ORIGINAL ARTICLE

Construction of minicircle DNA vectors capable of correcting familial hypercholesterolemia phenotype in a *LDLR*-deficient mouse model

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Familial hypercholesterolemia (FH) caused by defect in *low-density lipoprotein receptor* (*LDLR*) is a life-threatening disease with poor response to conventional treatments. Earlier gene therapy studies have generated promising results, but further development is hampered because the cells harboring the viral vectors were eliminated by host immune system soon after delivery, whereas the nonviral vectors were too bulky to be delivered to target cells. To overcome these problems, we constructed multiple minicircle (MC) DNA vectors to express the therapeutic LDLR. MC is an optimized nonviral vector that is capable of expressing high level of transgene product persistently. We found that among the seven MCs tested, the best is MC5 with multiple advanced features. First, the *LDLr* gene was placed under the control of sterol regulatory element (SRE) using *LDLr* gene promoter or apoprotein E (ApoE) promoter, allowing the transcription of the *LDLr* gene to be regulated by serum low-density lipoprotein (LDL) cholesterol as its functional gene counterpart. Second, a hepatic control region (HCR) was placed upstream of the promoter that serves as a controller to ensure liver-specific expression. Third, the modified Kozak sequence was placed in front of the *LDLr* gene start codon to enhance its translation efficiency. MC5 was 5.23 kb in size, and was capable of tight physiological control in intracellular LDL cholesterol level even when challenged with high dose of sterols *in vitro*. Importantly, it was able to correct the phenotype of *LDLR*-deficient mice C57BL/6 *LDLR*^{-/-} for more than 105 days without detectable toxicity. Therefore, this MC has the clinical application potential for treating FH.

INTRODUCTION

Familial hypercholesterolemia (FH) is an inherited metabolic disorder characterized by elevated levels of low-density lipoprotein (LDL) in plasma and an increased risk of atherosclerosis and coronary heart disease. Loss-of-function mutations of the *low-density lipoprotein receptor* (*LDLR*) gene are the most prevalent cause in either one (heterozygote) or both alleles (homozygote).[1–3] The function of the *LDLR* is to mediate the cellular uptake of the circulating LDL cholesterol (LDL-C) particle for catabolism. Therefore, in these patients especially in those of homozygotes, the LDL-C level in the circulation can reach extremely high level and result in the onset of cardiovascular diseases as early as in childhood. The prevalence of homozygous and heterozygous FH is ~1 in 1 million and 1 in 500 people worldwide, respectively. Patients with homozygous FH are minimally responsive to conventional LDL-lowering pharmacological therapies such as inhibitors of hydroxymethylglutaryl coenzyme A reductase (statins) that work by increasing LDLR-mediated LDL-C uptake. Currently, the standard of care for homozygous FH is apheresis, a physical method of purging plasma of LDL-C that can transiently reduce its level by >50%.[4] However, apheresis must be repeated every 1 to 2 weeks because re-accumulation of LDL-C in plasma always occurs shortly after the treatment.[5,6] Because of the difficulty in the treatment of this disease, alternative treatments are being searched vigorously.

Gene therapy, especially expression of *LDLR* in the liver, has been studied vigorously to correct this mono-gene disorder.

Liver is the main organ in maintaining cholesterol homeostasis. Although all cell types participate in cholesterol metabolism, 70% of circulating LDL-C is removed by the liver. Hepatocytes are the major cells in LDLR-mediating uptake of circulating LDL-C, and are the only cells that are capable of eliminating cholesterol through excreting bile. The regulation of the LDLR expression has been well documented.[2,7–9] When the protein SREBP (sterol regulatory element-binding protein 1 and 2) senses the cholesterol in the endoplasmic reticulum, it changes its conformation and binds to two other proteins: SCAP (SREBP cleavage activating protein) and Insig-1. When cholesterol levels fall, Insig-1 dissociates from the SREBP–SCAP complex, allowing the complex to migrate to the Golgi apparatus. Here SREBP-SCAP is cleaved by S1P and S2P (site-1 and -2 protease, two enzymes that are activated by SCAP), subsequently, the resulted SREBP migrates to the nucleus, and binds to the sterol regulatory element (SRE) to turn on the transcription of *LDLR*.

One of the desirable features for the gene therapy vectors is long-term expression of *LDLR* at physiological levels, but overexpression of this protein is cytotoxic.[10,11] In an early attempt, use of a 154-kb whole genomic locus of the *LDLR* leads to long-term and full complementation of the LDLR deficit in primary FH

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patient fibroblasts.[12] In a later experiment, the regulatory elements have been narrowed down to 10 kb upstream of the coding region.[11] However, the bulky vectors are hard to manipulate and deliver to human liver. Recently, adenovirus and adenovirus-associated virus vectors have been tried to express the receptor. The adenovirus vector was able to correct the LDLR deficit phenotype in a clinical trial. However, the therapeutic effect was transient because the host immune system rejected all the virus-residing hepatocytes. The adenovirus-associated virus vectors encoding the *LDLR* gene were also able to significantly reduce the LDL-C in a LDLR-deficient mouse model.[6] Nevertheless, their potential genomic toxicity may hamper clinical application.

