Original Article

Transplantation of chemically-induced liver progenitor cells ameliorates hepatic fibrosis in mice with diet-induced nonalcoholic steatohepatitis

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ABSTRACT

Background Can Background be changed from bold to normal text?: Chemically-induced liver progenitors (CLiPs) have promising applications in liver regenerative medicine. We aimed to clarify the efficacy of CLiPs for ameliorating fibrosis in a diet-induced nonalcoholic steatohepatitis rat model, since nonalcoholic fatty liver disease is currently recognized as the most common form of chronic liver disease in developed countries. Methods: Primary mature hepatocytes were isolated from 7-week-old male Wistar rats. To establish CLiPs, isolated hepatocytes were cultured in differentiation medium composed of Y-27632, A-83-01, and CHIR99021 (YAC medium). As an animal model that reproduces NASH pathophysiology, 6-week-old severe combined immunodeficient (SCID) mice were carefully selected and prepared and fed with choline-deficient, L-amino acid-deficient, high-fat diet (HFD). After 12 weeks’ HFD feeding, the mice were assigned to continue HFD with or without the administration of rat CLiPs (HFD + CLiPs and HFD-CLiPs, respectively). Rat CLiPs were administered from the spleen. Hepatic fibrosis was semi-quantitatively evaluated according to histology. Liver parenchyma and blood samples were collected for biochemical analyses. Results: Rat CLiPs were positive for CK19 and EpCAM were successfully delivered to the liver. At 8 weeks after CLiPs transplantation, the HFD þ CLiPs group showed significantly less positive staining than the HFD-CLiPs group. Alanine aminotransferase significantly improved in the HFD þ CLiPs group, as demonstrated by Azan staining and αSMA immunostaining. RT qPCR showed that the liver expression of MMP2 and 9 tended to be higher in the HFD þ CLiPs group. Conclusions: The anti-fibrotic effect of CLiPs was demonstrated in the immunodeficient NASH animal model and may have therapeutic applications in humans.

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1. Introduction

The possible application of cell therapy for end-stage liver disease has been investigated as an alternative treatment for patients indicated for liver transplantation. By stimulating endogenous regeneration and inhibiting fibrosis, cell therapy using liver progenitor cells may mitigate disease progression [1].

The efficacy of cell therapy for chronic liver disease has been explored. It has been based on the use of primary hepatocytes. Hepatocyte transplantation has been evaluated in numerous clinical trials, mostly with primary hepatocytes [2–4]. However, one of the important limitations in the application of human hepatocytes in cell therapy is the isolation of sufficient quantities of high-quality, metabolically-active cells. In addition, the availability of hepatocytes is limited due to a donor shortage and their properties, clinically effective hepatocyte transplantation using mature hepatocytes that cannot be proliferated in vitro is the primary limiting factor for clinical

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application. For this reason, alternative sources of cells are being explored. For example, hepatocytes have been induced from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) [5,6]. Complicated culture conditions must be applied to induce hepatocytes from pluripotent stem cells.

Recently, Katsuda et al. reported reprogramming mature hepatocytes into expandable bipotent progenitor cells using small molecules, including Y-27632 (Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor), and CHIR99021 (GSK3β inhibitor) in a rodent model [7]. Those cells were named chemically-induced liver progenitors (CLiPs). Subsequently, the successful establishment of CLiPs from human mature hepatocytes was reported [8]. In addition to bi-directional differentiation potential, and in contrast to mature hepatocytes, CLiPs can be stably cultured with maintained proliferative capability [9]. CLiPs have been reported to be expandable in vitro through more than 20 passages. Katsuda et al. also demonstrated that rat CLiPs extensively repopulated the injured liver of cDNA-uPA/SCID mice at [9]. CLiPs have been reported to be expandable in vitro through more than 20 passages. Katsuda et al. also demonstrated that rat CLiPs extensively repopulated the injured liver of cDNA-uPA/SCID mice at 70–95% repopulation efficiency without tumorigenic feature.

The repopulation efficiency of primary hepatocytes transplanted in injured mouse livers was reported to be 50%. On the other hand, the repopulation efficiency of laboratory-generated hepatocytes from pluripotent cells is limited, with repopulation efficiency generally <5% [10]. Considering the simple culture conditions without genetic manipulation and vigorous repopulation efficiency, CLiPs have advantages over pluripotent stem cells.

