological level, number of studies have identified IL-24 as a multi-functional protein, which affects a biological behavior of cancer.[14–19] These functions include apoptosis and/or autophagy induction, tumor angiogenesis inhibition, migration and invasion inhibition and bystander effect, whereas no effect was detected in healthy cells. According to previous studies, Phase I clinical trial by Ad.mda-7 (INGN 241), which employed intratumoral injection, was initiated to evaluate its safety profile and its biological effects, both locally and systemically in humans with multiple advanced cancers.[21] According to Fisher and colleagues,[22] future clinical trials with IL-24 should employ improved delivery vectors and should focus on systemically targeted delivery.

From our previous study, we have learned that IL-24 expression can be strongly associated with the survival rate and second primary malignancies occurrence in HNSCC.[20] In addition, we have showed that apoptotic protein expression in HNSCC cell could be induced by adenoviral delivery of IL-24 (Ad-IL24) *in vitro*.[20] Nevertheless, it was indispensable to continue working on discovering the safer and more efficient gene delivery methods due to safety reasons (possible cause of insertional mutagenesis and systemic inflammatory). In this study, we have developed a novel delivery system for antitumor gene IL-24 by using probiotic bacteria *B. breve* for tumor therapy *in vivo*.

Restriction and modify system was a key factor when constructing the shuttle vector between *E. coli* and *Bifidobacterium* strains.[23] *hup* promoter and *sec2* fragment were chosen from *B. breve* UCC2003 strain, in order to facilitate the target gene *IL-24* replication and secretion outside the cells. The addition of raffinose to the culture medium, tested as a good carbon source for *B. breve*, assisted to enhance the *B. breve* cell growth *in vitro*.[24,25] After construction of the transformants, no significant differences in cell growth and cell shapes were found between the wild type and recombinant strains. In addition, enzyme digestion of the plasmids extraction from the recombinant bacteria was completely in line with design.

*In vitro* expression of target gene tests showed that IL-24 were highly expressed in *B. breve-IL24* at mRNA level. In addition, the GFP protein expression from *B. breve-GFP* was tested *in vivo*. After subcutaneous injection, GFP signal was clearly visible as detected by whole-body fluorescence reflectance imaging device (Supplementary Figure S2 in Supplementary Information). To sum up, these data have demonstrated the ability of our new recombinant strains, *B. breve-IL24* and *B. breve-GFP*, to constitutively express IL-24 or GFP genes under the direct action of the *hup* promoter.

Moreover, the biodistributions of our recombinant *B. breve-GFP* and *B. breve-IL24* were further investigated *in vivo*. Seven days post bacteria injection, bacteria was detected only in the tumor tissue. These results have re-confirmed the recently published data by proving the specific capability of the *Bifidobacterium* strain to target and colonize the tumor tissue *in vivo*.[26] Furthermore, Cronin *et al.*[27] have recently discovered that *Bifidobacterium* may translocate from the gastrointestinal track, enter the bloodstream and target the tumor tissue in mice. However, the route and mechanism of this translocation still remain unclear. The initial research design of our study was to use the *GFP* reporter gene to investigate the biological behavior of *B. breve-GFP in vivo* and in real-time. Unfortunately, due to low wavelength (causing higher tissue scattering and autoflorescence *in vivo*), we were unable to detect the GFP signal in deep tissue. Recently, we have tested and monitored the biologic behavior of bacteria expressing a novel far red fluorescence reporter gene mKate2 in the gut of the mice.[28] Owing to lower autoflorescence and lower tissue scattering (above 600nm) in deep tissue, far red fluorescence is a suitable choice for our further examination of the route and mechanism of *B. breve* translocation and tumor targeting in the small animals such as mice.

Finally and most importantly, our new recombinant strain *B. breve-IL24* has shown to be an promising therapeutic approach for cancer, by promoting tumor apoptosis which in turn promotes inhibition of tumor growth *in vivo*. As expected, higher tumor growth inhibition rate was observed in the Drug group (treated with *B. breve-IL24*) compared with the Saline and *B. breve-GFP* group; even though, no statistical significance was observed in tumor size between *B. breve-GFP* and *B. breve-IL24* group. As illustrated in Results section, the average tumor size (including slower tumor growth rate) in *B. breve-IL24* was smaller compared with *B. breve-GFP* group. We also analyzed the bacterial therapy on

