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Culture and identification of biliary fibroblasts from Diannan small-ear pigs

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Abstract

This study amied to explore the culture method of biliary tract fibroblasts from Diannan small-ear pigs, and lay the foundation for further exploration on biliary tract benign stenosis. The biliary tract injury model was constructed via adopting Diannan small-ear pigs, and the tissue adherence method was adopted. After trying different medium and fetal bovine serum concentrations, the culture method of mature Diannan small-ear pig biliary tract fibroblast was explored. Additionally, vimentin and desmin was examined and identified as fibroblasts. The biliary tract fibroblasts that can be passaged were available to be cultured via tissue adherence method. Biliary tract fibroblast, a sort of cell with proliferation and multidirectional differentiation potential, is difficult to be cultured and passed on, and its successful culture lays a good foundation for further exploration on drug intervention.

Keywords: biliary tract fibroblasts; Diannan small-ear pigs; culture.

Pratica Application: Culture method of biliary tract fibroblasts from Diannan small-ear pigs.

1 Introduction

Benign biliary tract stenosis's treatment has always been the difficulties in hepatobiliary surgery. After repair of biliary tract injury (BTI) or anastomosis, fibrous tissue's hyperplasia results in biliary tract stenosis, which takes up a great proportion.

Studies have manifested the biliary tract scar formation's mechanism bears a resemblance to that of skin scar formation, in which the balance of cell proliferation with apoptosis is disrupted, and cell apoptosis is constrained, owing to the fibroblast proliferation and excessive secretion of matrix components like collagen (Geng et al., 2005). Up to now, no reliable artificial material is available to be adopted for biliary tract repair or reconstruction owing to biliary tract fibroblasts' particularity and the physical and chemical environment's complexity. Consequently, exploring mature biliary tract fibroblasts' culture method is the crucial foundation for figuring out benign biliary tract stenosis. Baoying Chen et al. have adopted enzymatic detachment method to culture rabbit biliary fibroblasts (Chen et al., 2004). In preliminary experiments, pancreatin and collagenase detachment methods were tried without success. Tissue adhesion method was adopted later, and different culture medium and fetal bovine serum (FBS) concentration were attempted, and mature Diannan small-ear pigs biliary tract fibroblasts' culture method was explored. It lays a good foundation for further exploration.

2 Materials and methods

2.1 Experimental model's construction

(1) Provision of a 14-months old Diannan small-ear pigs with weight of 16.9 kg and clean grade was implemented (the Experimental Animal Center of Kunming Medical University), license SCXK (Dian): No 2017-0004. Feeding conditions were as follows: one pig per cage, and free food and water. (2) After anesthesia took effect, the anterior wall of the common bile duct (CBD) was transversely cut in the middle section of CBD without exceeding 1/2 of the circumference. Full-thickness anastomosis of the CBD was sutured continuously with 5-0 absorbable suture with needle distance of about 0.1-0.2 cm and edge distance of approximately 0.2-0.3 cm. No bleeding and biliary leakage were presented after careful examination, a rubber drainage tube was placed near the Wen's foramen with close of the abdomen layer by layer. Fasting was implemented after the operation and given intramuscular injection of 1.6 million U penicillin. The drainage tube was removed 3 days after surgery, and the animals' feeding and mental status were observed (Figure 1).

2.2 Materials

(1) CO_2 incubator: Forma 311 scientific series II; Thermo Electron Corporation (2) 25 cm^3 culture flasks, 24-well and 96-well culture plates: Corning Corporation, USA, No. 430168; (3) Optical microscope: BX-60 type (Olympus, Tokyo, Japan); (4) Inverted phase contrast microscope: CKX41 type (Olympus); (5). Centrifuge: Germany BECMAN company; (6) Enzyme-linked immunoassay instrument: BIO- TEK; (7) Confocal Fluorescence Microscope: Leica.

