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Intestinal delivery of non-viral gene therapeutics: physiological barriers and preclinical models

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The future of nucleic acid-based therapeutics is dependent on achieving successful delivery. Recently, there has been an increasing interest in delivery via the gastrointestinal tract. Gene therapy via this route has many advantages, including non-invasive access and the versatility to treat local diseases, such as inflammatory bowel disease, as well as systemic diseases, such as haemophilia. However, the intestine presents several distinct barriers and, therefore, the design of robust non-viral delivery systems is key to future success. Several non-viral delivery strategies have provided evidence of activity *in vivo*. To facilitate the design of more efficient and safe gene medicines, more physiologically relevant models, at both the *in vitro* and *in vivo* levels, are essential.

Introduction

The prospect of somatic *in vivo* gene therapy as a treatment modality for both monogenetic and multifactorial diseases has generated significant interest [1]. Gene therapy has come to encompass the delivery of several distinct nucleic acids, including plasmid DNA (pDNA), antisense oligonucleotides (ASOs) and RNA interference (RNAi)-based systems [including small interfering RNAs (siRNAs), short hairpin RNAs (shRNAs) and microRNAs (miRNAs)] to target cells. DNA-based approaches offer an alternative to protein delivery and generally involve the expression of a transgene delivered by host cells. By contrast, both ASOs and RNAi approaches mediate their effects through sequence-specific silencing of endogenous gene expression, although the mechanisms by which they achieve this are different [2].

The use of the gastrointestinal tract (GIT) as a site for the local delivery of gene therapeutics or as a route of delivery to distant sites is an exciting prospect. Several characteristics of the GIT make it an attractive target for gene therapy applications. First, the gut is readily accessible either by oral, rectal or endoscopic methods, facilitating access to target tissues without the need for invasive surgery. The oral route is of particular interest owing to high patient compliance and reduced healthcare cost. Although intestinal gene therapy has been achieved by other routes (e.g. intravenous injection [3]), such studies are beyond the scope of this review. The large surface area of the gut means that a large population of cells are available for uptake [4]. Another advantage to

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gut gene delivery is the presence of stem cells in the crypts of Lieberkuhn. These might be of particular interest in certain gene therapy applications as their successful transfection could, in some circumstances, facilitate long-term expression of therapeutic genes. Finally, the gut epithelium is highly vascularised, being located only a few microns from an extensive capillary network [4]. This could provide access for therapeutic proteins synthesised in the gut (post gene transfer) to the systemic circulation and could be a promising strategy in the treatment of diseases such as haemophilia. The potential delivery of nucleic acid therapies themselves to distant disease sites (e.g. tumours) following transport across the intestinal epithelial barrier (by transcellular or paracellular routes) is also of interest (Fig. 1).

Local conditions that might be treatable by gene therapy include inflammatory bowel disease (IBD), familial adenomatous polyposis (FAP), intestinal cancers and the intestinal symptoms of cystic fibrosis [5]. The use of orally delivered DNA vaccines is also an area of intense interest given the presence of the gut-associated lymphoid tissue (GALT) and the range of pathogens to which the gut is exposed. Of particular interest to DNA vaccine applications are the antigen-sampling M-cells found in the follicle-associated epithelium (FAE) of lymphoid follicles and Peyer's patches.

In this review, we summarise some of the non-viral delivery strategies that have been utilised for intestinal gene delivery to date, as well as the various cellular targets for gene therapy. Extracellular and cellular barriers that must be overcome for optimal activity are also discussed. Furthermore, we describe various *in vitro* and *in vivo* models that might be useful for predicting the stability and/or effectiveness of nucleic acid therapies for IBD and intestinal cancer treatment.

Obstacles to intestinal gene delivery and cellular targets

Whereas the GIT presents several opportunities for gene therapy, several extracellular and cellular barriers exist that can limit therapeutic success (Fig. 1). An ideal gene delivery vector (GDV) would need to survive in the extracellular milieu and efficiently transfect or traverse the mucosal epithelium, depending on therapeutic strategy.

Extracellular barriers

If a gene therapeutic is administered orally, then the first major obstacle it faces is the harsh acidic (pH 1.5–1.9) environment of the stomach [6]. Indeed, nucleic acids are known to be denatured and depurinated over time in acidic gastric media, decreasing their effectiveness [7]. In addition, the presence of the proteolytic gastric enzyme pepsin might impact GDV stability. The fluid flow and peristaltic activity of the GIT might also reduce the contact time between GDVs and the epithelial layer, thereby limiting the opportunities for uptake. Nuclease enzymes are present in the GIT lumen and might degrade nucleic acids before cellular entry. Furthermore, the pancreatic secretions that enter the duodenum contain bile salts and many degradative enzymes, including amylase, trypsin and lipase. Additionally, pH values in the small and

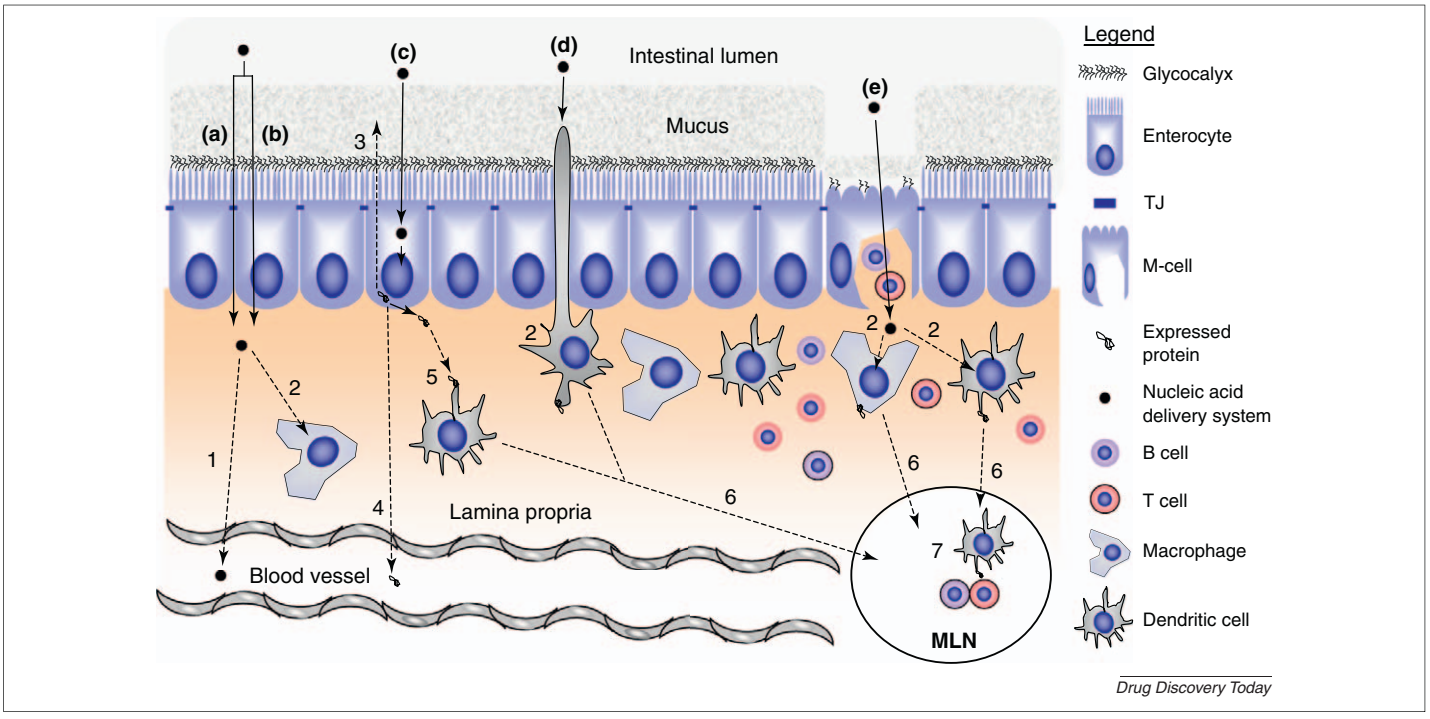

FIGURE 1

Uptake and/or transport of GDVs by the intestinal epithelium and induction of biological effect(s). GDVs can gain access to the lamina propria by **(a)** the paracellular route or via transcytosis through **(b)** enterocytes or **(e)** M-cells. Alternatively, dendritic cells potentially facilitate GDV transport across the epithelium **(d)**. The GDVs can subsequently (1) gain access to the systemic circulation or (2) transfect lamina propria cells. **(c)** GDVs can also transfect epithelial cells and expressed therapeutic proteins might enter the lumen (3) or be secreted basolaterally (4) and enter the bloodstream or (5) be processed by lamina propria cells. In terms of DNA vaccines, APCs that have the expressed antigenic protein [either through (2) their direct transfection or (5) protein processing] can migrate to the mesenteric lymph nodes (MLNs) (6) and induce T and B cell differentiation and/or activation (7). These immunocompetent cells can enter into the systemic circulation and mucosal tissues, generating systemic and mucosal immunity.

large intestine can be variable [8] and, therefore, GDV stability over a wide pH range is necessary. The gut flora, which is predominant in the distal ileum and colon, produces a range of enzymes, some of which might have GDV-degrading capacities.