To make a gene therapy vector with clinical application potential, we used minicircle (MC) to express the *LDLR* gene. MCs are a class of optimized nonviral vectors made by elimination of backbone DNA sequences from parental plasmids via DNA recombination. It has been shown that MCs free of the plasmid backbone are capable of stably expressing therapeutic level of gene product *in vivo*.[13–16] These vectors have been used for years in preclinical gene transfer research because of their 10- to 1000-fold enhancement compared with regular plasmids in long-term transgene expression in quiescent tissues *in vivo*[14] and *in vitro*.[17] Compared with plasmids, MC DNA benefits from higher transfection efficiencies because of its smaller sizes, longer ectopic expression because of its lower activation of exogenous silencing mechanisms[13] and improved resistance to the shearing forces because of its smaller sizes and super-coiled structures.[18] In the present study, a set of MCs were made with multiple enhancements. First, the *LDLR* gene control region was shortened from 10 to 2 kb, and the *LDLR* gene was placed under the control of hepatic control region (HCR) using *LDLr* gene promoter or apoprotein E (ApoE) promoter, allowing the transcription of the *LDLr* gene to be regulated by serum LDL-C as its functional gene counterpart. Second, a HCR was placed upstream of the promoter that serves as a controller to ensure liver-specific expression. Third, a modified Kozak sequence was used to increase translation efficiency. The resulted MC vectors were 3.85 to 5.23 kb in size and were tested to verify the ability in long-term expression of therapeutic level of LDLR and the function in sterol feedback control. We conducted vigorous *in vitro* and *in vivo* function tests, and found one of them demonstrated the desired features and had the great potential for clinical application in treating FH.

RESULTS

MC DNA constructs

The LDLR gene comprises 18 exons scattering in a 45-kb locus. It generates a 5.3-kb mRNA with a 2.6-kb coding sequence that gives rise to the final mature LDLR protein of 839 amino acids.[19] Early experiments have shown that vectors of 135 kb[12] or 10 kb[11] are able to confer the physiological regulation of LDLR expression and correct the LDLR-deficient phenotype. Because the bulky vectors are hard to construct and deliver, we set off to make vectors encoding much smaller control elements using the plasmid backbone-free MC technology (Figure 1a). A total of seven LDLR-expressing MCs were made (Figure 1b). MC1 encoded the *LDLR* promoter to drive the expression of *LDLR* gene with a wild-type Kozak sequence, and MC1 was used to serve as control. Compared with MC1, MC2 and MC3 carried the additional SRE, and the wild-type Kozak sequence was replaced with mutated counterpart in MC3. Compared with MC2 and MC3, MC4 and MC5 carried the additional HCR element. Compared with MC4 and MC5, the *LDLR* promoter was replaced with ApoE promoter in MC6 and MC7. The size of the MCs was between 3.85 and 5.23 kb.

Regulation of LDLR expression in HepG2 cells

MC1–7 were used to transfect the HCC cell line HepG2 and *LDLR* mRNA expression profiles were determined with or without treatments of sterol or statin (using the ZY781 as control that was free of the *LDLR* expression cassette). The results were presented in Figure 2a and Table 1. The value of the HepG2 harboring the control plasmid ZY781 without any treatment was set as 1 and used as reference for individual measurements. Unexpectedly, the treatments of sterol (SL) and statin (SN) resulted in only small response in *LDLR* mRNA in the HepG2 cells harboring ZY781 even though these cells carry the normal *LDLR* gene (as indicated by the small SN/SL ratio of 1.43, Table 1). Interestingly, all the cells transfected with the MC1 increased the expression level by 41 to 74%, but demonstrated a similar minor responsiveness pattern to the treatments (the *LDLR* was under the control of the 2 kb *LDLR* promoter without SRE, Figures 1b and 2b and Table 1). The results suggested that the transgene rendered an increased constitutive expression but not a physiological control. In all the cells transfected with MC2 to MC5, *LDLR* mRNA increased by 75 to 93%, whereas the NS/NL ratios increased by ⩾2-fold, suggesting that the SRE rendered the cells with higher level as well as the desired physiological control in LDLR expression. Because the difference in transgene expression level was negligible between the HCR-free MC2 and MC3 vs HCR-encoding MC4 and MC5 groups, this genetic element appeared to play an insignificant role in the regulation of the *LDLR* mRNA expression. In the MC6 and MC7 groups (where the *LDLR* promoter was replaced with ApoE promoter), *LDLR* mRNA increased by 8-fold to >10-fold, much more than that of the MC2 to MC5 groups. However, similar small SN/SL ratios in the MC6 and MC7 groups were seen as in control ZY781 and MC1 groups (Figure 2a and Table 1). These observations suggested that the ApoE promoter rendered a very strong constitutive expression of *LDLR* mRNA, but not the desired physiological control function in response to sterol and statin treatments.

