



## Original Article

# Comparative study of differentiating human pluripotent stem cells into vascular smooth muscle cells in hydrogel-based culture methods



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## ARTICLE INFO

## Article history:

Received 23 September 2022

Received in revised form

31 October 2022

Accepted 6 December 2022

## Keywords:

Human pluripotent stem cells

PNIPAAm-PEG hydrogel

Alginate hydrogel fiber

Vascular smooth muscle cells

Industrial production

## ABSTRACT

Vascular smooth muscle cells (VSMCs), which provides structural integrity and regulates the diameter of vasculature, are of great potential for modeling vascular-associated diseases and tissue engineering. Here, we presented a detailed comparison of differentiating human pluripotent stem cells (hPSCs) into VSMCs (hPSCs-VSMCs) in four different culture methods, including 2-dimensional (2D) culture, 3-dimensional (3D) PNIPAAm-PEG hydrogel culture, 3-dimensional (3D) alginate hydrogel culture, and transferring 3-dimensional alginate hydrogel culture to 2-dimensional (2D) culture. Both hydrogel-based culture methods could mimic *in vivo* microenvironment to protect cells from shear force, and avoid cells agglomeration, resulting in the extremely high culture efficiency (e.g., high viability, high purity and high yield) compared with 2D culture. We demonstrated hPSC-VSMCs produced from hydrogel-based culture methods had better contractile phenotypes and the potential of vasculature formation. The transcriptome analysis showed the hPSC-VSMCs derived from hydrogel-based culture methods displayed more upregulated genes in vasculature development, angiogenesis and blood vessel development, extracellular matrix compared with 2D culture. Taken together, hPSC-VSMCs produced from hydrogel-based culture system could be applied in various biomedical fields, and further indicated the suitable development of alginate hydrogel for industrial production by taking all aspects into consideration.

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Peer review under responsibility of the Japanese Society for Regenerative Medicine.

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## 1. Introduction

Vascular smooth muscle cells (VSMCs) are one of the major cell types of blood vessel, and they play a critical role in the development, function and maintenance of vasculature [1,2]. VSMCs hold the great potential of vascular-associated disease modeling and drug discovery [3–6], tissue engineering [7–9] and cytototherapy [10,11]. However, a big challenge for those application mentioned above is how to get enough VSMCs from *in vitro* differentiation or primary tissues [1,12,13]. Currently, human pluripotent stem cells

(hPSCs), possessing the ability of unlimited proliferate *in vitro* and differentiating into all somatic cell types of human body [14,15], could be used to resolve the obstacle. Especially, induced pluripotent stem cells (iPSCs) derived from patient cells or tissues retaining the patient's genetic information is suitable for modeling human diseases [16,17]. Recently, many protocols for effectively differentiating hPSCs into VSMCs have been developed, either through embryoid bodies [3,18,19] or monolayer culture [20,21]. The researchers are able to drive hPSCs differentiation into different regions of VSMCs [6,21–26]. For example, an efficient and simple protocol of VSMCs differentiation from hPSCs reported by Patsch has been established in 2D monolayer level in 6 days for a range of biological applications [20].

Although using the current protocols, producing large number of hPSC-VSMCs for various biological applications remains difficult. The traditional 2D monolayer cell culture generally produces a low cell yield [27,28]. The traditional 3D suspension culturing often form big agglomerates, which cause spontaneous differentiation in the central region of agglomerates, and limit cell proliferation [27], which are not suitable for large scale production. To resolve the limitations, we previously reported a high culture efficiency method for hPSCs expansion and differentiation [29–32]. Here, we represented and compared four different culture methods for hPSC derived VSMCs. Firstly, hPSCs are cultured in 3D PEG hydrogel and 3D alginate hydrogel, then they are differentiated into VSMCs. Secondly, we compared hPSC-VSMCs in different culture methods, including cell yield, differentiation efficiency, functional properties and the whole transcriptome gene expression. The results revealed that hPSC-VSMCs could be highly produced in the hydrogels-based culture methods, and hPSC-VSMCs produced from hydrogel-based culture methods had better contractile phenotypes and the potential of vasculature formation. The transcriptomic analysis showed the hydrogel-based culture methods displayed more upregulated genes in vasculature development, angiogenesis and blood vessel development, extracellular matrix compared with 2D culture. Taken all aspects into together, the alginate hydrogel-based culture methods will be suitable for the applications of hPSC-VSMCs in biological fields and industrial production.