The GIT is lined by a viscous sticky layer of mucus, which is capable of 'trapping' and removing foreign and hydrophobic particles (including GDVs) prior to reaching the underlying epithelium (Fig. 1). The chief components of mucus besides water are proteoglycan coated mucin proteins, which contribute a net negative charge to the mucous layer. The 'trapping' effect of mucus might be mediated by electrostatic and/or hydrophobic interactions. The thickness of the adherent mucous layer varies depending on the site in the GIT, being thicker in the colon than in the ileum [9]. Mucoadhesive delivery systems have received significant attention as they can help slow the transit time of particles through the GIT, by increasing particle adherence to mucus. The cationic polymer chitosan is one of the most commonly used oral GDVs [10] and achieves mucoadhesion mainly through electrostatic interactions. However, although mucoadhesive systems have shown potential, they might represent a limited approach to nucleic acid delivery. Indeed, given that mucus is secreted, shed and discarded over a relatively short time period (50–270 min) [11], a proportion of mucoadhesive GDVs might not traverse the mucous layer in time to reach the underlying epithelium. The shortcomings of mucoadhesive particles have prompted research into the development of muco-inert mucus-penetrating particles [9]. Furthermore, mucolytic agents [e.g. *N*-acetylcysteine (*N*-AC)] might facilitate improved GDV access to underlying target cells. In conditions such as ulcerative colitis, the mucus layer can be reduced or missing in areas of acute inflammation [12]. Another barrier to GDV delivery is the glycocalyx (Fig. 1); a glycoprotein and polysaccharide layer (400–500 nm thick) associated with the apical membrane of enterocytes [13]. It acts as a size-selective diffusional barrier preventing access of certain viruses, bacteria and particles to the underlying plasma membrane.

Cellular barriers and targets

The intestinal mucosa consists of three layers: a single layer of epithelial cells; the lamina propria (richly vascularised, with numerous immune cells); and the muscularis mucosa. The mucosal surface is associated with extensive amounts of immune inductive tissue (i.e. GALT), including lymphoid follicles and Peyer's patches, which have an important role in generating mucosal and systemic immunity. These structures are located beneath the epithelia between villi and are most abundant in the ileum.

The epithelial layer consists of enterocytes, goblet cells, M-cells, Paneth cells, intraepithelial lymphocytes and hormone-secreting endocrine cells. Several obstacles to GDV uptake are presented by the epithelial barrier. Depending on the gene therapy application, transfection or knockdown of a gene in the epithelial cells themselves might be the goal, or else direct access to the underlying lamina propria might be desired. In relation to the latter, GDVs primarily cross the epithelium either between the cells (paracellular route) or through the cells (transcellular route) (Fig. 1).

The paracellular transport of GDVs is limited by the presence of tight junctions (TJs) between cells. Indeed, the paracellular pore size in the human intestine generally lies within the 0.5–3 nm range [14], which is smaller than the size of most GDVs. However, the use of permeation enhancers could facilitate increased paracellular transport by disrupting TJs [15]. Indeed, the permeation enhancer sodium caprate, known to increase both paracellular and transcellular uptake [16], has been included in the formulations for the intestinal administration of ASOs in dogs [17] and pigs [18]. More recently, the modified ASO, ISIS 104838, was delivered orally to human volunteers as minitablets with sodium caprate, achieving an average plasma bioavailability of 9.5% [19]. A GDV itself might also have TJ destabilising effects (e.g. chitosan [20]). It is of note that in IBD, TJ permeability is increased as the epithelium is disrupted [21]. A major drawback associated with TJs is the fact that they represent <1% of the mucosal surface area of the intestine [22].

The transcellular route of transport is advantageous owing to the extensive surface area for uptake [23]. In terms of the transcellular transport of GDVs, the main mechanism is transcytosis (Fig. 1). This involves the endocytosis of the GDV at the apical membrane of the intestinal epithelial cells (IECs), its transport through the cell and across the basolateral membrane into the underlying lamina propria (Fig. 1). Several endocytic mechanisms exist by which GDVs can enter IECs [24]. Intracellular barriers also exist and include the presence of nucleases, the possibility of GDV recycling back to the lumen and nuclear uptake in the case of pDNA strategies where IEC transfection is desired.

Epithelial cells have a short lifetime of 5–7 days, being continuously shed and replaced. Therefore, repeated administrations of gene therapies might be necessary when these cells are targeted. The most common cells of the epithelial layer are the enterocytes (absorptive cells), representing approximately 90% of the epithelium [22]. Enterocytes might be particularly useful as 'protein factories' where the expressed therapeutic protein is secreted into the bloodstream (Fig. 1). However, several features might limit their role in gene therapy applications. The apical membrane of enterocytes is associated with a relatively thick glycocalyx, which might limit access to the underlying epithelia (Fig. 1). In addition, their apical membrane is relatively thick, has a high protein content and is less fluid than the cell membrane of other cell types. This reduced membrane fluidity might contribute to the low level of endocytic activity associated with these cells [24]. In addition, heparan sulphate proteoglycans (important in cationic vector binding to cells) have been reported to localise to the basolateral membrane of enterocytes upon differentiation [25]. Enterocyte apical membranes are characterised by a high number of microvilli, which are approximately 1 mm in length and approximately 50 nm in width [26]. Therefore, given that endocytosis occurs primarily at the base of microvilli [22], it has been proposed that particles >50 nm in diameter might not be efficiently endocytosed [27].

Goblet cells are polarised mucus-secreting cells and are the next most common cell type in the epithelial layer. They have fewer and more irregular microvilli than do enterocytes and have a poorly developed glycocalyx [26]. Although evidence of their transfection has been observed [28], their specific role as gene therapy targets is still unclear.

M-cells are primarily located in the FAE of Peyer's patches, to a certain degree in the colon and, more recently, have been reported in the villous epithelium [29–31]. Given their role in the transport of foreign material from the lumen to the immune cells of the

lamina propria, M-cells are characterised by a high level of endocytic activity and, thus, might be amenable to GDV uptake. Indeed, the apical membrane of M-cells is characterised by microfolds as opposed to the microvilli of enterocytes (Fig. 1). They also have a reduced glycocalyx [13] and mucous layer [24] and a lower level of alkaline phosphatase activity relative to enterocytes [26]. Furthermore, M-cells are reported to have a lower number of lysosomes [32], and so the intracellular degradation of GDV and/or nucleic acid might be avoided. Invagination of the basolateral membrane of M-cells results in a basolateral pocket that houses several lymphocytes and macrophages [24,33] (Fig. 1). This reduces the distance that GDVs need to travel to traverse the cell and their close proximity to antigen-presenting cells (APCs) means that M-cells might have particular potential as sites of DNA vaccine delivery (Fig. 1). The major stumbling block associated with M-cells is their low number in the GIT. Reports vary, but they are believed to constitute approximately 5% of the human FAE, and approximately 1% of the total intestinal epithelial layer [24]. However, their numbers can be altered in conditions of intestinal inflammation [34–36]. The capillary network underlying the FAE is less dense and less permeable than that underlying villi [37] and, therefore, M-cells might not represent the optimal delivery site for nucleic acid therapeutics or encoded proteins that require access to the bloodstream [38]. Rather, they might be most useful as a transcytotic route of delivery to lamina propria immune cells (Fig. 1).

Although the stem cells of the intestine represent an interesting target for long-term gene expression, their location at the base of intestinal crypts and the overlying thick mucous layer might hamper their transfection. In addition, DNA delivered with most non-viral GDV approaches is generally located episomally, and transgene loss upon cell division is a concern. Hence, transfection of stem cells with traditional non-viral methods is unlikely to confer long-term gene expression.

The immune cells of the lamina propria include T and B cells and APCs [dendritic cells (DCs) and macrophages] (Fig. 1). These cell types present targets and/or components for several gene therapy applications. Macrophages might be useful targets in conditions such as IBD, where they contribute to disease state in part by producing proinflammatory cytokines [e.g. tumour necrosis factor (TNF)-α and interleukin (IL)-6]. DCs might be particularly important cellular targets for DNA vaccine delivery owing to their essential role in initiating and directing antigen-specific T-cell responses. DCs might also have a role in the uptake of GDVs across the intestine (Fig. 1), as they can send their projections between epithelial cells into the lumen to sample bacteria and antigens [39]. Furthermore, similarly to M-cells, APCs have high endocytic activity and might thus be amenable to GDV uptake.