an additional tumor cell line (that is, melanoma cell line MDA-MB-435s). The tumor growth curve and *P*-value are provided in the Supplementary Data and Supplementary Figure S3 in Supplementary Information. As the results in HNSCC, the average tumor size (including slower tumor growth rate) in *B. breve-IL24* was smaller compared with *B. breve-GFP* group in melanoma. In addition, the apoptotic induction of *B. breve-IL24* was examined in real-time, by using [18F]-ML-10 tracer and a homemade PET/CT *in vivo* imaging device. [18F]-ML-10 is a PET imaging probe specifically designed to target and enter apoptotic cells *in vivo*, and only recently it was used for the first time in a human study.[29] In this study, we used [18F]-ML-10 to compare a tracer/tumor uptake in the Saline and Drug group mice bearing a similar tumor size. Our imaging data showed higher tracer uptake in the Drug group compared with the Saline group. Although the data have been obtained from a smaller number of mice, these preliminary results suggested strong apoptotic induction by *B. breve-IL24* therapy *in vivo*. Moreover, PET/[18F]-ML-10 may be a suitable imaging approach for further studying the apoptotic induction by *B. breve-IL24* during early stages (few days post injection) of tumor development *in vivo*.

In addition, further *ex vivo* analysis of tumor tissue has confirmed that *B. breve-IL24* could reduce the anti-apoptotic protein Bcl-2 and enhance pro-apoptotic protein Bim expression. Moreover, compared with Saline group, lower Bcl-2 and higher Bim expression were detected in tumor treated with *B. breve-GFP* negative control strain. This data indicated that the *B. breve* itself have the ability to influence the tumor microenvironment and activate the host immune *response in vivo*, which was already verified by other researchers. Sivan *et al.*[30] have demonstrated that oral administration of commensal *Bifidobacterium* alone could enhance antitumor immunity *in vivo* to the same degree as programmed cell death protein 1 ligand 1-specific antibody therapy. The underlying mechanisms could act trough altering dendritic cell activity.[30]

Moreover, elevated expression of total caspase 3 was observed in *B. breve-IL24* compared with other two groups. In addition, a shadow dye in a cleaved caspase-3 position (19 and 17 kDa) was observed on the same polyvinylidene difluoride membrane. Thus, we hypothesized that the high signal of pro-caspase-3 was shedding the weak signal of cleaved caspase-3. Therefore, we analyzed the expression of cleaved caspase-3 independently. Our data indicated a high expression of cleaved caspase-3 in *B. breve-IL24* group (Supplementary Figure S1 in Supplementary Information). To conclude, considering the weaker expression of cleaved caspase-3 compared with obvious higher expression of Bim and lower expression of Bcl-2 in the *B. breve-IL24* group, we concluded that the apoptosis effect by this strain may mainly rely on the mitochondrial control (Bcl-2 and Bim) of caspase-independent cell death.[31]

Currently, several engineering bacteria have been under clinical trial investigation. For example, BioMed Valley Discoveries, Inc. (Kansas City, MO, USA) is currently examining the safety and the anti-tumor activity of intratumoral administration of *Clostridium* Novyi-NT spores in patients with treatment-refractory solid tumor malignancies (https://clinicaltrials.gov/ct2/show/NCT01924689). In addition, Marina Biotech, Inc. (City of Industry, CA, USA), has recently developed a new drug CEQ508 (*E. coli* bacteria engineered using transkingdom RNA interference platform), which can be administrated orally, and is used for Familial Adenomatous Polyposis treatment in clinical practice (http://www.marinabio.com/files/5014/3880/5803/15-03-30_-_Marina_Biotech_Announces_FDA_Fast_Track_Designation_for_CEQ508.pdf). Recently, Cronin *et al.*[27] compared different administration routes of *B. breve UCC2003*, that is, oral intake vs intravessal injection using a murine animal model. Their data suggested that orally taken of *B. breve UCC2003* was similar with intravenous administration in subsequent homing to and growth specifically in tumors.[27] Moreover, we are currently investigating the oral intake of *B. breve-IL24 in vivo*.

To conclude, a new recombinant strain *B. breve-IL24* can induce apoptosis and consequently increase tumor growth inhibition in HNSCC mice model *in vivo*. In addition, *B. breve-IL24* is not affecting the healthy tissue, thus could offer a safe and clinically acceptable therapeutic approach for tumor therapy *in vivo*.

## MATERIALS AND METHODS

### Reagents

The HNSCC cell line (WSU-HN6), authenticated by short tandem repeat profiling (Supplementary Figure S4) and free of mycoplasma contamination, was obtained from Central Laboratory of Peking University School and Hospital of Stomatology. It was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen, Waltham, MA, USA) and 1% Penicillin/Streptomycin (Gibco, Waltham, MA, USA) in a humidified atmosphere containing 5% $CO_2$/95% air at 37 °C. *B. breve* (No. 1.3001) was purchased from China General Microbiological Culture Collection Center (Beijing, China) and routinely cultured for 72 h in MRS Broth (BD, Franklin Lakes, NJ, USA) or RCM (Oxoid, Waltham, MA, USA) plus Raffinose (Amresco, Solon, OH, USA) supplement and 0.5% L-cysteine•HCl (Sigma Aldrich, Damstadt, Germany) (MRSRC), under anaerobic environment at 37 °C. *E. coli* DH5α strain was cultivated in Luria-Bertan broth at 37 °C with vigorous shaking. Bacteria culture medium was supplemented with the following antibiotic concentration: 100 μg ml$^{-1}$ Ampicillin, 5 μg ml$^{-1}$ Tetracycline for *E. coli* and 5 μg ml$^{-1}$ Tetracycline for *Bifidobacterium* strain. All the restriction enzymes and T4 DNA ligase were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