Regulation of LDL uptake in HepG2 cells

In all the cells treated with MC2 to MC5 carrying both *LDLR* promoter and SRE, the SL/SN ratios of the LDL uptake capacity (Table 1 and Figures 2b and c) increased from <1.5 in ZY781 and MC1 groups up to 10.91 in MC2–5 groups. The SL/SN ratios of the LDL uptake capacity were much greater than that of both the intracellular LDLR mRNA and protein (Table 1 and Supplementary Figures 1a and b), especially in the MC3 and MC5 groups carrying the mutated Kozak sequence. The results suggested that these MCs had the optimal artificial promoter/enhancer, and that the mutated Kozak worked better than its wild-type counterpart. Both ApoE-encoding groups (MC6 and MC7 groups) demonstrated much smaller SN/SL ratios, although both demonstrated a much higher baseline LDL uptake capacity in the cells without treatment, suggesting both MCs had a poorer capacity in physiological control. Therefore, MC5 was chosen as the best vector to be further tested in preclinical study with the consideration of combining safety and strength of transgene expression (Table 1). MC7 was used as the control.

MC-mediated correction of LDLR-deficient phenotype in mice

To conduct the preclinical study, 0.3 nmol kg^{-1} body weight of MC5 and MC7 were injected into the livers of C57BL/6 LDLR$^{-/-}$ mice using established hydrodynamic procedure. Subsequently, the plasma LDL levels were determined periodically using the orbital blood.

As expected, the control mice receiving saline and control plasmid ZY781 demonstrated persistent high level of plasma cholesterol (~250–300 mg ml^{-1}) and high LDL level (~200–250 mg ml^{-1}) respectively. In contrast, the plasma cholesterol decreased to
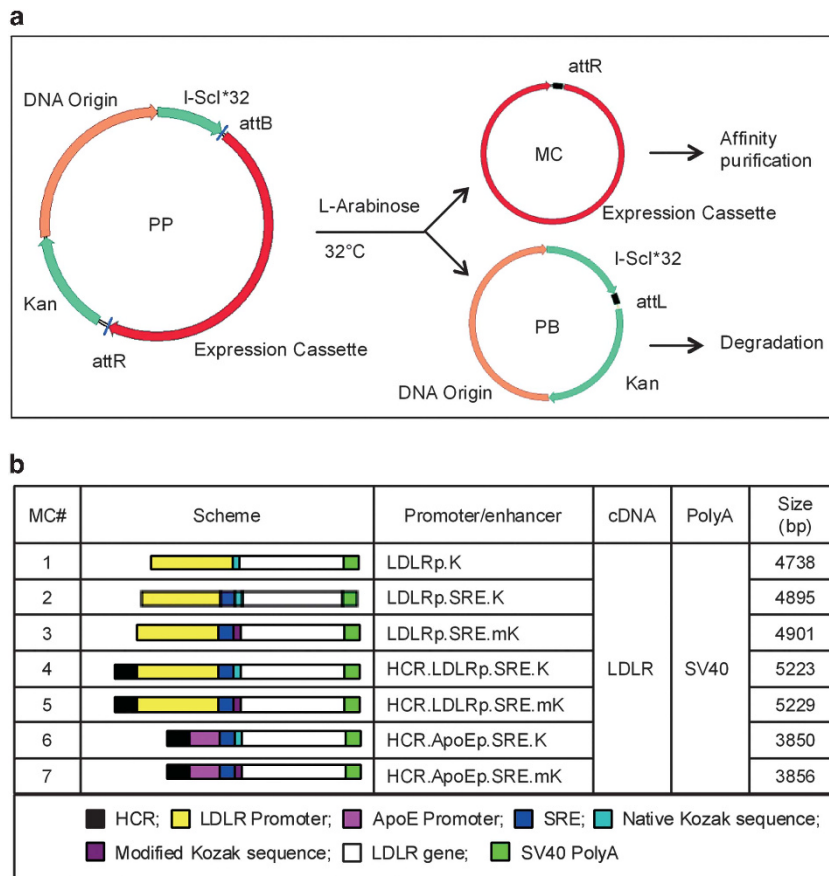
Figure 1. Schematic illustration of MC generation and composition of *LDLR* expression cassettes. (**a**) MC was made following established protocol.[13–15] MC generation was initiated by addition of L-arabinose to induce expression of DNA recombinase ΦC31 and homing endonuclease I-SceI encoded in the genome of bacterium strain ZYCY10P3S2T in the overnight culture. The ΦC31 mediated the recombination between attB and attP sites within the MC producing plasmid (PP), resulting in the MC and the plasmid backbone circle (PB), that was subsequently degraded by I-SceI. As the only episomal DNA circle, the MC was isolated using affinity column technology. (**b**) All *LDLR* expression cassettes carried the same *LDLR* complementary DNA (cDNA) plus a SV40 polyadenylation signal (poly A) and a promoter/enhancer comprising the promoter of either *LDLR* gene (*LDLR*p) or Apoprotein E gene (*ApoE*p), together with different elements, for example, HCR, SRE, native (K) or modified Kozak sequence (mK).