Delivery systems

Nucleic acids are hydrophilic, negatively charged, high molecular weight materials that are readily degraded by nucleases *in vivo*. Therefore, several delivery strategies have been investigated to improve both the stability and uptake of these therapeutic molecules. Viral approaches are generally the most efficient and widely used delivery systems, given their highly evolved natural pathways for infection. However, issues including immunogenicity and insertional mutagenesis have led to the development of non-viral approaches, which, although less efficient than virus-based strategies, are potentially less immunogenic and can incorporate larger genetic units. Non-viral approaches include the use of synthetic (lipids, polymers and organic nanoparticles) and bacterial delivery systems. To date, numerous non-viral agents have provided evidence of their ability to transfect the gut or gut-associated tissues with reporter plasmids (Table 1) as well as therapeutic nucleic acids *in vivo* (Table 2). However, cases of nucleic acid activity have also been reported *in vivo* without the use of such delivery systems. Indeed, an orally administered ASO (EN101) targeting an isoform of acetylcholinesterase reached phase IIa clinical trials for the treatment of myasthenia gravis, with a reduced disease score reported (http://www.amarincorp.com) [40].

In terms of lipid-based vectors, most *in vivo* studies have utilised commercially available cationic lipids, such as Lipofectin®, Lipofectamine™ 2000 and DOTAP (Tables 1 and 2). Liposomes have poor stability in the gut and this could explain why polymer-based approaches have been used more commonly [41]. Both condensing and non-condensing polymers have been utilised [42]. Condensing polymers are cationic in nature and interact electrostatically with nucleic acids to form polyplexes, whereas non-condensing polymers can interact with DNA by hydrogen bonding and/or van der Waals interactions. The cationic polysaccharide, chitosan, is among the most widely used condensing polymers for intestinal gene therapy research (Tables 1 and 2). It forms nanoparticulate complexes with nucleic acids and is biodegradable, biocompatible and relatively non-toxic in nature [43]. It also has mucoadhesive and TJ destabilising effects. Poly(lactide-co-glycolide) (PLGA) has been one of the most commonly used non-condensing polymers in intestinal gene delivery. It has generally been formulated as microparticles to deliver DNA vaccines (Table 2).

Two relatively recent intestinal non-viral strategies are of particular note [i.e. NiMOS (nanoparticles-in-microsphere oral system) developed by Amiji's group [44,45] and the GeRP (β1,3-D-glucan encapsulated siRNA particles) system developed by Czech's group [46]]. NiMOS consists of pDNA physically encapsulated by type B gelatin nanoparticles, which are further encapsulated within poly(epsilon-caprolactone) (PCL) microparticles. Upon reaching the intestine, PCL is degraded by intestinal lipases, releasing the nanoparticles, which are then available for uptake by cells of the small and large intestine. This system facilitates protection of the nanoparticles from the harsh environment of the stomach. GeRPs are 2–4 μm sized β1,3-D-glucan shells (prepared by chemically treating bakers yeast) containing an siRNA–PEI–tRNA core. These orally delivered vectors are phagocytosed by macrophages and DCs of the GALT through their interaction with the dectin-1 receptor, and have achieved significant targeted gene silencing *in vivo* post oral delivery. Access to these GALT cells is believed to be M-cell mediated. GeRPs are currently being investigated as oral delivery systems by RXi Pharmaceuticals (http://www.rxipharma.com).

Bacteria-mediated gene delivery can be achieved by bactofection or alternative gene therapy (AGT) strategies [47]. Bactofection is similar to traditional gene delivery and involves the delivery of pDNA by bacteria to host cells. In AGT, the bacteria themselves

TABLE 1

Synthetic non-viral GDVs used to deliver reporter genes to and/or via the intestine in animal models[a]

Vector	Animal	Reporter gene(s)	Delivery route	Refs.
Lipids				
Lipofectin®	Rat	β-Galactosidase	Intrarectal (enema)	[139]
Lipofectamine™	Rat	β-Galactosidase	Intrarectal (double-balloon catheter)	[28,89,140]
DOTMA–DOPE	Rabbit	CAT	Intraduodenal and intracolonic delivery (via laparotomy)	[141]
Lipofectamine™	Mouse	Luciferase	Oral	[142]
DOTAP-cholesterol and enhancer 2	Mouse	EGFP	Intracolonic (via laparotomy)	[143]
Polymers				
Fumaric-sebacic acid co-polymers (microspheres 0.1–10 mms)	Rat	β-Galactosidase	Oral	[144]
Chitosan nanoparticles	Rabbit	CAT	Intraduodenal and intracolonic delivery (via laparotomy)	[141]
	Mouse	β-Galactosidase	Oral	[145,147]
PEI	Rat	Luciferase	Intraduodenal (via laparotomy) and intestinal loop model	[62]
Chitosan and *N*-acetylated chitosan	Mouse	β-Galactosidase	Oral	[146]
Chitosan nanoparticles	Mouse	β-Galactosidase	Oral	[147]
PLGA microparticles containing PEI nanoparticles	Rat	β-Galactosidase	Oral	[148]
Non-ionic polymeric micelles (PEO–PPO–PEO)	Mouse	β-Galactosidase	Oral	[149]
Chitosan microparticles	Mouse	β-Galactosidase	Oral	[49]
PLA–CS nanoparticles and methoxypolyethyleneglycol–PLA–CS nanoparticles	Mouse	Luciferase	Oral	[142]
NiMOS-gelatin nanoparticles within biodegradable PCL microspheres	Rat	β-Galactosidase and EGFP	Oral	[44]

[a] *Abbreviations*: CAT, chloramphenicol acetyltransferase; DOPE, dioleoylphosphatidylethanolamine; DOTAP, 1,2-diacyl-3-trimethylammonium propane; DOTMA, *N*-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethyl ammonium chloride; EGFP, enhanced green fluorescent protein; PEI, polyethylenimine; PEO–PPO–PEO, poly(ethylene oxide)–poly(propylene oxide)–PEO; PLA–CS, polylactic acid–chitosan.

express the therapeutic protein or shRNA, either within or exterior to mammalian cells. A variety of bacterial species have been utilised for intestinal gene delivery, some of which are included in Table 2. Although a range of studies have utilised such bacterial gene therapy approaches, particularly in the area of DNA vaccines [48], the main focus of this review is synthetic delivery vectors.

In vitro models to predict *in vivo* effectiveness

Predicting the impact of extracellular conditions on GDV stability by in vitro *methods*

Cell culture transfection models often do not take into account the extracellular factors that can affect GDV and/or nucleic acid stability. Several media initially developed to assess the *in vivo* performance of drug products might be/have been useful in predicting non-viral GDV stability in the GIT lumen (Table 3).

Modelling the gastric environment is important in predicting the stability of orally administered GDVs. Several simulated gastric media have been developed (Table 3). Simulated mouse gastric fluid (pH 2.1) has also been used before *in vivo* testing (e.g. of chitosan–DNA microparticles [49]). Additionally, animal luminal fluids might also be useful for stability testing [20,50].

One of the major barriers, particularly with regard to liposomal delivery agents, is instability in the presence of bile salts in the small intestine [41,51]. Sodium taurocholate has been used as a model of bile salts to predict cationic liposome–DNA complex stability [52]. Additionally, human bile has also been used in GDV stability studies and was shown to reduce Lipofectamine™-mediated gene transfer *in vitro* [53]. Liposome stability can be improved by several methods, including PEGylation, incorporation of cholesterol and the use of saturated lipids with higher transition temperatures [54–56]. Although some studies have addressed the question of bile salt-associated liposome instability, bile salts associate with products of lipid digestion in the small intestine to form mixed micelles and, therefore, do not usually occur as simple bile salt micelles. Media simulating mixed micelles include fasted state simulated intestinal fluid (FaSSIF) and fed state simulated intestinal fluid (FeSSIF), which might be useful for GDV stability testing [57]. More biorelevant versions of these media, referred to as FaSSIF-V2 and FeSSIF-V2, have been recently developed [58]. A biocompatible media simulating mixed micelles has also been developed that could be particularly useful for transfection studies [59].