### Shuttle vector construction

*B. breve* UCC2003 was a kind gift from Prof. Douwe van Sinderen.[7] A backbone plasmid pAM4 was a kind gift from Prof. Baltasar Mayo.[32] Cloning vector pBluescript SK was stored in ACTA. Genomic DNA of *B. breve* UCC2003 was purified following the manufactures protocol (GeneJET Genomic DNA Purification Kit, Thermos Fisher), which was the template of hup promoter and sec2 fragment. IL-24 (Genebank NC_000001.11) fragment was PCR amplified using Human Universal cDNA (Clontech) as template. Briefly, a shuttle vector pLW5 was created as follows: promoter hup and signal peptide sec2 fragments were first sequentially cloned into pBluescript SK (pBSK) plasmid. IL24 gene was then cloned into pBSK-hup-sec2, by obtaining pBSK-hup-sec2-IL24 vector. Finally, the fragment hup-sec2-IL24 was digested and cloned into a backbone plasmid pAM4, which resulted in a shuttle vector pLW5. Additionally, pLW9 was constructed by cloning GFP into pLW5, which replaced sec2 and IL24 gene fragments.

### Transformation of *Bifidobacterium breve*

An overnight *B. breve* (No.1.3001) culture was first re-inoculated into fresh MRSRC medium (1:10) in anaerobic environment at 37 °C until the $OD_{600}$ of 0.4–0.6 was reached. The bacteria were then collected and centrifuged at 5000 rpm (4 °C), washed 3 times using washing buffer (0.5M sucrose+1 mM citrate acid) and finally re-suspended in 1:250 diluted washing buffer, resulting in *B. breve* competent cells.

0.5 μg plasmid (pAM4 or pLW5 or pLW9) was mixed with 50 μl competent cells in a pre-cooled Gene Pulser cuvette (Bio-rad, 1mm) and chilled on ice for 30 min before electroporation. A Gene Pulser Apparatus was employed and used at a high voltage of 15 KV/cm, 25 μF capacity and a parallel capacity of 200 Ω. 950 μl MRSRC medium was added to the cuvette immediately post electroporation and cultured for another 3 h at 37 °C in anaerobic environment to recover the antibiotic expression. The bacteria were then plated on the RCM agar plates supplement with 5 μg ml$^{-1}$ Tetracycline and cultured at 37 °C in anaerobic environment for 48–72 h.

### Culture of transfected *Bifidobacterium breve* and detection of positive clones

Transformed bacteria (*B. breve-Control*, *B. breve-GFP*, *B. breve-IL24*) were collected and cultivated in MRSRC medium supplement with 5 μg ml$^{-1}$ Tetracycline for 48–72 h, afterwards the plasmid DNA was extracted

(Tiangen, Beijing, China). XbaI and XhoI enzyme digestion was employed to test the extracted plasmid. Gram staining and growth curve were used to examine the bacteria shape and growth rate change post transformation.

### Real-time PCR

To investigate the expression of IL24 in new recombinant *Bifidobacterium* strains, total RNA was extracted by using RNAprep Pure Cell/Bacteria Kit (Tiangen, Beijing, China), which was reversely transcribed into double-stranded cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's protocols. Real-time PCR using SYBR green reagent (Roche, Indianapolis, IN, USA) was performed in an ABI Prism7000 Sequence Detection System (Applied Biosystems, Life Technologies, Warrington, UK). The primers for IL24 were as follows: IL24-F: TGCTGGAGTTCTACTTGAA, IL24-R: AGTTGTGACACGATGAGA. We choose uvrD as internal reference, which TTACCTATGATGCCTTCTTC as forward and CTGAGTGGCTGAGTATTC as reverse primers. The standard Real-time PCR conditions were 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. All reactions were performed in triplicate. The expression level of the target transcript in each sample was calculated using the comparative $2^{-\Delta\Delta Ct}$ method after normalization to uvrD expression. *B. breve-Control*, which was empty plasmid pAM4 transformants, was used as control group when comparing gene expression. The experiment was repeated three times.

