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New Technologies in Developing Recombinant Attenuated Bacteria for Cancer Therapy

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Abstract: Cancer has always been a global problem, with more cases of cancer patients being diagnosed every year. Conventional cancer treatments, including radiotherapy, chemotherapy and surgery, are still unable to bypass their obvious limitations, and developing effective targeted therapies is still required. More than one century ago, the doctor William B. Coley discovered that cancer patients had tumor regression by injection of *Streptococcus* bacteria. The studies of cancer therapy using bacterial microorganisms are now very widespread. In particular, the facultative anaerobic bacteria *Salmonella* Typhimurium is widely investigated as it can selectively colonize different types of tumors, locally deliver various anti-tumor drugs, and inhibit tumor growth. The exciting anti-tumor efficacy and safety observed in animal tumor models prompted the well-known attenuated *Salmonella* bacterial strain VNP20009 to be tested in human clinical trials in the early 21st century. Regrettably, no patients showed significant therapeutic effects and even bacterial colonization in tumor tissue was undetectable in most patients. *Salmonella* bacteria are still considered as a promising agent or vehicle for cancer therapy. Recent efforts have been focused on the generation of attenuated bacterial strains with higher targeting for tumor tissue, and optimization of the delivery of therapeutic anti-tumor cargoes into the tumor microenvironment. This review will summarize new technologies or approaches that may improve bacteria-mediated cancer therapy.

Keywords: cancer therapy, *Salmonella* Typhimurium, tumor-targeting, anti-tumor agents, lysis systems, membrane vesicles

Introduction

Although some progress has been made in cancer treatment and detection, cancer is still one of the leading causes of death worldwide. Conventional cancer therapies, including radiotherapy, chemotherapy and surgery, have their intrinsic limitations, such as high toxicity to normal tissue cells, the inability to treat deep tumor tissue and the possibility of developing drug resistance. Thus, safe and targeted cancer therapies are urgently needed to overcome these limitations. The potential of using microbes such as bacteria and oncolytic viruses to fight cancer has been documented for over one hundred years (Forbes et al., 2018). In the late 19th and early 20th centuries, New York doctor William B. Coley attempted to use live *Streptococcus pyogenes* and killed organisms (Coley's Toxins) to treat his patients (McCarthy, 2006). Unfortunately, his pioneering work was terminated by his boss later. In the middle of the last century, the studies of bacteria-mediated cancer therapy restarted and are very widespread now. Many facultative or obligate anaerobic bacteria, such as *Clostridium* (Malmgren & Flanigan, 1955), *Bifidobacterium* (Kohwi, Imai, Tamura, & Hashimoto, 1978), *Escherichia coli* (Stritzker et al., 2007) and *Salmonella* (Low et al., 1999), have been shown to have intrinsic tumor-targeting and tumor-killing activities. The development of molecular biology, the complete sequencing of many bacterial genomes and the establishment of genetic modification

methods have also greatly promoted the research of using bacteria and other microorganisms for cancer treatment.

Over the last three decades, the anti-tumor therapeutic potential of facultative anaerobic *Salmonella* Typhimurium has been studied extensively. Engineered *Salmonella* can directly exert the tumor-killing effects or act as a delivery vector for a wide variety of anti-tumor molecules. In the studies using animal tumor models, live attenuated *Salmonella* can selectively colonize tumor tissue after systemic infection and then produce anti-tumor drugs, thereby inhibiting tumor growth, extending animal survival and even eliminating tumors (Liang et al., 2019). Disappointingly, in human clinical trials was no significant anti-tumor effect observed in all cancer patients treated with engineered *Salmonella* VNP20009 (Toso et al., 2002). There are still many problems to be addressed before the application of *Salmonella* for clinical cancer treatment. For example, it is necessary to ensure effective bacterial colonization into tumor tissue as well as to enhance the anti-tumor ability of *Salmonella*. In this review, we will highlight new technologies or methods useful for *Salmonella*-mediated cancer therapy, which involves improving the tumor targeting and immunostimulatory ability of *Salmonella*, regulating the delivery and release of anti-tumor molecules, and developing non-living bacterial nanovesicle derivatives as biosafe delivery platforms.

Engineering *Salmonella* for enhancing their tumor targeting and immunostimulatory ability

2.1 Construction of auxotrophic mutants

The use of live bacteria in the treatment of cancer as well as prevention of other diseases always raises issues regarding the safety. If not adequately attenuated, live *Salmonella* may proliferate in the blood, release toxins, and even cause severe septic shock and death, especially when administered systemically. The reduction of bacterial virulence can be achieved by mutation of virulence-associated genes in the genome via site-directed gene mutation methods and transposon or chemical mutagenesis. Auxotrophic mutations are commonly introduced to generate bacterial mutants with high tumor targeting. For example, the well-known attenuated *Salmonella* Typhimurium strain VNP20009 is deleted of *msbB* and *purI*, of which the latter is responsible for adenine synthesis (Clairmont et al., 2000; Low et al., 1999). The tumor-targeting strain A1-R is auxotrophic for arginine and leucin (Hoffman, 2011; Zhao et al., 2006) and *aroA* mutants, such as the strain SL7207, are unable to synthesize aromatic amino acids (Berger et al., 2013; Hoiseth & Stocker, 1981; Liang et al., 2018; Meng et al., 2010; Sebastian et al., 2016; Yoon et al., 2017). Theoretically, these auxotrophic bacterial strains can obtain amino acids, purines, pyrimidines or other growth factors in the tumor microenvironment, while their fitness in normal tissues is reduced. It is possible that auxotrophic mutants are less able to multiply *in vivo*, even in the tumor microenvironment, compared to wild type

bacterial strains. The preferential tumor colonization of *Salmonella* with auxotrophic mutations can be determined through *in vivo* imaging and viable bacterial count (Li et al., 2012; Min, Nguyen, Kim, Hong, & Choy, 2008).

The strain A1-R was re-isolated from tumor tissue infected with its parent strain A1, which was previously selected after nitrosoguanidine (NTG) mutagenesis and auxotrophic for leucine and arginine (Zhao et al., 2005, 2006). Compared with A1, A1-R showed more efficient adhesion to tumor cells *in vitro* and improved tumor colonization *in vivo* (Zhao et al., 2006). In addition, A1-R monotherapy has been shown to be effective against primary and metastatic tumors of different types in clinically relevant mouse models (Hoffman, 2016). Thus, re-isolation of bacteria from tumor tissue after *in vivo* infection seems to be an approach to further enhance the tumor targeting of *Salmonella*. High throughput screening was used to screen *Salmonella* strains with reduced fitness in normal tissues but unaltered fitness in the tumor from thousands of candidate transposon-insertion mutants, offering an advantage over conventional methods (Arrach et al., 2010). In addition, through regulating the expression of essential genes under tumor-specific promoters, which can be specifically activated or repressed by factors in the tumor microenvironment, the tumor targeting of *Salmonella* could be improved as well. A good example is the "obligate anaerobic" strain YB-1, a derivative strain of SL7207, of which the gene *asd* is placed under a hypoxia-induced promoter. YB-1 is able to grow in the hypoxic and

necrotic regions of the tumor while being rapidly cleared from normal tissues, thereby exhibiting higher tumor targeting when compared to its parent strain (Yu et al., 2012). The genetic mutations commonly used to construct auxotrophic *Salmonella* bacterial strains for cancer therapy are listed in **Table 1**.

2.2 Surface display of tumor-specific antibodies/ligands

On the surface of tumor cells or endothelial cells involved in tumor angiogenesis are certain antigen proteins or receptors specifically expressed or overexpressed, which can also act as targets for cancer therapy. Progress has been made on the use of antibody-drug conjugates and modified nanocarriers for targeted drug delivery to the tumor (Beck, Goetsch, Dumontet, & Corvaia, 2017; Kumari, Ghosh, & Biswas, 2016). The display of tumor-specific antibodies or ligands on bacterial outer membrane surface is also potential to improve the ability of bacteria to attach to tumor cells, thereby reducing the toxicity to normal tissue cells. Notably, small-sized foreign proteins should be selected for surface display to avoid the disruption of membrane integrity as well as to ensure expected protein localization (Lee, Choi, & Xu, 2003).