2.3 Main reagents

(1) 10% glucose Dulbecco's Modified Eagle Medium (DMEM): American GIBCO Company (No.12100-046); (2) Standard FBS: American Solarbio Company; (3) TRITON x-100: American

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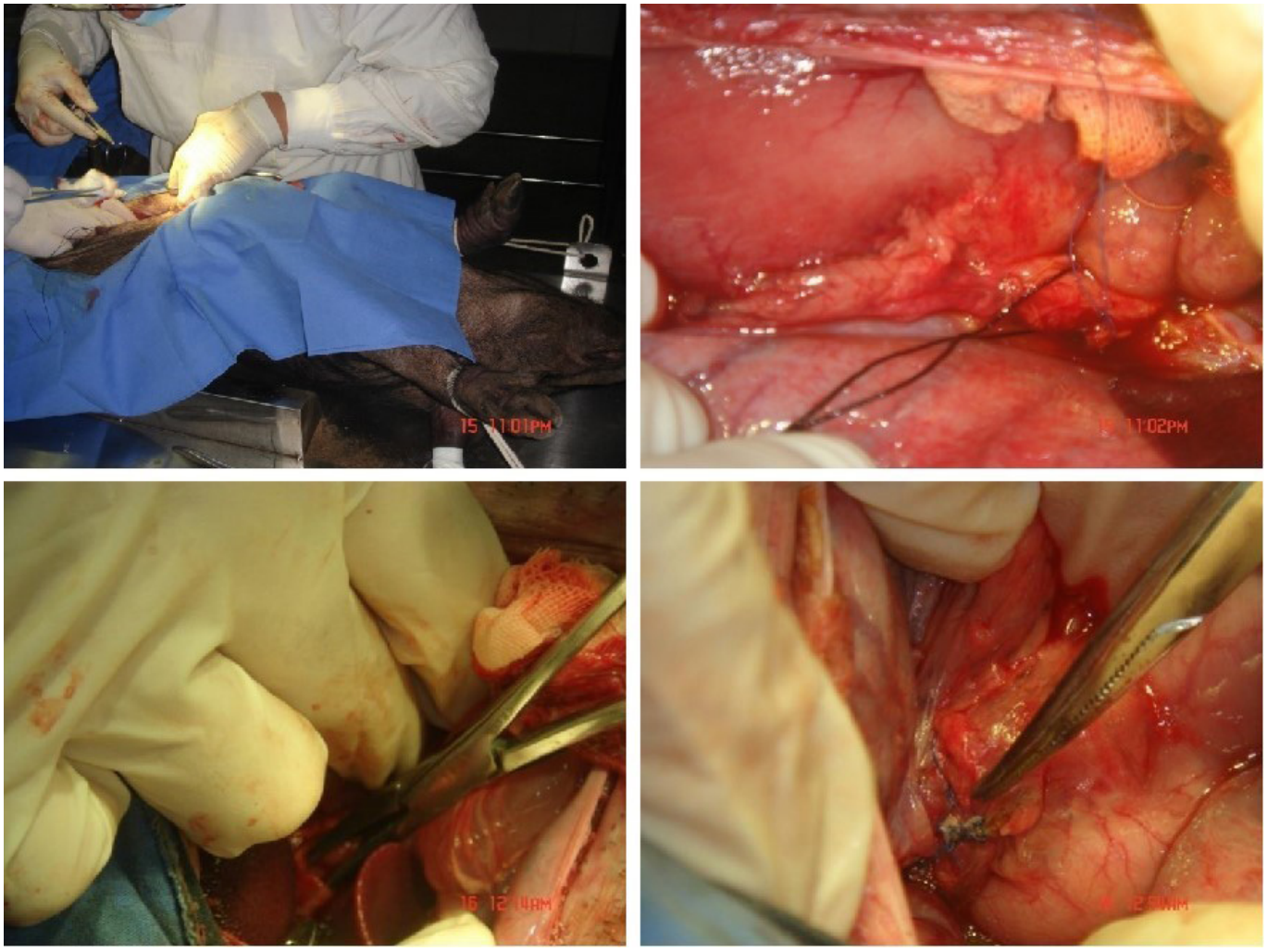

Figure 1. Taking of the sample 2 months after the operation, and cut of the biliary tract anastomosis and the upper and lower 1 cm bile duct tissue.

sigma Company; (4) Fluorescent CY3 labeled goat anti-mouse Immunoglobulin G (IgG): Wuhan Boster Bioengineering Co., Ltd. (No.BA1031); (5) Paraformaldehyde: Solarbio, USA; (6) Bovine serum albumin (BSA): Shanghai Huashun Biological Engineering Co., Ltd.