We conducted this study to clarify the efficacy of CLiPs for ameliorating fibrosis in an animal model. We adopted a diet-induced nonalcoholic steatohepatitis (NASH) rat model as a liver disease model. Because NASH is becoming a more common indication for liver transplantation and may surpass all other indications in the near future [11,12].

2. Methods

2.1. Isolation of primary rat hepatocytes

Primary mature hepatocytes were isolated from 7-week-old male Wistar rats (Charles River Laboratories Japan, Yokohama, Japan) using a modified two-step perfusion method in accordance with previous reports [13]. All of the experimental procedures involving animals were conducted in accordance with the institutional guidelines of Nagasaki University. This study was approved by the Animal Ethics Committee at Nagasaki University, Nagasaki, Japan [1907171547–2]. There are no human subjects in this article and informed consent is not applicable.

2.2. Reprogramming of rat hepatocytes into CLiPs

The differentiation protocol was as described previously [14–17]. In short, approximately $6 \times 10^8$ freshly isolated rat hepatocytes were seeded on 100 mm collagen-coated dishes (Asahi Techno Glass, Tokyo, Japan) in differentiation medium composed of DMEM/F12 consisting of 2.4 g/L NaHCO3 and L-glutamine added with 10 mM Y-27632 (AdooQ BioScience), 0.5 mM A-83-01 (Wako Pure Chemical), and 3 mM CHIR99021 (AdooQ BioScience) (YAC medium). Furthermore, the medium included 5 mM HepES, 30 mg/mL l-proline, 0.5 mg/mL BSA, 10 ng/mL epidermal growth factor, insulin-transferrin-serine-X, 0.1 mM dexamethasone (Dex), 10 mM nicotinamide, 1 mM ascorbic acid-2-phosphate, 100 U/mL penicillin, and 100 mg/mL streptomycin. The medium was changed 1 day after seeding and every other day thereafter. The cells achieved 90% confluence within 2 weeks. The features of CLiPs were evaluated by immunofluorescent staining (CK19, EpCAM). To confirm the ability of rCLiP to differentiate into mature hepatocytes, Oncostatin M and dexamethasone were added to rCLiP. The respective mature and undifferentiated hepatocyte components were examined using PCR. In this study, we evaluated SOX9 and EpCAM as characteristics of progenitor cells, CYP1A1 and Trypphantan 2,3-dioxygenase as characteristics of mature hepatocytes.

2.3. Animal model

Before initiating this study, we explored to find a best model to evaluate therapeutic effect of CLiP. With animal manufacture company (Charles River Laboratories Japan, Yokohama, Japan), we made 4 different potential animal models of NASH (a choline-deficient, l-amino acid-defined, high-fat diet (CDAHFD), High fat diet, Fat-fructose and cholesterol-rich diet, High sucrose-61% Fructose diet). The results are now shown in Supplementary Fig. 2, which shows evident fibrosis formation in the CDAHFD model even under SCID condition. Therefore, at least among our candidate of NASH models, SCID mice with MCD diet was used in our study to evaluate CLiPs’ therapeutic effect. In addition, under our quantification of hepatic fibrosis in Supplementary Fig. 3, amount of fibrosis in our CDAHFD model at 12 week showed equivalent percentage of fibrosis (15% of Azan positive) using an imaging analysis software (Win Tissue, MITANI Co., Tokyo, Japan) to clinical samples of F3–4 liver fibrosis of the patients, which were used for our previous study as a damaged liver [18]. Supplementary Table 1 shows biochemical parameters of each models at 12 weeks of each diet administration. Therefore, we would like to say that our model of CDAHFD model was valid in this study as far as diet induced NASH model was concerned.

CDAHFD model that reproduces the pathophysiology of NASH, 6-week-old CB17/1cr-Pkrdc <scid>/CrIcrj mice (Charles River Laboratories Japan, Yokohama, Japan) were prepared and fed with a choline-deficient, l-amino acid-defined, high-fat diet (CDAHFD) (A06071302; Research Diets, Inc.) [19]. To clarify sufficient duration of feeding CDAHFD for establishing sufficient fibrosis, an area of hepatic fibrosis was evaluated by measuring positive area with Azan staining in 10 random fields (x1000) of view per individual using an image analysis software program (Win ROOF, MITANI Co., Tokyo, Japan) at 6, 8 and 12 weeks. The area of positive AZAN staining chronologically increased with CDAHFD feeding, with the average area percentage exceeding 15% at 12 weeks of administration, approaching 17%, the area percentage of positive AZAN staining measured in human F3 or higher specimens. With these results, the duration of CDAHFD prior to CLiPs administration was set at 12 weeks.

