Cancer Letters 448 (2019) 168–181

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Mini-review

Genetically engineered *Salmonella* Typhimurium: Recent advances in cancer therapy

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ARTICLE INFO

Keywords:
Salmonella Typhimurium
Cancer therapy
Mechanisms
Anti-tumor agents
Combination therapy

ABSTRACT

Bacteria have been investigated as anti-tumor therapeutic agents for more than a century, since Coley first observed successful curing of a patient with inoperable cancer by injection of streptococcal organisms. Previous studies have demonstrated that some obligate or facultative anaerobes can selectively accumulate and proliferate within tumors and suppress their growth. Developments in molecular biology as well as the complete genome sequencing of many bacterial species have increased the applicability of bacterial organisms for cancer treatment. In particular, the facultative anaerobe *Salmonella* Typhimurium has been widely studied and genetically engineered to improve its tumor-targeting ability as well as to reduce bacterial virulence. Moreover, the effectiveness of engineered attenuated *S.* Typhimurium strains employed as live delivery vectors of various anti-tumor therapeutic agents or combined with other therapies has been evaluated in a large number of animal experiments. The well-known *S.* Typhimurium mutant VNP20009 and its derivative strain TAPET-CD have even been applied in human clinical trials. However, *Salmonella*-mediated cancer therapies have not achieved the expected success, except in animal experiments. Many problems remain to be solved to exploit more promising strategies for combatting cancer with *Salmonella* bacteria. Here, we summarize the promising studies regarding cancer therapy mediated by *Salmonella* bacteria and highlight the main mechanisms of *Salmonella* anti-tumor activities.

1. Introduction

The limitations of conventional anti-tumor therapies, such as high toxicity to normal tissue cells, the inability to treat deep tumor tissue and the possibility of producing drug resistance in tumor cells, have prompted the search for alternative approaches. Many facultative or obligate anaerobic bacteria, such as *Clostridium* [1], *Bifidobacterium* [2], *Escherichia* coli [3] and *Salmonella* [4], have been shown to possess inherent tumor-targeting and tumor-killing activities. It has been more than one hundred years since Coley's discovery that injection of streptococcal organisms into a patient with inoperable cancer successfully cured the patient [5]. The development of molecular biology, the complete sequencing of many bacterial genomes and the establishment of genetic modification methods have greatly increased the applicability of bacterial organisms for cancer therapy. In particular, the facultative anaerobe *Salmonella enterica* serovar Typhimurium (hereafter *S.* Typhimurium) has been extensively studied and considered as a potential anti-tumor agent, which either provides tumoricidal effects directly or is used to deliver various anti-tumor drugs. Live engineered attenuated *Salmonella* can selectively colonize tumors, inhibit tumor growth and prolong survival after systemic infection in animal tumor models. For example, the well-known genetically engineered *S.* Typhimurium strains, VNP20009 (purI$^-$/*msbB*$^-$), A1-R (*leu*$^-$/*arg*$^-$) and the ΔppGpp strain SHJ2037 (*relA*$^-$/*spoT*$^-$), were all attenuated by more than 10,000-fold compared with the wild-type strain [4,6,7] and had a tumor-to-liver ratio of bacterial colonization of greater than 1000:1 [8–10], with robust inhibitory effects on tumor growth and metastasis in animal models [10–12]. The application of tumor-targeting *Salmonella* as delivery vectors can overcome the penetration limitations and maximize the activities of chemotherapeutic drugs while reducing systemic toxicity to the host. Cytokines, cytotoxic agents, regulatory factors, prodrug-converting enzymes, and small interfering RNAs (siRNAs) are all potential targets that can be delivered by *Salmonella* bacterial vectors for cancer treatment. By regulating gene expression, it is possible to further restrict *Salmonella*-delivered anti-tumor agents accumulating at the tumor site and to control the timing of drug action. In addition, *Salmonella* and traditional therapies can synergize to improve anti-tumor efficacy, with *Salmonella* acting in

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https://doi.org/10.1016/j.canlet.2019.01.037
Received 23 November 2018; Received in revised form 27 January 2019; Accepted 30 January 2019
0304-3835/ Published by Elsevier B.V.

Table 1
Advantages or mechanisms of *Salmonella* bacteria as an anti-tumor agent.

Features	Description	References
Tumor-targeting	Upon systemic injection, *Salmonella* can accumulate in tumors at a ratio greater than 1000:1 compared to normal tissues, thus reducing toxicity to the host	[8,13–18]
Intratumoral penetration	Motile *Salmonella* can actively migrate away from the vasculature into the deep tumor tissue, thus overcoming the penetration limitation of chemotherapeutics	[13,16,17,19–21]
Killing of cancer cells	*Salmonella* have intrinsic activities of killing of cancer cells through inducing both cellular apoptosis and autophagy	[19,22–27,29–31]
Inhibition of angiogenesis	*Salmonella* can inhibit tumor angiogenesis, which involves inhibition of the expression of angiogenic factors, such as VEGF	[28,32–34]
Immune activation	*Salmonella* infection can stimulate the host anti-tumor immune responses inside tumor tissue, including inflammatory and T cell-dependent immune responses	[10,29,33,35–49]
Convenient modification of *Salmonella*	*Salmonella* with intrinsically pathogenic properties can be genetically engineered to efficiently attenuate bacterial virulence and to enhance the tumor-targeting ability, thus increasing its applicability for cancer treatment	Table 2
Delivery of anti-tumor agents	Various anti-tumor agents, such as cytokines, cytotoxic agents, regulating molecules, tumor-associated antigens, prodrug enzymes, and siRNA, can be delivered by recombinant attenuated *Salmonella* vectors and precise triggering or regulation of gene expression can minimize the systemic toxicity of these drugs to host	Table 3; [76,98,145,146,148,149,151–158]
Combination therapies	*Salmonella* treatment combined with chemotherapy or radiotherapy may have enhanced anti-tumor effects and decreased toxicity compared with either single treatment	[122,127–138,140–144]

areas of hypoxia and necrosis while chemotherapy or radiotherapy treat tumor tissues near blood vessels. However, many issues remain to be resolved before *Salmonella* bacteria are applied for clinical cancer treatment. For example, many studies have reported tumor specificity and tumor-suppressive effects of *Salmonella*, but the underlying mechanisms are not clearly understood. In the small number of human clinical trials conducted, although *Salmonella* bacteria could be administered safely, significant tumor-inhibitory effects similar to those observed in numerous animal experiments were not observed, suggesting that differences between animal and human tumors may influence the anti-tumor effectiveness of *Salmonella*. Here, we summarize the main anti-tumor mechanisms of *Salmonella* and promising studies using genetically modified *Salmonella* (alone or combined with other strategies) in cancer treatment (see Table 1).

2. The mechanisms of *Salmonella* as an anti-tumor agent

2.1. Tumor-targeting properties

One of the main advantages of bacteria-mediated cancer treatment is that some bacteria have the ability to specifically target tumors. Studies have shown that *Salmonella* can preferentially accumulate in tumors at a ratio of greater than 1000:1 compared with healthy tissues such as the liver and spleen [8]. However, the mechanisms of tumor-targeting by *Salmonella* are complex and not completely known. Tumor blood vessels are structurally irregular and leaky and commonly exhibit a defective cellular lining composed of disorganized, loosely connected, branched or overlapping endothelial cells. It was assumed that bacteria as well as many macromolecular chemotherapeutics enter tumor tissue via the openings between such disorganized endothelial cells, which are between 200 and 2000 nm in size. The escape of bacteria from the bloodstream into tumor tissue could be passive or active. According to an active mechanism, *Salmonella* could seek the openings described above by using its chemotactic systems and motility [13,14]. In the passive scenario, a small number of bacteria would fortuitously enter the tumor via these openings and then rapidly proliferate. *Salmonella* likely induces strong blood influx into tumor tissue by promoting the expression of blood vessel-disrupting factors; this influx further flushes bacteria into the tumor tissue [15]. In fact, these active and passive mechanisms are not mutually exclusive, and *Salmonella* may use both to specifically target the tumor.

Oxygen is one of the most important signals used by bacteria to sense the tumor microenvironment. Hypoxia is a well-characterized feature of most solid tumors and favors tumor-specific accumulation of anaerobes, including *Salmonella* bacteria. Forbes et al. also showed that motile *Salmonella* bacteria can move by chemotaxis to tumor tissue by sensing certain chemicals secreted by the tumor microenvironment. In detail, the aspartate receptor initiates chemotaxis toward tumor cylindroids, the serine receptor initiates penetration, and the ribose/galactose receptor directs *S.* Typhimurium toward the tumor necrosis region [13,16]. However, Stritzker et al. revealed that chemotaxis and motility do not play significant roles in bacterial colonization and distribution within the tumor. Although both chemotaxis and motility mutants showed significantly altered motility compared with wild-type *S.* Typhimurium in a soft-agar motility assay, the colonization efficiency and distribution patterns in tumor tissue did not reveal significant differences among these strains. Colonization and intratumoral migration upon intravenous injection instead appears to be a passive process that is influenced by the reticuloendothelial system of the host, the tumor microenvironment and bacterial metabolism [17]. Leschner et al. investigated the initial events of tumor colonization by auxotrophic *Salmonella* SL7202 and demonstrated that bacterial accumulation within tumor tissue was associated with strong hemorrhage promoted by tumor necrosis factor-α (TNF-α) induced by *Salmonella* infection. The effects of *Salmonella* bacteria in tumor blood vessels resemble those of vascular disrupting agents (VDAs) or TNF-α [15]. After systemic injection, *Salmonella* attached to the tumor vessel wall at a low frequency, and the number of bacteria adhering to the blood vessel was dependent on the blood velocity, indicating that hemodynamics also plays an important role in the initial interaction of bacteria with the tumor [16]. In addition, inside the hypoxic and necrotic microenvironment of tumor tissue, *Salmonella* may obtain nutrients needed for growth and avoid clearance by the host immune system [18]. These are another two potential mechanisms to explain the preferential accumulation of *Salmonella* in the tumor rather than normal tissues for longer periods.

2.2. Penetration within tumor tissue

Motility is another potential advantage of bacterial-mediated tumor therapy that enables the treatment of deep tumor tissue. The distribution of chemotherapy drugs inside the tumor depends on passive transport, and the drug concentration drops with distance from the vasculature. However, bacteria are complex living organisms that can acquire energy from the surrounding environment, and thus their transport is not entropically limited. Theoretically, bacteria with motility hold promise for overcoming the penetration limitation of chemotherapeutics by actively dispersing away from the vasculature into

deep tumor tissue.

Forbes et al. defined four discrete tumor regions in tumor tissue, including a highly vascularized edge, a necrotic core, a viable body and a transition zone between the core and the body. After systemic administration, *Salmonella* were observed to accumulate as colonies and disperse in all tumor regions [19]. During the initial infection, colonized *Salmonella* may have different phenotypes in tumor tissue, with the formation of large colonies near blood vessels (proliferating) and small colonies both near (inactive) and far (penetrating) from vessels [20]. The bacteria subsequently migrated away from the vasculature and specifically accumulated within the necrotic region of tumor tissue one week post infection [16]. In an *in vitro* model consisting of continuously perfused solid tumor tissue, motility strongly affected the spatial distribution of bacterial accumulation within tumor tissue, and bacterial strains that were more motile penetrated deeper [14]. Moreover, knocking out the ribose/galactose receptor improved bacterial accumulation in the quiescent zone [13,20]. By contrast, Stritzker et al. showed that intratumoral migration of *S.* Typhimurium is likely a passive process that is independent of motility as well as chemotaxis. Wild-type *S.* Typhimurium and chemotaxis and motility mutants similarly accumulated in both the necrotic region and the border zone between vital and necrotic tumor tissue two days after bacterial injection [17]. However, these studies employed different strains and time points post infection to investigate the tumor-colonization events of *Salmonella*.

In addition, the host immune system may affect the distribution of *Salmonella* bacteria in tumor tissue. For example, neutrophils prevent bacteria from spreading from necrotic into vital tumor tissue. The bacteria-containing necrotic region is separated from a rim of viable cells by a barrier of infiltrated host neutrophils. Depleting these host neutrophils would increase the number of intratumoral bacteria and enable partial spreading of bacteria to vital tumor tissue, with the majority of bacteria still located in the necrotic region [21].

2.3. Direct killing of cancer cells

Salmonella has been reported to have intrinsic anti-tumor activities, although the underlying mechanisms have not been identified clearly. *In vitro*, infection with *Salmonella* A1-R caused some cancer cells to die rapidly within 30 min, and most cancer cells died within 2 h [22]. Killing of cancer cells was also observed in live tumor-bearing mice treated with A1-R by multiphoton tomography, which showed that the bacteria-infected cancer cells greatly expanded, burst and thus lost viability [23].

Previous studies have shown that the induction of tumor cell apoptosis is associated with the accumulation of *Salmonella* in tumor tissue [19]. *Salmonella* in tumors may induce apoptosis through a variety of mechanisms, including competition with cancer cells for nutrients and release of bacterial toxins. After coculture with attenuated *Salmonella* bacteria *in vitro* at a multiplicity of infection (MOI) of 1000:1, tumor cells underwent increased apoptosis, as determined via TUNEL and caspase-3 (Cas-3) activity assays as well as Annexin-V detection [24,25]. *Salmonella* infection also induced increased apoptosis in tumor tissues *in vivo* [19]. In addition to induction of apoptosis in tumor cells, *Salmonella* can induce autophagy, a cellular process that mediates the degradation of long-lived proteins and unwanted organelles in the cytoplasm. Tumor-targeting *S.* Typhimurium A1-R and VNP20009 both induced autophagy in cancer cells, as assessed by puncta formation and the conversion of LC3 I (microtubule-associated protein 1 light chain 3) to lipidated LC3 II [26]. Lee et al. used the autophagy inhibitor 3-methyladenine and the apoptosis inhibitor Z-VAD-FMK to demonstrate that *Salmonella* treatment induces both autophagy and apoptosis, which partner to cause cell death. *Salmonella* induced autophagy in tumor cells in a dose- and time-dependent manner by downregulating the protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway. Infected tumor cells undergo increased autophagy when apoptosis is blocked [27].

The AKT/mTOR pathway plays an important role in cellular physiology and homeostasis. By downregulating this axis in tumor cells, *Salmonella* can reduce the expression of many oncoproteins, including hypoxia-inducible factors (HIF) [28], indoleamine 2,3-dioxygenase (IDO) [29], P-glycoprotein [30] (P-gp), and matrix metalloproteinase 9 (MMP-9) [31], and thus inhibit tumor angiogenesis, immunosuppression, drug resistance and metastasis.

