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Vascular Smooth Muscle Cells Derived from Inbred Swine Induced Pluripotent Stem Cells for Vascular Tissue Engineering

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Competing interests: L.E.N. is a founder and shareholder in Humacyte, Inc., which is a regenerative medicine company. Humacyte produces engineered blood vessels from allogeneic smooth muscle cells for vascular surgery. L.E.N.'s spouse has equity in Humacyte, and L.E.N. serves on Humacyte's Board of Directors. L.E.N. is an inventor on patents that are licensed to Humacyte and that produce royalties for L.E.N.. L.E.N. has received an unrestricted research gift to support research in her laboratory at Yale. Humacyte did not fund these studies, and Humacyte did not influence the conduct, description or interpretation of the findings in this report.

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Abstract

Development of autologous tissue-engineered vascular constructs using vascular smooth muscle cells (VSMCs) derived from human induced pluripotent stem cells (iPSCs) holds great potential in treating patients with vascular disease. However, preclinical, large animal iPSC-based cellular and tissue models are required to evaluate safety and efficacy prior to clinical application. Herein, swine iPSC (siPSC) lines were established by introducing doxycycline-inducible reprogramming factors into fetal fibroblasts from a line of inbred Massachusetts General Hospital miniature swine that accept tissue and organ transplants without immunosuppression within the line. Highly enriched, functional VSMCs were derived from siPSCs based on addition of ascorbic acid and inactivation of reprogramming factor via doxycycline withdrawal. Moreover, siPSC-VSMCs seeded onto biodegradable polyglycolic acid (PGA) scaffolds readily formed vascular tissues, which were implanted subcutaneously into immunodeficient mice and showed further maturation revealed by expression of the mature VSMC marker, smooth muscle myosin heavy chain. Finally, using a robust cellular self-assembly approach, we developed 3D scaffold-free tissue rings from siPSC-VSMCs that showed comparable mechanical properties and contractile function to those developed from swine primary VSMCs. These engineered vascular constructs, prepared from doxycycline-inducible inbred siPSCs, offer new opportunities for preclinical investigation of autologous human iPSC-based vascular tissues for patient treatment.

Keywords

Tissue engineering; vascular tissue; induced pluripotent stem cell; smooth muscle cell; inbred swine

Introduction

Every year millions of patients with cardiovascular disease require vascular constructs for bypass surgery or replacement of defective blood vessels in the United States [1–3]. Tissue-engineered vascular constructs grown using vascular smooth muscle cells (VSMCs) isolated from primary tissue hold great potential as tools for surgical replacement of the affected vessels in these patients [4–6]. However, the development of engineered vascular constructs for clinical application using primary VSMCs has been hampered by limited accessibility to patient VSMCs, finite expandability of primary VSMCs, and functional disparities between VSMCs derived from different donors [5–7].

Human induced pluripotent stem cells (iPSCs) can be derived from patients' own somatic cells by ectopic expression of stem cell factors, self-renew, and differentiate into virtually every cell type in the human body including functional VSMCs, thereby providing an unlimited cell source for generating autologous vascular constructs for disease treatment [8–13]. Human iPSC-based engineered vascular constructs may be of particular importance to patients with dysfunctional vascular cells due to diseases or aging. In such patients,

allogeneic, decellularized vascular grafts might not be an optimal treatment option since patients' own defective vascular cells might not effectively remodel the implanted grafts into functional vessels. Our recent studies revealed that human iPSC-based engineered vascular constructs remained intact and were patterned with active vascular remodeling after implantation into rat abdominal aorta, setting the stage for developing iPSC vascular tissue constructs for potential clinical use in the future [13]. However, before therapeutic application of autologous, patient-specific iPSC vascular tissue constructs, it is necessary to establish an autologous, large animal iPSC model to evaluate the safety and efficacy of these iPSC vascular constructs.

Preclinical evaluation of human iPSC-based vascular tissue constructs using a non-human primate model may provide useful information. However, such allogeneic, human to non-human primate implantation requires sustained immunosuppression, and does not mimic clinical application of autologous, patient-specific iPSC-based vascular constructs. Additionally, the non-human primate model may also be limited by significant economic burden and potential challenges of ethical issues. In contrast, swine are an excellent preclinical model for developing therapeutic applications due to their similarity to human physiology and organ size, affordability, and considerably lesser level of ethical concerns [14–16].

Generation of swine iPSCs (siPSCs) has been reported [15, 17, 18]. However, the continual expression of ectopic reprogramming factors in cells derived from these siPSCs may inhibit cellular differentiation and maturation and increase the risk of tumorigenesis. Additionally, siPSCs generated from swine somatic cells with outbred origin would require sustained immunosuppression when siPSC-derived tissue constructs are implanted *in vivo*, potentially confounding the interpretation of research results. Thus, an inbred siPSC line based on an inducible expression of reprogramming factors is needed to evaluate the safety and efficacy of iPSC-derived therapies.

In this study, we have established the first doxycycline-inducible siPSC lines from fetal fibroblast cells of the Massachusetts General Hospital (MGH) miniature swine that are extensively inbred and therefore accept tissue and organ transplants without immunosuppression [19]. Our results revealed that the use of a doxycycline-inducible approach greatly reduced the adverse effects caused by reprogramming transgenes, since removal of doxycycline during differentiation resulted in elimination of transgene expression and enhancement of differentiation into the three germ lineages. Functional VSMCs differentiated from inbred siPSCs can be utilized to develop vascular tissues for cell-based therapies in a preclinical swine model, which helps to derive fundamental knowledge for clinical application of autologous iPSC-derived VSMC tissue constructs in patients with vascular disease (Supplementary Fig. 1). However, an efficient approach to derive functional siPSC-VSMCs is lacking in the field. Based on the enhanced lineage differentiation via doxycycline withdrawal in combination of the addition of ascorbic acid, we developed a robust approach to derive functional VSMCs from siPSCs, and then generated for the first time swine vascular tissue constructs by either seeding siPSC-VSMCs onto biodegradable polyglycolic acid (PGA) mesh or allowing siPSC-VSMCs to self-assemble into tissue rings in agarose molds. Our studies have laid the groundwork for inbred siPSC-based tissue

engineering and implantation studies, which closely resemble patient-specific, autologous iPSC-based therapeutic applications. It is anticipated that the knowledge derived from inbred doxycycline-inducible siPSC model would be readily utilized to guide autologous, patient iPSC-based tissue engineering and therapeutic intervention during human clinical trials (Supplementary Fig. 1), thus providing the field of regenerative medicine with a powerful tool for preclinical investigations.

2. Materials and methods

2.1. Animal use

The study was approved by the Yale University Institutional Animal Care and Use Committee. All animal care complied with the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Culture of primary inbred swine embryonic fibroblasts

Swine embryonic fibroblasts (SEFs) (isolated from inbred miniature swine at generation eleven of inbreeding) were provided by Drs. David Sachs and Robert Hawley, Transplantation Biology Research Center, Massachusetts General Hospital, Harvard Medical School, Cambridge, MA [19]. SEFs were cultured in fibroblast medium (Dulbecco's Modified Eagle Medium (DMEM; high glucose, ThermoFisher) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), 2 mM L-glutamine (ThermoFisher), 1% (v/v) non-essential amino acid (NEAA, ThermoFisher), 1 mM sodium pyruvate (ThermoFisher) and 1% (v/v) penicillin/streptomycin (pen/strep, ThermoFisher)). Medium was changed daily, and the SEFs were routinely passaged at 80% confluence every 4 or 5 days.

2.3. Derivation and maintenance of inbred doxycycline-inducible siPSCs

The plasmid vectors including the Tet-hSTEMCCA-loxP polycistronic cassette encoding human OCT4, SOX2, KLF4 and c-MYC (hSTEMCCA) and the construct encoding the reverse transcription tetracycline activator (rtTA) were provided by Dr. Darrell Kotton, Center for Regenerative Medicine (CRoM) of Boston University and Boston Medical Center, Boston, MA. The hSTEMCCA and rtTA vectors were packaged into lentiviral particles and collected as previously described [10]. To generate siPSCs, 8,000 SEFs/cm² (below passage 3) were seeded onto 6-well dish two days prior to viral infection (day -2). On day 0, fibroblast medium was replaced with medium containing lentiviral particles (1 mL fresh fibroblast medium mixed with 1.4 mL viral supernatant containing hSTEMCCA and 0.6 mL supernatant containing rtTA, plus 5 µg/mL polybrene (SigmaAldrich)). Viral infection was subsequently repeated at 12 hours, 24 hours and 36 hours after the first infection. 48 hours after the first infection, medium was switched to pluripotency-promoting medium (Knockout DMEM (ThermoFisher), 10% Knockout Serum Replacement (KOSR, ThermoFisher), 10% (v/v) FBS, 20 ng/mL human leukemia growth factor (LIF, Peprotech), 20 ng/mL human basic fibroblast growth factor (bFGF, SigmaAldrich), 2 µg/mL doxycycline (Stemgent), 1% (v/v) NEAA, 1% (v/v) pen/strep, 2 mM L-glutamine and 0.1 mM β-mercaptoethanol (SigmaAldrich)) for 48 hours. Then the infected SEFs were dissociated on day 4 with 0.05% trypsin EDTA (SigmaAldrich) and seeded onto irradiated mouse embryonic fibroblasts (MEFs, 40,000 cells/cm² as feeder layer) at 4,000 cells/cm², and cultured in siPSC medium

(Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, ThermoFisher), 20% (v/v) KOSR, 20 ng/mL LIF, 20 ng/mL bFGF, 2 µg/mL doxycycline, 2 mM L-glutamine, 1% (v/v) NEAA, 1% (v/v) pen/strep and 0.1 mM β-mercaptoethanol). The medium was changed daily. Fire-polished Pasteur pipettes were used to dissociate desired colonies. The dissociated colonies were subcultured onto new MEF feeder in siPSC medium. When colonies reached 80% confluency, siPSCs were treated with 1mg/mL dispase (ThermoFisher), dissociated into small clusters by pipetting, and subcultured. Note that inbred siPSCs were cultured in the continual presence of 2 µg/mL doxycycline, which allows persistent expression of ectopic reprogramming factors to support pluripotency.

