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Original Article

Reprogramming canine cryopreserved hepatocytes to hepatic progenitor cells using small molecule compounds

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ABSTRACT

Introduction: Exploring techniques for differentiating and culturing canine hepatocytes serves as a means to establish systems for liver transplantation and drug metabolism testing. However, establishing consistent methods for culturing stable hepatocytes remains a challenge. Recently, several investigations have shown the reprogramming of mature hepatocytes into hepatic progenitor cells by applying specific small molecule compounds, including Y-27632, (a ROCK inhibitor), A-83-01 (a TGFβ inhibitor), and CHIR99021 (a GSK3 inhibitor) (termed YAC) in rat, mouse, and humans, respectively. However, reports or evidence of successful reprogramming using these small-molecule compounds in dogs are absent. This study aimed to induce the differentiation of mature canine hepatocytes into progenitor cells.
Methods: Cryopreserved canine hepatocytes (cHep) were cultured for 14 d in a YAC-supplemented hepatocyte growth medium. Subsequently, an assessment was conducted involving morphological observations, quantitative real-time polymerase chain reaction (qRT-PCR), and immunocytochemistry.
Results: Notably, cryopreserved cHep cells emerged and exhibited ongoing proliferation and concurrently developed colonies within the YAC-enriched culture. These observations indicated that the mature hepatocytes reprogrammed into hepatic progenitor cells. Moreover, qRT-PCR analysis revealed a notable enhancement in gene expression levels. Specifically, the genes encoding α-fetoprotein (AFP), epithelial cell adhesion molecule (EpCAM), Cytokeratin 19 (CK19) and SRY-box9 (Sox9) displayed approximately 12-, 2.2-, 517- and 2.9- increases in hepatic progenitor cells, respectively, on day 14 as compared to their state before induction of differentiation. Hepatocyte-related protein expression of AFP, EPCAM, SOX9 and CK19 was confirmed via immunocytochemistry on day 21. In contrast, ALB and MRP2, which are highly expressed in mature hepatocytes, were decreased compared to those before YAC addition, which is consistent with the characteristics of undifferentiated hepatocytes.
Conclusions: Herein, we effectively promoted the reprogramming of cryopreserved cHep cells into hepatic progenitor cells using three small-molecule compounds. The mRNA and protein expression analyses demonstrated increased levels of hepatic progenitor cells-specific markers, whereas markers related to mature hepatocytes decreased, suggesting that reprogramming cryopreserved cHep cells to hepatic progenitor cells was achieved using YAC. Therefore, cultivating liver progenitor cells holds the potential to offer valuable insights into the development of artificial livers for drug discovery research and transplantation therapy aimed at addressing liver diseases in dogs.

1. Introduction

Recent advances in veterinary technology have extended the lifespans of companion dogs. Consequently, the number of cases of liver disease has increased [1,2]. Some dogs have been indicated to develop various spontaneous liver diseases including hepatitis,

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cirrhosis, portosystemic shunt, and portal hypoplasia [1–5]. Liver dysfunction, including chronic hepatitis, hepatic fibrosis, and cirrhosis, is more common in canine liver diseases than in biliary diseases [1,2]. In dogs, liver fibrosis and cirrhosis concurrent with chronic hepatitis are often occurred [1]. Canine hepatitis has a poor prognosis, with a median mean survival of approximately one and a half years [2]. Particularly, new effective treatment methods for canine cirrhosis should be established as soon as possible because the prognosis has been reported to be only 22 d [2]. Some therapeutic drugs, including inflammatory, antioxidant, and antifibrotic agents, immune suppressive therapy, and gene therapy have been used for treating hepatitis and secondary liver fibrotic disease [2,6,7]; however, an effective treatment has not yet been established. Liver transplantation using primary cultured hepatocytes is a promising alternative to conventional living-donor liver transplantation. However, canine allogeneic liver transplantation, including living-donor liver transplantation, has not been established in veterinary medicine.

Lots of dogs are used as laboratory animals for drug development worldwide, and even healthy individuals are killed. Generally, several enzymes are present in the liver, including cytochrome 450 (CYP), which is involved in drug metabolism [8–10]. In the liver, 95 % of the drugs taken into the body by the action of these enzymes are metabolised into a form that is easily excreted through detoxification and degradation of toxic substances. Therefore, drug metabolism in the liver is essential to evaluate the drug metabolic toxicity during drug development [9]. Drug-induced liver injury (DILI) is a major cause of discontinuing drug development and market withdrawal in drug discovery research, and establishing tools for early DILI prediction is a crucial issue in drug development [9,10]. Therefore, primary hepatocytes are used to conduct drug metabolism studies during the early stages of drug development to evaluate drug toxicity. However, primary hepatocytes can only be cultured for one week and cannot be completely evaluated [9]. Therefore, a demand exists to produce an artificial liver that can be cultured in dogs for a long period; however, this has not yet been established. Therefore, several dogs are still used and euthanised as experimental animals for toxicity testing, which poses an ethical problem. Thus, constructing a test system that can appropriately evaluate drug toxicity is currently the most important issue.

In our previous study, bone marrow-derived mesenchymal stem cells were reported to be directly differentiated into induced hepatocyte-like cell by introducing the transcription factors Hnf4α and Forkhead box gene, which are related to hepatocyte differentiation, using a gene transfer-based differentiation induction method that has become mainstream in liver regeneration technology [11,12]. However, several issues are to be solved, such as the carcinogenicity of the viral vector used for introduction and the lack of structure of bile ducts and sinusoids other than hepatocytes.

Recently, the use of three small molecule compounds, Y-27632 (ROCK inhibitor), A-83-01 (TGFβ inhibitor), and CHIR99021 (GSK3 inhibitor) (termed YAC) in culture in rats and mice was reported to enable differentiation of mature hepatocytes into hepatic progenitor cells that are capable of self-renewal and differentiation into hepatocytes. These cells are called chemically induced liver progenitor (CLiP) cells and are being analysed at institutions worldwide as a scheme for artificial liver production [13]. Furthermore, creating CLiP cells has solved the previously impossible tasks of (1) differentiating mouse hepatocytes into bile duct epithelial cells and (2) simultaneously differentiating into liver progenitor cells with self-renewal abilities. Additionally, when CLiP cells were transplanted into mice with severe liver damage, 75%–90 % of the damaged liver was successfully transplanted and replaced by transplanted cells [14]. Therefore, this study aimed to reprogram cryopreserved hepatocytes into canine liver progenitor cells using small-molecule compounds.

2. Methods

2.1. Cell preparation

Canine cryopreserved hepatocytes (cHep) were obtained from Biopredic International (Brittany, France). The cells were derived from male beagles with an age/weight of 19 months/8.1 kg. The cells were stored in 0.5 mL vials with a minimum cell count of 5×10^6 cells.

2.2. Hepatocyte thawing and culture

Primary cHep cell cultures were used to establish the hepatic progenitor cells. The cells were cryopreserved in liquid nitrogen and thawed before use. cHep were placed in a thermostatic chamber at 37 °C for thawing. Thawed cells were suspended in William's E medium (Corning, NY, USA) supplemented with a Hepatocyte Plating Supplement Pack (CM3000) (Corning) and centrifuged at 65 g for 4 min. The supernatant was removed after centrifugation, and the cell sediment was suspended in William's E medium (Corning) supplemented with a Hepatocyte Maintenance Supplement Pack (CM4000) (Corning). After counting the number of viable cells using the trypan blue exclusion assay, the cells were seeded at a density of $0.5 \times 10^5/cm^2$ onto a Collagen I coated 12-well plate (IWAKI, Shizuoka, Japan) with a thin layer of Matrigel (Corning, NY, USA). Day 0 is the day the cells were seeded. Cells were cultured for 14 d at 5 % CO_2 and 37 °C.