## 2. Materials and methods

### 2.1. Cell culture

H9 hESCs (#WA09, WiCell) were purchased from WiCell Research Institute. H9 hESCs were maintained in 6-well plate coated with Matrigel in Essential 8™ medium. Cells were passaged every 4 days with 0.5 mM EDTA. Medium was changed daily. Cells were routinely checked for the expression of pluripotency markers, OCT4 and NANOG, and bacterial or mycoplasma contamination. HUVECs (#00191027, Lonza) were obtained from Lonza, and maintained in 10-cm dish with EGM™-2 Endothelial Cell Growth Medium-2 BulletKit™ (CC-3162). HUVECs were passaged every 3–4 days with 0.25% Trypsin–EDTA solution. Medium was changed daily.

### 2.2. Culturing hPSCs in 3D PNIPAAm-PEG hydrogels and alginate hydrogel fibers

The method for hPSCs culture in 3D PNIPAAm-PEG Hydrogels and alginate hydrogel fibers have been described in our previous publication [15,30,33]. The optimal concentration (10%) of PNIPAAm-PEG [15] and (1.5%) sodium alginate hydrogel [30] for hPSC biology and culture were determined in our previous studies. For 3D PNIPAAm-PEG Hydrogels culture, hPSCs were first dissociated into single cells, then mixed with 10% PNIPAAm-PEG solution dissolved in E8 medium on ice and cast on tissue culture plates,

then incubated at 37 °C for 10 min to form hydrogels before adding warm E8 medium containing 10 mM ROCK inhibitor (LC Laboratories, no. Y5301). The medium was changed daily. Cells were passaged every 5 days. To quantify cells, the medium was removed, and 2 mL ice cold PBS was added to dissolve the hydrogel for 5 min. Cell spheroids were collected by spinning at 100 g for 3 min. Cells were dissociated into single cells in Accutase at 37 °C for 10 min, and counted cell with the TC20 automated cell counter and passage cells for the following culture.

For alginate hydrogel fibers culture, a hyaluronic acid (HA) or methylcellulose (MC) solution containing single cells and an alginate solution was pumped into the central and side channel of the home-made micro-extruder, respectively, and extruded into a CaCl<sub>2</sub> buffer (100 mM) to make hydrogel fibers. Subsequently, the CaCl<sub>2</sub> buffer was replaced by cell culture medium. For a typical cell culture, 20 μL of cell solution in hydrogel fibers were suspended in 2 mL E8 medium in a 6-well plate and cultured in an incubator. Medium was changed daily. To quantify cells, medium was removed and alginate hydrogels were dissolved with 0.5 mM EDTA for 5 min. Cell mass was collected by centrifuging at 100 g for 5 min, treated with Accutase at 37 °C for 12 min and dissociated into single cells, and counted cell with the TC20 automated cell counter and passage cells for the following culture.

### 2.3. Making hPSC-VSMCs in 2D culture, 3D PEG hydrogel or 3D alginate hydrogel fibers

Briefly, for VSMCs differentiation, hPSCs were first expanded according to different culture methods. Then, the medium was replaced with N2B27 medium (1:1 mixture of DMEM/F12 (#SH30004.04, HyClone) with Glutamax-I (#35050061, Life Technologies) and Neurobasal medium (#21103049, Life Technologies) supplemented with N2 (#17502048, Life Technologies) and B27 minus vitamin A (#12587010, Life Technologies) with 8 μM CHIR99021 (#C6556, LC laboratories) and 25 ng/mL BMP4 (#314BP010, R&D Systems). After 3 days, the medium was replaced by N2B27 medium supplemented with 10 ng/mL PDGF-BB (#100-14 B, PeproTech) and 2 ng/mL ActivinA (#338-AC, R&D Systems). The medium was exchanged every day. VSMCs were harvested for analysis on day 6 or day 10.