Among conventional antibody-derived fragments capable of binding to the target antigen, single-chain variable fragment (scFv) is the smallest, which contains immunoglobulin heavy chain and light chain variable regions (VH and VL) linked by a peptide spacer (**Figure 1**). The molecular weight of scFv is only 25~30 kDa, which is one fifth of the intact antibody (≈150 kDa), but it still retains the specificity and

affinity to the antigen (Ahmad et al., 2012). Nanobody, also known as a single-domain antibody (sdAb), is the variable fragment (VHH) of camelid heavy chain antibodies (hcAbs). hcAbs are characterized by the absence of the light chain polypeptide and the first constant domain (CH1). Thus, nanobody in an hcAb is the structural and functional equivalent of the antigen-binding fragment (Fab) present in conventional antibodies (**Figure 1**). The advantageous properties of nanobodies include thermal and chemical resistance, high-solubility and stability, in addition to low molecular weight (≈15 kDa), specificity and affinity (Muyldermans, 2013). Since neither of scFv and nanobody has the fragment crystallizable (Fc) region of traditional antibodies, they cannot trigger antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) to target cells upon antigen binding, two mechanisms known to be important for tumor eradication. However, scFv and nanobody can be used in different platforms for targeted cancer therapy. They are able to act as antagonistic drugs to block tumor-promoting signaling pathways, as targeting moieties of anti-tumor drugs, or as targeting moieties displayed on the surface of drug delivery systems (Alibakhshi et al., 2017; Oliveira, Heukers, Sornkom, Kok, & van Bergen En Henegouwen, 2013). It was shown that VNP20009 displaying carcinoembryonic antigen (CEA)-specific scFv on the surface had increased targeting to CEA-expressing tumors (Bereta et al., 2007). Massa et al selected a nanobody against the human CD20 tumor antigen from a phage-display library and expressed it on *Salmonella* bacterial surface. Engineered *Salmonella* with the surface decoration

showed significantly improved tumor targeting and decreased accumulation in normal tissues, and caused tumor inhibition through targeted delivery of cytotoxic molecules (Massa, Paniccia, Monegal, de Marco, & Rescigno, 2013).

Different peptides containing the RGD (arginine-glycine-aspartate) sequence have been designed to improve the tumor targeting of drug nanocarriers including liposomes, nanoparticles and micelles (Danhier, Le Breton, & Preat, 2012). RGD peptides are well-studied high-affinity ligands for the αvβ3 integrin, which is overexpressed in proliferating endothelial cells of tumor vasculature and certain types of tumor cells (Weis & Cheresh, 2011). Recently, Park et al verified the applicability of RGD peptides for improving the tumor-targeting of *Salmonella*. By genetic insertion of the peptide RGD-4C into the extracellular domain of OmpA, a novel bacterial strain displaying RGD peptides on the outer membrane surface was generated with increased tumor targeting efficiency and tumor suppression efficacy (Seung-Hwan et al., 2016).

2.3 Integration of the navigator

Recently, using external energy to navigate engineered bacteria to the tumor site has also attracted attention of researchers. For example, magnetic guidance has been proposed for spatiotemporal control of bacteria programmed as delivery vehicles of anti-tumor drugs. Magnetotactic bacteria that have magnetic crystals within their unique intracellular organelles called magnetosomes, are rare living systems known to

utilize magnetism. Some studies have attempted to couple these magnetotactic bacteria to nanoparticles loaded with anti-tumor chemotherapeutics for cancer therapy (Felfoul et al., 2016). Now, it is also feasible to magnetize naturally non-magnetic and clinically translatable bacterial strains by overexpressing iron-storage ferritins or iron-binding proteins with iron supply during bacterial growth. Researchers have found that *E. coli* bacteria forming ferritin-enriched bodies inside their cytoplasm are of magnetic properties and can be spatially controlled by external magnetic fields (Aubry et al., 2020). Another strategy to make non-magnetic bacteria controllable by external magnetic force is to simply attach synthetic magnetic materials to their surface. When exposed to an external magnetic field, attached magnetic particles will orient with the field, and so will the bacteria. Park et al integrated into *E. coli* bacteria the microparticles that encapsulates the chemotherapeutic doxorubicin and magnetite nanoparticles, and found these “microswimmers” were able to deliver doxorubicin drug molecules to target breast cancer cells under magnetic guidance *in vitro*, demonstrating their potential use for targeted drug delivery (Park, Zhuang, Yasa, & Sitti, 2017).

2.4 Improvement of the immunostimulatory ability

The anti-tumor effects of *Salmonella* are considered to mainly depend on bacterial immunostimulatory ability, although alternative mechanisms may also contribute. *Salmonella* bacteria are able to sensitize the host immune system to

produce anti-tumor responses, through the recruitment of many immune cells such as neutrophils, macrophages, dendritic cells (DCs) and $CD8^+$ T cells and the induction of IL-1β, TNF-α and other proinflammatory cytokines into tumor tissue (Liang et al., 2019). Bacteria-derived immunostimulatory components such as pathogen-associated molecular patterns (PAMPs), including lipopolysaccharide (LPS) and flagellin, have been shown to possess anti-tumor activities (Cai et al., 2011; Kocijancic et al., 2017; Sfondrini et al., 2006). For example, similar to the treatment using viable *Salmonella* bacteria, systemic administration of purified *Salmonella* LPS could induce high levels of inflammatory cytokines (e.g. TNF-α) and tumor-specific $CD8^+$ T cell responses, resulting in the elimination of CT26 tumors and transient growth regression of more resistant RenCa tumors (Kocijancic et al., 2017).

LPS, also known as endotoxin, is an important outer membrane component of Gram-negative bacteria including *Salmonella*. As the most conserved portion of LPS, lipid A anchors LPS to the outer membrane of bacteria and is responsible for both the immunostimulatory and toxic activities of LPS. Lipid A is classically recognized by host immune cells via Toll-like receptor 4 (TLR4), a pattern recognition receptor (PRR) (Miyake, 2004). It was recently discovered that caspase-11 can respond to cytoplasmic lipid A independent of TLR4 to promote the innate immune response (Hagar, Powell, Aachoui, Ernst, & Miao, 2013; Kayagaki et al., 2013). Studies of the structure-activity relationship of lipid A indicate that the number, length and

symmetry of acyl chains in lipid A govern its stimulatory activity. For example, some lipid A derivatives containing fewer acyl chains (e.g. penta- and tetra-acylated lipid A) have shown to antagonize or poorly activate TLR4 and caspase-11 signal pathways (Matsuura, Kawasaki, Kawahara, & Mitsuyama, 2012; Ohto, Fukase, Miyake, & Shimizu, 2012; Teghanemt, Zhang, Levis, Weiss, & Gioannini, 2005). This may partially explain the failure of VNP20009 in human clinical trials, which predominantly synthesizes penta-acylated lipid A due to the mutation of *msbB*.

The biosynthesis and modification systems of lipid A in *Salmonella* has been illuminated clearly, which make it possible to optimize the lipid A structure to retain or improve its beneficial immunostimulaty activity while reduce the endotoxic activity. Wild type *S.* Typhimurium are able to modify the structure of lipid A to reduce immune recognition by the host and synthesize a heterogeneous mixture of lipid A with different amounts of acyl chains (Raetz, Reynolds, Trent, & Bishop, 2007). To maximize the immune stimulation of bacteria for cancer treatment, Weiss et al and our group have attempted to generate *S.* Typhimurium mutants that synthesize homogeneous hexa-acylated lipid A, through genetically deleting the three modification genes *pagL*, *pagP* and *lpxR* (Felgner et al., 2016; Liang et al., 2018; Sebastian et al., 2016). In addition to the composition of acyl chains, the phosphorylation state of lipid A also impacts its activities. The removal of one single phosphate group was shown to decrease the toxicity of lipid A by 100 to 1000 folds

while not obviously affecting the immunostimulating activity. An example is an approved vaccine adjuvant, 3-O-deacylated monophosphoryl lipid A (MPL), which is capable of activating T-cell effector responses to the coadministered antigens with good safety and tolerance (Reed, Bertholet, Coler, & Friede, 2009). This MPL immunostimulant is produced by hydrolysis of *Salmonella minnesota* LPS, resulting in the removal of the 3-position fatty acid group and the 1-position phosphate group (Baldridge & Crane, 1999). Expression of LpxE, an inner membrane phosphatase from *Francisella tularensis*, can enable *Salmonella* to synthesize monophosphorylated lipid A (**Figure 2**) (Kong et al., 2011). Our studies have shown that *Salmonella* synthesizing 1-monophosphorylated lipid A have significantly reduced virulence and endotoxic activity but still provide protection against the challenge of the highly virulent *Salmonella* in mouse model. Remarkably, this engineered *Salmonella* with modified lipid A are effective for delivering foreign antigens and conferring protection to the host (Kong et al., 2011).

Bacterial flagellin, a principal component of flagella, is a virulence factor present on nearly all motile bacteria. Toll-like receptor 5 (TLR5) on the surface of host cells is known to recognize bacterial flagellin. The interaction of TLR5 and flagellin activates signaling through the adapter MyD88 and proinflammatory transcription factor NF-kB and triggers innate immune responses. Bacterial flagellin could also potentiate tumor antigen-mediated protective antitumor responses and thereby served as a potent

adjuvant for cancer immunotherapy (Nguyen et al., 2013). Moreover, Min et al showed that engineered *Salmonella* secreting *Vibrio vulnificus* flagellin B effectively suppressed tumor growth and metastasis, and prolonged survival in mouse model (Zheng et al., 2017). These results suggest that bacterial PAMPs such as LPS and flagellin are also promising anti-tumor agents, which aid in cancer immunotherapy through enhancing the anti-tumor immune responses specific to *Salmonella*-delivered tumor antigens.

