Journal of Visualized Experiments

Isolation and Culture of Primary Aortic Endothelial Cells from Miniature Pigs

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59673R2
Full Title:	Isolation and Culture of Primary Aortic Endothelial Cells from Miniature Pigs
Keywords:	Miniature Pig, Porcine aortic endothelial cells, Primary cells isolation, Cell culture, CD31+ cells identification, Xenotransplantation
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Shenzhen, Guangdong, China

May 10th, 2019

Dear Editor,

Enclosed please find our revised manuscript entitled “Isolation and culture for primary aortic endothelial cells of Miniature Pig” (JoVE59673R1). We have fully addressed the reviewers’ comments and revised the manuscript, and reorganized the figures as the reviewers’ comments, and hope that our revised manuscript is now suitable for publication in *JoVE*. All major revisions to the text are highlighted in yellow. The essential steps of the protocol for the video are highlighted in green as a separate file for uploading. The following are the reorganization of figures compared to the original submission:

Figure 1: add Figure 1C and Figure 1D; Figure 2: Figure 1C to Figure 2A, Figure 2A to Figure 2B, Figure 2B to Figure 2D, Figure 2D-E to Figure 2H-I, add Figure 2C, 2E-G, delete Figure 2C.

If you have any questions, please let me know at your best convenience. Thank you for your great effort in our manuscript.

I look forward to hearing from you.
Yours sincerely,

Hanchao Gao

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TITLE:

Isolation and Culture of Primary Aortic Endothelial Cells from Miniature Pigs

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KEYWORDS:

$CD31^+$ cells, cell culture, miniature pig, porcine aortic endothelial cells, primary cell isolation, xenotransplantation

SUMMARY:

An effective enzymatic method for isolation of primary porcine aortic endothelial cells (pAECs) from miniature pigs is described. The isolated primary pAECs can be used to investigate the immune and coagulation response in xenotransplantation.

ABSTRACT:

Xenotransplantation is a promising way to resolve the shortage of human organs for patients with end-stage organ failure, and the pig is considered as a suitable organ source. Immune rejection and coagulation are two major hurdles for the success of xenotransplantation. Vascular endothelial cell (EC) injury and dysfunction are important for the development of the inflammation and coagulation responses in xenotransplantation. Thus, isolation of porcine aortic endothelial cells (pAECs) is necessary for investigating the immune rejection and coagulation responses. Here, we have developed a simple enzymatic approach for the isolation, characterization, and expansion of highly purified pAECs from miniature pigs. First, the miniature pig was anaesthetized with ketamine, and a length of aorta was excised. Second, the endothelial surface of aorta was exposed to 0.005% collagenase IV digestive solution for 15 min. Third, the endothelial surface of the aorta was scraped in only one direction with a cell scraper (<10 times), and was not compressed during the process of scraping. Finally, the isolated pAECs of Day 3, and after passage 1 and passage 2, were identified by flow cytometry with an anti-CD31 antibody. The percentages of CD31-positive cells were 97.4% ± 1.2%, 94.4% ± 1.1%, and 92.4% ± 1.7% (mean ± SD), respectively. The concentration of Collagenase IV, the digestive time, the direction, and frequency and intensity of scraping are critical for decreasing fibroblast contamination and obtaining high-purity and a large number of ECs. In conclusion, our enzymatic method is a highly-effective method for isolating ECs from the miniature pig aorta, and the cells can be expanded in vitro to investigate the immune and coagulation responses in xenotransplantation.

INTRODUCTION:

The shortage of available organs for transplantation is an outstanding problem world-wide[1]. According to the Red Cross Society of China, only a small number of patients with end-stage organ failure received a suitable organ in China in 2018.

Xenotransplantation is a promising way to resolve the problem of organ shortage. Pig organs are considered to be the most suitable organs for humans because of anatomic and physiologic similarities[2,3]. The failure of a pig xenograft is largely related to the primate immune rejection and coagulation responses. Porcine endothelial cells (ECs) are critical since these cells are the first to interact with the primate immune system, which includes antibody, complement, cytokines, and immune cells (e.g., T cells, B cells, and macrophages)[4,5]. Porcine ECs play a vital role in pig organ and islet xenotransplantation[6,7]. Thus, ECs are important cells for investigating the rejection and coagulation responses to a pig graft. Isolation of high-quality porcine ECs is required for xenotransplantation research.

In attempts to isolate ECs from different organs (e.g., heart, kidney, liver and aorta), several protocols have been reported in a xenotransplant setting[8-12]. However, keeping an ultrapure culture of isolated ECs is an outstanding problem in standard protocols. The increased concentration of the digested solution, inappropriate digestion time and scrape intensity may contribute to the increased fibroblast contamination in current studies[8,10,13]. Besides, the method of isolated pAECs from miniature pigs is less studied. Here, we describe an optimized enzymatic method to isolate highly-purified pAECs from miniature pigs (Wuzhishan or Bama).

Several steps in the protocol have been designed to reduce fibroblast contamination and obtain high-purity ECs.

PROTOCOL:

The use of animals was approved by the Ethics Review Committee of Shenzhen Second People's Hospital, in accordance with the principles of animal welfare.