2.3. Reprogramming of the hepatic progenitor cells

The basal medium for culturing hepatic progenitor cells was hepatocyte growth medium (HGM), namely, Dulbecco's modified Eagle's medium/Nutrient Mixture Ham F-12 (DMEM/F12; Gibco, Massachusetts, USA) supplemented with 2.4 g/L $NaHCO_3$ (FUJIFILM Wako, Osaka, Japan), 5 % fetal bovine serum (Sigma-Aldrich, St. Louis, Missouri, USA), × 1 L-glutamine (L-gle; FUJIFILM Wako), 5 mM HEPES (Gibco), 30 mg/L L-proline (Sigma-Aldrich), 0.05 % bovine serum albumin (BSA; Sigma-Aldrich), 20 ng/ml epidermal growth factor, × 1 insulin-transferrin-serine-X (FUJIFILM Wako), 10^{-7} M dexamethasone (FUJIFILM Wako), 10 mM nicotinamide (FUJIFILM Wako), 1 mM L-ascorbic acid 2-phosphate trisodium salt (FUJIFILM Wako), and × 1 penicillin–streptomycin–amphotericin B suspension (antibiotic/antimycotic solution) (FUJIFILM Wako). Once the cultured cells were established on the plate, a total medium change was performed by dividing the medium into HGM with (Hepatic Progenitor Differentiation Group) and without YAC (Control Group). During the culture period, the medium was changed every 2–3 d for a half-volume medium change. Passages were performed using accutase (Innovative Cell Technologies, San Diego, CA, USA) when the cultures reached 80 % confluence. Hepatic progenitor cells were cultured in YAC-supplemented HGM on day 14.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

Cells were collected on days 0 and 14 and total RNA was extracted using a RiboPure RNA Purification Kit (Thermo Fisher Scientific). After extraction, DNA was removed using a DNA-free DNA Removal kit (Thermo Fisher Scientific). After measuring the amount of extracted RNA with DU730 (Beckman Coulter, CA, USA), cDNA was synthesised by reverse transcription of total RNA using a PrimeScript RT reagent kit (Takara Bio, Shiga, Japan). Reverse transcription was performed using a Veriti 96 well Thermal Cycler

(Thermo Fisher Scientific). Expression levels of hepatocyte-related genes in cultured cells on days 0 and 14 were analysed using TB Green® Premix Ex Taq™ II (Takara Bio). Thermal Cycler Dice® Real-Time System II (Takara Bio) was used for RT-PCR reaction. RT-PCR was performed under the condition of 40 cycles of two steps consisting of 95 °C for 5 s and 60 °C for 30 s. Comparative quantification was performed for *albumin (ALB)*, *a1-antitrypsin (a1-AT)*, *tyrosine aminotransferase (TAT)*, and *multidrug resistance-associated protein 2 (MRP2)*, which are markers of mature hepatocytes; for *α-fetoprotein (AFP)*, *epithelial cell adhesion molecule (EpCAM)*, *Cytokeratin19 (CK19)*, and *SRY-box9 (Sox9)*, which are markers of immature hepatocytes; for *E-cadherin (CDH1)*, which is a cell adhesion factor; and for *cytochrome P450 2E1 (CYP2E1)* and *cytochrome P450 3A12 (CYP3A12)*, which are drug metabolising enzymes. β-actin was used as the endogenous control, and relative quantification was performed using the ΔΔCt method. Table 1 provides a list of the primer sequences and temperature settings used in the second step.

2.5. Heatmap generation

A heatmap was created using Heatmapper (Sasha Babicki et al., 2016). Clustering method was not applied to maintain simple data visualisation. The heatmap clearly indicated genes whose expression levels increased or decreased during the differentiation process from day 0 of the cultured cells to the hepatic progenitor cells. Red indicates higher expression, and green indicates lower expression.

2.6. Immunofluorescent cell staining

Day 0 and 14 cultured cells were seeded in 12-well plates. These cells were used for immunohistochemistry after adhesion and proliferation were confirmed, the ratio of each proteins expressed were compared with Day 3 and 21. The medium was aspirated, and the cells were washed three times with phosphate-buffered saline (PBS) to remove any remaining medium. The cells were then fixed with 4 % paraformaldehyde phosphate buffer for 10 min at room temperature (RT) and washed three times with PBS. Subsequently, membrane permeation was performed for 5 min at RT using PBS containing 0.1 % Triton X-100 (Gibco), followed by three PBS washes. Blocking was subsequently performed by adding PBS containing 5 % skim milk (FUJIFILM Wako) and 0.1 % Tween 20 (Sigma-Aldrich), and the cells were allowed to stand at RT for 10 min. After blocking, the wells were washed once with PBS. Then, the primary antibody diluted 500-fold in an antibody diluent with background reducing components (Dako; Agilent, CA, USA) was added, and the wells were incubated at 4 °C overnight.

For F-actin staining, cells were washed with PBS after blocking. These cells reacted with a solution of Alexa Fluor™ 488Phalloidin (A12379; Gibco) dissolved in methanol to a final concentration of 200 units/mL (equivalent to approximately 6.6 μM) in 200 μl of PBS containing 1 % BSA. Subsequently, they were allowed to react in the reaction solution for 20 min at RT. The samples were then washed with PBS and sealed in a VECTA SHIELD mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA, USA) for observation.

The primary antibodies used were goat anti-dog albumin (A40-113A; Bethyl Laboratories Inc., Montgomery, TX, USA), mouse anti-human E-cadherin (sc-8426; Santa Cruz Biotechnology, Dallas, TX, USA), mouse anti-human MRP2 (MON9027; Cell Sciences, Newbury Port, MA, USA), rabbit anti-human EPCAM (21050-1-AP; Proteintech Rosemont, IL, USA), and rabbit anti-human SOX9 (LS-B12075; LS Bio (Absolute Biotech), Boston, MA, USA), rabbit anti-human Ck19 (10712-1-AP; Proteintech), and goat anti-human AFP (sc-8108; Santa Cruz Biotechnology).

Isotype control antibodies including normal goat IgG control (AB-108-C; Bio-Techne, MN, USA), normal mouse IgG control (NI03-100UG; Sigma-Aldrich), or normal rabbit IgG control (NI01-100UG; Sigma-Aldrich) diluted to the same concentration was added and allowed to stand overnight at 4 °C. Subsequently, the cells were washed three times with PBS, and the appropriate secondary antibody for each was diluted 200-fold with an antibody diluent containing background-reducing components and incubated for 1 h at RT under shielded light.

Table 1
PCR primer sequence and amplification conditions.

