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Advances in *Escherichia coli* Nissle 1917 as a customizable drug delivery system for disease treatment and diagnosis strategies

Haojie Chen [a,1], Pengyu Lei [a,1], Hao Ji [a,1], Qinsi Yang [b], Bo Peng [b], Jiahui Ma [a], Yimeng Fang [a], Linkai Qu [a,c], Hua Li [d], Wei Wu [d,***], Libo Jin [a,e,**], Da Sun [a,f,*]

[a] Institute of Life Sciences & Biomedical Collaborative Innovation Center of Zhejiang Province, Wenzhou University, Wenzhou, 325035, China
[b] Wenzhou Institute, University of Chinese Academy of Sciences, Wenzhou, 325000, China
[c] College of Life Sciences, Jilin Agricultural University, Changchun, 130118, China
[d] Key Laboratory for Biorheological Science and Technology of Ministry of Education, State and Local Joint Engineering Laboratory for Vascular Implants, Bioengineering College of Chongqing University, Chongqing, 400044, China
[e] Wenzhou City and WenZhouOuTai Medical Laboratory Co.,Ltd Joint Doctoral Innovation Station, Wenzhou Association for Science and Technology, Wenzhou, 325000, China
[f] Wenzhou City and Kunlong Technology Co., Ltd., Joint Doctoral Innovation Station, Wenzhou Association for Science and Technology, Wenzhou, 325000, China

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ABSTRACT

With the in-depth and comprehensive study of bacteria and their related ecosystems in the human body, bacterial-based drug delivery system has become an emerging biomimetic platform that can retain the innate biological functions. Benefiting from its good biocompatibility and ideal targeting ability as a biological carrier, *Escherichia coli* Nissle 1917 (ECN) has been focused on the treatment strategies of inflammatory bowel disease and tumor. The advantage of a bacterial carrier is that it can express exogenous protein while also acting as a natural capsule by releasing drug slowly as a result of its own colonization impact. In order to survive in harsh environments such as the digestive tract and tumor microenvironment, ECN can be modified or genetically engineered to enhance its function and host adaptability. The adoption of ECN carries or expresses drugs which are essential for accurate diagnosis and treatment. This review briefly describes the properties of ECN, the relationship between ECN and inflammation and tumor, and the strategy of using surface modification and genetic engineering to modify ECN as a delivery carrier for disease treatment.

1. Introduction

Nano drug delivery systems are used in order to achieve targeted therapeutic effects in various diseases (such as tumors, inflammation, etc.), enhance efficacy, and reduce the systemic toxic and side effects of drug [1–3]. To achieve good drug adaptability *in vivo*, in addition to chemical materials, natural bionic delivery systems similar to cell membranes or bacteria have also been developed [4–6]. In contrast to ligand targeting of cell membrane and avoiding immune response *in vivo*, the bacterial delivery system provides additional functionality through drugs or modification, provided that bacteria maintain their original functions (for example, inhibit cancer by competing with tumor cells) [7–9]. Human diseases are closely related to bacteria. Since the development of chemical biotechnology and genetic engineering technology, bacteria-based drug delivery systems for diagnosis and treatment have become a hot topic in biomedical research. Taking advantage of these characteristics, the first tumor immunotherapy with bacterial therapy appeared in 1890, which treated osteosarcoma by injecting *Streptococcus* into patients [10]. Many subsequent studies have demonstrated that many types of bacteria, including *Escherichia coli* (*E. coli*), *Salmonella typhimurium*, and *Clostridium Novi* can colonize tumor lesions [11].

* Corresponding author. Institute of Life Sciences & Biomedical Collaborative Innovation Center of Zhejiang Province, Wenzhou University, Wenzhou, 325035, China.
** Corresponding author. Institute of Life Sciences & Biomedical Collaborative Innovation Center of Zhejiang Province, Wenzhou University, Wenzhou, 325035, China.
*** Corresponding author. Key Laboratory for Biorheological Science and Technology of Ministry of Education, State and Local Joint Engineering Laboratory for Vascular Implants, Bioengineering College of Chongqing University, Chongqing, 400044, China.
E-mail addresses: david2015@cqu.edu.cn (W. Wu), 20160121@wzu.edu.cn (L. Jin), sunday@wzu.edu.cn (D. Sun).
[1] These authors contributed equally to this work.

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Abbreviations:

Escherichia coli Nissle 1917 ECN
inflammatory bowel disease IBD
irritable bowel syndrome IBS
tight junction TJ
reticuloendothelial system RES
polylactic acid-glycolic acid PLGA
tannic acid TA
polydopamine PDA
poly (ethylene glycol) polylactide PELA
yeast membrane YM
microbacterin Mc
Staphylococcus aureusahemolysin SAH
insulin-like growth factor-1 IGF-1
induced nitric oxide synthase INOS
3-hydroxybutyrate 3HB
butyryl CoA dehydrogenase BCD
butyryl CoA transfer BUT
extracellular vesicles EVs
pH(low) insert the peptide PHLIP
Epothilone B Epo B
zoledronic acid ZOL
intestinal epithelial cells IECs
β-D-1-Thiogalactopyranoside IPTG
superparamagnetic iron oxide nanoparticles SPION
hyaluronic acid-poly (propylene sulfide) HA-PPS
poly-norepinephrine NE
liposomal dioleoyl phosphate DOPA
lipid membrane coated bacteria LCB
ATP-binding cassette ABC
N-hydroxysuccinimide NHS
5-fluorouracil 5-FU
Growth Factor EGF
Cholera toxin subunit B CTB
Cell membrane coated bacteria CMCB

Compared with other engineering bacteria, *E. coli* Nissle 1917 (ECN) has good antibacterial, anti-inflammatory, regulating intestinal flora and facultative anaerobic characteristics [12–14]. According to some studies, the effect of ECN on treating Inflammatory bowel disease (IBD) is comparable to that of Mesalazine (it is more effective when used in combination) [15]. It is marketed as the main component of drug Mutaflor® for the treatment of IBD [16]. This anaerobic bacterium can colonize the hypoxic area of the tumor or the colon [17]. At present, ECN is one of the most popular engineering bacteria used in the treatment of tumors and gastrointestinal diseases [18–20].

Therefore, this review focuses on the research progress of ECN as a drug delivery system in recent years, summarizes the drug delivery systems for various diseases constructed with ECN, as well as its advantages and characteristics. In addition, we emphasize in particular the

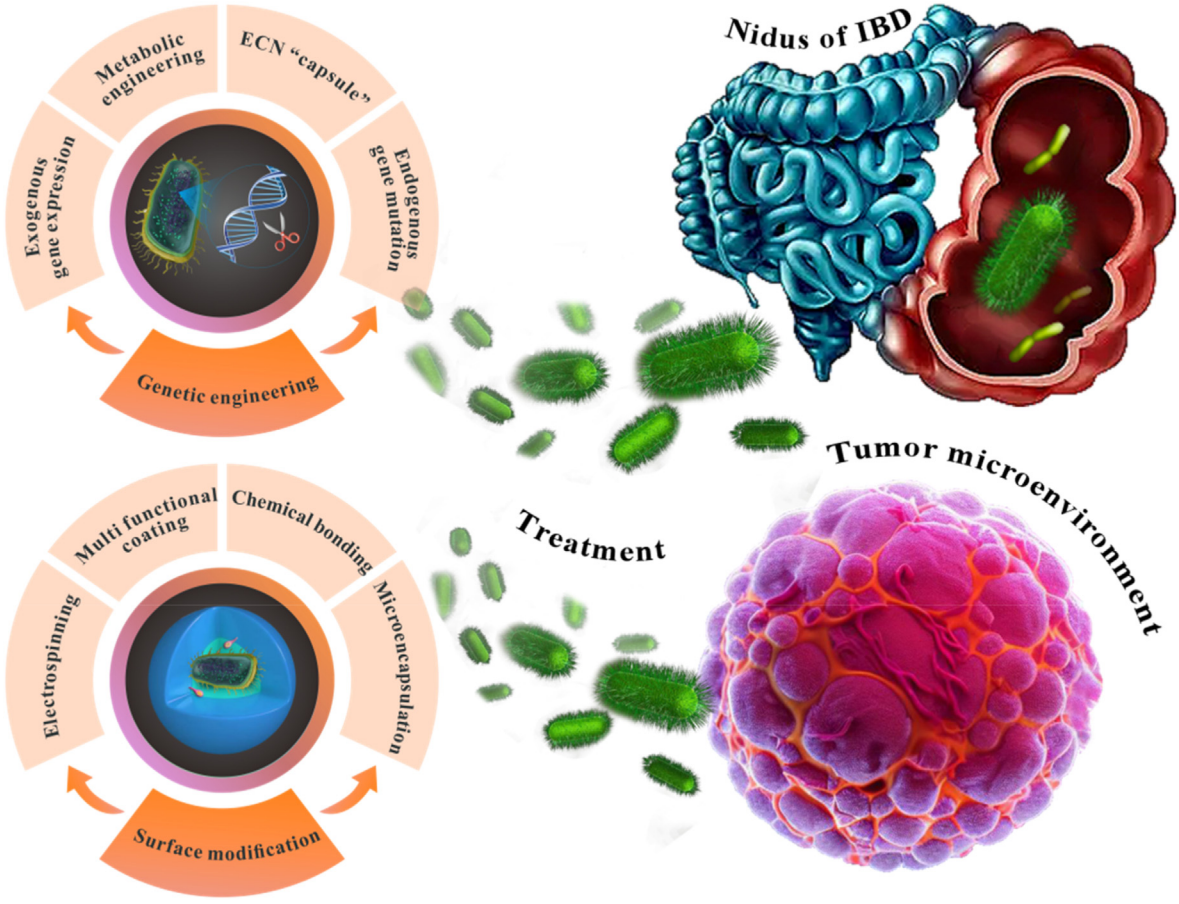

Fig. 1. This paper mainly introduces the strategies of engineered ECN for the treatment of IBD and tumor. ECN prepared by surface modification means, such as electrospinning, multifunctional coating, chemical bonding and microencapsulation. ECN prepared by means of genetic engineering, such as endogenous gene mutation, ECN "capsule", metabolic engineering and exogenic gene expression.

application strategies and functions of surface modification and genetic engineering to ECN based drug delivery systems, as well as their potential for future development. Various modification strategies of ECN are shown in Fig. 1.

2. Characteristics of ECN

2.1. ECN and intestinal microflora

A large number of *in vitro* experiments have proved that ECN has antibacterial effect [21]. The biofilm formed by ECN attached to silica gel material has a strong antibacterial effect on *E. faecalis* 210, even better than the flagellar enhanced transgenic ECN, which may be due to the quorum sensing of biofilm [22]. In addition, *in vitro* studies showed that etiology toxin produced by harmful bacteria *Vibrio* cholerae can be reduced by autoinducer 2 expressed by ECN through quorum sensing [23]. *In vitro* experiments on urinary tract pathogens showed that ECN could inhibit *Pseudomonas*, *E. coli*, *Enterococcus* and *Staphylococcus* to different degrees, and 100% inhibited Klebsiella and Enterobacter [24]. In addition to *in vitro* experiments, ECN can regulate the intestinal microbiota disorder caused by IBD [25]. IBD is often accompanied by intestinal microbiota disorders, including the decrease of *Streptomyces* and the increase of *Proteus* bacteria [26,27]. ECN adheres to intestinal epithelial cells (IECs) through its F1 fimbriae and H1 flagella [28]. In parallel, local continuous secretion of antibiotics (such as bacteriocin MccM and MccH47) is employed to antagonize *Salmonella* [29]. Further, ECN is capable of producing six types of siderophore (namely catecholate enterocin and chelate, isoxime oximate oxytocin, mixed iron carrier Yersenin, ChuA protein, and EfeU protein) to compete with other bacteria for iron as shown in Fig. 2A [30]. ECN can also inhibit the colonization of pathogenic bacteria (such as enterohemorrhagic *Escherichia coli*) and the production of toxins (such as Shiga toxinproducing *Escherichia coli*), thereby maintaining the stability of the intestinal flora [31,32]. In addition, ECN can promote intestinal peristalsis [33].

