Clinical Study Protocol

A pilot study for the safety and expression of dystrophin in skeletal muscle after SPOT-mRNA03 administration in Duchenne Muscular Dystrophy (DMD) patients

NCT Number:	**Not yet assigned**
Project Number：	**SPOT-MG02**
Protocol Number：	**FM-T3-SH**
Institution：	**Shanghai Children's Medical Center**
Principal Investigator：	**Wang Jiwen**
Co-sponsor：	**Shanghai Siponuoyin Biotechnology Co., Ltd**
CRO：	**Beijing Leadingpharm Medicine Technology Development Co., Ltd**
Version：	**V2.1**
Date：	**March 20, 2025**

CONFIDENTIAL

Investigator's Statement

I the undersigned, have reviewed this protocol (protocol no: FM-T3-SH, Version: v2.1, Date: March 20,2025) and I agree to conduct this protocol in accordance with International Conference on Harmonization (ICH) guidelines on Good Clinical Practice (GCP) and the Declaration of Helsinki. This protocol will be carried out upon the approval from the Ethics Committee (EC) or Institutional Review Board (IRB).

I understand that the protocol may not be modified without written approval of the sponsor and that all changes to the protocol must be submitted to the applicable regulatory authorities and the EC/IRB prior to implementation.

Principal Investigator:

_____

Signature *Date (mm/dd/yyyy)*

Name:

_____

Please Print

Institution:

_____

Collaborator's Statement

I the undersigned, have reviewed this protocol (protocol no: FM-T3-SH, Version: v2.1, Date: March 20,2025) and I agree to conduct this protocol in accordance with International Conference on Harmonization (ICH) guidelines on Good Clinical Practice (GCP) and the Declaration of Helsinki. I will fulfill the responsibilities of the sponsor including initiation, monitoring, and funding of this trial. I agree to conduct this clinical study in accordance with the design and provisions in the protocol.

Signature of Responsible Party:

_____

Signature *Date (mm/dd/yyyy)*

Name:

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Please Print

Collaborator:

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Abbreviation List

Abbreviations	Full Name
AE	Adverse Event
AR	Adverse Reaction
BMI	Body Mass Index
CBC	Complete Blood Count
CN	Copy Number
CRF	Case Report Form
CRO	Contract Research Organization
CRU	Clinical Research Unit
CTCAE	Common Terminology Criteria For Adverse Events
D/d	Day
DMD	Duchenne Muscular Dystrophy
EC	Ethics Committee
ECG	12-Lead Electrocardiogram
ELISA	Enzyme-Linked Immunosorbent Assay
EMG	Electromyography
EOS	End Of Study
EVs	Extracellular Vesicles
FAS	Full Analysis Set
FIH	First In Human
h	Hour
IC	Informed Consent
IIT	Investigator-Initiated Clinical Trial
IRB	Institutional Review Board
ISRs	Injection Site Reactions
IV	Intravenous
GCP	Good Clinical Practice
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
NMPA	National Medical Products Administration
NSAA	North Star Ambulatory Assessment
PET	Pulmonary Function Test
PK	Pharmacokinetics
PD	Pharmacodynamics

Abbreviations	Full Name
PP	Per-Protocol Set
qPCR	Quantitative Polymerase Chain Reaction
SAE	Serious Adverse Event
SAS	Safety Analysis Set
SAP	Statistical Analysis Plan
TRAEs	Treatment Related Adverse Reactions
W/w	Week

Synopsis

Title:	A pilot study for the safety and expression of dystrophin in skeletal muscle after SPOT-mRNA03 administration in Duchenne Muscular Dystrophy (DMD) patients
Project Number:	SPOT-MG02
Protocol Number:	FM-T3-SH
Collaborator:	Shanghai Siponuoyin Biotechnology Co., Ltd
Clinical Trial Phase:	Investigator-initiated clinical trial (IIT)
Investigational Drug:	SPOT-mRNA03 injection
Indication:	Duchenne Muscular Dystrophy（DMD）
Study Population	Ambulatory boys with DMD, aged 2~6 years
Sample Size	6 subjects
Duration of Study	The screening period is 0-30 days, with 4 weeks of treatment period and a follow-up period of 6 months.
Objectives	Primary objective: To evaluate the safety and tolerability of SPOT-mRNA03 administered by intravenous infusion (IV) to DMD patients. Secondary objectives: ● To evaluate the changes of dystrophin mRNA in serum and muscles of DMD patients after IV infusion of SPOT-mRNA03. ● To evaluate the changes in dystrophin expression and engraftment in muscles of DMD patients after IV infusion of SPOT-mRNA03. ● To evaluate the changes in serum anti-dystrophin antibodies and cytokines in DMD patients after IV infusion of SPOT-mRNA03.

Endpoints	Primary endpoints: ● Collection and quantification of adverse events (description of event, time of onset and resolution, assessment of severity and causal relationship to the investigational drug), as well as the data of physical examination, vital signs, 12-lead ECG, clinical laboratory test results (hematology, biochemistry, urinalysis) in this study. Secondary endpoints: ● Changes of dystrophin mRNA concentration in serum before and different timepoints after the initial administration (see Table 5 for schedule) as well as before and after the 2nd, 4th, 6th, and 8th administration and during follow-up. And the changes in Dystrophin mRNA concentration in muscles at baseline and D29 after the completion of the last dose (if the biopsy time is between 12-24 hours after the last dose) (by qPCR). ● Changes in the expression and engraftment level of dystrophin in muscle biopsies at baseline and D29 (after the completion of the last dose) (by Western Blot and IHC methods). ● Changes in anti-dystrophin antibodies and cytokines in the serum at baseline and before the 2nd, 4th, 6th, and 8th administration and during follow-up (see Table 5 for schedule).
Study Description	This is a FIH, open-label, single-arm, and single-center exploratory clinical study of SPOT-mRNA03 administered via IV infusion for DMD patients. The primary objective of this study is to evaluate the safety and tolerability of SPOT-mRNA03 for DMD patients. And the secondary objectives are to preliminarily investigate the concentration change of dystrophin mRNA, the expression and engraftment level changes of dystrophin protein, as well as changes in cytokines and immunogenicity. The study has two ascending dose cohorts: I cohort：5.0×10^9 CN dystrophin mRNA / kg II cohort：5.0×10^{10} CN dystrophin mRNA / kg The study will have a screening period of 30 days, during which

	patients or their legal guardian written informed consent will be obtained before screening assessments and eligibility will be determined. A total of 6 DMD patients will participate in the study. there are 3 patients in each dose cohort [No previous treatment with corticosteroids]. All subjects started taking 0.05-0.1mg/kg/day (adjusted according to the actual clinical situation) tacrolimus or sirolimus orally at D-3 (3 days before initial dose of SPOT-mRNA03) for 4 weeks. All subjects are first administered via intravenous infusion on D1, and then administered twice a week (once every 4 days) for a total of 8 doses. Four weeks after the initial administration of the subjects in the previous dose cohort, if there are no serious adverse events related to the treatment, it will be determined that the subjects in next dose cohort could be administered after discussion between the investigators and the sponsor. Safety evaluations on subjects are conducted during each administration and follow-up. Muscle biopsies are performed at baseline and D29 after administration (see Table 5 for details). Samples for biopsy will be taken from gastrocnemius/biceps brachii muscle at baseline and from the gastrocnemius and biceps brachii muscles on D29. Western blot (WB) is used to detect the expression of dystrophin protein. Immunohistochemistry (IHC) is used to detect fiber intensity and percentage of dystrophin positive fibers, and qPCR is used to detect changes in dystrophin mRNA at baseline and D29 (if the biopsy time is between 12~24 hours after the last dose). The blood samples are collected for detection of dystrophin mRNA, cytokines and immunogenicity (see Table 5 for details) besides for laboratory testing. Perform MRI and EMG examinations at baseline and at week 16 after administration.
Investigational Drug	**SPOT-mRNA03：** Colorless and odorless clear liquid; **Active Pharmaceutical Ingredients:** Dystrophin mRNA-loaded EVs. **Pharmaceutical excipients：** CryoStorCS10 (containing 10% dimethyl sulfoxide (DMSO)), phospate-buffered saline (PBS), human

	serum albumin (HSA), (pharmaecuetical grade) **Strength：** 1.0×10^{10} CN dystrophin mRNA / mL **Storage：** -60℃ ~ -90℃
Route of Administration	Intravenous infusion: twice a week (once every 4 days). Each subject will receive a total of 8 treatments. Each vial of SPOT-mRNA03 needs to slowly thaw at 4 ℃/ice before use. SPOT-mRNA03 after thawing should be used as soon as possible. The total volume of infusion is 40 mL. Choose an appropriate intravenous infusion site based on the subject's venous condition, with an infusion time of 60~120 minutes. If the subject experiences mild allergic reactions such as dizziness, chills, rash or extravasation during the infusion process, the infusion should be immediately suspended. After recovery, the investigator will determine whether to continue the administration. The infusion speed can be appropriately reduced (as determined by the investigator), and the infusion time can be correspondingly extended (as determined by the investigator). After completion of infusion, record the actual dosage and start and end time of infusion. Take photos for the infusion site before and after each infusion, and observe the infusion reaction.
Concomitant Medication	The newly generated dystrophin will be recognized by the immune system of the subjects as an exogenous protein, triggering an immune response. Any immune response to dystrophin can affect its engraftment and potentially induce further muscle damage, reversing any benefits that patients should have received. Therefore, the subjects should begin to orally take 0.05-0.1mg/kg (adjusted according to the actual clinical situation) tacrolimus or sirolimus daily at D-3 (before the initial administration of SPOT-mRNA03) for 4 weeks.
Inclusion Criteria	**Only those who meet all the following criteria can be enrolled:** 1) According to the requirements of the region/country and/or IRB/IEC, the patient and/or legal guardian have signed a written

	informed consent form and are aware of all relevant study content. 2) Ambulatory boys aged 2 to 6 years, inclusive. 3) Confirmed diagnosis of DMD through multiplex ligation-dependent probe amplification (MLPA) and whole-exome sequencing. 4) Tolerance for muscle biopsy under anesthesia with no absolute contraindications to the procedure. 5) Heart, liver, lung, and kidney functions are sufficient: a) The left ventricular ejection fraction (LVEF) should be ≥ 50%; b) Forced vital capacity (FVC) ＞ 50% of the expected value, and do not require nighttime ventilation; c) Patient's glomerular filtration rate (GFR) > 30 mL/min/1.73 m^2
Exclusion Criteria	**Those who meet any one of the following criteria must be excluded:** 1) Complications other than DMD that may cause muscle weakness and/or motor dysfunction. 2) There are severe intellectual disabilities (such as severe autism, severe cognitive impairment, and severe behavioral disorders) that, according to the investigator's judgment, can affect the study. 3) Hospitalization for respiratory failure within 8 weeks prior to screening. 4) Asthma or underlying lung diseases that are poorly controlled, such as bronchitis, bronchiectasis, emphysema, or recurrent infectious pneumonia that investigator believes may affect respiratory function. 5) Severe uncontrolled heart failure (NYHA III-IV), including any of the following conditions： a) Intravenous administration of diuretics or positive inotropic drugs is required within 8 weeks prior to screening. b) Hospitalization due to worsening heart failure or arrhythmia within 8 weeks prior to screening.