2.4. Inhibition of angiogenesis

Angiogenesis is a key factor in the progression and eventual metastasis of tumors. In addition to increasing tumor cell death, *Salmonella* can inhibit angiogenesis or destroy blood vessels within tumor tissue to delay tumor growth [28,32]. Both *in vitro* and *in vivo*, *Salmonella* infection can reduce the expression of vascular endothelial growth factor (VEGF), an important angiogenic factor [28]. The molecular mechanism by which *Salmonella* regulates VEGF involves inhibition of HIF-1α expression via downregulation of the AKT/mTOR pathway. In melanoma cells, *Salmonella* infection can also induce the upregulation of connexin 43 (Cx43) [33], a protein recently shown to inhibit tumor angiogenesis via downregulation of HIF-1α and VEGF [34]. Moreover, the vessel-destruction efficacy of *Salmonella* in tumors correlated with the extent of tumor vascularity. Tumors with abundant blood vessels responded to bacterial therapy earlier than those with sparse vessels [32].

2.5. Sensitization of the host immune system

Salmonella-induced tumor suppression is not only mediated by the intrinsic anti-tumor activity of *Salmonella* itself but also by the host immune responses, including the inflammatory and T cell-dependent immune responses. One of the major obstacles to achieve effective anti-tumor therapy is the immunosuppressive environment generated by tumors, which includes myeloid-derived suppressor cells and regulatory T cells. Attenuated microorganisms including *Salmonella* are promising for inducing a tumor microenvironment shift from immunosuppressive to immunogenic [35]. *Salmonella* infection in tumors can reduce the expression of the immunosuppressive molecule IDO, which is responsible for the activation of regulatory T cells (T_{reg}), inactivation of effective T cells and even apoptosis of immune cells [29,36]. Hong et al. revealed that intratumoral injection of recombinant attenuated *Salmonella* could elicit transformation of immunosuppressive myeloid-derived suppressor cells into TNF-α-secreting cells with characteristics of neutrophils and reduce the generation of T_{reg} cells, thereby significantly inhibiting tumor growth [37]. Kaimala et al. also demonstrated that *Salmonella* infection in tumor-bearing mice led to phenotypic and functional maturation of intratumoral myeloid cells, making them less suppressive [38]. Liu and Chopra showed that the anti-tumor immune responses induced by *Salmonella*, in which lipopolysaccharide (LPS) and Braun lipoprotein (Lpp) played a critical role, were related to the downregulation of $CD44^{high}$ and $CD4^+CD25^+$ T_{reg} cells [39]. A significantly increased proportion of M1-like macrophages and reduced T_{reg} cells were also observed in tumors colonized by ΔppGpp *Salmonella* during the period of tumor suppression [40].

Many bacterial components, including pathogen-associated molecular patterns (PAMPs), LPS, and flagella, have been shown to possess anti-tumor activities [41–43]. For example, Weiss et al. reported that systemic therapy using purified *Salmonella* LPS could induce high levels of TNF-α and tumor-specific $CD8^+$ T cell responses and result in the clearance of CT26 tumors, similar to viable *Salmonella* [41]. Peritumoral administration of flagellin could also significantly inhibit the growth of an immunogenic tumor, which was associated with an increased IFN-γ:IL-4 ratio and a decreased frequency of $CD4^+CD25^+$ T_{reg} cells [43]. These results are consistent with the hypothesis that the intrinsic anti-tumor effects of *Salmonella* depend on bacterial

immunogenicity or adjuvanticity, although alternate mechanisms may also contribute. Several studies have shown that *Salmonella* can induce anti-tumor responses by recruiting immune cells into tumor tissue, such as neutrophils, macrophages, dendritic cells (DCs) and $CD8^+$ T cells [40,44], as well as by inducing abundant expression of IL-1β, TNF-α and other proinflammatory cytokines [40,45,46]. In response to bacterial colonization, increased IL-1β and TNF-α cytokines are mainly produced by $CD11c^+$ DCs and $CD68^+$ macrophages, respectively, and both are associated with tumor regression. The levels of both IL-1β and TNF-α returned to normal when the tumors started to regrow [40]. *Salmonella* treatment can also significantly upregulate interferon-gamma (IFN-γ) [45,46], a multifunctional cytokine that can mediate anti-tumor effects directly by inhibiting tumor cell growth and/or indirectly by recruiting and activating leukocytes involved in the innate as well as the adaptive immune responses. In contrast to wild-type mice, tumor growth was unimpeded after *Salmonella* treatment in $MyD88^{-/-}$ mice, which lack a critical adaptor molecule required for most TLR signaling pathways [38]; in addition, wild-type mice showed more efficient tumor inhibition than TLR4-deficient mice [46]. Zheng et al. also found that the antitumor activity of ΔppGpp *S.* Typhimurium was completely abrogated in $TLR4^{-/-}$ and $MyD88^{-/-}$ mice and that colonized *Salmonella* bacteria were not able to induce the infiltration of monocytes/macrophages and neutrophils into tumors in $TLR4^{-/-}$ mice when compared with wild-type mice [10]. Furthermore, there was significant up-regulation of IFN-γ, IFN-inducible chemokines CXCL9 (MIG) and CXCL10 (IP-10), and increased apoptosis of cancer cells in wild-type mice compared with TLR4-deficient mice [46]. Thus, it can be speculated that *Salmonella*-induced host anti-tumor responses at least partially depend on a functional MyD88-TLR4 pathway. In addition, it has been found recently that *Salmonella* colonization of tumors promotes the secretion of inflammatory cytokines such as IL-1β and IL-18 via activating inflammasome pathways. Upon direct interaction with cancer cells damaged by *Salmonella*, bone marrow-derived macrophages were shown to express inflammasome-related proteins including NLRP3, IPAF and active caspase-1 p10, in which ATP released by damaged cancer cells may act as an upstream signal of inflammasome activation [47].

Studies have indicated that *Salmonella* infection can induce cross-presentation of tumor antigens and subsequent tumor-specific immune responses. In detail, infection with *Salmonella* in melanoma cells induces the upregulation of connexin 43 (Cx43), a ubiquitous protein that is normally lost during melanoma progression. Functional gap junctions (i.e., Cx43) established between tumor cells and adjacent DCs then transfer preprocessed antigen peptides from tumor cells to DCs. The antigen peptides are subsequently presented on the surface of the DCs and activate cytotoxic T cells, which are able to kill tumor cells at the primary site and prevent metastasis formation [33]. Moreover, the host anti-tumor response may be enhanced by the killing of tumor cells recognized as infected rather than as malignant. Avogadri et al. showed that *Salmonella*-infected melanoma cells could present antigenic determinants of bacterial origin and thus became targets of *Salmonella*-specific T cells [48]. During the clearance of killed cancer cells, tumor debris is generated in large quantities and taken up by antigen-presenting cells, including macrophage and DCs. The processed tumor antigens are then presented to naive T cells for stimulation of tumor-specific T cells. Lee et al. compared the anti-tumor effects of systemic *Salmonella* infection in wild-type, $CD4^+$ T cell-deficient and $CD8^+$ T cell-deficient mice bearing syngeneic tumors. Upon *Salmonella* injection, more efficient inhibition of tumor growth was observed in wild-type mice compared with T cell-deficient mice, indicating that the anti-tumor effects of *Salmonella* are associated with host T cells [49]. Another study demonstrated that *Salmonella*-mediated anti-tumor effects were independent of T lymphocytes and T-dependent antibody responses, with identical inhibition of tumor growth observed in C57BL/6 (wild-type), NMRI/nude (deficient in T lymphocytes) and $CD154^{-/-}$ (featuring a disrupted costimulatory pathway required for T-B cell interactions and immunoglobulin isotype-switching) mice [38]. However, in most of these studies, the research focus and experimental materials, including bacterial strains, mouse strains and tumor lines, differ, which may explain the divergent and even contradictory anti-tumor mechanisms of *Salmonella* observed.

3. Exploration of *Salmonella* for cancer therapy

3.1. Modification of Salmonella

Considering the intrinsically pathogenic properties of *Salmonella* bacteria, efficient attenuation of virulence is necessary for safety. For example, the well-known *S.* Typhimurium strain VNP20009 ($purI^-$/$msbB^-$) has been widely studied in animal tumor models and tested in Phase I clinical trials [8,50–53]. Chromosomal deletion of *purI* creates a requirement for an external source of adenine, and deletion of *msbB* prevents the addition of a terminal myristyl group to the lipid A domain of lipopolysaccharide. The virulence of VNP20009 is greatly attenuated, as reflected by its maximum tolerated dose (MTD) in monkeys, pigs and mice as well as the lethal dose in mice, which is 50,000 times lower than that of the wild-type *S.* Typhimurium strain 14028 [54]. The LD_{50} of VNP20009 was increased more than 10,000-fold compared with the wild-type strain. This attenuated strain resulted in dramatically lowered TNF-α induction in mice and pigs, thus reducing the possibility of lipopolysaccharide-induced septic shock. Importantly, in tumor-bearing mice, VNP20009 preferentially accumulates in tumor tissue over the liver at a ratio of 1000:1 and restrains tumor growth upon systemic administration [8,55]. Based on its attenuation, tumor-targeting and tumor-inhibiting properties observed in animal models, VNP20009 was subsequently applied to human clinical trials in 1999. Disappointingly, VNP20009 showed insufficient tumor colonization, and none of the patients experienced obvious tumor regression [53]. *S.* Typhimurium strain A1-R, which is auxotrophic for leucine and arginine, was obtained after nitrosoguanidine (NTG) mutagenesis (A1) and reisolation from A1-infected tumors [6,9]. Upon intravenous injection, tumor-seeking A1-R suppressed the growth of different types of tumors, including prostate, breast and pancreatic cancers and spinal cord glioma [6,9,22,56], and eradicate cancer metastases [57–59]. The vaccine potential of aro^- auxotrophic *Salmonella* for cancer treatment has been investigated as well [60–63]. For example, the *aroA*-deficient strains SL3261 and SL7207 and the *aroA*/*aroD* double mutant BRD509, derived from highly virulent *S.* Typhimurium SL1344, have been tested for the delivery of various anti-tumor agents in mouse models [64–68]. By placing the essential gene *asd* under a hypoxia-induced promoter, an 'obligate' anaerobic strain derived from SL7207 was generated [69]. This novel strain, termed YB1, can survive only in anaerobic conditions, such as the hypoxic and necrotic regions inside the tumor, and is rapidly eliminated from normal tissues under aerobic conditions because Δ*asd Salmonella* bacteria lyse during growth without a supply of exogenous diaminopimelic acid (DAP) [25,70,71]. The balanced-lethal strategy based on the essential gene *asd* can also be used to ensure the stability of expression plasmids carried by *Salmonella* bacteria (balanced-lethal host-vector system) *in vivo* [72,73].

The *relA* and *spoT* double mutant strain of S.Typhimurium termed SHJ2037, which is defective in ppGpp synthesis, has been widely investigated for cancer treatment. This ΔppGpp strain is avirulent and capable of suppressing tumor growth by monotherapy and eradicating tumors by delivering various cytotoxic agents in mice [7,40,47,74–78]. Deletion of the *phoP*/*phoQ* operon, which is a typical bacterial two-component regulatory system, significantly reduces bacterial survival in macrophages as well as bacterial dispersion in healthy tissues while not affecting anti-tumor effects [79]. Mutations such as *htrA*, SPI-2 and *STM3120* are also potential candidates to reduce bacterial fitness in normal tissues while retaining fitness in tumors [80]. Modification of the structure of LPS can influence the immunostimulatory effects as well as the virulence of *Salmonella* bacteria. Frahm et al. showed that

Table 2
Genetic modifications of *Salmonella* bacteria used for cancer treatment.

Genes mutated or modified	Strains involved	Description	References
msbB/PurI	VNP20009 TAPET-CD	The deletion of *msbB* modifies the Lipid-A structure, reducing bacterial ability of TNF-α induction and mutation of *purI* results in bacterial deficiency in adenine synthesis	[4,8,11,50–55,79,90–92,97–99,108,120,122,123,127,140]; [116,117,159]
leu/arg	A1-R	The mutations of *leu* and *arg* generate the auxotrophic strain defective in the synthesis of leucine and arginine	[6,9,22,56–59,130–132,134,143,144]
aro-	SL3261 SL7207 YB1 BRD509 et al.	The genes *aroA* and *aroD* are responsible for the biosynthesis of aromatic amino acids	[64–68,95,96,137,154]; [60,61,103,109,115,135]; [25,69–71]; [89,93,94,112,142]; [44,63,85–87,118,119,136]
relA/spoT	SHJ2037	*Salmonella* lacking both RelA and SpoT are unable to produce ppGpp, a global regulator involving bacterial adaptation of extreme environment	[7,10–12,30,40,47,74–78,141,147]
phoP/phoQ	LH430 VNP (PhoP/Q$^-$)	The knock-out of PhoP and PhoQ that regulate acid phosphatase synthesis significantly reduces bacterial survival in macrophages	[106,111,121,124,125]; [79]
purD/htrA	MvP728	The gene *purD* encodes 5′- phosphoribosyl- glycinamide synthetase involved in purine biosynthesis; *htr*A encodes heat-shock proteins, which are produced in response to a variety of stressful stimuli	[113,126]
cya/crp	x4550	The two genes *cya* and *crp* encode cAMP (cyclic adenosine monophosphate) synthetase and cAMP receptor protein, respectively	[88]
asd	YB1 ST8	The gene *asd* is necessary for the synthesis of DAP; *Salmonella asd* mutants will lyse during growth unless exogenous DAP is supplied	[25,69–71]; [87]
pagP/pagL/lpxR	SF200 S634	The triple mutant strain harbors homogeneous hexa-acylated lipid A that should have potent immune-stimulation to host	[83]; [84]
rfa-	SF200	Mutation in *rfaG* or *rfaD* generates *Salmonella* strains harboring highly truncated LPS as well as attenuated bacterial virulence	[81,83]
dam	RE88	The gene *dam* encodes DNA adenine methylase	[85]
sptP	SB824	SptP is a effector protein of *Salmonella* pathogenicity island 1 (SPI-1), which acts as protein tyrosine phosphatase/GTPase activating proteins	[86]
gmd	ST8	The gene *gmd* is in colanic acid gene cluster and encodes GDP-mannose 4,6-dehydratase	[87]

rfaG and *rfaD* mutants harboring highly truncated LPS had attenuated virulence but also impaired anti-tumor abilities. Chromosomal integration of the LPS biosynthesis gene into the araBAD locus may balance bacterial attenuation and therapeutic benefit [81]. Studies of the structure-activity relationship of lipid A indicate that factors including the number, length and symmetry of fatty acid chains of lipid A govern its immunological activity [82]. The failure of clinical human trials using VNP20009 may be partially attributable to its generation of penta-acylated lipid A, which is an antagonist for TLR4 and caspase-11. Hexa-acylated lipid A stimulates TLR-4 with high affinity. By deleting the three modification genes *pagP*, *pagL* and *lpxR*, Weiss et al. and our group generated *S.* Typhimurium mutants that harbor homogeneous hexa-acylated lipid A in an attempt to maximize bacterial immune stimulation for cancer treatment [83,84]. Mutations in other genes, including *dam* [85], *sptP* [86] and *gmd* [87], have also been introduced into *Salmonella* strains to construct anti-tumor vaccine vectors. The widely studied *S.* Typhimurium bacterial strains for cancer treatment and corresponding genetic modifications are listed in Table 2.