2.4. Culture of primary swine vascular smooth muscle cells, human umbilical vein endothelial cells and HEK293T cells

Primary swine aortic vascular smooth muscle cells (VSMCs) and human umbilical vein endothelial cells (HUVECs) were provided by Dr. Laura Niklason, Department of Biomedical Engineering, Department of Anesthesiology, Yale University, New Haven, CT [20]. Primary swine VSMCs within passage five were maintained in smooth muscle growth medium-2 (SmGM-2, Lonza), and the medium was replenished every other day. HUVECs within passage five were cultured in VasculLife VEGF Endothelial Medium (Lifeline Cell Technology), and the medium was changed every other day.

Human embryonic kidney 293T cells (HEK293T) were cultured in medium containing DMEM (high glucose) supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 1% (v/v) NEAA, 1 mM sodium pyruvate and 1% (v/v) pen/strep. Medium was changed every other day.

2.5. Spontaneous differentiation of doxycycline-inducible siPSCs in vitro

siPSC colonies were treated with dispase, collected and further dissociated into single cells by accutase (SigmaAldrich). The collected siPSCs were subjected to floating culture with siPSC medium containing 5 µM ROCK inhibitor (Calbiochem) in low attachment 6-well plate (Corning). 24 hours later (day 1), small embryoid bodies (EBs) were formed. Then siPSC medium was changed to a mixture of siPSC medium with EB medium (DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1% (v/v) NEAA, 1% (v/v) pen/strep, and 0.012 mM β-mercaptoethanol) in 1:2 (volume) for 24 hours. From day 2 100% EB medium was used and changed daily. On day 6, EBs were transferred to 0.1% gelatin (SigmaAldrich)-coated culture dish. EBs attached overnight and started cell outgrowth. Attached cells were cultured in EB medium with daily medium change until day 12.

2.6. VSMC differentiation of doxycycline-inducible siPSCs

Spontaneous differentiation of siPSCs was completed via EB formation for 12 days as described above. The attached cells were dissociated with 0.05% trypsin EDTA, re-seeded on culture plates coated with growth factor-reduced Matrigel (Corning, 1:120 diluted in DMEM) at 50,000 cell/cm², and cultured with VSMC growth medium (SmGM-2 (Lonza, U.S.A.) plus 50 µg/mL of ascorbic acid (SigmaAldrich)) until day 19. The medium was changed every other day. To induce maturation, siPSC-VSMCs were subcultured on day 19 with VSMC maturation medium (DMEM supplemented with 5% (v/v) FBS, 2 mM L-

glutamine, 1% (v/v) NEAA, 1% (v/v) pen/strep, and 0.012 mM β -mercaptoethanol) for 7 days. The maturation medium was changed every other day.

2.7. Alkaline phosphatase activity assay and immunostaining

Alkaline phosphatase activity was evaluated by using alkaline phosphatase staining kit II (Stemgent) according to the manufacturer's instructions. To conduct immunostaining, cells were fixed with 4% paraformaldehyde (PFA, Electron Microscopy Sciences) and blocked by 10% goat serum (ThermoFisher) in PBST buffer (Dulbecco's Phosphate-Buffered Saline (PBS, ThermoFisher) with 0.1% triton X-100 (SigmaAldrich)) for 30 minutes at room temperature. Cells were then incubated with primary antibody in PBST containing 1% goat serum at 4°C overnight. Cells were then washed and incubated with secondary antibody (1:1000 in PBST with 1% goat serum) for one hour at room temperature and washes with PBS. Nuclei were counterstained with DAPI (ThermoFisher). Stained samples were analyzed using a fluorescent microscope (Leica). Primary and secondary antibodies were listed in Supplementary Table 1. Alkaline phosphatase activity and immunostaining assays were repeated for at least three times for each experiment.

2.8. Karyotyping analysis

As previously described, SEFs or siPSCs were cultured on gelatin or Matrigel-coated cover slips and sent to the laboratory of Yale Cytogenetic Service [10]. Karyotyping analysis was completed via standard G-banding chromosome analysis according to the standard procedures. Ten cells were randomly selected and analyzed.

2.9. Teratoma formation assay

Teratoma formation assay was performed as previously described [10]. Briefly, undifferentiated siPSCs were cultured on MEFs for 4–5 days and dissociated. 1–5 million siPSCs were injected subcutaneously or intramuscularly into NOD/SCID or Rag2/Il2rg double knockout mice (Taconic). The injected mice were observed for at most three months for the tumor formation.

2.10. RNA extraction, reverse transcription and quantitative polymerase chain reaction (qRT-PCR)

RNA extraction and reverse transcription were completed using NucleoSpin RNA XS Total RNA Isolation Kit (Macherey-Nagel) and iScript cDNA synthesis Kit (Bio-Rad), respectively, following the manufacturer's instruction. Quantitative polymerase chain reaction was performed using Bio-Rad IQ SYBR green supermix (Bio-Rad) according to the manufacturer's instructions. Expression of candidate genes was normalized to swine GAPDH. Three biological replicates were completed for expression analysis of each gene. Primer sequences are listed in Supplementary Table 2.

2.11. Flow cytometry analysis

Single cell suspensions were fixed with 4% PFA for 10 minutes at room temperature (R.T.) and then blocked with 5% BSA in PBST for 30 minutes at R.T.. Samples were next incubated with 1 μ g/mL primary antibody overnight at 4°C. Mouse and rabbit IgG isotype

controls (Thermo Scientific) were used for flow cytometry. On the next day, samples were incubated with secondary antibody (1:1000) for 60-min at R.T.. Cell fluorescence was measured by LSRII flow cytometer (BD biosciences) and analyzed by Flow-Jo software (Treestar). Three biological replicates were completed for analysis of each gene.

2.12. Contractility assay

siPSC-VSMCs or swine primary VSMCs in growth medium or maturation medium were treated with 1 mM carbachol (Abcam) or 80 mM KCl (SigmaAldrich) or PBS as control for 30 minutes. Reduction of cell surface area was recorded as series of time-lapse images (images taken every 6 seconds). The changes of surface area were analyzed with ImageJ software. Three independent batches of siPSC-VSMCs or primary VSMCs were subjected to contractility assay for each treatment, and the surface area changes of 10 randomly selected siPSC-VSMCs in each batch for each treatment were recorded and analyzed, respectively.

2.13. Tube formation assay

48-well cell culture dish was coated with Matrigel (non-diluted) at 37°C for 1-hour. A total of 100,000 cells of human umbilical vein endothelial cells (HUVECs), or HUVECs mixed with HEK293T, siPSC-VSMCs (immature or mature), or swine primary VSMCs (immature or mature) at the ratio of 2:1, were seeded in Matrigel-coated wells with complete VasculLife VEGF Endothelial Medium (Lifeline Cell Technology). At least three phase-contrast images of randomly selected areas in each well were taken 12 hours after cell seeding, and the tube length and number of nodes per field were evaluated by ImageJ software. Three independent batches of cells for each group were subjected to the assay described above.

2.14. Matrigel plug assay

One and a half million siPSC-VSMCs grown in maturation medium were mixed within 300 μ L growth factor-reduced Matrigel supplemented with 500 ng/mL bFGF. NOD/SCID mice were anesthetized using isoflurane, and cell-containing Matrigel or plain Matrigel (300 μ L) was implanted into subcutaneous tissue on the groin. After 10 days, mice were sacrificed, and the Matrigel plugs were isolated and fixed with 4% PFA for 30 minutes at room temperature, followed by incubation with 15% sucrose (SigmaAldrich) in PBS for 12 hours at 4°C, immersion in Tissue-Tek O.C.T compound (Sakura Finetek) inside Cryomold (Sakura Finetek), and freezing on dry ice. Animal surgery was performed under stereomicroscope (Leica, Model MST34). The Matrigel assays with both Matrigel only group and Matrigel containing siPSC-VSMCs group were repeated on three mice independently.