1. Preparation of animals, medium, and buffers

1.1. Prepare the miniature pig.

NOTE: All miniature pigs were Chinese Wuzhishan or Bama pigs (male). The age and weight of pigs were 100 days ± 8 days and 5.7 kg ± 1.0 kg (mean ± SD, n = 3), respectively.

1.2. Prepare the culture medium: endothelial cell medium (ECM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% (v/v) penicillin/streptomycin (P/S) and 1% (v/v) endothelial cell growth supplement (ECGS).

1.3. Prepare the washing buffer: 1x PBS solution (pH 7.4) with 1% (v/v) P/S.

1.4. Prepare a 0.005% collagenase IV digestive solution: 1 mg of collagenase IV powder in 20 mL of PBS solution. Pre-warm the collagenase digestive solution at 37 °C prior to the digestion.

1.5. Prepare the stopping buffer: Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS and 1% P/S.

2. Surgery and preparation for porcine aortic endothelial cell isolation

2.1. Sterilize the operating room with UV light 1 h before performing surgery.

2.2. After anaesthetizing the miniature pig with 15 mg/kg compound ketamine (volume: 100 µL/kg) in intramuscular injection for 10−30 min (**Figure 1A**), confirm porcine anesthetization by checking porcine pain reaction and respiratory rate. Wash the porcine abdominal wall 3 times with 75% medical alcohol, and subsequently wash it once with tincture of iodine (**Table of Materials**).

2.3. Cut the abdomen with a scalpel, expose the inferior vena cava and consequently inject heparin sodium (5000 U, **Table of Materials**) into the inferior vena cava with 1 mL syringe.

2.4. Five minutes later, insert a catheter to the abdominal aorta to blood, cut the skin of chest and incise the diaphragm with a scalpel and subsequently cut the left ventricle off.

2.5. Excise the ribs with bone forceps (**Table of Materials**), and expose the heart and lungs. Find

the aorta posterior to the heart and lungs and clamp the two ends. Wash the aorta once with pre-cooled washing buffer (**Figure 1B,C**).

2.6. Cut out the aorta with scissors and keep the aorta clamped at each end. Excise excess tissue around the aorta with sterile forceps and scissors. Put the aorta into a 50 mL centrifuge tube, wash the aorta with pre-cooled washing buffer 3x (30 mL per wash), and transfer the aorta to the laboratory (**Figure 1D**).

3. Isolation of porcine aortic endothelial cells

3.1. Under aseptic conditions, remove the pig aorta from the washing buffer, and place it on a 150 mm Petri dish (**Figure 2A**).

3.2. Gently cut off the two ends of the aorta and excise excess tissue around aorta with sterile forceps and scissors again (**Figure 2B**). Wash the outside and inside of the aorta (over the culture dish at room temperature) with 20−50 mL of washing buffer.

NOTE: Try to only cut the area where the clamps were placed during surgery since some ECs were damaged in this area, and remove excess tissues and arterial side branches.

3.3. Pass a surgical suture through the aorta and then tie one end of the aorta using the surgical suture. Keep the surgical suture in the inside of the aorta (**Figure 2C,D**).

3.4. Gently fix the aorta near the tied end with forceps, and then pull the surgical suture slowly to reverse the aorta until the endothelial surface of the aorta is exposed (**Figure 2E-G**).

NOTE: Ensure to fix the aorta near the tied end and do not fix the aorta tightly, or else the aorta cannot be reversed by pulling the surgical suture.

3.5. Wash the endothelial surface of the aorta with washing buffer 3x (10 mL per wash), and then discard this solution. Place the aorta into a 15 mL centrifuge tube, and cover the aorta with 10 mL of 0.005% collagenase IV digestive solution in the tube (**Figure 2H**).

NOTE: Pre-warm the 0.005% collagenase IV digestive solution at 37 °C before digestion.

3.6. Incubate at 37 °C for 15 min. Place the digested aorta and digestive solution into a 100 mm culture dish and stop the effects of 0.005% collagenase IV by adding 10−15 mL of pre-cooled stopping buffer.

NOTE: The recommended digestion time is between 10 and 20 minutes. Make sure the endothelial surface of the aorta is covered by the stopping buffer.

3.7. Gently scrape pAECs off the inside surface of the aorta using a cell scraper (**Figure 2I**). Wash the scraped aorta 3x with washing buffer (5 mL per time). Put the solution from the culture dish

into a 50 mL centrifuge tube. Wash the bottom of the culture dish 2x with washing buffer (5 mL per time), and put the solution into a 50 mL centrifuge tube again.

NOTE: Scrape in one direction gently and do not compress. Do not touch the tissue near the edges and holes. Scraping 5−8x is recommended.

3.8. Centrifuge the tube at 600 x *g* for 6 min at 4 °C. Discard the supernatant and leave 10 mL of solution at the bottom of a 50 mL centrifuge tube. Add 20 mL of washing buffer to the 50 mL centrifuge tube, and resuspend the pellets. Avoid bubbles during this step.