	6) Abnormal laboratory values considered clinically significant: a) GGT > 3 × upper limit of normal b) Bilirubin ≥ 3.0 mg/dL c) Creatinine ≥ 1.8 mg/dL d) Hemoglobin < 8 or > 18 g/dL e) White blood cell count > 18,500/μL 7) There are arrhythmias that require antiarrhythmic treatment. 8) Subjects who are undergoing immunosuppressive therapy. 9) Has used other gene therapy, investigational drugs, or any treatment aimed at increasing dystrophin expression. 10) Subjects with a history of major surgeries within 12 weeks prior to the initial infusion or planning to undergo major surgeries (such as scoliosis surgery) during this study. 11) Subjects who are allergic to investigational products or local anesthetic drugs or have a history of severe allergies or genetic allergic reactions. 12) Within 6 months prior to the initial infusion, the subjects are exposed to another investigational drug or are participating in an intervention clinical trial. 13) Subjects with positive of hepatitis B core antibody or hepatitis C antibody or HIV antibody during screening. 14) Investigator believes that the presence of any other serious diseases, medical conditions, or chronic drug treatment needs can pose unnecessary risks to gene transfer.
Withdrawal Criteria	1) Considering the safety, the investigator believes that withdrawing from the study is in the best interest of the subjects. 2) Subjects with poor compliance cannot insist on completing the trial as planned or have other circumstances that may affect the judgment of the research results. 3) The subject voluntarily requested to withdraw from the trial and withdraw the informed consent.

End of Study	After the enrolled subjects complete all administration and follow-up at week 28, the study ends.
Muscle Sample Collection	Muscle biopsy will be performed at baseline and D29 (after the last dose). Samples of each subject for biopsy will be taken from gastrocnemius/biceps brachii muscle at baseline (one site) and from the gastrocnemius and biceps brachii muscles on D29 (two sites). Select a suitable location of the gastrocnemius muscle/biceps brachii muscle, confirm the puncture site, disinfect with iodine, and after local anesthesia, use puncture needle to penetrate the tissue and muscle, extract muscle tissue with a size of 0.5 cm × 1 cm × 0.5 cm and sent to a pathological laboratory for muscle biopsy. Camera the biopsy site before and after each muscle biopsy. The expression of dystrophin in muscle tissue will be detected using the following methods: • Evaluate expression level of dystrophin (measured by western blot) at baseline and D29 (after the completion of the last dose). • Evaluate engraftment of dystrophin (measured by immunofluorescence for fiber intensity and percentage of dystrophin positive fibers) at baseline and D29 (after the completion of the last dose). The concentration of dystrophin mRNA in muscle tissue will be detected: • Evaluate changes in dystrophin mRNA (detected by qPCR) at baseline and D29 (if the biopsy time is between 12~24 hours after the last dose).
Safety Assessment	Vital signs (pulse, respiratory rate, blood pressure, temperature) and physical examination will be performed at each visit. Safety assessments including laboratory tests (hematology, chemistry, urinalysis) and 12-lead ECG will be performed on some visits (see Table 5 for details). ISRs (redness, swelling, heat, pain, and itching) will be observed within 30 minutes each post-treatment.

	According to the Common Terminology Criteria for Adverse Events (CTCAE, version 5.0), all adverse events that occur throughout the study period will be evaluated and graded. The investigator will collect a description of the events, time of onset and resolution, assessment of severity and causal relationship to SPOT-mRNA03.
Pharmacokinetics Assessment	Using qPCR to detect the changes of dystrophin mRNA in serum samples of subjects. Blood samples for PK test will be collected before and 0h+10min after the 1st, 2nd, 4th, 6th, and 8th infusion. Intensive blood collection will be carried out at 1 h ± 10 min, 2 h ± 20 min, 8 h ± 1 h, 24 h ± 1 h, 48 h ± 2 h, and 72 h ± 3 h after the initial infusion. PK blood samples will also be collected during follow-up. If dystrophin mRNA cannot be detected at W8 follow-up, blood samples may not be collected for the test in subsequent follow-up.
Pharmacodynamic Assessment	Muscle biopsies will be performed at the baseline and D29 after the last dose to detect the expression of dystrophin in the muscle. Quantity of dystrophin is measured by western blot of biopsied muscle tissue. Levels are described as change from baseline, in order to remove any background signal in the assay. Levels are expressed as a percent of control (i.e., as a percent of the level of normal, wild-type dystrophin present in muscle tissue of healthy individuals without DMD or Becker muscular dystrophy). Perform MRI and electromyogram examinations at baseline and W16 to assess the health of muscles and the nerve cells that control them.
Immunogenicity	• Cytokine test includes TNF-α, INF-γ, IL-2, IL-6, and IL-10. Collect blood samples before administration and during follow-up (see Table 5 for detailed schedule). If cytokine levels return to normal at W8 follow-up, blood samples may not be collected for the test in subsequent follow-up. • Anti-dystrophin antibody level can be measured by ELISA. Collect blood samples at baseline (if does not exceed 7 days from baseline to the first dose, no additional collection is required

	before the firs dose) and before administration, and follow-up (see Table 5 for detailed schedule). If anti-dystrophin antibody cannot be detected at W8 follow-up, blood samples may not be collected for the test in subsequent follow-up.
Statistical Analysis of Population	1) Full analysis set (FAS): FAS will be used to report medication compliance and to summarize demographic characteristics (age, gender, race, height and weight) and background characteristics (medical history, concomitant medications, and physical examination, etc.) for all randomized subjects by treatment group. 2) Safety analysis set (SAS): All subjects who have been randomized into cohorts, have used the investigational drug, and have safety evaluation data post-treatment constitute the safety population of this study. The safety population will be used for safety analysis.
Statistical Method	Due to the small sample size and open-label design of this study, only descriptive statistics will be conducted for all endpoints, including the number of subjects, mean and standard deviation, minimum and maximum values of continuous variables, and the number and percentage of categorical variables.

1. Background

Duchenne muscular dystrophy (DMD) is the most common X-linked recessive male hereditary disease and the most common fatal monogenic disease. The incidence rate of DMD in male newborns is about 1/3500. Approximately 400-500 cases of DMD are born in China every year, with a total of approximately 70.000 confirmed cases, making it one of the countries with the highest number of DMD patients in the world. There is no obvious geographical distribution characteristic of DMD patients in China [1].

1.1. Dystrophin gene and its mutation types

DMD is caused by mutations in the gene encoding the dystrophin protein. This gene is located in the Xp21.2 region of chromosome, with a total length of 2.3 Mb and 79 exons. The mRNA sequence encoded by this gene has a total length of 11 Kb. Dystrophin gene is the largest known human gene [2][3][4]. The normal dystrophin protein size generated by the translation of this gene in muscle cells is 427 kDa, and it is expressed in muscle cells throughout the body, including the heart, skeletal muscles, smooth muscles, retina, and brain [5].

The mutation types in the dystrophin gene are mainly exon deletions/duplications, while mutations in large segments (≥ 1 exon) with deletions or duplications account for about 79% (of which deletions account for about 68% and duplications account for about 11%). The most common hotspots of deletions and duplications are located in exons 45-55 and 3-9 of the gene [2][4]. Other types of small mutations (<1 exon) account for approximately 21% (of which point mutations account for about 11%, small deletions account for about 5%, small insertions account for about 2%, and splice site mutations account for about 3%) [4][5]. These point mutations are distributed throughout the entire DMD gene and there are no obvious hotspots. DMD patients are unable to synthesize normal dystrophin in their bodies due to mutations in the dystrophin gene.

The phenotype of DMD mainly depends on whether mutations in the dystrophin gene have disrupted the open reading frame. Out-frame mutations lead to transcription and translation disorders of the dystrophin gene, resulting in a more severe DMD phenotype. In-frame mutations, while retaining an open reading frame, can transcribe and translate into truncated, partially functional dystrophin proteins, resulting in milder phenotypes, such as Becker muscular dystrophy (BMD) [2].

1.2. Clinical therapy for DMD patients

Based on the guidelines of the Neurology Branch of the Chinese Medical Association [7], the therapy of DMD patients mainly involves Multi-Disciplinary Treatment (MDT)

symptomatic treatment, usually starting in boys aged 4 or above. MDT mainly includes drug treatment, rehabilitation treatment, treatment of respiratory complications, treatment of heart disease, surgical orthodontic treatment, and other treatments etc [1]. Although advances in multidisciplinary nursing and disease management strategies for DMD have slowed down disease progression, the disease is still incurable [6][8][9][10].

At present, the drug treatment for DMD patients mainly is glucocorticoids, such as prednisone (0.75mg/kg/d), which can improve the strength and lung function of DMD patients, reduce the need for spinal surgery, and delay the occurrence of cardiomyopathy. But hormone therapy may cause many side effects, including obesity, Cushing's face, hirsutism, osteoporosis, and so on. To reduce side effects, the dose can be reduced by one-third if necessary [7].

In recent years, in addition to hormone therapy, with the increasing maturity of core technologies such as gene delivery vector technology and gene editing technology, gene therapy has also been gradually developing and heating up. Due to its potential to cure rare diseases in one go, it has long received global attention and is known as the "dawn of rare disease treatment".

1.3. Gene therapy for DMD

The DMD gene therapy strategy is designed for different types of gene mutations aimed at restoring dystrophin expression in muscle tissue. It mainly includes readthrough therapy [11], exon skipping therapy [12], vector mediated gene replacement therapy [13], clustered regularly interspaced short palindromic repeat，CRISPR/ CRISPR-associated nuclease 9，(Cas9）gene editing therapy [14].

The above four therapies are suitable for DMD patients with different mutation types, and each has its own advantages and disadvantages, see Table 1.

Table 1 Summary of gene therapy strategies for DMD [15]

Therapy strategy	Mechanism of action	Applicable to patients	Advantage	Disadvantage	R&D progress
Readthrough	Prevent signal recognition of termination codon and induce read-through.	Nonsense mutation	Good safety and tolerability	Applicable to few patients and limited clinical benefits	Some drugs approved by EMA
Exon skipping therapy	Antisense oligonucleotides mask mRNA splicing signals and achieve exon skipping.	Exon deletion	Good safety and tolerability	Limited clinical benefits，frequent administration requirement and low myocardial	Some drugs have been approved by FDA, and some clinical trials are ongoing.

				transduction efficiency	
Vector mediated dystrophin gene replacement therapy	AAV loaded micro-dystrophin	AAV antibody negative	Restore stable high expression of dystrophin	High demand for AAV, time-consuming manufacturing, high cost, and susceptibility to immune reactions	Accompanied by questioning and controversy, the gene therapy drug-Elevidys, jointly developed by Sarepta & Roche, has been approved for market by FDA.
CRISPR/Cas9 gene editing	AAV loaded CRISPR/Cas9 precise repair gene mutation	AAV antibody negative	one-time treatment	High demand for AAV, time-consuming manufacturing, high cost, susceptibility to immune reactions, and off-target risk	The most studies are in the animal experimental stage, and one clinical trial has been terminated

EMA：European Medicines Agency，FDA：U.S. Food and Drug Administration，AAV：Adeno-associated virus，CRISPR：Clustered regularly interspaced short palindromic repeat，Cas9：CRISPR-associated nuclease 9

1.4. Approved drugs for DMD and their scope of application

In recent years, the approved drugs for DMD treatment are mainly steroid and gene therapy products. See Table 2.