2.2. ECN and intestinal epithelial barrier

As shown in Fig. 2B, the pili and flagella expressed by ECN directly stimulate intestinal epithelial cells (IECs) to produce human β-defensin [34]. Human β-defensin can directly kill bacteria and regulate immunity [35]. The level of β-defensin in the mucosal barrier of patients with IBD is often abnormal [36]. ECN avoids inflammation caused by mucosal

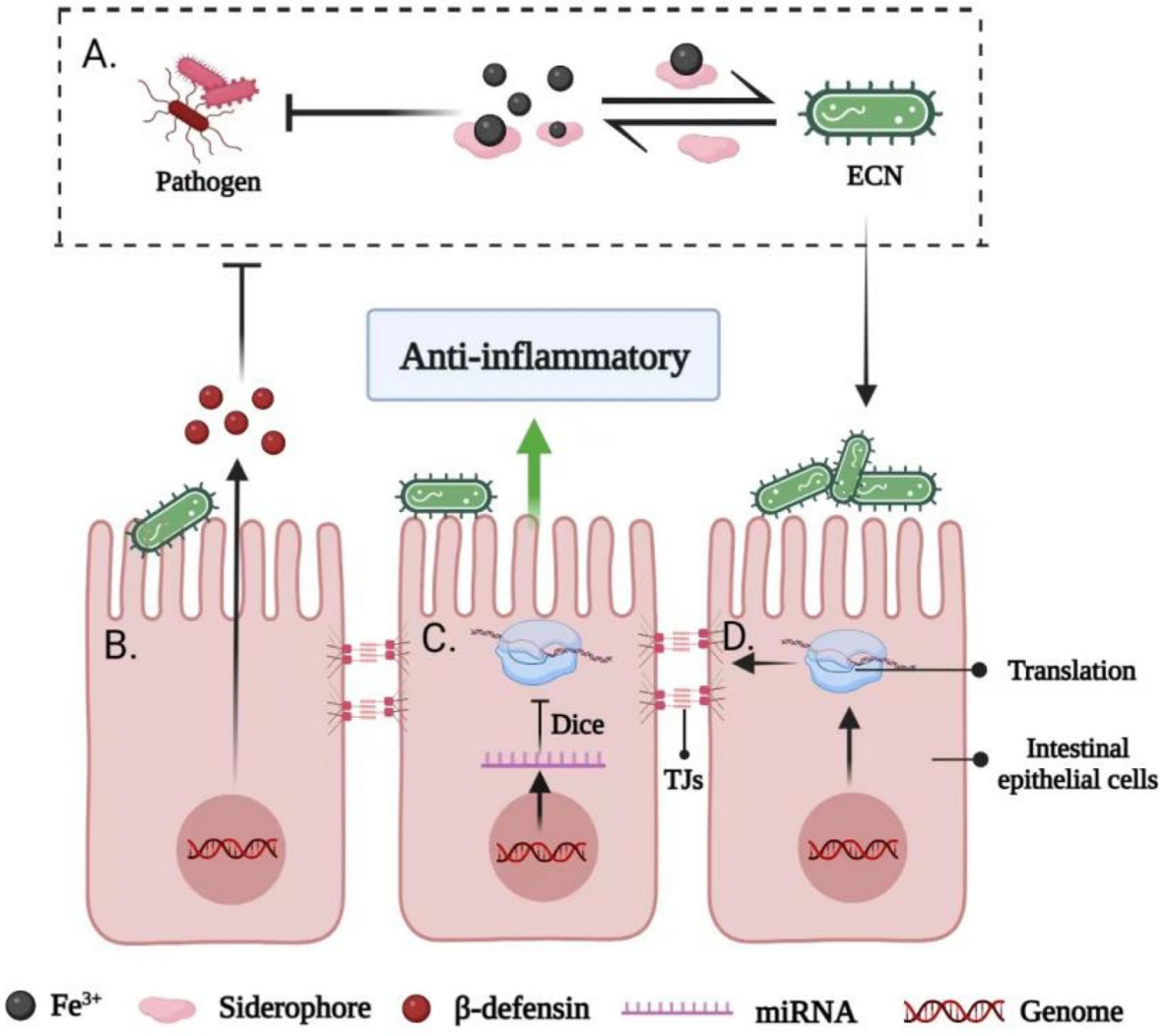

Fig. 2. The anti-inflammatory effect of ECN on IECs in IBD. ECN relieves inflammation in four ways: A. Indirect anti-inflammation by competing with harmful bacteria for iron element to inhibit the growth of harmful bacteria; B. Stimulate intestinal epithelial cell production β-defensin resist harmful bacteria and thus indirectly resist inflammation; C. Promote the production of miRNA by epithelial cells and inhibit the expression of pro-inflammatory factors; D. Stimulate epithelial cells to repair TJ to reduce intestinal leakage.

bacterial attachment and invasion to some extent [37]. Furthermore, it has been demonstrated that ECN regulates inflammation by affecting the expression of miRNA in tissues. In mice with 2,4-dinitrobenzenesulphonate enteritis, ECN decreased the expression of miRNA-155 and miRNA-223 in intestinal tissue, and these two indexes were positively correlated with pro-inflammatory factors IL-1β and TNF-α. At the same time, ECN up-regulated miRNA-143, which has the effect of suppressing innate immunity. As shown in Fig. 2C, ECN can restore the expression of miRNA-143 and miRNA-150 by down-regulating the expression of matrix metalloproteinase-2 and TNF-α [38]. In addition, in patients with IBD, the intestinal epithelial barrier is destroyed due to the damage of tight junction (TJ) within IECs as well as an increase in permeability between the IECs, which makes pathogen or substances readily able to enter the systemic circulation. Experimental studies in mice showed that ECN can promote the expression of TJ protein Zonula occludens-1 in IECs [39,40]. In the T84 cell model infected with enteropathogenic *E. coli*, ECN can promote the expression of TJ protein ZO-2 parallel to its redistribution at the cell boundary [41]. All of these effects of ECN repair

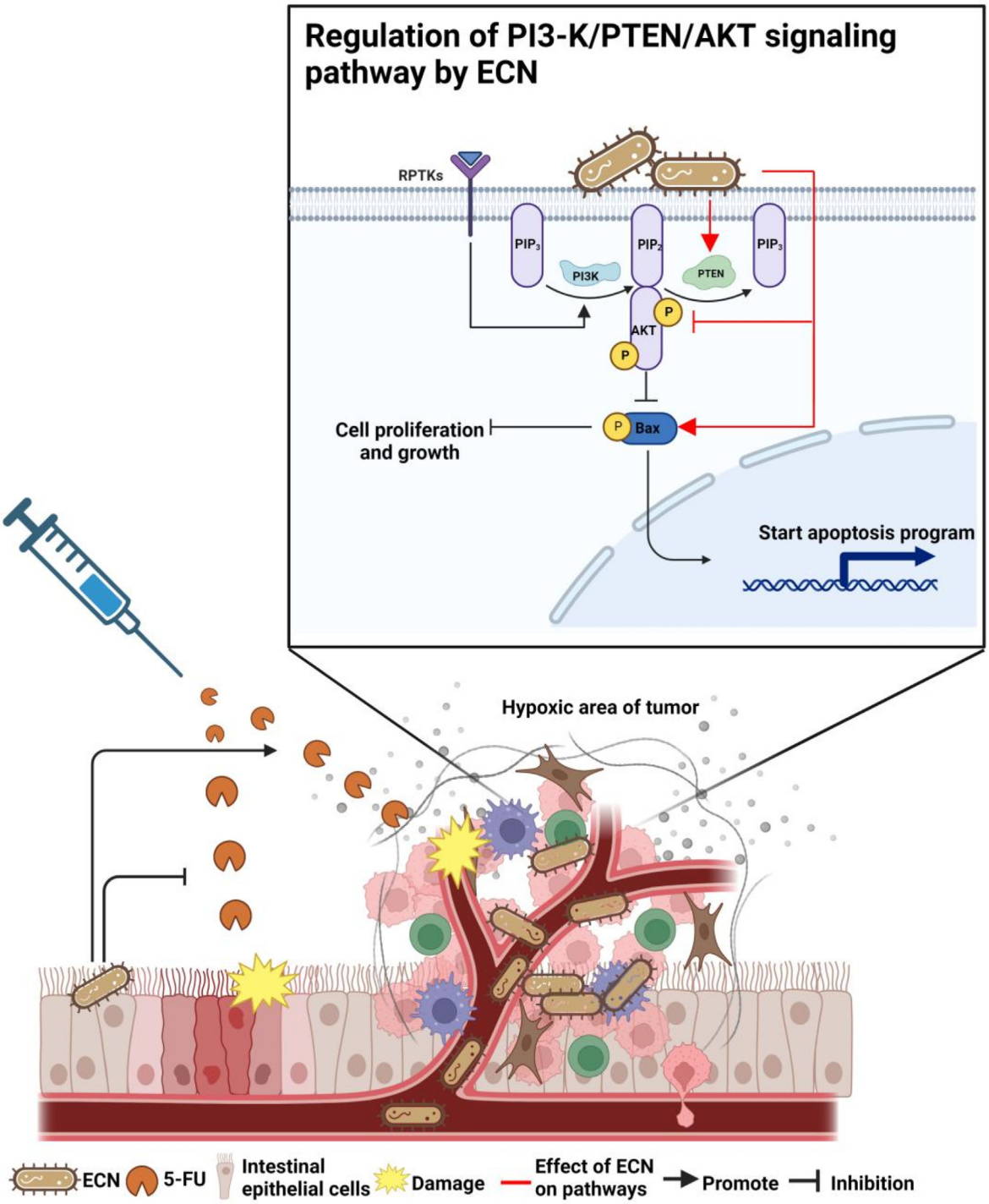

Fig. 3. The mechanism of ECN antitumor effects. In addition to inhibiting epithelial cell damage caused by 5-FU, ECN can also promote the killing of tumor cells. Tumors are mostly killed by controlling the PI3K/PTEN/AKT signaling pathway, inhibiting cell growth and promoting apoptosis by up-regulating PTEN and Bax, and down-regulating AKT.