3.9. Centrifuge at 600 x *g* for 6 min at 4 °C. Slowly discard the supernatant. Resuspend the cell pellets with 1 mL of culture medium and mix well.

NOTE: The obtained average number of ECs per cm aorta is $1.9 \times 10^5 \pm 1.4 \times 10^4$ (mean ± SD, n = 3).

3.10. Count the cells with a cell counter. Plate and culture the cells in a vessel without coating any materials according to the cell number. If the cell number is less than or equal to 1.0×10^6, culture cells in a 25 cm^2 flask. If the cell number is larger than 1.0×10^6, culture cells in several 25 cm^2 flasks (1.0×10^6 cells per flask). Place the flask in an incubator (without shaking) at 37 °C with 5% CO_2 and replace the medium every 2−3 days.

NOTE：Some cells are damaged by the cell scraper. A cell viability assay found the percentage of live cells to be 95.7% ± 1.7% (mean ± SD, n = 3). The doubling time of the isolated cells is about 1−2 days.

4. Harvest and characterization of pure endothelial cells

4.1. Leave the cells grow for about 4−5 days. When the cells reach 80% confluence (**Figure 3A**), digest the cells with 0.25% trypsin and passage the cells. Then, collect some of the isolated cells (Day 3, Passage 1 and Passage 2) and identify by flow cytometry (with an anti-CD31 antibody).

4.2. Label isolated pAECs (cell number: 1×10^5) (Day 3, Passage 1 and Passage 2) with anti-porcine CD31-fluorescein isothiocyanate (FITC) conjugated antibody (10 µL antibody for per 100 µL cells, stained for 30 min at 4 °C) for flow cytometry analysis.

4.3. Gate total live cell population with a forward scattered plot, unstained sample as a negative control. Then present the CD31 positive cells of the gated cells with histogram.

NOTE: The efficacy of CD31-positive cells is 97.4% ± 1.2% (Day 3), 94.4% ± 1.1% (P1) and 92.4% ± 1.7% (P2) (mean ± SD), respectively (**Figure 3B**).

REPRESENTATIVE RESULTS:

Our method aims to provide an effective way to isolate highly-purified ECs from the aortas from

miniature pigs. The process of aorta surgery is shown in **Figure 1**. The first step is that the whole aorta is excised from the pig. Preventing other cell or bacterial contamination is very important. Thus, do not injure other organs or tissues in case untargeted cells or bacteria contaminate the aorta, and wash the aorta with pre-cooled washing buffer 3x (**Figure 1B-D**). Another critical step to maintain cell viability is to minimize the period of time between obtaining the surgical specimen and the isolation procedure.

The processes involved in the purification of pAECs are shown in **Figure 2**. Our method is different from others since one end of the aorta is tied off and the endothelial surface is exposed **(Figure 2C-G).** Subsequently, the endothelial surface of the entire aorta is digested with pre-warmed collagenase digestive solution (5-10 mL) in a 15 mL centrifuge tube **(Figure 2H)**. Compared to other methods, the endothelial surface is more completely, and preferentially, exposed to the digestive solution because of the inversion of the aorta and submersion into the digestive solution (without bubbles). After the digestion is stopped with stopping buffer, the endothelial surface of the aorta must be gently scraped in only one direction with a cell scraper **(Figure 2I)**. The direction of scraping is important to avoid damage to the ECs. Do not touch the holes and cut edges of the digested aorta.

After isolation, the ECs are inspected on Days 0, 1, and 2, and after passage 1 (P1) and passage 2 (P2) **(Figure 3A)**. The isolated ECs are identified by flow cytometry using an anti-CD31-FITC antibody. Flow cytometry analysis has indicated that the percentages of CD31-positive cells are 97.4% ± 1.2% (Day3), 94.4% ± 1.1% (P1) and 92.4% ± 1.7% (P2) (mean ± SD), respectively **(Figure 3B)**. Thus, the isolation of pAECs can be successfully achieved with this protocol.

FIGURE LEGENDS:

Figure 1: The surgical procedure and excision of the aorta from a miniature pig. (**A**) Photograph of a miniature pig (Bama). (**B**) The aorta is exposed after laparotomy and thoracotomy. (**C**) The aorta is clamped at each end. (**D**) The aorta is placed in a 50 mL tube with cold washing buffer.

Figure 2: Porcine aorta digestion and pAECs isolation. (A) The aorta is placed in a 150 mm Petri dish with cold washing buffer. (**B**) Photograph of porcine aorta after removing connective tissues. (**C**) The glass bar tied with a sterile surgical suture to pass through the porcine aorta. (**D**) One end of the aorta is tied with a sterile surgical suture, which is kept on the inside of the aorta. (**E,F**) The aorta is reversed by pulling the surgical suture. (**G**) The endothelial surface of porcine aorta is exposed. (**H**) The digested aorta. (**I**) Scraping the endothelial surface of the aorta with a cell scraper.

Figure 3: Identification of highly-purified pAECs by flow cytometry with an anti-CD31 monoclonal antibody. (**A**) Representative images of isolated pAECs on Days 0, 1, and 2, and after passage 1 and passage 2 (200x magnification). (**B**) Flow cytometry analysis of pAECs (Day 3, passage 1 and 2) with anti-CD31-FITC antibody. Statistical data are presented in the bottom right. Data are representative of three independent experiments (mean ± SD).