3.2. Delivery of anti-tumor agents

Despite the notable successes achieved in studies using bacteria for cancer treatment, bacteria are often insufficient for the complete eradication of experimental tumors. To enhance their anti-tumor therapeutic efficacy, engineered attenuated *Salmonella* have been employed as live delivery vectors of various anti-tumor agents, such as cytokines, cytotoxic agents, regulating molecules, tumor-associated antigens or antibodies, prodrug enzymes, and siRNAs. After *Salmonella* colonize tumor tissue, anti-tumor therapeutic molecules can be directly expressed by engineered bacteria (prokaryotic expression system) or expressed after the corresponding encoding DNA is transferred into host cells such as cancer cells and macrophages (eukaryotic expression system). The delivery of these exogenous drugs by *Salmonella* is expected to produce maximum anti-tumor activities at the site of the tumor, and to have minimum toxic side effects on normal tissues when the drug concentration to be reduced by 1000-fold.

Cytokines can induce killing of tumor cells by promoting the activation, proliferation and migration of immune cells. Sorenson et al. [88] demonstrated that attenuated *Salmonella* expressing interleukin-2 (IL-2) can effectively induce local and systemic natural killer (NK) cell proliferation and reduce metastatic osteosarcoma. Another study showed that anti-tumor responses induced by IL-2-expressing *Salmonella* were correlated with decreased angiogenesis and increased necrosis within tumor tissue [89]. Intravenous administration of attenuated *Salmonella* expressing IL-18 was associated with increased accumulation of T-lymphocytes and NK cells, massive infiltration of granulocytes, and an increased production of several cytokines in tumors [90]. Treatment of tumor-bearing mice with engineered attenuated *Salmonella* expressing LIGHT or CCL21 also induced leukocyte infiltration and inhibit tumor growth [91,92], with little evidence of systemic toxicity. Yoon et al. showed that subcutaneous administration of *S.* Typhimurium carrying prokaryotic plasmids encoding IFN-γ or TNF-α efficiently inhibited tumor growth and prolonged the survival of C57BL/6 mice bearing B16F10 melanoma [93,94]. Gene therapy using human and mouse IL-12 and GM-CSF delivered orally by *Salmonella* was shown to elicit strong protection against cancer development [67,95]. However, IL-12 and GM-CSF did not appear to cooperate with each other in mediating anti-tumor therapy [67]. Agorio et al. showed that a single dose of *Salmonella* carrying eukaryotic plasmids encoding IL-4 or IL-18 resulted in retardation of tumor growth and prolonged survival of mice bearing melanoma [96].

Using tumor-targeting *Salmonella* as delivery vectors, cytotoxic agents can exert direct killing activities on tumor cells while reducing toxicity to the host. For example, cytolysin A (ClyA), which is found in *E. coli* and *Salmonella enterica* serovars Typhi and Paratyphi A, can cause pore formation in mammalian cell membranes and thus induce cell apoptosis. Treatment of tumor-bearing mice with ΔppGpp *Salmonella* expressing ClyA led to significant tumor shrinkage and, in some cases, tumor eradication [78]. In addition, *Salmonella* expressing *Pseudomonas* exotoxin PE38 or the mitochondrial target domain of Noxa can significantly induce cancer cell apoptosis and inhibit cancer cell proliferation, leading to suppression of tumor growth [74,77,97]. FasL (Fas

ligand) and TRAIL (TNF-related apoptosis-inducing ligand) are cytotoxic agents belonging to the tumor necrosis factor-α (TNF-α) family. These agents induce apoptosis through a death receptor pathway and selectively exert cytotoxic effects on tumor cells, but both have a shorter circulating half-life and significant hepatotoxicity after systemic administration. Using tumor-targeting *Salmonella* to deliver these agents can reduce their toxic side effects on normal tissue cells while maintaining high intratumoral drug concentrations [98,99]. Flagellin is an excellent adjuvant for anti-tumor immunotherapy because it can activate host innate immune responses via the Toll-like receptor 5 (TLR5) signaling pathway [100]. Min et al. showed that engineered *Salmonella* bacteria secreting flagellin B (FlaB) effectively suppressed tumor growth and metastasis in mouse models [10]. CD40L gene therapy orally delivered by attenuated *Salmonella* also produced significant protection against $CD40^+$ B cell lymphoma [101]. The use of attenuated *Salmonella* vectors allowed precise expression of the diphtheria toxin A chain (DTA) gene in the tumor microenvironment and eradicated large tumors established in immunocompetent animals [102]. Gene therapies based on TRAIL and chicken anemia virus VP3 were also effective in inducing enhanced apoptosis in cancer cells [103].

Certain molecules have been designed to regulate tumor cell growth or inhibit angiogenesis in tumor tissues. L-asparaginase (L-ASNase), which primarily converts asparagine to aspartate, is an anti-tumor protein used to treat acute lymphoblastic leukemia. The activity of L-ASNase delivered by tumor-targeting ΔppGpp *Salmonella* has recently been evaluated. Originally, the expression of L-ASNase was inducible under the control of the *araBAD* promoter (P_{BAD}) of the *E. coli* arabinose operon [75]. To further improve performance, P_{BAD} was incorporated into the quorum-sensing machinery. In this new system, recombinant *Salmonella* in tumors expressed and secreted active L-ASNase in a cell mass-dependent manner, yielding significant anti-tumor effects [76]. Angiogenesis, the formation of new capillaries from the pre-existing vasculature, is critical for tumor growth and metastasis, and thus inhibition of angiogenesis inside tumor tissue is another promising direction for cancer therapy. Endostatin, a 20-kDa C-terminal fragment of type XVIII collagen, is a powerful inhibitor of angiogenesis. Delivery of endostatin fused with the secretion signal of the type III secretion protein SopA by engineered *Salmonella* was efficient for endostatin secretion and diffusion throughout tumor tissue [87]. We recently engineered an *aroA*-deficient and lipid A-modified *S*. Typhimurium mutant strain expressing endostatin and employed it in mouse colon carcinoma and melanoma models. Suppressed tumor growth and prolonged survival of mice were observed, accompanied by increased cell apoptosis and decreased microvessel density (MVD) inside tumor tissue [84]. In addition, the delivery of eukaryotic plasmids carrying the gene encoding anti-angiogenic endostatin or thrombospondin by tumor-targeting *Salmonella* bacteria was shown to significantly inhibit tumor angiogenesis and cause tumor-killing effects [104,105]. Engineered attenuated *Salmonella* harboring eukaryotic coexpression plasmids for endostatin and STAT3-specific siRNA also conferred significant tumor-suppression effects in mouse tumor models [106]. The overexpression of the RBM5 (RNA-binding motif protein 5) gene delivered by *Salmonella* within tumor tissue was capable of enhancing the apoptosis of cancer cells in tumor xenografts [107]. Vaccination with recombinant attenuated *Salmonella* harboring the 4-1BBL gene efficiently enhanced T cell immunity and inhibited the development of carcinogen-induced colorectal cancers in rats [66].

Tumor-associated antigens delivered by *Salmonella* can sensitize the host immune system to produce tumor-specific immune responses, and the resultant immune protection can be directed against tumors expressing the corresponding antigens. Moreover, displaying antibodies or ligands that specifically bind to tumor-associated antigens on the bacterial surface can significantly improve the tumor-targeting ability of *Salmonella*. A strain derived from VNP20009 expressing carcinoembryonic antigen (CEA)-specific antibodies on its surface was shown to possess enhanced tumor-targeting ability compared with its parent strain [108]. Similarly, the binding specificity of *Salmonella* to $CD20^+$ cancer cells was significantly improved by expressing a specific antibody directed to the tumor-associated antigen CD20 on the bacterial surface [109]. RGD-displaying *Salmonella* bound strongly to cancer cells overexpressing αvβ3 but weakly to αvβ3-negative cancer cells, showing strong targeting efficiency [110]. Recombinant toxins containing transforming growth factor (TGF-α) delivered by attenuated *Salmonella* were shown to specifically kill cancer cells overexpressing epidermal growth factor receptor (EGFR) [77,97]. NY-ESO-1 is a germ cell protein that is often expressed by tumor cells but not normal somatic cells. Oral administration of *S.* Typhimurium expressing NY-ESO-1 fused to the type III secretion protein SopE resulted in the regression of established NY-ESO-1-expressing tumors in mice [111]. *S.* Typhimurium expressing an HPV16-E7 fusion protein induced anti-tumor immune responses through secretion of IFN-γ and TNF-α cytokines and exhibited enhanced anti-tumor therapeutic efficacy in cervical cancer-bearing mice [112]. Fimbrial surface display systems can be used to present specific epitopes of tumor antigens by *Salmonella* and to induce efficient epitope-specific immune responses *in vivo* [64]. Survivin is another ideal tumor-associated antigen that is overexpressed by nearly all solid tumors but has limited expression in normal tissues. Delivery of a survivin DNA vaccine by oral gavage of *Salmonella* SL7207 or MvP728 induced strong and effective anti-tumor immune responses *in vivo* [61,113]. The vascular endothelial growth factor receptor 2 (VEGFR-2), also called fetal liver kinase 1 (FLK-1) in mice, plays an important role in the proliferation of tumor endothelial cells. Studies have shown that an orally administered DNA encoding the extracellular domain (ECD) of FLK1 delivered by attenuated *S.* Typhimurium could break immune tolerance to FLK1, elicit effective protective anti-tumor immunity and inhibit metastasis of Lewis lung carcinoma in mice [68]. Gene therapies of prostate cancer-specific antigen (PCSA) [60], α-fetoprotein (AFP) [114] and melanoma antigen (Melan-A) [115] delivered by attenuated *S.* Typhimurium led to significant retardation of tumor growth by breaking host immune tolerance to these antigens. The suppression of tumor-associated macrophages (TAMs) in tumor stroma is also a strategy to inhibit tumor growth and dissemination. Legumain, an asparaginyl endopeptidase overexpressed on TAMs, has been targeted in cancer gene therapy delivered by *Salmonella* [85].

Enzymatic proteins expressed by engineered *Salmonella* can convert nontoxic prodrugs into anti-tumor toxic drugs at the tumor site when the corresponding prodrugs are also given, thereby minimizing systemic toxicity. This strategy was used to introduce *E.* coli cytosine deaminase (CD), which activates nontoxic 5-fluorocytosine (5- FC) to anti-tumor 5-fluorouracil (5-FU), into VNP20009 by chromosomal insertion [116]. This CD-containing strain, designated TAPET-CD, maintains the properties of VNP20009, including preferential accumulation in tumors, and selectively produces high levels of CD in tumors compared with normal tissues. Coadministration of TAPET-CD and 5-FC induced prolonged high concentrations of 5-FU in tumor tissue, causing a reduction of tumor size. To prevent killing of *Salmonella* producing enzymes by the accumulation of toxic 5-FU, a 5-FU resistant strain with an *upp* mutation was recently constructed [117]. Another prodrug enzyme, herpes simplex virus thymidine kinase (HSV-TK), can convert ganciclovir into a deoxyguanosine triphosphate analog that induces cell apoptosis upon DNA incorporation. Tumor-targeting *Salmonella* engineered to carry the prodrug-converting enzyme HSV-TK effectively treated human lymphoma xenografts when coadministered intratumorally or intravenously with ganciclovir in mice [109]. Oral administration of attenuated *S.* Typhimurium carrying eukaryotic plasmids encoding *E.* coli purine nucleoside phosphorylase (ePNP) could convert two prodrugs, 6-methylpurine 2′-deoxyriboside (MePdR) and 6-methoxypurine 2′-deoxyriboside (MoPdR), into the toxic substances 6-methylpurine (MeP) and 6-methoxypurine (MoP), respectively, leading to anti-tumor effects in terms of tumor growth and survival rate in tumor-bearing mice [118,119]. The PNP gene of *S.*

Typhimurium (sPNP) has 96% sequence homology with ePNP and has the ability to convert 6MePdR to 6MeP. Chen et al. showed that combined administration of VNP20009 expressing the endogenous PNP gene followed by 6MePdR treatment significantly delayed the growth of B16F10 tumors [120].

RNA interference (RNAi), in which double-stranded RNAs mediate sequence-specific gene silencing, has provided a new strategy for cancer treatment. However, the greatest challenge is how to efficiently deliver small interfering RNAs (siRNAs) to target cancer cells. The efficiency of using attenuated *S.* Typhimurium as delivery vectors of siRNAs to specifically downregulate the expression of tumor-promoting factors has been tested *in vitro* and *in vivo*. The transcription factor STAT3 (signal transducer and activator of transcription 3) [121], the anti-apoptosis protein Bcl-2 (B cell lymphoma-2) [65], the cell cycle-associated protein PLK1 (polo-like kinase 1) [102], Sox2 (sex determining region Y-box 2) [122] and the immunosuppressive molecule IDO [123] are usually overexpressed in tumors and promote tumorigenesis. Due to their association with cancer progression, these molecules can serve as ideal targets for enhancing anti-tumor responses. Attenuated *S.* Typhimurium carrying a plasmid-based Stat3-specific siRNA significantly inhibited tumor growth, reduced cancer metastasis, and extended the survival of tumor-bearing mice compared with bacterial treatment alone [121,124]. Oral administration of *S.* Typhimurium carrying a Bcl-2-specific siRNA induced significant gene silencing in murine melanoma cells and elicited remarkable anti-tumor effects [65]. Intravenous injection of the clinically relevant *S.* Typhimurium strain VNP20009 carrying plasmids encoding an IDO-specific siRNA elicited substantial control of aggressive B16F10 melanomas [123]. The use of DNA vectors delivered by attenuated *S.* Typhimurium may increase the effectiveness of siRNA targeting specific tumor-promoting factors when combined with other anti-tumor agents. Attenuated *S.* Typhimurium harboring eukaryotic coexpression plasmids encoding Stat3-specific siRNA and endostatin showed superior anti-tumor effects in mice bearing implanted prostate cancer or hepatoma [106,125], resulting in regulation of various immune cells and cytokines, a decrease in cell proliferation, induction of cell apoptosis and inhibition of angiogenesis. Oral administration of *S.* Typhimurium MvP728 ($purD^{-}/htrA^{-}$) overexpressing codon-optimized survivin inhibited the growth of murine melanoma, and additional treatment with VNP20009 carrying plasmids encoding STAT3-siRNA by intravenous injection enhanced the anti-tumor efficacy [126].

Anti-tumor therapeutic agents delivered by engineered attenuated *Salmonella* bacteria are listed in Table 3.