Optimizing bacterial delivery of anti-tumor cargoes

3.1 Regulation of gene expression

One of the advantages of live attenuated *Salmonella* as a delivery vector for anti-tumor agents is that bacteria with active metabolism and autonomous motility can selectively colonize and disperse inside tumor tissue, thereby continuously releasing drugs to maintain high drug concentration for action. The use of live *Salmonella* vector to deliver anti-tumor drugs has shown various degrees of therapeutic efficacy in animal experiments. However, assuming that the colonization ratio of the tumor to normal tissues is 1000:1, a considerable number of *Salmonella* will still colonize normal tissues while most of them selectively accumulate at the tumor site. This case further necessitates the regulation of the production of anti-tumor agents from bacteria, especially for those with common toxicity. The convenience of molecular biology allows us to easily insert a specific promoter sequence upstream of the gene

encoding an anti-tumor agent of interest, so that we can control the gene transcription by adding or removing external inducers in the growth environment of bacteria. Fine tuning regulation of drug production *in vivo* can maximize drug activities and reduce unwanted toxicity to healthy tissues at the correct time and location.

The strategies of gene regulation mainly include three types: extracellular triggering, environmental sensing and self-regulation. L-arabinose and acetylsalicylic acid (ASA) are common non-toxic biological triggers for gene expression under the control of promoters P_{BAD} and P_m, respectively (Loessner et al., 2007; Royo et al., 2007). The divergent promoters (P_{tetA} and P_{tetR}) derived from a repressor-regulated tetracycline efflux system enable bilateral dual gene expression in response to clinically relevant inducer doxycycline (Jiang et al., 2013). Although these inducers can be used conveniently in *in vitro* tests, their limitation of tissue diffusion cannot guarantee the expression of regulated genes in all regions of tumor tissue by colonized *Salmonella*. Radiation can also act as an inducer for certain promoters and is not subject to *in vivo* tissue diffusion limitation. For example, the prokaryotic promoter P_{recA} is capable of timely regulating drug expression with activation upon irradiation, despite the damage of radiation itself to the body. After systemic administration of recombinant *Salmonella* carrying P_{recA}-regulated TRAIL (TNF-related apoptosis-inducing ligand), 2 Gy of γ-irradiation was shown to trigger the expression of TRAIL, and elicited a significant delay in the growth of mammary tumors and a

reduced risk of death (Ganai, Arenas, & Forbes, 2009). *In vivo* regulation of gene expression by the light seems to be safer than by radiation. Ohlendorf et al have established the plasmids P_{Dusk} and P_{Dawn}, which employ blue light photoreceptors to confer light-repressed or light-induced gene expression in *E.coli* with high reactivity to the illumination (Ohlendorf, Vidavski, Eldar, Moffat, & Moglich, 2012). It remains to be tested whether these plasmids are applicable for *in vivo* control of drug production. Zheng and his colleagues have designed a biotic/abiotic hybrid system for light-triggered cytotoxic molecule production by attaching to the surface of *E. coli* bacteria photo-catalytic nanoparticles that, upon light exposure, release photoelectrons to promote the enzymatic reduction of anti-neoplastic NO from endogenous $NO3^{-}$. In a mouse model of breast cancer, these engineered bacteria could accumulate throughout the tumor and resulted in around 80% inhibition of tumor growth after subsequent optically controlled NO generation (Zheng et al., 2018).

The characteristics of tumor tissue that differ from normal tissues, such as hypoxia, low pH and necrosis, can also be utilized for targeted tumor therapy. Theoretically, the expression systems activated by hypoxia or low pH are able to restrict gene expression mainly in tumor tissue. In order to select *Salmonella* promoters that are preferentially activated in the tumor, Arrach et al. inserted a random library of promoter-free *Salmonella* DNA fragments upstream of the green fluorescent protein (GFP) gene and identified two positive clones that were induced

under hypoxic conditions, which contain *pflE* and *ansB* promoter regions, respectively (Arrach, Zhao, Porwollik, Hoffman, & McClelland, 2008). Similarly, Flentie et al. analyzed a library of 7,400 independent *Salmonella* mutants that contained transposon insertions of the promoter-free bacterial luciferase in the genome, and identified five genes specifically activated by the acidic microenvironment associated with cancer cells (Flentie et al., 2012). The *STM1787* promoter was found to be highly activated in *in vitro* low pH conditions and in tumor tissue, and *Salmonella* expressing Shiga toxin from this promoter could provide potent and selective antitumor activity (Flentie et al., 2012). However, it should be noted that the promoters activated in tumors identified by library screening don't guarantee the specificity of activation by hypoxic or low pH, and the effectiveness of these promoters needs to be further tested. Other modified hypoxia-inducible promoters, such as P_{HIP-1} and P_{FF+20*}, have also been tested in animal tumor models for delivery anti-tumor agents (Mengesha et al., 2014; Ryan et al., 2009). The promoters that respond to specific factors in the tumor microenvironment are promising for reducing the expression of regulated genes in non-malignant tissues and thereby relieving health burden of the host. However, gene expression regulated by hypoxic- or low pH-inducible promoters may be silent in some regions of tumor tissue, such as the viable region, due to the lack of specific microenvironment for promoter activation. As a result, some tumor cells will not be treated and continue to proliferate, resulting in tumor recurrence.

The third regulation mode is quorum sensing (QS), which depends on population density and no external inducer (Garg, Manchanda, & Kumar, 2014). In bacterial QS system, bacteria can produce, release and sense small signaling molecules called autoinducers; when bacterial density reaches a threshold level, the regulated genes will be activated, resulting in corresponding transformation of bacterial phenotypes, such as biofilm, virulence factor and bioluminescence. The QS phenomenon was first discovered on the bioluminescent bacteria *Vibrio fischeri* (Nealson, Platt, & Hastings, 1970). The behavior of bioluminescence is regulated by the *lux* operon, of which genes *luxA-E* encode all enzymes and substrates required for luminescence production, and *luxI* and *luxR* act as regulatory genes (Engebrecht & Silverman, 1984). In this QS system, the autoinducer is a small molecule N-acyl-homoserine lactone (AHL), which is synthesized by *luxI*-encoded enzyme LuxI. When bacterial population is at low density, AHL molecules synthesized by a basal level of LuxI rapidly diffuse out and are diluted in the extracellular environment. Following the increase of population density, AHL molecules accumulate intracellularly due to the reduced diffusion gradient across cell membranes (Kaplan & Greenberg, 1985). When the signaling molecules reach a critical threshold concentration, they are bound to the receptor protein LuxR and this complex activates the transcription of *LuxI* and the genes for luminescence from promoter P_{luxI} (**Figure 3-A**). It was hypothesized that the QS system would allow bacteria specifically produce therapeutic cargoes within tumor tissue while a significant decrease of bacterial colonization in normal tissues

would reduce the likelihood of turning on QS switch. This hypothesis was first verified by Forbes and colleagues. They integrated attenuated *Salmonella* with a quorum-sensing system for the expression of GFP reporter and showed that GFP expression was triggered in response to high bacterial density *in vitro* and only in the tumor but not healthy tissues after bacterial infection in tumor-bearing mice (Swofford, Van Dessel, & Forbes, 2015). Kim et al also showed that expression of an anti-tumor protein L-ASNase by attenuated *Salmonella* in a bacterial density-dependent manner yielded significant anticancer effects in mouse model of colon adenocarcinoma (Kim et al., 2018). These results suggest that tumor-targeting bacteria integrated with QS genetic circuits that regulate the production of therapeutic payloads can serve as an ideal drug-delivery system for cancer therapy and obviate the necessity of administration of an exogenous inducer to induce gene expression.