	Gene	F/R	Sequence (5'→3')	Product size (bp)	Annealing Temperature (°C)
1	*β-actin*	F	GATGAGGCCCAGAGCAAGAG	77	65
		R	TCGTCCCAGTTGGTGACGAT		
2	*ALB*	F	GTTCCTGGGCACGTTTTTGTATGA	278	
		R	CTTGGGGTGCTTTCTTGGTGTAAC		
3	*AFP*	F	CTTTGGCTGCCCACTATGGC	182	
		R	AACCGTTATGGCTCGGAAGGT		
4	*a1-AT*	F	GCTGTCCAGGAGACGGATGAT	157	
		R	CAAAGGCTGTAGCGATGCTCA		
5	*EPCAM*	F	AGCGAGACCTGAGGGAGCTT	102	
		R	GTGGTGGTGCCATTGCACTG		
6	*TAT*	F	TTCTGGCTGTGGCTGCAAGG	181	
		R	AGGATCCAGCCCAACCTCCA		
7	*SOX9*	F	AAGAAAGACCACCCGGATTACA	154	
		R	CGGAGGAGGAGTGCGGCGAGT		
8	*CK19*	F	GCCCAGCTGAGCGATGTGC	86	
		R	TGCTCCAGCCGTGACTTGATGT		
9	*CDH1*	F	GGTGCTCACATTTCCCAGTT	100	60
		R	AAATGGGCCTTTCTCGTTT		
10	*CYP2E1*	F	CAGGACACGATGTTCAGAGGA	116	
		R	CTGGCTTGAACTTCTCTGGATC		
11	*CYP3A12*	F	AAGGACTTCCTTTTGTTCTTCAAGAAA	86	
		R	CCTACATGAGTGAAACCACATAATCAA		
12	*MRP2*	F	ATAGGGCAGAGGCAGCTACT	177	
		R	GGTGTGTAGCCTGTGAGCAA		

F: forward primer, R: reverse primer

The secondary antibodies used were donkey anti-goat IgG (H + L) Alexa Fluor 488 (A-11055; Waltham, MA, USA), goat anti-mouse IgG (H + L) Alexa Fluor 488 (A-11001), and goat anti-rabbit IgG (H + L) Alexa Fluor 594 (A-11012). Finally, the cells were washed three times with PBS and encapsulated in a VECTA SHIELD mounting medium containing DAPI (Vector Laboratories, Inc.). The cells were assessed using a fluorescence microscope. Table 2 shows the antibodies used. The antigen-positive cells on day 3 and day 21 were compared using a *t*-test.

3. Results

3.1. Morphological evaluation of the hepatic progenitor cells

Cell morphology was evaluated in the hepatic progenitor cells differentiation-induction group and in the control group (Fig. 1). On Day 3, adhesion to the plate was observed in both the cell culture groups. After seven days of culture, the control group exhibited one or two oval cells with nuclei; however, no colony formation w hepatic progenitor cells as observed. Subsequently, the cytoplasm of the cultured cells elongated to the periphery, and the cells in this group did not proliferate. In the hepatic progenitor differentiation induction groups, including YAC, cells with one or two nuclei formed colonies. Cultured cells continued to proliferate on day 14. Colonies on day 14 contained relatively small oval cells with a single nucleus and a nucleus-to-cytoplasm ratio (N/C ratio) of approximately 1:1 and relatively large cells with a wide cytoplasm range, one to several nuclei, and polygonal shapes.

3.2. Relative quantification of liver-related genes using qRT-PCR

mRNA expression levels were assessed using qRT-PCR in the hepatic progenitor cells and control groups on days 0 and 14 (Fig. 2). The expression levels of *AFP, EpCAM, CK19*, and *Sox9* increased by 12-, 2.2-, 517-, and 2.9-fold, respectively. These findings suggested that hepatocytes were induced to differentiate into hepatic progenitor cells with undifferentiated gene expression by YAC addition. Compared to day 0 hepatocytes, day 14 hepatic

Table 2
List of antibodies used for immunofluorescent cell staining.

	Antibody	clone number	Company	Location
Primary antibodies	mouse anti-Human E-cadherin	G-10	Santa Cruz Biotechnology	Dallas,TX,USA
	mouse anti-Human MRP2	M2III-6	Cell Sciences	Newburyport,MA,USA
	goat anti-Dog Albumin	A40-113A	Bethyl Laboratories Inc.	Montgomery,TX,USA
	rabbit anti-Human EPCAM	21050-1-AP	Proteintech	Rosemont,IL,USA
	rabbit anti-Human SOX9	LS-B12075	LS Bio(Absolute Biotech)	Boston,MA,USA
	rabbit anti-Human CK19	10712-1-AP	Proteintech	Rosemont,IL,USA
	goat anti-Human AFP	sc-8108	Santa Cruz Biotechnology	Dallas,TX,USA
(Isotype control)	Normal goat IgG	AB-108-C	R&D systems	Minneapolis,MN,USA
	Normal mouse IgG	NI03-100UG	Calbiochem(Merk)	Darmstadt,DEU
	Normai Rabbit IgG	NI01-100UG	Calbiochem(Merk)	Darmstadt,DEU
Secondary antibodies	donkey anti-Goat IgG(H+L) Alexa Fluor 488	A11055	Invitrogen (Thermo Fisher Scientific)	Waltham,MA,USA
	goat anti-Mouse IgG(H+L) Alexa Fluor488	A11001	Invitrogen (Thermo Fisher Scientific)	Waltham,MA,USA
	goat anti-Rabbit IgG(H+L) Alexa Fluor594	A11012	Invitrogen (Thermo Fisher Scientific)	Waltham,MA,USA

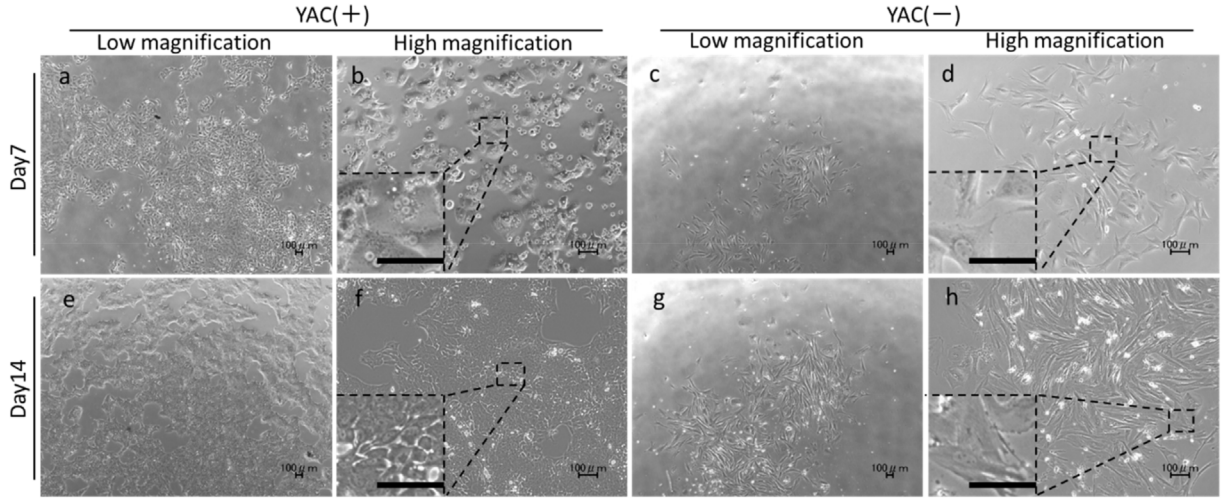

Fig. 1. Morphological evaluation.
Morphological findings of the control group using the inverted microscope included a: low magnification, b: high magnification on day 7, e: low magnification and f: high magnification on day 14. After 7 d of culture, cultured cells with one or two nuclei were identified, and colonies were formed. Cultured cells continued to proliferate on day 14. After 14 d of culture, cultured cells contained relatively small oval cells with a single nucleus and a nucleus-to-cytoplasm ratio (N/C ratio) of approximately 1:1, and relatively large cells with a wide cytoplasm range, one to several nuclei, and polygonal shapes.
Morphological findings of the control group using the inverted microscope included c: low magnification, d: high magnification on day 7 g: low magnification, and h: high magnification on day 14. After 7 d of culture, the cells exhibited one or two oval cells with nuclei. After 14 d of culture, the cytoplasm of the cultured cells elongated to the periphery, and the cells in this group did not proliferate.
The area enclosed by the dashed line is magnified in the lower left of each panel. Scale bar: 100 μm.