DISCUSSION:

Endothelial cells are commonly used in research of vascular dysfunction, diabetes, tissue regeneration, transplantation, and cancers[14-18]. To understand and characterize the biology and function of ECs in these diseases, numerous methods to isolate the ECs of different diseased organs or tissues have been reported[8,19-24]. Recently, an increasing number of reports have demonstrated that porcine ECs have various functions in immune rejection and coagulation of xenotransplantation[6,25]. However, the isolation of highly-purified aortic endothelial cells from miniature pigs has been reported less frequently. Here, we describe a stable and easy method for the isolation of aortic endothelial cells from miniature pigs.

Collagenase can degrade different tissues, and is superior to trypsin or other digestive solutions[26-29]. We used 0.005% collagenase IV to dissociate pAECs from a small pig aorta. Importantly, the concentration of the digestive solution should be optimized to different pigs. Collagenase digestive solutions at 0.025%, 0.05% and 0.2% have been used respectively in different pigs, according to age and breed[10,13,30]. Here, we recommend the optimal concentration of collagenase IV to be 0.005%, and the optimal digestive time to be 15 min. The time should not be <10 min or >20 min, which is consistent with previous studies[8,30]. The concentration of collagenase IV and the digestive time are critical for obtaining high-purity and large numbers of ECs. Lower concentrations of collagenase IV or shorter digestive times will result in fewer ECs. Higher concentrations of collagenase IV or longer digestive times will lead to more fibroblast contamination.

Cell damage and fibroblast contamination are two problems in the isolation of pAECs. In order to reduce cell damage and fibroblast contamination, we adopted a method in which the ECs were scraped gently with a cell scraper. The direction, frequency, and intensity of scraping are critical to prevent cell damage and fibroblast contamination. First, we scraped the endothelial surface of the aorta in one direction only. Second, we recommend that the scraping frequency be less than 10 times (5 to 8 times is recommended), and the intensity must be gentle. Finally, the areas surrounding the holes of arterial side branches and the cut edges of the aorta (which might lead to fibroblast cells and more damage cells) should not be scraped.

A higher collagenase concentration, longer digestion time, and increased frequency and intensity of scraping may increase fibroblast contamination. Fibroblasts may rapidly outgrow the CD31-positive cells, thus reducing the purity of the isolated ECs[31]. Comparing to existing protocols, although the protocol has been carefully designed to prevent fibroblast contamination, and the CD31-positive cells on Day 3 reached 97.4% ± 1.2% (mean ± SD), the percentage of CD31 positive cells slowly decreased following culture. If the isolated ECs are used to carry out experiments after 5 passages, we suggest that the cells should be sorted with a flow cytometry cell sorter[10].

Usually, we not only isolate the endothelial cells, but also get other organs for xenotransplantation research, such as the pancreas and kidney. According to our experience, it would better to isolate the pancreas and lung first, and then perform the procurement of liver

and kidney. Even after that it is not too late to isolate the heart and aorta, if the surgeon is quick enough to finish all of the isolation in less than half an hour. During the process, it is very critical to keep the surgical area sterile and cold. The cold PBS with antibiotics was poured to the target organ to clean the possible contamination and to keep the tissues in low temperature. It is also necessary to prevent the coagulation by injecting heparin after anesthesia of animal. The clot in the micro vascular vessel would induce cell death and inflammation of organs with more capillaries, such as the pancreas, the lung and the liver. We always inject heparin to the inferior vena cava and insert a catheter to the abdominal aorta to blood. This will effectively prevent coagulation related cell death.

In conclusion, we provide an effective method to isolate highly-purified pAECs from miniature pigs. The isolated pAECs are beneficial for xenotransplantation research. Although we have not isolated the pAECs from a large pig using the protocol, we believe we will obtain highly purified ECs from a large pig with the protocol by modifying some critical steps.

ACKNOWLEDGMENTS:

The work was supported by grants from Natural Science Foundation of Guangdong Province, Grant/Award Number: 2016A030313028; Medical Scientific Research Foundation of Guangdong Province, Grant/Award Number: B2018003; Shenzhen Foundation of Science and Technology, Grant/Award Number: JCYJ20160425103000011, JCYJ20160428142040945; Shenzhen Longhua District Foundation of Science and Technology, Grant/Award Number: 2017013; National Key R&D Program of China, Key R&D Program of China, Grant/Award Number: 2017YFC1103704; Sanming Project of Medicine in Shenzhen, Grant/Award Number: SZSM201412020. We thank Hancheng Zhang and Zhicheng Zou from Shenzhen University for assisting in the preparation of the manuscript.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