2.4 Methods

Placing of the biliary tract scar tissue was in normal saline, placing was on the ice, and electroblot was onto the culture room. Rinse was with phosphate buffer saline (PBS) (PH = 7.4) buffer covering 1% double antibodies for three times, then careful removal of serous membrane and adipose tissue was with ophthalmic scissors, and rinse of the tissue was with PBS (PH = 7.4) buffer covering 1% double antibodies until the liquid was clear without floating matter. Electroblot of the processed tissue was onto a clean petri dish. Cut was into 0.5 mm^2-1 mm^2 pieces with ophthalmic scissors, and seeding of the tissue pieces was into a clean culture flask or petri dish with a straw. The gap between the tissue masses was about 1 cm. Placing was in an incubator. After the tissue mass was slightly dry and adherent to the wall, addition of 5 mL DMEM low-sugar culture solution covering 10% imported FBS to the bottom of the culture bottle. The culture flask was carefully overturned to render the culture solution to submerge the adherent tissue. Placing of the culture flask was in an incubator. Observation was implemented on the third day, and then the fluid was changed every 2-3 days. When the cells grew and fused into sections with the density of about 80%, rinse was with calcium and magnesium ion-free PBS twice, addition of PBS detachment solution covering 0.25% trypsin and 0.02% ethylene diamine tetraacetic acid, and the cells were contracted into round shape after detachment. Addition of DMEM covering 10% FBS was to terminate detachment, and passage was carried out at a ratio of 1 : 2. Fibroblasts deriving from biliary tract scars were available to be passaged in about 4-5 days.

2.5 Identification

Detection of vimentin and desmin was via Cellular immunofluorescence to identify cells. Specific steps were as follows:

Seeding of sections: Seeding of the cells was in a six-well plate with a cover glass until the cells reached 80%→Rinse with PBS 2-3 times→Fixation with 4% paraformaldehyde for

20 min→Rinse with PBS 3 times→Block of sections: BSA is diluted with PBS to concentration of 3% for 1 h→Rinse with PBS 5-7 times→Addition of primary antibodies (diluted with PBS to 1 : 400) 1.5 mL/well, overnight at 4 °C.→TRITON x-100 is diluted with PBS into 0.05% solution and washes 3 times, 3 minutes each time→Addition of secondary antibodies: Fluorescent CY3 labeled goat anti-mouse IgG, 1.5 mL per well, incubation is implemented at 37 °C for 1 h→TRITON x-100 and PBS is diluted to 0.05% solution and washes 5 times, 5 min each time→Removal of the cover glass and placing on the glass slide→Fluorescence staining: Staining with 5 × 4', 6-diamidino-2-phenylindole (DAPI) 10 μL/slice, and incubation is implemented overnight at 4 °C→Rinse with PBS 3 times, and dehydration with ethanol gradient of four concentration gradients of 50%, 70%, 95% and 100%→Dropwise addition of fluorescent mounting solution to block sections→Observation under a confocal fluorescence microscope and photography of sections.

3 Results

3.1 Observation with inverted microscope

After 5-7 days of primary culture, new cells were presented, which were fibroblast type with adherent growth. Initially, cell morphology was diversified with long spindle shape and thick protuberances (Figure 2). After 14 days of separation and purification, they became a long fusiform and strip shape, and fused into sections (Figure 3). Cells deriving from biliary tract scars grew faster vs. the cells deriving from normal tissues.

3.2 Immunofluorescence identification

Biliary tract fibroblast vimentin imunofluorescence staining was positive, the nucleus surrounding cytoplasm was red filamentous network shape with the positive rate of over 95%, while nucleus of desmin was blue, and no red substance was presented in the cytoplasm with the negative rate of over 95% (Figures 4-5).