2.15. Preparation of polyglycolic acid (PGA) scaffolds

Nonwoven-PGA polymer mesh (0.3 mm $\times$ 150 mg/cc, 20 cm $\times$ 30 cm sheet, BIOFELT) was cut into 5 mm $\times$ 5 mm squares and prepared for cell seeding as described previously [13, 21]. Briefly, the PGA squares were immersed in 1.0 N NaOH (SigmaAldrich) for 1 min to increase the fiber wettability. Meshes were then rinsed extensively in distilled water to remove the residual NaOH, sterilized with 70% ethanol, and air-dried overnight under sterile

conditions. Before seeding the cells, the PGA squares were coated with 0.1% gelatin at 37°C for 1 hour and then air-dried for 2–3 hours.

2.16. Lentivirus-mediated cell labeling by green fluorescent protein (GFP)

To label the siPSC-VSMCs or swine primary VSMCs, cells were cultured in growth medium and infected with lentiviral particles that contained GFP (gift from Dr. William Sessa, Vascular Biology and Therapeutics Program and Department of Pharmacology, Yale University, New Haven, CT). Briefly, HEK293T cells were co-transfected with plasmids of pMSCV-GFP lentiviral vector along with lentivirus packaging vectors pMD2.G and psPAX2. 48 and 72 h later, culture supernatants containing viral particles were collected, filtered through 0.45-mm membranes (Millipore), and used immediately. The expression of GFP in infected cells was observed 48-hour post infection.

2.17. Generation and subcutaneous implantation of tissue by culturing cells on PGA scaffolds

siPSC-VSMCs or primary swine VSMCs maintained in SmGM-2 were harvested for tissue generation. The pre-treated PGA squares were transferred into 24-well low attachment dish (Corning). 40 μ L of SmGM-2 containing 0.4 million siPSC-VSMCs or primary swine VSMCs were dropped on the PGA square and incubated at 37°C and 5% CO₂ for one hour. The well was then filled with 1 mL of SmGM-2 and cultured overnight. On the next day, the medium was changed to collagen-promoting medium (complete SmGM-2, supplemented with 50 μ g/mL ascorbic acid, 50 μ g/mL proline (SigmaAldrich), 20 μ g/mL alanine (SigmaAldrich), 50 μ g/mL glycine (SigmaAldrich), 3 ng/mL CuSO₄ (SigmaAldrich)), and the medium was changed every other day. The tissues were cultured for 14 days and collected for both histological analysis and implantation into Rag2/Il2rg double knockout mice. PGA scaffolds without cell seeding maintained in the collagen-promoting medium for 14 days were used as the blank control.

For subcutaneous implantation into the rodent model, the Rag2/Il2rg double knockout mice were anesthetized using isoflurane, and the tissue was subcutaneously implanted into the groin. Animal surgery was performed under stereomicroscope (Leica, Model MST34). 14 days after implantation, mice were sacrificed, and the tissues were isolated, fixed with 10% Neutral Buffered Formalin (10% NBF, ThermoFisher) for overnight at room temperature, and transferred to Yale Pathology Tissue Services in 70% ethanol for paraffin embedding and histological analysis. Implantation was independently repeated on three mice for both siPSC-VSMC group and primary VSMC group.

For subcutaneous implantation into the swine model, five pieces of the tissue with GFP-labeled siPSC-VSMCs or swine primary VSMCs on PGA scaffold (5 mm $\times$ 5 mm, cultured for 10 days *in vitro*) were subcutaneously engrafted into one swine (Yorkshire, male, 46 kg, 3 months) for explantation after one week. In brief, swine was anesthetized with isoflurane and monitored according to standard operative protocols. After adequate anesthesia, a vertical midline incision was made in the neck and dissection carried down to the strap muscles; approximately 15 cm length was mobilized on each side. Five pieces of tissue were placed on each strap muscle, with 2 cm between each one, and sutured in place with a 3–0

Vicryl suture. The skin was then closed in multiple layers, and the animal allowed to recover from anesthesia. After one week, the animal was re-anesthetized and the surgical site re-examined; each tissue was identified by the presence of the suture and then explanted for histological analysis described above. Afterwards the animal was euthanized.

2.18. Ring-shape vascular tissue fabrication

Polydimethylsiloxane (PDMS) molds were provided by Dr. Marsha Rolle, Department of Biomedical Engineering, Worcester Polytechnic Institute, Worcester, MA and used to make agarose cell seeding wells as described previously with minor modifications [22]. Briefly, custom polymer templates (VeroWhite Plus with glossy finish) were created by 3D printing (Objet260 Connex Rapid Prototype Machine at Worcester Polytechnic Institute) with 5 annular wells, each with a 2-mm diameter post, and each mold can be fitted into a well of 6-well plate. The PDMS mold was cured on the 3D-printed template, and was then used to cast wells using 2% agarose (ThermoFisher) solution made in DMEM. The agarose molds were next equilibrated in collagen-promoting medium overnight. To fabricate the ring-shaped tissue, 1.2 million siPSC-VSMCs on day 15 of differentiation or primary swine VSMCs were mixed in 50 mL of collagen-promoting medium plus 5 mM blebbistatin (SigmaAldrich) and added into the wells in agarose mold. After 24 hours, medium was changed to fresh collagen-promoting medium. Medium was next changed every other day, and the rings were harvested 10-day after cell seeding.

2.19. Preparation of vessel rings from swine coronary arteries

The swine coronary arteries (including left anterior descending coronary arteries and right coronary arteries) were isolated from Yorkshire pigs (male, 3-month-old) within 20 min after euthanization. Coronary arteries were immediately immersed into cold (4°C) PBS. After removing the adherent connective tissue and fat tissue, the segments of arteries with inner diameters of approximately 2mm were cut into vessel rings with 0.5 mm in length for histological analysis or evaluation of mechanical strength and contractility. The vessel rings from three independent branches of coronary arteries of Yorkshire swine were subjected to evaluation of mechanical strength and contraction.

2.20. Tissue immunohistochemistry and histology

For immunohistochemistry, frozen blocks were sectioned at 5 μ m intervals using Micromcryostat. For frozen-tissue sections (Matrigel plug assay), slides were fixed in acetone for 10 min at -20°C. For paraffin sections (cell/PGA tissue implantation assay and ring assay), slides were dewaxed in xylene, and antigen retrieval was performed with boiling for 20 min in citrate buffer (10 mM, pH 6.0) and rehydrated. After three-time washing with Tris-buffered saline (TBS), tissue sections were incubated with the following primary antibodies diluted in blocking solution (10% (w/v) bovine serum albumin (BSA, SigmaAldrich) or 10% (v/v) horse serum (SigmaAldrich) in PBS) overnight at 4°C in a humidified chamber. Sections were then washed three times with TBS and incubated with appropriate Alexa Fluor 488, 594, or 633 conjugated secondary antibodies diluted 1:200 in blocking solution for one hour at room temperature, followed by three additional washes and mounting on slides with Prolong Gold mounting reagent with DAPI (ThermoFisher). All

immunofluorescence micrographs were captured under inverted microscope (Zeiss, Axiovert 200 M) with Volocity software.

For hematoxylin and eosin and Masson's Trichrome staining, tissue samples were paraffin-embedded and cut into sections of 5 μm by Yale Pathology Tissue Services based on a standard protocol.

2.21. Mechanical testing for tissue-engineered rings or native vessel rings

The mechanical strength of siPSC-VSMC- or primary swine VSMC-derived tissue-engineered rings or swine coronary arterial vessel rings with comparable dimensions was analyzed using an Instron 5848 microtester (Instron) equipped with a 10N load cell. Rings were mounted between two stainless steel pins, one of which was attached to actuator and the other to the load cell. Samples were cyclically pre-stretched for 3 cycles to 10% strain and then stretched until failure to evaluate ultimate tensile strength (UTS). Engineering stress was calculated by dividing tensile force (F) to total cross-sectional area ($A = 2\pi r^2$; assuming ring cross-section to be circular, r is half of the ring thickness and cross-sectional area of the ring is multiplied by two to consider both sides of the ring). The maximum stress, failure strain, maximum modulus, functional stiffness, and toughness of the rings were calculated and plotted according to the method described previously [22]. Three independent batches of tissue-engineered rings or native vessel rings from three independent branches of coronary arteries of Yorkshire swine were subjected to tensile stress tests.

2.22. Contractility testing for tissue-engineered rings or native vessel rings

In order to measure the contractility, swine tissue-engineered rings or native coronary arterial vessel rings with comparable dimensions were transferred into a temperature-controlled perfusion bath. Tissue rings or vessel rings were held by two motorized micromanipulators with hooks at the end, which makes the ring suspended between an anchoring point at one side and a force transducer (KG7, SI Heidelberg) at the other side (see Figure 8C) [12]. During force measurements, rings were incubated in freshly bubbled Tyrode's solution (NaCl 140mM, KCl 5.4mM, MgCl_2 1mM, HEPES 25mM, Glucose 10mM and CaCl_2 1.8mM, pH 7.3) at 37°C. Force measurements were taken at the original (slack) length, and the manipulators were next moved apart by a distance of 1.5 mm in order to measure the second reference force before stimulation of agonist. Carbachol solution was used in a final concentration of 1mM, while KCl solution was 80mM to induce the ring contraction. The readings of force measurement were recorded for 15 minutes. Customized Matlab software was employed to quantify the changes in the force development. Rings with PBS treatment were run to get the baseline value of change in the force and deducted from the initial value of treatment groups in order to derive the final changes in force. Ultimate changes in tension (Pa) were determined by dividing the force by the cross sectional area of the corresponding ring. The cross-section was determined using optical coherence tomography (Ganymede-II- HR, Thorlabs). An index of refraction of 1.38 was utilized for each of the rings. Images were taken, and their cross-sectional area was determined using Image J and averaged to obtain the overall cross sectional area of the rings. Tissue rings were derived from siPSC-VSMCs or swine primary VSMCs, and three independent batches of tissue-engineered rings or native vessel rings from three independent branches of

coronary arteries of Yorkshire swine for each group were subjected to contractility tests for carbachol or KCl treatment and analyzed.