For transferring 3D alginate hydrogel culture to 2D culture, hPSC-VSMCs derived from alginate hydrogel were dissociated into single cell and replated into 2D culture for 6 days.

### 2.4. Immunocytochemistry

For 2D immunostaining, the 2D cells were fixed with 4% paraformaldehyde (PFA) at room temperature for 20 min, permeabilized with 0.25% Triton X-100 for 30 min, and blocked with 5% donkey serum for 1 h before incubating with primary antibodies: α-SMA (sc-130616, Santa Cruz), SM22A (ab14106, Abcam), Human-PDGFBR (PA5-14718, ThermoFisher), Human-CD31 (555444, BD Biosciences) at 4 °C overnight. After extensive washing, secondary antibodies (715-545-151, 711-585-152, 711-165-152, 705-605-147, Jackson ImmunoResearch) and 10 μM 4', 6-Diamidino-2-Phenylindole (DAPI) in 2% BSA were added and incubated at room temperature for 4 h. Cells were washed with PBS for 3 times before imaging with A1 confocal microscope. For cell mass immunostaining, after fixation for 30 min, cell masses were incubated with PBS +0.25% Triton X-100 + 5% (vol/vol) goat serum + primary antibodies at 4 °C for 48 h. After extensive washing, secondary antibodies in 2% BSA were added and incubated at 4 °C for 24 h. Cells were washed with PBS three times before imaging with a confocal microscope. For 3D cell staining, VSMCs derived from 3D PEG-hydrogel and 3D-alginate hydrogel

were dissociated into single cells and replated into well-plate for 2 h, followed by fixed with 4% PFA, then performed the above immunostaining procedure.

### 2.5. Co-culture assay of HUVECs and hPSC-VSMCs

200  $\mu$ L of Matrigel was added into each well of a 12-well plate and incubated for 30 min at 37 °C to allow the formation of a thin layer of hydrogel. For the functional tube formation and association assays *in vitro*, the HUVECs and the hPSC-VSMCs were prestained with DiI (red) and DiO (green), respectively, according to the manufacturer's instructions. Cells were grown in EGM-2 medium supplemented with 50 ng/mL VEGF-A. For the experiment,  $2.0 \times 10^4$  HUVECs/cm<sup>2</sup> and  $2.0 \times 10^4$  hPSC-VSMCs/cm<sup>2</sup> were co-cultured for 24 h in the incubator. Cells were fixed with 2% PFA for 10 min and analyzed using Zeiss fluorescence microscope.

### 2.6. Fibronectin production

After 5 days of differentiation, all VSMCs were seeded in N2B27 containing 10 ng/mL PDGF-BB at 40,000 cells/cm<sup>2</sup> on gelatin-coated wells. After 24 h, the medium was changed to N2B27 with 10 ng/mL PDGF-BB supplemented with DMSO or 2.5 ng/mL TGF- $\beta$ . After 24 h, cells were washed with PBS, fixed with 4% PFA for 10 min at room temperature, and immunofluorescence staining of deposited Fibronectin (ab2413, Abcam) was performed.