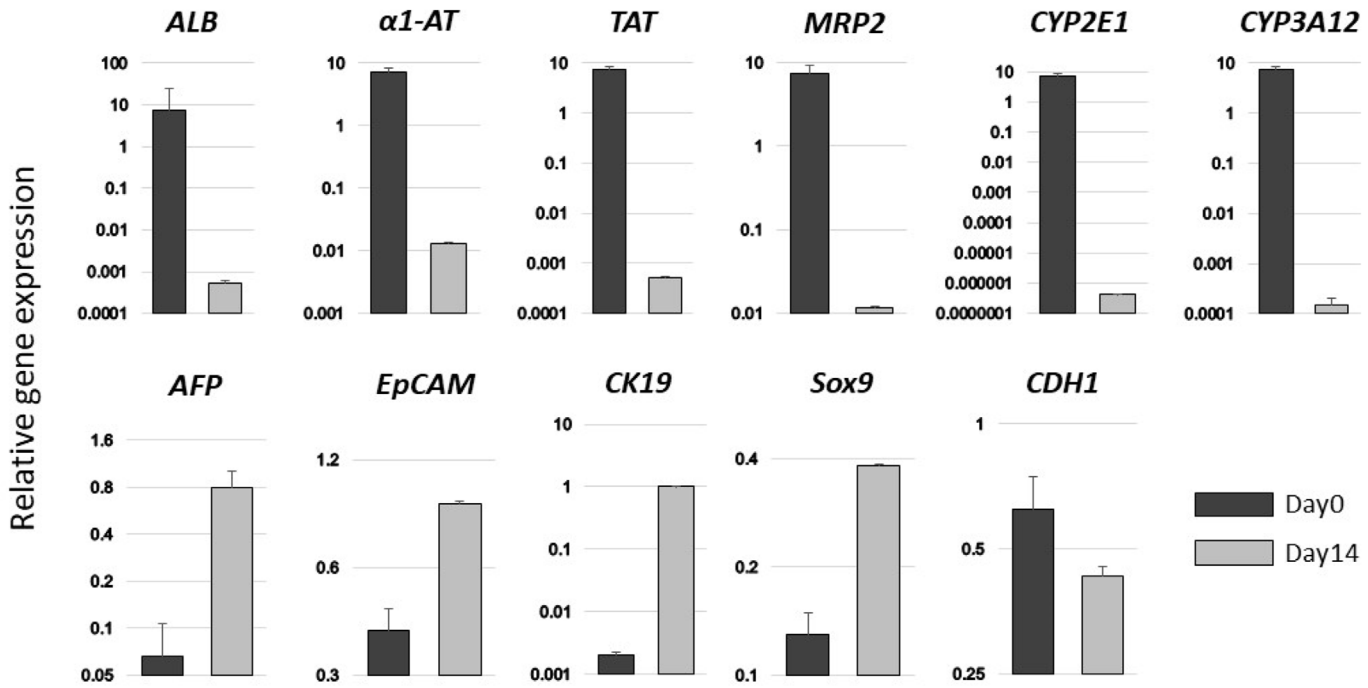

Fig. 2. Liver-related gene expression analysis using comparative quantitative real-time polymerase chain reaction.
Expression levels of liver-related genes were compared between day 0 and day 14. Compared to day 0 hepatocytes, day 14 cells exhibited increased *AFP, EpCAM, CK19,* and *Sox9* mRNA expression.

progenitor cells exhibited decreased *ALB, α1-AT, TAT, MRP2, CYP2E1, CYP3A12 and CDH1* mRNA expression. The gene expression levels are shown as a heat map for improved visual recognition in Fig. 3. Red and green indicate higher and lower expressions, respectively.

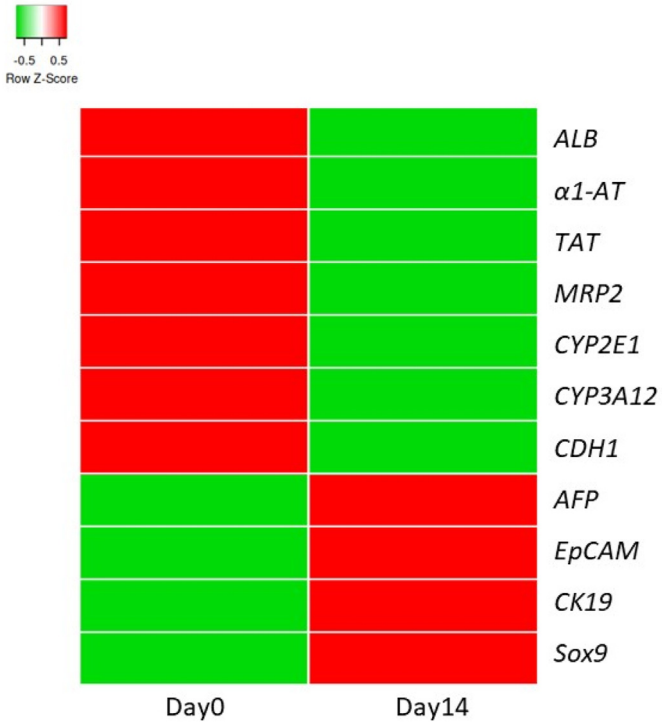

Fig. 3. Heatmap.
Heatmap showing the expression of liver-related genes as assessed using qualitative real-time polymerase chain reaction.
Red and green colours indicate higher and lower expression, respectively.

3.3. Immunofluorescent cell staining

The proteins expressed in the hepatic progenitor cells were characterised using immunofluorescent cell staining (Fig. 4). ALB and MRP2 were expressed in the day 0 hepatocytes and AFP, EPCAM, SOX9, and CK19 were almost undetectable. CK19, AFP, EPCAM, and SOX9 proteins were expressed in the day 21 hepatic progenitor cells, all of which were clearly increased compared with the levels before YAC addition. Furthermore, hepatic progenitor cells stained with F-actin exhibited small ovoid/spindle-shaped morphology (Fig. 5). Contrastingly, ALB and MRP2 were weakly expressed compared with the day 0 hepatocytes, and the expression of these proteins was attenuated. Particularly, ALB-, EPCAM-, SOX19-and CK19-positive cells were numerous at the margins of the hepatocyte progenitor cell colonies. Immunocytochemistry at day 21 revealed that the percentage of cells marked with ALB, MRP2, AFP, EPCAM, SOX9 and CK19 was 25.27 ± 6.27 %, 24.61 ± 7.25 %, 79.59 ± 4.14 %, 63.97 ± 16.72 %, 64.95 ± 6.27 % and 74.50 ± 12.47 %, respectively (Fig. 6).