3.3. Combination therapy

Despite the numerous advantages of *Salmonella*-mediated cancer therapy compared with conventional treatments, *Salmonella* itself is generally insufficient to completely suppress tumor growth or metastasis. Hence, many studies have suggested that the combination of *Salmonella* with other therapies may be more effective for cancer treatment.

Bacterial immunotherapy combined with specific chemotherapy may enhance anti-tumor efficacy and decrease toxicity compared with single therapy with bacteria or chemotherapeutics. For example, combination treatment with VNP20009 and cyclophosphamide led to a more significant decrease in intratumoral microvessel density and serum vascular endothelial growth factor (VEGF) levels compared with either single treatment without obvious enhanced toxicity in a murine melanoma model [127,128]. VNP20009 harboring expression plasmids for siRNA targeting Sox2 were shown to be efficient for the treatment of lung cancer when combined with the anti-angiogenic agent HM-3 [122]. The anti-inflammatory and anti-angiogenic compound triptolide also significantly enhanced the anti-tumor effects of VNP20009 by modulating tumor angiogenesis and host immune responses [129]. The combination of *S.* Typhimurium A1-R and low-dose chemotherapeutic drugs, such as temozolomide, doxorubicin and anti-angiogenic agents, also significantly suppressed the growth of tumors with fewer side effects in patient-derived orthotopic xenograft (PDOX) models [130–132]. The effectiveness of a novel strategy of “decoy, trap and shoot chemotherapy” was recently demonstrated. In detail, stomach cancer was first treated *in vivo* with *S.* Typhimurium A1-R to decoy quiescent cancer cells to enter the cell cycle. Subsequent administration of recombinant methioninase selectively trapped the decoyed cancer cells in S/G0 phase, and cisplatinum or paclitaxel was then administered to kill the decoyed and trapped cancer cells to completely regress tumor growth [133,134]. Multidrug resistance (MDR) is a major cause of failures of chemotherapy in human malignancy, and the presence of the plasma membrane MDR protein P-glycoprotein (P-gp) is highly relevant. *Salmonella* that colonized in the tumor site could decrease the expression of P-gp and enhance the therapeutic effects of 5-fluorouracil [30]. Inhibiting P-gp expression in tumor cells using RNA interference can also effectively reverse MDR in different kinds of tumor cells. A MDR1-specific siRNA delivered by attenuated *Salmonella* enhanced the anti-tumor effects of cisplatin chemotherapy against cisplatin-resistant ovarian carcinoma [135]. Daily treatment with topical imiquimod in combination with one intratumoral injection of attenuated *S.* Typhimurium LVR01 induced anti-tumor immune responses that prolonged the survival of melanoma-bearing mice and reduced the occurrence of distant metastases [136]. *Salmonella* bacterial immunotherapy combined with a low dose of anti-tumor chemotherapeutic drugs effectively delayed tumor growth and was less toxic than chemotherapy at the maximum tolerated dose [137]. Forbes et al. showed that concurrently administering lipid A with VNP20009 increased intratumoral bacterial accumulation without affecting the tumor-targeting ability of *Salmonella* [138]. However, attenuated *Salmonella* VNP20009, which synthesizes penta-acylated lipid A, did not accumulate in tumors as effectively as *Salmonella* with functional *msbB* [139].

The effectiveness of combination treatment using *Salmonella* and radiotherapy has been investigated as well. In melanoma tumor models, coadministration of VNP20009 and X-rays produced significantly greater anti-tumor effects than either treatment alone [140]. Combined treatment with 21 Gy of X-ray radiotherapy and ΔppGpp *S.* Typhimurium expressing ClyA also significantly delayed tumor growth compared with bacterial therapy alone [141]. Attenuated *Salmonella* BRD509 plus γ-radiation suppressed the growth of B16F10 melanoma more significantly than single treatment with either *Salmonella* or γ-radiation and prolonged overall survival by 90% in tumor-bearing mice over 55 days [142].

Excision of primary tumors is associated with increased systemic metastatic burden, and postoperative metastases usually exhibit increased proliferation. Postoperative A1-R treatment significantly inhibited surgery-induced breast cancer metastasis [143]. After bright-light surgery (BLS) of liver metastasis, treatment with A1-R was highly effective in increasing survival and disease-free survival [144]. These results suggest the future clinical potential of *Salmonella* treatment after surgical resection of tumors.

3.4. Triggering of gene expression

A controllable system of gene expression is used to manage the timing and location of drug production *in vivo*. Precise triggering of the expression of anti-tumor drugs can maximize their therapeutic effects while minimizing systemic toxicity. Some anti-tumor molecules, such as cytotoxic agents that are toxic to tumor cells as well as normal tissue cells, require strict control of production rather than constitutive expression. Insertion of a specific promoter sequence upstream of the gene encoding a specific anti-cancer agent enables the control of gene transcription by external signals. Strategies for gene regulation or triggering mainly belong to three categories: extracellular triggering, environmental sensing [145] and self-induction [146].

External triggering factors include L-arabinose, salicylate and γ-

Table 3
Anti-tumor therapeutic agents delivered by engineered attenuated *Salmonella* bacteria.

Anti-tumor agents	Description	References
Prokaryotic expression		
Cytokines		
IL-2	A signaling molecule, regulates the activities of lymphocytes	[88,89]
IL-18	A signaling molecule, stimulates NK cells and T cells to release IFN-γ	[90]
LIGHT	A TNF-family cytokine, has recently been recognized as a growth factor for DCs	[91]
CCL21	A chemokine, controls the migration of T cells, DCs and NK cells	[92]
IFN-γ	A cytokine, plays a central role in the induction of host anti-tumor immune responses, including inhibition of tumor cell growth and/or recruitment and activation of neutrophils, NK cells and macrophages	[93]
TNF-α	A cytokine, involves in both innate and specific acquired immunity and has the ability to induce apoptosis of tumor associated cells	[88]
Cytotoxic agents		
Cytolysin A	A pore-forming hemolytic protein found in *E.* coli and *Salmonella enterica* serovars Typhi and Paratyphi	[78]
Noxa	A pro-apoptotic protein containing the BH3 domain and MTD, mediates apoptosis via activation of mitochondrial damage and the intrinsic apoptosis signaling pathway	[74]
PE38	A modified *Pseudomonas* exotoxin A, acts by inactivating protein synthesis in mammalian cells	[77]
TRAIL	A pro-apoptotic molecule, induces tumor cell apoptosis through the death receptor pathway	[98]
FasL	A membrane protein that belongs to the TNF family of proteins, induces the apoptosis of Fas-expressing cells after binding to its receptor Fas	[99]
FlaB	Flagellin B of *Vibrio* vulnificus, is the natural ligand for TLR5	[10]
Regulator		
L-asparaginase	An anti-tumor protein, has the glutaminase activity and promotes apoptosis	[75]
Endostatin	One powerful inhibitor of angiogenesis, can block the proliferation and migration of endothelial cells and induce apoptosis after its binding to a variety of receptors on the surface of endothelial cells	[84,87]
Tumor-associated antigens/antibodies		
CEA-scFv	Specific single chain antibody fragment of CEA, one TAA abundantly expressed in a large number of human carcinomas	[108]
RGD	A tumor-homing peptide, specifically binds to αvβ3 integrin which is widely overexpressed on cancer cells and blood vessels during cancer angiogenesis	[110]
CD20-specific antibody	A single-domain (VHH) antibody, is directed to the TAA CD20	[109]
TGF-α	A natural ligand for the EGFR, which is expressed by many tumor cells at high levels	[77,97]
NY-ESO-1	A germ cell protein, is often expressed by tumor cells but not normal somatic cells	[111]
HPV16-E7	Human papillomavirus (HPV) is the leading cause of cervical cancer; E7 is an oncogenic protein, continuously expressed in carcinomas containing HPV and their metastatic lesions	[112]
Prodrug enzymes		
Cytosine deaminase	An enzyme found in bacteria and fungi but not in mammalian cells, can activate nontoxic 5-FC to anti-tumor 5-FU that disrupts RNA and DNA synthesis	[116,117]
HSV-TK	An enzyme, can convert prochemotherapeutic ganciclovir into a deoxyguanosine triphosphate analog that induces cell apoptosis	[109]
Eukaryotic expression		
Cytokines		
IL-12	A signaling molecule, stimulates the proliferation of activated NK and T cells and induces IFN-g production from these cells	[67]
GM-CSF	A signaling molecule, activates neutrophils, eosinophils and macrophages to lyse tumor cells directly, or mediates antibody- dependent cellular cytotoxicity	[67]
IL-4	A signaling molecule, induces differentiation of naive helper T cells (Th_0 cells) to Th_2 cells	[96]
IL-18	A signaling molecule, induces IFN-γ secretion from NK and T cells	[96]
Cytotoxic agents		
CD40L	A cellular surface molecule, is required to activate antigen presenting cells (APCs) and has growth-suppressive effects on malignant B cells	[101]
Diphtheria toxin	An exotoxin secreted by *Corynebacterium diphtheriae*	[102]
Apoptin	The protein apoptin is encoded by the VP3 virus, which selectively induces apoptosis in human tumor cell lines from various tissues	[103]
Regulators		
Endostatin	A powerful inhibitor of angiogenesis	[104,106]
Thrombospondin	An endogenous angiogenic inhibitor	[98]
RBM5	An RNA binding protein, can modulate apoptosis and cell cycle arrest through pre-mRNA splicing of multiple target genes, such as p53	[107]
4-1BBL	An regulator of T cell immunity, can stimulate T cell cytokine production and proliferation, and prolong T cell survival through interaction with 4-1BB on activated T cells	[66]
Tumor-associated antigens		
Survivin	An apoptosis inhibitor, functions to inhibit caspase activation, leading to negative regulation of apoptosis or programmed cell death	[61,113]
FLK-1	FLK-1 also known as VEGFR-2, is exclusively expressed in endothelial cells and plays a pivotal role in endothelial cell differentiation and proliferation during tumor angiogenesis	[68]
PCSA	Prostate cancer specific antigen	[60]
AFP	An hepatocellular carcinoma-specific (HCC)-specific antigen, is frequently overexpressed in HCC or embryonal malignancies	[114]
Legumain	An asparaginyl endopeptidase, is shown to highly upregulated in many tumors	[85]
Prodrug enzymes		
PNR	An enzyme, can convert two prodrugs MePdR and MoPdR into toxic substances MeP and MoP, respectively	[118–120]
siRNA		
STAT3-specific	Stat3, of which chronic activation is related to abnormal cell proliferation and malignant transformation	[121,124]
Bcl-2-specific	Bcl-2, an anti-apoptosis protein, is expressed in more than 90% of all malignant melanomas	[65]
PLK1-specific	PLK1, a regulatory serine/threonin kinase involved in the cell cycle	[102]
Sox2- specific	Sox2, is expressed at high level in cancer stem cells and necessary for the migration and growth of cancer cells	[122]
IDO-specific	IDO, a tryptophan-catabolizing enzyme that acts as a potent suppressor of adaptive immunity	[123]
MDR1-specific	MDR1, encodes the plasma membrane glycoprotein P-gp, protecting a tumor cell population against numerous drugs differing in the chemical structure	[135]

irradiation. Both L-arabinose and acetyl salicylic acid (ASA) are suitable nontoxic biological triggers that can be used to positively control gene expression under the P_{BAD} promoter and Pm promoter, respectively [147–149]. The P_{BAD} system responds to the extracellular inducer L-arabinose via the regulatory protein AraC, which is tightly regulated. Intravenous administration of L-arabinose can activate gene expression in tumors colonized with *Salmonella* carrying P_{BAD} expression plasmids. The ASA expression system in attenuated *Salmonella* is also efficient in selectively regulating the expression of the 5-FC-converting enzyme cytosine deaminase *in vivo*. Induction with ASA before 5-FC administration resulted in a significant reduction of tumor growth [149]. A repressor-regulated tetracycline efflux system has been explored to control the expression of a therapeutic gene and an imaging reporter gene by divergent promoters (tetAP and tetRP) in response to extracellular tetracycline or doxycycline. The advantage of this approach is that this system can work by using very low dose of clinically relevant inducer doxycycline [150]. For temporal and spatial control of gene expression, the prokaryotic radiation-inducible RecA promoter may be another feasible choice [98,151]. γ-radiation causes DNA damage and activates RecA, which promotes lysis of the repressor lexA, inducing gene expression. A major advantage of radiation over molecular triggering is that radiation can penetrate directly into tumor tissue and is not subject to the diffusion limit. Systemic injection of *Salmonella* and induction of TRAIL expression using 2 Gy of γ-irradiation caused a significant delay in the growth of mammary tumors and reduced the risk of death by 76% [98]. Moreover, the RecA promoter was modified to increase radiation responsiveness by incorporating an extra Cheo box in the promoter region [152].

The characteristics of the tumor microenvironment differ from those of normal tissues, including hypoxia, low pH and necrosis, which can be utilized to improve the tumor specificity of *Salmonella* colonization as well as drug delivery. For example, hypoxia-inducible expression systems have been used to restrict gene expression by *Salmonella* vectors to the hypoxic region of tumor tissue. The hypoxia-inducible promoters HIP-1 and FF + 20* are both fumarate and nitrate reduction regulator (FNR)-regulated promoters characterized by the presence of FNR-binding sites [153,154]. FNR is a hypoxic sensor as well as a hypoxia-responsive transcription factor that regulates the global response to the transition between aerobic and anaerobic growth in *Salmonella*. In the absence of oxygen, iron-sulfide clusters induce the formation of FNR homodimers that bind to specific DNA sites and promote transcription. Under aerobic conditions, the iron-sulfide clusters are disassembled, and the FNR dimers dissociate to form non-DNA-binding monomers. To monitor *Salmonella* promoters that are preferentially activated in tumors, Arrach et al. cloned a random library of *Salmonella* DNA fragments upstream of the green fluorescent protein (GFP) gene without the promoter, and two clones that contained promoter regions of *pflE* and *ansB* were identified as induced under hypoxic conditions. In some cases, promoter activation within tumors may be unrelated to hypoxia [155]. Flentie et al. analyzed a library of 7,400 independent *Salmonella* mutants containing transposon insertions of promoterless bacterial luciferase in the chromosome, and five promoter sequences were found to be specifically activated by the acidic microenvironment associated with cancer cells *in vitro* and tumors *in vivo*. *Salmonella* expressing *Shiga* toxin under the pH-sensitive STM1787 promoter provided potent and selective anti-tumor activity [156].