Table 2 Approved drugs for the treatment of DMD [16][17]

Drug classification	Drug name	R&D company	Approval time	Indication
Exon skipping therapy（ASO）	Casimersen	Sarepta	2021	DMD patients with exon 45 gene mutation
	Viltolarsen	Nippon Shinyaku Co Ltd	2020	DMD patients with exon 53 gene mutation
	Golodirsen	Sarepta	2019	DMD patients with exon 53 gene mutation
	Eteplirsen	Sarepta	2016	DMD patients with exon 51 gene mutation
Corticosteroid	Deflazacort	PTCT	2017/2019	DMD patients aged 2 years and above
	Vamorolone	Catalyst Pharms	2023	DMD patients aged 2 years and above
Readthrough	Ataluren	PTCT	2014	Ambulatory DMD patients with nonsense mutation
AAV vector mediated dystrophin gene replacement therapy	Elevidys	Sarepta	2023	Used to treat DMD outpatient patients aged 4-5 with confirmed mutations

1.4.1. Steroid drugs

In 2017, the corticosteroid drug Deflazacort was approved by the US Food and Drug

Administration (FDA) for the treatment of DMD patients. On October 26, 2023, the FDA approved the marketing application of Vamorolone oral suspension 40 mg/mL for the treatment of DMD patients aged 2 years and above. Vamorolone is a first-in-class steroid drug, which differs from normal steroid drugs in that it can selectively activate certain signaling pathways of steroids, resulting in better safety [16].

1.4.2. Gene therapy for DMD

In 2014, EMA approved the drug Ataluren (trade name: Translarna) to enter the European market. This drug can reduce the sensitivity of ribosomes to early termination codons, allowing mRNA to continue translation without stopping at the termination codon during translation (i.e. "readthrough"). It can be used to treat gene nonsense mutations, with approximately 10% of DMD patients [17].

The four exon skipping therapies approved by the FDA for the treatment of DMD are Eteplarirsen, Golodirsen, Viltolasen, and Casimersen. These drugs can only be used for DMD patients with specific gene mutations: Eteplarirsen (Exonys 51), approved for marketing on September 19, 2016, is used to treat approximately 13% of DMD patients with exon 51 mutations [19]. Golodirsen (Vyondys 53), approved for sale on December 12, 2019, and Viltolarsen, approved for sale in Japan and the United States in March and August 2020, respectively, are used to treat approximately 8% of DMD patients with exon 53 mutations [20][21]. Casimersen (Amondys 45), approved for marketing on February 25, 2021, is used to treat approximately 8% of DMD patients with exon 45 mutations [22]. These four drugs are all ASO that modify the splicing of dystrophin mRNA to translate into truncated, partially functional dystrophin, transforming more severe DMD phenotype into lighter BMD phenotype [23][24]. These types of drugs have a low affinity for proteins, so they will be quickly cleared in the body after systemic injection. The biggest challenge for this type of drug currently is the difficulty of entering the cell, therefore, it is necessary to administer larger drug doses or increase the frequency of administration to improve bioavailability. All four drugs require regular intravenous injection [25]. More importantly, these products are conditionally marketed and have not yet completed the required confirmatory clinical studies, so their clinical benefits are not fully understood.

On June 22, 2023, the FDA accelerated the approval of SRP-9001 (trade name: Elevidys) of Sarepta Therapeutics for the treatment of DMD patients aged 4-5. SRP-9001 (AAV loading micro-dystrophin) has also become the world's first disposable gene therapy for DMD. This drug uses the AAVrh74 vector. The gene loading capacity of this type of vector is limited, and

it cannot load entire dystrophin gene. Therefore, the main functional regions of dystrophin gene can only be selected for Elevidys design based on existing studies. After administration of Elevidys, the final product generated in the body, the micro-dystrophin (138 kDa), only contains the selected domain of the normal wild-type dystrophin (427 kDa). The micro-dystrophin lacks the domain that can bind to neuronal nitric oxide synthase and α- syntrophic factors, which of them can protect muscle cells by synergistically regulating blood flow. So this gene therapy method can only alleviate the symptoms of DMD patients. Although in theory, the function of the micro-dystrophin may be similar to that of normal dystrophin, but it is a novel protein that does not exist in nature. It is different from both the normal wild-type and the endogenous shortened form of dystrophin in BMD patients, as well as the internally truncated dystrophin produced through exon skipping drug therapy expression. The relationship between micro-dystrophin and various shortened forms of dystrophin is still unclear, and whether its actual performance is close enough to that of natural dystrophin remains questionable. In addition, there is no clear answer to how high the expression level of micro-dystrophin can benefit patients clinically. Meanwhile, due to the lack of epidemiological data on this protein, it is not clear how it affects the pathophysiology of DMD patients [26].

The use of AAVrh74 vector also limits the clinically treatable population, requiring patients with anti-AAVrh74 antibody titers<1:400 to be selected. The existing clinical studies of Elevidys have shown that after large-scale administration, the titer of anti-AAVrh74 antibody in patients significantly increases. Therefore, even if the patient's initial dose is insufficient or there is no therapeutic efficacy after treatment, they cannot accept additional doses of the same drug for re-administration. And due to the possibility of immune cross reactivity with other AAV subtypes, DMD patients who have not benefited from Elevidys treatment may not be able to accept gene therapy based on other serotype AAV vectors in the future [26].

In addition, multiple experts have raised doubts about the structure, animal study, and clinical trial results of Sarepta's micro-dystrophin. They questioned whether there is a significant correlation between the micro-dystrophin level and muscle performance in DMD patients. Although the FDA has approved the product for market through accelerated approval, it requires further post market study to be conducted [26].

At present, there are no approved DMD gene therapy in China. From the above description, although gene therapy brings hope for the clinical treatment of DMD patients, the gene therapy products currently on the market have different shortcomings. Existing clinical studies have shown that they cannot meet the clinical treatment needs of DMD patients.

Therefore, R&D personnel still need to make efforts, as well as a large amount of funding to develop more promising and effective gene therapy products for the clinical treatment of DMD patients.

1.5. Global ongoing study on DMD treatment drugs

According to the Clinicaltrials.gov [18], there are a total of 381 clinical trials for the treatment of DMD (2023/8/29). The clinical trial information of new drugs that are currently ongoing or in recruitment status is detailed in Table 3. Whether from the approved DMD drugs or the current number of ongoing new drugs, the industry's enthusiasm for DMD drug development is gradually increasing.

Table 3 Global information of the ongoing new drugs for DMD

Drug name	R&D company	Treatment strategies	Phase	ID
CAP-1002	Capricor Inc	Cell therapy	III	NCT05126758
CRD-TMH-001	Cure Rare Disease	Gene editing	I	NCT05514249
PF-06939926	Pfizer	Gene replacement therapy	III	NCT04281485
SGT-001	Solid Biosciences		I/II	NCT03368742
GNT 0004	Genethon-Sarepta		III	/
RGX-202	REGENXBIO		II	NCT05693142
AOC 1044	Avidity Biosciences	Exon skipping therapy	II	NCT05670730
WVE-N531	Wave Life Sciences		II	NCT04906460
DYNE-251	Dyne Therapeutics		II	NCT05524883
SQY51	Sqy Therapeutics		II	NCT05753462
Brogidirsen	NS Pharma		II	NCT05996003
ENTR-601-44	Entrada Therapeutics		II	/
PGN-EDO51	PepGen		II	NCT06079736
Renadirsen	Daiichi Sankyo		II	NCT04433234
Vesleteplirsen	Sarepta Therapeutics		II	NCT04004065
TAS-205	Taiho Pharmaceutical Co., Ltd.	Chemical drug	III	NCT04587908
EDG-5506	Edgewise		III	NCT05540860
Givinostat	Italfarmaco Group		NDA	NCT01761292
Edasalonexent	Catabasis		III	NCT03703882
Ezutromid	Summit Therapeutics		II	NCT02858362
Pamrevlumab	FibroGen	Monoclonal antibody	III（Failed）	NCT04632940

2. SPOT-mRNA03 Introduction

2.1. SPOT-mRNA03 design

SPOT-mRNA03 is a gene therapy product for DMD, using the patented technology of Spot Biotech - Cellular nanoporation (CNP) (which can rearrange cell membrane molecules and improve membrane permeability through high-intensity electric field, thereby constructing a channel for introducing exogenous molecules into cells [27]) for transfecting human

dermal fibroblasts (HDFs), introducing target mRNA molecules and molecular targeted peptides (which can be co-expressed with transmembrane proteins of EVs). Compared with the traditional transfection technology, this technology can significantly improve the transfection efficiency, promote the cells to load about 14kb of full-length dystrophin mRNA molecules encoding dystrophin with high flux, and simultaneously generate a large number of EVs loaded with target dystrophin mRNA. After transfection, collect the supernatant containing the target product, purify and concentrate it, add phosphate buffer, cell cryopreservation solution CS10, and human serum albumin to prepare SPOT-mRNA03 injection.

2.2. Mechanism of Action of SPOT-mRNA03

SPOT-mRNA03 is an EVs loaded full-length dystrophin mRNA molecule, and the EVs membrane surface expresses a molecular targeted peptide, which can be used as a gene therapy product for DMD. In order to improve the targeting ability of SPOT-mRNA03 into cells, the molecular targeting peptide was co-transfected with the transmembrane protein of EVs, thus introducing the molecular targeting peptide onto the EVs membrane. After intravenous injection of SPOT-mRNA03 into DMD patients, the targeting ability of SPOT-mRNA03, endowed by molecular targeting peptides on the EVs membrane, can guide SPOT-mRNA03 into the patient's muscle cells, allowing dystrophin deficient receptor cells to utilize intracellular ribosomes and amino acids to translate and secrete endogenous full-length intact dystrophin. The generated dystrophin can tightly bind with various proteins in the inner, transmembrane, and outer regions of the cell membrane, such as sarcoglycan and dystroglycan, and are interrelated to form a whole inside and outside the cell membrane. It maintains material exchange and connection inside and outside the cell membrane, protects the integrity and stability of the cell membrane structure, and thus protects muscles from damage and/or promotes muscle repair and regeneration.

3. Risk and Benefit Assessment

3.1 Risk Assessment

The main components of SPOT-mRNA03 are EVs with molecular targeted peptides secreted by fibroblasts and full-length dystrophin mRNA molecules loaded on them. After administration, SPOT-mRNA03 can target muscle cells and generate dystrophin. The main component of SPOT-mRNA03, EVs, can be secreted by all cells in the human body, with low immunogenicity and good biocompatibility. It is hard to induce the production of antibodies and to cause immunogenicity in the body. EVs of SPOT-mRNA03, integrating corresponding

molecular targeted peptides on the lipid layer membrane, possess strong targeting ability, which can directly deliver loaded dystrophin mRNA to target cells. The dystrophin mRNA sequence loaded on SPOT-mRNA03 is the same as the mRNA molecular sequence encoding dystrophin in the human body. In addition, the mRNA molecule can only be delivered to the cytoplasm through EVs, without entering the nucleus, so there is no risk of foreign gene integration. Therefore, compared to AAV gene therapy products, SPOT-mRNA03 has higher safety.