2.23. Statistical analysis

All graph illustrations and statistical analyses were completed using GraphPad Prism6 software. Unpaired two-tailed Student's t test was used to determine the significance of difference between the controls and the experimental groups. One-way ANOVA was used for comparison among multiple groups. P value less than 0.05 was considered significant. Numerical data were reported in the format of mean $\pm$ S.E.M from three independent experiments.

3. Results

3.1. Generation and characterization of doxycycline-inducible inbred siPSCs

The swine embryonic fibroblasts (SEFs) were isolated from Massachusetts General Hospital (MGH) inbred miniature swine at generation eleven of inbreeding and exhibited normal karyotype (Supplementary Fig. 2). To generate siPSCs, SEFs were infected with lentiviral particles containing hSTEMCCA and reverse transcription transactivator (rtTA). As illustrated in Figure 1A, the hSTEMCCA cassette encodes human OCT4, SOX2, KLF4, and c-MYC in a doxycycline-inducible manner and also contains CRE recombinase-excisable loxP sites. siPSC-like colonies appeared 10 days post infection, and were picked and expanded in the presence of doxycycline. Five siPSC clones were initially selected based on compact colony morphology with clear boundary, positive alkaline phosphatase (AP) activity, and presence of pluripotency markers NANOG and SSEA-4 (Fig. 1B–C and Supplementary Fig. 3B). Among the five selected siPSC clones, clone 4 and 10 were subsequently focused for further pluripotency characterization and VSMC differentiation in the subsequent studies. These inbred siPSCs were also positive for surface marker TRA-1-60, but negative for both TRA-1-81 and SSEA-1 (Supplementary Fig. 3A–B). In the presence of doxycycline, siPSCs were capable of long-term self-renewal for over 60 passages (Supplementary Fig. 4). Additionally, inbred siPSCs from clone 4 were subjected to cytogenetic analysis and showed normal karyotype (Fig. 1D).

Embryoid body (EB) formation based on three-dimensional cell aggregation was next used to evaluate the differentiation potential of siPSCs. After 2 weeks of spontaneous differentiation, both clones 4 and 10 siPSCs gave rise to cell derivatives from three germ layers (nestin for ectoderm, desmin for mesoderm and α -fetoprotein (AFP) for endoderm) in the absence of doxycycline (Fig. 1E and Supplementary Fig. 3C), suggesting competent differentiation capacity of the inbred siPSCs.

3.2. Doxycycline-induced activation of ectopic reprogramming factors hindered differentiation of inbred siPSCs

Next, we determined the effect of ectopic reprogramming factors on pluripotency and differentiation of doxycycline-inducible siPSCs. Doxycycline withdrawal for 7 days resulted in a loss of colonial morphology, decreased alkaline phosphatase activity, and disappearance of expression of NANOG and SSEA-4 (Fig. 2A). Additionally, withdrawal of doxycycline

for 4 days led to a marked reduction of expression of human exogenous OCT4, SOX2, c-MYC and KLF4 (Fig. 2B). Interestingly, while the expression of swine endogenous OCT4, KLF4 and NANOG was downregulated 4 days after doxycycline withdrawal, the expression of swine endogenous SOX2 and c-MYC appeared to be upregulated (Supplementary Fig. 5A), possibly due to the induction of early neuroectodermal/progenitor lineage that may maintain a high expression level of SOX2 and c-MYC[23, 24]. Notably, a marked reduction of all five swine endogenous pluripotency markers including SOX2 and c-MYC was observed upon a 12-day spontaneous differentiation of siPSCs in the absence of doxycycline and self-renewal growth factors (LIF and bFGF) (Supplementary Fig. 5B). These results suggest that the pluripotency of siPSCs is dependent on the continued expression of doxycycline-inducible reprogramming factors.

To assess the effect of the presence of the ectopic reprogramming factors on differentiation, siPSCs were subjected to EB-based spontaneous differentiation in the presence or absence of doxycycline, and expression of differentiation markers was evaluated (Fig. 2C). Inclusion of doxycycline in siPSCs culture resulted in significantly lower levels of expression of genes indicative of ectodermal (nestin), endodermal (AFP) and mesodermal (desmin) differentiation compared with those in siPSCs cultured in the absence of doxycycline (Fig. 2D), suggesting that activation of ectopic reprogramming factor in siPSCs markedly hampers lineage differentiation. Based on this observation, it appears that doxycycline withdrawal would be a productive means to enhance lineage differentiation of siPSCs.

3.3. Derivation of VSMCs from siPSCs and evaluation of VSMC gene expression in siPSC-VSMCs

Functional VSMCs derived from inbred doxycycline-inducible siPSCs can be employed to engineer vascular tissue constructs for cell-based regenerative therapies in a preclinical swine model, thereby generating important information for therapeutic application of autologous iPSC-derived VSMC tissue for treatment in patients with vascular disease (Supplementary Fig. 1). However, an efficient approach to derive functional siPSC-VSMCs has not been reported. Previous studies reported the EB approach-based generation of VSMCs from human ESCs or iPSCs [8, 10]. However, such an approach failed to result in the production of healthy siPSC-EBs and sufficient siPSC-VSMCs. Substantial modifications were made to enhance the viability of EBs by using pure siPSC self-renewal medium with the ROCK kinase inhibitor Y-27632 and siPSC self-renewal medium-containing EB differentiation medium (volume ratio at 1:2) to culture day 1 and day 2 EBs (details see Materials and Methods), respectively. Moreover, ascorbic acid was supplemented in VSMC growth medium and maturation medium (Fig. 3A), since it has been implicated in VSMC differentiation [25, 26]. As illustrated in Figure 3A, differentiation of siPSCs was initiated by EB formation in suspension and outgrowth after EB adhesion to plates (day 0 to 12). VSMC lineage commitment was induced by dissociating day 12 cells and then culturing in VSMC growth medium (SmGM-2 medium) supplemented with ascorbic acid (day 12 to 19). To generate functionally more mature VSMCs, day 19 siPSC-VSMCs were further cultured in the maturation medium containing ascorbic acid but lacking exogenous epidermal growth factor (EGF), bFGF and insulin (day 19 to 26).

Since constant activation of ectopic reprogramming factors blocked three-germ layer lineage commitment in day 12 siPSC differentiation culture (Fig. 2D), the effect of the presence of reprogramming factors on VSMC differentiation was next explored in day 26 differentiation culture. Activation of reprogramming factors by doxycycline appeared to significantly block VSMC differentiation, since the majority of cells maintained undifferentiated, colony-like morphology, and failed to express the VSMC marker calponin (CNN1) (Supplementary Fig. 6). Thus, doxycycline was omitted in differentiation media during the derivation of siPSC-VSMC. Additionally, ascorbic acid appeared to be beneficial during siPSC-VSMC derivation, since the proliferation rate of siPSC-VSMCs cultured in the presence of ascorbic acid was higher than that of cells cultured in the absence ascorbic acid (Supplementary Fig. 7A). Interestingly, ascorbic acid did not seem to affect the proliferation of swine primary VSMCs (Supplementary Fig. 7A), raising the possibility that intrinsic cell proliferation machinery in primary VSMCs is insensitive to ascorbic acid stimulation. Notably, ascorbic acid appeared to enhance the collagen matrix production in both siPSC-VSMCs and swine primary VSMCs (Supplementary Fig. 7B). We thus established the optimized VSMC derivation from siPSCs by omitting doxycycline in the differentiation culture and including ascorbic acid in the VSMC growth medium and maturation medium (Fig. 3A).

siPSC-VSMCs cultured in SmGM growth medium (day 19) appeared as rhomboid shaped, while siPSC-VSMCs cultured in maturation medium (day 26; siPSC-VSMC-M) showed an elongated, spindle-like shape (Fig. 3B), indicative of a more mature VSMC phenotype [27]. VSMC marker expression during siPSC-VSMC differentiation and maturation was next assessed by qRT-PCR. siPSC-VSMCs grown in SmGM growth medium (siPSC-VSMCs-G) were early stage VSMCs, and expressed VSMC markers including α -smooth muscle actin (α -SMA), transgelin (SM22 α), CNN1, caldesmon (CALD), smoothelin and myocardin, as well as extracellular matrix (ECM) markers such as elastin, collagen type I (COL1) and fibronectin (Fig. 3C and Supplementary Fig. 8). Further culture of early stage siPSC-VSMCs in maturation medium resulted in significantly increased expression levels of α -SMA, CALD, smooth muscle myosin heavy chain (SMMHC), myocardin, fibronectin and COL1, and these more mature VSMCs are termed siPSC-VSMCs-M (Fig. 3C and Supplementary Fig. 8). In comparison with primary swine VSMCs cultured in maturation medium (primary VSMC-M), siPSC-VSMCs-M showed higher expression levels of α -SMA, SM22 α , CNN1, CALD, SMMHC and fibronectin, lower expression levels of smoothelin, myocardin and elastin, and similar expression level of COL1 (Fig. 3C and Supplementary Fig. 8). These results reveal the feasibility of derivation of siPSC-VSMCs in a stepwise, VSMC lineage specification and maturation manner.