### Animal experiments

This study was approved by the Medical Ethical Committee of Peking University Health Science Center (No. LA2016276). Balb/c male nude mice, 6–8 weeks old, weighing 19–21 g, were obtained from Vital River Laboratories, China. All the animals were housed in an environment with the temperature of 22 ± 1 ºC, relative humidity of 50 ± 1% and a light/dark cycle of 12/12 h. All animal studies (including the mice euthanasia procedure) were done in compliance with the regulations and guidelines of Peking University Institutional Animal Care and Use Committee. When compared three groups' tumor inhibition efficiency, the sample size was calculated according to one-way ANOVA (two-sided) model formulas provided in the website http://powerandsamplesize.com/Calculators/Compare-k-Means/1-Way-ANOVA-Pairwise. $\alpha = 0.05$ and $\beta = 0.9$ was chosen. The sample size was limited to seven to ten after calculating to allow for the detection of highly significant differences.

### Biodistribution study

12 mice were subcutaneously injected with $5 \times 10^6$ WSU-HN6 cells on the mice back (close to the right shoulder). When the tumor tissue reached approximately 5 mm in diameter, mice were randomly dividend into the *B. breve-GFP* and *B. breve-IL24* group, 6 mice per group. $1 \times 10^8$ viable recombinant bacteria were then collected, washed 3 times, re-suspended in 200 µl phosphate buffered saline (PBS) and injected by tail vain. The biodistribution experiment was performed on day 1, 3 and 7 post bacteria injection. During each time-point, 2 mice per group were euthanized, the main organs were dissected, weighed, and homogenized after added 9 parts of PBS containing 0.1% Tween 20 per g of per organ (dilution 1:10), assuming that the volume of 1 g of organ corresponds to 1 ml of PBS. Next they were plated on RCM agar plus tetracycline plates in different dilution times (1:10, 1:100, 1:1000).

### Cancer therapy *in vivo*

30 mice were subcutaneously injected with $1x10^6$ WSU-HN6 cells on the mice back. When the tumor tissue reached approximately 50 $mm^3$ in size, mice were randomly divided into three groups (Drug, GFP and Saline group), 10 mice per group. There was no significance difference of the average tumor sizes among three groups after random allocation. The therapy was given by tail vein twice per week, for a total of 2 weeks, as follows: $1 \times 10^8$ *B. breve-IL24* in 200 µl for the Drug group; $1 \times 10^8$ *B. breve-GFP* in 200 µl for the GFP group; 200 µl PBS for the Saline group. Tumor size was measured by researcher blinded to the group allocation twice a week post bacteria injection, using standard caliper tumor volume calculation method $V_{tumor} = (\text{length} \times \text{width}^2)/2$. After treatments all mice were euthanized and tumor tissues were dissected and quick frozen in liquid nitrogen, and then stored at − 80 °C for future western bolt assay. This experiment was run in triplicate.

### PET/CT imaging

The tumor apoptosis were analyzed by our homemade small PET/CT device, using [18F]-ML-10, which is a novel PET tracer for apoptosis.[33] Briefly, 0.3 mCi [18F]-ML-10 was first injected by tail vein (3 mice/group). 10 min later, mice was injected with Avertin anesthesia (2.5%, 0.5 ml per mice, i.p.) and palced on the animal bed in the dorsal position. During the next 54 min, the whole body PET/CT images were acquired as follows: 2 bed for CT scanning (15 min/ bed) and 3 bed for PET scanning (8 min/bed).

### Western blot

Tumor tissues were carefully dissected and stored in liquid nitrogen, followed by lysing in RIPA buffer (Applygen, Beijing, China) containing proteinase inhibitors and phosphatase inhibitors. After measuring protein concentration using the BCA kit (Thermo Fisher Scientific, Waltham, MA, USA), equal amounts of protein samples were separated by 15 % SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes by wet blotting. The membranes were blocked in 5 % non-fat dry milk for 1 h and probed with antibodies against caspase-3 (8G10) (CST 9665, 1/1000), cleaved caspase-3(Asp175) (CST9661, 1/1000), Bim (GTX27888, 1/1000), Bcl-2 (CST3498, 1/1000) and GAPDH (CST8884, 1/1000) separately at 4 °C overnight. After incubation with peroxidase-linked secondary antibodies for 1 h at room temperature, the enhanced chemiluminescent reagent was used to visualize the immune-reactive proteins.

### Statistical analysis

Real-time PCR results and tumor size were summarized as mean ± s.d. Regarding IL-24 expression and tumor size, comparisons were made using one-way analysis of variance (two-side) after normal distribution test and homogeneity of variance analysis. Depending on sample variance, least significant difference was used for multiple comparisons and no adjustments were made in tumor size analysis and Games–Howell's correction was applied for multiple comparisons in IL-24 expression level. All calculations and analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). When assessing, investigator was blind to the group allocation. A $P$-value $< 0.05$ was considered statistically significant.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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