According to the mechanism of action of SPOT-mRNA03, endogenous full-length intact dystrophin can be generated and secreted in dystrophin deficient receptor. Because there are not the intact dystrophin proteins in DMD patients, it may be recognized by the immune system as an exogenous protein in the patient's body, leading to a more severe immune response and potentially inducing further muscle damage, reversing any benefits that the patient should have received. There have been reports in other clinical studies of gene therapy for DMD [27]. According to reports, independent clinical studies of three different AAV products have shown a total of approximately 200 patients, of which 5 experienced severe immune reactions. Symptom onset occurred 3 to 6 weeks after administration: all five patients had severe weakness of the proximal and distal limb muscles that led to loss of ambulation, as well as weakness of the bulbar and respiratory muscles, which led to receipt of transient ventilatory support in three of the patients (two with noninvasive ventilation and one with endotracheal intubation). In addition, all patients had symptoms of myositis, and three patients also experienced symptoms of myocarditis. Therefore, various immunomodulatory treatments were used among the trials, including pulse dose glucocorticoids, intravenous immunoglobulin, plasmapheresis, and tacrolimus. The above clinical symptoms were resolved within 3 months after treatment. To prevent the occurrence of immune response, this study orally administered tacrolimus or sirolimus daily for 4 weeks starting from D-3 before treatment of the study drug. Since these two drugs are immunosuppressants, it is necessary to closely monitor the adverse reactions of medication to prevent the occurrence of infections. The detailed adverse reactions of this drug can be found in **Error! Reference source not found.**.

In addition, SPOT-mRNA03 belongs to a hypertonic solution with high osmotic pressure (>600 Osm/L) and may lead to extravasation and phlebitis if via intravenous infusion. Therefore, appropriate venous access should be selected for administration, and appropriate vascular access device should be used. During the infusion process, administer slowly,

carefully observe the patient's infusion site, evaluate the infusion site reaction, and strictly prevent adverse events such as extravasation and venous infusion complications. If extravasation is observed, stop administration immediately.

The preclinical sin- and multi- dose toxicity study of SPOT-mRNA03 showed that SPOT-mRNA03 had no significant systemic adverse reactions in the animal body. At present, there are no similar products to SPOT-mRNA03 on the market, and there are also no corresponding reports of ongoing clinical trials. There are over 400 registrations or related clinical trials based on EVs, most of which focus on the diagnosis, treatment, or prognostic strategies for various cancers, cardiovascular diseases, and neurodegenerative diseases. So far, these clinical trials have reported that EVs based therapies have no significant toxicity or adverse effects, indicating the safety and feasibility of the technology.

In summary, from the perspective of product design, production, and mechanism of action, the most likely adverse reactions caused by SPOT-mRNA03 infusion in DMD patients are infusion reaction and immune reaction, which may pose safety risks in the clinical application of this product. However, the toxicity studies of SPOT-mRNA03 and other relevant reports of gene therapy indicate that the safety risks of SPOT-mRNA03 in clinical application are controllable.

3.2 Benefit Assessment

SPOT-mRNA03 is the latest gene therapy product for DMD. After administration to DMD patients, the molecular targeting peptide on the EVs membrane can guide SPOT-mRNA03 to enter the patient's muscle cells, allowing dystrophin deficient receptor cells to utilize intracellular ribosomes and amino acids to translate and secrete endogenous full-length intact dystrophin. Dystrophin is a cytoskeletal protein that has an anti-mechanical stretching effect and can prevent damage to muscle cells during contraction. The generated dystrophin can tightly bind with various proteins in the inner, transmembrane, and outer regions of the cell membrane, such as sarcoglycan and dystroglycan, and are interrelated to form a whole inside and outside the cell membrane. It maintains material exchange and connection inside and outside the cell membrane, protects the integrity and stability of the cell membrane structure, and thus protects muscles from damage and/or promotes muscle repair and regeneration. Therefore, SPOT-mRNA03 to DMD patients can theoretically alleviate the clinical symptoms and improve the quality of life of DMD patients.

Since SPOT-mRNA03 is completely different from existing gene therapy products on the market, it is the first to achieve in vivo targeted delivery of full-length dystrophin mRNA

molecules, which can generate full-length and complete dystrophin in the body. Therefore, SPOT-mRNA03 based on dystrophin mRNA loaded on EVs can provide a new and effective protein replacement therapy for DMD patients, making clinical cure of DMD possible.

4. Study Objectives and Endpoints

Table 4 The objectives and endpoints of this study

Objectives	Endpoints
Primary objective:	Primary endpoints:
• To evaluate the safety and tolerability of SPOT-mRNA03 administered by intravenous infusion (IV) to DMD patients.	• Collection and quantification of adverse events (description of event, time of onset and resolution, assessment of severity and causal relationship to the investigational drug), as well as the data of physical examination, vital signs, 12-lead ECG, clinical laboratory test results (hematology, biochemistry, urinalysis) in this study.
Secondary objectives:	Secondary endpoints:
• To evaluate the changes of dystrophin mRNA in serum and muscles of DMD patients after IV infusion of SPOT-mRNA03.	• Changes of dystrophin mRNA concentration in serum before and different timepoints after the initial administration (see Table 5 for schedule) as well as before and after the 2nd, 4th, 6th, and 8th administration and during follow-up. And the changes in dystrophin mRNA concentration in muscles at baseline and D29 after the completion of the last dose (if the biopsy time is between 12-24 hours after the last dose) (by qPCR).
• To evaluate the changes in dystrophin expression and engraftment in muscles of DMD patients after IV infusion of SPOT-mRNA03.	• Changes in the expression and engraftment level of dystrophin in muscle biopsies at baseline and D29 (after the completion of the last dose) (by Western Blot and IHC methods).
• To evaluate the changes in serum anti-dystrophin antibodies and cytokines in DMD patients after IV infusion of SPOT-mRNA03.	• Changes in anti-dystrophin antibodies and cytokines in the serum at baseline and before the 2nd, 4th, 6th, and 8th administration and during follow-up (see Table 5 for schedule).

5. Study Design

5.1. Overall Design

This is a FIH, open-label, single-arm, and single-center exploratory clinical study of SPOT-mRNA03 administered via IV infusion for DMD patients. The primary objective of this study is to evaluate the safety and tolerability of SPOT-mRNA03 for DMD patients. And the

secondary objectives are to preliminarily investigate the concentration change of dystrophin mRNA, the expression and engraftment level changes of dystrophin protein, as well as changes in cytokines and immunogenicity. The study has two ascending dose cohorts:

The study has two ascending dose cohorts:

I cohort：5.0×10^9 CN Dystrophin mRNA / kg

II cohort：5.0×10^{10} CN Dystrophin mRNA / kg

The study will have a screening period of 30 days, during which patients or their legal guardian written informed consent will be obtained before screening assessments and eligibility will be determined. A total of 6 DMD patients will participate in the study. there are 3 patients in each dose cohort [No previous treatment with corticosteroids]. All subjects started taking 0.05-0.1mg/kg/d (adjusted according to the actual clinical situation) tacrolimus or sirolimus orally at D-3 (3 days before initial dose of SPOT-mRNA03) for 4 weeks. All subjects are first administered via intravenous infusion on D1, and then administered twice a week (once every 4 days) for a total of 8 doses. Four weeks after the initial administration of the subjects in the previous dose cohort, if there are no serious adverse events related to the treatment, it will be determined that the subjects in next dose cohort could be administered after discussion between the investigators and the sponsor.

Safety evaluations on subjects are conducted during each administration and follow-up. Muscle biopsies are performed at baseline and D29 after administration (see Table 5 for details). Samples for muscle biopsy will be taken from gastrocnemius/biceps brachii muscle at baseline and from the gastrocnemius and biceps brachii muscles on D29. Western blot (WB) is used to detect the expression and engraftment of dystrophin protein. Immunohistochemistry (IHC) is used to detect fiber intensity and percentage of dystrophin positive fibers, and qPCR is used to detect changes in dystrophin mRNA at baseline and D29 (if the biopsy time is between 12~24 hours after the last dose).

The blood samples are collected for detection of dystrophin mRNA, cytokines and immunogenicity (see Table 5 for details) besides for laboratory testing. Perform MRI and EMG examinations at baseline and at week 16 after administration.

5.2. Duration of Study

The screening period is 0-30 days, with 4 weeks of treatment period and a follow-up period of 6 months.

5.3. Termination Criteria

1) According to CTCAE v5.0 criteria, during the dose escalation process, more than

1/2 of the subjects have grade 2 or above liver, kidney, cardiac and hematological TRAEs, or more than 1/3 of the subjects have other system AEs of grade 3 or above occur, the trial should be terminated.

2) The collaborator requests to terminate the study due to financial or management reasons, etc.
3) Termination of the trial is requested by IRB/EC.

5.4. Schedule of Events

Table 5 Schedule of Events

Study Period	**Screening**	**Baseline**	**Treatment**								**Follow-up**					
Visit Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Visit Time[a]	D-30~D-8	D-7~D-1	D1	D5	D9	D13	D17	D21	D25	D29	W8	W12	W16	W20	W24	W28
Visit Window (Days)				±1	±1	±1	±1	±1	±1	±1	±3	±3	±5			
Informed Consent	X															
Demographics	X															
Medical History[b]	X															
Vital Signs[c]	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Physical Examination[d]	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
ECHO[e]	X												X			
12-lead ECG [f]	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
PFT[g]	X	X											X			
Chest X-Ray	X															
Haematology[h]	X	X		X		X		X		X			X			X
Biochemistry[i]	X	X		X		X		X		X			X			X
Urinalysis[j]	X	X		X		X		X		X			X			X
Hepatitis B & C, HIV[k]	X															
Coagulation[l]	X															
SPOT-mRNA03 Administration			X	X	X	X	X	X	X	X						
EMG		X											X			
MRI[m]		X											X			
PK[n]			X	X		X		X		X	X	X	X			
Muscle Biopsy[o]		X								X						
Photography[p]		X	X	X	X	X	X	X	X	X						

Study Period	**Screening**	**Baseline**	**Treatment**								**Follow-up**					
Visit Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Visit Time[a]	D-30~D-8	D-7~D-1	D1	D5	D9	D13	D17	D21	D25	D29	W8	W12	W16	W20	W24	W28
Visit Window (Days)				±1	±1	±1	±1	±1	±1	±1	±3	±3	±5			
BiomarkerTesting[q]			X	X		X		X		X	X	X	X			
Immunogenicity[r]		X	X	X		X		X		X	X	X	X	X	X	X
NSAA[s]		X											X			X
Administration of tacrolimus/sirolimus		Starting from D-3, take 0.05-0.1mg/kg tacrolimus/sirolimus orally daily for 4 weeks														
Adverse Events	Continuous monitoring records from initial administration to last visit															
Concomitant Medications	Continuous recording from signing informed consent to the last follow-up															

ABBREVIATIONS: D = study day; W = study week; ECG = electrocardiogram; ECHO = echocardiogram; PFT = pulmonary function test; EMG = electromyography; MRI = magnetic resonance imaging; EOS = end of study; AE = adverse events. Unscheduled tests may be performed at the discretion of the Investigator if clinically indicated and required for safety.