3.4. Functional characterization of siPSC-VSMCs

Next we examined VSMC marker expression in siPSC-VSMCs at the protein level. siPSC-VSMCs in growth or maturation medium expressed VSMC markers including CNN1, α -SMA and SM22 α , as well as the matrix protein collagen type I (Fig. 4A and Supplementary Fig. 9). Consistent with the qRT-PCR results (Fig. 3C), siPSC-VSMCs grown in the maturation medium showed higher numbers of cells expressing SMMHC than those cultured in growth medium (Fig. 4A and Supplementary Fig. 9). Additionally, flow cytometry analysis revealed that $98.2\% \pm 0.9\%$ and $54.0\% \pm 1.9\%$ of siPSC-VSMCs grown in the

maturation medium were positive for CNN1 and SMMHC, respectively, a VSMC marker expression pattern comparable to that of primary swine VSMCs ($98.2\% \pm 0.1\%$ for CNN1 and $47.7\% \pm 1.3\%$ for SMMHC) (Fig. 4B–C and Supplementary Fig. 10).

We then examined the contractile function of siPSC-VSMCs. siPSC-VSMCs or swine primary VSMCs cultured in growth medium (siPSC-VSMC-G; immature) or maturation medium (siPSC-VSMC-M; mature) were treated with vasoconstrictors carbachol and KCl, respectively, and cell contraction was monitored for 30 minutes (Fig. 5A, Supplementary Fig. 11 and Supplementary Movies 1–8). The percentages of decreased surface area in both immature siPSC-VSMCs ($9.9\% \pm 0.8\%$ for carbachol and $9.9\% \pm 0.7\%$ for KCl treatment) and mature siPSC-VSMCs ($16.8\% \pm 1.6\%$ for carbachol and $19.1\% \pm 2.3\%$ for KCl treatment) were significantly higher than those in the respective vehicle control treatment group ($4.9\% \pm 0.6\%$ in immature siPSC-VSMCs and $6.8\% \pm 1.2\%$ in mature siPSC-VSMCs) (Fig. 5B). Similarly, both immature swine primary VSMCs (primary VSMC-G) and mature swine primary VSMCs (primary VSMC-M) also exhibited significantly higher contraction in the presence of carbachol ($12.4\% \pm 1.3\%$ for primary VSMC-G and $17.6\% \pm 1.0\%$ for primary VSMC-M) and KCl ($8.4\% \pm 1.2\%$ for primary VSMC-G and $15.9\% \pm 0.7\%$ for primary VSMC-M), when compared to the respective vehicle control treatment group ($3.8\% \pm 0.8\%$ in VSMC-G and $5.1\% \pm 1.5\%$ in VSMC-M) (Fig. 5B). Additionally, no significant differences were observed between the contractility of siPSC-VSMCs and primary VSMCs grown in maturation medium (Fig. 5B). These results revealed that siPSC-VSMCs are capable of responding to vasoconstrictive stimulus signals.

We next examined the vasculogenic potential of siPSC-VSMCs by assessing their abilities in cooperating with human umbilical vein endothelial cells (HUVECs) to form capillary-like structures that mimic tube formation *in vivo*. It was previously reported that the human iPSC-derived VSMC-like cells may enhance the formation of endothelial progenitor cell (EPC)-based capillary-like structures in the presence of Matrigel by increasing the tube length and decreasing the numbers of nodes [28]. HUVECs (EC) only, or HUVECs mixed with either siPSC-VSMC-G, siPSC-VSMC-M, or human embryonic kidney 293T cells (HEK293T) at 2:1 ratio on Matrigel, with the same total cell number of 100,000 were cultured on Matrigel-coated culture wells. After 12 hours, the capillary-like networks were formed in all groups (Supplementary Fig. 12A). Interestingly, both siPSC-VSMC-G and siPSC-VSMC-M induced the formation of capillary-like networks with increased tube length, fewer nodes and less complexity, compared with those based on HUVEC only (Supplementary Fig. 12A–B). Moreover, siPSC-VSMC-M appeared to further increase tube length and decrease the number of nodes of HUVEC-based capillary-like networks in comparison with siPSC-VSMC-G (Supplementary Fig. 12A–B). In contrast, HEK293T cells did not affect the physical arrangement of HUVEC networks (Supplementary Fig. 12A–B). These results suggest that siPSC-VSMCs, especially those grown in maturation medium, may be able to support the formation of an EC-based capillary-like network.

Next, we evaluated the capability of siPSC-VSMCs in cooperating with murine host endothelial cells (ECs) in the capillary-like network formation in the Matrigel assay *in vivo*. It was reported that the VSMCs derived from human iPSCs can be recruited to the host murine ECs in the capillary-like structure in Matrigel *in vivo* [11]. siPSC-VSMCs grown in

maturation medium were derived and mixed with bFGF in Matrigel, followed by subcutaneous implantation into immunodeficient mice. Based on the representative sections with the infiltrated host blood vessels, $27.8\% \pm 4.2\%$ of the swine iPSC-VSMCs labeled by swine antigen appeared to associate with host CD31⁺ ECs (Supplementary Fig. 12C–D). These results raised the possibility that siPSC-VSMCs have the ability to support the vessel formation of endothelial cells.

3.5. Generation of vascular tissue by culturing siPSC-VSMCs on a biodegradable scaffold

We next examined vascular tissue formation, collagenous matrix deposition and the maintenance of VSMC phenotype for swine iPSC-VSMCs or swine primary VSMCs seeded onto the biodegradable polyglycolic acid (PGA) scaffolds in a murine subcutaneous engraftment model, which has been commonly used to assess the potential of VSMC-based tissue formation and collagen production, as well as VSMC phenotypic expression [29, 30]. Since collagen production plays an essential role during vascular tissue formation, we established a collagen-promoting medium by supplementing SmGM-2 growth medium with ascorbic acid and other reagents favoring collagen synthesis and deposition (Supplementary Fig. 13). The plain PGA scaffolds without cell seeding were maintained in the collagen-promoting medium for two weeks as control. siPSC-VSMCs or swine primary VSMCs cultured in SmGM-2 growth medium were seeded on the 5 mm × 5 mm PGA mesh scaffolds and further cultured in collagen-promoting medium for two weeks (Fig. 6A). Oval-shaped, opaque vascular tissue formed (Supplementary Fig. 14A), and histological analysis revealed that the vascular tissues derived from siPSC-VSMCs were highly cellularized with expression and deposition of collagen, comparable to those derived from swine primary VSMCs (Supplementary Fig. 14B–C). siPSC-VSMCs and swine primary VSMCs also maintained the expression of VSMC markers including α -SMA, CNN1 and collagen type I within the vascular tissues without expression of SMMHC (Supplementary Fig. 14C).

The vascular tissues derived from siPSC-VSMCs or swine primary VSMCs or PGA mesh without cell seeding were next implanted into immunodeficient mice subcutaneously for two weeks (Fig. 6A). Histological analysis of the explanted tissues developed from siPSC-VSMCs or swine primary VSMCs showed the cellularization, collagen deposition within the tissues, and minimal non-degraded PGA remnants (Fig. 6B). Notably, siPSC-VSMC- or swine primary VSMC-based vascular tissues appeared to undergo further maturation after implantation *in vivo*, since they displayed the presence of the mature VSMC marker SMMHC (Fig. 6C). In contrast, the plain PGA scaffold control displayed infiltration of host cells that were negative for both SMMHC and swine-specific surface antigen, as well as large amounts of PGA remnants (Fig. 6B–C). These results suggest that siPSC-VSMCs are capable of generating vascular tissues on biodegradable PGA scaffolds, and maintain the VSMC characteristics both *in vitro* and *in vivo*.

3.6. Evaluating the feasibility of scaffold-free, vascular tissue generation using siPSC-VSMCs

Since human primary VSMCs are capable of forming ring-like vascular tissues without the assistance of scaffold materials [22, 31, 32], we next investigated whether siPSC-VSMCs are able to self-assemble and generate scaffold-free vascular tissue-engineered rings, which

could be used for measurements of mechanical properties and contractile function and as the modular building blocks to generate tubular tissue constructs by ring fusion. Ring-shape vascular tissues were generated by seeding siPSC-VSMCs or swine primary VSMCs in SmGM-2 collagen-promoting medium including 50 µg/mL ascorbic acid, as well as 5 µM cell survival-promoting small molecule blebbistatin, in agarose molds via cell self-assembly for 10 days (Fig. 7A–B). Both siPSC-VSMC- and swine primary VSMC-derived tissue-engineered rings were highly cellularized and showed collagen deposition and expression of VSMC marker CNN1 (Fig. 7C–D). Swine arterial rings with comparable dimensions as the tissue-engineered rings were also isolated from swine coronary arteries for assessment (Supplementary Fig. 15A). Considerable deposition of collagen and expression of VSMC marker CNN1 were observed in the arterial vessel rings (Supplementary Fig. 15B–C).