Salmonella integrated with a quorum-sensing (QS) system is a promising tool for cancer treatment. This system can target therapeutic drugs to tumors while reducing unwanted side effects in healthy tissues. The lux QS system derived from the marine bacterium *Vibrio fischeri* consists of two genes that encode the autoinducer synthesis protein LuxI and transcriptional regulator protein LuxR. At low population density, low-level expression of LuxI results in the synthesis of the autoinducer N-3(oxohexanoyl) homoserine lactone (3OC6HSL), which freely diffuses out of cells. As the population density increases, intracellular 3OC6HSL activates LuxR, creating a positive feedback loop that increases the production of any gene incorporated into the operon. Clustering of bacterial cells prevents dilution of the autoinducer 3OC6HSL and improves QS activation. A decrease in the concentration of signaling molecules will reduce the likelihood of activating the QS switch. Thus, it was hypothesized that quorum-sensing *Salmonella* bacteria only initiate drug expression in tightly packed colonies present within tumors. This hypothesis was validated by Forbes and colleagues [146], who injected attenuated *Salmonella* integrated with the lux QS system and GFP reporter into tumor-bearing mice and determined the bacterial density in tumors and the liver and the bacterial spatial distribution and GFP expression within tumors. When the P_{BAD} promoter was integrated into the quorum-sensing machinery to generate a “cell communication system”, protein production and sensitivity to the inducer agent L-arabinose were significantly increased [157]. Similar strategies were used to express active L-ASNase by ΔppGpp *Salmonella* in a cell mass-dependent manner in tumors [76]. These results suggest that expression of therapeutic proteins by tumor-targeting *Salmonella* vectors under the control of the QS autoinduction system combined with a remote control system represents a promising approach for cancer treatment. To control population levels and facilitate drug delivery using bacteria, a synchronized lysis circuit (SLC) was engineered consisting of a common promoter, pLuxI that drives the expression of both the autoinducer (positive feedback) and a bacteriophage lysis gene (negative feedback). The bacterial population dynamics arising from the SLC can be conceptualized as a slow build-up of the signaling molecule (AHL) to a threshold level, followed by a lysis event that rapidly prunes the population and enables the release of bacterial contents. Following the lysis of most quorum-sensing *Salmonella*, a small number of surviving bacteria reseed the growing population, thus leading to pulsatile cycles. The pore-forming toxin HlyE incorporated into the SLC strain could be efficiently released following the cyclical lysis of bacteria, resulting in killing of cancer cells *in vitro* [158]. The SLC may be a promising tool for drug delivery that does not require additional engineering for protein secretion.

3.5. Clinical trials

Salmonella-mediated cancer therapies have been implemented in a small number of human trials. Based on preclinical success, VNP20009 entered Phase I human clinical trials in 1999 and was administered to 24 patients with metastatic melanoma and 1 patient with metastatic renal cell carcinoma intravenously [53]. The results showed that VNP20009 could be safely used in cancer patients, and only three cases of tumor colonization were observed at the highest tolerated dose. No patients experienced objective tumor regression, including those with *Salmonella*-colonized tumors [53]. Additional studies are required to reduce dose-related toxicity and to improve tumor localization of *Salmonella* bacteria. VNP20009 was further modified by chromosomal insertion of a gene encoding *E. coli* cytosine deaminase (CD) to generate a strain designated TAPET-CD [116]. A pilot trial in three refractory cancer patients was performed to investigate the feasibility and effectiveness of intratumoral injection of TAPET-CD [159]. No significant adverse events clearly attributable to TAPET-CD were observed, and two patients showed bacterial colonization within tumor tissue that persisted for at least 15 days after the initial injection. In these two patients, the conversion of 5-FC to 5-FU as a result of corresponding prodrug expression was demonstrated with a tumor-to-plasma ratio of 5-FU of 3:1, indicating significantly increased levels of 5-FU at the site of TAPET-CD colonization and insignificant systemic spread of the injected bacteria. By contrast, the ratio was less than 1.0 in the one patient who did not show intratumoral bacterial colonization [159]. These clinical data indicate that differences between animal and human tumors may influence the anti-tumor effectiveness of engineered *Salmonella* bacteria, such as the tumor growth rate, architecture and blood supply, the entry of bacteria into tumors, the growth of bacteria within tumors, and the clearance of bacteria from the peripheral circulation

and from tumors.

4. Conclusions and future directions

Bacteria-mediated anti-tumor therapies have made great progress over the past two decades, and the facultative anaerobe *Salmonella* Typhimurium is a particularly promising anti-tumor agent. However, some basic problems remain to be solved before engineered anti-tumor *Salmonella* can be applied clinically. First, safety is a prerequisite for all cancer treatments, including *Salmonella*-mediated therapy. If not adequately attenuated, systemically administered live *Salmonella* bacteria may proliferate in the blood, release bacterial toxins and even cause severe septic shock. Through genetic modification, such as deletion of genes responsible for the biosynthesis of LPS, amino acids, or purines, the virulence of *Salmonella* can be reduced to varying degrees [4,6,160]. However, excessive attenuation of virulence also affects bacterial invasion and tumor colonization, as well as immune-stimulating ability. For example, the insufficient tumor colonization of VNP20009 in clinical trials was attributed to its excessive attenuation due to the *msbB* mutation, which reduces TNF-α induction [138]. In addition to directly deleting genes from the bacterial genome, regulated delayed attenuation may be considered to ensure that *Salmonella* survive the stresses encountered in the host during the early stage of infection, especially after oral administration. The main principle of this system is to replace the constitutive promoter of certain virulence-related or essential genes with an inducible promoter, such as the promoter P_{BAD}. The key gene can be expressed normally under induction conditions *in vitro*, and *Salmonella* can infect the host as the wild-type strain. Once the *Salmonella* reaches the host environment lacking the induction conditions *in vivo*, the expression level of the gene under regulation is greatly reduced (basic expression), and thus the bacteria show weakened pathogenicity [161]. It is important to achieve a balance by developing bacterial strains with moderate virulence that can be safely applied without impairing anti-tumor activities. Lipid A is an important virulence factor for *Salmonella* as well as other gram-negative bacteria and is a natural ligand for host TLR4. Because the host innate immune responses activated by colonized *Salmonella* via the lipid A/TLR4 pathway play a key role in anti-tumor responses, it is necessary to screen out superior lipid A structures for effective immune stimulation. Our group has constructed *Salmonella* strains harboring modified lipid A and verified that the structure of lipid A, including the number of acylated chains and the presence or absence of modification groups, affects bacterial pathogenicity as well as the ability to stimulate innate immunity [162–164]. In addition, immunocompromised patients may be unable to completely clear *Salmonella* when the treatment needs to be terminated. Thus, the possibility that these genetically modified bacteria might reverse their attenuated phenotypes poses a real risk for these patients. In theory, the use of antibiotics can remove residual *Salmonella* in the body, but the possibility of bacteria “naturally” acquiring antibiotic resistance cannot be ruled out. In this case, a regulation system designed to cause programmed bacterial cell lysis may be one solution [165].

With a 1000-fold difference in the ability to colonize tumors and normal tissues, a considerable number of bacteria can still infect normal tissues such as the liver and spleen and cause obvious toxic side effects in the host, especially during the initiation of infection. To minimize the spread of *Salmonella* in blood and normal tissues, the tumor-targeting ability of *Salmonella* must be further improved. Auxotrophic mutants of *Salmonella* constructed via transposon-mediated random mutation or suicide plasmid-mediated site-directed mutagenesis have been shown to invade and destroy a broad number of cancer cell types *in vitro* and to specifically colonize tumors over normal tissues and inhibit tumor progression *in vivo*. Hypoxia-inducible promoters can be used to improve the tumor-colonization specificity of *Salmonella* by regulating bacterial essential genes. Under this regulation system activated by low oxygen concentration, *Salmonella* bacteria grow normally in the hypoxic microenvironment of the tumor, while their viability in normal tissues is greatly reduced [69]. By displaying antibodies or ligands specific for tumor-associated antigens on the surface, bacterial adhesion to cancer cells and the specificity of colonizing tumors can also be significantly increased [77,97,108–110].

The use of *Salmonella* bacteria as live delivery vectors for various anti-tumor therapeutic agents can synergistically increase anti-tumor efficacy compared with single therapy using *Salmonella*. On the one hand, *Salmonella* itself can overcome the penetration limitation of chemotherapeutic drugs to colonize the necrotic and hypoxic areas of tumor tissue and has intrinsic tumor-killing, vessel-destruction and immune-stimulation activities. On the other hand, live *Salmonella* bacteria are able to proliferate and continuously express anti-tumor proteins or deliver DNA vaccines to host cells, thus allowing drug concentrations in tumors to be maintained at high levels. Moreover, by regulating the expression of anti-tumor agents delivered by *Salmonella* vectors, especially toxic agents that are harmful to normal tissue cells, the specificity of drug action toward the tumor can be improved, and toxic side effects can be minimized. For example, using hypoxia-inducible promoters, including HIP-1 and FF + 20*, anti-tumor drugs are mainly produced in the hypoxic region of the tumor after the accumulation of *Salmonella* vectors, with little production in normal tissues. The anti-tumor effects of *Salmonella* combined with other therapies have also been investigated in tumor models. For example, the use of *Salmonella* to deliver specific siRNAs targeting multiple drug-resistance genes can increase tumor sensitivity to chemotherapeutic drugs [135]. The combination of *Salmonella* with radiotherapy or surgery has also been tested. In addition, the clinical challenge of insufficient tumor colonization by VNP20009 may be solved through coadministration with lipid A [138]. Moreover, *Salmonella* can be genetically engineered to target tumor cells, tumor blood vessels or immune cells, which should result in differential anti-tumor activities, and combined treatment with these engineered *Salmonella* bacteria may yield significant tumor-suppressive efficacy.

Although many studies have observed tumor-targeting and tumor-inhibitory activities of *Salmonella*, the underlying mechanisms are still not understood well. Different studies have used different bacterial strains, mouse strains, tumor cell lines, and even different infection procedures including routes, doses and repeat times of injection, further increasing the complexity of elucidating *Salmonella* anti-tumor mechanisms. The mechanism explaining the preference of *Salmonella* for tumor tissue after systemic infection remains controversial. Systematic studies are needed to clarify how *Salmonella* bacteria cause tumor cell death, destroy the local vasculature, and sensitize the host immune system after colonization inside tumor tissue. Greater knowledge of these mechanisms will facilitate the development of safer and more effective *Salmonella* bacterial strains for cancer treatment. Most importantly, the promising results regarding the anti-tumor effects of genetically engineered *Salmonella* observed in a large number of preclinical animal experiments have not been validated in human clinical trials, suggesting that there are some differences between human and animal tumors. Animal tumor models that can adequately simulate tumors in clinical cancer patients should be considered and applied in future preclinical tests. In addition, most of engineered *Salmonella* species used for cancer treatment belong to broad-host-range *S.* Typhimurium, which typically invade only local intestinal epithelial tissue and have inability to penetrate the deeper tissues of humans, therefore human-restricted serovars Typhi and Paratyphi A of *Salmonella enterica* may be considered for genetic modification and human clinical trials.

Although *Salmonella*-mediated cancer therapy has not achieved the expected success in human clinical trials, *Salmonella* is worthy of further study because of its numerous potential advantages over conventional anti-tumor treatments. It is reasonable to believe that with the further development of related research and modern biotechnology, a major breakthrough will be achieved in the application of *Salmonella*

bacteria for cancer treatment.

Conflicts of interest

The authors have no conflicts of interest.

Acknowledgements

This work is supported by National Natural Science Foundation of China (grant No. 31570928), Chongqing natural science foundation (grant No. csct2018jcyjA1294) and the Fundamental Research Funds for the Central Universities (SWU117062, SWU117061). All contributors who provided help during the research have been listed.