The inclusion of pancreatin in simulated intestinal fluid (SIF) provides a source of several digestive enzymes, including lipase, amylase and trypsin. Pancreatin-containing media have been used to predict the enzymatic stability of a range of delivery systems, including liposomes and polymers [51,60]. Furthermore, depending on the GDV being used, other enzymes not present in pan-

TABLE 2

Synthetic and bacterial vectors used to deliver therapeutic nucleic acids and DNA vaccines to and/or via the intestine in animal models[a]

Vector	Animal (disease model)	Therapy	Delivery route	Comments	Refs.
Lipids					
DC-Chol/DOPE	Mouse (cftr^{tm1Hgu} mutant)	pDNA (*CFTR* gene)	Intrarectal	CFTR mRNA expression in rectum; cAMP mediated chloride transport increased by ~20% in ileum and rectum	[129]
Lipofectin®	ApcMin mice	pDNA (*Apc* gene)	Intrarectal enema	*Apc* expression in colon; no significant therapeutic effect	[150,151]
			Oral	*Apc* expression in small intestine; 54% reduction in number of polyps	[124]
Various mixtures of different lipids (Lipodine™)	Mouse (DNA vaccine)	pDNA (HBV HBsAg)	Oral (intragastric)	HBsAg–sIgA response	[152]
Lipofectamine™	ApcMin mice	pDNA (*Apc* gene)	Oral	*Apc* expression not detected in either small or large intestine; ~25% reduction in number of polyps	[153]
Lipofectamine™ 2000	Mouse (DSS colitis)	siRNA (TNF-α)	Intrarectal	Silenced TNF-α in colon; reduced histopathological disease	[154]
DOTAP	Healthy rat	pDNA (insulin gene under the control of GIP promoter)	Duodenum via laparotomy	Insulin expression detected in duodenum	[155]
DOTAP-cholesterol and enhancer 2	Mouse (DSS colitis)	pDNA (IL-22 gene)	Colon via laparotomy	Alleviated disease symptoms; enhanced mucus production	[143]
Lipofectamine™ 2000	Mouse (DNA vaccine delivered before *Mycobacterium tuberculosis* challenge)	pDNA (Ag85a)	Oral (gastric juice pre-neutralised)	*Ag85a* expression in small intestinal cells; enhanced host defence against TB	[156]
Polymers					
PLG microparticles	Mouse (DNA vaccine delivered before rotavirus challenge)	pDNA (VP6, VP4 and VP7 DNA vaccines)	Oral (gastric pH neutralised)	Generated rotavirus-specific antibodies and reduced faecal rotavirus antigen	[157,158]
Chitosan nanoparticles	Mouse (allergen gene immunisation pre-sensitisation and anaphylaxis induction with crude peanut extract and Arah-2)	pDNA (Arah-2 peanut allergen gene)	Oral	Increased sIgA in faecal extracts and serum IgG2a; delayed and/or reduced severity of anaphylaxis	[145]
PLG microparticles	Mouse (DNA vaccine delivered before immune challenge with env-expressing vaccinia virus)	pDNA (HIV env glycoprotein)	Intragastric inoculation	Expression of env glycoprotein in small and large intestine; systemic and mucosal immune responses; resistance to mucosal viral transmission	[159]
PLGA microparticles	Rhesus macaques (DNA vaccine)	pDNA (encoding numerous CTL epitopes)	Intrarectal	Improved CTL response post vaccinia virus immunisation	[160]
Chitosan nanoparticles	Mouse	pDNA (*mEpo* gene)	Oral	Increase in haematocrit	[147]
PLGA microparticles	Mouse (DNA vaccine)	pDNA (HBV HBsAg)	Oral (gastric pH neutralised)	HBsAg expression in GALT; antigen-specific antibody response systemically and mucosally; higher antibody response than IM-administered naked pDNA	[161]
NiMOS–gelatin nanoparticles within biodegradable PCL microspheres	Mouse (TNBS colitis)	pDNA (IL-10 gene)	Oral	Increased IL-10 expression in colon; reduced levels of proinflammatory cytokines; reversal of disease state	[45]
Chitosan nanoparticles	Mouse (haemophilia A)	pDNA (Factor VIII gene)	Oral	Phenotypic correction in 13/20 mice	[131]

TABLE 2 (*Continued*)

Vector	Animal (disease model)	Therapy	Delivery route	Comments	Refs.
	OVA-induced allergic mice	pDNA (TGF-β gene)	Oral	Increased TGF-β expression in small intestine; increased OVA-IgA; decreased serum OVA-IgE; reduced histamine release by mast cells into GIT	[162]
Non-ionic polymeric micelles (PEO–PPO–PEO)	Rat (hypoparathyroidism model)	pDNA (PTH gene)	Oral	Increased levels of serum PTH and calcium; improved survival rate	[163]
Galactosylated chitosan	Mouse (TNBS and $CD^+CD45RB^{hi}$ T cell colitis)	ASO (TNF-α)	Intrarectal	Targeted macrophages, silenced TNF-α; reduced disease state	[104]
Bacteria					
Salmonella typhimurium	Mouse (DNA vaccine delivered before challenge with lethal doses of *Listeria monocytogenes*)	pDNA [ActA and Listeriolysin (*Listeria monocytogenes* virulence factors)]	Oral (in sodium bicarbonate buffer)	Humoral and cellular immune responses; increased survival rates post challenge with *Listeria monocytogenes*	[164]
Lactococcus lactis[b]	Mouse (DSS and IL-$10^{-/-}$ colitis)	DNA (IL-10)	Oral (intragastric inoculation)	50% decrease in DSS colitis pathological symptoms; prevented onset of colitis in IL-$10^{-/-}$ mice; IL-10 expression detected in colon	[100]
S. typhimurium	Mouse (subcutaneously injected with tumour cells before oral therapy)	DNA (CD40 ligand gene)	Oral	CD40L expression in Peyer's patches; significant protection against tumour challenge; increased survival	[109]
Bifidobacterium longum[b]	Mouse (with HepG2 subcutaneous tumours)	Bacteria expressing endostatin	Oral	Inhibited tumour growth by ~50% and prolonged survival time	[110]
Escherichia coli	Mouse (DNBS colitis)	pDNA (TGF-β1 gene)	Oral	TGF-β1 expression; reduced disease state; use of IL-8 inflammation driven promoter increased intestinal specificity of expression	[105]
E. coli[b]	Healthy mice	Bacteria producing shRNA against β-catenin	Oral	Significant gene silencing in intestinal epithelium	[128]
L. lactis	Mouse (DNA vaccine)	pDNA (BLG)	Oral	BLG expression in small intestine; BLG-IgG2a response; reduced levels of serum BLG-IgE post sensitisation with milk proteins	[165]
Bifidobacterium breve[b]	Mice (with B16-F10 subcutaneous tumours)	Bacteria expressing lux reporter	Oral	Bacteria localise in tumour post oral delivery and express reporter gene	[111]
Other					
N/A	SCID and/or nude mice (with subcutaneous tumours)	ASOs (RIα subunit of protein kinase A)	Oral	Inhibited tumour growth; prolonged survival time	[112,113]
GeRPs	Mouse (LPS-induced inflammatory response)	siRNA (MAP4k4 and TNF-α)	Oral	Targeted macrophages, silencing of MAP4k4 and TNF-α, reduced levels of LPS-induced death	[46]
Thioketal nanoparticles (DOTAP–siRNA complexes within ROS labile PPADT microparticles)	Mouse (DSS colitis)	siRNA (TNF-α)	Oral	Improved colonic targeting; silencing of TNF-α and other proinflammatory cytokines; protected mice from DSS colitis	[106]

[a] *Abbreviations*: Ag85a, mycobacterium antigen Ag85a; BLG, β-lactoglobulin; CFTR, cystic fibrosis transmembrane conductance regulator; CTL, cytotoxic T lymphocyte; DC-Chol, 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol hydrochloride; env, envelope; GIP, glucose-dependent insulinotropic polypeptide; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IgE, immunoglobulin E; IgG, immunoglobulin G; IM, intramuscular; LPS, lipopolysaccharide; *mEpo*, erythropoietin gene; OVA, ovalbumin; PLG, poly(lactide-coglycolide); PTH, parathyroid hormone; SCID, severe combined immunodeficiency; sIgA, secretory immunoglobulin A.
[b] Bacterially expressed product (AGT).