We further examined the mechanical properties of tissue-engineered rings by using the Instron microtester system to derive the stress-strain plots (representative plots were shown in Fig. 8A). A series of mechanical parameters were analyzed, including maximum stress, failure strain, maximum tangent modulus (the maximum slope of the linear region of the curve), functional stiffness (the maximum slope of the force-displacement curve multiplying the gauge length) and toughness (the area under the curve, reflecting the ability of energy absorption before tissue breaking). As shown in Figure 8B, the mechanical properties, including maximum stress, failure strain, maximum tangent modulus, functional stiffness and toughness, of tissues derived from siPSC-VSMCs appeared to be statistically comparable to those of tissues derived from swine primary VSMCs. Not surprisingly, swine native arterial rings displayed markedly higher mechanical strength than tissue-engineered rings derived from either siPSC-VSMCs or primary VSMCs (Supplementary Fig. 15D), potentially due to the fact that the tissue-engineered rings were derived based on the self-aggregation of the cells cultured under the static condition for a mere 10-day, while the native vessel rings were derived from the coronary arteries of swine of 3-month-old, which underwent a much longer duration for vessel formation under continuous cyclic stretching. These results suggest that the tissue-engineered rings based on siPSC-VSMCs maintained comparable mechanical strength to those made from swine primary VSMCs, and that future optimizations of culture medium and duration and inclusion of cyclic stretching may be required for the generation of mechanically more robust tissue-engineered rings.

Next we evaluated the contractility of the siPSC-VSMC-based tissue rings in the presence of vasoconstrictors carbachol or KCl using our previously described approach based on direct force measurement by a force transducer [12]. siPSC-VSMC and swine primary VSMC tissue-engineered rings showed similar contraction in response to carbachol treatment (48.64 ± 11.19 Pa vs. 37.23 ± 13.95 Pa, respectively; no significant difference, $P=0.78$) (Fig. 8C–D). Additionally, KCl stimulation resulted in statistically comparable contractility between siPSC-VSMC and swine primary VSMC tissue-engineered rings (29.83 ± 23.34 Pa vs. 49.50 ± 6.01 Pa, respectively; no significant difference, $P=0.12$) (Fig. 8C–D). As expected, swine native arterial rings exhibited markedly stronger contractility than tissue-engineered rings derived from either siPSC-VSMCs or primary VSMCs (1185.65 ± 494.25 Pa for carbachol and 890.98 ± 602.30 Pa for KCl; Supplementary Fig. 15E–F), likely due to a highly elevated level of maturation of VSMCs in the native swine arterial vessels engendered by prolonged duration of vessel formation under continuous pulsatile stretching. These results suggest that

siPSC-VSMC- and swine primary VSMC-based tissue rings exhibit similar contractile function in response to vasoconstrictors, and that optimized medium components and duration of ring formation in the presence of pulsatile biomechanical stretching may be necessary to enhance the contractile function of tissue-engineered rings in future endeavors.

Discussion

The current study is the first report of generation of siPSC lines using doxycycline-inducible reprogramming factors from inbred MGH miniature swine that can accept organ grafts from each other without immunosuppression. The inhibition of three-germ layer differentiation caused by the continual presence of reprogramming factors clearly revealed an advantage of generation of siPSC lines in which reprogramming transgenes can be turned off during differentiation. This approach allowed us to achieve high efficiency production of functional differentiated cells from doxycycline-inducible siPSCs. By inactivating reprogramming transgenes and inclusion of ascorbic acid during differentiation, we have developed for the first time a robust approach to derive highly enriched, VSMCs from siPSCs that showed functional contractility and potential in supporting vessel formation of endothelial cells. siPSC-VSMCs appeared to be readily compatible with biodegradable PGA scaffolds, forming cellularized vascular tissue with collagenous matrix and maintaining VSMC characteristics prior to and after subcutaneous implantation in mice. Besides vascular tissue formation supported by PGA scaffolds, siPSC-VSMCs efficiently formed 3D tissue-engineered rings with comparable biomechanical and contractile properties to those derived from swine primary VSMCs via a scaffold-free, cellular self-assembly approach. The unlimited supply of functional, inbred siPSC-VSMCs and the efficient formation of vascular tissue constructs from siPSC-VSMCs may enable investigation of safety and efficacy of autologous hiPSC-based vascular tissues as a therapeutic intervention in a preclinical inbred swine model.

Tissue-engineered vascular grafts have been generated by seeding allogenic human primary VSMCs onto polyglycolic acid (PGA) scaffolds and culturing in bioreactors for 8–10 weeks [5, 13, 20, 33, 34]. Importantly, decellularization of vascular grafts can eliminate or greatly reduce cell-based immunogenicity and enable a longer-term storage, thus providing off-the-shelf product for current clinical trials [20, 35]. However, the generation of vascular grafts using human primary VSMCs is hampered not only by limited proliferation potential of donor cells, but also by batch-to-batch variations in collagen matrix production [5]. Also, in patients with defective vascular cells due to aging or genetic mutations, decellularized vascular tissue constructs may not be a highly effective therapeutic intervention, since patients' own vascular cells may not be able to effectively remodel the implanted vascular constructs. Since Yamanaka reprogramming factors (OCT4, SOX2, c-MYC and KLF4) reactivates the pluripotency program after inducing patients' somatic cells into iPSCs, and the iPSC-derived vascular cells mimic patients' early life counterparts, the aging-related cellular defects may be minimized. Additionally, genetic mutations can be readily corrected in patient iPSCs by CRISPR-Cas9-based gene editing. Thus, autologous, hiPSC-based engineered vascular constructs may provide a unique opportunity to treat patients with dysfunctional vascular cells. Since the time required to make human iPSC-based vascular grafts is about five months starting from procurement of patient blood or skin biopsy, this

approach would be more suited to treat patients with chronic vascular diseases, which may allow sufficient time for iPSC generation and vascular graft engineering. For patients with acute vascular diseases, immunosuppression combined with the production of allogeneic, off-the-shelf vascular grafts from banked human iPSC-VSMCs that could cover major subtypes of major histocompatibility complex (MHC) molecules might be one viable treatment option. Before clinical applications in patients, preclinical animal studies are required to investigate the efficacy and safety of autologous or allogeneic, iPSC-based therapies.

Currently, the animal model that can in principle be used to investigate autologous, iPSC-based therapies is the C57/B16 inbred mouse model. However, since mouse cardiovascular physiology is markedly different from that of humans, iPSC lines derived from inbred, larger animals whose cardiovascular physiology closely resembles that of humans, is sorely needed to provide preclinical knowledge of autologous, iPSC-based therapies (Supplementary Fig. 1) [14, 36]. Thus, the generation of doxycycline-inducible iPSC lines from inbred MGH miniature swine represents an important step forward, as such siPSC lines would enable the generation of vascular tissues and testing in multiple swine without immunosuppression, as the inbred model would overcome the issue of histoincompatibility between individuals. It has been postulated that patient-specific, autologous iPSC-based therapies would overcome the issue of immune rejection, a challenge frequently seen in allogeneic cell-based therapies, and thus result in better cell engraftment and prolonged therapeutic efficacy. However, rigorous basic and translational studies need to be performed to investigate this hypothesis as well as long-term safety and host and iPSC-graft interaction in a preclinical animal model before human clinical trials. The availability of inbred siPSC lines, siPSC-VSMC-based tissue constructs and inbred swine animal models thus provide the scientific community an exciting opportunity to directly investigate autologous iPSC-based therapies for vascular disease in a preclinical large animal model and thus help to advance the cell-based regenerative medicine field.

The generation of siPSC lines in a doxycycline-inducible manner in this study allows the maintenance of pluripotency in the presence of doxycycline, while allowing efficient differentiation by removing doxycycline. Consistent with our previous studies, reprogramming transgene expression appeared to markedly inhibit differentiation [37]. Thus, reprogramming genes were inactivated via doxycycline withdrawal during differentiation. Additionally, ascorbic acid was included during VSMC differentiation step and found to significantly enhance collagen type I production and VSMC proliferation. It is likely that the enhanced production of collagen type I by ascorbic acid might result in upregulated collagen type I/integrin activity, which could activate cell cycle machinery of VSMCs, leading to a higher cellular proliferation rate. By transgene inactivation and addition of ascorbic acid, we developed for the first time a robust approach to derive high-purity siPSC-VSMCs without the need for cell sorting. siPSC-VSMCs express typical VSMC markers CNN1, SM22 α and SMMHC, secrete extracellular matrix collagen type I, and show contractile phenotype in response to vasoconstrictors carbachol and KCl. Additionally, siPSC-VSMCs appear to be able to enhance the formation of endothelial cell (EC)-based capillary-like structures with increased tube length and decreased numbers of nodes and complexity *in vitro*, potentially due to the effect of siPSC-VSMCs in promoting the maturation of ECs by secreted cytokines

and/or in stabilizing EC capillary networks via physical interaction. Moreover, subcutaneous implantation of siPSC-VSMCs into immunodeficient mice revealed that siPSC-VSMCs appear to have the potential to cooperate with murine host ECs during vessel formation in the Matrigel plug. Future endeavors will be made to develop more robust assays to markedly enhance the new vessel formation within the Matrigel, which would assist the analysis of the potential of siPSC-VSMCs in modulating the size and/or function of EC vessels. For examples, co-implantation of HUVEC and siPSC-VSMCs and/or extending the duration of Matrigel plug implantation to 4 weeks could be explored, as VSMC/EC co-delivery and 4-week implantation approaches were employed previously [9, 38].