As an animal model of NASH, we fed a CDAHFD to 6-week-old CB17/1cr-Pkrdc <scid>/CrIcrj mice for 12 weeks. After 12 weeks of CDAHFD feeding, mice CLiPs were administered, and the mice were divided into the following four groups according to diet (with/without the continuation of CDAHFD) and the administration or non-administration of CLiPs: The mice were grouped as follows: a, continuation of high-fat diet with the administration of CLiPs; b, continuation of high-fat diet without the administration of CLiPs; c, discontinuation of high-fat diet with the administration of CLiPs; d, discontinuation of high-fat diet without the administration of CLiPs (Fig. 1). At 8 weeks after the administration of CLiPs, the mice were sacrificed.

2.4. Administration of rat CLiPs to SCID mice with NASH

Mice were laparotomized under anesthesia, and $4.0 \times 10^5$ CLiPs (approximately 0.1% of mouse liver weight) were administered to the spleen in the administration group. At eight weeks after the administration of CLiPs, the mice were sacrificed by collecting blood from the inferior vena cava, and the liver was removed. Mice
fed with a CDAHFD for 8 weeks were divided into two groups according to whether or not CDAHFD feeding was continued. The two groups were further grouped according to the presence or absence of CLiPs treatment, and a comparative study of the four groups was conducted.

2.5. Blood sampling

Blood samples were centrifuged at 3000 rpm for 10 min to extract serum components. Each sample was cooled and stored at −80 °C until measurement. The following items were evaluated: aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (TC), albumin (Alb), free fatty acid (FFA), and choline esterase (ChE).

2.6. Histopathological evaluation

The liver was fixed with formalin and sliced to a thickness of 5 μm. Azan staining and αSMA staining were performed to evaluate fibrosis. In addition, immunohistochemistry with rat-positive/mouse-negative CK19 (NBP2-29804, Novus Biologicals USA, USA) were performed to identify rat-derived cells, which were used to establish rat CLiPs. We performed immunohistochemistry with rat anti–CK19 antibody in mice not treated with rat CLiP and confirmed negative results. With that result, we consider the cells that were positive in the rat CLiP group to be rat CLiP. The positive cell rate was measured in 10 random fields (×1000) of view per individual using an image analysis software program (Win ROOF, MITANI Co., Tokyo, Japan).

2.7. RNA extraction and reverse transcription-quantitative (RT-q) PCR

The statements of αSMA, Collagen-1, MMP-2, and MMP-9 in the collected liver tissue were analyzed by RT-qPCR. MMP-2 and MMP-9 were evaluated as possible factors of anti-fibrotic effect induced by CLiPs administration. ISOGEN (Code No. 311–02501 NIPPON GENE CO.) was used as the reagent for RNA extraction. Total RNA was dissolved in water by recovering with isopropanol precipitation after phenol/chloroform extraction and replacement with ethanol. cDNA synthesis was performed for 15 min at 42 °C using 2 μg total RNA as a template, RT primer and QuantiTect reverse transcriptase (Superscript III; Thermo Fisher Scientific, Inc.). Each primer was purchased from Takara Bio Inc. (αSMA: Catalog No. MA139213, Collagen-1: MA107374, MMP-2: MA175979, MMP-9: MA097597, 18sRNA: MA050364), but the primer sequences were not disclosed. For qPCR, in each reaction, 1 U LightCycler SYBR Green 1 Master mix (Roche Diagnostics) was used and the cycling conditions were as follows: pre-incubation for 3 min at 95 °C; followed by 45 cycles of 15 s at 95 °C, 1 min at 60 °C and 30 s at 40 °C. The gene expression was calculated according to the 2–ΔΔCq method [10]. PCR was performed in duplicate.