3.2 Bacterial lysis systems

Effective delivery of antitumor drugs by live attenuated *Salmonella* requires not only successful bacterial colonization at the tumor site, but also the sufficient release of drugs after production. Bacteria normally don’t secrete proteins into the extracellular environment except for a few classes of proteins such as exotoxin. Proteins secreted into the periplasm, especially those of low molecular weight, may leak into extracellular environment possibly due to increased permeability of bacterial cell membrane. Now there are a number of methods to promote extracellular secretion

of recombinant proteins from *E. coli*, which are probably feasible for *Salmonella* as well (Choi & Lee, 2004). For example, through fusing the protein of choice to a carrier protein that is normally secreted extracellularly (e.g. hemolysin toxin) or adding the signal sequences, more extracellular secretion of proteins can be achieved (Choi & Lee, 2004). Alternatively, co-expression of a protein that compromises the membrane integrity of bacterial cells or forms a tunnel spanning the inner and outer membranes, can also cause the efflux of cytoplasmic components including expressed proteins. For example, the protein encoded by *E* gene derived from the bacteriophage φX174 can form a transmembrane tunnel, which allows for the release of bacterial cytoplasm into the environment. Recently, Din and colleagues have designed a quorum sensing-regulated and *E* gene-mediated bacterial lysis system called "synchronized lysis circuit" (SLC) for the delivery of therapeutic anti-tumor cargoes (Din et al., 2016). For the regulation of bacterial lysis, this SLC utilizes a promoter mentioned above, P_{luxI} that drives the expression of both the autoinducer AHL (positive feedback) and the bacteriophage lysis gene *E* (negative feedback). The dynamic of bacterial population can be conceptualized as pulsating cycles. With the proliferation of bacteria, the signaling molecules AHL gradually accumulate to the threshold level. Then, the promoter P_{luxI} is activated and drives the transcription of *E*, thereby triggering the lysis of most bacterial cells and release of intracellular contents. A few surviving bacteria reseed the population and start the next cycle (**Figure 3-A**). When a therapeutic cargo is integrated into the SLC strain under the control of P_{luxI} or

a constitutive promoter, it can be effectively released after production due to the periodic lysis of bacteria and thereby exerts its anti-tumor activity (Chowdhury et al., 2019; Din et al., 2016). Since bacterial population is pruned after each lysis event, this paradigm can also prevent uncontrollable growth of bacteria in the tumor or elsewhere and diminish an undesirable host response.

Curtiss et al previously developed an arabinose-regulated delayed lysis system for antigen or DNA vaccine delivery by *Salmonella* and for achieving biological containment (Kong et al., 2008, 2012). This lysis system is dependent on the regulation of *asd* and *murA*, encoding enzymes for synthesis of diaminopimelic acid (DAP) and muramic acid respectively, two essential components of the peptidoglycan. The main genetic engineering is composed of two parts. The first component is *S.* Typhimurium mutant strain, with the deletion of *asd*, regulation of *murA* under the arabinose-activated promoter P_{BAD} and an insertion of P_{BAD}-regulated *c2* gene in the chromosome, which encodes C2 repressor that inhibits the transcription from the promoter P_R. The second one is the plasmid, which contains *murA* and *asd* regulated by P_{BAD} and transcribes antisense mRNA of *asd* and *murA* from promoter P_R. Recombinant *Salmonella* are able to grow normally when cultured *in vitro* with the addition of the inducer arabinose. Since arabinose is not available in host tissues after *Salmonella* infection, the transcription of *asd* and *murA* will gradually cease and the translation of residual mRNA will be blocked by the synthesis of their antisense

mRNA, which eventually lead to bacterial cell death by lysis (**Figure 3-B**). For the delivery and release of antigens expressed by *Salmonella*, antigen gene was inserted into the plasmid under a promoter P_{trc} for expression (W. Kong et al., 2012). To develop this system for DNA vaccine delivery, a plasmid that contains the elements required for the regulated delayed lysis and a eukaryotic promoter for antigen expression by host cells was constructed. Additional genetic modifications were also involved to maximize the uptake or internalization of *Salmonella* bacteria by host antigen-presenting cells (APCs), to enable endosomal escape to release DNA vaccines into the host cell cytosol, and to improve DNA trafficking to the nucleus and resistance to the attack from mammalian nucleases. Unlike the bacterial lysis system regulated by quorum sensing, this one is arabinose-dependent and leads to the programmed lysis of bacteria with no survivors *in vivo*.

3.3 Different routes for Salmonella infection and anti-tumor cargo delivery

In preclinical studies, intravenous (i.v) and intraperitoneal (i.p) routes are usually adopted for *in vivo* infection of genetically engineered attenuated bacteria, which act as monotherapy or a live vector for delivering various anti-tumor drugs (Liang et al., 2019). Although the mechanism of *Salmonella* targeting the tumor is still controversial, the high permeability of tumor blood vessels is generally considered to promote the entry of bacteria into tumor tissue. Tumor vasculature is typically irregular and leaky, and usually exhibits disordered arrangement of endothelial cells.

It is assumed that the openings between these endothelial cells permit access of many macromolecular chemotherapeutics as well as bacteria to tumor tissue. As far as *Salmonella* bacteria are concerned, they may complete tumor colonization via such openings in tumor vasculature, taking advantage of bacterial chemotactic systems and motility (actively) (Kasinskas & Forbes, 2007; Toley & Forbes, 2012), and/or being flushed into tumor tissue by strong blood influx induced by bacterial infection (passively) (Leschner et al., 2009). The studies of *Salmonella* strains A1-R and SL7207 have shown that systemic infection through i.v or i.p route elicits more effective tissue colonization and tumor suppression than oral administration (Crull, Bumann, & Weiss, 2011; Zhang et al., 2012). The virulence of *Salmonella* bacteria can be highly reduced with a safe dose of up to 5×10^7 CFU per mouse, which is at least 100,000 times the LD_{50} of the wild type strain in mouse models, and in clinical trials, the maximum tolerated dose of VNP20009 through 30-minute intravenous bolus infusions is as high as 3×10^8 CFU/ m^2 for patients with metastasis (Toso et al., 2002). However, systemic infection of live *Salmonella* bacteria with the pathogenic nature still has a high risk of toxicity, especially for cancer patients with poor immunity.

Intratumoral (i.t) injection allows higher administration dosage by releasing bacteria directly into tumor tissue. In another clinic trial for testing TAPET-CD, a VNP20009-derived strain that was integrated with cytosine deaminase (CD) gene in

the genome, two of three treated patients showed bacterial colonization in the tumor after i.t injection. Regrettably, this trial was discontinued for nonmedical issues and the therapeutic effects remained unclear (Nemunaitis et al., 2003). Recent studies have also shown that i.t infection exhibits similar anti-tumor therapeutic efficacy and reduced toxic-side effects when compared to i.v administration. Surprisingly, untreated tumor masses could also be targeted by *Salmonella* bacteria following i.t infection (Kocijancic, Felgner, et al., 2017). However, the i.t route for *Salmonella* infection is not applicable to cancer patients with hard-to-reach tumors or highly dispersed metastasis.

The noninvasive oral route for administration of live attenuated bacteria offers the advantages of safety and better patient compliance. Saltzman et al have developed an attenuated *S.* Typhimurium strain carrying the human gene for interleukin-2 (renamed *Salmonella*-IL2 or Saltikva) (Saltzman et al., 1996). When *Salmonella*-IL2 was administered orally to cancer patients in Phase I trial, no toxicity or adverse events were observed, although there was no survival advantage (Gniadek et al., 2020; Batist et al., 2020). Given the ability of *Salmonella* to infect gut-associated and internal lymphoid tissues after oral administration, *Salmonella* has been widely exploited as a delivery system for the antigens of cancer or infectious diseases, both as bacterially expressed proteins and eukaryotic DNA plasmids (Parsa & Pfeifer, 2007). As a facultative intracellular bacterium, *Salmonella* can be taken up by

phagocytic cells including professional APCs, such as macrophage and dendritic cells, improve intracellular delivery of chosen antigens for effective antigen processing and induce co-stimulatory molecule production through their pathogen-associated molecular patterns (PAMPs) (Parsa & Pfeifer, 2007). Oral immunization of tumor-associated antigens delivered by attenuated *Salmonella* bacteria is promising for stimulating tumor-specific immune responses by breaking host immune tolerance, and thereby protecting the host from the challenge of tumor cells or inhibiting the growth of established tumors. For example, Oral administration of *S.* Typhimurium strain engineered to deliver tumor antigen NY-ESO-1 through type III secretion system could regress established NY-ESO-1-expressing tumors in mice (Nishikawa et al., 2006). The DNA vaccine of VEGFR-2 (also known as flk-1) orally delivered by *S.* Typhimurium was shown to elicit antigen-specific cytotoxic T cell immunity and suppression of tumor angiogenesis, leading to effective inhibition of tumor growth and metastasis in animal tumor models (Niethammer et al., 2002; Wieckowski et al., 2017). Moreover, an analogous human-specific vaccine using *Salmonella* Typhi Ty21a as the delivery vector has been tested in clinical trials, showing no dose-limiting toxicities and a significant reduction of tumor perfusion in vaccinated patients accompanied by increased levels of serum biomarkers indicative of the anti-angiogenic activity (Schmitz-Winnenthal et al., 2015).