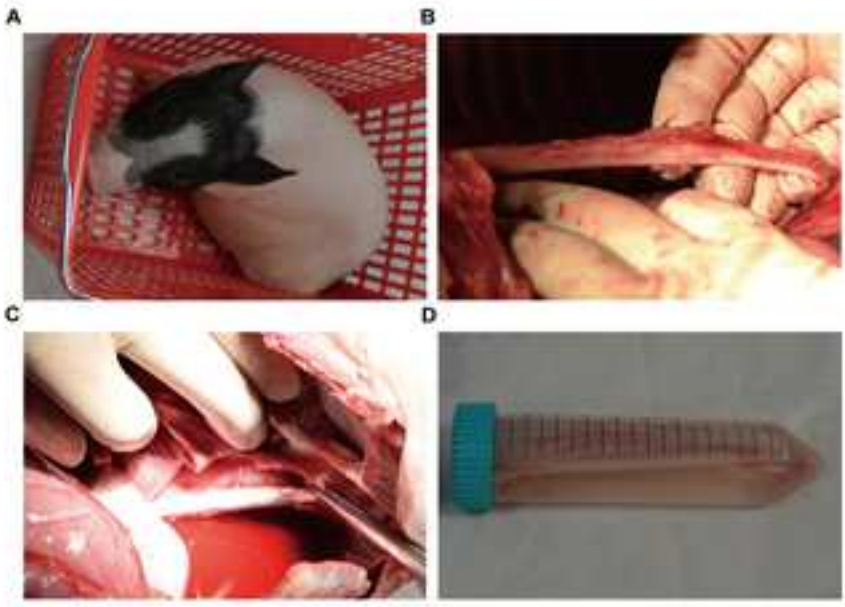
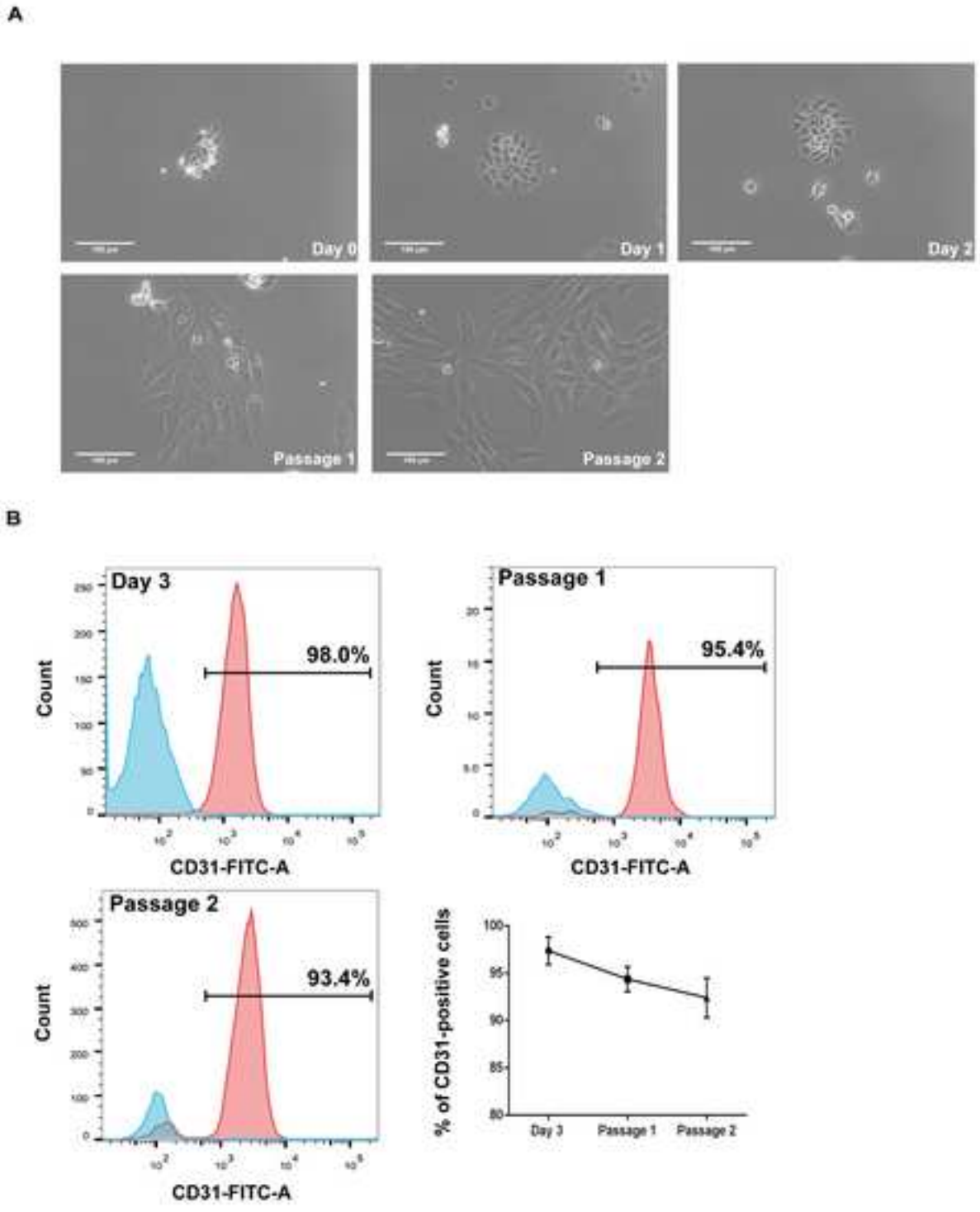