TJ. Studies showed that ECN inhibited the activation of RHOA/ROCK2/MLC signaling pathway (the activation of which will cause changes in the distribution of TJ proteins and the destruction of barrier function) by up-regulating the expression of TLR-4, thus repairing the destruction of intestinal epithelial barrier, as shown in Fig. 2D [42,43]. The semi-rough Lipopolysaccharide (LPS) from ECN was, however, capable of promoting several pro-inflammatory factors, including IL-1, IL-6, IL-12, IL-18, IFN-α and TNF-α to slightly increase in difference *in vitro* cell experiment. In another study on the effect of ECN on human blood mononuclear cells, the results showed that the LPS of ECN activated less the expression of CCL24 (eosinophil chemotactic protein), while the ECN lysate could promote the expression of anti-inflammatory IL-10 much more than the IL-12p40 it induced. This immune regulation did not originate from the LPS of ECN, which still needs further study [44]. Additionally, the flagella of ECN can also combine with TLR-5 to produce the pro-inflammatory factor IL-8 [45]. But in another study, ECN flagellin was found to harbor a substantially longer hypervariable region compared to other commensal *E. coli*, and this region mediated symbiotic properties through stronger activation of TLR-5, thereby resulting in IL-22-mediated protection of mice against DSS-induced colitis [46]. It is therefore possible to use ECN low immunogenicity as a vaccine against allergic reactions. But its powerful anti-inflammatory effect is often applied to treat inflammatory reactions caused by other antigens [30,47]. In conclusion, ECN plays an important and positive role in repairing first-line immunity and regulating innate immunity.

2.3. ECN and tumor

Nowadays, surface modifications of drug molecules with micelles, polymers, liposomes and nanoparticles are designed to increase stability *in vivo* and prolong blood circulation time, but it is difficult to penetrate the barrier into tumor necrotic and hypoxic zones [48–50]. Most drug delivery systems are captured by the reticuloendothelial system (RES), and only 0.7% dose accumulates at the tumor site [51]. Upon systemic administration, ECN colonizes anaerobic tumor tissue in animal models, making it a promising probiotic for the treatment of tumors. ECN does not destroy or induce autologous peripheral T cell tolerance in an autoimmune environment [52]. An experiment showed that ECN could colonize in osteosarcoma cells (about 10^9 CFU/g tumor tissue) after intravenous injection in mice, and the colonization time was as long as 8 d. Experiment *in vitro* showed that both ECN and heat-inactivated ECN could induce apoptosis of colon cancer HT-29 Human colon cancer cells via PI3K/PTEN/AKT signaling pathway, which resulted in colon cancer cells undergoing apoptosis by up-regulating PTEN and BCL2-associated x (Bax) and down-regulation of AKT, as indicated in Fig. 3. ECN supernatant was shown in another study to be effective in reducing the viability of Caco-2 cancer cells and significantly reducing the activity of caspase3/7 to reduce damage to IEC-6 cells caused by 5-fluorouracil (5-FU) chemotherapeutic drug [53]. The exact mechanism by which ECN inhibits cancer has not yet been clarified, and further research is required [54].

3. Surface modified ECN

Oral administration is a major feature of bacterial carriers. By modifying the surface of ECN, the tolerance to gastrointestinal environment (low pH gastric juice, low temperature and low osmotic pressure) can be increased, and the material can also be modified to increase its targeting and therapeutic effect. Common bacterial surface modifications include microencapsulation (such as layer by layer encapsulation, a multilayer structure formed by electrostatic self-assembly), electrospinning, surface grafting functional groups to bind to various materials and simple mechanical extrusion encapsulation. The purpose of these modifications is to increase adhesion, tolerance to gastrointestinal environment, better targeting and better therapeutic effect. This article lists some ECN with surface modification, as shown in Table 1.

3.1. Polymer coated ECN

Different packaging technologies of bacteria in the market (such as spray drying, freeze drying or fluidized bed drying) have limitations, because the probiotics encapsulated by these technologies will be released to the target site or product quickly and lost before reaching the lesion site. Hydrogel, a clinically approved medical material with excellent biocompatibility, can be utilized to encapsulate bacteria in order to address the aforementioned issues. Polymeric acid glycolic acid

Table 1
Surface modified ECN.

Surface modification materials	Surface modification method	Disease model	main functions	Reference
Alginate and chitosan nanoparticles	Microencapsulation	/	It can resist the acidic environment of gastrointestinal tract, avoid the influence of high temperature, and enhance the colonization effect.	[55]
Coated with chitosan and sodium alginate, and then coated with $CaCl_2$	Microencapsulation	TNBS-induced colitis in rats	It can resist the acidic environment of gastrointestinal tract, prevent the mechanical damage of gastrointestinal peristalsis to ECN, and enhance the anti-inflammatory effect.	[56]
Mucins and TA	Microencapsulation	The DSS induces the enteritis in the mice	Mucins enhanced the colonization effect of ECN, and TA removed the superoxide radicals produced by inflammation.	[57]
PELA surface grafted functional groups (mannose group) or aminofibers	Electrospinning	/	Synthetic fibers were used for the immobilization of ECN to improve the targeted colonization and survival of ECN.	[58]
Polydopamine microtubules of the glycan-loaded urokinase	Electrospinning	Mice with hindlimb vein thrombosis	ECN as a driving device improved the bioavailability of urokinase for thrombosis.	[59]
Biotin was bound to ECN by amide bonding by NHS, and then bound to SA bound to streptavidin	Chemical method	Mice infused with bacterial gavage	To enhance the fixed value effect and time of bacteria in the intestine, it can combine various kinds of targets on the surface of various bacteria for lesion targeting and treatment.	[60]
liposome	Self-assembly package	*Salmonella* typhimurium-induced colitis in mice	Increased the tolerance of ECN to the gastrointestinal environment and enhanced the anti-inflammatory effect of ECN.	[61]
Erythrocyte membrane	Multifunctional coating for simple mechanical extrusion	Breast cancer 4T1 tumor-bearing mice	It provided ECN with a camouflage shell to escape immunity, reduced the bacterial clearance in the blood, reduced the efflux of cytotoxins of ECN, and enhanced the therapeutic effect of ECN for tumors.	[61]
Yeast membrane coating	Multifunctional coating for simple mechanical extrusion	Enteritidis infected with oral *Salmonella* mice	It can induce markedly stimulated immunity to actively regulate intestinal flora, maintain intestinal homeostasis, and prevent anaerobic bacteria from destroying intestinal epithelium.	[62]
PDA and chitosan	Co-deposited with modified multifunctional coatings	DSS-induced enteritis in mice	It resisted the acidic environment of the gastrointestinal tract and enhanced the colonization effect.	[63]

(PLGA) copolymer and alginates, as well as polysaccharides like chitosan, are commonly utilized to encase bacteria in the form of a hydrogel. For microencapsulating ECN, Mawad A used alginate and chitosan nanoparticles. In addition to being acid insoluble, these materials are capable of withstanding the acidic environment present in the gastrointestinal tract. The results showed that microencapsulated drugs could be stored at high temperature for up to 48 h, thus enhancing aggregation and colonization [55]. As shown in Fig. 4A. Xiaoming Luo prepared GECN by surface modification. ECN was coated with chitosan and sodium alginate (layer by layer) to protect it against gastric acid, and then coated with $CaCl_2$ in order to prevent mechanical damage caused by gastrointestinal peristalsis. The anti-inflammatory effect of GECN was significantly enhanced (including reducing the expression of pro-inflammatory cytokines (MPO, TNF-α, IL-6) and increasing the expression of anti-inflammatory cytokines (IL-10)). The experimental results are shown in Fig. 5A. In addition, an *in vitro* simulated gastric environment experiment showed that GECN survived significantly longer [56]. By coating layer by layer technique, another ECN (ECN@TA-Ca^{2+}@Mucin) was developed that is coated with mucin and tannic acid (TA). Through hydrogen bonds, disulfide bonds, and hydrophobic forces, mucins enhance colonization, whereas TA in the inclusion layer scavenged superoxide radicals. Researchers demonstrated that the constructed ECN was more resistant to the acidic environment of the gastrointestinal tract, promoted ECN colonization, and alleviated intestinal inflammation in mice induced with Dextran Sulfate Sodium (DSS) [57]. In addition to coating layer by layer, there is also surface coprecipitation technology. After the surface modification of ECN by using Michael addition reaction to co-deposit polydopamine (PDA) and chitosan to form a Plastic-Clad Silica (PCS) layer, the growth in simulated intestinal fluid containing trypsin (pH = 6.8) showed that ECN@PCS retained the original proliferation ability. At the same time, the PCS coating greatly enhanced the survival rate of bacteria to gastrointestinal stressors, and the targeting of chitosan increased the accumulation of this system in the mouse intestine [63]. In addition to these commonly used hydrogel material modifications, there are other chemical polymer modifications. In one study of oral administration of ECN-L, ECNs were coated with a PH-sensitive intestinal soluble Eudradit polymer, L100-55, which formed a stable layer on the bacterial surface when pH was reduced to≈5.0 (calcium ions were electrostatic adsorbed to ECNs, providing the cross-linking sites needed for self-assembly of L100-55). After passing through the difficult intestinal environment and reaching a pH > 5.5, ECN-L dissolved, which was shown to increase ECN utilization [64]. Jun Liu et al. applied tannic acid (TA) and enteric-soluble L100 as capsules for ECNs to address delivery challenges after oral administration, similarly enhancing intestinal environmental tolerance of ECNs. In addition, *in vitro* experiments showed that TA enhanced the adhesion of ECN in mouse intestinal tract [65]. These coating materials provide support for ECN in targeting or intestinal tolerance.

Encapsulated ECNs are often used to overcome the difficult intestinal environment, either to enhance targeting or to achieve the purpose of spatial and temporal release. Encapsulated ECNs are often applied to overcome the difficult intestinal environment, either to enhance targeting or to achieve the purpose of spatial and temporal release. This dictates that the polymer needs to be biocompatible while protecting the