140–200 mg dl^{-1}, and LDL decreased to 90–150 mg dl^{-1} in the mice receiving either MC5 or MC7 without significant difference between the two groups (Figure 3). Furthermore, the levels of high-density lipoprotein, triglycerides and alanine transaminase were the same as the controls (Supplementary Figure 2). These results suggested that these two MCs were able to correct the defective phenotypes in *LDLR*-deficient mice without detectable toxicity.

DISCUSSION

LDLR-deficient phenotype mice (C57BL/6 LDLr$^{-/-}$) have been used as a model of treating FH for 20 years. C57BL/6 LDLr$^{-/-}$ mice have mildly increased plasma cholesterol levels of ~200 mg dl^{-1} on a chow diet that is approximately threefold higher than that in C57BL/6 mice. When the mice were fed a cholesterol-rich diet (0.2% cholesterol, 10% coconut oil), cholesterol levels increased to 400 mg dl^{-1}.[20] Expression of *LDLR* gene is effective to reduce cholesterol level, not only for the mice on a cholesterol-rich diet, but also for the mice on a standard diet.[21] The C57BL/6 LDLr$^{-/-}$ mice on a standard diet in this study demonstrated a persistent high level of plasma cholesterol at ~250–300 mg ml^{-1}. The plasma cholesterol and LDL levels are fluctuating for FH patients. Sterol and statin treatment provided fluctuating plasma cholesterol and LDL levels for *in vitro* and *in vivo* test of regulation of the LDLR expression. Long-term physiologically regulated expression

of the LDLR *in vivo* was confirmed by Hibbitt *et al.*[11] Using luciferase expression and specific LDL binding and internalization assays, they have shown *in vitro* that the genomic promoter element confers long-term, physiologically regulated gene expression and complementation of receptor deficiency in culture for 240 cell generations. This was demonstrated in the presence of sterols or statins, modifiers of LDLR promoter activity. *In vivo*, they demonstrated efficient liver-specific delivery and expression of luciferase following hydrodynamic tail vein injection, and confirmed that expression from the LDLR promoter element is sensitive to statin administration. The LDLR promoter region including elements essential for physiologically regulated expression (especially SRE) was used in our study for regulation of LDLR expression.