a) Study drug will be started administration on Day 1 (t = 0). Day 1 is defined as the day of first administration of study drug. The time of the study drug first administration is defined as t = 0. Baseline will be the last-performed assessment before the first administration of study drug. There is no Day 0; the day before Day 1 is Day -1.

b) A full medical history will be obtained from subjects to determine eligibility. If AEs to be recorded from time of dosing rather than from signing consent to be determined Any medical events occurring in the Screening period until first administration of study drug will be recorded as medical history. Current medical history related to DMD (gene detection results and corresponding treatments information), as well as other treatment history within 6 months will be collected.

c) Vital signs include blood pressure, pulse, respiration, and body temperature. During the treatment period, vital sign test will be performed within 1 hour before each administration. In addition, vital sign testing will also be conducted 30 minutes (± 10 minutes), 2 hours, and 4 hours (± 30 minutes) after the initial administration.

d) Physical examination includes height, weight, skin and mucous membranes, lymph nodes, head and neck, chest, abdomen, spine, limbs and joints, musculoskeletal system, and nervous system. It will be performed before administration in each cycle. Calculate the individual dosage based on the baseline body weight and height values. Only the height and weight values measured during the screening period are used to calculate BMI and determine whether participants can be enrolled. BMI = weight (kg)/height ^{2} (m^2).

e) ECHO will be performed during screening and EOS.

f) 12-lead ECG testing is required before and 2 hours (± 1 hour) after D1 administration, and others are before administration. When the ECG measurement results are abnormal (including QTcF interval abnormalities, such as QTcF>500 ms or relative baseline increase>60 ms) or other abnormalities have clinical significance, an additional 2 ECG measurements are required, with a minimum interval of about 2 minutes between repeated measurements.

g) Pulmonary function tests include forced vital capacity (FVC), maximum inspiratory pressure (MIP), and maximum expiratory pressure (MEP). It will be performed during screening, baseline, and at W16 visits).。

h) Haematology includes white blood cell count (WBC), neutrophil count (NEUT #), eosinophil count (EO #), basophil count (BASO #), lymphocyte count (LYMPH #), red blood cell count (RBC), hemoglobin (HGB), platelet count (PLT), and hematocrit (HCT) (the test results within 7 days before administration can be accepted).

i) Biochemistry includes bilirubin (total, indirect and direct), total protein, albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), creatinine (Cr), blood urea (Urea), blood glucose (GLU), serum sodium (Na+), serum potassium (K^+), serum calcium ($Ca2^+$), serum magnesium (Mg^{2+}), serum chloride (CL^-), lactate dehydrogenase (LDH), serum creatine kinase (CK), serum creatine kinase MB isoenzyme (CKMB) and c-reactive protein (CRP). Calculation of eGFR [CKD-EPI]. (the test results within 7 days before administration can be accepted).

j) Urinalysis includes Urinary bilirubin (UBG), urinary bilirubin (BIL), urinary ketone body (KET), urinary occult blood (BLD), urinary protein (PRO), urinary nitrite (NIT), urinary glucose (GLUU), urinary specific gravity (SG), urinary pH (pH), red blood cells (RBC), and white blood cells (WBC). (the test results within 7 days before administration can be accepted).

k) Hepatitis B & C and HIV serology tests at Screening will include hepatitis B surface antigen (HBs Ag), anti-hepatitis B surface antibodies (anti-HBs Ab) anti-hepatitis B core antibodies (anti-HBc Ab), hepatitis B e antigen (HBe Ag), anti-hepatitis B e antibodies (anti-HBe Ab), hepatitis C antibodies, anti-HIV1 and anti-HIV2 antibodies. Any positive result is exclusionary. (the test results within 14 days before screening can be accepted).

l) Coagulation includes prothrombin time (PT), activated partial thromboplastin time (APTT) and international normalized ratio (INR). The examination results of coagulation within 14 days before screening can be accepted.

m) Only conscious sedation will be used during the cardiac MRI. If general anesthesia is required, the cardiac MRI will not be performed.

n) The blood sample for the PK test will be collected before and 0h+10min after the 1st, 2nd, 4th, 6th, and 8th administration. Intensive blood collection will be performed before and 1h ± 10min, 2h ± 20min, 8h ± 1h, 24h ± 1h, 48h ± 2h, and 72h ± 3h after the initial administration. PK blood samples will also be collected at each follow-up. If dystrophin mRNA cannot be detected at W8 follow-up, blood samples may not be collected for this test in subsequent follow-up.

o) Three subjects from each cohort will undergo muscle biopsy at baseline and on D29 (after administration). Perform biopsy sampling from the gastrocnemius/biceps brachii muscle at baseline and from the gastrocnemius and biceps brachii muscles on D29 (after the last dose).

p) Photography: Take photos of the biopsy site before and after each biopsy. Take photos of the infusion site approximately 1 hour after each administration and observe the reaction at the infusion site.

q) Cytokine test: TNF-α, INF-γ, IL-2, IL-6, and IL-10. Collect blood samples before the 1st, 2nd, 4th, 6th, and 8th administration. If the cytokine levels return to normal at W8 follow-up, blood samples may not be collected for this test during subsequent follow-up.

r) Immunogenicity: anti-dystrophin antibody test. Collect blood samples at baseline/before the initial dose, before the 2nd, 4th, 6th, and 8th dose (if no more than 7 days from baseline to the initial dose, no additional collection is required before the initial dose) and during each follow-up. If the anti-dystrophin antibody cannot be detected in the follow-up samples at week 8, blood samples may not be collected for this test during subsequent follow-up.

s) NSAA：North Star Ambulatory Assessment, conducted at baseline, W16, and W28 to assess differences in ambulatory function before and after dosing. The NSAA examination includes: standing, walking, rising from a chair, stepping onto a raised surface, jumping, running, standing on one leg, heel walking, descending stairs, ascending stairs, lifting a leg, and hopping on one leg.

6. Study Population

6.1 Inclusion criteria

Only those who meet all the following criteria can be enrolled:

1) According to the requirements of the region/country and/or IRB/IEC, the patient and/or legal guardian have signed a written informed consent form and are aware of all relevant study content.
2) Ambulatory boys aged 2 to 6 years, inclusive.
3) Confirmed diagnosis of DMD through multiplex ligation-dependent probe amplification (MLPA) and whole-exome sequencing.
4) Tolerance for muscle biopsy under anesthesia with no absolute contraindications to the procedure
5) Heart, liver, lung, and kidney functions are sufficient:
    a) The left ventricular ejection fraction (LVEF) should be ≥ 50%;
    b) Forced vital capacity (FVC) ＞ 50% of the expected value, and do not require nighttime ventilation;
    c) Patient's glomerular filtration rate (GFR)>30 mL/min/1.73 m^2

6.2 Exclusion Criteria

Those who meet any one of the following criteria must be excluded:

1) Complications other than DMD that may cause muscle weakness and/or motor dysfunction.
2) There are severe intellectual disabilities (such as severe autism, severe cognitive impairment, and severe behavioral disorders) that, according to the investigator's judgment, can affect the study.
3) Hospitalization for respiratory failure within 8 weeks prior to screening.
4) Asthma or underlying lung diseases that are poorly controlled, such as bronchitis, bronchiectasis, emphysema, or recurrent infectious pneumonia that investigator believes may affect respiratory function.
5) Severe uncontrolled heart failure (NYHA III-IV), including any of the following conditions：

a) Intravenous administration of diuretics or positive inotropic drugs is required within 8 weeks prior to screening.

b) Hospitalization due to worsening heart failure or arrhythmia within 8 weeks prior to screening.

6) Abnormal laboratory values considered clinically significant:

a) GGT > 3 × upper limit of normal

b) Bilirubin ≥ 3.0 mg/dL

c) Creatinine ≥ 1.8 mg/dL

d) Hemoglobin < 8 or > 18 g/dL

e) White blood cell count > 18,500/μL

7) There are arrhythmias that require antiarrhythmic treatment.

8) Subjects who are undergoing immunosuppressive therapy.

9) Has used other gene therapy, investigational drugs, or any treatment aimed at increasing dystrophin expression.

10) Subjects with a history of major surgeries within 12 weeks prior to the initial infusion or planning to undergo major surgeries (such as scoliosis surgery) during this study.

11) Subjects who are allergic to investigational products or local anesthetic drugs or have a history of severe allergies or genetic allergic reactions.

12) Within 6 months prior to the initial infusion, the subjects are exposed to another investigational drug or are participating in an intervention clinical trial.

13) Subjects with positive of hepatitis B core antibody or hepatitis C antibody or HIV antibody during screening.

14) Investigator believes that the presence of any other serious diseases, medical conditions, or chronic drug treatment needs can pose unnecessary risks to gene transfer.

7. Subject Coding and Grouping

This study is an open-label, single-arm study.

There are two ascending dose cohorts in this study:

I cohort：5.0×10^9 CN Dystrophin mRNA / kg

II cohort：5.0×10^{10} CN Dystrophin mRNA / kg

Four weeks after the initial administration of the subjects in the previous dose cohort, if there are no serious adverse events related to the treatment, it will be determined that the subjects in next dose cohort could be administered after discussion between the investigators and the sponsor.

This study is a first-in-human, open-label study of SPOT-mRNA03, with only 6 subjects, so the subjects will be coded, grouped, and dosed only based on the time of participant screening and enrollment. Once the subjects sign the informed consent form, they will receive a unique screening number 01, 02, 03 **----** in order, and so on. After enrollment, obtain the unique enrollment numbers AZ01, AZ02, AZ03 **----** in order, and so on.

8. Study Procedures

8.1. Treatment and Follow-up

Those subjects who are eligibility will receive an enrollment number and participate in subsequent clinical items. The detailed items that subjects need to participate in at each visit are as follows.

8.1.1. Screening (D-30 ~ -8)

1) Sign informed consent form: explain the study process to the subjects or legal guardian and obtain a written informed consent form from the subjects.
2) Assign screening numbers in the order of obtaining written informed consent.
3) Demographics: gender, age, race, height, weight.
4) Disease history：medical history and surgical history within the 6 months prior to screening.
5) Medical history：drug allergy history and medication history within the 6 months prior to screening.
6) Physical examination: height, weight, skin and mucous membranes, lymph nodes, head and neck, chest, abdomen, spine, limbs and joints, musculoskeletal system, and nervous system.
7) Vital signs: blood pressure, pulse, respiration, and body temperature.
8) ECHO and 12-lead ECG：the ECHO and ECG records will be checked by an investigator to determine if the patient meets the inclusion and exclusion criteria.
9) Pulmonary function tests: forced vital capacity (FVC), maximum inspiratory pressure (MIP), and maximum expiratory pressure (MEP).
10) Chest X-Ray.

11) Lab tests: the items include Haematology, Biochemistry, Urinalysis, Coagulation, and Hepatitis B & C, HIV examination.
12) Collect concomitant medication information.

Determine the eligibility of subjects based on the test results during the screening period. Eligible subjects will participate in the baseline examinations listed below.