Besides, many means for attenuation of *Salmonella* may diminish its ability to tolerate hostile stresses encountered in the gastrointestinal tract after oral immunization, or impair the ability to invade into and survive in gut-associated and internal lymphoid tissues, collectively leading to inadequate induction of desired protective immunity. To achieve a balance between attenuation of the virulence and retention of bacterial invasion and colonization, a strategy called "regulated delayed attenuation" has been developed (Curtiss et al., 2009, 2010). The virulence-associated genes are placed under the control of tightly regulated P_{BAD} promoter for genetically engineering bacterial strains. Resultant strains can normally express regulated genes *in vitro* in the presence of the inducer arabinose, and gene expression will gradually be turned off with the consumption and no supply of arabinose *in vivo*. Thus, Bacteria have the characteristics similar to the wild-type strain at the time of oral inoculation, and then, after colonization of lymphoid tissues, gradually lose virulence traits due to cell division (Curtiss et al., 2009; Li et al., 2009). Another approach is to coat attenuated bacteria with synthetic nanoparticles self-assembled from cationic polymers (Hu et al., 2015). Owing to the electronegative nature of bacterial cell wall, the positively charged nanoparticles can readily attach to *Salmonella* surface via electrostatic interaction and form a dense coating layer with protective effects. The surface coating is able to enhance acid tolerance of *Salmonella* bacteria in stomach and intestines, and aid in phagosomal escape after bacteria are captured by phagocytes. As a result, engineered *Salmonella* bacteria coated with cationic

nanoparticles disseminate into blood with higher concentration of viable bacteria after oral administration, allowing more bacteria to colonize lymphoid tissues (Hu et al., 2015).

Bacterial derivatives for cancer therapy

The use of live bacteria for cancer therapy is usually associated with safety concerns. An alternative approach for therapeutic cargo delivery is to use non-living bacterial derivatives, such as bacterial ghosts, minicells and outer membrane vesicles. These nano-scale vesicles are able to preferentially accumulate in tumor tissue with the leaky vasculature and inadequate lymphatic drainage through the enhanced permeability and retention (EPR) effect (Matsumura & Maeda, 1986). Moreover, the display of targeting moieties on the surface through *in vitro* attachment or genetic modification of parental bacterial cells provides these vesicles as delivery vectors with the active mechanism of targeting tumor cells, thereby eliciting less systemic toxicity and better therapeutic efficacy.

4.1 Ghosts

Bacterial ghosts (BGs) are essentially empty cell envelopes of Gram-negative bacteria and generally produced after the removal of cytoplasmic contents by the expression of cloned lysis gene *E* of bacteriophage φX174 (**Figure 4-A**). The small-sized polypeptide encoded by *E* gene actually is a membrane protein capable of oligomerizing into a transmembrane tunnel that spans the inner and outer membrane.

After the tunnel structure forms, bacterial cytoplasm is released into the extracellular environment, leaving behind cell envelopes known as BGs (Witte & Lubitz, 1989; Witte et al., 1990). Since this process does not cause any denaturation to envelope structures, one of the main characteristics of BGs is the preservation of surface immunostimulatory elements, such as LPS, peptidoglycan and flagella, showing a potential for BGs used as a candidate vaccine against respective pathogens (Hoseini Shahidi, Hashemi Tabar, Bassami, Jamshidi, & Dehghani, 2019; Vinod et al., 2017). Moreover, bacterial ghosts with the perfect adjuvant activity and a high loading capacity can serve as a carrier for delivery of heterologous antigens and DNA vaccines (Hajam, Dar, Won, & Lee, 2017). Due to the particular nature of BGs and the components of many PAMPs including LPS and flagella, BGs are able to be effectively recognized and taken up by APCs and subsequently evoke specific immune responses against delivered antigens. Using recombinant DNA technology, foreign antigen proteins can be integrated into bacterial cell envelopes including outer membrane, periplasm and inner membrane, and still keep preserved in BGs after protein E-mediated bacterial lysis (Hur & Lee, 2015). For the delivery of DNA vaccines as well as other anti-tumor compounds of interest, BGs with the intracellular lumen have an outstanding loading capacity. Up to 6,000 midsize plasmid copies per BG can be loaded through resuspension of lyophilized BGs in the solution of DNA plasmids and additional washing steps to remove unbound plasmids. Moreover, it was shown that BGs could deliver DNA vaccines to APCs effectively, increase the

transfection efficiency of antigen genes and induce more potent immune responses than naked DNA (Wen et al., 2012).

4.2 Minicells

Bacteria-derived minicells were first observed and described by Howard Adler and his colleagues in 1967 (Adler, Fisher, Cohen, & Hardigree, 1967). They are anucleate, non-living and nano-sized cells (approximately 400 nm in diameter), and are generally produced through derepressing the polar sites of cell fission via genetic mutations in genes that control the normal division of bacterial cells, such as *minCDE* (Lutkenhaus, 2007). Like the parent bacterial cells, minicells contain membranes, peptidoglycans, ribosomes, RNA, proteins, and usually plasmids, except for the genome (Farley, Hu, Margolin, & Liu, 2016). Minicells derived from *Salmonella* or other bacteria have attracted the attention as a novel drug delivery system for cancer therapy (**Figure 4-B**). Bacterial minicells can be loaded with different kinds of anti-tumor molecules including chemotherapeutics and/or functional nucleic acids (e.g. siRNAs) in high concentrations, thereby increasing the flexibility of drug delivery and potential therapeutic effects. After intravenous administration, minicells take advantage of the EPR effect to accumulate inside tumor tissue (passively), and target tumor cells in tumor microenvironment via bispecific antibodies (BsAbs) attached on the surface of minicells (actively). In general, one arm of BsAbs binds to the O-antigen component on minicell surface and another one is specific for certain

receptors or other proteins (e.g. EGFR) on tumor cell surface (MacDiarmid & Brahmbhatt, 2011). Folic acid is also a cost-effective choice for minicell surface modification, which conjugates easily with variety of biomaterials including minicells and targets folate receptors overexpressed in majority of malignancies (Jivrajani & Nivsarkar, 2016). Protein display technology can also endow minicells with the specific targeting ability, with an example of *E. coli* Nissle 1917 (EcN)-derived minicells displaying a low-pH insertion peptide, which are able to selectively target the acidic microenvironments and thereby deliver packaged chemotherapeutic drugs to in the necrotic and hypoxic regions of tumor tissue (Zhang et al., 2018). After extravasation from the defective tumor vasculature into the tissue, minicells modified with BsAbs can selectively bind to tumor cell surface via the receptor-specific antibody and are then internalized through membrane receptor-mediated endocytosis. Thereafter, the functional cargoes are released following intracellular degradation of minicells (MacDiarmid et al., 2007). In addition, minicells are able to maintain other cellular activities, including ATP synthesis, transcription of plasmid DNA and translation of mRNA. It was shown that minicells could inject proteins directly into targeted cells via *Salmonella* T3SS system (Carleton, Lara-Tejero, Liu, & Galan, 2013).

The studies of MacDiarmid et al revealed that, with intravenous administration of BsAb-modified minicells carrying doxorubicin or paclitaxel, tumor growth

inhibition was more significant than with the treatment of 1875-fold and 8000-fold higher amounts of free chemotherapeutics, proving the high delivery efficiency of minicells in animal tumor models (MacDiarmid et al., 2007). It was also shown that repeated i.v administration of minicells only stimulated weak immune responses against LPS, one dominant surface antigen, and did not result in immune exclusion of subsequent doses. Dual sequential treatments with tumor-targeting minicells packaged with siRNA designed for knocking down a multidrug resistance protein followed by cytotoxic drug-loaded minicells could eliminate previously drug-resistant tumors and enable complete survival without adverse toxicity in mice (MacDiarmid et al., 2009). In the first-in-man clinical trial, BsAb-conjugated minicells packaged with the chemotherapeutic paclitaxel, have shown the safety and modest clinical efficacy (Solomon et al., 2015).

4.3 OMVs

Bacterial outer membrane vesicles (OMVs), are nano-scale (50~250 nm) spherical bilayered proteoliposomes naturally produced from nearly all Gram-negative bacteria through budding from the outer membrane, and consist of phospholipids, outer membrane proteins (OMPs), LPS and periplasm constituents (Beveridge, 1999). In general, OMVs seem to be constitutively released by Gram-negative bacteria during different growth phases and enhanced OMVs production can be induced by addition of specific signals, such as temperature stress,

amino acid limitation and antibiotics (van der Pol, Stork, & van der Ley, 2015). Non-living OMVs are considered safe compared with live attenuated bacteria and gain extensive interests as vaccines or delivery vehicles. In fact, one OMV-based vaccine "Bexsero" is being clinically used as meningococcal group B vaccine for individuals from the age of two months, indicating that the methods of safety and quality control have already been demonstrated for human use of OMVs (Acevedo et al., 2014). There have been some attempts to use bacterial OMVs for cancer immunotherapy. It was shown that bacterial OMV monotherapy was highly effective for less destructive tumors, such as CT26 and MC38 adenocarcinoma, with interferon-γ dependent antitumor effects (Kim, Park, et al., 2017). Recently, Grandi et al demonstrated that immunization with OMVs decorated with the B cell epitope provided mice with strong protection from tumor challenge and that 100% protection was achieved with OMVs carrying both B cell and $CD4^+$ T cell epitopes, two antigenic epitopes present in B16F10EGFRvⅢ tumor cells(Grandi et al., 2017).