2.8. Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde for 10 min at room temperature (RT), permeabilized with 0.1% Triton X-100 (Sigma Aldrich, Tokyo, Japan) in PBS for 10 min, and then blocked with PBS containing 1% BSA for 1 h at RT. Cells were incubated with primary antibodies at 4 °C, followed by 45 cycles of 15 s at 95 °C, 1 min at 60 °C and 30 s at 40 °C. The gene expression was calculated according to the 2–ΔΔCq method [10]. PCR was performed in duplicate.

2.9. Statistical analysis

All data are presented as the mean ± standard deviation. The Mann–Whitney U test and Kruskal–Wallis test were used for comparisons among the groups. P values of <0.05 were considered
statistically significant. All statistical analyses was performed using SPSS version 21.

3. Results

3.1. Reprogramming rat hepatocytes to rat CLiPs

Rat CLiPs were successfully established by reprogramming isolated mature hepatocytes from rats. As we reported earlier [20], although the isolated hepatocytes adhered to each other and maintained their unique shape on the first day of culture, small cells appeared after the fourth day of culture and reached 80% confluence on the 14th day. Immunostaining of the cells on day 14 was positive for Ck19 and ki67 (Fig. 2). These cells that are positive for the two markers CK19 and Ki67 are considered CLiPs.

Furthermore, after the differentiation of these cells, genes characteristic of progenitor cells, such as SOX9 and EpCAM, were downregulated, while the expression of CYP1A1 and Tryptophan 2,3-dioxygenase, which are found in mature hepatocytes, increased (Supplementary Fig. 1).

3.2. The presence of rat CLiPs in the liver following transsplenic administration

In normal SCID mice, rat-positive/mouse-negative CK19-positive cells (rat CLiP) were observed on days 1, 3, or 7, and similar cells were also observed in the hepatic vessels on day 1. Following the administration of CLiPs to NASH model mice, rat CLiPs were observed in the liver and under the spleen capsule on day 1, and the CLiPs were also observed in the liver on day 12. Rat CLiPs had already reached the liver parenchyma on Day 1 after transplantation and were still present sporadically in the liver parenchyma, ranging from Zone 1 to nearly Zone 3, with no specific location identified.

It was found that the administered rat CLiPs were viable in the liver parenchyma for a long period of time and were widely present from the portal venous region to the central venous region.

3.3. Blood analysis

The blood analysis revealed no significant differences in AST, TC, ALB, FFA, or ChE according to the administration or non-administration of rat CLiPs. In contrast, the administration of rat CLiPs was associated with a significant improvement in ALT in mice that were fed a high-fat diet (Fig. 4).

These results suggest that rat CLiPs may reduce cellular damage due to inflammation in the liver parenchyma of mice on a high-fat diet.

3.4. Histopathological evaluation

Immunostaining of rat-positive/mouse-negative CK19 revealed positive cells in the liver of some mice that received CLiPs, which were sacrificed at 8 weeks after the administration (Fig. 5). Rat-positive/mouse-negative CK19-positive cells were found sporadically in the liver parenchyma, ranging from Zone 1 to nearly Zone 3, with no specific location identified.

It was found that the administered rat CLiPs were viable in the liver parenchyma for a long period of time and were widely present from the portal venous region to the central venous region.

Azan staining revealed significantly less positive staining in the group with CLiPs administration in comparison to the group without CLiPs administration, with or without high-fat diet (Fig. 6).

In mice that continued to receive the high fat diet, the positive area ratio on αSMA immunostaining was significantly reduced in the rat CLiPs-treated group (Fig. 7). In the mice in which the high-fat diet was discontinued, the degree of fibrosis itself was weak, and no significant difference was observed between the mice with and without the administration of CLiPs. Histological findings suggested that Rat CLiPs may have reduced the persistent fibrosis caused by continued high-fat diet. On the other hand, fibrosis in the group that discontinued the high-fat diet was reversible, and no
Fig. 3. Immunohistochemistry with rat-positive/mouse-negative CK19 antibody in mice with continuous MCD feeding and the administration of rat CLiPs.

Fig. 4. Blood test results. SCD shows the data of SCID mice before the administration of the special diet. NM shows data of SCID mice after 12 weeks of special diet before the administration of CLiPs. The mice were grouped as follows: a, continuation of high-fat diet with the administration of CLiPs; b, continuation of high-fat diet without the administration of CLiPs; c, discontinuation of high-fat diet with the administration of CLiPs; d, discontinuation of high-fat diet without the administration of CLiPs. The data are shown as the mean ± SEM of three independent experiments. *p < 0.05, **p < 0.01, Mann–Whitney U test.
significantly different effect was observed in the present model to the extent of further promoting fibrosis improvement.