8.1.2. Baseline (D-7 ~ -1)

1) Vital signs and physical examination
2) 12-lead ECG
3) PFT
4) Lab tests: Haematology, Biochemistry and Urinalysis (if the screening period test is conducted within 7 days before administration, this test may not be performed here).
5) Starting from D-3, take 1mg/kg tacrolimus/sirolimus orally daily.
6) EMG
7) MRI
8) Sampling for muscle biopsy is performed from the gastrocnemius/biceps brachii muscle, and the biopsy site is photographed before and after biopsy.
9) Collect blood samples required for immunogenicity testing.
10) Collect concomitant medication information.
11) Collect adverse events information (due to the use of tacrolimus/sirolimus, collect AEs from D-3).
12) NSAA: To evaluate subjects' motor function and ambulatory capacity. It's included 12 functional activities: standing, walking, rising from a chair, stepping onto a raised surface, jumping, running, standing on one leg, heel walking, descending stairs, ascending stairs, active straight leg raise, and hopping on one leg.

8.1.3. Treatment (D1 ~ 29)

The subjects participating in the baseline will receive treatments of SPOT-mRNA03 via IV on D1.

Before the initial administration on D1

1) Vital signs and physical examination.

2) 12-lead ECG
3) Collect blood samples for PK and Biomarker tests, if blood samples for immunogenicity testing were not collected during the baseline, they should also be collected at this time.
4) Take 0.05-0.1mg/kg tacrolimus/sirolimus orally daily.
5) Collect concomitant medication information.
6) Collect adverse events information.

Calculate the dosage of SPOT-mRNA03 based on the concentration of SPOT-mRNA03, subject weight, and dose cohort participating in the study.

After the initial administration on D1

1) Take photos of the infusion site and observe the infusion response within 4 hours.
2) Vital signs: testing will be conducted 30 min (± 10 min), 1 h (± 10 min), 2 h (± 30 min), and 4 h (± 30 min) after the initial infusion.
3) The blood samples required for PK testing will be collected densely at timepoints of 0h +10min, 1h ± 10min, 2h ± 20min, 8h ± 1h, 24h ± 1h, 48h ± 2h, 72h ± 3h after the initial infusion, respectively.
4) 12-lead ECG needs to be tested 2 hours (± 1 hour) after the end of infusion.
5) Collect adverse events information.

Other treatment cycles

1) Vital signs: vital signs should be tested within 1 hour before and after each administration.
2) Physical examination should be performed prior to each administration.
3) 12-lead ECG should be performed prior to each administration.
4) Lab tests: perform before the 2nd, 4th, 6th, and 8th administration, respectively, including Haematology, Biochemistry and Urinalysis.
5) Take photos of the infusion site, within 4 hours after each administration, closely observe whether the subjects have any infusion reactions.
6) Take 1mg/kg tacrolimus/sirolimus orally daily for 4 weeks.
7) Samples for muscle biopsy will be taken from the gastrocnemius muscle and biceps brachii muscle, and the sampling site will be photographed before and after sampling.
8) Blood samples for PK, Biomarker and Immunogenicity testing will be collected

before the 2nd, 4th, 6th, and 8th administration, respectively. And blood samples for PK test will also be collected after 0h + 10min of the 2nd, 4th, 6th, and 8th administration.

9) Collect concomitant medication information.
10) Collect adverse events information.

Inform the subjects and guardians of the specific time for the next follow-up at the end of each follow-up.

8.1.4. Follow-up

The subjects will be followed up for various tests and sample collection at weeks 8, 12, 16, 20, 24 and 28 after the initial administration, as follows:

1) Perform vital signs, physical examination, and 12-lead ECG at each follow-up.
2) Blood sample collection for PK, biomarkers, and immunogenicity testing (if any one of dystrophin mRNA, cytokine, or anti-dystrophin antibody cannot be detected in the blood sample of W8 or can be detected but returns to normal, further blood sample collection for this test is not necessary in subsequent follow-up).
3) Lab tests (Haematology, Biochemistry and Urinalysis) and NSAA at W16 and W28
4) ECHO, EMG, MRI, and PFT tests will be conducted at W16.
5) Collect concomitant medication information.
6) Collect adverse events information.

The study will be ended after completing follow-up at W28.

8.2. Supply of Investigational drugs/treatments

8.2.1. Name, Source and Strength

【Generic Name】Dystrophin mRNA Injection

【Product code】SPOT-mRNA03

【Strength】****E10 Dystrophin mRNA / 10mL / vial

【Expiry Date】3 month

【Active Pharmaceutical Ingredients】Dystrophin mRNA-loaded EVs

【Pharmaceutical excipients】CryoStorCS10 frozen storage solution (containing 10% dimethyl sulfoxide (DMSO)), phosphate-buffered saline (PBS), human serum

albumin (HSA)

【Storage】-60℃~ -90℃

8.2.2. Preparation and Storage

SPOT-mRNA03 needs to be transported on dry ice and stored at -60 ℃ to -90 ℃. It is expected to be stored for 3 months.

8.2.3. Route of Administration and Dosage Adjustment

Route of Administration

Intravenous infusion: twice a week (once every 4 days). Each subject will receive a total of 8 treatments.

Each bag of SPOT-mRNA03 needs to slowly thaw at 4 ℃/ice before use. If there are no special requirements, SPOT-mRNA03 after fusion should be used as soon as possible.

Dosage Adjustment

Calculate the dosage of SPOT-mRNA03 based on the concentration of SPOT-mRNA03, subject weight, and dose cohort participating in the study. The total volume of infusion is 40 mL.

Choose an appropriate intravenous infusion site based on the subject's personal and venous condition, with an infusion time of 60~120 minutes. If the subject experiences mild allergic reactions such as dizziness, chills, rash or extravasation during the infusion process, the infusion should be immediately suspended. After recovery, the investigator will determine whether to continue the administration. The infusion speed can be appropriately reduced (as determined by the investigator), and the infusion time can be correspondingly extended (as determined by the investigator). After completion of infusion, record the actual dosage and start and end time of infusion.

Photograph

Take photos for the infusion site before and after each infusion, and carefully observe the infusion reaction.

8.3. Concomitant medication

The newly generated dystrophin will be recognized by the immune system of the subjects as an exogenous protein, triggering an immune response. Any immune response to Dystrophin can affect its engraftment and potentially induce further muscle damage,

reversing any benefits that patients should have received. Therefore, the subjects should begin to orally take 0.05-0.1 mg/kg tacrolimus or sirolimus daily for 4 weeks at D-3 before SPOT-mRNA03 administration.

Tacrolimus/Sirolimus are both potent immunosuppressants, and there is a possibility of clinical side effects after use, which require experienced clinical physicians to handle. Along with its needed effects, tacrolimus may cause some unwanted effects. Although not all of these side effects may occur, if they do occur, they may need medical attention. Check with your doctor immediately if any of the following side effects occur while taking tacrolimus/sirolimus.

1) Cardiovascular system symptoms: including hypertension, angina, palpitations, heart failure, etc.
2) Sensory system symptoms: Some patients have tremors, headaches, insomnia, depression, hallucinations, forgetfulness, etc.
3) Digestive system symptoms: including constipation, diarrhea, nausea, vomiting, weight changes, difficulty swallowing, stomach pain, etc.
4) Respiratory symptoms: Some patients have asthma, respiratory failure, lung function damage, pulmonary fibrosis, etc.
5) Skin symptoms: may cause itching, sweating, rash, hirsutism, etc.
6) Symptoms of blood and lymphatic system: leukopenia, anemia, thrombocytopenia, bone marrow suppression, etc. Daily manifestations can include sudden high fever, pale complexion, mental fatigue, limb weakness, memory loss, bleeding, etc.
7) Renal dysfunction: renal failure, tubular necrosis, oliguria, etc. Absence of urine and uremia are rare.
8) Other: blurred vision, photophobia, tinnitus, edema, local pain, urinary and fecal incontinence, thyroid abnormalities, etc.

8.4. Patient Compliance and Withdrawal

8.4.1. Screening Failure

Screening failure is defined as the subject who signed the informed consent form not being eligibility for the study according to the results of various examination during screening

period. Subjects who failed the screening should not participate in this trial, and the reasons for the screening failure must be recorded in the original records.

8.4.2. Withdrawal Criteria

1) Considering the safety, the investigator believes that withdrawing from the study is in the best interest of the subjects.
2) Subjects with poor compliance cannot insist on completing the trial as planned or have other circumstances that may affect the judgment of the research results.
3) The subject voluntarily requested to withdraw from the trial and withdraw the informed consent.

8.4.3. Management of Subjects During the Study

Participants in this study should follow the process arrangement of the study. At each visit, participants should be arranged to participate in relevant study items as required. Inform the subjects and guardians of the specific time and the testing items to be implemented for the next follow-up at the end of each follow-up. At each visit, the study logs of the subjects should be checked.

Participants can withdraw from this study at any time during the trial process. investigators should understand the reasons for subjects withdrawing from the trial and whether any adverse events have occurred. If any adverse events occur, follow-up should be conducted. Participants who withdraw early should complete the corresponding experimental procedures (including physical examinations, vital signs, ECG, and laboratory tests) as much as possible according to the protocol requirements.

Subjects who withdrew from the study due to adverse events or abnormal safety evaluations with clinical significance should be followed up by the investigators until the adverse events are resolved or stabilized until the investigators deems follow-up unnecessary or lost.

The date and reason for early withdrawal from the study must be recorded in the corresponding case report form (CRF). Adverse events, concomitant medications, tracking of abnormal laboratory test results, and the end page of CRF must be completed as much as possible.

9. Study Assessment

9.1 PK Assessment

9.1.1 Assessment Indicators

Application of qPCR method to detect changes in dystrophin mRNA concentration in the serum of subjects. Intensive collection of blood samples after the initial administration for detecting single-dose PK. Subsequent blood collection is used to detect steady-state PK after multiple administration.

PK indicators: Cmax (peak concentration), Tmax (peak time), C trough, ss (steady-state minimum blood drug concentration), Css,max (steady-state maximum blood drug concentration), AUC0-t (area under the curve of the last measurable concentration time point) and t1/2 (elimination phase half-life).

9.1.2 Timepoints of Sampling

The blood samples for PK test will be collected before and 0 h +10 min after the 1st, 2nd, 4th, 6th, and 8th infusion. Intensive blood collection will be carried out at 1 h ± 10 min, 2 h ± 20 min, 8 h ± 1 h, 24 h ± 1 h, 48 h ± 2 h, and 72 h ± 3 h after the initial infusion. PK blood sample collection is also performed during the follow-up (if dystrophin mRNA cannot be detected in the blood samples at W8, blood samples for PK testing do not need to be collected again during subsequent follow-up).

9.2 Pharmacodynamic Assessment

9.2.1 Dystrophin Expression

1) **Assessment Indicators**

Quantity of dystrophin is measured by western blot of biopsied muscle. Immunohistochemistry (IHC) is used to detect fiber intensity and percentage of dystrophin positive fibers. Levels are described as change from baseline, in order to remove any background signal in the assay. Levels are expressed as a percent of control (i.e., as a percent of the level of normal, wild-type dystrophin present in muscle tissue of healthy individuals without DMD or Becker muscular dystrophy).