In addition, OMVs of bacterial origin can be genetically modified to load therapeutic proteins or to display targeting ligands and easily isolated by ultracentrifugation, obviating the need for *in vitro* package or surface attachment. For example, genetic fusion to the C-terminus of ClyA enables exogenous proteins to be effectively transported to OMVs (Kim et al., 2008). In this way, the display of a human epidermal growth factor receptor 2 (HER2)-specific affibody on OMV surface

was achieved and functional siRNA molecules delivered by these tumor-targeting OMVs were shown to cause the silencing of the target gene and significant regression of tumor growth in mice bearing HER2-overexpressed tumors (Gujrati et al., 2014). Moreover, OMVs can be bioengineered to target various tumors with different surface markers and thereby become attractive vehicles for targeted delivery of functional proteins, chemotherapeutics and nucleic acids to the tumor (**Figure 4-C**).

4.4 Protoplasts

By definition, bacterial protoplasts are cells with their walls removed. Most of bacterial toxins located on the outer wall can be removed by various methods to form less toxic protoplasts, such as the use of enzymes to degrade membrane components or genetic engineering of the parent strain to prevent cell wall synthesis (Kim et al., 2015; Lederberg, 1956). Moreover, being of bacterial origin, protoplasts can also genetically modified to express tumor-targeting moieties on the surface of the inner membrane and act as a universal carrier for various anti-tumor molecules of interest (**Figure 4-D**). Kim et al generated bacterial protoplast derived nanovesicles (PDNVs) displaying epidermal growth factor (EGF) as the targeting moiety to tumor cells expressing the EGF receptor on the surface, through engineering parental bacterial cells to express EGF fused with a inner membrane lipoprotein and treating bacterial cells with lysozyme to form the protoplasts. When these EGF-displaying PDNVs were loaded with the chemotherapeutic doxorubicin or idarubicin and injected

intravenously to tumor-bearing mice, packaged drugs could accumulate in tumor tissue and effectively inhibit tumor growth without notable adverse effects (Kim, Dinh, et al., 2017). So far, there has not been much information regarding the applicability of bacterial protoplasts in cancer treatment.

Conclusions and future perspective

The potential of *Salmonella* bacteria for tumor therapy has been observed in preclinical studies except in human clinical trials. Some problems still remain to be solved before the clinical application of *Salmonella* bacteria for cancer treatment. The use of live bacteria always raises safety concerns. Due to the pathogenic nature of *Salmonella*, attenuation is essential to reduce toxic-side effects caused by bacterial infection on the host. However, the anti-tumor effectiveness of *Salmonella* seems to be largely related to bacterial pathogenesis or virulence as well. Excessive attenuation will also impair bacterial colonization of tissues and *in vivo* anti-tumor effects including the induction of tumor cell apoptosis and reverse of the immunosuppression in the tumor microenvironment. Therefore, it is desirable to achieve a balance between attenuation of the virulence and retention of the colonization ability. With the development of bioengineering technology, it is now easier to construct strains with different genetic mutations, or screen strains with different phenotypes using high-throughput methods. Bacterial strains with auxotrophic mutations are unable to synthesize specific factors needed for growth, and thus have reduced fitness in normal

tissues but unchanged adaptability in tumor tissue. Alternatively, engineering the bacteria to display small-sized targeting moieties on the outer membrane surface, such as RGD peptides and antibody fragments (e.g. scFv and nanobody), may also improve the tumor targeting and safety of bacteria. In addition, it is possible to enhance bacterial immunostimulaty activity while reduce the endotoxic activity through optimizing the structure of lipid A.

The reduction in the antitumor efficacy due to attenuation of bacterial virulence can be compensated by additionally equipping bacteria with therapeutic payloads. Attenuated *Salmonella* can be utilized as live vectors to deliver therapeutic molecules of different characteristics and thereby exert superior anti-tumor effectiveness compared to the monotherapy with bacteria or free drugs. In this case, the administration dosage of engineered bacteria can be reduced appropriately to minimize the toxicity to the host. We should also realize that only *Salmonella* and/or one single anti-tumor drug may be not sufficient for complete tumor suppression. The simultaneous delivery of multiple therapeutic agents by bacteria vectors for targeting different tumor-promoting signaling pathways or killing both tumor cells and their supporting cells (e.g. proliferating tumor vascular endothelial cells), makes it possible to overcome the development of drug resistance and thereby to eliminate tumors. Bacteria can also be coupled to micro- or nano-materials of different properties, such as drug-loaded, photo-catalytic and/or magnetic-sensing nanoparticles, taking

advantage of the net negative charge on the surface of most bacteria (Park et al., 2017; Zheng et al., 2018). In addition, bacteria-mediated cancer therapy combined with other treatments that have been clinically proven effective but with certain shortcomings, including chemotherapy, radiotherapy and immunotherapy, may synergistically produce surprising therapeutic effects (Liang et al., 2019). This strategy has attracted more attentions recently. For example, *Salmonella* bacterial immunotherapy combined with a low dose of the chemotherapeutic agent doxorubicin effectively delayed tumor growth and was less toxic than chemotherapy at the maximum tolerated dose chemotherapy in an autochthonous murine model of breast cancer (Saltzman, Augustin, Leonard, Mertensotto, & Schottel, 2018).

The routes of *Salmonella* administration also affect the overall anti-tumor efficacy as well as the safety. Preclinical studies have shown that systemic (through intravenous or intraperitoneal injection) and direct intratumoral route elicits more effective tissue colonization and tumor suppression than oral administration. The noninvasive oral route of administration offers the advantages of safety, which is more applicable for clinical cancer patients. As facultative intracellular bacteria, *Salmonella* are able to deliver expressed antigens or carried DNA vaccines to APCs, stimulate the host to produce antigen-specific immune responses and are considered as candidate oral vaccine delivery vectors (Parsa & Pfeifer, 2007). Cancer immunotherapy that uses orally administrated *Salmonella* to deliver tumor-associated antigens has made

some progress, with candidate vaccines already tested in clinical trials (Niethammer et al., 2002; Nishikawa et al., 2006; Schmitz-Winnenthal et al., 2015; Wieckowski et al., 2017). To improve the efficacy and safety of vaccines orally delivered by *Salmonella*, bacterial regulated delayed attenuation and delayed lysis systems have been developed (Curtiss et al., 2010). The delayed attention system, which places bacterial virulence-associated genes under the control of tightly regulated P_{BAD} promoter, is to ensure that *Salmonella* can tolerate the pressure encountered in the gastrointestinal tract after oral administration and successfully colonize lymphoid tissues to deliver the antigens to APCs (Curtiss et al., 2009; Li et al., 2009). Coating attenuated bacteria with synthetic nanoparticles self-assembled from cationic polymers could also enhance acid tolerance of *Salmonella* bacteria in stomach and intestines, and aid in phagosomal escape after bacteria are captured by phagocytes, allowing more bacteria to colonize lymphoid tissues (Hu et al., 2015). The delayed lysis system is designed for programmed death of all bacterial cells administrated into the host, so as to achieve more effective antigen release and delivery as well as to confer biological containment (Kong et al., 2008, 2012). Systemic infection of *Salmonella* through the intravenous or intraperitoneal route is usually chosen for bacterial monotherapy or delivery of various anti-tumor drugs and causes higher efficiency of tumor colonization than oral administration. However, infection of live bacteria through systemic routes has a risk of toxicity. Especially for the delivery of anti-tumor agents with unspecific toxicity, the damage to normal tissues cannot be ruled out, despite that

Salmonella bacteria can be genetically engineered with high tumor targeting. It is desirable that the production and action of chosen drugs can be restricted to the tumor site but not healthy tissues, thereby improving the safety during cancer treatment. In recent years, many regulation systems have been designed for the purposeful control of antitumor drug delivery by live bacterial vectors. The promoters that are specifically activated in the tumor microenvironment or by external triggers without tissue diffusion limitation can be utilized to minimize gene expression in non-target tissues, thereby reducing unwanted toxicity to these tissues. The quorum sensing regulated system, which actives gene expression when the bacterial density reaches a certain threshold level, is also promising for targeted drug delivery by *Salmonella* bacteria, considering the fact that engineered *Salmonella* are able to accumulate in the tumor with a bacterial density 1,000 times higher than that in normal tissues. Moreover, when bacteria are integrated with a lysis gene under the control of QS switch, bacteria will exhibit a phenotype of cyclical lysis dependent on bacterial density. Hence, bacterial expressed anti-tumor molecules or carried DNA plasmids can be effectively released without the need of additional engineering for secretion and uncontrollable bacterial proliferation is avoided.