Gene transfer constitutes an important strategy for treating FH because most of the patients are resulted from loss-of-function mutations of *LDLR* and resistant to convenient treatments. Previous gene therapy studies have generated promising results, but further development is hampered because the cells harboring the viral vectors were eliminated by host immune system soon after delivery,[10,11] whereas the nonviral vectors were too bulky to be delivered to target cells.[2] In the present study, we overcame these problems by using the optimized nonviral vector MC[13–15] expressing the functioning *LDLR*. We made seven MCs using artificial promoter/enhancers comprising different regulatory elements to drive the expression of LDLR, and tested them through vigorous
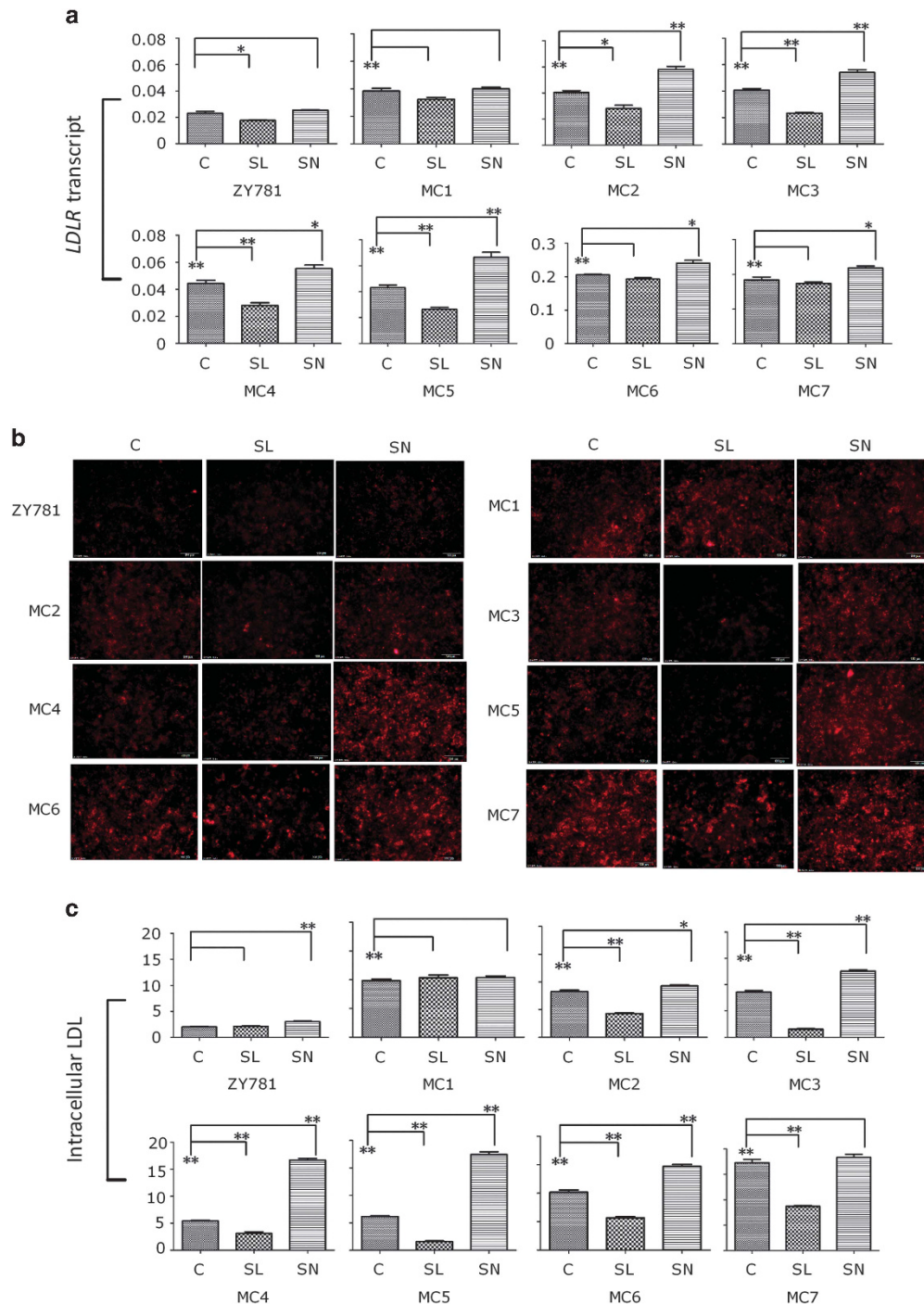
Figure 2. Expression of the *LDLR* and uptake of LDL-C in HepG2 cells harboring different MC vectors. (**a**) *LDLR* gene expression. HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) at a density of 5×10^4 cells per well in 24-well plates. After 24 h, the cells were transfected with 0.5 μg of individual MCs, MC1–7, or the control plasmid, the MC producing plasmid ZY781 without an expression cassette. At 6 h after the transfection, groups of the cells were treated with sterol (10 μg ml^{-1} medium of cholesterol plus 0.5 μg ml^{-1} medium of 25-hydroxycholesterol) or statin (0.75 μg ml^{-1} medium of statin) for 36 h before the cells were processed for determination of *LDLR* mRNA by quantitative PCR (qPCR). (**b**) Visualization of intracellular LDL. HepG2 cells were treated as described in (**a**). The intracellular LDL was illustrated by fluorescence technique. (**c**) LDL uptake capacity in HepG2 cells. It was actually the quantity of the intracellular LDL as determined by computer-aided quantification of the antibody fluorescence illustration of the intracellular LDL. Similar to that in the demonstration of LDLR mRNA (**a**) and protein (Supplementary Figure 1), LDL uptake capacity in HepG2 cells of ZY781 group served as the reference for determination of the regulation function of different MC vectors ($*P < 0.05$; $**P < 0.01$). C, control; SL, sterol; SN, statin.

in vitro and *in vivo* function tests. We determined MC5 as the best candidate for clinical application in treating FH because it demonstrated the ideal features. MC5 was 5.23 kb in size within the low end of most nonviral vectors, so that it was convenient

for delivery. The gene regulatory region comprised multiple well-characterized elements. The SRE in concert with the 2 kb 5′-untranslated region of *LDLR* was used to confer tightly physiological control in LDLR expression. The HCR was used to
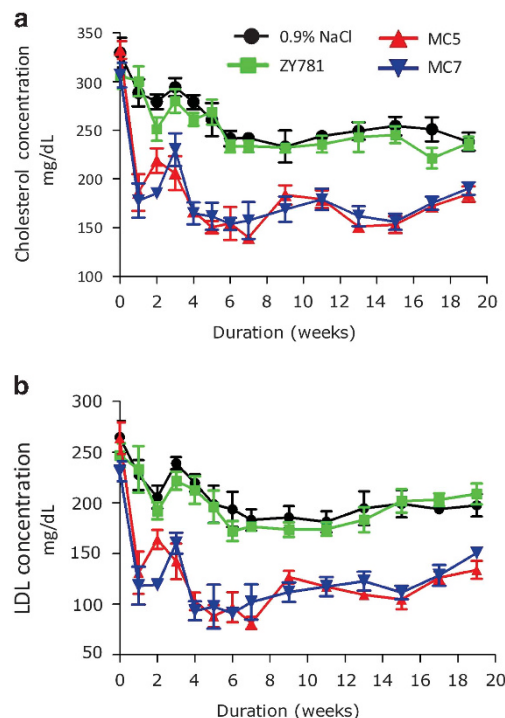
Table 1. Determination of minicircle (MC) functions in HepG2 cells