4. Discussion

Herein, we demonstrated that canine cryopreserved hepatocytes could be reprogrammed into hepatic progenitor cells by culturing in a YAC-supplemented medium. To the best of our knowledge, this is the first report on the reprogramming of canine hepatic progenitor cells using low-molecular-weight compounds. Furthermore, we demonstrated a straightforward and cost-effective method for cultivating canine hepatic progenitor cells using low-molecular-weight compounds. These findings indicated the potential utility of canine hepatic progenitor cells as a foundational technology for developing a feasible toxicity assessment system and producing transplantable cultured hepatocytes. In liver regenerative medicine, several studies have been conducted on inducing differentiation into the liver progenitor cells and

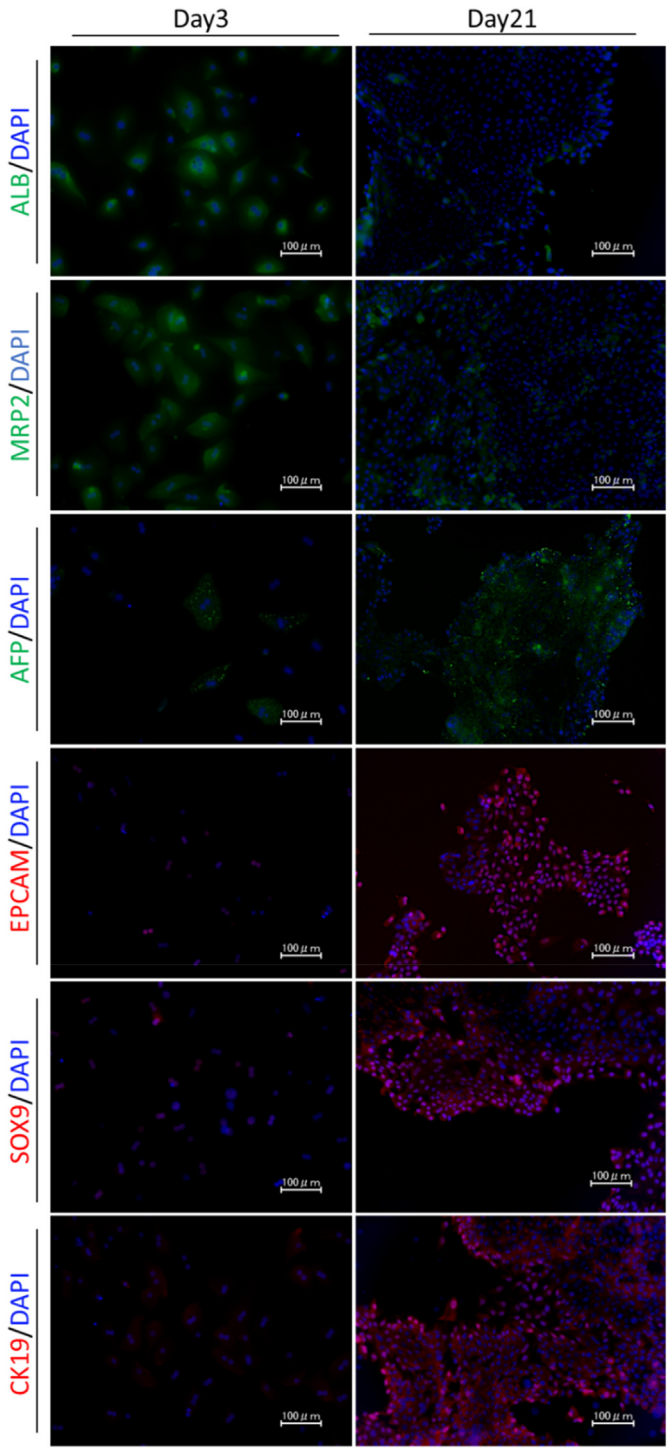

Fig. 4. Immunofluorescent cell staining.
Immunostaining demonstrated cHep cell differentiation into hepatic progenitor cells. On day 21, ALB and MRP2 were weakly expressed compared to day 3 hepatocytes, and CK19, AFP, EPCAM, and SOX9 proteins were highly expressed. Nuclei were stained with 4′,6-diamidino-2-phenylindole. Scale bar: 100 μm.

improving their culture in rats, humans, and dogs [15–17]. Previously, Arends et al. reported that canine liver progenitor cells (LPC) can be isolated from the liver tissue of healthy dogs and cultured [15]. Clonal expansion of LPCs was observed after culturing in hepatocyte-specific medium for 2 weeks. Furthermore, LPCs exhibited a small cell morphology with a high N/C ratio in the monolayers, which expressed keratin 7 (K7) using immunohistochemistry. The morphological findings of LPCs were similar to those of hepatic progenitor cells in this study; however, the hepatic progenitor cell attributes remain unclear. In the present study, the same small-molecule compounds used in other species were able to reprogram hepatic progenitor cells from mature canine hepatocytes. In studies that used other species, small molecule compounds have been reported to contribute to the induction and maintenance of hepatic stem cell pluripotency in rats [16] and humans [17]. In rats, isolated hepatocytes were reprogrammed to CLiPs by culturing them in a differentiation medium (YAC medium) consisting of Y-27632, A-83-01 and CHIR99021 [16]. The generated CLiPs were positive for Ck19, a marker of hepatic progenitor cells and exhibited high proliferation. In humans [17], reprogramming infant human hepatocytes to CLiPs has been reported using a similar cocktail of molecules (YAC). Furthermore, a cocktail of small molecules, Y-27632, A-83-01 and CHIR99021, was recently reported to convert mature rat and mouse hepatocytes into proliferative and bipotent cells under *in vitro* conditions [18]. Based on this study, we believe that YAC can induce canine hepatic progenitor cells from mature hepatocyte, as in other species.

The cell morphology of hepatic progenitor cells was similar to that of dogs, humans, and mice.

The proportion of large and small cells differed according to the colony size. Previously, the presence of small hepatocytes with high proliferative capacity in the liver has been reported in rats [19]. A chronically injured liver has a reduced proliferative capacity, and as the symptoms progress with inadequate regeneration, small hepatocytes begin to proliferate compared to mature, functional hepatocytes. These hepatocytes are believed to be extremely small and dormant in the normal liver since they are rarely found in the normal state. Furthermore, they are activated and contribute to regeneration only when the liver is affected by chronic hepatitis. However, studies since 2013 have revealed that these cells are not activated from dormancy but are rather altered from mature hepatocytes in *in vitro* conditions [20–22]. Hepatic progenitor cells are hepatoblasts that are found in the fetal liver and are capable of differentiating into hepatocytes and bile duct epithelial cells. These small hepatocytes have progenitor-like properties as they are capable of proliferating in *in vitro* and *in vivo* conditions and differentiating into hepatocytes and bile duct epithelial cells despite being located in the mature liver. On day 14, most of the colonies contained a mixture of large and small cells, with a greater proportion of small cells. Hepatic progenitor cells often give rise to cells called “oval cells” [23]. These cells are thought to be present in the liver as hepatic stem cells, which can differentiate into hepatocytes and biliary epithelial cells. In addition to oval cells [24], other cells, including hepatic progenitor cells [18] and small hepatocytes [19], are involved in liver regeneration. Hepatocyte-like cells induced to differentiate from human pluripotent stem cells using small-molecule compounds exhibit rapid proliferation and cytoplasmic enlargement of individual cells to form a cuboidal shape with well-defined boundaries [25]. This morphological finding in this study is similar to that reported by Katsuda et al. [18] for primary cultured small mouse hepatocytes. The findings of this study indicated that no observable morphological distinctions were found between the liver progenitor cells of dogs and those found in the other examined animal species.