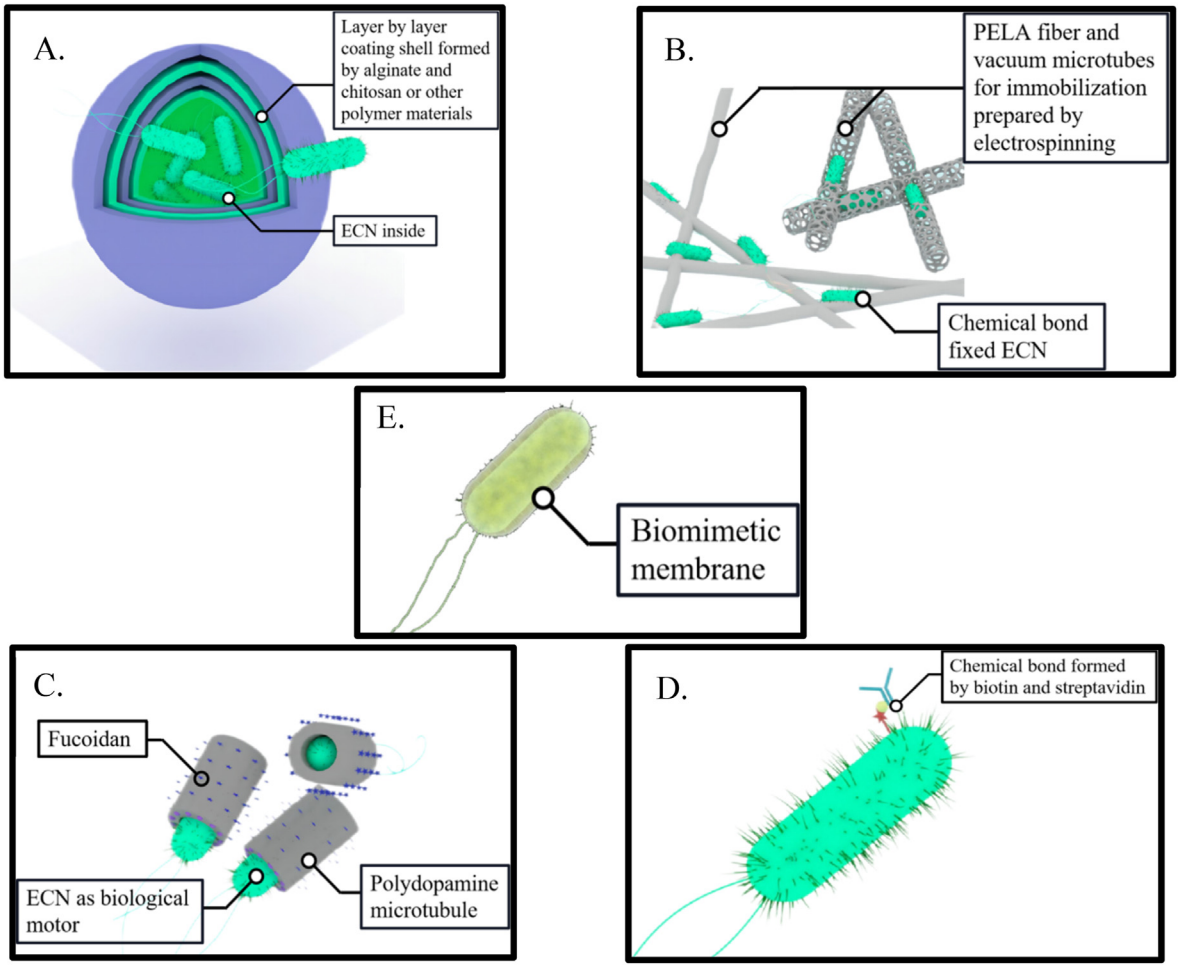

Fig. 4. A. Preparation of ECN layer by layer. B. Aggregated internally or externally adhered ECN prepared by electrospinning. C. ECN driven motor. D. Streptavidin drugs and biotinylated ECN. E. Various membrane coated ECN.

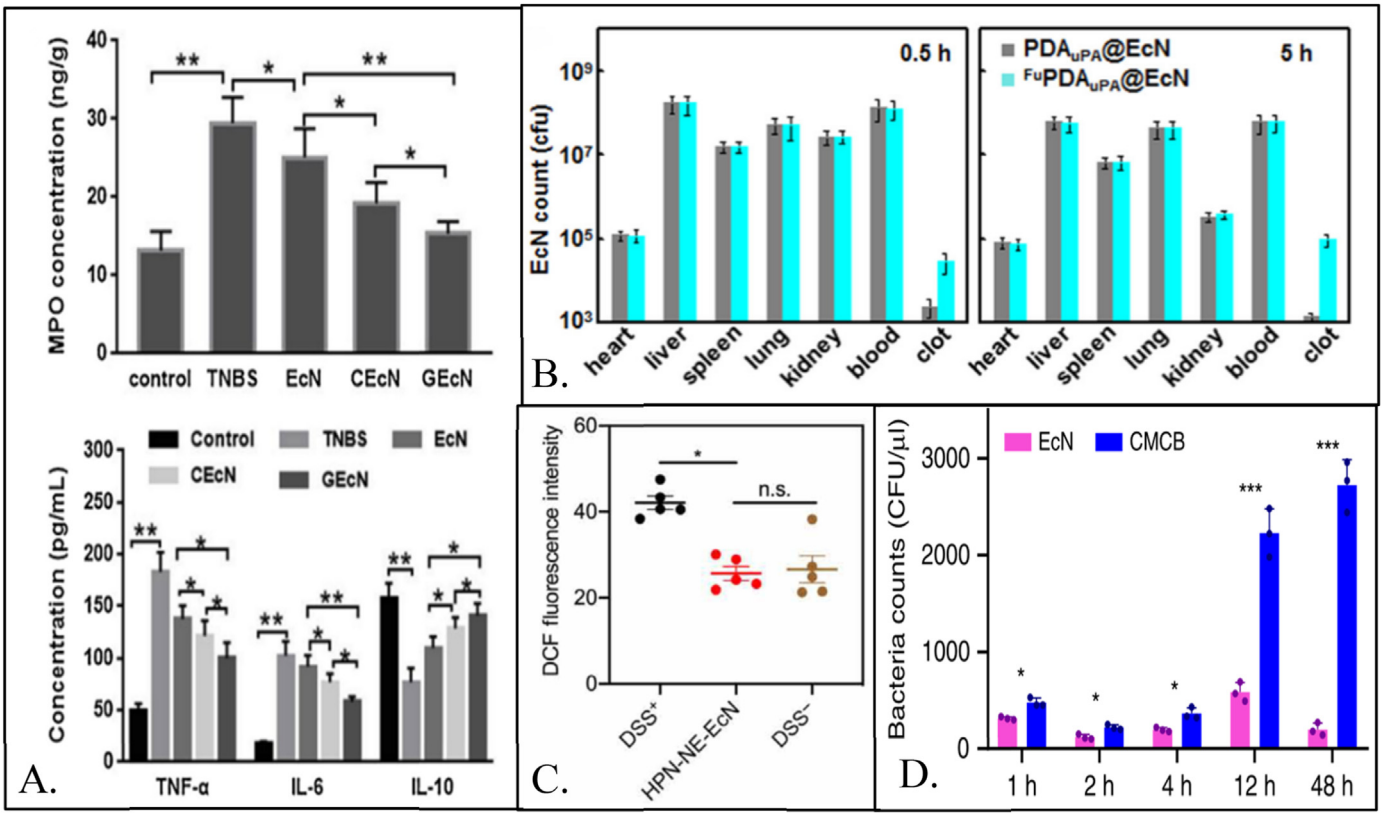

Fig. 5. A. *In vitro* experiments in mice showed that GECN significantly decreased intestinal inflammatory factors MPO, TNF-α, IL-6 and promoted the level of anti-inflammatory factor IL-10 in TNBS-induced colitis. Adapted reprinted with permission from Ref. [56], Copyright @ 2022 Elsevier Ltd. All rights reserved (License Number : 5,457,111,172,821); B. Results showing the levels of ECN in liver, heart, lung, spleen, kidney, blood and thrombus after administration of this ECN and control ECN in mice showed that this modification at 0.5 h and 5 h, the thrombus ECN levels were increased 10-fold and 3-fold, respectively. Adapted reprinted with permission from Ref. [59], Copyright @ 2022 Elsevier Ltd. All rights reserved (License Number: 5,457,130,583,213); C. DSS-induced colitis mice experiments showed DCF fluorescence intensity in colonic tissue after incubation with DCFH-DA (ROS probe), and HPN-NE-ECN significantly reduced ROS. Adapted reprinted with permission from Ref. [64], based on Creative Commons Attribution-NonCommercial 4.0 International Public License (CC BY-NC 4.0) © 2022 Jun Liu et al. D. ECN was injected into the tail vein of mice, then diluted to 10^{-2} after orbital blood sampling at different time periods and spread on LB agar plates. The CMCB bacterial counts were significantly increased. Adapted reprinted with permission from Ref. [61], based on Creative Commons Attribution License (CC BY 4.0), Copyright © 2019 Zhenping Cao et al.

internal ECN without inhibiting the normal physiological activity of the ECN, whose primary therapeutic function depends on the ECN.

3.2. ECN modified by electrospinning

Surface modification of various materials is commonly achieved through electrospinning. Songhai Xie prepared poly (ethylene glycol) polylactide (PELA) fiber by single nozzle electrospinning. ECN was captured into PELA fiber by coaxial electrospinning technology to maintain its viability against solvent and mechanical interference. By grafting mannose groups onto the PELA surface, bacteria can be immobilized on the fibers using affinity adsorption between bacteria and mannose groups. The other was to graft the bacteria onto the fiber through the glutaraldehyde connection between the aminated fiber and the amino group on the bacterial surface. As shown in Fig. 4B, both affinity adsorption and covalent binding can immobilize ECN on fiber surfaces. Compared to covalent binding, ECN affinity adsorption exhibits significantly higher immobilization efficiency, and *in vitro* experiment showed that bacteria survive more effectively, providing a means for centralized targeted diagnosis and treatment of ECN by modifying the surface of bacteria [58]. Additionally, the author prepared polydopamine microtubules (also prepared by electrospinning) that were surface modified with fucoidan and loaded with urokinase, and contained ECN inside the tubes. Using ECN as the driving device, fucoidan was the target, urokinase was the drug. The experimental results are shown in Fig. 5B [59]. Electrostatic spinning provides plasticity to the material, which provides more possibilities for the immobilization and surface engineering of ECNs, which can form multiple physical structures of ECNs for better drug delivery, immobilization and targeting.

3.3. Chemically bonded ECN

Another method for combining exogenous substances with ECN is shown in Fig. 4D. Vargason, Ava M et al. used the *N*-hydroxysuccinimide (NHS) method to bind biotin to ECN surface by forming an amide bond, and then bound the monoclonal antibody synthetic adhesin (SA) linked to streptavidin with biotinylated ECN. The SA is a synthetic adhesin that enhances bacterial colonization, and experiment showed that SA-ECN increases both bacterial abundance and colonization time in the mouse intestine as compared to ECN. This is also widely applied in other bacteria, where multifunctional biohybrid microswimmers carrying drugs to kill tumors were prepared by attaching erythrocytes loaded with anticancer adriamycin drug molecules and superparamagnetic iron oxide nanoparticles (SPION) to bioengineered bacteria through a biotin-affinity-biotin binding complex [66]. It is expected that this technique of biotin conjugation with streptavidin, which is capable of conjugating a wide range of targets to the surface of a wide range of bacterial membranes for lesion targeting and therapy, will continue to be developed in the future [60]. Jun Liu et al. synthesized hyaluronic acid-poly (propylene sulfide) (HA-PPS) polymer with ROS-responsive linker attached to

the polymer surface by NHS activation. The PPS polymer contains multiple ROS-responsive sites and is capable of scavenging multiple ROS molecules. The other end of the ROS-responsive linker is coupled to an ECN surface encapsulated with poly-norepinephrine (NE) wrapped around the surface of ECN, which enhances the targeting and environmental tolerance of ECN colon tissue. When reaching the location with more ROS, the ROS-responsive linker disintegrates and ECN releases HA-PPS to eliminate ROS for the treatment of IBD. When more ROS is reached, ROS-responsive linker dissolves and ECN releases HA-PPS to eliminate ROS and treat IBD. *In vitro* and *in vivo* experiments have shown that this modification increases ECN and prolongs the retention time of ECN in the intestine, thereby improving the abundance and diversity of intestinal flora and enhancing the effect of ECN in the prevention and treatment of IBD, and the experimental results are shown in Fig. 5C [67]. Chemically bonded ECNs make it possible to carry a variety of bioactive substances, even the size of cells, which provides unlimited possibilities for the combination of engineered nanocarriers and bacterial targeting.