2) **Sampling for Muscle Biopsy**

Select a suitable location of the gastrocnemius muscle/biceps brachii muscle, confirm the puncture site, disinfect with iodine, and after local anesthesia, use puncture needle to

penetrate the tissue and muscle, extract muscle tissue with a size of 0.5 cm × 1 cm × 0.5 cm and sent to a pathological laboratory for biopsy. Camera the biopsy site before and after each muscle biopsy.

3) **Timepoints of Sampling**

Samples of each subject for biopsy will be taken from gastrocnemius or biceps brachii muscle at baseline (one site) and from the gastrocnemius and biceps brachii muscles on D29 (two sites).

4) **Detection Method**

The expression of dystrophin muscle tissue is detected by the following methods:

- Evaluate expression level of dystrophin (measured by western blot) at baseline and D29 (after the completion of the last dose).
- Evaluate engraftment of dystrophin (measured by immunofluorescence for fiber intensity and percentage of dystrophin positive fibers) at baseline and D29 (after the completion of the last dose).

The concentration of dystrophin mRNA in muscle tissue will be detected:

- Evaluate changes in dystrophin mRNA (detected by qPCR) at baseline and D29 dystrophin protein (if the biopsy time is between 12~24 hours after the last dose).

9.2.2 Electromyography

Perform electromyography at baseline and at W16 after the initial administration to assess muscle health status.

9.2.3 MRI

Perform electromyography at baseline and at W16 after the initial administration to assess muscle health status.

9.3 Immunogenicity Assessment

9.3.1 Assessment Indicators

1) Changes of concentration of cytokine including TNF-α, INF-γ, IL-2, IL-6, and IL-10 will be detected.
2) Changes of anti-dystrophin antibody level can be measured.

9.3.2 Timepoints of Sampling

Cytokine test: TNF-α, INF-γ, IL-2, IL-6, and IL-10. Collect blood samples before the initial administration and before the 1st, 2nd, 4th, 6th, and 8th administration. If the cytokine levels return to normal at W8 follow-up, blood samples may not be collected for this test during subsequent follow-up.

Anti-dystrophin antibody test. Collect blood samples at baseline/before the initial dose, before the 2nd, 4th, 6th, and 8th dose (if no more than 7 days from baseline to the initial dose, no additional collection is required before the initial dose) and at each follow-up. If the anti-dystrophin antibody cannot be detected in the follow-up samples at week 8, blood samples may not be collected for this test during subsequent follow-up.

9.3.3 Detection Method

Immunogenicity and cytokine tests will be detected by ELISA method.

9.4 Safety Assessment

9.4.1 Safety Parameters

All AEs and clinically significant laboratory abnormalities will be graded according to Common Terminology Criteria for AEs, Version 5.0 dated 27 November 2017.

Safety parameters include vital signs (including temperature, pulse, respiratory rate, blood pressure), physical examination (including general condition, mucous membranes, lymph nodes, head and neck, abdomen, musculoskeletal, respiratory rate, cardiovascular, nervous and psychiatric conditions) and laboratory tests (including hematology, urinalysis, blood chemistry) and 12-lead ECG. In case of any abnormality, the corresponding indicators shall be rechecked.

Evaluate the TRAEs occurred during the clinical study, record its clinical manifestation, severity, occurrence time, duration, treatment method and prognosis, and determine its correlation with the investigational drug.

9.4.2 Baseline Signs and Symptoms

The baseline value serves as the starting point for clinical evaluation, and a series of physical examinations or laboratory tests are conducted to confirm the premedication status of the subjects, in order to facilitate comparison between post-treatment and pre-treatment. All subjects are required to undergo baseline measurement, which should be

done before randomization. However, if the examination is invasive (such as biopsy), it should be performed after chemical examination as much as possible to reduce harm to the subjects. In addition, the baseline value should be as close as possible to the initial treatment. Before the study begins, conduct a comprehensive physical and laboratory examination of the study subjects, and record relevant data. Inquire and record in detail the lifestyle and past medication history of the study subjects. Based on the collected data, determine the baseline status of each study subject and record it in the CRF.

9.4.3 Laboratory Safety Assessment

According to the study Schedule in Table 5, list the ***Hematology***, ***blood biochemistry, Urinalysis, coagulation, and serum pathogen test*** for each subject. If applicable, it will also be marked as above or below the corresponding normal range. Evaluate changes in laboratory results before and after administration. We will also compare the indicators before and after the study to discover whether the parameters have returned to the pre study level.

1) ***Haematology***: white blood cell count (WBC), neutrophil count (NEUT #), eosinophil count (EO #), basophil count (BASO #), lymphocyte count (LYMPH #), red blood cell count (RBC), hemoglobin (HGB), platelet count (PLT), and hematocrit (HCT) (the test results within 7 days before administration can be accepted).
2) ***Urinalysis:*** Urinary bilirubin (UBG), urinary bilirubin (BIL), urinary ketone body (KET), urinary occult blood (BLD), urinary protein (PRO), urinary nitrite (NIT), urinary glucose (GLUU), urinary specific gravity (SG), urinary pH (pH), red blood cells (RBC), and white blood cells (WBC).
3) ***Biochemistry:*** bilirubin (total, indirect and direct), total protein, albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), creatinine (Cr), blood urea (Urea), blood glucose (GLU), serum sodium (Na+), serum potassium (K^+), serum calcium ($Ca2^+$), serum magnesium (Mg^{2+}), serum chloride (CL^-), lactate dehydrogenase (LDH), serum creatine kinase (CK), serum creatine kinase MB isoenzyme (CKMB) and c-reactive protein (CRP). Calculation of eGFR [CKD-EPI].
4) ***Coagulation:*** prothrombin time (PT), activated partial thromboplastin time

(APTT) and international normalized ratio (INR).

5) ***Hepatitis B & C and HIV serology tests***: hepatitis B surface antigen (HBs Ag), anti-hepatitis B surface antibodies (anti-HBs Ab) anti-hepatitis B core antibodies (anti-HBc Ab), hepatitis B e antigen (HBe Ag), anti-hepatitis B e antibodies (anti-HBe Ab), hepatitis C antibodies, anti-HIV1 and anti-HIV2 antibodies.

9.4.4 Vital Signs and Physical Examination

Perform vital signs and physical examinations at each visit and record any clinically significant changes. The changes in vital signs from baseline to the EOS will be provided, and the incidence of significant abnormalities in all vital signs will be summarized and listed. The safety assessment of vital signs and physical examinations will only be provided in the list.

Vital signs: temperature, resting pulse, blood pressure, and respiratory rate. Vital signs will be recorded at every visit.

Physical examination: Including general condition, mucous membranes, lymph nodes, head and neck, abdomen, musculoskeletal, respiratory rate, cardiovascular, nervous and psychiatric conditions. Weight and height (Screening visit only) should be recorded and calculated for BMI. BMI=weight (kg) /height^2 (m^2).

9.4.5 12-Lead ECG

A 12-lead ECG will be recorded measuring the RR, PR, QRS, and QT interval durations. Summarize the changes in ECG parameters during the study period relative to baseline values and present abnormal results. All ECG data will also be provided in the subject list.

9.4.6. NSAA Scale

NSAA (North Star Ambulatory Assessment) is a validated rating scale used to assess the impact of Duchenne Muscular Dystrophy on patients' ambulatory function. Assessment items include: standing/walking/rising from a chair/stepping onto a raised surface/jumping/running/standing on one leg/heel walking/descending stairs/ascending stairs/lifting a leg/hopping on one leg.

Subjects complete NSAA at baseline to record pre-dosing motor function scores.Post-dosing, subjects complete NSAA twice at Week 16 and Week 28 to record scores again.

Clinical performance is based on NSAA scores. Higher scores indicate better functional performance.Subjects' NSAA results will be compared with previous scores to track

improvement, maintenance, or decline in motor function over time.

Scoring and Results:

Score 0: Unable to complete task

Score 1: Completes with assistance, or completes independently with difficulty

Score 2: Completes independently without difficulty

Age-Specific Maximum Scores:

Ages 2-3: 8 items, maximum 16 points

Ages 3-4: 13 items, maximum 26 points

Ages 4-6: Maximum 45 points

NSAA Assessed Skills		Patient scores
Stand	Stand barefoot for as long and still as possible without external support.	
Walk	Walk forward for at least 10 steps (about 8-10 feet) with a consistent heel-to-toe gait.	
Rise from chair	Begin seated with arms crossed over chest, then stand up from the chair without uncrossing arms.	
Climb step (right leg)	Step onto a box step at least 15cm high with right (or dominant) foot.	
Climb step (left leg)	Step onto a box step at least 15cm high with left (or non-dominant) foot, joining the other.	
Gets to sitting	Lay flat on the floor with arms by side and move to a sitting position without turning towards the floor or using both hands to get up. Using one hand is permissible to achieve the top score.	
Jump	Stand on the floor with both feet together and jump as high as possible with minimal forward movement.	
Run	Run as fast as possible for about 32 feet. To achieve the highest score, both feet must clear the ground when running.	
Stand on right leg	Run as fast as possible for about 32 feet. To achieve the highest score, both feet must clear the ground when running.	
Stand on left leg	Stand on left leg, with arms down, for as long as possible.	

Descend box step (right leg)	Facing forward, step down from the box with right (or dominant) foot.	
Descend box step (left leg)	Facing forward, step down from the box with left (or non-dominant) foot, joining the other.	
Stand on heels	Lean back onto heels for three counts while barefoot. To achieve a top score, both feet must be lifted at the same time using clear dorsiflexion (raising the foot towards the shin).	
Rise from floor	Lay flat on back and stand up as quickly as possible without rolling into a four-point kneeling or prone position (Gower's maneuver).	
Lift head	Lay flat on the floor with arms crossed across the chest and hands resting below the shoulder. Then, lift head, touching chin to chest, while keeping arms folded.	
Hop on right leg	Stand on right leg and hop one-legged without landing on both feet.	
Hop on left leg	Stand on left leg and hop one-legged without landing on both feet.	
NSAA TOTAL SCORE		

10. Adverse Event Reporting

10.1. Definition of Adverse Event

10.1.1. Definition

Adverse Event：An adverse event (AE) is defined as any reaction, side effect, or untoward event that occurs during the course of the clinical trial whether or not the event is considered related to the study drug.

Serious adverse events: A serious adverse event (SAE) is defined as any serious adverse medical events such as death, life-threatening, permanent or severe disability or loss of function of the subject after receiving the investigational drug, the subject's need for hospitalization or extended hospitalization time, as well as congenital abnormalities or birth defects.

10.1.2. Categorization of AEs

All AEs and clinically significant laboratory abnormalities will be graded according to Common Terminology Criteria for AEs, Version 5.0 dated 27 November 2017. For any

term that is not specifically listed on the CTCAE scale, intensity will be assigned a grade of 1 through 5 using the CTCAE guidelines Table 6。

Table 6 The CTCAE displays grade 1 through 5 criteria of adverse event

Grades	Criteria
Grade 1	Mild； asymptomatic or mild symptoms; clinical or diagnostic observation only; intervention not indicated
Grade 2	Moderate; minimal, local or noninvasive intervention indicated; limiting age-appropriate instrumental activities of daily living (ADL)*.
Grade 3	Severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self care ADL△.
Grade 4	Life-threatening consequences; urgent intervention indicated.
Grade 5	Death related to AE.