A prerequisite for generating vascular tissue constructs to replace the diseased vessels is to obtain biodegradable scaffolds to support VSMC growth and matrix production. Our studies revealed that siPSC-VSMCs readily populated within the polyglycolic acid (PGA) biodegradable scaffolds, secreted collagen extracellular matrix, and maintained expression of VSMC markers, including α -SMA and CNN1 but not mature VSMC marker SMMHC, during a 2-week culture *in vitro*. Interestingly, after such 2-week, PGA-supported vascular tissue constructs were implanted in a subcutaneous murine model *in vivo* for two weeks, the PGA remnants observed in the vascular tissue prior to implantation largely disappeared, and the mature SMMHC marker was abundantly expressed in siPSC-VSMCs. PGA degradation products, including major glycolic acid monomer and oligo-glycolic acid, were reported to affect local pH and osmolarity, which may dedifferentiate VSMCs and maintain them in an immature state [39]. It is likely that *in vivo* environment and completion of PGA degradation after implantation *in vivo* allowed siPSC-VSMCs to mature into SMMHC-expressing state. The potential dedifferentiation effect due to PGA degradation during tissue construction could also be readily alleviated by inclusion of TGF- β 1, a growth factor that induces VSMC maturation and is commonly used during vascular tissue engineering [9, 13]. Since application of pulsatile radial stress can significantly improve cell alignment and mechanic strength [4], the effect of cyclic stretching on siPSC-VSMC-based vascular graft construction will be explored during future endeavors. In addition, other potential scaffold materials that may produce degradation products with minimal effect on VSMCs could be considered in future vessel engineering work. For example, it was reported that material composed of 87% glycolide, 7% trimethylene carbonate, and 6% polyethylene glycol degrades more extensively than PGA in the presence of VSMCs, leading to enhanced VSMC proliferation and collagen deposition[34]. Additionally, esterified hyaluronic acid, a material commonly used in vascular tissue engineering, was also reported to be degraded by newly formed tissue, and the degradation products appear to be readily biocompatible for VSMCs [40, 41]. In summary, a robust inbred siPSC-VSMC production and a highly supportive PGA scaffold from our studies thus set the stage for developing tissue-engineered vascular grafts by seeding swine iPSC-VSMCs onto PGA scaffolds in bioreactors in the future.

To evaluate the feasibility of engrafting the siPSC-based engineered tissue in a large-animal model, we have developed a subcutaneous implantation approach in swine (Supplementary Fig. 16 A). Vascular engineered tissues produced by seeding green fluorescent protein (GFP)-labelled siPSC-VSMCs or swine primary VSMCs onto PGA scaffolds were placed on strap muscle, and explanted for histological analysis one week after tissue implantation

(Supplementary Fig. 16 A-C). GFP⁺ cells appeared to engraft and had the ability to express the mature SMC marker SM-MHC (57.9% ± 10.1% and 62.1% ± 12.3% GFP⁺SM-MHC⁺ cells amongst GFP⁺ cells) in the siPSC-VSMC- and swine primary VSMC-derived tissues, respectively (Supplementary Fig. 16D). The availability of this large animal implantation model would allow comprehensive implantation studies of siPSC in inbred syngeneic swine hosts in the future. In summary, a robust inbred siPSC-VSMC production and a highly supportive PGA scaffold from our studies thus set the stage for developing tissue-engineered vascular grafts by seeding swine iPSC-VSMCs onto PGA scaffolds in bioreactors in the future. Such inbred swine engineered vascular grafts can be implanted as end-to-side grafts to the swine common carotid artery to mimic clinical vascular bypass, as performed in our previous report [20], thus providing important knowledge for preclinical investigation of autologous, iPSC-based therapeutic interventions in patients with vascular diseases.

Besides forming vascular tissue with the support of PGA scaffolds, siPSC-VSMCs have the ability to generate 3D tissue rings via a robust scaffold-free, cellular self-assembly approach. The fabricated vascular tissue ring provided us a way to assess biomechanical properties of siPSC-VSMCs and their cell-derived extracellular matrix. Our results revealed that biomechanical properties including maximum stress, failure strain, maximum modulus, functional stiffness and toughness of 3D tissue rings made from siPSC-VSMCs were comparable to those made from swine primary VSMCs. Moreover, the rings derived from siPSC-VSMC displayed comparable contractility in response to vasoconstrictor to that of rings developed from swine primary VSMCs. These results further support the notion that the siPSC-VSMCs derived in this study are functionally comparable to swine primary VSMCs and that siPSC-VSMCs represent an excellent cell source for vascular tissue engineering. Besides providing a useful tool to readily measure biomechanical properties of tissue constructs, 3D tissue rings can potentially be used to screen for optimal culture condition for vascular tissue engineering. In addition, self-assembled 3D tissue rings can be used as modules for fusion into tubular structures and provide an intriguing opportunity to develop swine inbred tissue-engineered vascular grafts for vessel replacement in the inbred swine model in the future [22]. Unsurprisingly, rings derived from native swine arteries with dimensions similar to tissue-engineered rings based on siPSC-VSMCs or swine primary VSMCs displayed markedly higher levels of mechanical strength, as well as stronger contractility in response to vasoconstrictor. These differences are potentially due to substantially higher deposition of extracellular matrix components such as collagen and elastin, as well as a highly elevated level of maturation of VSMCs in the native swine vessels engendered by prolonged duration of vessel formation under continuous mechanical stretching caused by pulsatile blood flow [4, 27]. Future endeavors will be focused on generating robust tissue-engineered blood vessels by seeding siPSC-VSMCs onto biodegradable polyglycolic acid scaffolds in pulsatile bioreactors for an extended culture duration (8–10 weeks), an approach employed in our previous studies [20]. The mechanical and contractile properties of rings derived from such robust siPSC-VSMC-based TEBVs are expected to be greatly enhanced and may approach those of swine native vessel rings.

In our recent proof-of-principle human iPSC-VSMC-based vascular graft studies, there did not appear to be appreciable hyperplastic effect or teratoma formation of iPSC-VSMCs after implantation into the rat abdominal aorta [13]. In future studies, we will evaluate the

proliferation of siPSC-VSMs in engineered vascular graft after implantation into the rodent and inbred swine models. In case swine iPSC-VSMCs may show hyperplastic effect after a prolonged period of implantation, reducing the dosage of serum and growth factors could be considered to induce further maturation and quiescence of VSMC in bioreactors before implantation. Moreover, genetic engineering of siPSCs to express VSMC maturation factors, such as myocardin [42] or adiponectin [43, 44], may help to induce VSMC quiescence, alleviating the potential hyperproliferative effect.

In contrast to murine and human iPSC pluripotency, siPSC pluripotency appears to require constant expression of ectopic reprogramming transgenes, and transgene-free siPSC generation using Sendai virus or other methods is currently not feasible [15, 17, 18, 45]. Thus, we have derived siPSCs from inbred MGH swine fibroblasts using doxycycline-inducible vectors, reducing adverse effects caused by reprogramming transgenes via simple doxycycline withdrawal during differentiation. While we have demonstrated that this approach is compatible with the generation of VSMC, its applicability in the generation of other lineages derived from human and mouse iPSC lines remains to be established. Additionally, inbred swine embryonic fibroblasts were employed for cellular reprogramming in this study. In our future endeavors, we will derive iPSCs from adult inbred swine fibroblasts and investigate whether adult fibroblast-derived iPSCs behave and differentiate similarly as embryonic fibroblast-derived iPSCs. Typically, iPSCs derived from either murine embryonic or adult fibroblasts possess similar differentiation potential once they are reprogrammed into pluripotent cells [46, 47]. Moreover, iPSCs were successfully generated from human adult fibroblasts, and the age does not appear to affect iPSC derivation and lineage differentiation [37, 48]. Additionally, iPSC lines derived from perinatal and adult human subjects also behave and differentiate into VSMCs similarly in our previous study [12]. Interestingly, siPSCs injected into immunocompromised mice in this study did not produce obvious teratomas (data not shown), possibly due to reprogramming transgene-dependence of pluripotency and rapid loss of expression of ectopic reprogramming factor after injection into mice. In future work, reprogramming transgenes could in principle be removed from differentiated cells by CRE recombinase-triggered excision (see cartoon in Fig. 1A). Additionally, the availability of doxycycline-inducible siPSC lines will allow the screening of agents that could support pluripotency in the absence of continued expression of ectopic reprogramming factors, and active ongoing efforts in our group are in progress to approach this goal. More thoroughly reprogrammed siPSC lines independent of transgene expression cultured under more optimized conditions through future endeavors are expected to solve such challenges and to generate functionally more optimal, differentiated cells for therapeutic investigation.