3.4. ECN wrapped in cell membrane

In order to avoid the complex process flow of traditional surface modification process, Jina Liu's team developed the ECN of multifunctional biological coating, as shown in Fig. 4E. This method decorates various cell membranes (including bacteria, fungal, and mammalian cells) on bacterial surfaces, increasing bacterial accessibility (e.g., shielding immunogenicity) and versatility while simplifying the preparation process. As a result of the coating, the gut microbiota encapsulated with the coating can be used in the oral treatment of IBD. ECN and erythrocyte membrane were mechanically extruded and simply fused to prepare ECN (cell membrane coated bacteria) (CMCB) encapsulated by erythrocyte membrane. By reducing the clearing of bacterial particles from the blood, this CMCB provided ECN with a camouflaged coating that avoided immune recognition and inhibits engulfment by RES, while encapsulating the ECN minimized cytotoxin efflux. Compared with uncoated ECN, ECN (CMCB) achieve 14 times higher blood reservation 48 h post-injection, and the experimental results are shown in Fig. 5D. In the breast cancer 4T1 tumor bearing mouse model experiment, inflammatory factors including IFN-γ, IL-1β and TNF-α were measured. These indexes in serum decreased, while those in tumor increased [61]. The author also mechanically squeezed the yeast membrane (YM) embedded with Dectin-1 protein on the surface with ECN, making the ECN surface coated with YM. Experimental results indicated that the ECN@YM in the gastrointestinal tract in the list for ECN group peyer's patches colonize more, and this method can increase the efficiency of ECN absorption by M cells *in vivo* and *in vitro*. ECN@YM induced a significant immune response 4 h after administration, and intestinal sigA concentration was significantly increased compared with the other groups. In conclusion, the use of ECN@YM prevented the destruction of the intestinal epithelium by anaerobic bacteria [62]. Jina Liu et al. used a natural liposome package in addition to synthetic liposomal dioleoyl phosphate (DOPA) and cholesterol vortex to encapsulate ECN. lipid membrane coated bacteria (LCB) was prepared by encapsulating ECN with liposome self-assembly, which increased the tolerance of ECN to gastrointestinal environment. LCB can significantly reduce inflammation in mice with *Salmonella typhimurium* induced colitis, which was reflected in lower levels of cytokines in serum, including IL-6 and IFN-γ [68].

The aim of either natural or artificial biofilm wrapped ECN is to reduce bacterial immunology and increase its invisibility to increase its *in vivo* circulation time. These methods are simple and convenient and escape the *in vivo* immune clearance mechanism, but the problem of long-term bacterial accumulation still needs further study.

4. Diagnosis and treatment of various diseases using genetically engineered ECN

Currently, ECN is mainly edited using biological tools, including recombinant plasmid transfer, Red/ET homologous recombination, and CRISPR-Cas9 [69]. Most gene circuits are constructed on plasmids, which are distributed in two generations along with the process of bacterial cell division. It should be noted, however, that this distribution is uneven, resulting in the gradual loss of plasmids in bacteria. There are various degrees of applicability of plasmids to different bacterial strains, but recent studies have shown that the wild-type ECN already has two occulted plasmids, pMUT1 and pMUT2. Moreover, the two plasmids have been sequenced and proved to be genetically stable and untransferable, which provides materials for subsequent gene editing of ECN plasmids [70,71]. To achieve genetic stability, some people construct nutrient-deficient strains to form a lethal equilibrium system by expressing nutrients through plasmids in addition to antibiotic-resistant strains [72]. Alternatively, construct long-lived toxin (hok) and short-lived antitoxin (sok) systems, and in the event that the plasmid is lost, the bacteria will be directly killed by the sok system [73]. It is also possible to clone the exogenous gene directly into the ECN genome without the risk of plasmid loss. Table 2 summarizes the current ECN of genetic engineering in this review.

4.1. ECN of gene mutation

Different mutant strains of ECN have different effects on different diseases. Mutations in the *kfiB* gene of the K5 capsule biosynthesis cluster (*kfiABCD*) of ECN resulted in significantly increased adhesion to Caco-2 cells. The kfiB mutant was 2.2 times more adherent to Caco-2 cells than the wild type ECN, and cytotoxicity and apoptosis were also enhanced. Currently, there is no clear role for *KfiB* in the biosynthesis of K5 capsules, so it might be worthwhile to investigate the role which it plays in the interaction between ECN and Caco-2 [88]. The *KfiC* gene is a determinant of K5 capsular CAP, a biopolymer shell on the extracellular membrane that protects microorganisms from different environmental conditions and reduces susceptibility to antimicrobial agents. ECN Δ*KfiC* is more immunogenic than ECN and easier for macrophages to clear, so changing the thickness of CAP will determine the *in vitro* circulation time for ECN. Researchers have used this mutant of ECN to overexpress CAP to improve its immunogenicity and maximum tolerance *in vivo*, enhancing the target dose and in situ trafficking of the drug, and ultimately improving its safety and tumor colonization efficiency.

In addition, many studies have shown that the genetic toxicity of ECN is related to its antibacterial properties. ECN produces microbacterin (Mc) with the assistance of C1bP, which promotes the output of the iron carrier Mc. Mutation of the *C*-terminal of this peptidase, rather than deletion of the C1bP, can cause ECN to lose its genetic toxicity and maintain its antibacterial activity, thereby enhancing its safety for research and use [89]. In such studies, ECN mutants were screened in order to improve their functionality, adaptability, or practicality. Further research is needed to determine how this endogenous mutation affects ECN and its role in disease. Perhaps this mutation will be the key to improving ECN function or therapeutic efficacy.

4.2. Gene engineering ECN for disease treatment and diagnosis

4.2.1. ECN engineering bacteria with different promoters

Traditional plasmid induction systems generally require injecting or taking the inducer orally in order to achieve time-space administration of ECN. This *in vivo* remote control has proved to be effective. Holger Loessner et al. injected tumor-bearing mice with an ECN containing reporter gene, followed by oral or systemic administration of inducers (such as L-arabinose, L-rhamnose, or dehydrated tetracycline). Observations showed that ECN colonized the intestinal tract and tumor, and the high dose of ECN colonized the gallbladder as well [90]. As a result of the first pass effect of the gastrointestinal tract, the induction effect of inducers was often greatly reduced, making it difficult to control bacterial expression accurately. Furthermore, the injection of inducer has a certain dose limit, and side effects of injection and oral administration of inducer

Table 2
ECN for disease diagnosis and treatment.

Plasmid	Induction conditions	The role of genes	Disease model	Gene modification site	Appliance	References
pDAWN-SAH	Blue light induction	SAH is a water-soluble and pore forming hemolytic exotoxin, which can cause membrane damage and induce cell apoptosis.	SW480 colon cancer cells	Plasmid	Antitumor therapy for colorectal cancer	[74]
pDAWN-mil-10	NIR light induction	Secreted IL-10 treats intestinal inflammation in UC mice and protects intestinal mucosa from damage	DSS-induced enteritis in mice	Plasmid	Diagnosis and treatment of UC	[75]
pEAS106/pAR1219	IPTG induction	The secreted β-defensin is used to counter harmful bacteria and compensate for the decrease of β-defensin expression in CD patients to treat CD	/	Plasmid	Treatment of CD	[76]
ECN-SJ16	/	A Sj16 protein secreted by *Schistosoma japonicum*, which has the function of immune regulation and regulation of intestinal flora, and can alleviate colitis.	DSS-induced colitis in mice	Chromosome	Treat IBD	[77]
pET28-Pvhb-pelB-SUMO-Tum 5-MMP-p53	Hypoxia induction	P53 is a tumor suppressor protein, which directly induces apoptosis of tumor cells. Anti angiogenic protein Tum-5, which inhibits angiogenesis in tumor region to play an anti-tumor role	SMMC-7721 tumor bearing BALB/c nude mice	Plasmid	ECN delivers therapeutic protein to solid tumor area for cancer treatment	[78]
pSEVA131-AND	Nitrate and thiosulfate induction	The gene circuit was constructed with AND logic gates, and sfgfp was expressed only when two kinds of induction existed at the same time. The two markers of IBD were proportional to the fluorescence intensity.	DSS-induced colitis in mice	Plasmid	Diagnose IBD	[79]
PROP-Z ((pTKW106alp7A))	IPTG induction	The expressed lacZ cleaves the substrate of systemic injection, and the lysate is filtered into urine through the kidney for detection. The luxCDABE box makes ECN self fluorescent	Balb/c mice of liver tumors	Plasmid and chromosome	Non invasive detection of liver cancer	[80]
ECNL4	Hypoxia induction	3HB promotes the growth of probiotics, improves intestinal microenvironment and alleviates enteritis	DSS-induced colitis in mice	Chromosome	Treatment of colitis	[81]
ECN-BCD-BUT	/	Carrying BCD and BUT genes at the same time can promote the production of butyric acid in ECN. This short chain fatty acid (SCFA) can prevent and improve colitis	DSS-induced colitis in mice	Chromosome	Treatment of colitis	[82]
SYNB1020	Hypoxia induction	The Arginine repressor is deleted, and the *N*-acetylglutamate synthetase ArgA is inserted into the genome to convert NH3 into L-arginine (*l*-arg) to reduce blood ammonia	Mice [C57BL/6]	Chromosome	Treatment of hyperammonemia	[83]
pSB-AN	IPTG induction	Ethanol dehydrogenase 2 gene, aldehyde dehydrogenase 2 gene, NAD synthase gene and NADH oxidase gene promote the degradation of ethanol and acetaldehyde in the liver	Alcohol fed mice	Plasmid	Reversing liver and intestinal injury induced by alcoholism	[84]
pSTBlue-BETAipi	/	Generated in the intestine β- Carotene can restore functional mucosal immunity and treat diarrhea caused by vitamin A deficiency	/	Plasmid	Treatment of diarrhea caused by vitamin A deficiency	[85]
ΔECN ((pET28a-pHLIP))	IPTG induction	Knock out minCD gene and enhance minE to produce External vesicle for DOX targeted delivery	4T1 breast cancer mouse model	Plasmid and Chromosome	Cancer treatment	[86]
ECN Z/F @Au	NIR light induction	Gene E expression producing bacteria ghost is used for the spatiotemporal release of 5-FU and ZOL	Tumor bearing mice	Plasmid	Cancer treatment	[87]

are unknown. In order to overcome this limitation, researchers have developed other conditionally induced ECN plasmids.

As shown in Fig. 6A variety of ECN containing several promoter plasmids are listed below in order to address different disease types or to achieve precise spatiotemporal delivery: (1) The oxygen regulated promoter (pvhb) of *Vitreoscilla* hemoglobin gene has been used to directly express exogenous proteins at high levels. Pvhb is often used for genetically engineered ECN to express exogenous proteins in hypoxic environments (such as intestinal tract and tumor microenvironment) [91,92]. (2) In addition, optogenetics technology provides another means for ECN to induce the expression of exogenous genes. *In vitro*, the blue light-sensitive pDAWN system and the red light-sensitive cph8 system can accurately regulate the expression of foreign genes in bacteria. Siamak Alizadeh et al. cloned *Staphylococcus* aureusahemolysin (SAH) into pDAWN after codon optimization, and transformed it into ECN. Using this method, ECN can be induced with only 400–480 nm blue light (37.1 mW/cm^2) or in dark conditions at 25 °C for 24 h [74]. However, blue light cannot penetrate the human body, making *in vivo* experiment difficult. Therefore, Meihui Cui modified the ECN surface with nanomaterials and upconversion nanorod, and changed the ECN with pDAWN into a near-infrared light (980 nm) responsive ECN to achieve the space-time secretion of exogenous proteins for disease diagnosis and treatment [75]. (3) For the treatment of hyperglycemia, a specific inducible promoter was proposed for ECN. Zahra Bazi proposed constructing ECN that express heterozygous gene CTB-IGF-1 using glucose-sensitive promoter. The insulin-like growth factor-1 (IGF-1) is a polypeptide that alleviates diabetes. A domain of Cholera toxin subunit B (CTB) is capable of targeting IECs. As a result of enzyme activity, IGF-1 is able to penetrate IECs and eventually enter the blood, thereby regulating the level of intestinal blood glucose [93]. In the future, engineering ECN of different induction conditions will emerge according to the treatment needs of patients.