References

[1] R.A. Malmgren, C.C. Flanigan, Localization of the vegetative form of *Clostridium tetani* in mouse tumors following intravenous spore administration, Cancer Res. 15 (7) (1955) 473–478.
[2] Y. Kohwi, K. Imai, Z. Tamura, Y. Hashimoto, Antitumor effect of *Bifidobacterium infantis* in mice, Gan 69 (5) (1978) 613–618.
[3] J. Stritzker, S. Weibel, P.J. Hill, T.A. Oelschlaeger, W. Goebel, A.A. Szalay, Tumor-specific colonization, tissue distribution, and gene induction by probiotic *Escherichia coli* Nissle 1917 in live mice, Int. J. Med. Microbiol. 297 (3) (2007) 151–162.
[4] K.B. Low, M. Ittensohn, T. Le, J. Platt, S. Sodi, M. Amoss, et al., Lipid A mutant *Salmonella* with suppressed virulence and TNFα induction retain tumor-targeting *in vivo*, Nat. Biotechnol. 17 (1999) 37–41.
[5] E.F. McCarthy, The toxins of William B. Coley and the treatment of bone and soft-tissue sarcomas, Iowa Orthop. J. 26 (2006) 154–158.
[6] M. Zhao, M. Yang, H. Ma, X. Li, X. Tan, S. Li, et al., Targeted therapy with a *Salmonella* Typhimurium leucine-arginine auxotroph cures orthotopic human breast tumors in nude mice, Cancer Res. 66 (15) (2006) 7647–7652.
[7] H.S. Na, H.J. Kim, H.C. Lee, Y. Hong, J.H. Rhee, H.E. Choy, Immune response induced by *Salmonella* Typhimurium defective in ppGpp synthesis, Vaccine 24 (12) (2006) 2027–2034.
[8] C. Clairmont, K.C. Lee, J. Pike, M. Ittensohn, K.B. Low, J. Pawelek, et al., Biodistribution and genetic stability of the novel antitumor agent VNP20009, a genetically modified strain of *Salmonella* Typhimurium, J. Infect. Dis. 181 (6) (2000) 1996–2002.
[9] M. Zhao, M. Yang, X.M. Li, P. Jiang, E. Baranov, S. Li, et al., Tumor-targeting bacterial therapy with amino acid auxotrophs of GFP-expressing *Salmonella* Typhimurium, Proc. Natl. Acad. Sci. U. S. A. 102 (3) (2005) 755–760.
[10] J.H. Zheng, V.H. Nguyen, S.N. Jiang, S.H. Park, W. Tan, et al., Two-step enhanced cancer immunotherapy with engineered *Salmonella* Typhimurium secreting heterologous flagellin, Sci. Transl. Med. 9 (376) (2017).
[11] X. Luo, Z. Li, S. Lin, T. Le, M. Ittensohn, D. Bermudes, et al., Antitumor effect of VNP20009, an attenuated *Salmonella*, in murine tumor models, Oncol. Res. 12 (11) (2001) 501–508.
[12] R.M. Hoffman, Tumor-seeking *Salmonella* amino acid auxotrophs, Curr. Opin. Biotechnol. 22 (6) (2011) 917–923.
[13] R.W. Kasinskas, N.S. Forbes, *Salmonella* Typhimurium lacking ribose chemoreceptors localize in tumor quiescence and induce apoptosis, Cancer Res. 67 (7) (2007) 3201–3209.
[14] B.J. Toley, N.S. Forbes, Motility is critical for effective distribution and accumulation of bacteria in tumor tissue, Integr. Biol. 4 (2) (2012) 165–176.
[15] S. Leschner, K. Westphal, N. Dietrich, N. Viegas, J. Jablonska, M. Lyszkiewicz, et al., Tumor invasion of *Salmonella* enterica serovar Typhimurium is accompanied by strong hemorrhage promoted by TNF-alpha, PLoS One 4 (8) (2009) e6692.
[16] N.S. Forbes, L.L. Munn, D. Fukumura, R.K. Jain, Sparse initial entrapment of systemically injected *Salmonella* Typhimurium leads to heterogeneous accumulation within tumors, Cancer Res. 63 (17) (2003) 5188–5193.
[17] J. Stritzker, S. Weibel, C. Seubert, A. Gotz, A. Tresch, N. van Rooijen, et al., Enterobacterial tumor colonization in mice depends on bacterial metabolism and macrophages but is independent of chemotaxis and motility, Int. J. Med. Microbiol. 300 (7) (2010) 449–456.
[18] M. Sznol, S.L. Lin, D. Bermudes, L.M. Zheng, I. King, Use of preferentially replicating bacteria for the treatment of cancer, J. Clin. Invest. 105 (8) (2000) 1027–1030.
[19] S. Ganai, R.B. Arenas, J.P. Sauer, B. Bentley, N.S. Forbes, In tumors *Salmonella* migrate away from vasculature toward the transition zone and induce apoptosis, Cancer Gene Ther. 18 (7) (2011) 457–466.
[20] M. Zhang, N.S. Forbes, *Trg*-deficient *Salmonella* colonize quiescent tumor regions by exclusively penetrating or proliferating, J. Contr. Release 199 (2015) 180–189.
[21] K. Westphal, S. Leschner, J. Jablonska, H. Loessner, S. Weiss, Containment of tumor-colonizing bacteria by host neutrophils, Cancer Res. 68 (8) (2008) 2952–2960.
[22] H. Kimura, L. Zhang, M. Zhao, K. Hayashi, H. Tsuchiya, K. Tomita, et al., Targeted therapy of spinal cord glioma with a genetically modified *Salmonella* Typhimurium, Cell prolif 43 (1) (2010) 41–48.
[23] A. Uchugonova, M. Zhao, Y. Zhang, M. Weinigel, K. König, R.M. Hoffman, Cancer-cell killing by engineered *Salmonella* imaged by multiphoton tomography in live mice, Anticancer Res. 32 (10) (2012) 4331–4337.
[24] B. Li, H. He, S. Zhang, W. Zhao, N. Li, R. Shao, *Salmonella* Typhimurium strain SL7207 induces apoptosis and inhibits the growth of HepG2 hepatoma cells *in vitro* and *in vivo*, Acta Pharm. Sin. B 2 (6) (2012) 562–568.
[25] C.X. Li, B. Yu, L. Shi, W. Geng, Q.B. Lin, C.C. Ling, et al., Obligate' anaerobic *Salmonella* strain YB1 suppresses liver tumor growth and metastasis in nude mice, Oncol. Lett. 13 (1) (2017) 177–183.
[26] B. Liu, Y. Jiang, T. Dong, M. Zhao, J. Wu, L. Li, et al., Blockage of autophagy pathway enhances *Salmonella* tumor-targeting, Oncotarget 7 (16) (2016) 22873–22882.
[27] C.H. Lee, S.T. Lin, J.J. Liu, W.W. Chang, J.L. Hsieh, W.K. Wang, *Salmonella* induce autophagy in melanoma by the downregulation of AKT/mTOR pathway, Gene Ther. 21 (3) (2014) 309–316.
[28] D.G. Tu, W.W. Chang, S.T. Lin, C.Y. Kuo, Y.T. Tsao, C.H. Lee, *Salmonella* inhibits tumor angiogenesis by downregulation of vascular endothelial growth factor, Oncotarget 7 (25) (2016) 37513–37523.
[29] Y.D. Kuan, C.H. Lee, *Salmonella* overcomes tumor immune tolerance by inhibition of tumor indoleamine 2, 3-dioxygenase 1 expression, Oncotarget 7 (1) (2016) 374–385.
[30] C.J. Yang, W.W. Chang, S.T. Lin, M.C. Chen, C.H. Lee, *Salmonella* overcomes drug resistance in tumor through P-glycoprotein downregulation, Int. J. Med. Sci. 15 (6) (2018) 574–579.
[31] Y.T. Tsao, C.Y. Kuo, S.P. Cheng, C.H. Lee, Downregulations of AKT/mTOR signaling pathway for *Salmonella*-mediated suppression of matrix metalloproteinases-9 expression in mouse tumor models, Int. J. Mol. Sci. 19 (6) (2018) 1630.
[32] F. Liu, L. Zhang, R.M. Hoffman, M. Zhao, Vessel destruction by tumor-targeting *Salmonella* Typhimurium A1-R is enhanced by high tumor vascularity, Cell Cycle 9 (22) (2010) 4518–4524.
[33] F. Saccheri, C. Pozzi, F. Avogadri, S. Barozzi, M. Faretta, P. Fusi, et al., Bacteria-induced gap junctions in tumors favor antigen cross-presentation and antitumor immunity, Sci. Transl. Med. 2 (44) (2010) 44ra57.
[34] W.K. Wang, M.C. Chen, H.F. Leong, Y.L. Kuo, C.Y. Kuo, C.H. Lee, Connexin 43 suppresses tumor angiogenesis by down-regulation of vascular endothelial growth factor via hypoxic-induced factor-1alpha, Int. J. Mol. Sci. 16 (1) (2014) 439–451.
[35] S.Y. Chang, Y.J. Kim, H.J. Ko, Potential therapeutic anti-tumor effect of a *Salmonella*-based vaccine, Hum. Vaccines Immunother. 9 (8) (2013) 1654–1660.
[36] W.-K. Wang, M.-F. Lu, Y.-D. Kuan, C.-H. Lee, The treatment of mouse colorectal cancer by oral delivery tumor-targeting *Salmonella*, Am. J. Canc. Res. 5 (7) (2015) 2222–2228.
[37] F. Avogadri, D. Mittal, F. Saccheri, M. Sarrafiore, M. Ciocca, P. Larghi, et al., Intratumoral *Salmonella* Typhimurium induces a systemic anti-tumor immune response that is directed by low-dose radiation to treat distal disease, Eur. J. Immunol. 38 (7) (2008) 1937–1947.
[38] S. Kaimala, Y.A. Mohamed, N. Nader, J. Issac, E. Elkord, S. Chouaib, et al., *Salmonella*-mediated tumor regression involves targeting of tumor myeloid suppressor cells causing a shift to M1-like phenotype and reduction in suppressive capacity, Cancer Immunol. Immunother. 63 (6) (2014) 587–599.
[39] T. Liu, A.K. Chopra, An enteric pathogen *Salmonella* enterica serovar Typhimurium suppresses tumor growth by downregulating $CD44^{high}$ and $CD4^{+}$ T regulatory (Treg) cell expression in mice: the critical role of lipopolysaccharide and Braun lipoprotein in modulating tumor growth, Cancer Gene Ther. 17 (2) (2010) 97–108.
[40] J.E. Kim, T.X. Phan, V.H. Nguyen, H.V. Dinh-Vu, J.H. Zheng, M. Yun, et al., *Salmonella* Typhimurium suppresses tumor growth via the pro-inflammatory cytokine interleukin-1beta, Theranostics 5 (12) (2015) 1328–1342.
[41] D. Kocijancic, S. Leschner, S. Felgner, R.M. Komoll, M. Frahm, V. Pawar, et al., Therapeutic benefit of *Salmonella* attributed to LPS and TNF-alpha is exhaustible and dictated by tumor susceptibility, Oncotarget 8 (22) (2017) 36492–36508.
[42] Z. Cai, A. Sanchez, Z. Shi, T. Zhang, M. Liu, D. Zhang, Activation of Toll-like receptor 5 on breast cancer cells by flagellin suppresses cell proliferation and tumor growth, Cancer Res. 71 (7) (2011) 2466–2475.
[43] L. Sfondrini, A. Rossini, D. Besusso, A. Merlo, E. Tagliabue, S. Menard, et al., Antitumor activity of the TLR-5 ligand flagellin in mouse models of cancer, J. Immunol. 176 (11) (2006) 6624–6630.
[44] S. Grille, M. Moreno, T. Bascuas, J.M. Marques, N. Munoz, D. Lens, et al., *Salmonella* enterica serovar Typhimurium immunotherapy for B-cell lymphoma induces broad anti-tumour immunity with therapeutic effect, Immunology 143 (3) (2014) 428–437.
[45] B. Chirullo, S. Ammendola, L. Leonardi, R. Falcini, P. Petrucci, C. Pistoia, et al., Attenuated mutant strain of *Salmonella* Typhimurium lacking the ZnuABC transporter contrasts tumor growth promoting anti-cancer immune response, Oncotarget 6 (19) (2015) 17648–17660.
[46] C.H. Lee, C.L. Wu, A.L. Shiau, Toll-like receptor 4 mediates an antitumor host response induced by *Salmonella* choleraesuis, Clin. Cancer Res. 14 (6) (2008) 1905–1912.
[47] T.X. Phan, V.H. Nguyen, M.T. Duong, Y. Hong, H.E. Choy, J.J. Min, Activation of inflammasome by attenuated *Salmonella* Typhimurium in bacteria-mediated cancer therapy, Microbiol. Immunol. 59 (11) (2015) 664–675.
[48] F. Avogadri, C. Martinoli, L. Petrovska, C. Chiodoni, P. Transidico, V. Bronte, et al., Cancer immunotherapy based on killing of *Salmonella*-infected tumor cells, Cancer Res. 65 (9) (2005) 3920–3927.
[49] C.H. Lee, J.L. Hsieh, C.L. Wu, H.C. Hsu, A.L. Shiau, B cells are required for tumor-targeting *Salmonella* in host, Appl. Microbiol. Biotechnol. 92 (6) (2011) 1251–1260.
[50] S.A. Rosenberg, P.J. Spiess, D.E. Kleiner, Antitumor effects in mice of the intravenous injection of attenuated *Salmonella* Typhimurium, J. Immunother. 25 (3)