TABLE 3

***In vitro* models of gastrointestinal fluids**[a]

Model media	Description	Refs.
Gastric fluids		
Simulated gastric fluid	pH 1.2 and pepsin	[167]
FaSSGF	pH 1.6, pepsin and low levels of bile salt and lecithin	[168]
FeSSGF	pH 5.0 and milk	[58]
Intestinal fluids		
SIF	pH 6.8 and pancreatin	[167]
FaSSIF	pH 6.5, 3 mM bile salt and 0.75 mM lecithin	[57,169]
FeSSIF	pH 6.5, 15 mM bile salt and 3.75 mM lecithin	[57,169]
FaSSIF-V2	pH 6.5, 3 mM bile salt and 0.2 mM lecithin	[58]
FeSSIF-V2	pH 5.8, 10 mM bile salt, 2 mM lecithin and lipolysis products	[58]
Leibovitz (L)-15 SIF	pH 6.0, 5 mM bile salt, 1.25 mM lysophosphatidylcholine and lipolysis products	[59]

[a] Adapted from [166].

creatin might impact stability (e.g. lysozyme-mediated degradation of chitosan [61]). A common obstacle to nucleic acid delivery to the gut is the high concentration of luminal nucleases (the estimated nucleolytic activity of porcine small intestinal juice is 20 U/ml deoxyribonuclease I) [7]. Several other studies have assessed the ability of GDVs to protect their DNA cargo from DNase-mediated degradation, with significant nuclease resistance reported [44,49,61].

In vitro studies have examined the impact of mucus on GDV activity. As discussed below, mucus-producing subclones of Ht29 cells are useful tools in predicting the effect of mucus on GDV transport [62]. Alternatively, *in vitro* prepared simulated mucus can be added directly to IEC monolayers [63]. However, this methodology overestimates the thickness of the mucous layer. Directly mixing non-viral GDVs with mucin has also enabled researchers to examine the effects of mucin on transfection as well as on the size and charge of transfection complexes [64,65]. Several more sophisticated methods have also been utilised to measure transport through mucus directly. Indeed, fluorescence recovery after photobleaching (FRAP) techniques have been used to examine the diffusion of DNA in bovine cervical mucus [66]. Alternatively, Hanes and co-workers have developed a multiple-particle tracking system that can examine the influence of mucus at the individual particle level, providing such information as the number of particles adherent to mucus at a given time [9].

Cell culture models

Most studies assessing GDV transfection activity *in vitro* utilise rapidly growing immortalised cells. However, these are poor models of the intestinal epithelium. Therefore, several differentiated cell culture models have been developed that might be more physiologically relevant and might improve *in vitro–in vivo* correlation of data.

Caco-2 cells

The Caco-2 cell line was first established during the 1970s from a human colon adenocarcinoma and is one of the most well-established intestinal cell models [67]. These cells differentiate over 2–3 weeks in a Transwell™ filter culture to form a monolayer of highly polarised cells with TJs, a glycocalyx and apical microvilli (i.e. characteristics of mature enterocytes). In addition, they express several membrane and cytosolic enzymes common to intestinal enterocytes *in vivo*. The Caco-2 Transwell™ system separates apical and basolateral compartments and has been traditionally used to predict the intestinal permeability of drugs [67]. In terms of gene delivery studies, the Caco-2 Transwell™ system could similarly be used to assess the transcellular transport capacity of GDVs.

Studies have assessed the effect of Caco-2 cell differentiation on the transfection efficiency of apically administered synthetic GDVs [27,62,68]. Caco-2 cells become increasingly resistant to transfection as they differentiate. The differentiation-induced drop in transfection was associated with a decrease in particle uptake, albeit not at a level that could entirely explain the reported drop in transfection. Given the non-dividing nature of differentiated cells, poor nuclear accessibility is likely to contribute to the drop in transfection. Differentiated Caco-2 cells have also been useful in quantifying the level of expressed proteins that gain access to the basolateral compartment [20]. Measurement of the transepithelial electrical resistance (TEER) of Caco-2 monolayers can also provide information on monolayer integrity and/or the TJ-destabilising activity of GDVs. The differentiated Caco-2 cell model has also been used to assess transgene expression levels mediated by bacteria [69].

Ht29 and Caco-2/Ht29 co-cultures

Some limitations of the Caco-2 cell line include the absence of a mucus layer and the fact that it represents only the enterocytes of the intestinal epithelium. Several clones of the Ht29 cell line (a human colon carcinoma) have been established, including the Ht29GlucH clone, which has been reported to form monolayers with a high proportion of goblet cells capable of secreting mucin molecules [70]. Indeed, after 4 weeks in culture, a mucous layer approximately 40–60 μm thick with >95% coverage was reported overlying the cells. The Ht29GlucH model, as well as co-cultures of Caco-2 and Ht29GlucH cells, has been used to assess the effect of mucus on drug transport [71–73]. Cryan and O'Driscoll utilised these co-cultures to assess the transfection activity of several non-viral GDVs [62]. Interestingly, they reported that removal of the mucus layer by *N*-AC treatment reduced the transfection activity of cationic synthetic vectors, whereas *N*-AC treatment of Caco-2

cell monolayers had no effect. However, this study did not take into account the turnover time of intestinal mucus.

A more phenotypically stable sub-clone, termed Ht29MTXE12, was developed to overcome problems associated with the Ht29GlucH sub-clone, including the multilayer growth of cells [74]. Once differentiated, these cells are defined by TJ formation as well as a mucous layer 142 ± 51 μm thick [75], which is comparable to that found in the human colon. This could provide a more reliable cell model in future gene therapy studies. Indeed, Kissel's group utilised this model to predict the effect of mucus on nanoparticle uptake by IECs [76]. This cell culture model has also been used to assess the mucoadhesion of polymers, and data correlated well with measurements from rat intestinal tissue [77].

FAE model

Given that M-cells might provide a more favourable means of GDV access to underlying cells, *in vitro* models of the FAE could be useful. These are based on the principle that lymphocytes convert enterocytes to M-cells, as demonstrated by Kerneis *et al.*, who first developed an *in vitro* FAE model [78]. Caco-2 cells are cultured upside down on Transwell™ filters for 14 days before addition of mouse Peyer's patch lymphocytes, which intercalate into the Caco-2 monolayer and induce M-cell formation. Gullberg *et al.* developed an alternative and simpler model by culturing physically separated Caco-2 cells and human Raji B-cells [79]. Similarly, des Rieux *et al.* developed a FAE model using inverted Transwell™ with Raji B-cells in direct contact with Caco-2 cells [80]. These latter two models are advantageous in that the cells used are of human origin; avoiding the use of primary murine lymphocytes. These FAE models share several characteristics of the FAE of Peyer's patches *in vivo* with M-cells, having fewer or no microvilli, as well as reduced alkaline phosphatase activity. FAE models have been used in an effort to identify human M-cell cell surface targets [81]. In addition, these FAE models have proved useful in examining the association and uptake of nanoparticles and microparticles [79,82–84]. In general, the FAE models are characterised by higher levels of transport than is the Caco-2 model, which resembles the situation *in vivo*. FAE models might be useful in assessing the contribution of M-cells to transfection and/or transepithelial transport, and in the development of M-cell targeted GDVs. Indeed, a recent *in vitro* study demonstrated that adenovirus-mediated transfection of FAE was higher than in differentiated Caco-2 monolayers [85].

IEC–macrophage co-culture systems

In addition to the differentiated monolayer cell models of the intestine, multi-layer cell culture systems offer another dimension to gene delivery research. This involves the culture of an epithelial monolayer on the semipermeable membrane of a Transwell™ plate and the culture of another cell type of interest in the basolateral compartment. Such systems might be useful in applications where the target cells are located beneath the intestinal epithelium. A multi-layer cell model that could be of particular interest to intestinal gene delivery research is the IEC–macrophage co-culture system. In principle, IECs (e.g. Caco-2 or T84 cells) are cultured on the Transwell™ membrane until fully differentiated, and a human or murine macrophage cell line (e.g. THP-1 or RAW264.7 cells) or primary isolated human mononuclear cells are cultured on the basolateral side of the Transwell™ plate [86–88]. The basolateral macrophages are generally stimulated with lipopolysaccharide (LPS), resulting in the production of TNF-α by macrophages and IL-8 by IECs. In addition, the barrier integrity of IECs has been reported to decrease under these conditions. Notably, increased cytokine production and barrier dysfunction are characteristic of IBD [21]. This co-culture model has numerous applications. The ability of GDVs targeting macrophages to traverse the 'leaky' epithelium and silence macrophage-associated proinflammatory cytokine expression (e.g. TNF-α) could be assessed, as could the delivery of DNA-based vaccines to basolateral macrophages. The introduction of Caco-2/Ht29 and FAE co-cultures to the epithelial layer is another possibility.

In vivo models

Although *in vitro* models are useful, they do not completely represent the complex environment of the intestine. Several studies have tested the ability of non-viral GDVs to mediate a local and/or distant biological response, be it transgene expression, gene silencing, and/or therapeutic effect(s) following intestinal delivery (Tables 1 and 2).