Vector code	Treatment	LDLR mRNA		LDLR protein		LDL uptake capacity	
		Level	SN/SL ratio	Level	SN/SL ratio	Level	SN/SL ratio
ZY781	C	1.00[a]		1.00		1.00	
	SL	0.77		0.84		1.05	
	SN	1.10	1.43	1.31	1.56	1.49	1.42
MC1	C	1.67		1.43		4.79	
	SL	1.41		1.47		5.04	
	SN	1.74	1.23	1.37	0.93	5.06	1.00
MC2	C	1.75		1.38		4.03	
	SL	1.22		0.92		2.08	
	SN	2.52	2.07	2.05	2.23	4.53	2.18
MC3	C	1.77		1.37		4.19	
	SL	1.01		0.55		0.74	
	SN	2.35	2.33	1.75	3.18	6.13	8.28
MC4	C	1.93		1.94		2.66	
	SL	1.22		1.26		1.51	
	SN	2.41	1.98	2.32	1.84	8.15	5.40
MC5	C	1.87		1.65		3.00	
	SL	1.14		1.04		0.78	
	SN	2.90	2.54	2.00	1.92	8.51	10.91
MC6	C	8.94		2.71		4.97	
	SL	8.38		2.4		2.75	
	SN	10.46	1.25	2.88	1.20	7.19	2.61
MC7	C	7.97		2.98		8.44	
	SL	7.54		2.42		4.25	
	SN	9.46	1.25	3.38	1.40	8.96	2.11

Abbreviations: C, control; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; SL, sterol; SN, statin. [a]Strength of LDLR gene expression and LDL uptake was expressed using the value of HepG2 harboring plasmid ZY781 without any treatment as 1.

Figure 3. MC vector-mediated correction of phenotype of LDLR-deficient mice. MCs (MC5 and MC7) encoding different *LDLR* expression cassettes were injected into livers of 6-week-old female LDLR-deficient mice. The serum levels of cholesterol and LDL-C were monitored periodically using the retro–orbital blood. The charts demonstrating the (**a**) serum cholesterol levels and (**b**) serum LDL levels, respectively; $n = 4$.

provide the liver specificity and greatly enhanced transgene expression. The modified Kozek sequence was used for optimal ribosomal entry. The experimental results confirmed the desired built-in features. It demonstrated the capacity to express high levels of LDLR transcripts and proteins, and importantly to respond to treatments of sterol and statin (as evidenced by the highest SN/SL ratio among all the constructed MCs). More importantly, MC5 was able to correct the LDL-defective phenotype of *LDLR*-deficient mice for up to 105 days without detectable toxicity. The plasma cholesterol level was maintained at 140–200 mg dl^{-1} and LDL level was maintained at 90–150 mg dl^{-1}, and the levels were also in normal human levels. Although the MC5-harboring mice were not challenged with high cholesterol diet, the HepG2 cells responded well to the treatments of high-level sterol as well as statin, suggesting its capacity in meeting the challenge. Because cholesterol is an essential component of the body, the gene therapy vector must have a tight physiological regulatory mechanism in addition to correct the LDLR-deficient phenotype. In previous studies, this has been achieved using a 145 or 10-kb *LDLR* locus to control the expression of LDLR. The present study demonstrated that the 5.23 kb sequence of MC5 was able to function as well as the lengthy LDLR locus.

This discrepancy between mRNA and protein existed in our *in vitro* test. The intracellular LDLR protein expression pattern was quite different from that of the mRNA (Table 1 and Supplementary Figures 1a and b). A much bigger different response of HepG2 to SLandSN treatments was seen in the LDLR protein (Supplementary Figures 1a and b). The ratio of LDLR protein is increased for MC2 and MC3, but decreased for MC4 and MC5. Interestingly, the 7.5-fold to >10-fold increase in *LDLR* mRNA in MC6 and MC7 groups (Figure 2a) was not seen in protein (2.4- to 3.4-fold), suggesting the existence of an effective post-transcriptional

control in prevention of overexpression of *LDLR* in the cells. LDL uptake level (Table 1 and Figures 2b and c) was reflected by increased SN/SL ratios in MC2–5. The mechanism of regulation of the LDLR expression is mentioned in the Introduction. When cholesterol levels are high in the endoplasmic reticulum (sterol treatment), transcription of *LDLR* is rather low; when the cholesterol levels fall (statin treatment), high-level transcription of *LDLR* is turned on. However, the mechanism of regulation of the LDLR expression is rather complicated. Especially, post-transcriptional control and modification in prevention of overexpression of *LDLR* existed in the cells.