(2002) 218–225.
[51] L.J. Jia, D.P. Wei, Q.M. Sun, Y. Huang, Q. Wu, Z.C. Hua, Oral delivery of tumor-targeting *Salmonella* for cancer therapy in murine tumor models, Cancer Sci. 98 (7) (2007) 1107–1112.
[52] D.H. Thamm, I.D. Kurzman, I. King, Z. Li, M. Sznol, R.R. Dubielzig, et al., Systemic administration of an attenuated, tumor-targeting *Salmonella* Typhimurium to dogs with spontaneous neoplasia: phase I evaluation, Clin. Cancer Res. 11 (13) (2005) 4827–4834.
[53] J.F. Toso, Phase I study of the intravenous administration of attenuated *Salmonella* Typhimurium to patients with metastatic melanoma, J. Clin. Oncol. 20 (1) (2002) 142–152.
[54] K.C. Lee, L.-M. Zheng, X. Luo, C. Clairmont, J. Fischer, D. Margitich, et al., Comparative evaluation of the acute toxic effects in monkeys, pigs and mice of a genetically engineered *Salmonella* strain (VNP20009) being developed as an antitumor agent, Int. J. Toxicol. 19 (1) (2016) 19–25.
[55] K.B. Low, M. Ittensohn, X. Luo, L.-M. Zheng, I. King, J.M. Pawelek, et al., Construction of VNP20009: a novel, genetically stable Antibiotic-sensitive strain of tumor-targeting *Salmonella* for parenteral administration in humans, Methods Mol. Med. 90 (2004) 47–60.
[56] C. Nagakura, K. Hayashi, M. Zhao, K. Yamauchi, N. Yamamoto, H. Tsuchiya, et al., Efficacy of a genetically-modified *Salmonella* Typhimurium in an orthotopic human pancreatic cancer in nude mice, Anticancer Res. 29 (6) (2009) 1873–1878.
[57] K. Hayashi, M. Zhao, K. Yamauchi, N. Yamamoto, H. Tsuchiya, K. Tomita, et al., Cancer metastasis directly eradicated by targeted therapy with a modified *Salmonella* Typhimurium, J. Cell. Biochem. 106 (6) (2009) 992–998.
[58] K. Hayashi, M. Zhao, K. Yamauchi, N. Yamamoto, H. Tsuchiya, K. Tomita, et al., Systemic targeting of primary bone tumor and lung metastasis of high-grade osteosarcoma in nude mice with a tumor-selective strain of *Salmonella* Typhimurium, Cell Cycle 8 (6) (2009) 870–875.
[59] M. Zhao, J. Geller, H. Ma, M. Yang, S. Penman, R.M. Hoffman, Monotherapy with a tumor-targeting mutant of *Salmonella* Typhimurium cures orthotopic metastatic mouse models of human prostate cancer, Proc. Natl. Acad. Sci. U. S. A. 104 (24) (2007) 10170–10174.
[60] S. Ahmad, G. Casey, M. Cronin, S. Rajendran, P. Sweeney, M. Tangney, et al., Induction of effective antitumor response after mucosal bacterial vector mediated DNA vaccination with endogenous prostate cancer specific antigen, J. Urol. 186 (2) (2011) 687–693.
[61] E. Berger, R. Soldati, N. Huebener, O. Hohn, A. Stermann, T. Durmus, et al., *Salmonella* SL7207 application is the most effective DNA vaccine delivery method for successful tumor eradication in a murine model for neuroblastoma, Cancer Lett. 331 (2) (2013) 167–173.
[62] K.K. Feng, H.Y. Zhao, J. Chen, D.X. Yao, X.B. Jiang, W. Zhou, Anti-angiogenesis effect on glioma of attenuated*Salmonella* Typhimurium vaccine strain with flk-1 gene, J. Huazhong Univ. Sci. Med. 24 (4) (2004) 389–391.
[63] R.A. Kazmierczak, B. Gentry, T. Mumm, H. Schatten, A. Eisenstark, *Salmonella* bacterial monotherapy reduces autochthonous prostate tumor burden in the TRAMP mouse model, PLoS One 11 (8) (2016) e0160926.
[64] J.Z. Meng, Y.J. Dong, H. Huang, S. Li, Y. Zhong, S.L. Liu, et al., Oral vaccination with attenuated *Salmonella* enterica strains encoding T-cell epitopes from tumor antigen NY-ESO-1 induces specific cytotoxic T-lymphocyte responses, Clin. Vaccine Immunol. 17 (6) (2010) 889–894.
[65] N. Yang, X. Zhu, L. Chen, S. Li, D. Ren, Oral administration of attenuated *S.* Typhimurium carrying shRNA-expressing vectors as a cancer therapeutic, Cancer Biol. Ther. 7 (1) (2014) 145–151.
[66] J. Ye, L. Li, Y. Zhang, X. Zhang, D. Ren, W. Chen, Recombinant *Salmonella*-based 4-1BBL vaccine enhances T cell immunity and inhibits the development of colorectal cancer in rats: *in vivo* effects of vaccine containing 4-1BBL, J. Biomed. Sci. 20 (2013) 8.
[67] L. Yuhua, G. Kunyuan, C. Hui, X. Yongmei, S. Chaoyang, T. Xun, et al., Oral cytokine gene therapy against murine tumor using attenuated *Salmonella* Typhimurium, Int. J. Cancer 94 (3) (2001) 438–443.
[68] S.G. Zuo, Y. Chen, Z.P. Wu, X. Liu, C. Liu, Y.C. Zhou, et al., Orally administered DNA vaccine delivery by attenuated *Salmonella* Typhimurium targeting fetal liver kinase 1 inhibits murine Lewis lung carcinoma growth and metastasis, Biol. Pharm. Bull. 33 (2) (2010) 174–182.
[69] B. Yu, M. Yang, L. Shi, Y. Yao, Q. Jiang, X. Li, et al., Explicit hypoxia targeting with tumor suppression by creating an "obligate" anaerobic *Salmonella* Typhimurium strain, Sci. Rep. 2 (2012) 436.
[70] B.T. Ning, B. Yu, S. Chan, J.L. Chan, J.D. Huang, G.C. Chan, Treatment of neuroblastoma with an engineered "obligate" anaerobic *Salmonella* Typhimurium strain YB1, J. Canc. 8 (9) (2017) 1609–1618.
[71] B. Yu, L. Shi, B.Z. Zhang, K.E. Zhang, X. Peng, H.B. Niu, et al., Obligate anaerobic *Salmonella* Typhimurium strain YB1 treatment on xenograft tumor in immunocompetent mouse model, Oncol. Lett. 10 (2) (2015) 1069–1074.
[72] S. Wang, Q. Kong, R. Curtiss 3rd, New technologies in developing recombinant attenuated *Salmonella* vaccine vectors, Microb. Pathog. 58 (2013) 17–28.
[73] J.J. Min, V.H. Nguyen, H.J. Kim, Y. Hong, H.E. Choy, Quantitative bioluminescence imaging of tumor-targeting bacteria in living animals, Nat. Protoc. 3 (4) (2008) 629–636.
[74] J.H. Jeong, K. Kim, D. Lim, K. Jeong, Y. Hong, V.H. Nguyen, et al., Anti-tumoral effect of the mitochondrial target domain of Noxa delivered by an engineered *Salmonella* Typhimurium, PLoS One 9 (1) (2014) e80050.
[75] K. Kim, J.H. Jeong, D. Lim, Y. Hong, H.J. Lim, G.J. Kim, et al., L-Asparaginase delivered by *Salmonella* Typhimurium suppresses solid tumors, Mol. Ther. Oncolytics 2 (2015) 15007.
[76] K. Kim, S.Y. Min, H.D. Lim, S.H. You, D. Lim, J.H. Jeong, et al., Cell mass-dependent expression of an anticancer protein drug by tumor-targeted *Salmonella*, Oncotarget 9 (9) (2018) 8548–8559.
[77] D. Lim, K.S. Kim, H. Kim, K.C. Ko, J.J. Song, J.H. Choi, et al., Anti-tumor activity of an immunotoxin (TGFalpha-PE38) delivered by attenuated *Salmonella* Typhimurium, Oncotarget 8 (23) (2017) 37550–37560.
[78] V.H. Nguyen, H.S. Kim, J.M. Ha, Y. Hong, H.E. Choy, J.J. Min, Genetically engineered *Salmonella* Typhimurium as an imageable therapeutic probe for cancer, Cancer Res. 70 (1) (2010) 18–23.
[79] X. Cheng, X. Zhang, Y. Zhou, C. Zhang, Z.C. Hua, A *Salmonella* Typhimurium mutant strain capable of RNAi delivery: higher tumor-targeting and lower toxicity, Cancer Biol. Ther. 15 (8) (2014) 1068–1076.
[80] N. Arrach, P. Cheng, M. Zhao, C.A. Santiviago, R.M. Hoffman, M. McClelland, High-throughput screening for *Salmonella* avirulent mutants that retain targeting of solid tumors, Cancer Res. 70 (6) (2010) 2165–2170.
[81] M. Frahm, S. Felgner, D. Kocijancic, M. Rohde, M. Hensel, R.III Curtiss, et al., Efficiency of conditionally attenuated *Salmonella* enterica serovar Typhimurium in bacterium-mediated tumor therapy, mBio 6 (2) (2015) e00254-15.
[82] A. Teghanemt, D. Zhang, E.N. Levis, J.P. Weiss, T.L. Gioannini, Molecular basis of reduced potency of underacylated endotoxins, J. Immunol. 175 (7) (2005) 4669–4676.
[83] S. Felgner, M. Frahm, D. Kocijancic, M. Rohde, D. Eckweiler, A. Bielecka, et al., *aroA*-deficient *Salmonella* enterica serovar Typhimurium is more than a metabolically attenuated mutant, mBio 7 (5) (2016) e01220-16.
[84] K. Liang, Q. Liu, P. Li, Y. Han, X. Bian, Y. Tang, et al., Endostatin gene therapy delivered by attenuated *Salmonella* Typhimurium in murine tumor models, Cancer Gene Ther. 25 (7-8) (2018) 167–183.
[85] S. Lewen, H. Zhou, H.D. Hu, T. Cheng, D. Markowitz, R.A. Reisfeld, et al., A Legumain-based minigene vaccine targets the tumor stroma and suppresses breast cancer growth and angiogenesis, Cancer Immunol. Immunother. 57 (4) (2008) 507–515.
[86] E. Roider, S. Jellbauer, B. Kohn, C. Berchtold, M. Partilla, D.H. Busch, et al., Invasion and destruction of a murine fibrosarcoma by *Salmonella*-induced effector $CD8^+$ T cells as a therapeutic intervention against cancer, Cancer Immunol. Immunother. 60 (3) (2011) 371–380.
[87] L. Shi, B. Yu, C.H. Cai, J.D. Huang, Angiogenic inhibitors delivered by the type III secretion system of tumor-targeting *Salmonella* Typhimurium safely shrink tumors in mice, Amb. Express 6 (1) (2016) 56.
[88] B.S. Sorenson, K.L. Banton, N.L. Frykman, A.S. Leonard, D.A. Saltzman, Attenuated *Salmonella* Typhimurium with IL-2 gene reduces pulmonary metastases in murine osteosarcoma, Clin. Orthop. Relat. Res. 466 (6) (2008) 1285–1291.
[89] B.K. Al-Ramadi, M.J. Fernandez-Cabezudo, H. El-Hasasna, S. Al-Salam, G. Bashir, S. Chouaib, Potent anti-tumor activity of systemically-administered IL2-expressing *Salmonella* correlates with decreased angiogenesis and enhanced tumor apoptosis, Clin. Immunol. 130 (1) (2009) 89–97.
[90] M. Loeffler, G. Le'Negrate, M. Krajewska, J.C. Reed, IL-18-producing *Salmonella* inhibit tumor growth, Cancer Gene Ther. 15 (12) (2008) 787–794.
[91] M. Loeffler, G. Le'Negrate, M. Krajewska, J.C. Reed, Attenuated *Salmonella* engineered to produce human cytokine LIGHT inhibit tumor growth, Proc. Natl. Acad. Sci. U. S. A. 104 (31) (2007) 12879–12883.
[92] M. Loeffler, G. Le'Negrate, M. Krajewska, J.C. Reed, *Salmonella* Typhimurium engineered to produce CCL21 inhibit tumor growth, Cancer Immunol. Immunother. 58 (5) (2009) 769–775.
[93] W. Yoon, Y.C. Park, J. Kim, Y.S. Chae, J.H. Byeon, S.H. Min, S. Park, Y. Yoo, Y.K. Park, B.M. Kim, Application of genetically engineered *Salmonella* Typhimurium for interferon-gamma-induced therapy against melanoma, Eur. J. Cancer 70 (2017) 48–61.
[94] W.S. Yoon, Y.S. Chae, J. Hong, Y.K. Park, Antitumor therapeutic effects of a genetically engineered *Salmonella* Typhimurium harboring TNF-alpha in mice, Appl. Microbiol. Biotechnol. 89 (6) (2011) 1807–1819.
[95] Y.H. Li, H. Chen, K.Y. Guo, Y.M. Xie, F. Wang, K.C. Xie, et al., Prophylaxis of tumor through oral administration of IL-12 GM-CSF gene carried by live attenuated *Salmonella*, Chin. Sci. Bull. 46 (13) (2001) 1107–1111.
[96] C. Agorio, F. Schreiber, M. Sheppard, P. Mastroeni, M. Fernandez, M.A. Martinez, J.A. Chabalgoity, Live attenuated *Salmonella* as a vector for oral cytokine gene therapy in melanoma, J. Gene Med. 9 (5) (2007) 416–423.
[97] D. Quintero, J. Carrafa, L. Vincent, D. Bermudes, EGFR-targeted Chimeras of Pseudomonas ToxA released into the extracellular milieu by attenuated *Salmonella* selectively kill tumor cells, Biotechnol. Bioeng. 113 (12) (2016) 2698–2711.
[98] S. Ganai, R.B. Arenas, N.S. Forbes, Tumour-targeted delivery of TRAIL using *Salmonella* Typhimurium enhances breast cancer survival in mice, Br. J. Canc. 101 (10) (2009) 1683–1691.
[99] M. Loeffler, G. Le'Negrate, M. Krajewska, J.C. Reed, Inhibition of tumor growth using *Salmonella* expressing Fas ligand, J. Natl. Cancer Inst. 100 (15) (2008) 1113–1116.
[100] C.T. Nguyen, S.H. Hong, J.I. Sin, H.V. Vu, K. Jeong, K.O. Cho, et al., Flagellin enhances tumor-specific $CD8^+$ T cell immune responses through TLR5 stimulation in a therapeutic cancer vaccine model, Vaccine 31 (37) (2013) 3879–3887.
[101] M. Urashima, H. Suzuki, Y. Yuza, M. Akiyama, N. Ohno, Y. Eto, An oral CD40 ligand gene therapy against lymphoma using attenuated *Salmonella* Typhimurium, Blood 95 (4) (2000) 1258–1263.
[102] L. Shi, B. Yu, C.H. Cai, W. Huang, B.J. Zheng, D.K. Smith, et al., Combined prokaryotic-eukaryotic delivery and expression of therapeutic factors through a primed autocatalytic positive-feedback loop, J. Contr. Release 222 (2016) 130–140.
[103] H.D. Cao, Y.X. Yang, L. Lü, S.N. Liu, P.L. Wang, X.H. Tao, et al., Attenuated *Salmonella* Typhimurium carrying TRAIL and VP3 genes inhibits the growth of