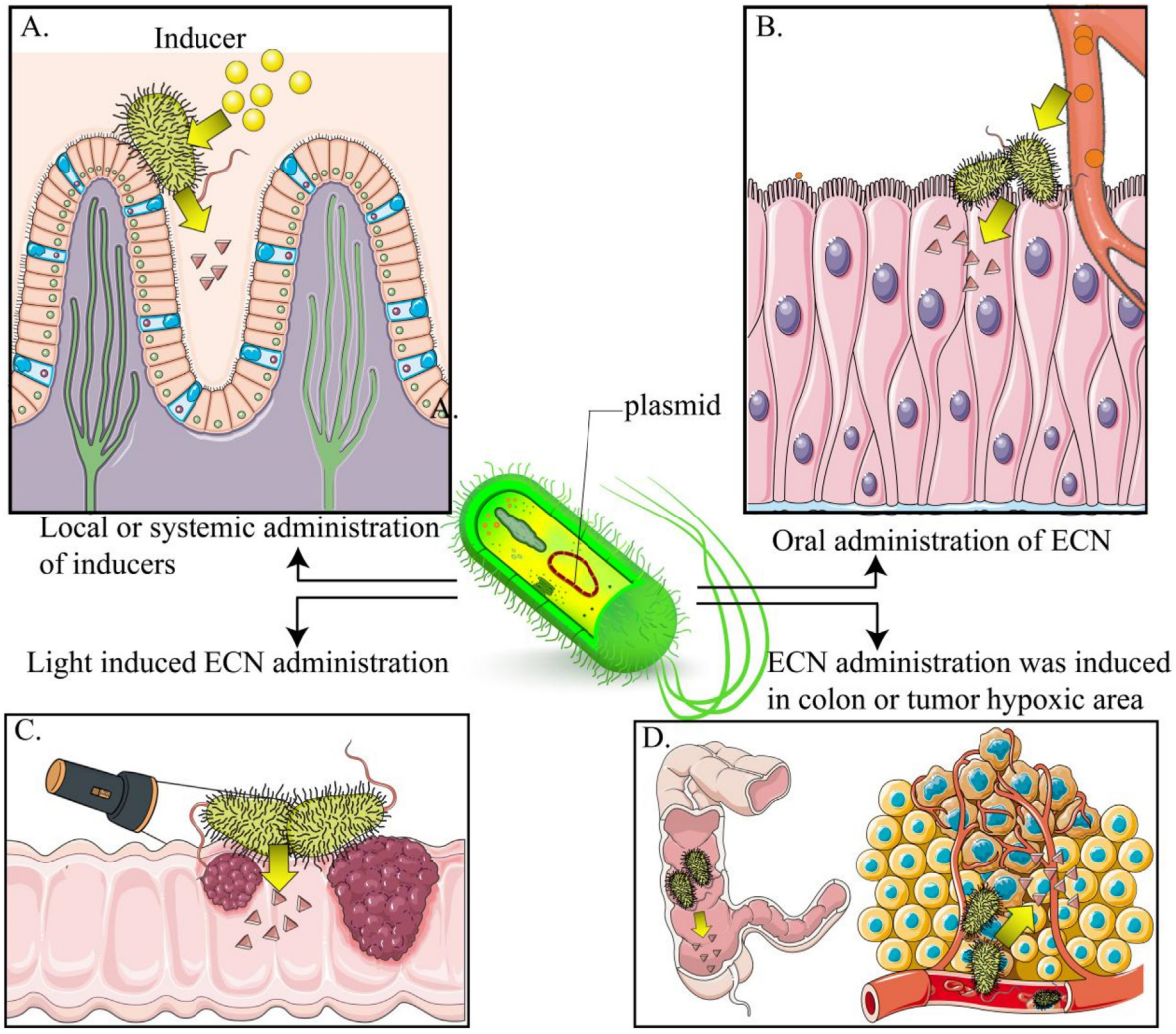

Fig. 6. A. Induction by traditional oral or in situ injections of inducers; B. Hypoxia (colon or tumor area) induction; C. Induced by light of specific wavelength (such as near-infrared); D. Induction of hyperglycemic regions.

As promoters in different species become more complex and research advances, the selection of switch elements that can be used in bacterial programming will increase. It is the primary problem of engineering bacteria to choose appropriate promoters in accordance with the characteristics of diseases or the needs of treatment.

4.2.2. Genetically engineered ECN expresses exogenous protein to treat IBD and tumor

The genetically engineered ECN can express therapeutic proteins to treat diseases, such as IBD. Due to the lack of antimicrobial peptides (α-defensins and β-defensins) in the intestines of IBD patients, the researchers constructed plasmid containing the β-defensins gene and transformed it into ECN mutant strain. In addition to the efficient secretion of β-defensins, bacteriostatic experiment showed that it can effectively inhibit the activity of pathogenic *Salmonella* and *Listeria*, which is a potential and feasible treatment for IBD [76]. In order to secrete Epidermal Growth Factor (EGF) in the intestine through targeted colonization of ECN, Mira Yu inserted the human EGF gene into the genome of ECN. In a mouse intestinal ulcer model, EGF-ECN alleviated intestinal ulcers both during prevention and post-injury treatment, and promoted the repair of the intestinal epithelial layer and proliferation of crypt progenitor cells [94]. ATP-binding cassette (ABC) transporter-linked secretion of human EGF from EGF-ECN has been developed to enhance the beneficial effects of the EGF receptor. Fig. 7A shows the inserted components on the chromosome. Lifu Wang et al. constructed ECN-Sj16 strains by inserting the gene *Sj16* with anti-inflammatory properties into pGEX-4T-1 and secreting SJ16 into the extracellular space of ECN. The HlyA secretory system is often cloned into the genome of bacteria. For secreting exogenous proteins into ECN extracellular space, the author used the HlyA secretory system (including the HlyA, HlyB, and HlyD genes from *Yersinia* pestophila) [77,95]. As a result of the DSS-induced enteritis mice experiment, ECN-Sj16 changed the microflora of the intestinal tract. Through Ruminococcaceae/Butyrate/Retinoic acid axis, Treg cells were increased, Th17 was decreased, and inflammation was alleviated, Fig. 8A illustrates the experimental results. Fig. 7B illustrates the information and mode of action of the inserted components on the plasmid.

Genetically engineered ECN can also encode toxic proteins to kill tumors. It is difficult for chemotherapy drugs to penetrate the blood-brain barrier and enter the necrosis and hypoxia areas of solid tumors, whereas ECN promotes selective proliferation in these areas. Researchers cloned tumor suppressor protein P53 and antiangiogenic protein Tum-5 into hypoxia expression vector plasmids, and then transformed it into ECN. The result of *in vitro* experiment showed that the bifunctional Tum5-p53 protein produced by ECN significantly inhibited the growth of human liver cancer SMMC-7721 cells and human cervical cancer HeLa cells. The experiment of tumor bearing mice showed that ECN treatment can induce significant expression of caspase-3 in tumor tissue and induce apoptosis of cancer cells [78]. Vascular growth is critical to tumor growth and metastasis. He L et al. constructed a plasmid containing an