As shown in Figure 2 and Table 1, *LDLR* mRNA, LDLR protein and LDL uptake capacity of ApoE promoter groups (MC6 and MC7) were much higher than that of other LDLR promoter groups (MC2–5). On the contrary, the SN/SL ratios of ApoE promoter groups (MC6 and MC7) were much lower than that of other LDLR promoter groups (MC3 and MC5). These observations suggested that the ApoE promoter rendered a very strong constitutive expression of *LDLR*, and responded poorly to sterol and statin treatments *in vitro* test. However, the strong constitutive expression of *LDLR* of ApoE promoter was useful to reduce high level of plasma cholesterol and LDL levels, seen in *in vivo* test. Interestingly, the MC7 demonstrated a same capacity as MC5 in correcting the *LDLR*-deficient phenotype in the mice. This MC expressed much higher levels of LDLR transcript *in vitro* (up to 7.8-fold) and was capable of responding to treatment of statin as well as MC5, but responded to treatment of sterol challenge poorly. Expression of LDLR is highly regulated and excess receptor expression is cytotoxic.[11] Therefore, it may not be a good candidate for clinical application, although this has to be tested by further experiment.

Among the risks that World Health Organization (WHO),[22] Food and Drug Administration (FDA)[23] and European Medicines Agency (EMEA)[24] list for the use of DNA vaccines is the hazard of

integration into recipient's chromosomal DNA, with the resulting risk of insertional mutagenesis or spreading of antibiotic resistance genes. The probability of chromosomal integration increases if the introduced plasmid DNA has been linearized. Any plasmid preparation will contain plasmid DNA with different topologies: super-coiled material, open circular and linear. This is the reason why the regulatory authorities require the plasmid preparation intended for vaccination or gene therapy to contain a high percentage of super-coiled material.[18] FDA recommends a super-coiled plasmid fraction above 80%.[23] A major advantage of vaccination with circular plasmid molecules MC DNAs (rather than linear plasmid vectors or viral vaccines) is that there is considered to be no risk of genome integration. In a recent study, Stenler *et al.*[25] have investigated the fate of MC DNAs and plasmid DNA after delivery through mouse hide using pneumatics. What is perhaps most noteworthy is that the plasmid DNA is partially destroyed and does no longer meet the FDA requirements of an 80% super-coiled fraction. The MC DNAs construct fared much better, with the nicked fraction being 10 times lower than for the full-length plasmid construct.[25] In another study, circular vectors were considerably more resistant to shearing than linear vectors of the same length, and DNA super-coiling afforded additional protection. These results show the potential of shear-resistant Minivector DNAs to overcome one of the major challenges associated with gene therapy delivery.[26]

The most important—and most difficult—challenge in gene therapy is the issue of delivery. Not only must the therapy evade the reticuloendothelial system as it circulates after systemic administration, but it must also cross several barriers before it arrives in the cytoplasm or nucleus of its target cells.[27,28] Various strategies (for example, biomaterial-based vectors and biologically derived vectors) have been developed to deliver gene cargos efficiently into target cells by nonviral vectors that have attracted much attention in recent years. Rationally designed nonviral vectors have exhibited improved *in vivo* stability and pharmaco-kinetics, little reticuloendothelial system uptake, high tumor accumulation, target specificity, efficient endosome release and nuclear transcription of the encapsulated therapeutic nucleic acids. Even so, we have no doubt that the development of safe, stable, effective and tumor-specific nanoparticles for systemic administration remains an unmet goal for successful clinical applications of cancer gene therapeutics.[28] Delivery is also the bottleneck of MCs for clinical gene transfer. Our ongoing attempts (erythrocyte ghost, nanomaterials) may be useful for clinical gene transfer.

In summary, we made a MC that demonstrated the desired features in correcting the LDLR-deficient phenotype and had the clinical application potential in treating FH. Currently, we are working on DNA delivery technology to further test whether MC5 is able to reverse atherosclerosis using appropriate animal models.

MATERIALS AND METHODS

Cell lines and cell culture
HepG2 cells, a human hepatoma cell line, was cultured in Dulbecco's modified Eagle's medium with high glucose supplement (GIBCO, Grand Island, UK) containing 10% fetal bovine serum, 100 units per ml of penicillin and streptomycin, and incubated at 37 °C and 5% CO_2. Sterol media also contained 10 μg ml^{-1} of cholesterol or 0.5 μg ml^{-1} of 25-hydroxycholesterol, whereas statin medium included 0.75 μg ml^{-1} of statin.