*Instrumental ADL refer to preparing meals, shopping for groceries or clothes, using the telephone, managing money, etc.

△Self care ADL refer to bathing, dressing and undressing, feeding self, using the toilet, taking medications, and not bedridden.

10.1.3. Causes of AEs

According to the standards formulated by the Adverse Drug Reaction Monitoring Center of the Ministry of Health, the five-level classification is "Definitely related, Probably related, Possibly related, Unlikely related, Unrelated". The first three levels were judged to be related to the investigational drug. The judgment criteria of the five-level classification method on AEs are shown in Table 7 below：

(1) Whether there is a reasonable order of time.

(2) Whether it is consistent with the known AR type of the investigational drug.

(3) Whether it can be explained by the volunteers' clinical status, concomitant medication, combined therapy and previous therapy.

(4) If the investigational drug is stopped or the dosage is reduced, whether it is reduced or disappeared.

(5) Whether the same reaction occurs again after repeated exposure to the same drug.

Table 7 Causality or relatedness assessment criteria in AEs and investigational drugs

Analysis results	Indicators				
	1	2	3	4	5
Definitely Related	+	+	-	+	+
Probably Related	+	+	-	+	?
Possibly Related	+	+	±	±	?
Unlikely related	+	-	±	±	?
Unrelated	-	-	+	-	-

The relationship between AEs (including SAEs) and drugs should be determined as far as possible. If it is believed that it is “Definitely related, Probably related and Possibly related”, it should be considered as an adverse reaction caused by investigational drug, and whether it is a SAE should be considered according to the severity. Adverse reactions should be listed and classified according to organ system or adverse event syndrome.

10.2. Adverse Event Recording and Reporting

The investigator should tell the subject who is required to truthfully feedback the changes after the medication but should avoid inductive questions. While observing the curative effect, the investigator should pay close attention to the AEs or unexpected toxic and side effects (including symptoms, signs and laboratory tests), analyze the causality, make judgments, track the observation and record, and count the incidence of adverse events.
For the AEs occurred during the trail, the investigator should record the time, severity, duration, treatment measures, outcome, etc. of the occurrence in the CRF, judge the correlation with the investigational drug, sign and indicate the date. In case of SAE in the trail, the investigator must immediately take measures to protect the safety of the subjects, and report to the department responsible for adverse drug reaction reporting in the study site in a timely manner. Then the adverse drug reaction report form should carefully be filled in according to their opinions. Finally, continue reporting should follow the site's process.

10.3. Risk Prevention and Handling

When AEs occur, the investigator can decide whether to terminate the trial based on the severity, and the researcher will provide corresponding treatment in a timely manner according to the subject's condition. In case of SAEs, Emergency handling measures must be taken to protect the safety of the subjects. All AEs should be tracked and followed up, with detailed records of the handling process and results, until they are properly resolved or the condition stabilizes. If there are laboratory abnormalities, they should be traced back to normal or baseline levels. The follow-up can be selected based on the severity of AEs, including hospitalization, outpatient, home visits, phone calls, mail, and other forms.

11. Statistical Analysis

11.1. Sample Size

The study is an IIT and no sample size estimation is conducted. The number of subjects is determined based on the study objectives and relevant guiding principles. Six healthy subjects are planned to be enrolled, and those who have not been administered after randomization will be replaced.

11.2. Populations for Analysis

Ambulatory boys with confirmed DMD by clinical diagnosis and genetic testing, aged 2~6years.

The age, gender, and race of the subjects, as well as their height and weight, should be recorded. Information about allergies/drug sensitivity or drug abuse should be obtained. All the information of these subjects should be recorded in descriptive language in the table.

11.2.1 Full Analysis Set

Full analysis set (FAS): FAS will be used to report medication compliance and to summarize demographic characteristics (age, gender, race, height and weight) and background characteristics (medical history, concomitant medications, and physical examination, etc.) for all randomized subjects by treatment group.

11.2.2 Per-Protocol Set

Per-protocol set (PP)：Per-protocol analysis is a comparison of treatment groups that includes only those patients who completed the treatment originally allocated.

11.2.3 Safety Analysis Set

Safety analysis set (SAS): All subjects who have been randomized into cohorts, have used the investigational drug, and have safety evaluation data post-treatment constitute the safety population of this study. The safety population will be used for safety analysis.

11.2.4 Pharmacokinetic Dataset

The pharmacokinetic dataset (PKS) consists of all enrolled subjects who have received at least one study drug and have post-treatment PK evaluation data, forming the PK analysis set for this study. In the PK analysis set, different pharmacokinetic parameters may include

different numbers of subjects based on the actual completion of the study.

11.3. Efficacy Analysis and Statistical Methods

Due to the small sample size and open-label design of this study, only descriptive statistics will be conducted for all endpoints, including the number of subjects, mean and standard deviation, minimum and maximum values of continuous variables, and the number and percentage of categorical variables.

Using descriptive statistical methods, describe the number and proportion of subjects entering each statistical analysis set by treatment group, the number and proportion of participants who completed the trial and withdrew midway, and the reasons for withdrawing midway (and their proportion). Describe demographic and other baseline characteristics by treatment group.

11.3.1 Analysis of Primary Endpoints

The primary endpoint is safety. Using a safety analysis set, summarize the baseline data, post-treatment data, and change from baseline data by follow-up and treatment group based on laboratory tests, vital signs, electrocardiograms, and other safety data. A cross table will be used to describe the changes from baseline to each follow-up after administration regarding the normality and clinical significance of various examination results used as categorical data.

11.3.2 Analysis of Secondary Endpoints

1) **PK Parameter Indicators and Assessment**

The main pharmacokinetic parameters include t1/2, AUC, Vss, CL, Cmax, Tmax, etc. Descriptive statistical analysis of pharmacokinetic parameters for different dose groups or queues, calculating the arithmetic mean, standard deviation, coefficient of variation, median, maximum, minimum, and geometric mean of pharmacokinetic parameters for each dose group or queue.

2) **PD Assessment**

Statistically describe each pharmacodynamic indicator according to its data characteristics, and compare the changes after administration with the baseline characteristics. Analyze pharmacodynamic parameters according to actual needs.

3) **Immunogenicity Assessment**

Descriptive statistical analysis was conducted to compare the changes in cytokine production and anti-dystrophin antibodies in subjects at different time points after treatment with the baseline period.

11.4. Safety Analysis and Statistical Methods

Using the safety analysis set, AEs, ARs, and SAEs of each treatment group will be summarized and utilized. Compare the incidence rates among different treatment groups using the χ^2-test/Fisher exact probability method. Adverse events and reactions will also be standardized medical codes based on System Organ Class and Preferred Term.

All completed examination items including physical examination, 12-lead ECG, lab tests (Haematology, Urinalysis, Biochemistry, coagulation function, etc.), and descriptive statistics are listed in the form of a cross tabulation of pre- and post- treatment (based on the judgment of clinical doctors). The items of abnormal ECG, physical examination and laboratory test at each visit shall be listed in the form of a list. Descriptive recording of vital sign data, including values at each timepoint and their relative changes relative to baseline.

List all AEs, including subject number, dosage group, AE name, description, start date, stop date, NCI-CTCAE level, severity of AEs, relationship with study drug, impact on study drug, outcome, etc.

11.5. Mid-term Analysis

Due to the small sample size of this study, which is only an exploratory IIT clinical study, no criteria for mid-term analysis/stop analysis are set. Both parties can obtain study data. If there are SARs during the study, both parties can negotiate and decide on the continued implementation of the study.

11.6. Data Monitoring Committee

Due to the small sample size and short study duration, which is only an exploratory IIT clinical study, no data monitoring committee was established.

12. Data Collection and Management

12.1. CRF/EDC

Due to the small sample size in this study, the data will be entered into a paper CRF.

Clinical investigators or data entry personnel (clinical coordinators) designated by the investigators shall promptly and accurately input source data into CRF. The data entry personnel or investigators can modify the data after verifying it, and the modified data needs to be filled in the modification reason on the CRF. The investigators has signature permission for all final data.

12.2. Data Management

1) Investigators should keep all detailed original files of the subjects to ensure that the data is accurate, complete, and timely. The original documents, medical records, etc. should be clear, detailed, and easily identifiable by personnel participating in this clinical trial.
2) The data in CRF can only be modified by investigators or authorized personnel.
3) During the monitoring visit, the inspector can contact the staff of the research center, obtain source documents, and provide an appropriate environment to complete the review of study related documents. The monitor will meet regularly with the investigators during the study to provide feedback on the progress of the study.
4) The inspector will compare the CRF data with hospital records (source files). The nature and location of all source files will be clarified to ensure understanding of all sources of raw data required for filling out CRF. Supervisors can also contact the study center to review these data sources.
5) After the study is completed, archive the CRF as needed. The experimental data should be retained for 5 years after the end of the study. But if there are requirements in current regulations or agreements with partners, these materials should be kept for a longer period of time. The collaborators will notify the investigators in writing when these materials will no longer need to be preserved.

13. Ethical Considerations

13.1. Ethics Committee/Institutional Review Board

This protocol, written informed consent form, and materials directly related to the subjects must be submitted to the ethics committee for written approval before the study can be officially conducted. Investigators must submit their annual study report to the ethics committee at least annually (if applicable). When the study is terminated and/or completed,

the investigators must notify the ethics committee in writing. Investigators must promptly report all changes that have occurred in their study work to the ethics committee (such as revisions to relevant study documents such as protocols and/or informed consent forms), and these changes must not be implemented without approval from the ethics committee, unless they are made to eliminate obvious and direct risks to the subjects. When such situations occur, the ethics committee will be notified.

13.2. Informed Consent

In this study, all subjects are required to voluntarily sign a written informed consent form. Investigators must provide informed consent forms that are easy to understand and approved by the ethics committee to the subjects or their guardians and give them sufficient time to consider this study. Before obtaining a signed written informed consent form from the subject, the subject is not allowed to join the study. During the study, all updated versions of the informed consent form and written information will be provided to the participants. The informed consent form should be kept as an important document for clinical trials for future reference.

13.3. Protocol Amendment

The protocol amendment may affect the legal and ethical status of the study, as well as the statistical evaluation and the possibility of achieving the primary purpose of the study.

The terms of the protocol and its annexes must be strictly observed, except in emergency situations. If amendment is required, it must be provided in writing according to the corresponding SOP of the study site and approved. The protocol amendment will be presented to the investigator and explained. All protocol amendments must be submitted to the EC for review and approval before implementation, as well as to the management department for approval/notification when appropriate. Major amendments to the protocol must obtain the approval of Haining Spot Biotech Co., Ltd., the site’s EC and the management department (if required) before implementation.

14. Confidentiality

Only investigators and monitors participating in clinical trials may have access to the personal medical records of the subjects, and they will sign a "Researcher Declaration" or

"Confidentiality Commitment" that includes confidential information. Data processing will adopt a "data anonymity" approach, omitting information that can identify the individual identity of the subjects. The medical records of the subjects will be kept in the data archive room of the clinical trial center.

The results of this study may be published in medical journals, but we will keep patient information confidential in accordance with legal requirements, unless personal information of patients is not disclosed due to relevant legal requirements. When necessary, government management departments, hospital ethics committees, and their relevant personnel may access patient information in accordance with regulations.

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