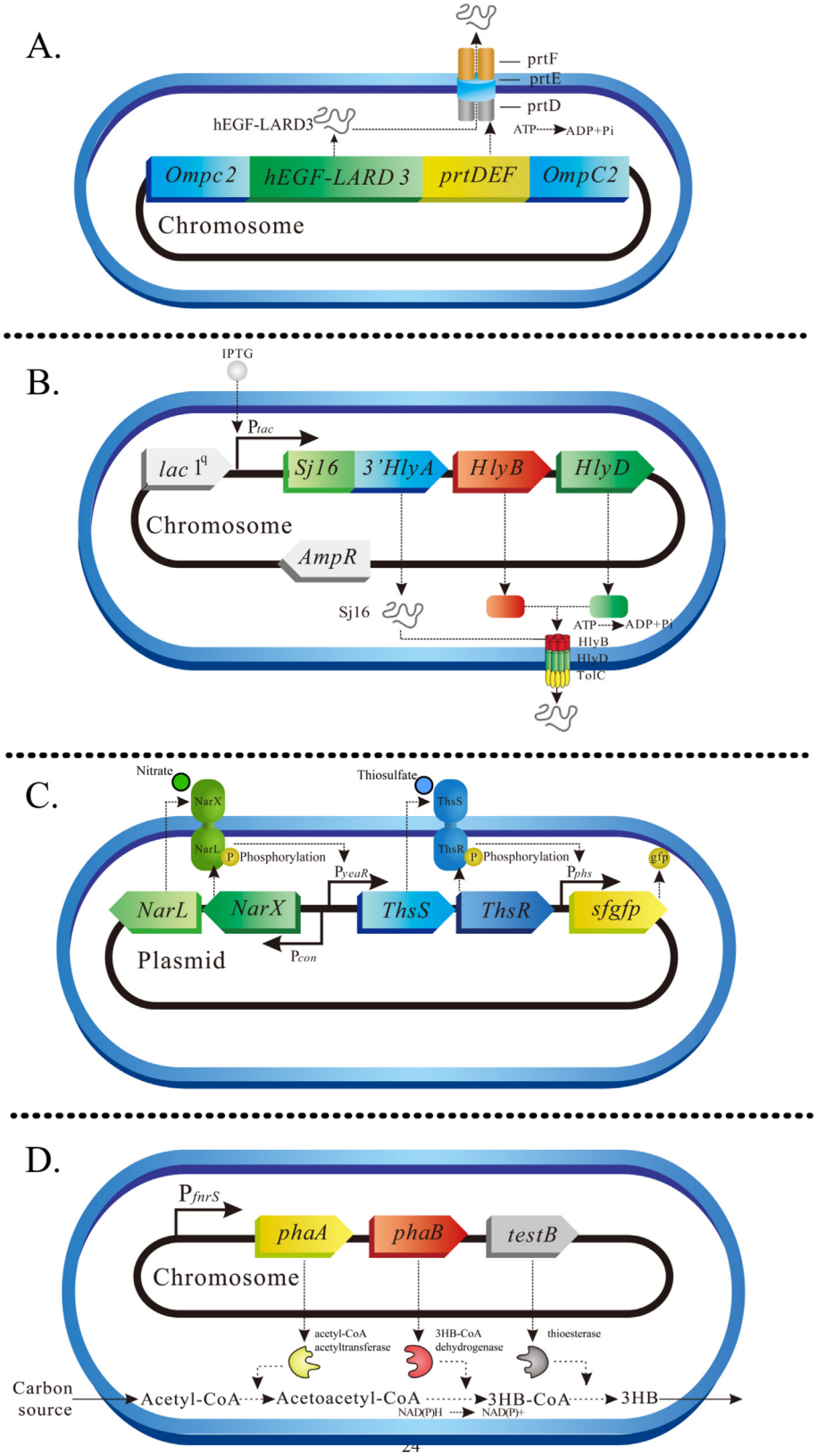

Fig. 7. A. Plant ABC transporter system prtDEF is inserted into the genome of ECN. PrtDEF consists of three functional units: outer membrane protein (prtF), membrane fusion protein (PrtE) and ATP binding cassette (PrtD). In particular, LARD3 binds to the intracellular part of prtDEF, and consumes ATP through recombination transmembrane protein to guide the target protein hEGF to be excreted out of the cell. Intestinal ulcers can be treated with hEGF; B. This plasmid contains genes of HlyA type secretion system and exogenous protein SJ16. Under the condition of Isopropyl β-D-1-Thiogalactopyranoside (IPTG) induction, ECN secretes Sj16 into ECN extracellular space through the efflux system. The HlyA type secretion system is composed of the inner membrane components HlyB and HlyD and the outer membrane component TolC. Because the inner and outer membranes form membrane pores, the fusion protein of the target protein and HlyA is excreted from the cell by consuming ATP. Colitis can be treated with SJ16; C. Dual response system of Boolean AND logic gates. The constitutively expressed NarXL protein is phosphorylated in the presence of nitrate and activates ThsSR protein expression via the yeaR promoter when nitrate is present. When thiosulfate is present, the expressed ThsSR protein is phosphorylated and the sfGFP reporter is activated via the PhsA promoter. Flow cytometry was used to measure the fluorescence intensity of bacteria containing the reporter protein; D. Engineering ECN with a hypoxia-induced 3HB synthesis pathway. A 3HB synthesis system contains the genes phaA, phaB, and tesB, which encode acetyl-CoA acetyltransferase, 3HB-CoA dehydrogenase, and thioesterase. Colitis is treated with 3HB efflux. This process consumes NAD(P)H.

anti-angiogenic active fragment (Tum-5) of the hypoxia promoter pvhb and transformed it into ECN. It was observed that ECN (Tum-5) had a significant inhibitory effect on B16 melanoma in mice. The experimental results are shown in Fig. 8B. In addition, many inflammatory cells were infiltrating the tumor area, which contributed to tumor immunotherapy [92]. Siamak Alizadeh cloned the SAH gene into pDAWN and then transformed it into ECN. The supernatant of induced ECN was sterilized and used for *in vitro* cell experiments, indicating that SAH produced by ECN has high cytotoxicity to SW480 colon cancer cells [74]. Tetsuhiro HarimotoIn et al. prepared IPTG-induced ECN ICAP based on ECNΔKFIC,

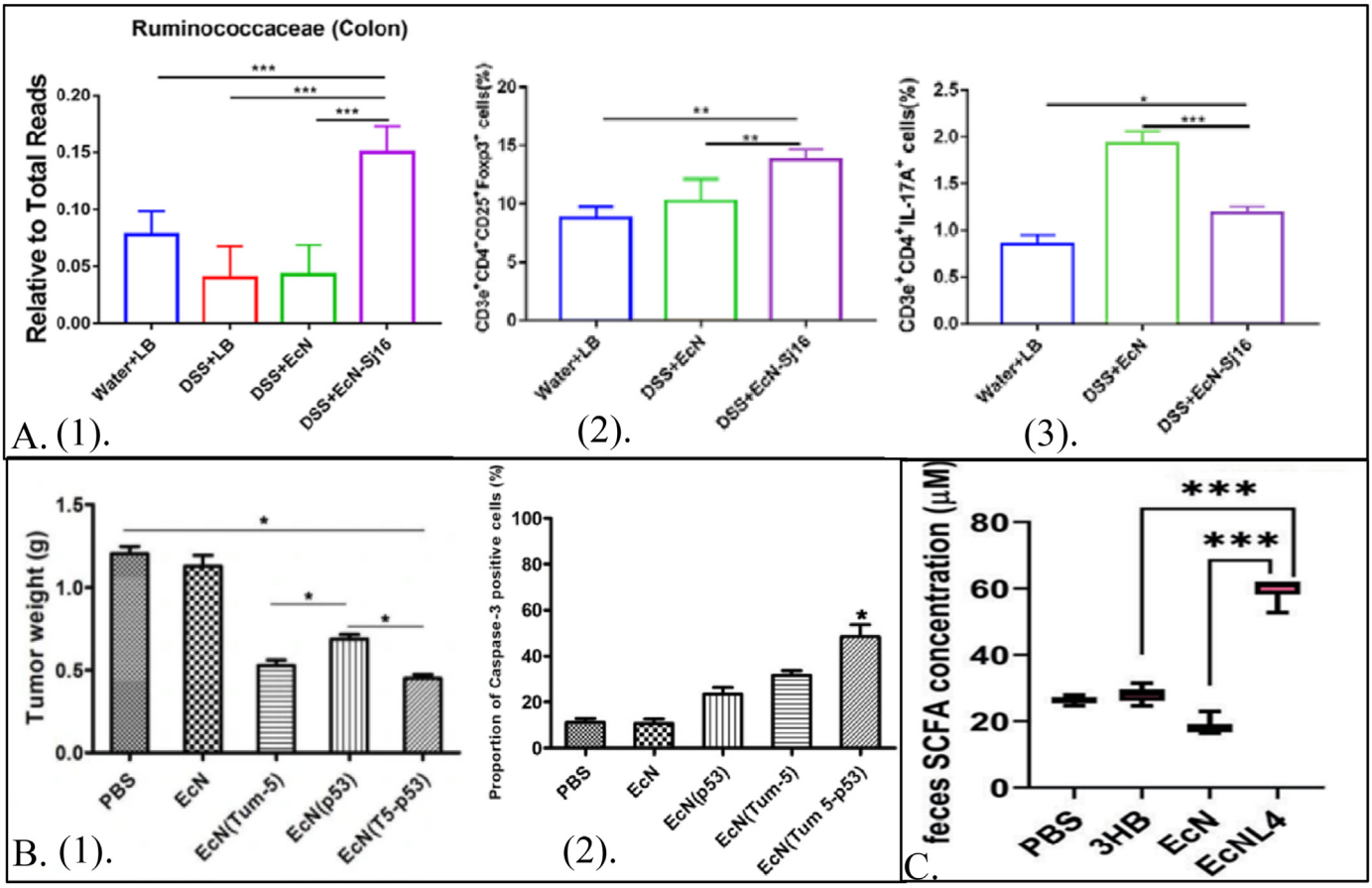

Fig. 8. A. (1) ECN-Sj16 increased the abundance of Ruminococcaceae in the colon of DSS mice. (2) and (3) indicate that ECN-Sj16 reduces the differentiation of naiveT cells into Th17 cells and increases the differentiation of Treg cells to treat inflammation. Adapted reprinted with permission from Ref. [73], based on Creative Commons Attribution License (CC BY 4.0), Copyright © 2021 Lifu Wang et al. B. BALB/c nude mice bearing SMMC-7721 tumor were injected with PBS, ECN, ECN (p53), ECN (Tum-5) or ECN (Tum 5-p53) intravenously every week. After 21 days, ECN (Tum 5-p53) group significantly inhibited the tumor weight of SMMC-7721. Caspase-3 quantitative analysis of tumor immunofluorescence analysis showed that ECN (Tum 5-p53) produced the most caspase-3, indicating that cell death was induced. Adapted reprinted with permission from Ref. [74], based on Creative Commons Attribution License (CC BY 4.0), Copyright © 2019 Lian He et al. C. The ECNL4 prepared by metabolic engineering finally increased the short-chain fatty acids in the feces of DSS mice, and then improved the intestinal flora. Adapted reprinted with permission from Ref. [77], based on Creative Commons Attribution License (CC BY 4.0), copyright © The Author(s), under exclusive licence to CSI and USTC 2021.

and then transferred it into AHL-induced system capable of producing antitumor drugs. The production of theta toxin in primary and distal tumors was significantly reduced by IPTG-containing water and orthotopic injection of AHL in a mouse model. ECN system effectively kills distal tumors while avoiding inflammation caused by overgrowth of ECN in non-target tissues. In a word, the genetically engineered ECN improves the effect of tumor treatment and has the function of tumor targeting to achieve precise treatment.

Based on the programmability of ECN, efflux proteins can be used to treat colitis or inhibit tumor growth, as has been demonstrated in several animal studies. However, the specificity of this ECN therapy, as well as its clinical effects on non-lesions, are still unknown. Specifically, overdosing of efflux proteins, non-target effects, and potential effects on the gut environment and microbiota must be carefully evaluated before safe application is possible.

4.2.3. Genetically engineered ECN as a biosensor for monitoring the course of treatment of enteritis and tumor diseases

By constructing gene circuits, engineered ECN can also be used as a diagnostic tool for IBD, as shown in Fig. 7C. When IBD occurs, induced nitric oxide synthase (INOS) in the body will produce nitrate, whereas the ROS created by inflammation will produce thiosulfate. Therefore, nitrate and thiosulfate can be used as markers of inflammatory diseases. In order to prepare the biosensor, Seung Gyun Woo et al. cloned the two-component gene circuit composed of (NarX and NarL) and (ThsS and ThsR) into the plasmid of ECN. By responding to the two-component (nitrate and thiosulfate) to regulate the transcription of the promoter P*yeaR* and P*phsA* in the form of two input AND logic gates, the expression of the sfgfp (report gene) was finally regulated, and the degree of IBD is measured by detecting the green fluorescence intensity [79]. False positives such as some nitrate ingestion are avoided because of the two-component response. Thus, the biosensor has a good compliance and sensitive response, and the gene that replaces the sfgfp with an anti-inflammatory agent can also be used to treat IBD. However, this still needs to face clinical issues, which need to address how to respond quickly and facilitate the detection of reported products.

Based on the characteristics of ECN colonizing in anaerobic zone, a more mature kit for clinical detection of liver metastasis of tumor was proposed. Tal Danino et al. prepared PROP-Z plasmid and transformed it into ECN for bioluminescence imaging of the tumor site, and determined the extent of liver metastasis through urine analysis. After oral administration of engineered ECN that can produce Lac-Z cleaving enzyme, LuGal (a soluble combination of fluorescein and galactose) was administered intravenously. The ECN colonized in liver metastases can effectively cleave LuGal, convert it into fluorescein, and finally excrete it *in vitro* with urine after renal filtration. The detection of fluorescein in urine

using a luciferase kit can be used to diagnose the risk of tumor metastasis to the liver. Finally, tumor metastasis can be observed by small animal imaging *in vivo*. The oral ECN enters the blood circulation and colonizes the liver via the enterogenous vein under the natural reticuloendothelium filtration. According to the experimental results of liver metastatic tumor mice, the colonization efficiency of ECN in tumor metastatic foci was 88%, and the concentration of bacteria in blood and off target organs was low, which would not result in systemic reactions. At the same time, this plasmid contains the hok/sok system and alp7 gene (used to encode the filaments that push the plasmid to the two poles of the cell) to ensure that the plasmid will not be lost during bacterial division. In order to avoid the immune reaction caused by ECN, the elimination of ECN can be achieved by applying antibiotics. As a result, this is a highly sensitive, non-operative, and non-radioactive method of detecting liver metastases, which can be applied clinically, as well as for colon tumor [80].