Construction of MC vectors
Because the three SREs (within the 500-bp region upstream of the *LDLR* transcript) were critical for blood LDL-C-mediated physiological control of the expression of LDLR, we assumed that the 2 kb region that harbors promoters of most genes was able to render the physiological control of *LDLR* gene, and used it to construct the MC vectors. The plasmid ZY781

(pMC.BESX)[15] was used to make a set of MC-producing plasmids encoding the following genetic elements amplified by PCR reactions: the human LDL receptor complementary DNA,[10,29] the SRE,[30] the HCR,[31] the *LDLR* gene promoter[30] or ApoE;[32] and the modified Kozak sequence[33] (Figure 1b and Supplementary Tables 1 and 2).

Production of MC vector
All the MCs were generated following the protocol of the ZYCY10P3S2T system[15] with modifications. Briefly, a seed culture was prepared using one transformed colony in 20 ml TB with kanamycin (50 μg ml^{-1}) in a 250 ml conical flask and cultured at 37 °C with shaking at 250 r.p.m. until reaching the logarithmic phase (OD A600 = 2.0). Subsequently, an expanded culture was composed by adding 4 ml of the seed culture to 200 ml TB containing kanamycin (50 μg ml^{-1}) in a 500-ml flask. The culture was incubated at 37 °C with shaking at 250 r.p.m. for 10 h to the stationary phase. At this point in time, MC formation reaction was initiated by adding equal volume of induction mix comprising LB medium (200 ml) and 40 μl of 20% filtered L-arabinose that was adjusted to pH 7.0 using appropriate volume of sodium hydroxide (1 mol l^{-1}). The reaction continued with shaking at 250 r.p.m., 30 °C for additional 5 h before being processed to isolate MC DNA from bacterial lysates using commercially available affinity column (Qiagen kit, Invitrogen, Catalog Number 12183, Carlsbad, CA, USA). The generated MC DNAs were also adjusted to 1 μg μl^{-1} using TE buffer.

Quantification of *LDLR* transcript by quantitative reverse transcription-PCR
HepG2 cells were cultured in Dulbecco's modified Eagle's medium at a density of 5×10^4 cells per well in 24-well plates at 37 °C. At 24 h after seeding, the cells were transfected with 0.5 μg of each MC using Superfect (Invitrogen, Shanghai, China) according to the vendor's instruction. After incubating for 6 h, the transfection medium was replaced with Dulbecco's modified Eagle's medium, sterol medium or statin medium. After 36 h, the cells were processed for quantifying *LDLR* transcript via reverse transcription-PCR using Takara Primescript RT Reagent Kit (Takara, Dalian, China) following the vendor's protocol. The PCR primer sets LDLR-F plus LDLR-R and Actin-F plus Actin-R (Supplementary Table 1) were specific for LDLR and β-actin genes shared by all templates. Quantitative PCR was performed using the Qiagen QuantiFast SYBR Green PCR kit (Invitrogen) with the Roche system 480 (Shanghai, China) according to the vendor's protocol. All samples were normalized to internal controls and fold changes were calculated through relative quantification ($2^{-\Delta\Delta Ct}$).

Determination of intracellular *LDLR* distribution and LDL-C uptake capacity
After 36 h of incubation with sterol and statin, HepG2 transfected with individual MCs was processed to illustrate intracellular LDLR and cellular uptake of LDL using Cayman LDL Uptake Assay Kit (Cayman, Ann Arbor, MI, USA) according to the vendor's instruction. The kit employs DyLight 549 as a fluorescent probe to illustrate and quantify intracellular LDL. An LDLR-specific polyclonal antibody and a DyLight 488-conjugated secondary antibody were included for visualizing the intracellular LDLR. The intracellular fluorescence intensity is measured using the software program (ImageJ, Annatto Beach, CA, USA).

MC-mediated correction of disease phenotype of LDLR-deficient mice
The 6-week old (~20 g) female LDLR-deficient mice C57BL/6 LDLR$^{-/-}$[20] were purchased from Model Animal Research Center of Nanjing University (Nanjing, China) and maintained on a standard diet with free access to water throughout the experiments. The animals were treated following the guideline of the Animal Ethic Committee of SIAT of Chinese Academy of Sciences. The mice received MC5, MC7 or ZY781, 0.3 nmol kg^{-1} DNA in 2 ml saline each, respectively, via hydrodynamic tail vein injection technique. The control mice received equal volume of saline. Blood was collected by the retro–orbital procedure and plasma was immediately isolated by centrifugation at 1100 g for 10 min before being stored at − 20 °C until analysis. Plasma cholesterol, LDL, high-density lipoprotein, triglycerides and alanine transaminase levels were determined using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the vendor's instruction.

Statistical analysis

Statistical analysis was performed using Student's *t*-test or analysis of variance. Data are presented as mean $\pm$ s.d.

CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

Z-YC perceived the concept and worked with XHH and RJ to design the experiments and write the manuscript. XHH, RJ, TYW, XYG, YSC and C-YH conducted the experiments.

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