gastric cancer cells *in vitro* and *in vivo*, Tumori J 96 (2) (2010) 296–303.
[104] C.H. Lee, C.L. Wu, A.L. Shiau, Endostatin gene therapy delivered by *Salmonella* Choleraesuis in murine tumor models, J. Gene Med. 6 (12) (2004) 1382.
[105] C.H. Lee, C.L. Wu, A.L. Shiau, Systemic administration of attenuated *Salmonella* Choleraesuis carrying thrombospondin-1 gene leads to tumor-specific transgene expression, delayed tumor growth and prolonged survival in the murine melanoma model, Cancer Gene Ther. 12 (2) (2005) 175–184.
[106] H. Jia, Y. Li, T. Zhao, X. Li, J. Hu, D. Yin, et al., Antitumor effects of Stat3-siRNA and endostatin combined therapies, delivered by attenuated *Salmonella*, on orthotopically implanted hepatocarcinoma, Cancer Immunol. Immunother. 61 (11) (2012) 1977–1987.
[107] C. Shao, B. Yang, L. Zhao, S. Wang, J. Zhang, K. Wang, Tumor suppressor gene RBM5 delivered by attenuated *Salmonella* inhibits lung adenocarcinoma through diverse apoptotic signaling pathways, World J. Surg. Oncol. 11 (2013) 123.
[108] M. Bereta, A. Hayhurst, M. Gajda, P. Chorobik, M. Targosz, J. Marcinkiewicz, et al., Improving tumor targeting and therapeutic potential of *Salmonella* VNP20009 by displaying cell surface CEA-specific antibodies, Vaccine 25 (21) (2007) 4183–4192.
[109] P.E. Massa, A. Paniccia, A. Monegal, A. De Marco, M. Rescigno, *Salmonella* engineered to express CD20-targeting antibodies and a drug-converting enzyme can eradicate human lymphomas, Blood 122 (5) (2013) 705–714.
[110] S.-H. Park, J.H. Zheng, V.H. Nguyen, S.-N. Jiang, D.-Y. Kim, M. Szardenings, et al., RGD peptide cell-surface display enhances the targeting and therapeutic efficacy of attenuated *Salmonella*-mediated cancer therapy, Theranostics 6 (10) (2016) 1672–1682.
[111] S.-H. Park, J.H. Zheng, V.H. Nguyen, S.-N. Jiang, D.-Y. Kim, M. Szardenings, et al., RGD peptide cell-surface display enhances the targeting and therapeutic efficacy of attenuated *Salmonella*-mediated cancer therapy, Theranostics 6 (10) (2016) 1672–1682.
[112] W. Yoon, J.H. Choi, S. Kim, Y.K. Park, Engineered *Salmonella* Typhimurium expressing E7 fusion protein, derived from human papillomavirus, inhibits tumor growth in cervical tumor-bearing mice, Biotechnol. Lett. 36 (2) (2014) 349–356.
[113] G. Xiong, M.I. Husseiny, L. Song, A. Erdreich-Epstein, G.M. Shackleford, R.C. Seeger, et al., Novel cancer vaccine based on genes of *Salmonella* pathogenicity island 2, Int. J. Cancer 126 (11) (2010) 2622–2634.
[114] C.K. Chou, J.Y. Hung, J.C. Liu, C.T. Chen, M.C. Hung, An attenuated *Salmonella* oral DNA vaccine prevents the growth of hepatocellular carcinoma and colon cancer that express alpha-fetoprotein, Cancer Gene Ther. 13 (8) (2006) 746–752.
[115] Y. Mei, L. Zhao, Y. Liu, H. Gong, Y. Song, L. Lei, et al., Combining DNA vaccine and AIDA-1 in attenuated *Salmonella* activates tumor-specific $CD4^+$ and $CD8^+$ T-cell responses, Canc. Immunol. Res. 5 (6) (2017) 503–514.
[116] I. King, D. Bermudes, S. Lin, M. Belcourt, J. Pike, K. Troy, et al., Tumor-targeted *Salmonella* expressing cytosine deaminase as an anticancer agent, Hum. Gene Ther. 13 (10) (2002) 1225–1233.
[117] B. Mesa-Pereira, C. Medina, E.M. Camacho, A. Flores, E. Santero, Improved cytotoxic effects of *Salmonella*-producing cytosine deaminase in tumour cells, Microb. Biotechnol. 8 (1) (2015) 169–176.
[118] W. Fu, H. Lan, S. Li, X. Han, T. Gao, D. Ren, Synergistic antitumor efficacy of suicide/ePNP gene and 6-methylpurine 2'-deoxyriboside via *Salmonella* against murine tumors, Cancer Gene Ther. 15 (7) (2008) 474–484.
[119] W. Fu, H. Lan, S. Liang, T. Gao, D. Ren, Suicide gene/prodrug therapy using *Salmonella*-mediated delivery of *Escherichia coli* purine nucleoside phosphorylase gene and 6-methoxypurine 2'-deoxyriboside in murine mammary carcinoma 4T1 model, Cancer Sci. 99 (6) (2008) 1172–1179.
[120] G. Chen, B. Tang, B.Y. Yang, J.X. Chen, J.H. Zhou, J.H. Li, et al., Tumor-targeting *Salmonella* Typhimurium, a natural tool for activation of prodrug 6MePdR and their combination therapy in murine melanoma model, Appl. Microbiol. Biotechnol. 97 (10) (2013) 4393–4401.
[121] L. Zhang, L. Gao, L. Zhao, B. Guo, K. Ji, Y. Tian, et al., Intratumoral delivery and suppression of prostate tumor growth by attenuated *Salmonella* enterica serovar Typhimurium carrying plasmid-based small interfering RNAs, Cancer Res. 67 (12) (2007) 5859–5864.
[122] C. Zhao, J. He, H. Cheng, Z. Zhu, H. Xu, Enhanced therapeutic effect of an anti-angiogenesis peptide on lung cancer *in vivo* combined with *Salmonella* VNP20009 carrying a Sox2 shRNA construct, J. Exp. Clin. Canc. Res. 35 (1) (2016) 107.
[123] C.A. Blache, E.R. Manuel, T.I. Kaltcheva, A.N. Wong, J.D. Ellenhorn, B.R. Blazar, et al., Systemic delivery of *Salmonella* Typhimurium transformed with Ido shRNA enhances intratumoral vector colonization and suppresses tumor growth, Cancer Res. 72 (24) (2012) 6447–6456.
[124] Y. Tian, B. Guo, H. Jia, K. Ji, Y. Sun, Y. Li, et al., Targeted therapy via oral administration of attenuated *Salmonella* expression plasmid-vectored Stat3-shRNA cures orthotopically transplanted mouse HCC, Cancer Gene Ther. 19 (6) (2012) 393–401.
[125] X. Li, Y. Li, B. Wang, K. Ji, Z. Liang, B. Guo, et al., Delivery of the co-expression plasmid pEndo-Si-Stat3 by attenuated *Salmonella* serovar Typhimurium for prostate cancer treatment, J. Canc. Res. Clin. Oncol. 139 (6) (2013) 971–980.
[126] E.R. Manuel, C.A. Blache, R. Paquette, T.I. Kaltcheva, H. Ishizaki, J.D. Ellenhorn, et al., Enhancement of cancer vaccine therapy by systemic delivery of a tumor-targeting *Salmonella*-based STAT3 shRNA suppresses the growth of established melanoma tumors, Cancer Res. 71 (12) (2011) 4183–4191.
[127] L.J. Jia, D.P. Wei, Q.M. Sun, G.H. Jin, S.F. Li, Y. Huang, et al., Tumor-targeting *Salmonella* Typhimurium improves cyclophosphamide chemotherapy at maximum tolerated dose and low-dose metronomic regimens in a murine melanoma model, Int. J. Cancer 121 (3) (2007) 666–674.
[128] X. Zhang, X. Cheng, Y. Lai, Y. Zhou, W. Cao, Z.C. Hua, *Salmonella* VNP20009-mediated RNA interference of ABCB5 moderated chemoresistance of melanoma stem cell and suppressed tumor growth more potently, Oncotarget 7 (12) (2016) 14940–14950.
[129] J. Chen, Y. Qiao, B. Tang, G. Chen, X. Liu, B. Yang, et al., Modulation of *Salmonella* tumor-colonization and intratumoral anti-angiogenesis by triptolide and its mechanism, Theranostics 7 (8) (2017) 2250–2260.
[130] Y. Hiroshima, Y. Zhang, T. Murakami, A. Maawy, S. Miwa, M. Yamamoto, et al., Efficacy of tumor-targeting *Salmonella* Typhimurium A1-R in combination with anti-angiogenesis therapy on a pancreatic cancer patient-derived orthotopic xenograft (PDOX) and cell line mouse models, Oncotarget 5 (23) (2014) 12346–12357.
[131] K. Kawaguchi, K. Igarashi, T. Murakami, B. Chmielowski, T. Kiyuna, M. Zhao, et al., Tumor-targeting *Salmonella* Typhimurium A1-R combined with temozolomide regresses malignant melanoma with a BRAF-V600E mutation in a patient-derived orthotopic xenograft (PDOX) model, Oncotarget 7 (52) (2016) 85929–85936.
[132] T. Murakami, J. DeLong, F.C. Eilber, M. Zhao, Y. Zhang, N. Zhang, et al., Tumor-targeting *Salmonella* Typhimurium A1-R in combination with doxorubicin eradicate soft tissue sarcoma in a patient-derived orthotopic xenograft (PDOX) model, Oncotarget 7 (11) (2016) 12783–12790.
[133] K. Igarashi, K. Kawaguchi, T. Kiyuna, K. Miyake, M. Miyake, S. Li, et al., Tumor-targeting *Salmonella* Typhimurium A1-R combined with recombinant methioninase and cisplatinum eradicates an osteosarcoma cisplatinum-resistant lung metastasis in a patient-derived orthotopic xenograft (PDOX) mouse model: decoy, trap and kill chemotherapy moves toward the clinic, Cell Cycle 17 (6) (2018) 801–809.
[134] S. Yano, K. Takehara, M. Zhao, Y. Tan, Q. Han, S. Li, et al., Tumor-specific cell-cycle decoy by *Salmonella* Typhimurium A1-R combined with tumor-selective cell-cycle trap by methioninase overcome tumor intrinsic chemoresistance as visualized by FUCCI imaging, Cell Cycle 15 (13) (2016) 1715–1723.
[135] J. Deng, Y. Guo, Z. Jiang, M. Yang, H. Li, J. Wang, Enhancement of ovarian cancer chemotherapy by delivery of multidrug-resistance gene small interfering RNA using tumor targeting *Salmonella*, J. Obstet. Gynaecol. Res. 41 (4) (2015) 615–622.
[136] M. Vola, A. Monaco, T. Bascuas, G. Rimsky, C.I. Agorio, J.A. Chabalgoity, et al., TLR7 agonist in combination with *Salmonella* as an effective antimelanoma immunotherapy, Immunotherapy 10 (8) (2018) 665–679.
[137] D. Saltzman, L. Augustin, A. Leonard, M. Mertensotto, J. Schottel, Low dose chemotherapy combined with attenuated *Salmonella* decreases tumor burden and is less toxic than high dose chemotherapy in an autochthonous murine model of breast cancer, Surgery 163 (3) (2018) 509–514.
[138] M. Zhang, C.A. Swofford, N.S. Forbes, Lipid A controls the robustness of intratumoral accumulation of attenuated *Salmonella* in mice, Int. J. Cancer 135 (3) (2014) 647–657.
[139] K. Crull, D. Bumann, S. Weiss, Influence of infection route and virulence factors on colonization of solid tumors by *Salmonella* enterica serovar Typhimurium, FEMS Immunol. Med. Microbiol. 62 (1) (2011) 75–83.
[140] J. Platt, S. Sodi, M. Kelley, S. Rockwell, D. Bermudes, K.B. Low, et al., Antitumour effects of genetically engineered *Salmonella* in combination with radiation, Eur. J. Cancer 36 (18) (2000) 2397–2402.
[141] X. Liu, S. Jiang, L. Piao, F. Yuan, Radiotherapy combined with an engineered *Salmonella* Typhimurium inhibits tumor growth in a mouse model of colon cancer, Exp. Anim. 65 (4) (2016) 413–418.
[142] W.S. Yoon, S. Kim, S. Seo, Y. Park, *Salmonella* Typhimurium with gamma-radiation induced H2AX phosphorylation and apoptosis in melanoma, Biosci. Biotechnol. Biochem. 78 (6) (2014) 1082–1085.
[143] Y. Zhang, N. Zhang, R.M. Hoffman, M. Zhao, *Salmonella* Typhimurium A1-R inhibits surgically induced breast cancer metastasis, Cancer Res. 74 (19 Supplement) (2014) 712.
[144] T. Murakami, Y. Hiroshima, M. Zhao, Y. Zhang, T. Chishima, K. Tanaka, et al., Adjuvant treatment with tumor-targeting *Salmonella* Typhimurium A1-R reduces recurrence and increases survival after liver metastasis resection in an orthotopic nude mouse model, Oncotarget 6 (39) (2015) 41856–41862.
[145] N.S. Forbes, Engineering the perfect (bacterial) cancer therapy, Nat. Rev. Canc. 10 (11) (2010) 785–794.
[146] C.A. Swofford, N. Van Dessel, N.S. Forbes, Quorum-sensing *Salmonella* selectively trigger protein expression within tumors, Proc. Natl. Acad. Sci. U. S. A. 112 (11) (2015) 3457–3462.
[147] H. Hong, D. Lim, G.J. Kim, S.H. Park, H. Sik Kim, Y. Hong, et al., Targeted deletion of the ara operon of *Salmonella* Typhimurium enhances L-arabinose accumulation and drives PBAD-promoted expression of anti-cancer toxins and imaging agents, Cell Cycle 13 (19) (2014) 3112–3120.
[148] H. Loessner, A. Endmann, S. Leschner, K. Westphal, M. Rohde, T. Miloud, et al., Remote control of tumour-targeted *Salmonella* enterica serovar Typhimurium by the use of L-arabinose as inducer of bacterial gene expression *in vivo*, Cell Microbiol. 9 (6) (2007) 1529–1537.
[149] J.L. Royo, P.D. Becker, E.M. Camacho, A. Cebolla, C. Link, E. Santero, et al., *In vivo* gene regulation in *Salmonella* spp. by a salicylate-dependent control circuit, Nat. Methods 4 (11) (2007) 937–942.
[150] S.N. Jiang, S.H. Park, H.J. Lee, J.H. Zheng, H.S. Kim, H.S. Bom, et al., Engineering of bacteria for the visualization of targeted delivery of a cytolytic anticancer agent, Mol. Ther. 21 (11) (2013) 1985–1995.
[151] S. Nuyts, L. Van Mellaert, J. Theys, W. Landuyt, P. Lambin, J. Anné, The use of radiation-induced bacterial promoters in anaerobic conditions: a means to control gene expression in *Clostridium*-mediated therapy for cancer, Radiat. Res. 155 (5) (2001) 716–723.
[152] S. Nuyts, L. Van Mellaert, S. Barbe, E. Lammertyn, J. Theys, W. Landuyt, et al., Insertion or deletion of the Cheo box modifies radiation inducibility of Clostridium

promoters, Appl. Environ. Microbiol. 67 (10) (2001) 4464–4470.
[153] A. Mengesha, L. Dubois, P. Lambin, W. Landuyt, R.K. Chiu, B.G. Wouters, et al., Development of a flexible and potent hypoxia-inducible promoter for tumor-targeted gene expression in attenuated *Salmonella*, Cancer Biol. Ther. 5 (9) (2014) 1120–1128.
[154] R.M. Ryan, J. Green, P.J. Williams, S. Tazzyman, S. Hunt, J.H. Harmey, et al., Bacterial delivery of a novel cytolysin to hypoxic areas of solid tumors, Gene Ther. 16 (3) (2009) 329–339.
[155] N. Arrach, M. Zhao, S. Porwollik, R.M. Hoffman, M. McClelland, *Salmonella* promoters preferentially activated inside tumors, Cancer Res. 68 (12) (2008) 4827–4832.
[156] K. Flentie, B. Kocher, S.T. Gammon, D.V. Novack, J.S. McKinney, D. Piwnica-Worms, A bioluminescent transposon reporter-trap identifies tumor-specific microenvironment-induced promoters in *Salmonella* for conditional bacterial-based tumor therapy, Cancer Discov. 2 (7) (2012) 624–637.
[157] Y. Dai, B.J. Toley, C.A. Swofford, N.S. Forbes, Construction of an inducible cell-communication system that amplifies *Salmonella* gene expression in tumor tissue, Biotechnol. Bioeng. 110 (6) (2013) 1769–1781.
[158] M.O. Din, T. Danino, A. Prindle, M. Skalak, J. Selimkhanov, K. Allen, et al., Synchronized cycles of bacterial lysis for *in vivo* delivery, Nature 536 (7614) (2016) 81–85.
[159] J. Nemunaitis, C. Cunningham, N. Senzer, J. Kuhn, J. Cramm, C. Litz, et al., Pilot trial of genetically modified, attenuated *Salmonella* expressing the *E. coli* cytosine deaminase gene in refractory cancer patients, Cancer Gene Ther. 10 (10) (2003) 737–744.
[160] Q. Kong, J. Yang, Q. Liu, P. Alamuri, K.L. Roland, R.III Curtiss, Effect of deletion of genes involved in lipopolysaccharide core and O-antigen synthesis on virulence and immunogenicity of *Salmonella* enterica serovar Typhimurium, Infect. Immun. 79 (10) (2011) 4227–4239.
[161] R.III Curtiss, W. Xin, Y. Li, W. Kong, S.-Y. Wanda, B. Gunn, et al., New technologies in using recombinant attenuated *Salmonella* vaccine vectors, Crit. Rev. Immunol. 30 (3) (2010) 255–270.
[162] Q. Kong, D.A. Six, Q. Liu, L. Gu, K.L. Roland, C.R. Raetz, et al., Palmitoylation state impacts induction of innate and acquired immunity by the *Salmonella* enterica serovar Typhimurium *msbB* mutant, Infect. Immun. 79 (12) (2011) 5027–5038.
[163] Q. Kong, D.A. Six, K.L. Roland, Q. Liu, L. Gu, C.M. Reynolds, et al., *Salmonella* synthesizing 1-dephosphorylated lipopolysaccharide exhibits low endotoxic activity while retaining its immunogenicity, J. Immunol. 187 (1) (2011) 412–423.
[164] Q. Kong, D.A. Six, Q. Liu, L. Gu, S. Wang, P. Alamuri, et al., Phosphate groups of lipid A are essential for *Salmonella* enterica serovar Typhimurium virulence and affect innate and adaptive immunity, Infect. Immun. 80 (9) (2012) 3215–3224.
[165] W. Kong, S.Y. Wanda, X. Zhang, W. Bollen, S.A. Tinge, K.L. Roland, et al., Regulated programmed lysis of recombinant *Salmonella* in host tissues to release protective antigens and confer biological containment, Proc. Natl. Acad. Sci. U. S. A. 105 (27) (2008) 9361–9366.