In situ *models*

Although many of these studies involve direct *in vivo* application of GDVs, be it by oral or rectal routes, several *in situ* strategies are also worth noting. These involve the direct delivery of GDVs to isolated intestinal segments of animals under anaesthesia. The use of a balloon catheter is one such method. This involves inserting a double-balloon catheter into the colon of anaesthetised rats, which, upon inflation, creates a space in the colon into which the GDV is administered, facilitating increased GDV contact time with the mucosa. Using this method, successful transfection of the colonic epithelium with various synthetic GDVs has been reported [28,89]. Intestinal loop models have also been utilised in gene therapy research, which involve injection of GDVs into ligated isolated segments of intestine. This model has been used with both viral [90,91] and non-viral GDVs [62]. Although these *in situ* approaches might facilitate increased transfection activity, they are invasive and less patient friendly; and are, therefore, more unlikely to be utilised in human studies.

Animal disease models

Animal disease models are important tools in developing novel therapeutics and can facilitate assessment of the curative potential of nucleic acid therapies through assessment of disease symptoms. In addition, the influence of the disease state on gene expression and/or knockdown efficiency can also be determined. In such models, indicators of disease severity should be predetermined to enable fair assessment of the therapeutic benefits of different gene therapy strategies.

IBD

Crohn's disease and ulcerative colitis are the two major forms of IBD. Crohn's disease can potentially affect any part of the GIT, but most cases occur in the distal ileum or the proximal colon. By contrast, ulcerative colitis affects only the large intestine [92]. Several lines of evidence suggest that the pathogenesis of IBD is dependent on the interaction between local immune and environmental factors in genetically susceptible individuals [93].

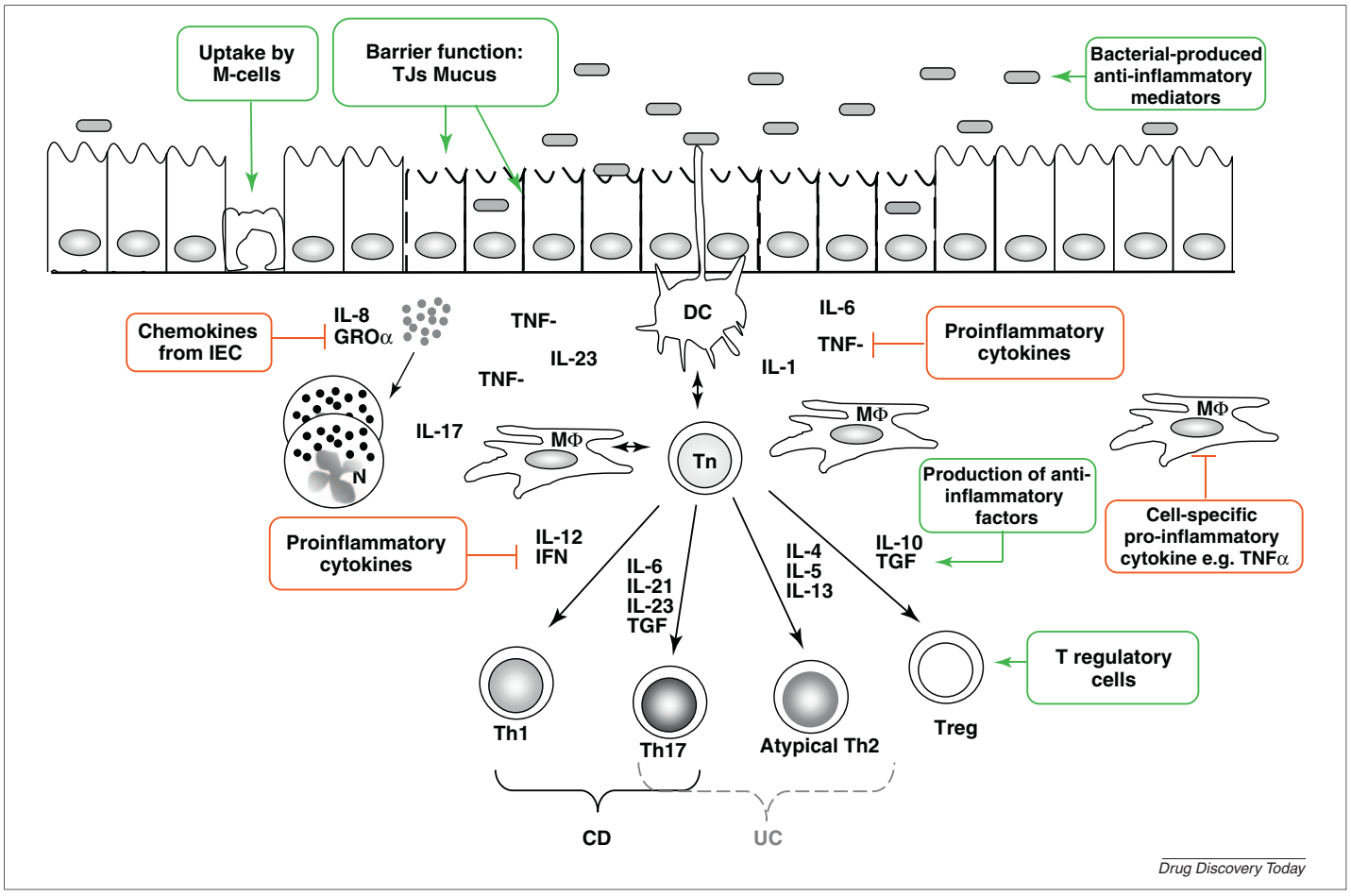

FIGURE 2

Schematic view of IBD pathology and potential gene therapy targets. In IBD, the commensal and pathogenic bacteria, and a combination of both internal and external stimuli, induce cytokine and chemokine production by IECs, DCs and macrophages (MΦ). Different cytokines regulate the differentiation of naïve T cells (Tn) into T effector cells. The cytokines IFN-γ and IL-12 induce T helper 1 (Th1) cells, whereas IL-6, IL-21, IL23 and TGF-β induce Th17 cells. The immune response in Crohn's disease (CD) is directed by Th1 and Th17 cells. By contrast, the immune response in ulcerative colitis (UC) is an atypical Th2 response with high production of IL-5, IL-10 and IL-13 together with Th17 cells. An enhancement in T regulatory cells (T reg), differentiated by IL-10 and TGF, exists in both diseases. Production of the proinflammatory cytokine, IL-17, induces cytokine production from MΦ as well as neutrophil (N) recruitment. Endothelial cell-produced cytokines and chemokines also lead to N recruitment. Production of chemokines, such as IL-8 and growth-regulated oncogene (GRO)-α, from IECs can attract N to the intestinal wall. The epithelial barrier is also affected by altered TJ permeability, goblet cell numbers and mucus production. Potential targets for gene therapy are highlighted in boxes outlined in either red or green. Inhibition of proinflammatory cytokines and chemokines, such as TNF-α, IL-6, IL-17, IL-23 and IL-8, can dampen inflammation by inhibiting the activation of MΦ and DCs or by reducing N influx. Silencing of MΦ-produced TNF-α could provide a targeted strategy. Another approach could be to potentiate the production of regulatory or anti-inflammatory cytokines, such as IL-10. Barrier function might also be improved through enhanced mucus production (e.g. through expression of IL-22 in IECs) or stabilised expression of TJ proteins. The protein p120-catenin was recently shown to be essential in maintaining barrier function and might be an important gene therapy target [170]. In addition, increased levels of M-cell-mediated uptake might facilitate access to underlying immune cells.

Inflammation in Crohn's disease is characterised by high production of the cytokines interferon (IFN)-γ, IL-17 and TNF-α [representing a T helper1 (Th1)–Th17 response]. In ulcerative colitis, the immune response is characterised by Th17 and an atypical Th2, with high production of IL-5, IL-10 and IL-13 (Fig. 2) [94]. Currently, most treatments in patients with IBD are primarily aiming to suppress disease severity and to prolong remission time. Given problems associated with traditional immunosuppressive drug treatments, it has been proposed that gene therapy could have a role in IBD treatment [95]. Fig. 2 outlines several potential targets for gene therapy intervention.

There are currently over 40 mouse models of intestinal inflammation described, divided into chemically induced models, spontaneous models (most owing to genetic manipulation) and models dependent on transfer of cells to immunodeficient recipients [96]. Although no animal model represents all aspects of human IBD, such models are useful tools. The most commonly used models in non-viral gene delivery studies are based on colitis induction in rats and mice by chemical agents, such as dextran sodium sulphate (DSS) administered in drinking water [97], and trinitrobenzene sulfonic acid (TNBS) administered intrarectally [98]. Another widely used IBD model is the IL-10 knockout (IL-$10^{-/-}$) mouse [99]. Diseased mice are characterised by disrupted epithelium, infiltration of immune cells and luminal bacterial translocation; resembling several features of the human diseases. The severity of colitis can be evaluated by several methods, such as: assessing clinical signs of disease including body weight loss, stool consistency and rectal bleeding; macroscopic markers of disease, includ-

ing colon macroscopic score, colon length and weight; and colon markers of inflammation, including proinflammatory cytokines, colon histology and myeloperoxidase activity.