Herein, canine hepatic progenitor exhibited gene expression levels consistent with the characteristics of hepatic progenitor cells reported in a previous study [18]. Herein, the gene expression levels of *ALB, α1-AT, TAT, MRP2, AFP, EpCAM, CK19, Sox9, CDH1, CYP2E1*, and *CYP3A12*, which are liver-related genes, were evaluated using qRT-PCR. A increase in *AFP* and *Sox9* expression was observed, which are specifically expressed in liver progenitor cells [23,24,26].

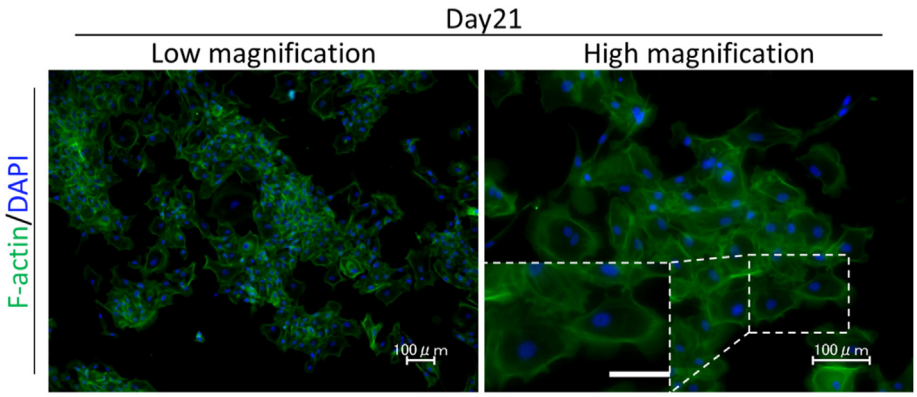

Fig. 5. F-actin staining.
Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). F-actin staining revealed the cytoskeletal structure. Scale bar: 100 μm.

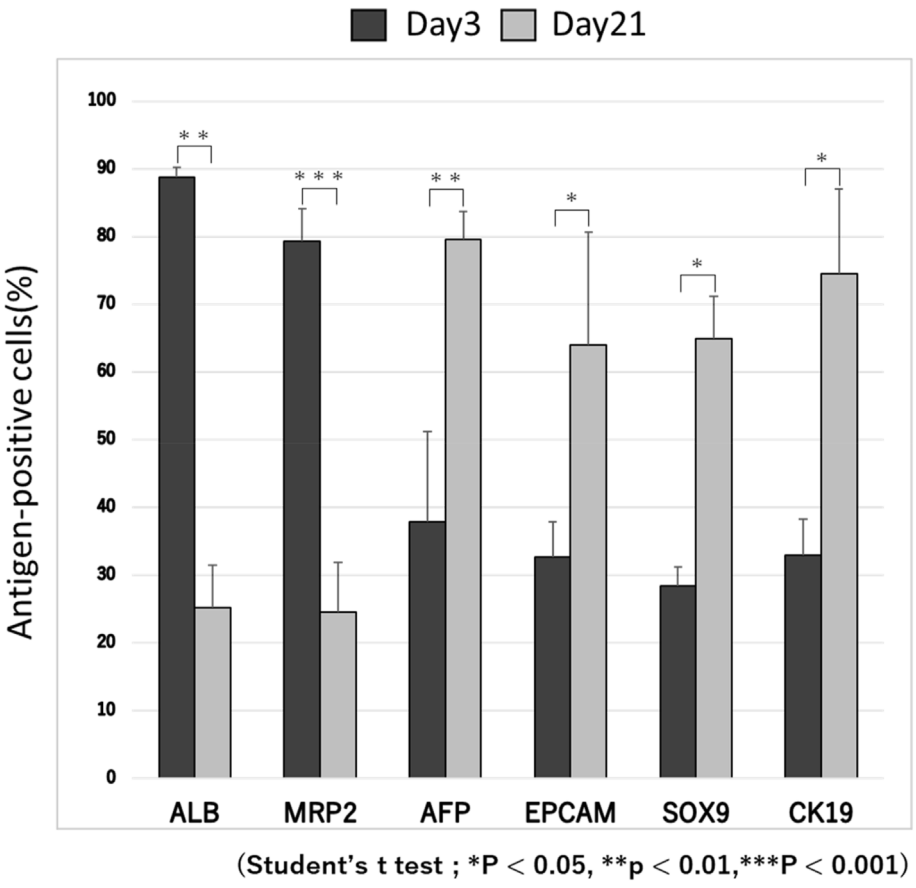

Fig. 6. Antigen-positive cells before and after inducing differentiation.
The graph shows the percentage of cells stained with ALB, MRP2, AFP, EpCAM, SOX9, and CK19 in mature hepatocytes (day 3) and hepatic progenitor cells (day 21) (400–800 cells were counted for each cell). The antigen-positive cells on day 3 and day 21 were compared using a *t*-test and a statistically significant difference was considered when *p < 0.05.

Additionally, the expression levels of *EpCAM* and *CK19* increased. Increased mouse *CK19* expression has been reported in liver stem cells that combine hepatocyte and bile duct epithelial cell expression types and express *ALB, CK19*, and *AFP* [27,28]. The increased *EpCAM* expression in CLiP cells was also validated based on previous study [18]. Additionally, hepatic progenitor cells exhibited decreased *ALB, α1-AT, TAT, MRP2, CYP2E1*, and *CYP3A12* expression, which are markers of liver function expressed in mature hepatocytes. Hepatic progenitor cells have decreased hepatic function in contrast to the increased undifferentiated marker expression [29]. The decrease in hepatic functional markers was similar to the change in gene expression in the hepatic progenitor cells. These results support the hypothesis that the hepatic progenitor cells produced in this study have the characteristics of progenitor cells.

Immunohistochemistry analysis revealed that hepatocyte characteristics were preserved following the induction of differentiation, despite the transition to progenitor cells. Since the proteins ALB and MRP2 exhibited reduced expression, whereas AFP, EPCAM, SOX9 and CK19 showed increased expression, these results were confirmed using mRNA expression analysis. In previous

studies, certain proteins have been identified as markers of liver progenitor cells in dogs. Our previous study showed that AFP was expressed in immature hepatocytes [30]. Furthermore, immunohistochemistry analysis demonstrated positive SOX9 expression in canine hepatocellular carcinoma [31]. Additionally, immunofluorescence staining for EpCAM in canine fetal tissue and isolated cultured cells was positive, demonstrating the presence of hepatic progenitor cells [32]. In other species, the hepatic progenitor cell markers AFP, EpCAM and CK19 are expressed in CLiP cells derived from mature rat hepatocytes [33], and similar results were obtained in the present study. Herein, we observed that the protein expression pattern of liver progenitor cells in dogs closely resembled that of known liver progenitor cells in dogs and other animals. However, the fundamental characteristics of canine liver progenitor cells remain largely unexplored and poorly understood. Consequently, extensive research is warranted to comprehensively elucidate the unique attributes and functional properties of canine liver progenitor cells.