Figure 2

Figure 3

Figure 3



Name of Material/Equipment	Company	Catalog Number
20 x PBS solution (pH 7.4,Nuclease free)	Sangon Biotech	B540627#
75% Medical alcohol	Guilin LiFeng Medical Supplies Co.,Ltd.China	
BD FACSAria II	BD Bioscience	
Boneforceps	Beijing HeLi KeChuang Technology Development CO.,Ltd. China	HL-YGQ
BOON Disposable Syringe (10ml)	Jiangsu Yile medical Article Co., Ltd. China	
CD31-FITC antibody	Bio-Rad	MCA1746F
Cell scraper	Corning	3010#
Collagenase IV	Sigma-Aldrich	C5138#-1G
Compound ketamine injection	Veterinary Pharmaceutical Factory of Shenyang, China	
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	Life Technologies	D1306#
DMEM	Life Technologies	11965118#
ECGS	Sciencell	1052#
ECM	Sciencell	1001#
Eppendorf Snap-Cap Microtube(1.5mL)	AXYGEN	MCT-150-C#
Falcon 100mm Cell Culture Dish	Corning	353003#
Fetal Bovine Serum	GIBCO	10270-106#
Flowjo v10.0		
Forceps	ShangHai medical instruments Co.,Ltd.China	
Heparin sodium	Jiangsu WanBang biopharmaceutical Co.,Ltd.China	
Iodine tincture	Guilin LiFeng Medical Supplies Co.,Ltd.China	
Miniature Pig (Bama or Wuzhishan)	Kang Yi Ecological Agriculture Co., Ltd, China	
Mshot microscope	Guangzhou Micro-shot Technology Co., Ltd.	M152
Penicillin/Streptomycin	Life Technologies	15070063#
Petri Dishes (150 x 15 mm)	Biologixgroup	66-1515#
Rectangular Canted Neck Cell Culture Flask with Vent Cap （T25）	Corning	3289#
Scissors	ShangHai medical instruments Co.,Ltd.China	

Serological Transfer Pipettes (10ml)	JET Biofil	GSP010010#
Sterile Pasteur Pipette	GeneBrick	GY0025#
Sterile Syringe Filter (0.22μm)	Millipore	SLGV033RS#
Surgical scalpel	ShangHai medical instruments Co.,Ltd.China	22#
Syringe（1mL）	Shengguang Medical Instrument Co., Ltd.China	
Trypsin-EDTA (0.25%), phenol red	GIBCO	25200056#

Comments/Description

Ketamine Hydrochloride：0.3g/2ml,Xylazine hydrochloride:0.3g/2ml, Phenacetin hydrochloride：1mg/2ml

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Title of Article:	Isolation and culture of primary aortic endothelial cells from miniature pigs
Author(s):	Yanli Zhao, Chengjiang Zhao, David K.C. Cooper, Ying Lu, Kewang Luo, Huiyun Wang, Pengfei Chen, Changchun Zeng, Shaodong Luan, Lisha Mou, Hanchao Gao

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Date:	2018.01.09

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May 10th, 2019

Dear Editor，

Enclosed please find our revised manuscript entitled “Isolation and culture for primary aortic endothelial cells of Miniature Pig” (JoVE59673R1). We thank the reviewers for the important and insightful comments. We appreciate the opportunity you offered us to revise our manuscript. We have now carefully revised the manuscript according to all the comments of the referees, and hope that our revised manuscript is now fully acceptable to you for publication in ‘*JoVE*’. All major revisions to the text are highlighted in yellow. The essential steps of the protocol for the video are highlighted in green as a separate file for uploading.

Our point-by-point responses are listed below.

Sincerely,

Hanchao Gao

Editorial comments:

Changes to be made by the author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: We have carefully checked spelling and grammar in the revised manuscript.

2. Introduction: Please expand to include the advantages of the presented method over alternative techniques with applicable references to previous studies, description of the context of the technique in the wider body of literature and information that can help readers to determine if the method is appropriate for their application.

Response: Yes. It is a good suggestion for our manuscript. We have added the content in the revised manuscript. (Page 4, lines 95-99)

"However, to keep an ultra pure culture of isolated ECs is an outstanding problem in standard protocols. The higher concentration of digested solution, inappropriate digested time and scraped intensity may contribute to the increased fibroblast contamination in the present studies.[1-3] Besides, the method of isolated pAECs from miniature pigs is less studied."

3. Section 1: Please list approximate volumes for all buffers and stock solutions to be set up.

Response: Yes, we have revised the manuscript as suggested.

4. 2.2: Please mention how proper anesthetization is confirmed.

Response: After compound ketamine injection for 10-30 mins, confirm porcine anesthetization with checking porcine pain reaction and respiratory rate. We have

specifically demonstrated compound ketamine in Table of Materials and revised the sentence of Section 2 as the following.