Because of the direct effect of ECN on IBD and tumor, ECN can be used as a biosensor for real-time and accurate diagnosis of disease during drug treatment, the sensor has good programmability, safety, specificity and compliance, non-invasive diagnosis and oral administration of drugs for patients to facilitate the way.

4.3. Metabolically engineered ECN produces drugs that treat diseases

As a bacteria carrier, ECN can also be used in metabolic engineering, which can colonize target sites in the human body and be continuously administered. The use of synthetic biology to construct metabolic pathways not found in ECN is commonly used in the treatment of IBD. Xu Yan et al. inserted exogenous 3-hydroxybutyrate (3HB) (a therapeutic agent for colitis) synthetic pathway into the ECN genome, and constructed ECNL4 of the hypoxia promoter pfnrS, which maximized 3HB production in the intestinal anaerobic environment. Components inserted on plasmid are shown in Fig. 7D. The DSS induced colitis experiment in mice demonstrated that the strain could produce 3HB continuously through colonization to treat intestinal inflammation directly. As a result, engineered bacteria can also regulate the intestinal tract by increasing the abundance of AKK probiotics, producing a large number of short chain fatty acids, and indirectly alleviating intestinal inflammation. In comparison with the conventional oral dose of 3HB, modified ECNL4 is more effective in the treatment of intestinal inflammation, the experimental results are shown in Fig. 8C [81]. Young Tae Park cloned butyryl CoA dehydrogenase (BCD) and butyryl CoA: acetate CoA transfer (BUT), two enzymes of probiotic (*Faecalibacterium* prausnitzii), into ECN carrying plasmids. The experiment in UC mice showed that ECN-BCD-BUT had a certain preventive effect on colitis [82].

Metabolically engineered ECN can also be used for cancer treatment. Chung-Jen Chiang also constructed ECN-BUT. *In vitro* experiment showed that butyrate could induce non-P53 mitochondrial apoptosis in tumor cells. The ECN-BUT injected into tumor bearing mice resulted in tumor specific colonization, which reduced tumor volume by 70% [96]. In a word, this metabolic engineering ECN shows a good therapeutic effect on IBD and tumors.

In order to treat hyperammonemia, Caroline B. Kurtz et al. edited the ECN genome in order to obtain the SYNB1020 strain. The researchers deleted the gene encoding ArgR (an arginine repressor), and the *thyA* gene (which would limit replication in and out of the host when thymidine concentration was low), and then cloned the *argAfbr* (an *N*-acetylglutamate synthase) gene containing the pfnrS hypoxia promoter into the *malE* and *malK* gene regions of the genome. *In vitro* experiment indicated that this system produced L-arg and consumed NH_3. *In vivo* experiment showed that SYNB1020 improved the survival rate of mice lacking ornithine transcarbamylase, and reduced hyperammonemia in mice with thioacetamide induced liver injury [83].

ECN can also be used to treat liver damage caused by alcoholism. Hua Cao et al. cloned alcohol dehydrogenase 2 gene, aldehyde dehydrogenase 2 gene, NAD synthase gene and NADH oxidase gene in the plasmid. In the human body, the first two are used to convert ethanol into acetaldehyde and then into non-toxic acetate. However, excessive drinking will lead to the destruction of these two enzymes and eventually lead to the accumulation of acetaldehyde. The latter two are used to produce more NAD^+ and enhance the activity of the first two dehydrogenases. The engineered ECN is used to promote the degradation of ethanol and acetaldehyde in the liver. In the experiment of alcohol induced liver injury in mice, the engineered ECN can resist oxidative stress, lipid peroxidation and inflammation, improve liver injury, increase the abundance of AKK in intestinal flora, and reverse the liver injury and intestinal injury caused by alcoholism [84].

Jennifer K. Miller used engineered ECN to stimulate mucosal immunity and relieve diarrhea caused by vitamin A deficiency. He cloned the genes of β-carotene synthesis pathway enzymes (crtE, crtB, crtI and crtY) encoded by *Pantoea* agglomerans and IPI (An enzyme used for isomerization of isopentenyldiphosphate) into the ECN plasmid. β-Carotene is metabolized by the body to produce vitamin A, which is used to treat diarrhea in children. Experimental results showed that the weight of β-carotene was about 0.6 μg/mg of dry bacteria. *In vitro* experiment showed that in the presence of micromolar levels of β-carotene, ECN-BETA retained its ability to stimulate dendritic cells in mice without causing immunosuppression, which preserved the beneficial immunostimulatory properties of probiotics [85].

Compared with traditional drugs, the drugs produced by metabolic engineering ECN are more effective than traditional drugs in terms of precision, duration and control. Because the traditional medicine is consumed by enterohepatic circulation after oral administration, the effect of the drug is greatly reduced. But the metabolic engineering ECN has the characteristics of targeted colonization and sustainable drug delivery, which makes it an alternative to clinical drug therapy.

4.4. Engineered ECN "capsule" for disease treatment

ECN itself can be used as a drug or as a drug carrying "capsule". ECN extracellular vesicles (EVs) can be obtained through genetic engineering. It is a feasible method to combine chemotherapy drugs with tumor therapy. For example, Taxol packaged *Salmonella* typhimurium EVs have achieved success in Phase I clinical trials [97]. By knocking out the minCD gene of ECN genome and increasing the expression of MinE in ECN, a large number of EVs were produced. By protein display technology in the membrane surface show a pH (low) insert the peptide (PHLIP), which has a stronger ability to target tumor cells in an acidic microenvironment. While single chemotherapy drugs cannot reach the necrosis or anoxic zone of breast cancer in situ, EVs which carry chemotherapy drugs can reach position. At the same time, the drug loading method is simple, and only needs to co incubate the drug with the microcell. Yunfei Zhang targeted EVs loaded with DOX to breast cancer cells through PHLIP. In the 4T1 breast cancer mouse model, this invasion led to significant inhibition of the growth of breast tumors in situ [86].

Similar to the EVs drug delivery system, there is also the bacterial ghost (BG) system. BG is a hollow shell of gram-negative bacteria with a pore [98–100]. Wenxing Zhu co cultured HeLa cells with ECN ghost carrying Epothilone B (Epo B). Result showed that the release of cytochrome C and the activation of caspase-3 were more significant after treatment, which suggested that EpoB@BG may more effectively induce apoptosis of HeLa cells mediated by mitochondrial pathway. ECN ghost may provide an effective drug delivery carrier for candidate drugs for cancer treatment [101]. Songhai Xie et al. loaded the chemotherapy drug 5-FU and macrophage phenotype regulator zoledronic acid (ZOL) into ECN through electroporation, and then modified the surface of Au nanorods. After the modified ECN entered the animal body, under the near-infrared radiation, the light energy was converted into heat energy through the Au nanorods, and the ECN was transformed into BG through the induced expression of temperature controlled plasmid, and the drug was released from the pore of BG. According to animal experiment, the local release of ZOL from this system enhanced the effective polarization

of macrophages towards M1 phenotypes and significantly enhanced the production of proinflammatory cytokines, thus inhibiting tumor growth in a synergistic manner. This study combines chemotherapy, immunotherapy and photothermal effect, and is a potential and effective cancer treatment method [102]. In addition, BG can be preserved for a long time at room temperature as a dead shell.

Whatever the ECN EVs or BG, its purpose is to reduce the cytotoxicity of the drug loaded on non-target cells and improve drug targeting. In terms of carrying, both are carried by simple mechanical oscillation at room temperature [103,104]. Considering that ECN's probiotic properties are rapidly eliminated from non-target tissues, and that ECN has no side effects on the human body, these ECN based delivery systems are anticipated to enter clinical trials in the near future [105]. Similar bacterial membrane "capsules" also include outer membrane vesicle (OMV) and smell, which have been studied in other bacteria. However, there are still challenges for the stable large-scale production of such "capsules", and the long-term preservation of these active substances is also a key issue.

5. Summarization and prospect

Nanodrugs are a hot research area combining genetic engineering, synthetic biology, photogenetics, tumor immunotherapy, and photodynamics. By using genetic engineering or material engineering, bacteria can be endowed with additional functions while maintaining the original functions of bacteria. As an example, it can facilitate the delivery of drugs over time, enhance their protection, reduce drug use and toxicity, enhance targeting, or integrate their production and delivery together. In the end, the bacterial delivery system is designed to be used as a sensor to diagnose diseases or to enhance the therapeutic effect of drugs. The ECN bacterial vector works well for targeting intestinal diseases. In order to develop ECN as a molecular delivery system for drugs, genetic engineering or surface modification with materials is the primary method. The problem of developing ECN delivery systems with targeted drug release conditions and efficacy depends on the disease, the drug delivery mode, and the individual characteristics of the patient.

The ECN drug molecule delivery carrier has the characteristics of good compliance, lasting efficacy, precise treatment, etc., however, there are still a number of problems that must be resolved before it can be put on the market. A few engineered ECN strains that are effective in animal experiment are not ideal in clinical trials, such as SYNB1020, an ECN strain that has been tested in clinical trials [106]. There is evidence that it is capable of converting ammonia into L-arginine, which is expected to treat hyperammonemia in animals, but in clinical trials it has not been successful. SYNB1618, developed by the company for the treatment of phenylketonuria, has achieved good results in phase II clinical trials [107]. There are also concerns regarding the stability and safety of recombinant strains in industrial production as plasmids of bacteria are unstable by heredity, and the antibiotic gene of plasmids is easily transferred. Some problems, however, can be solved by cloning foreign genes into the ECN genome. Additionally, it would be necessary to conduct a number of animal experiment in order to determine whether the insertion of foreign genes can affect the metabolism, growth, and reproduction of bacteria, as well as the effects on host. ECN (the pks locus containing colistin in the genome) has been shown to be genotoxic in some studies, leading to mutations or DNA strand breaks in the host. ECN, however, was not shown to induce DNA strand breaks in recent studies and to have no detectable genotoxic potential in experimental animals. ECN's long-term safety is controversial. ECN is genotoxic due to its antibacterial properties, which requires that the virulence factor of ECN be decoupled from its antibacterial properties. A point mutation in the *C1bP* gene has been shown to inactivate genotoxicity while maintaining antibacterial activity in known studies. This problem may be solved by future research on virulence factors and mutant strains. It is still necessary to study the mechanism of action of ECN in the treatment of various diseases. The large-scale industrial production of drug molecular delivery systems prepared by ECN will also face unknown challenges.

Credit author statement

Haojie Chen: Visualization, Writing- Original draft preparation. Pengyu Lei: Writing- Original draft preparation. Hao Ji: Visualization. Qinsi Yang: Writing- Reviewing and Editing. Bo Peng: Writing- Reviewing and Editing. Jiahui Ma: Writing- Reviewing and Editing. Yimeng Fang: Visualization. Linkai Qu: Writing- Reviewing and Editing. Hua Li: Writing- Reviewing and Editing. Wei Wu: Conceptualization. Libo Jin: Conceptualization, Writing- Reviewing and Editing. Da Sun: Conceptualization, Visualization, Writing- Reviewing and Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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