3.5. RT-q PCR

Although no statistically significant difference was observed in the qPCR results, the expression of Collagen-1 tended to be lower and the expression levels of MMP-2/9 tended to be higher in the rat CLiPs-treated group (Fig. 8).

The PCR study was conducted to investigate the effect of MMP2 and MMP9 as a possible mechanism of the fibrosis-reducing effect of rat CLiPs observed in the histological evaluation, but the mechanism could not be determined.

4. Discussion

This is the first study to show the efficacy of CLiPs for ameliorating fibrosis in an animal model reproducing NASH. The significant difference in the expression of aSMA between mice with and without the administration of CLiPs seems to be related to the mechanism of through which fibrosis was ameliorated by CLiPs.

Furthermore, ALT significantly decreased in rats with CLiPs administration. ALT has been reported as a biomarker of treatment response in NASH [21,22]. Hoofnagle et al. have shown that decline in serum ALT matters in NASH because it is associated with improvement in liver histology [23]. Taken together, these results suggest a possible mechanism by which CLiP has an anti-inflammatory effect, resulting in suppression of HSC and improvement of fibrosis. As per our personal communication with co-author (T.O.), intraportal administration of human CLiP increased the hepatic MMP-2 mRNA level in NOD/SCID mice with chronic liver damage with CCl4. In addition, various miRNAs including miR-16 and miR-223 those have been reported to have anti-inflammatory effect were found in the exosome in the CLiPs (personal communication with T.O.).

In this study, we were able to generate CLiPs from rat mature hepatocytes by culturing them according to the previously
described protocol [14,20]. The CLiPs were positive for CK19, which is a marker of hepatic progenitor cells and also showed high cell proliferation. Since previous report proved that successful repopulation of rat CLiPs transplanted into immunodeficient mice with chronic liver injury, we adopted the combination in this study [7].

Azan staining and αSMA staining, which were performed for the evaluation of liver fibrosis, both showed significant differences depending on the administration or non-administration of rat CLiPs. It is considered that rat CLiPs probably suppress hepatic fibrosis by suppressing the activation of αSMA-positive Kupffer cells. In addition, since liver fibrosis is associated with a complex pathophysiological process that causes the excessive accumulation of extracellular matrix proteins, how the CLiPs that remain in the liver affect the suppression of hepatic fibrosis has not been completely clarified. Whether fibrosis is improved by the influence of a large amount of rat CLiPs thought to have existed in the early stage, or whether rat CLiPs that continue to exist, even in a small amount, continuously release exosomes and other products to improve fibrosis over time.

Recent studies to have shown that exosomes function as mediators for intercellular transfer and contain all of the necessary signals to induce fibrosis, including macrophage activation and cytokine

**Fig. 6.** (A) Evaluation of fibrosis by Azan staining. The mice were grouped as follows: a, continuation of high-fat diet with the administration of CLiPs; b, continuation of high-fat diet without the administration of CLiPs; c, discontinuation of high-fat diet with the administration of CLiPs; d, discontinuation of high-fat diet without the administration of CLiPs. (B) Positive area ratio measurement on Azan-stained sections. The data are shown as the mean ± SEM of three independent experiments. **p < 0.01, Mann–Whitney U test.**
secretion, ECM remodeling and modulation of hepatic stellate cells [24]. Micro RNAs have been reported to be upregulated in activated HSCs and play a role in regulating the deposition of extracellular matrix proteins (e.g., collagen and \( \alpha \)-SMA), leading to the altered expression of MMPs [25]. To date, the relationship between exosomes derived from CliPs have been reported [26]. In xenotransplantation model, Rong et al. found that human derived exosomes treatment significantly decreased \( \alpha \)-SMA expression level both in vivo and in vitro [27]. The authors addressed that the human mesenchymal stem cell (MSC)-derived exosomes inhibit HSC activation via inhibition of Wnt/\( \beta \)-catenin signaling. In this study, rat CliPs were transplanted into mice, which are also rodents, but this is a xenotransplantation, and it is possible that exosomes from other species contributed to the improvement of fibrosis, as in the aforementioned report on the combination of humans and rats. In the current study, \( \alpha \)-SMA expression significantly decreased in mice fed with high fat diet by transplantation of CliPs possibly due to exosomes from CliPs. In the present study, there was no significant change in MMP values in the CliPs-treated group, but this result would be reasonable if the improvement in fibrosis by CliPs was mainly due to exosomes. One limitation of this study is that the exosomes were not measured in this study. Future studies on exosome isolation from rat CliPs and studies using exosomes may help to elucidate the mechanism. Furthermore, MMP2 and MMP9 were measured in the samples taken from the liver of 8-week post-CliPs administration. At 8 weeks after CliPs administration, CliPs in the