“2.2 After anaesthetizing the miniature pig with 15 mg/kg compound ketamine (Volumes: 100μL /kg) in intramuscular injection for 10-30 mins (see Table of Materials) (Figure 1A), confirm porcine anesthetization with checking porcine pain reaction and respiratory rate.” (Page 5, lines 135-137)

5. Section 4: Please describe the gating strategies. Please also specify the temperature at which the staining is done. Also please split this step into two or three sub-steps so that each individual step contains only 2-3 actions.

Response: Yes. It is a good suggestion. We have revised the manuscript. (Page 8, lines 210-224).

“4. Harvest and Characterization of Pure Endothelial Cells

4.1 Leave the cells grow for about 4-5 days. When the cells reach 80% confluence (Figure 3A), the cells were then digested with 0.25% trypsin and passaged. Then, some of the isolated cells (Day 3, Passage 1 and Passage 2) were collected and identified by flow cytometry (with an anti-CD31 antibody).

4.2 Label isolated pAECs (Cell number: $1x10^5$) (Day 3, Passage 1 and Passage 2) with anti-porcine CD31-fluorescein isothiocyanate (FITC) conjugated antibody (10 μL antibody for per 100 μL cells, stained for 30min at 4℃) for flow cytometry analysis.

4.3 Gate total live cell population with a forward scattered plot, unstained sample as a negative control. And then the CD31 positive cells of the gated cells were presented with histogram. The efficacy of CD31-positive cells is 97.4% ± 1.2% (Day3), 94.4% ± 1.1% (P1) and 92.4% ± 1.7% (P2) (mean ± SD), respectively (Figure 3B).”

6. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

Response: We have revised the manuscript (Section 2 and Section 3) as suggested. (Pages 5-8, lines 131-208)

7. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Response: We have highlighted the essential steps in green and we will upload the manuscript as a separate file.

8. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia.

Response: We have revised the filmed manuscript as requested.

9. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Response: We have revised the filmed manuscript as suggested.

10. Table of Materials: Please remove trademark (™), registered (®) and copyright symbols.

Response: We have revised the Table of Materials as requested.

References:

1 Beigi, F. *et al.* Optimized method for isolating highly purified and functional porcine aortic endothelial and smooth muscle cells. *Journal of cellular physiology.* **232** (11), 3139-3145, (2017).

2 Bernardini, C. *et al.* Heat shock protein 70, heat shock protein 32, and vascular endothelial growth factor production and their effects on lipopolysaccharide-induced apoptosis in porcine aortic endothelial cells. *Cell stress & chaperones.* **10** (4), 340-348, (2005).

3 Zhang, J. *et al.* Potential Antigens Involved in Delayed Xenograft Rejection in a Ggta1/Cmah Dko Pig-to-Monkey Model. *Scientific reports.* **7** (1), 10024, (2017).

Reviewers' comments:

Reviewer #3:

Manuscript Summary:

The manuscript clearly describes an optimised procedure for the isolation of endothelial cells from porcine aorta. The authors highlighted the critical points of the protocol to reduce fibroblast contamination consisting in the right concentration of the Collagenase IV, the digestive time, the direction, frequency and intensity of the scraping. The manuscript might be useful for the scientific community working with primary endothelial cells from large vessels.

Major Concerns:
N/A

Minor Concerns:
Line 225: Please specify whether the flasks are coated (fibronectin, collagen or gelatin) before cell seeding.

Response: The flasks are not coated before cell seeding in the protocol. We have added the content in the revised manuscript. (Page 8, lines 201-202)

Reviewer #4:

Manuscript Summary:
The authors clearly outline the protocol that they utilize to isolate endothelial cells and prevent fibroblast contamination. In particular, their discussion of inverting the aorta to preferentially expose the endothelium to the collangenase is an insightful recommendation. Furthermore, they nicely demonstrate a high level of success from the utilizing this method.

Major Concerns:

A few points of clarification that would be beneficial:

1) the method that they outline focuses on using animals solely for the isolation of the endothelial cells. Since the animals utilized for xenotransplantation often include the removal of multiple organs in addition to the isolation of the aorta, it would be beneficial for them to include a small discussion about how they perform their aorta procurement with simultaneous organ procurements.

Response: We have added a small discussion in the revised manuscript. (Page 12, lines 330-341)

"Usually, we not only isolate the endothelial cells, but also get other organs for xenotransplantation research, such as the pancreas and kidney. According to our experience, it would better isolate the pancreas and lung first, and then perform the procurement of liver and kidney. Even after that it is not too late to isolate the heart and aorta, if the surgeon is quick enough to finish all the isolation in less than half an hour. During the process, it is very critical to keep surgical area sterile and cold. The cold PBS with antibiotics was poured to the target organ to clean the possible contamination and to keep the tissues in low temperature. It is also necessary to prevent the coagulation by injecting the heparin after the anesthesia of animal. The clot in micro vascular vessel would induce the cell death and inflammation of organ with more capillaries, such as pancreas, lung and liver. We always inject the heparin to inferior vena cava and insert a catheter to the abdominal aorta to blood. This will effectively prevent the coagulation related cell death."