The ability to induce the differentiation of hepatic progenitor cells from cryopreserved hepatocytes has the following advantages: Advances in regenerative medicine: Hepatic progenitor cells are the population that can induce liver regeneration and play an essential role in treating liver injury and diseases. The easy availability of hepatic progenitor cells from cryopreserved hepatocytes is expected to advance regenerative medicine, including treatment development for liver diseases and injuries and transplantation medicine improvement. Improving biopharmaceutical productivity: Hepatic progenitor cells may be used to produce biopharmaceuticals with liver-specific drug metabolism and detoxification properties because of their cellular functions in the liver. The ability to culture cryopreserved cells for long periods can improve biopharmaceutical production, potentially resolving supply shortages and cost issues. Research reproducibility and standardisation: The use of cryopreserved cells allows researchers to conveniently perform culture tests. In research and experiments, the use of the same lot of cells improves the reproducibility of the experimental results and provides reliable data. Moreover, sharing and exchanging cells between different laboratories has become easier, and the use of standardised cells in regenerative medicine and clinical applications contributes to improved treatment safety and efficacy. Reducing costs and addressing ethical problems: Long-term storage of hepatocytes through cryopreservation allows for a continuous cell supply. Consequently, the cost of cell procurement for experiments and therapies can be reduced. Furthermore, from an ethical standpoint, this approach reduces the problems associated with harvesting and supplying cells from living organisms.

Moreover, considering that reprogramming hepatocytes obtained via liver biopsy into hepatic progenitor cells was not possible in this study, this should be considered in the future. Particularly, liver biopsy by true cutting is minimally invasive and would contribute to solving the ethical problems associated with cell experiments using laboratory animals. Once a technique for creating canine CLiP cells is established, artificial livers derived from canine livers can be used to advance the analysis of pathogenic mechanisms and the development of therapeutic agents. Research on assessing drug toxicity and metabolic processes using hepatocytes obtained via liver biopsy allows for safe drug development and toxicological risk assessment. Additionally, culturing patient-specific hepatocytes allows for a further accurate understanding of the liver function and disease characteristics of individual patients. Consequently, analysing individualised pathogenic mechanisms, developing therapeutic methods, and tailoring appropriate drug treatments for each patient will be possible. Moreover, it is expected to contribute considerably to speeding up and reducing the cost of therapeutic drug development, as well as improving animal welfare.

The characteristics of the canine progenitor cells produced in this study were similar to those of CLiPs generated using YAC. These results confirm that cHep cells can be easily and efficiently cultured in large quantities into hepatic progenitor cells by adding YAC. Furthermore, differentiation into cHep cells required the presence of all three YAC small molecules, and employing one or two of these small molecules proved inadequate to achieve differentiation into hepatic progenitor cells (data not shown). In the near future, we believe that cHep cells can be applied to developing toxicity assessment tools and transplantation medicine, and can serve as a cell source for liver regenerative medicine. Previous studies have not established an efficient and simple method for increasing the number of hepatic progenitor cells; however, Katsuda et al. successfully cultured various stem and progenitor cells, including rat ES cells, using four small-molecule compounds [22,34]. Furthermore, if small hepatocytes in the liver have the properties of hepatic progenitor cells, a stable culture of hepatocytes might be possible using these small-molecule compounds. Additionally, reprogramming of mature hepatocytes to CLiP cells in mice and rats has been attempted. Consequently, CLiP cells directly demonstrated reprogramming of mature hepatocytes *in vitro* and demonstrated an ameliorative effect on liver injury, as CLiP cells were viable in liver-damaged mice [14]. In summary, establishing canine hepatic progenitor cells is expected to enable the stable culture of canine mature hepatocytes and the application of liver transplantation by hepatic progenitor re-maturation in the future.

This study had four main limitations. First, the final confirmation that the hepatic progenitor-like cells generated are CLiP cells requires proof that the cells can be induced to again differentiate into mature hepatocytes and bile duct epithelial cells. Second, only a small number of genes were analysed in the hepatic progenitor cells, and the expression levels of liver-related genes and other molecules were not comprehensively analysed, which will require microarray analysis in the future. Third, whether all the generated hepatic progenitor cells have uniform properties is unclear; therefore, analysis using single-cell sorting is necessary. Fourth, hepatic progenitor cells were not generated from primary cultured hepatocytes obtained by biopsy.

5. Conclusions

Herein, to the best of our knowledge, we demonstrated for the first time that cryopreserved adult canine hepatocytes can be reprogrammed into hepatic progenitor cells and proliferated *in vitro* using three small-molecule compounds named YAC. The results of this study have made it possible to address an important issue in hepatocyte research, namely the culture and *in vitro* hepatocyte propagation. The canine hepatic progenitor cells established in this study are expected to be cultured and characterised for re-maturation and used in drug toxicity testing for drug discovery research and transplantation therapy for treating canine liver disease. Further studies are needed to evaluate the drug metabolism of hepatic progenitor cells, generate and analyse cultured liver tissue by 3D culture, and evaluate the replacement rate and function after *in vivo* transplantation.

Declaration of competing interest

We have no conflicts of interest directly relevant to the content of this article.

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References

[1] Eulenberg VM, Lidbury JA. Hepatic fibrosis in dogs. J Vet Intern Med 2018;32:26–41. https://doi.org/10.1111/jvim.14891.

[2] Webster CRL, Center SA, Cullen JM, Penninck DG, Richter KP, Twedt DC, et al. ACVIM consensus statement on the diagnosis and treatment of chronic hepatitis in dogs. J Vet Intern Med 2019;33:1173–200. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6524396/.

[3] Devriendt N, Serrano G, Paepe D, de Rooster H. Liver function tests in dogs with congenital portosystemic shunts and their potential to determine persistent shunting after surgical attenuation. Vet J 2020;261:105478. https://www.sciencedirect.com/science/article/pii/S1090023320300551?via%3Dihub.

[4] Konstantinidis AO, Patsikas MN, Papazoglou LG, Adamama-Moraitou KK. Congenital portosystemic shunts in dogs and cats: classification, pathophysiology, clinical presentation and diagnosis. Vet Sci 2023;10:160. https://www.mdpi.com/2306-7381/10/2/160.

[5] Akiyoshi M, Hisasue M, Akiyoshi M. Clinicopathological findings and prognosis in canine cases diagnosed as primary hypoplasia of the portal vein. Front Vet Sci 2017;4:224. https://www.frontiersin.org/articles/10.3389/fvets.2017.00224/full.

[6] Ullal T, Ambrosini Y, Rao S, Webster CRL, Twedt D. Retrospective evaluation of cyclosporine in the treatment of presumed idiopathic chronic hepatitis in dogs. J Vet Intern Med 2019;33:2046–56. https://onlinelibrary.wiley.com/doi/10.1111/jvim.15591.

[7] Kruitwagen HS, Oosterhoff LA, van Wolferen ME, Chen C, Nantasanti Assawarachan S, et al. Long-term survival of transplanted autologous canine liver organoids in a COMMD1-deficient dog model of metabolic liver disease. Cells 2020;9:410. https://www.mdpi.com/2073-4409/9/2/410.

[8] Kaplowitz N. Idiosyncratic drug hepatotoxicity. Nat Rev Drug Discov 2005;4:489–99. https://doi.org/10.1038/nrd1750.

[9] Li AP, Lu C, Brent JA, Pham C, Fackett A, Ruegg CE, et al. Cryopreserved human hepatocytes: characterization of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug-drug interaction potential. Comparative Study 1999;121:17–35. https://doi.org/10.1016/S0009-2797(99)00088-5.

[10] Stresser DM, Turner SD, Blanchard AP, Miller VP, Crespi CL. Cytochrome P450 fluorometric substrates: identification of isoform-selective probes for rat CYP2D2 and human CYP3A4. Drug Metab Dispos 2002;30:845–52. https://doi.org/10.1124/dmd.30.7.845.