4 Discussion and conclusion

Fibroblasts, the extremely prevalent cells in connective tissue, differentiate from mesenchymal cells in the embryo and are available to synthesize and secrete collagen fibers, reticular fibers, elastic fibers and organic matrix (Lew et al., 2020). In the meantime, fibroblasts, the functionally active cells, undergo hyperplasia, chemotaxis or phenotypic transformation under multiple factors' influence. Recently, fibroblasts are gradually recognized to be nearly associated with scar contracture (McGeer & McGeer, 1999). Scars are an inevitable product in the process of tissue repair after trauma. The formation of scars is nearly associated with local inflammatory cells and a sequence of growth factors. In the meantime, the cells' excessive proliferation and apoptosis are restrained (Lu et al., 2007). Researches have illuminated formation mechanism of benign biliary stenosis bears a resemblance to skin scars' formation of skin scars, in which the balance of cell proliferation with apoptosis is destroyed and cell apoptosis is constrained, owing to the fibroblast proliferation and excessive secretion of matrix components like collagen.

The walls of the extrahepatic bile duct and the common bile duct are composed of mucosa, fibromuscular layer and outer membrane from the inside to the outside. In physiological conditions, the innermost mucosal layer is immediately in contact with bile. After BTI, numerous fibroblasts at rest are situated in the fibromuscular layer below the mucous membrane. After BTI, the destruction of the mucous membrane exposes the fibromuscular layer to the chronic inflammatory environment created via bile components (Geng et al., 2005). Fibroblasts with static boundary are available to differentiate into contractile phenotype with specific α smooth muscle actin under cytokines' control, namely myofibroblast (MFB), leading to the entire granulation tissue's contraction. Consequently, fibroblasts are a kind of cells with the proliferation and multidirectional differentiation potential. It participates in diversified physiological and pathological reaction, and exerts the crucial action in benign biliary stenosis's occurrence and advancement (Chen et al., 2004), construct a canine BTI model to observe biliary tract scars' microscopic and ultrastructure, and maintain MFB rarely presented in the normal biliary tract wall, while it was persisted in biliary tract scars. Meanwhile, they also consider persistence of fibroblasts and its contraction ability are critical factors leading to biliary tract scar stenosis.

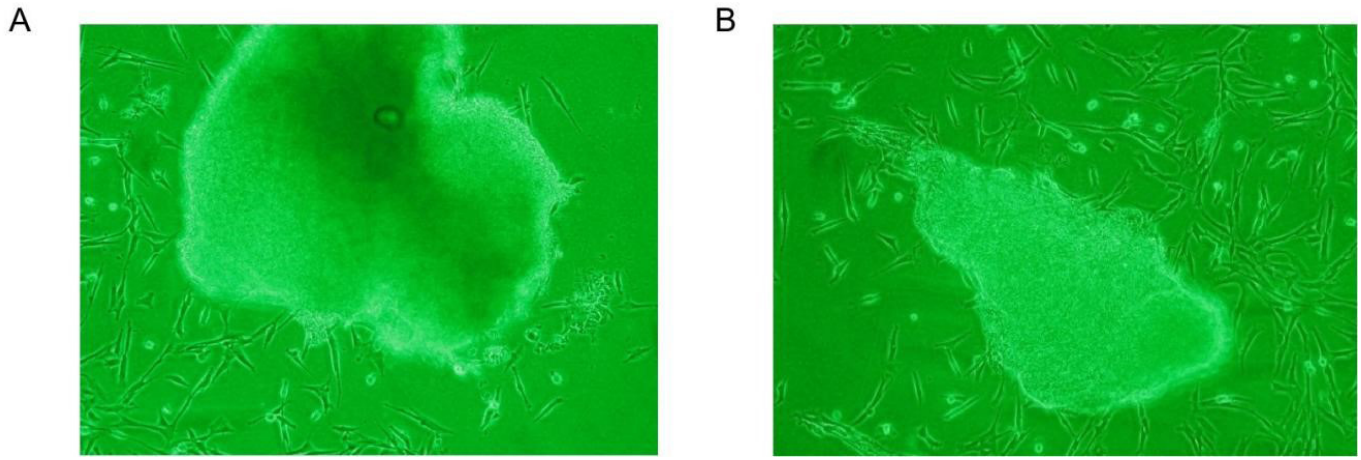

Figure 2. Cells start to grow after 7 days of primary culture: A is 7 days; B is 10 days. 10 × 10 times.