Amiji's group utilised TNBS-induced colitis mice to assess the therapeutic activity (as determined by the above parameters) of IL-10 gene therapy with NiMOS [45]. IL-10 is an anti-inflammatory cytokine that acts, in part, by inhibiting the production of proinflammatory cytokines by macrophages and by inducing T regulatory cells. Indeed, IL-10 expression was associated with a decrease in the level of proinflammatory cytokines along with significant therapeutic effects. Similarly, IL-10-producing *Lactococcus lactis* were reported to have a therapeutic benefit in DSS-induced colitis and prevented colitis development in IL-$10^{-/-}$ mice [100]. More recently, a clinical trial in human patients with Crohn's disease using this approach was undertaken, with reduced disease activity reported post treatment [101]. Non-viral approaches to silence the expression of disease-associated genes have also been assessed. Indeed, an ASO against ICAM-1 (intercellular adhesion molecule-1) has been developed by Isis (ISIS 2302-Alicaforsen; http://www.isis.stfc.ac.uk) and has demonstrated some beneficial effects in patients with ulcerative colitis [102]. Alicaforsen was administered rectally via enema, possibly because oral delivery of an ASO against ICAM-1 was reported to be ineffective in DSS mice [103]. More recently, galactosylated chitosan complexed with ASO against TNF-α successfully silenced its expression in macrophages in TNBS-induced colitis mice and had significant therapeutic benefit [104]. This recent study is of particular significance to non-viral intestinal gene delivery because a specific cell type is actively targeted. Indeed, increasing the specificity of gene delivery might be an important step in facilitating safer and more effective IBD treatments.

Another potential targeting strategy utilised by Castagliuolo *et al.* is the use of inflammation inducible promoters, such as the IL-8 promoter [105]. Indeed, they reported transforming growth factor (TGF)-β1 transgene expression mainly in the inflamed colonic mucosa using *Escherichia coli* as the GDV. A more formulation-based approach to achieve targeted siRNA delivery for IBD treatment has been recently reported by Wilson and colleagues [106]. Here, TNF-α siRNA was pre-complexed with DOTAP, and these complexes were formulated within the reactive oxygen species (ROS)-sensitive polymer, poly-(1,4-phenyleneacetone dimethylene thioketal) (PPADT). This polymer prevented complex degradation in GIT fluids. However, increased ROS levels at sites of intestinal inflammation led to polymer degradation and the selective release of therapeutic siRNA. Using this approach, increased colonic targeting and significant reductions in TNF-α expression were reported in DSS colitis mice following oral delivery.

Administration of LPS, a component of the outer membrane of Gram negative bacteria, induces endotoxemia. This acute inflammation model has been used to test nucleic acid-based treatments for inflammation. Indeed, orally delivered GeRPs containing siRNA against mitogen-activated protein kinase kinase kinase kinase 4 (Map4k4) mediated significant protection against LPS-induced lethality in mice [46]. This was the first demonstration of effective oral siRNA delivery *in vivo*.

Cancer and familial adenomatous polyposis

Non-viral gene delivery to the intestine has potential in the treatment of local intestinal cancers as well as distant cancer sites. Colorectal cancer is one of the most common forms of cancer and several gene therapeutic strategies have been proposed as treatments (Fig. 3) [107,108]. The pathogenesis of spontaneous and colitis-associated colon cancer are summarised in Fig. 3. To date, most cancer models used are based on local tumour induction following subcutaneous injection of cancer cells (xenografts or allografts). Most synthetic GDV approaches have assessed therapeutic effect following intravenous or intratumoural injection. However, orally delivered bacteria (bactofection and AGT) have shown antitumour activity in subcutaneous cancer models as assessed by tumour volume and mouse survival [109,110]. Furthermore, a recent study provided direct evidence of reporter gene expression in tumours following oral administration of *Bifidobacterium breve* [111]. Therefore, orally administered gene therapies have potential in treating distant cancer sites. Indeed, orally administered ASOs targeting the RIα subunit of protein kinase A have also demonstrated antitumour activity in nude mice bearing subcutaneous xenografts [112,113].

Although several local intestinal cancer models exist [114], few non-viral studies have utilised them. Given its high incidence, models of colon cancer are more likely to be studied. As mice and rats do not generally spontaneously develop colon cancer, induction of tumour growth is required. This can be achieved by genetic or chemical methods. Similar to the induction of subcutaneous tumour growth described above, cancer cells can also be injected into the large intestine, inducing local tumour growth as well as metastases in other tissues [115]. The use of the carcinogen azoxymethane (AOM) or its precursor 1,2-dimethylhdrazine (DMH) is among the most commonly used chemical approaches, with AOM being used more routinely owing to its higher potency [116,117]. Repetitive AOM and DMH administration (generally over a period of 10 weeks) has been reported to primarily induce colonic tumours in several animal species, but has been generally used in mice and rats. The carcinogen-induced colonic tumours resemble human colon cancer in terms of their histology and morphology and, similar to human tumours, are often mutated on genes encoding K-ras and β-catenin (Fig. 3) [118]. However, several discrepancies also exist. Indeed, whereas metastasis is common in human colorectal cancer, there is low incidence in DMH-induced and AOM-induced cancer [119]. Furthermore, induced tumours, unlike many human cancers, are only infrequently mutated at the adenomatous polyposis coli (*Apc*) gene and are not mutated at the *P53* gene [118,120]. Although it is believed that human colon cancers develop from polyps, no evidence of a benign polyp to rat colon adenocarcinoma transition has been reported in either AOM-treated or DMH-treated rodents [119]. A model of colitis-associated colon tumour development with aberrant β-catenin, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) (Fig. 3) has also been developed that combines AOM and DSS [121]. Several other chemical inducers of colon carcinomas have also been used and include amino(α)carboline and 2-amino-l-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) [122].

Genetic mouse models that spontaneously develop intestinal cancer have also been developed. Mutations in the *Apc* gene are associated with the development of colon cancer as well as a condition called familial adenomatous polyposis (FAP). FAP is an inherited condition characterised by the development of