Conclusions

In summary, our results underscored the feasibility of engineering vascular tissue from doxycycline-inducible inbred siPSC-VSMCs. Inbred siPSCs were generated by using doxycycline-inducible reprogramming factors, and highly homogenous, functional VSMCs were efficiently derived from siPSCs for the first time. siPSC-VSMCs seeded into biodegradable PGA scaffolds grew into vascular tissue that contained collagen matrix and supported VSMC further maturation after subcutaneous implantation. Moreover, siPSC-

VSMCs were able to form highly cellularized, collagen-containing 3D tissue rings that showed biomechanical properties and contractile phenotypes comparable to those of swine primary VSMC-derived tissue rings. Thus, our findings pave the way for construction of inbred siPSC-based vascular tissue constructs for vascular repair and replacement in the inbred swine model, which serves as an excellent preclinical model and provides essential knowledge for autologous, hiPSC-based therapeutic interventions. We anticipate that inbred siPSC-based tissue engineering and inbred swine model will be of general interest to the scientific community and help to move the cell-based regenerative medicine field forward.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Generation and characterization of inbred swine induced pluripotent stem cells (siPSCs, clone 4)

(A) Strategy of the establishment of inbred siPSCs with doxycycline-inducible hSTEMCCA vector encoding human OCT4, KLF4, SOX2 and c-MYC, and rtTA vector encoding the reverse tetracycline transactivator. **(B)** Typical colony morphology and alkaline phosphatase (AP) activity in siPSCs at passage 5 (P5) cultured in the presence of 2 µg/mL doxycycline. Scale bar: 250 µm. **(C)** Immunofluorescence of siPSCs for the pluripotency markers including SSEA-4 and NANOG cultured in 2 µg/mL doxycycline-containing medium. DNA (nuclear) was counterstained by DAPI. Scale bar: 250 µm. **(D)** Karyotype analysis of siPSCs. **(E)** Immunofluorescence of spontaneously differentiated siPSCs for nestin (ectodermal marker), desmin (mesodermal marker) and α-fetoprotein (AFP, endodermal marker) cultured in the absence of doxycycline. Scale bar: 250 µm.

Figure 2. Doxycycline-induced activation of reprogramming factors maintains the pluripotency but inhibits the differentiation of siPSCs (clone 4)

(A) The morphology, alkaline phosphatase (AP) activity, and the expression of NANOG and SSEA-4 in siPSCs cultured for 7 days in the presence or absence of doxycycline (day 7 DOX+ or day 7 DOX-). DNA (nuclear) was counterstained by DAPI. Scale bar: 250 μ m.

(B) qRT-PCR analysis of the exogenous human OCT4, SOX2, c-MYC and KLF4 in siPSCs cultured in the presence or absence of doxycycline (DOX+ or DOX-) for 4 days. Gene expression levels were normalized to the house-keeping gene GAPDH (n=3; *: p<0.05 vs. day 0).

(C) Strategy of embryoid bodies-based differentiation of siPSCs. (D) qRT-PCR analysis of the swine differentiation markers (nestin for ectoderm, desmin for mesoderm and alpha-fetoprotein (AFP) for endoderm) in differentiated siPSCs on day 12 during spontaneous differentiation, in the presence or absence of doxycycline (DOX+ or DOX-). Gene expression levels were normalized to swine GAPDH (n=3; *: p<0.05).

Figure 3. Generation of VSMCs from siPSCs (clone 4) (siPSC-VSMCs) and evaluation of VSMC gene expression

(A) Schematic illustration of VSMC differentiation of inbred siPSCs. (B) Morphological changes during VSMCs differentiation of siPSCs in the absence of doxycycline on day 6 (floating EBs), day 12 (attached EBs), day 19 (siPSC-VSMCs in growth medium) and day 26 (siPSC-VSMCs in maturation medium). Scale bar: 250 μ m. (C) qRT-PCR analysis of VSMC markers (α -smooth muscle actin (α -SMA), calponin (CNN1), transgelin (SM22 α), caldesmon (CALD), smooth muscle myosin heavy chain (SMMHC) and smoothelin) in

undifferentiated siPSCs, siPSC-VSMCs in growth medium on day 19 (siPSC-VSMC-G), siPSC-VSMCs in maturation medium on day 26 (siPSC-VSMC-M), primary swine VSMCs maintained in growth medium (primary VSMC-G), and primary swine VSMCs maintained in maturation medium (primary VSMC-M). Gene expression levels were normalized to the swine house-keeping gene GAPDH (n=3; *: p<0.05; N.S: not significant).

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Figure 4. Characterization of VSMCs derived from siPSCs (clone 4)

(A) Immunostaining of VSMC markers (α-SMA, CNN1, SM22α, SMMHC and collagen type I (COL1)) in VSMCs derived from siPSCs in growth medium or maturation medium, and swine primary VSMCs in growth medium or maturation medium. DNA (nuclear) was counterstained by DAPI. Scale bar: 250 μm. (B) Representative plots of percentage of CNN1-positive or SMMHC-positive siPSC-VSMCs in maturation medium (siPSC-VSMCs-M) or swine primary VSMCs in maturation medium (primary VSMCs-M) by flow cytometry analysis. (C) Quantification of percentage of CNN1-positive or SMMHC-positive siPSC-VSMCs or swine primary VSMCs in maturation medium by flow cytometry analysis (n=3; N.S: not significant).

Figure 5. Contractility analysis of siPSC-VSMCs

(A) siPSC-VSMCs (derived from clone 4 siPSC line) cultured in growth medium (siPSC-VSMC-G) or maturation medium (siPSC-VSMC-M) or swine primary VSMCs cultured in growth medium (primary VSMC-G) or maturation medium (primary VSMC-M) contracted in response to 30-minute treatment (before and after 30 minutes) by carbachol (upper panels) or KCl (lower panels), as indicated by the arrows. Scale bar: 250 μ m. (B) Quantification of reduced cell area of siPSC-VSMCs and swine primary VSMCs in response

to vehicle (control), carbachol or KCl (n=3; *: $p<0.05$ vs. control; #: $p<0.05$ between indicated groups; N.S: not significant between indicated groups).

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Figure 6. Engineered tissues generated from siPSC-VSMCs cultured on biodegradable polyglycolic acid (PGA) scaffolds

(A) Illustrative scheme of the method used to establish tissue patches from siPSC-VSMCs or swine primary VSMCs growing on biodegradable PGA scaffolds. (B) H&E staining and Masson's Trichrome staining of the explanted tissues derived from siPSC-VSMCs (generated from clone 4 siPSC line) or swine primary VSMCs seeded onto PGA scaffold or plain PGA scaffold without cell seeding, after 2-week-subcutaneous implantation (day 28) into immunodeficient mice. The arrows indicate PGA remnants. Scale bar: 100 μ m. (C) Immunohistological staining of the explanted tissues derived from siPSC-VSMCs or swine primary VSMCs seeded onto PGA scaffold or plain PGA scaffold without cell seeding. The section was stained with smooth muscle myosin heavy chain (SMMHC) and swine specific surface antigen (swine). DNA (nuclear) was counterstained by DAPI. Scale bar: 100 μ m.

Figure 7. Vascular tissue rings fabricated from siPSC-VSMCs

(A) Illustrative scheme of the method used to establish vascular tissue rings from siPSC-VSMCs or swine primary VSMCs. (B) Morphology of the tissue rings from siPSC-VSMCs (derived from clone 4 siPSC line). The ring in the agarose mold on the top view (left), 45-degree view (middle) and the size of the ring were displayed (right). The rings in the molds were indicated by the arrows. (C) H&E staining and Masson's Trichrome staining of the tissue rings made from siPSC-VSMCs or swine primary VSMCs. Scale bar: 100 μ m. (D) Immunohistological staining of the tissue rings made from siPSC-VSMCs or swine primary VSMCs. The sections were stained with collagen type I (COL1) and calponin (CNN1). DNA (nuclear) was counterstained by DAPI. Scale bar: 100 μ m.

Figure 8. Mechanical and contractile properties of the siPSC-VSMC-derived tissue rings
(A) Representative stress-strain curve plots of tissue rings (cultured for 10 days) fabricated from siPSC-VSMCs (derived from clone 4 siPSC line) or swine primary VSMCs. **(B)** Mechanical parameters, including maximum stress, failure strain, maximum modulus, functional stiffness, and toughness were compared between the siPSC-VSMC and swine primary VSMC derived tissue rings (n=3; N.S: not significant). **(C)** Representative photograph of tissue ring (cultured for 10 days) fabricated from siPSC-VSMCs (derived from clone 4 siPSC line) mounted on micromanipulators and immersed in temperature-controlled perfusion bath containing Tyrode's solution for contractility assay. The red arrows indicate the tissue ring from siPSC-VSMCs, and the blue arrows indicate the arm of the

force transducer. **(D)** Contractility changes (Pascal) of tissue rings (cultured for 10 days) fabricated from siPSC-VSMCs (derived from clone 4 siPSC line) or swine primary VSMCs in response to the vasoconstrictor carbachol or KCl for 15 minutes (n=3; N.S: not significant). PBS was used as negative control.

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