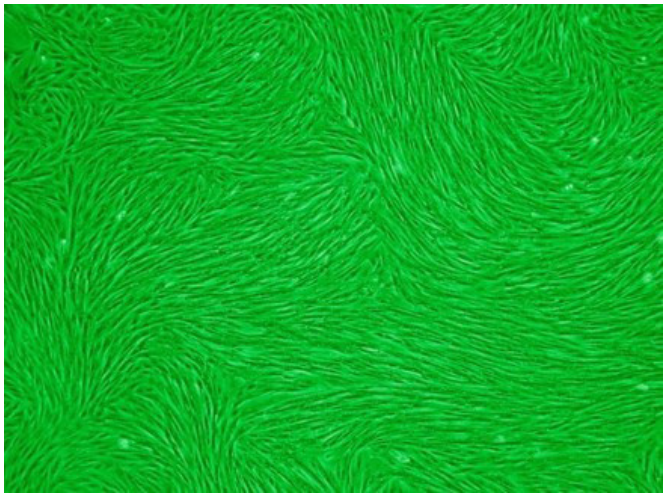

Figure 3. Cell condition and 10 × 10 times after 14 days of primary culture.

The culture of biliary tract fibroblasts is a relatively difficult part, specifically fibroblasts deriving from normal biliary tract, which might be linked with the species and the characteristics of biliary tract cells, and is also a crucial step in exploring benign biliary tract stenosis. Mastering the imperative culture method of biliary tract fibroblasts in animal models provides the foundation for later research on benign biliary tract stenosis, and offers experimental objects for subsequent in *vitro* drugs or gene therapy to interfere with the reduction of fibroblast proliferation. In the light of literature search, the culture of porcine biliary fibroblasts has hardly been reported at home and abroad.

In biliary fibroblasts' primary culture from Diannan small-ear pigs, the following points are worth noting: 1: Try to choose animals that are younger in age. After multiple experiments, the younger the animal is, the easier it is for the cells to dissociate, and the more vigorous cell growth is. 2: After the tissue mass is seeded into the culture flask, it should be placed in an incubator to boost its adherence. 3: After adding the appropriate amount of

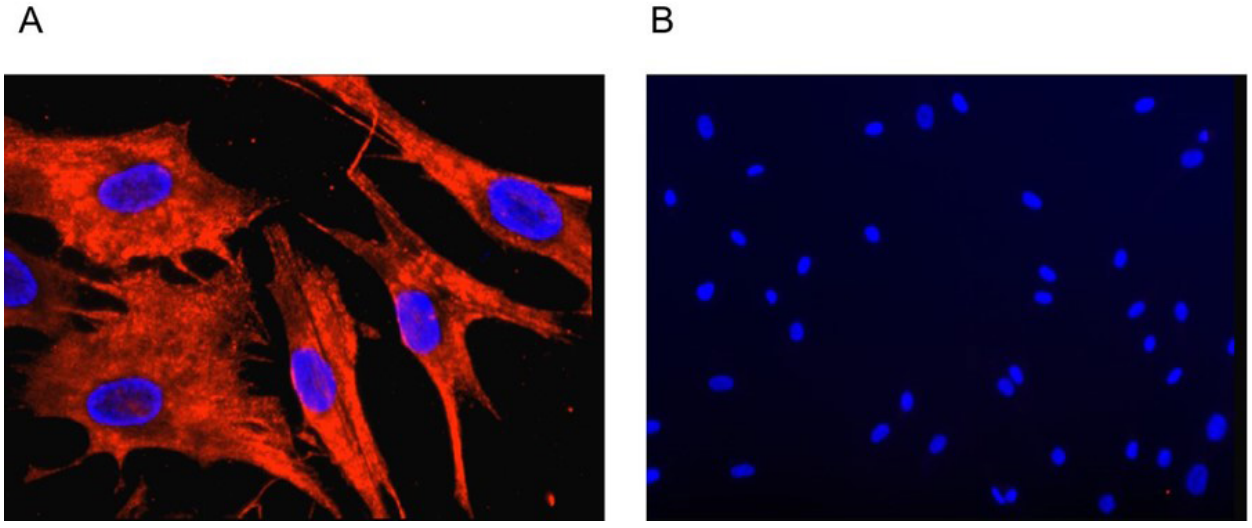

Figure 4. Cellular immunofluorescence detection of vimentin A: positive vimentin. B: the control group: negative vimentin.