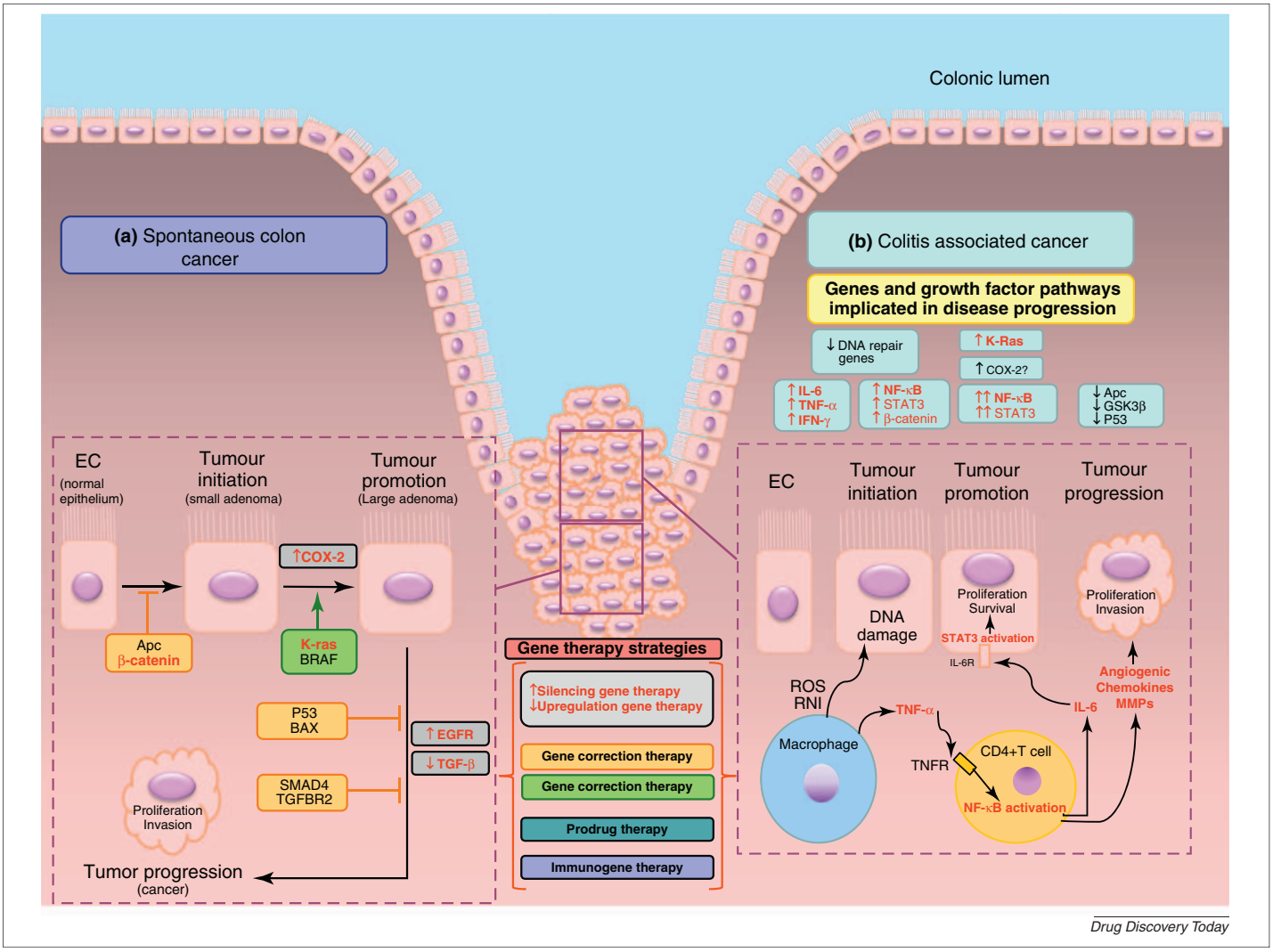

FIGURE 3

Mechanisms of **(a)** spontaneous colorectal cancer and **(b)** colitis-associated cancer development and gene therapy strategies. The initial step in spontaneous colorectal cancer is associated with mutations of Apc and deregulation of β-catenin. Larger adenomas and early carcinomas acquire mutations in the GTPase K-ras, followed by loss of chromosome 18q with SMAD4, which is downstream of TGF-β downregulation, and P53 mutations. Epidermal growth factor receptor (EGFR) is upregulated and mediates signalling by activating the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signalling cascades. Colitis associated-cancer is mediated by cells of the innate immune system (e.g. macrophages) through release of ROS or nitrogen intermediates (RNI), which lead to DNA damage in healthy epithelial cells by mutations in oncogenes and tumour suppressor genes (e.g. *Apc*, *P53* and *K-ras*) and genomic instability. Persistent inflammation owing to high cytokine production facilitates tumour promotion, leading to the recruitment of adaptive immune cells into the site of inflammation. CD4+ effector T cells release tumour-promoting cytokines (e.g. IL-6), angiogenic chemokines and matrix metalloproteases (MMPs), enabling tumour progression. Several gene therapy strategies for colon cancer treatment exist. (i) Gene knockdown/upregulation/correction: gene knockdown or upregulation can be achieved through delivery of DNA, shRNA, ASOs or siRNAs. Both the P53 tumour suppressor gene and K-ras proto-oncogene are mutated in ~50% of colorectal tumours and are, therefore, attractive targets. (ii) Prodrug therapy involves gene transfer of a viral or bacterial enzyme into tumour cells, which then converts an inactive prodrug into a short-lived toxic metabolite, leading to tumour-cell death. Enzyme–prodrug combinations include: thymidine kinase and ganciclovir; and cytosine deaminase and 5-fluorocytosine. (iii) Immunogene therapy involves activation of an immune response against cancer cells. Several strategies of immunogene therapy have been explored: cytokine gene transfer to tumour or T cell, co-stimulation with B7 and vaccination against tumour-associated antigens or epitopes.

numerous polyps in the colon. Some of these polyps can subsequently become malignant adenocarcinomas. Several mouse models based on mutations in the *Apc* gene have been developed. The first of these, the multiple intestinal neoplasia (ApcMin) mouse, was identified following random mutagenesis [123]. ApcMin mice develop numerous intestinal polyps and are a useful model for FAP and cancer development. Non-viral studies have utilised the ApcMin mouse model (Table 2). In one such study, Lipofectin® complexed with a plasmid containing a functional *Apc* gene was administered orally to ApcMin mice and a 54% reduction in the number of polyps was reported [124]. One of the main limitations of this model is that most polyps develop in the small intestine rather than the colon, which is the main site of polyp formation in human FAP. Numerous other mouse models based on different mutations in the *Apc* gene have also been developed [114,125]. One such mutant *Apc* model was constructed in which multiple polyps form in the distal colon [126], which might be a more useful model in future studies. Mice models of hereditary nonpolyposis colorectal cancer, with mutations in mismatch repair genes, have also been developed [127].

In terms of FAP and human gene therapy, a recent study utilising orally delivered *Escherichia coli* expressing shRNA against β-catenin showed significant silencing activity in healthy mice [128]. Recently, a clinical trial using this approach has been approved for use in the treatment of FAP (http://www.cequent-pharma.com). This is the first clinical trial to use orally delivered RNAi technology.

Other disease models

Several other animal disease models have been used successfully in non-viral gene therapy research to/via the intestine (Table 2) and include models of cystic fibrosis [129], duodenal ulcers [130], and haemophilia [131]. Models of diabetes may be useful in future work given that gut K cells have been identified as potential targets for insulin gene therapy in Type 1 diabetes [132].

Conclusions and future directions

The non-viral delivery of gene therapeutics to the GIT is a significant challenge given the host of barriers that exist at both the extracellular and cellular level. Modelling of these systems is desirable and can facilitate elucidation of the main contributory factors impacting activity. Indeed, several *in vitro* models exist that provide an opportunity for the preliminary testing of GDV activity and/or stability. Furthermore, *in vivo* studies have verified that transfection and/or translocation of the GIT can be achieved by non-viral means. Animal disease models will become increasingly important in the future as more research groups make the step from simple proof-of-concept to therapeutic studies. Importantly, care must be taken when extrapolating data from small animal studies, as interspecies differences can have a significant bearing on activity. Indeed, the pH of the luminal contents of mice and rats is different to that generally found in humans [133]. Of particular note is the difference in gastric pH: approximately pH 1–2 in humans and approximately pH 3–5 in mice and rats. Therefore, the gastric stability of orally delivered GDVs might be overestimated in rodents. Furthermore, the proportion of M-cells in the FAE varies depending on the model animal used (e.g. the FAE of rabbits consists of ~50% M-cells, whereas that of humans contains only ~5% [24,37]). Therefore, the contribution of M-cells might also be overestimated in some animal models.

Strategies to improve GDV activity and/or stability will need to be considered to achieve optimal intestinal delivery. Notably, PEGylation can help prevent GDV aggregation and interaction with mucus components [64]. In addition, PEGylation has been reported to confer increased stability in gastric and pancreatic fluids [134], as well as improved resistance to nuclease attack [135]. An alternative strategy to achieve increased nuclease resistance is replacement of nuclease labile 'hot spot' regions in the pDNA sequence [136,137]. Furthermore, a more targeted approach might need to be adopted to increase the activity and reduce the likelihood of unwanted side effects associated with gene therapies. Targeting ligands on the surface of nucleic acid carriers could provide a means of transfecting specific cell types *in vivo*. Examples of their use thus far include the targeting of GALT macrophages by GeRPs–siRNA and galactosylated chitosan–ASO nanoparticles. Several targeting group strategies to increase the uptake of drug-loaded nanoparticles by enterocytes and M-cells have been developed [24], some of which might be applicable in gene therapy targeting. More elaborate formulation strategies could provide a platform for increasing both the specificity and stability of GDVs *in vivo*. This might involve the encapsulation of nanoparticles (containing nucleic acid) within microscale oral delivery devices [138]. Indeed, this strategy has already accomplished some success, as demonstrated with lipase labile NiMOS utilised by Amiji and co-workers [44]. The inclusion of nanoparticles (containing nucleic acid) within other environmentally sensitive (e.g. pH or colonic enzymes) microsphere systems also has significant potential [38,138]. Indeed, the increased ROS levels associated with areas of intestinal inflammation have recently been exploited as a means of achieving the selective release of therapeutic siRNA at disease sites [106].

Whereas most studies to date have focused on DNA delivery, it is likely that future studies will focus more on RNAi-based approaches to treat disease. Indeed, siRNAs are highly potent molecules that are effective at low concentrations and do not require nuclear entry. Another area of interest is miRNAs. These are short non-protein coding RNAs of 20–22 nucleotides known to alter gene expression at the post-transcriptional level. Their biological importance in the pathogenesis of gastrointestinal inflammatory conditions and cancer is currently emerging, which could result in several therapeutic opportunities in the future [94].

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