**Fig. 7.** (A) \( \alpha \)-SMA immunostaining. The mice were grouped as follows: a, continuation of high-fat diet with the administration of CliPs; b, continuation of high-fat diet without the administration of CliPs; c, discontinuation of high-fat diet with the administration of CliPs, d, discontinuation of high-fat diet without the administration of CliPs. (B) Positive area ratio measurement of \( \alpha \)-SMA-stained sections. The data are shown as the mean ± SEM of three independent experiments. **\( p < 0.01 \), Mann–Whitney U test.
liver were sporadic, but in the early post-CLiPs treatment period, when there were still more numbers of CLiPs and fibrosis was severer, MMP2 and MMP9 expression may be different. Future evaluation of chronological changes in MMPs after CLiPs administration will also help to elucidate the mechanism of amelioration of hepatic fibrosis by CLiPs. The mechanism through which CLiPs ameliorated fibrosis in the animal model reproducing NASH is a complex process that involves cytokines and exosomes, which needs to be clarified in further studies.

One of the limitations of this study is that we did not clarify the ideal amount of rat CLiPs for the improvement fibrosis in the mouse model of NASH. Furthermore, the fate of transplanted rat CLiPs has not been clarified. In the immuno-deficient SCID mouse model in this study, the elimination of transplanted rat CLiPs by natural killer cells was possible [28]. Although we proved the successful transplantation of CLiPs into the liver until 2 weeks, we did not chronologically sacrificed mice for clarifying exact location of CLiPs. The chronological evaluation of existence of transplanted rat CLiPs may lead to clarifying the mechanism of the anti-fibrosis effect of rat CLiPs.

Our group has established a technique for generation of CLiP based on the report by Katsuda et al. [7,16]. In previous reports by Katsuda et al., CLiP was evaluated by Afp, Cd90, Foxj1, Hnf1b, Hnf6, Cd44 and Itga6 as undifferentiated cell components. In addition, as evaluation of mature hepatocyte components, the expression of Alb, Ttr, Cnx32, Hnf4z, G6pc, Tat, C/ebp-z, Cyp3a1, and Cyp3a2 were observed [7]. Although the present study was conducted according to the protocol already established for generation of CLiP in our group, the number of evaluation items was small. Therefore, if the above-mentioned items were evaluated, the evaluation of CLiP would have been more elaborate, which is a limitation of the present study.

In this study, we recognized the effect of CLiP in reducing fibrosis in the NASH model. It is important to examine whether the fibrosis-reducing effect of CLiP is similar in other fibrosis models, such as drug-induced liver injury models in order to clarify the therapeutic effect of CLiP and its mechanism. We have recognized ameliorative effect by transplantation of CLiP in a drug-induced liver injury model with concanavalin A (unpublished data). These results will lead to the accumulation of evidence to consider the potential therapeutic application of CLiP.

In conclusion, the anti-fibrotic effect of CLiPs was shown in this study with a rat model of NASH. Future studies are expected to elucidate the mechanism of antifibrosis by CLiPs and the optimal dosage of CLiPs, which could lead to potential therapeutic applications.

Ethical approval

This study was approved by the Animal Ethics Committee at Nagasaki University, Nagasaki, Japan [1907171547-2].

Statement of animal rights

All of the experimental procedures involving animals were conducted in accordance with the institutional guidelines of Nagasaki University, Japan and approved by the Animal Ethics Committee at Nagasaki University, Nagasaki, Japan [1907171547-2].

Statement of informed consent

There are no human subjects in this article and informed consent is not applicable.

Conflict of Interest

The authors declare no conflicts of interest in association with the present study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2022.11.001.

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