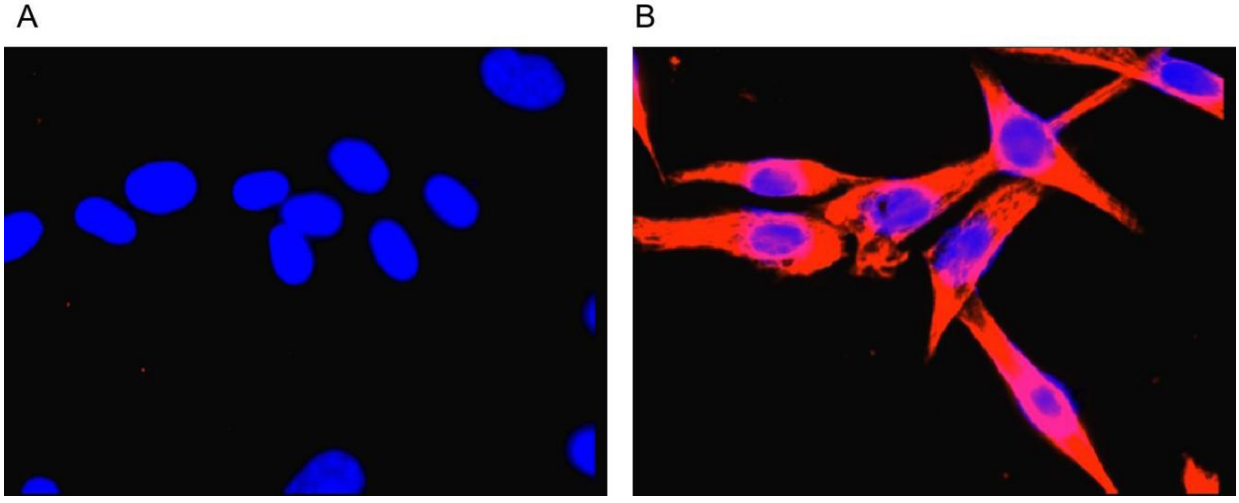

Figure 5. Cellular immunofluorescence test of desmin A: negative desmin. B: positive control for desmin adopting known striated muscle cells. 10 × 40 times.

culture solution, the culture flask should be carefully overturned to submerge the tissue mass. Fibroblasts start to grow slowly and are adherent growth type. Consequently, the culture flask should be allowed to stand for 3 days after cell seeding to avoid oscillations. 4: Porcine biliary fibroblasts cultured in *vitro* is provided with a limited lifespan. The longer the culture time is, the more unstable the cells are. Generally, signs of cell senescence like vacuoles were presented after 8-10 generations of culture. To ensure the experiment's accuracy, selection of 3-5 generations of cells of well-grown is for the experiment. 5: Strictly complying with aseptic operation to avoid contamination (Ferreira et al., 2016).

Vimentin, desmin or keratin are all cytoskeleton intermediate filaments' structural proteins. Different cells are provided with different intermediate filaments and tissue specificity, becoming relatively specific markers for the classification and identification of different types of cells (Strehl et al., 2002). Vimentin, a type of intermediate fibrin, exists in mesenchymal derived cells (like fibroblasts or vascular smooth muscle) with molecular weight of about 53KD. One end of vimentin is connected to the nuclear membrane, and the other end is linked to desmosomes or semi-desmosomes on the cell surface. Vimentin filaments primarily form a network around the nucleus, offering mechanical support to the nucleus and stabilizing its position in the cell (Shen et al., 2022). While the desmin basically lies in mature muscle cells. Adoption of vimentin and desmin was as the markers for cell identification.

In this experiment, porcine biliary fibroblasts deriving from normal biliary tract and biliary tract scar were successfully isolated, purified, cultured and identified. The cell yield obtained is large, which is a reliable technique and is available to be adopted to explore the pathogenesis of benign biliary tract stenosis in *vitro*, offering conditions for the next experiment.

Conflict of interest

Authors declared no conflict of interest.

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Not applicable.

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