Nanovesicle structures derived from *Salmonella* and other bacteria can also be used as biocompatible nanocarriers for the delivery of functional therapeutic molecules in cancer therapy. These non-living nanovesicles have the significant

advantage of safety compared to live bacteria. Especially, bacterial ghost, minicells and OMVs contain outer membrane immunostimulatory components of the parent strain, including LPS, flagella and outer membrane proteins. The good adjuvant properties make them useful for delivering foreign antigens in immunotherapy of cancer or infectious diseases. In addition, these nanovesicles can be loaded with chemotherapeutic drugs, functional proteins and nucleic acids, through *in vitro* coincubation or electroporation. Moreover, through genetically engineering parental bacterial cells, the nanovesicles derived from them will have similar characteristics, eliminating the need for additional treatment. For example, the bacterial strain displaying targeting ligands on the surface is generated to obtain nanovesicles with higher targeting to cancer cells. After systemic injection, these nanovesicles protect the drugs from being quickly cleared and take advantage of the EPR effect of tumor tissue to achieve targeted drug delivery. In the tumor microenvironment, surface-modified nanovesicles specifically adhere to tumor cells and are internalized through receptor-mediated endocytosis, followed by being destroyed to release packaged drugs. Tumor-associated antigens can also be expressed by parental bacterial cells at different sites including the outer membrane, periplasm and inner membrane, so that derived nanovesicles also contain these antigens useful for cancer immunotherapy.

Salmonella-mediated cancer therapies have been implemented in a small number of human trials with little success (Toso et al., 2002; Nemunaitis et al., 2003; Gniadek et al., 2020; Batist et al., 2020). In preclinical studies showing promising results regarding the anti-tumor effects of genetically engineered *Salmonella*, the xenograft model which involves the implantation of cultured tumor cell lines into immuno-deficient mice has often been used (Ruggeri, Camp, & Miknyoczki, 2014). However, this model is not considered to reflect the diversity and complexity of tumors at present. There are some differences between tumors from this type of xenograft models and human patients, 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 tumors. Therefore, animal tumor models that can adequately mimic tumors in clinical cancer patients should be considered and applied in future preclinical tests. Patient-derived xenograft (PDX) model, which involves the implantation of patient-derived tumor tissue into immune-deficient mice, is expected to resolve this issue (Goto, 2020; Tentler et al., 2012). Compared with conventional preclinical tumor models involving the implantation of cancer cell lines into mice, PDX models preserve tumor heterogeneity and tumor microenvironment including stroma and vasculature and more closely resemble tumors in patients. Such a tumor model is promising to improve the predictability of clinical therapeutic responses as well as to screen out drugs or agents with real anti-tumor activities including genetically modified *Salmonella*.

For the clinical application of bacteria-mediated cancer therapy, it is necessary to further improve our knowledge on bacterial toxicity, colonization efficiency, and the efficacy and mechanisms of tumor suppression upon different infection routes. Future studies should build animal models that mimic human tumors better, test more attenuated bacterial strains, and screen more drugs or functional molecules that have anti-tumor activities.

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Table 1: Auxotrophic *Salmonella* bacterial strains used for cancer therapy and mutations involved

Strains	**Gene mutation**	**Gene functions**	**References**
VNP20009, TAPET-CD, MVP728	*pur I*$^{-}$, *purD*$^{-}$	Adenine synthesis	(Clairmont et al., 2000; Mesa-Pereira, Medina, Camacho, Flores, & Santero, 2015; Nemunaitis et al., 2003; Toso et al., 2002; Xiong et al., 2010)
A1-R	*leuC/D*$^{-}$, *argB*$^{-}$	Leucine and arginine synthesis	(Hoffman, 2011; Zhao et al., 2005, 2006)
SL3261, SL7207, BRD509	*aroA*$^{-}$, *aroD*$^{-}$	Aromatic amino acid synthesis	(Berger et al., 2013; Hoiseth & Stocker, 1981; Liang et al., 2018; Meng et al., 2010; Sebastian et al., 2016; Yoon et al., 2017)
Y1-B	*asd*::P*pepT asd*	DAP synthesis	(Yu et al., 2012)

Table 2: Candidate promoters for *in vivo* regulation of gene expression and targeted cancer therapy

Promoters	**Inducers**	**References**
Extracellular triggering		
P_{BAD}	L-arabinose	(Loessner et al., 2007)
P_m	Acetylsalicylic acid	(Royo et al., 2007)
P_{tetA}/P_{tetR}	Doxycycline or tetracycline	(Jiang et al., 2013)
PrecA	Radiation	(Ganai et al., 2009)
P_{Dawn}	blue light	(Ohlendorf et al., 2012)
Environmental sensing		
Salmonella STM1787 promoter	Low pH	(Flentie et al., 2012)
P_{HIP-1}	Hypoxia	(Mengesha et al., 2014)
P_{FF+20*}	Hypoxia	(Ryan et al., 2009)
Self-regulation		
P_{luxI}	AHL (auto-inducer)	(Swofford et al., 2015; Kim et al., 2018; Din et al., 2016; Chowdhury et al., 2019)

Figure legends

Figure 1. Schematic representation of small-sized antibody fragments scFv and nanobody. Conventional antibodies or immunoglobulins are composed of two heavy chains and two light chains. The variable domain of the antibody is composed of non-covalently associated variable regions of the heavy chain (VH) and light chain (VL). VH and VL (inside the green oval) can be genetically fused into a single-chain variable fragment (scFv) via a short linker peptide. scFv with a molecular weight of 25~30 kDa usually retains the specificity of the original antibody, despite the removal of the constant regions and the introduction of the linker peptide. Camelid heavy chain antibodies (hcAbs) are only composed of two heavy chains that have lost one of their constant domains (CH1). A single monomeric variable domain (VHH) of one hcAb is called single-domain antibody (sdAb) or nanobody, with a low molecular weight of only 15 kDa. Nanobodies have been shown to be as specific as a regular antibody or even more robust in some cases.

Figure 1

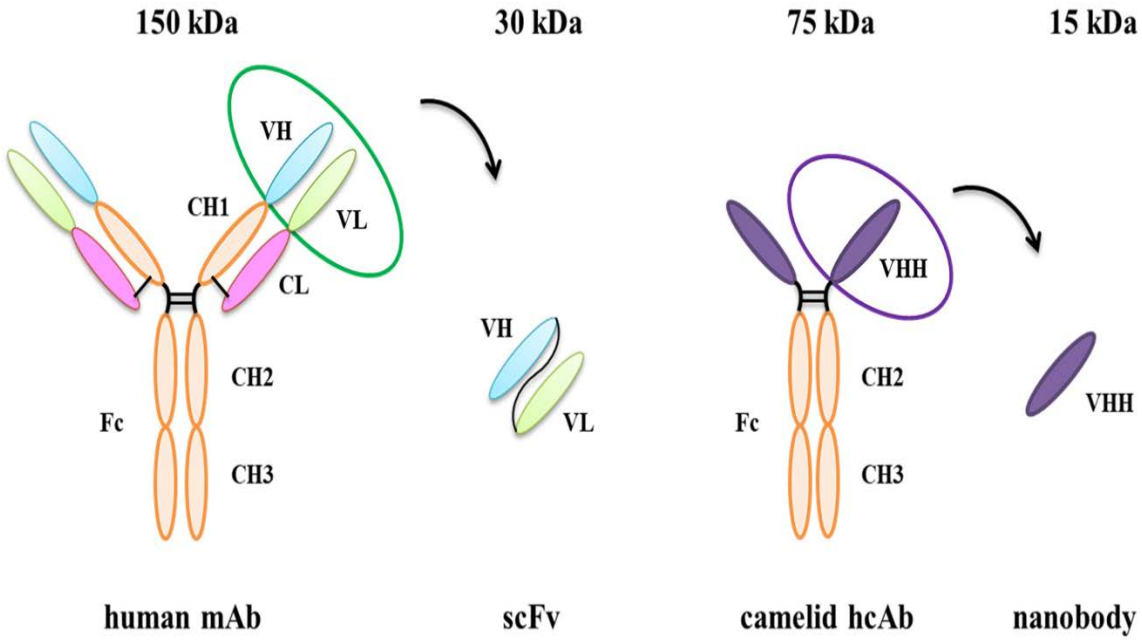
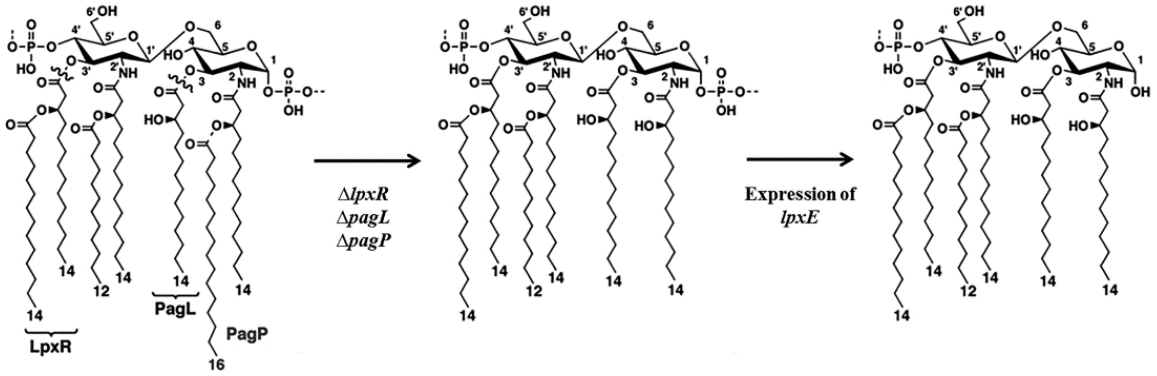