[11] Nitta S, Hisasue M, Horiguchi Y, Yamada Y, Kikuchi K, Kubo T, et al. Three-dimensional spheroid culture of canine hepatocyte-like cells derived from bone marrow mesenchymal stem cells. Regen Ther 2020;15:210–5. https://doi.org/10.1016/j.reth.2020.09.002.

[12] Nitta S, Kusakari Y, Yamada Y, Kubo T, Neo S, Igarashi H, et al. Conversion of mesenchymal stem cells into a canine hepatocyte-like cells by Foxa1 and Hnf4a. Regen Ther 2020;14:165–76. https://doi.org/10.1016/j.reth.2020.01.003.

[13] Huang Y, Sakai Y, Hara T, Katsuda T, Ochiya T, Gu WL, et al. Differentiation of chemically induced liver progenitor cells to cholangiocytes: investigation of the optimal conditions. J Biosci Bioeng 2020;130:545–52. https://doi.org/10.1016/j.jbiosc.2020.07.009.

[14] Katsuda T, Ochiya T. Chemically induced liver progenitors (CLiPs): a novel cell source for hepatocytes and biliary epithelial cells. Methods Mol Biol 2019;1905:117–30. https://link.springer.com/protocol/10.1007/978-1-4939-8961-4_11.

[15] Arends B, Spee B, Schotanus BA, Roskams T, van den Ingh TS, Penning LC, et al. In vitro differentiation of liver progenitor cells derived from healthy dog livers. Stem Cells Dev 2009;18:351–8. https://doi.org/10.1089/scd.2008.0043.

[16] Murakami S, Soyama A, Miyamoto D, Hara T, Matsuguma K, Imamura H, et al. Transplantation of chemically-induced liver progenitor cells ameliorates hepatic fibrosis in mice with diet-induced nonalcoholic steatohepatitis. Regen Ther 2022;21:574–83. https://doi.org/10.1016/j.reth.2022.11.001.

[17] Katsuda T, Matsuzaki J, Yamaguchi T, Yamada Y, Prieto-Vila M, Hosaka K, et al. Generation of human hepatic progenitor cells with regenerative and metabolic capacities from primary hepatocytes. Elife 2019;8:e47313. https://doi.org/10.7554/eLife.47313.

[18] Katsuda T, Kawamata M, Hagiwara K, Takahashi RU, Yamamoto Y, Camargo FD, et al. Conversion of terminally committed hepatocytes to culturable bipotent progenitor cells with regenerative capacity. Cell Stem Cell 2017;20:41–55. https://doi.org/10.1016/j.stem.2016.10.007.

[19] Mitaka T, Mikami M, Sattler GL, Pitot HC, Mochizuki Y. Small cell colonies appear in the primary culture of adult rat hepatocytes in the presence of nicotinamide and epidermal growth factor. Hepatology 1992;16:440–7. https://doi.org/10.1002/hep.1840160224.

[20] Kim Y, Kang K, Lee SB, Seo D, Yoon S, Kim SJ, et al. Small molecule-mediated reprogramming of human hepatocytes into bipotent progenitor cells. J Hepatol 2019;70:97–107. https://doi.org/10.1016/j.jhep.2018.09.007.

[21] Gentric G, Desdouets C. Polyploidization in liver tissue. Am J Pathol 2014;184:322–31. https://doi.org/10.1016/j.ajpath.2013.06.035.

[22] Tarlow BD, Pelz C, Naugler WE, Wakefield L, Wilson EM, Finegold MJ, et al. Bipotential adult liver progenitors are derived from chronically injured mature hepatocytes. Cell Stem Cell 2014;15:605–18. https://doi.org/10.1016/j.stem.2014.09.008.

[23] Yang XY, Li XL, Xue Y, Zhang CX, Wang Y, Hu X, et al. Bone marrow cells differentiation into organ cells using stem cell therapy. Eur Rev Med Pharmacol Sci 2016;20:2899–907. https://www.europeanreview.org/article/11118.

[24] Farber E. Similarities in the sequence of early histological changes induced in the liver of the rat by ethionine, 2-acetylamino-fluorene, and 30 -methyl4-dimethylaminoazobenzene. Cancer Res 1956;16:142–8. https://pubmed.ncbi.nlm.nih.gov/13293655/.

[25] Asumda FZ, Hatzistergos KE, Dykxhoorn DM, Jakubski S, Edwards J, Thomas E, et al. Differentiation of hepatocyte-like cells from human pluripotent stem cells using small molecules. Differentiation 2018;101:16–24. https://doi.org/10.1016/j.diff.2018.03.002.

[26] Furuyama K, Kawaguchi Y, Akiyama H, Horiguchi M, Kodama S, Kuhara T, et al. Continuous cell supply from a Sox9-expressing progenitor zone in adult liver, exocrine pancreas and intestine. Nat Genet 2011;43:34–41. https://www.nature.com/articles/ng.722#citeas.

[27] Carpentier R, Suñer RE, van Hul N, Kopp JL, Beaudry JB, Cordi S, et al. Embryonic ductal plate cells give rise to cholangiocytes, periportal hepatocytes, and adult liver progenitor cells. Gastroenterology 2011;141:1432–8. https://doi.org/10.1053/j.gastro.2011.06.049.

[28] Shiojiri N. Development and differentiation of bile ducts in the mammalian liver. Microsc Res Tech 1997;39:328–35. https://doi.org/10.1002/(SICI)1097-0029(19971115)39:4<328::AID-JEMT3>3.0.CO;2-D.

[29] Hee Hong D, Lee C, Kim Y, Lee SB, Han SC, Kim SJ, et al. Generation of hepatic progenitor cells from the primary hepatocytes of nonhuman primates using small molecules. Tissue Eng Regen Med 2021;18:305–13. https://link.springer.com/article/10.1007/s13770-020-00327-8.

[30] Kawarai S, Hashizaki K, Kitao S, Nagano S, Madarame H, Neo S, et al. Establishment and characterization of primary canine hepatocellular carcinoma cell lines producing alpha-fetoprotein. Vet Immunol Immunopathol 2006;113:30–6. https://doi.org/10.1016/j.vetimm.2006.03.006.

[31] Tsuji Y, Kuramochi M, Izawa T, Akiyoshi H, Yamate J, Kuwamura M. Hepatic myoepithelial carcinoma in a dog: immunohistochemical comparison with other canine hepatic carcinomas. Vet Pathol 2019;56:889–94. https://doi.org/10.1177/0300985819854439.

[32] Tavares MR, de Castro RVG, Pieri NCG, Cruz NRN, Martins DS, Ambrósio CE, et al. Identification of hepatic progenitor cells in the canine fetal liver. Res Vet Sci 2020;133:239–45. https://doi.org/10.1016/j.rvsc.2020.09.012.

[33] Huang Y, Sakai Y, Hara T, Katsuda T, Ochiya T, Gu WL, et al. Bioengineering of a CLiP-derived tubular biliary-duct-like structure for bile transport in vitro. Biotechnol Bioeng 2021;118:2572–84. https://doi.org/10.1002/bit.27773.

[34] Chen Q, Kon J, Ooe H, Sasaki K, Mitaka T. Selective proliferation of rat hepatocyte progenitor cells in serum-free culture. Nat Protoc 2007;2:1197–205. https://www.nature.com/articles/nprot.2007.118.