2) They highlight several steps that they feel are critical to optimal isolation of the

endothelial cells, such as scraping the endothelial surface in a single direction, avoiding scraping over side branches, length of time of exposure to collagenase, etc., but do not provide any data to support these claims.

Response: It is a good suggestion for our manuscript. We have isolated the PAECs for many times and found that scraping the endothelial surface in a single direction will decrease the cell death. Besides, we also found that avoiding scraping over side branches can prevent fibroblast contamination since the area surrounding side branches would have many untargeted cells. In fact, we also tried different digested time and the concentration of collagenase and we found the indicated time and concentration of collagenase in the present protocol was better to prevent the fibroblast contamination and obtain more PAECs. We obtained these valuable experiences from many times isolation.

3) In the 'Representative Results' section, the authors state that the 'aortic digestion is more complete since the endothelial surface of the aorta is filled with pre-warmed digestive solution'. This is mildly confusing since the protocol does not contain a step where the aorta is filled with the digestive solution at any point. It should state that the endothelial surface is more completely, and preferentially, exposed to the digestive solution because of the inversion of the aorta and submersion into the digestive solution.

Response: Yes. It is great helpful for our manuscript. We have revised the manuscript as suggested. (Page 9, lines 247-249)

4) The concentration stated for the endothelial cell growth supplement (ECGS) should be more clearly stated. It is not listed in the Table of Materials and is stated

as 1% (vol/vol) in the text of the protocol. The final concentration should be listed as mass/volume for this reagent.

Response: We have added ECGS in the Table of Materials. ECGS which was purchased from Sciencell (1052#) was lipid but not solid, so we stated the reagent as volume/volume.

Reviewer #5:

Manuscript Summary:
in the manuscript a method for isolation of primary porcine aortic endothelial cells from miniature pigs is described.

Major Concerns:
The description of some details should be implemented
1 Surgery and Preparation for Porcine Aortic Endothelial Cell Isolation.
Specify if the animals are alive or not. If not, the euthanasia should be described. Was any kind of analgesia used?

Response: Yes. It is a good suggestion for our manuscript. The anaesthetized pig was sacrificed by cutting left ventricle off before the procurement of porcine aorta. And Xylazine hydrochloride as an analgesia drug has been used in this process since the compound ketamine injection contains Xylazine hydrochloride.

We have revised step 2.4 as requested. (Page 6, lines 144-145)

“2.4 Five minutes later, insert a catheter to the abdominal aorta to blood, cut the skin of chest and incise the diaphragm with a scalpel and subsequently cut left ventricle off.”

Lines 143, 144. Cut the abdomen with a scalpel, expose the inferior vena cava and consequently inject heparin sodium (5000U, see Table of Materials) into the inferior vena cava. By measn of what?

Response: We injected heparin sodium into the inferior vena cava with 1mL syringe. We have revised the manuscript. (Page 6, line 142)

Lines 151, 152. Find the aorta posterior to the heart and lungs, and clamp the two ends. Wash the aorta with pre-cooled washing buffer x1 (Figure 1B). Shouldn't it be Fig. 1 C? May be an additional fig should be added

Response: Yes. We have added an additional fig (Figure 1C) in the revised Figure 1. And the continuous actions will be showed in the video. (Page 6, line 149)

Lines 173, 174. Pass through the aorta with a surgical suture and then tie one end of the aorta using the surgical suture, and keep the surgical suture in the inside of the aorta (Figure 2B). An additional figure should be added.

Response: We tied a surgical suture to the glass bar and consequently pass through the aorta. The fig (Figure 2C) has been added in the revised Figure 2, and the continuous action will be showed in the video. (Page 6, line 168)

Lines 176, 177. Gently fix the aorta near the tied end with forceps, and then pull

the surgical suture slowly to reverse the aorta until the endothelial surface of the aorta is exposed (Figure 2C). An additional figure should be added.

Response: We have added some additional figures (Figure 2E, Figure 2F and Figure 2G) in the revised Figure 2, and the continuous action will be showed in the video. (Page 6, line 171)

Lines 184, 185. Place the aorta into a 15 mL Falcon tube, which is filled with 0.005% Collagenase IV digestive solution (Figure 2D). Should the aorta be covered with solution? specify

Response: Yes. We have revised it as the following. (Page 7, lines 176-177)

"3.5 Wash the endothelial surface of the aorta with washing buffer x3 (10 mL per time), and then discard this solution. Place the aorta into a 15 mL Falcon tube, and cover the aorta with 0.005% Collagenase IV digestive solution in the tube (Volumes: 10mL) (Figure 2H)."

Line 235. When the cells reach 80% confluence (about 4-5 days) and passage the cells.. the sentence is not clear. Which is the cost of the entire process?

Response: We have revised Step 4.1 as the following (Page 8, lines 212-215).

"4.1 Leave the cells grow for about 4-5 days. When the cells reach 80% confluence (Figure 3A), the cells were then digested with 0.25% trypsin and passaged. Then, some of the isolated cells (Day 3, Passage 1 and Passage 2) were collected and identified by flow cytometry (with an anti-CD31 antibody)."

Minor Concerns:

The English should be checked

Response: Yes. We have carefully checked the English in the revised manuscript