Figure 2. Lipid A structures of wild type *Salmonella* bacteria and Δ*pagL*Δ*pagP* Δ*lpxR* mutants with or without *lpxE* expression. Wild type *Salmonella* are able to modify the lipid A structure and synthesize a heterogeneous mixture of lipid A, which are predominantly hexa- and hepta-acylated (left). LpxR and PagL catalyze the removal of the 3'-acyloxyacyl and the 3-hydroxymyristoyl chains from lipid A, respectively. PagP is responsible for the addition of palmitate to the 2 position R-3-hydroxymyristoyl chain. The mutation of the three genes *lpxR*, *pagL* and *pagP* disables these modifications involving acyl chains, resulting in the synthesis of homogenous hexa-acylated lipid A in *Salmonella* (middle). Heterologous expression of *lpxE* dererived from *Francisella tularensis* leads to nearly quantitative dephosphorylation of lipid A at the 1-position (right).

Figure 2

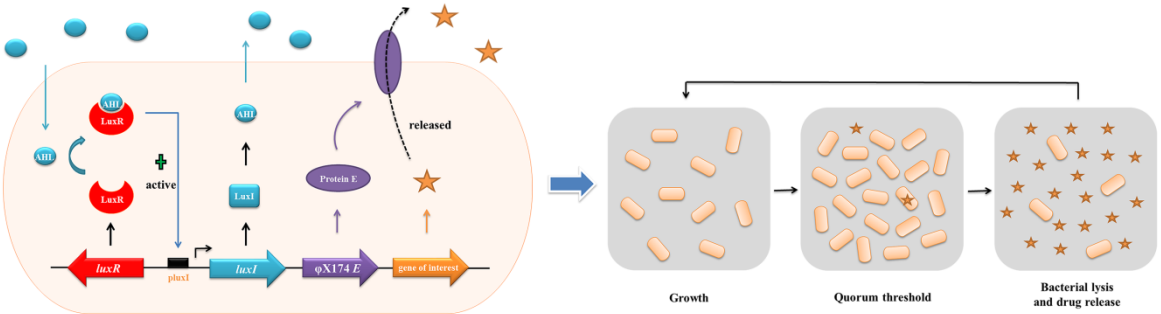


Lipid A of WT *Salmonella* **Hexa-acylated lipid A** **Hexa-acylated MPL**

Figure 3. The mechanisms of two bacterial lysis systems used for effective delivery of anti-tumor cargoes. A. The quorum-sensing lysis system uses the promoter P_{luxI} to regulate the expression of both the autoinducer AHL (positive feedback) and the bacteriophage lysis gene *E* (negative feedback). When bacterial density reaches a critical threshold level, P_{luxI} is activated by LuxR combined with AHL and drives the transcription of downstream genes including *luxI* and *E*, resulting in the production of more AHL and the lysis of most bacterial cells. Therapeutic cargoes integrated into the strain under the control of P_{luxI} or a constitutive promoter can thus be effectively released after production. A small number of surviving bacteria then reseed the population and produce AHL anew, allowing the process of bacterial proliferation and lysis to be repeated cyclically. B. The arabinose-dependent delayed lysis system is designed for antigen delivery after programmed bacterial lysis with no survivors. In the presence of arabinose, plasmid-encoded *asd* and *murA* and genome-encoded *murA* and *c2* are transcribed from their respective P_{BAD} promoters, which allows for bacterial growth with the synthesis of DAP and muramic acid and repression of the P_R promoter by C2. In the absence of arabinose, these P_{BAD} promoters will no longer be active. The concentrations of Asd, MurA and C2 decrease because of no further synthesis and cell division. The synthesis of DAP and muramic acid is reduced, and the P_R promoter is derepressed and drives the synthesis of antisense mRNA to blocks translation of residual mRNA of *asd* and *murA*. The peptidoglycan layer of the cell wall cannot be properly synthesized, eventually leading to bacterial cell death by lysis.

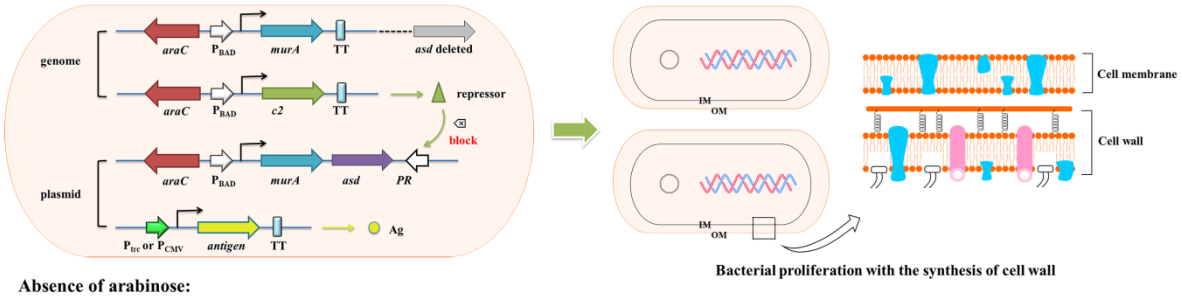
Figure 3

A. Bacterial lysis system regulated by quorum sensing



B. Bacterial delayed lysis system dependent on arabinose

Presence of arabinose:



Absence of arabinose:

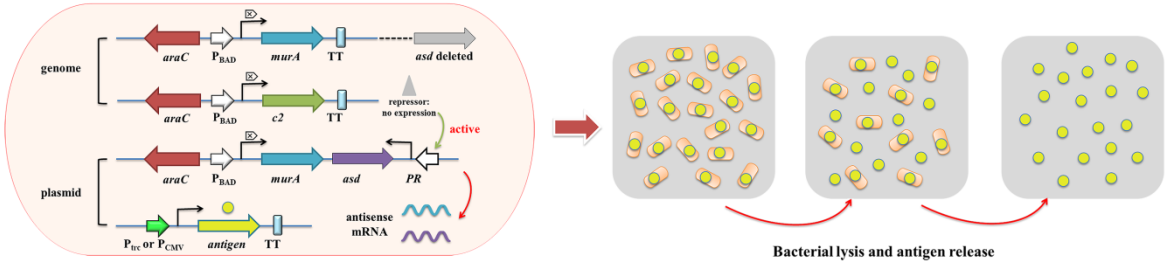


Figure 4. Bacterial derivatives used as delivery vehicles of anti-tumor cargoes. A. Bacterial ghosts are empty cell shells formed by overexpressing the bacteriophage *E* gene. Since the preservation of the outer membrane components of parent bacterial cells, bacterial ghosts have good adjuvant activity to attract immune cells and act as delivery vectors for foreign antigens or DNA vaccines. B. Bacterial minicells are usually produced by the mutation of chromosomal *minCDE*. Minicells attached with bispecific antibodies possess an active tumor-targeting mechanism and can be used to deliver therapeutic drugs to tumor cells. C. OMVs are naturally released from the outer membrane of Gram-negative bacteria. OMVs are able to display tumor-targeting moieties on their surface by genetic modification of the parent bacteria and then used for targeted delivery of anti-tumor drugs or other functional molecules. D. Bacterial protoplasts are cells with their cell wall removed. Protoplast-derived nanovesicles can be obtained from bacteria that are genetically engineered to express tumor-targeting moieties on the plasma membrane, which allows for the use of protoplasts for targeted drug delivery in cancer treatment.