### 2.7. Western blot analysis

After 5 days of differentiation, all VSMCs were seeded in N2B27 containing 10 ng/mL PDGF-BB at 40,000 cells/cm<sup>2</sup> on gelatin-coated wells. After 24 h, the medium was changed to N2B27 with 10 ng/mL PDGF-BB supplemented with DMSO or 2.5 ng/mL TGF- $\beta$ . After 24 h, cells were washed with PBS, then were harvested and cell lysates were prepared using the Pierce IP™ lysis buffer (cat # 87788, ThermoFisher) supplemented with Halt™ Protease & Phosphatase Single-Use Inhibitor Cocktail (100x, cat # 78442, ThermoFisher). 30  $\mu$ g of total proteins for each sample were subjected to SDS-PAGE analysis on a 4%–12% NuPAGE™ Bis-Tris protein gel (cat # NP0321BOX, ThermoFisher). GAPDH was used as a loading control. The membrane was blocked in TBST (Thermo Scientific) containing 5% nonfat dried milk (Applygen) and subsequently incubated with anti-Fibronectin antibody (1:3000, MA5-11981, ThermoFisher) at 4 °C overnight. The membrane was washed three times with TBST for 10 min and incubated with an anti-mouse/rabbit IgG secondary antibody conjugated to horseradish peroxidase (IgG-HRP; 1:3000; Santa Cruz) for 1 h at room temperature. Then, the membrane was washed three times with TBST for 10 min and imaged using a gel imaging system (Tanon).

### 2.8. Contraction study

VSMCs from 3D methods were first dissociated into single cells and replated into 2D plate. All VSMCs were seeded in N2B27 containing 2 ng/mL ActivinA and 2  $\mu$ g/mL at 40,000 cells/cm<sup>2</sup> on Collagen-coated wells according to previous studies [20,34]. After 48 h, they were stained with 2.5  $\mu$ M Fluo-4 AM (#50018, Biotium) at 37 °C for 1 h. Contraction was induced by treating the cells with 100  $\mu$ M carbachol (#2810, Tocris). Contraction images of VSMCs were acquired by a Zeiss fluorescence microscope. The fluorescence intensity of intracellular calcium flux, cell surface area (mm<sup>2</sup>) and percent change of cell surface area was assessed by choosing the same cell before and after drug treatment using ImageJ software.

### 2.9. Matrigel plug assay

Animal procedures were performed in accordance with an IACUC-approved protocol reviewed by the University of Nebraska–Lincoln Animal Care and Use Committee. 6–8 week old female SCID mice (Charles River Laboratory) were used. HUVECs and hPSC-VSMCs at a ratio of 1:1 were added to the Matrigel mixture to a final concentration of 10 million cells/mL. The Matrigel mixture (300  $\mu$ L) was then immediately engrafted subcutaneously into the dorsal flank of the mouse. Two implants were engrafted per animal. Implants were recovered after 14 days, then the implants were excised. They were fixed in 4% PFA. Immunostaining of human CD31 (HUVECs) and human PDGFRB (hPSC-VSMCs) was performed to analyze the tube formation potential *in vivo*. Three mice for each group were used for the Matrigel plug assay. Mean number of vessel-associated VSMCs per mm vessel length was assessed by ImageJ software.

### 2.10. RNA sequencing and data analysis

Total RNA of all VSMCs were prepared with RNeasy mini kit (cat # 74104 QIAGEN) according to the manufacturer's instruction. Prior to RNA sequencing, magnetic beads coated with anti-CD144 antibodies were added to remove CD144+ hPSC-ECs with a magnetic cell separator. VSMCs reached 95% purity after purification. Libraries were prepared with TruSeq Stranded mRNA Library Prep Kit and sequenced with Illumina NextSeq 500.20 million 75 bp paired-end reads were generated for each sample. The thresholds for differential expression were set at fold-change >2 and adjusted P-values <0.001 for the null hypothesis. Methods for the data processing, heatmap generating, PCA analysis, differential gene expression analysis have been described in our previous publication [30].

### 2.11. Statistical analysis

The data are presented as the mean  $\pm$  standard deviation (SD) from three independent experiments. We used an unpaired t-test to compare two groups and one-way ANOVA to compare more than two groups. A sample size of 3 was selected so that at a significance level of 0.05 there at least 95% chance of detecting two SD's difference in outcome between the groups. All data were processed using GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA).

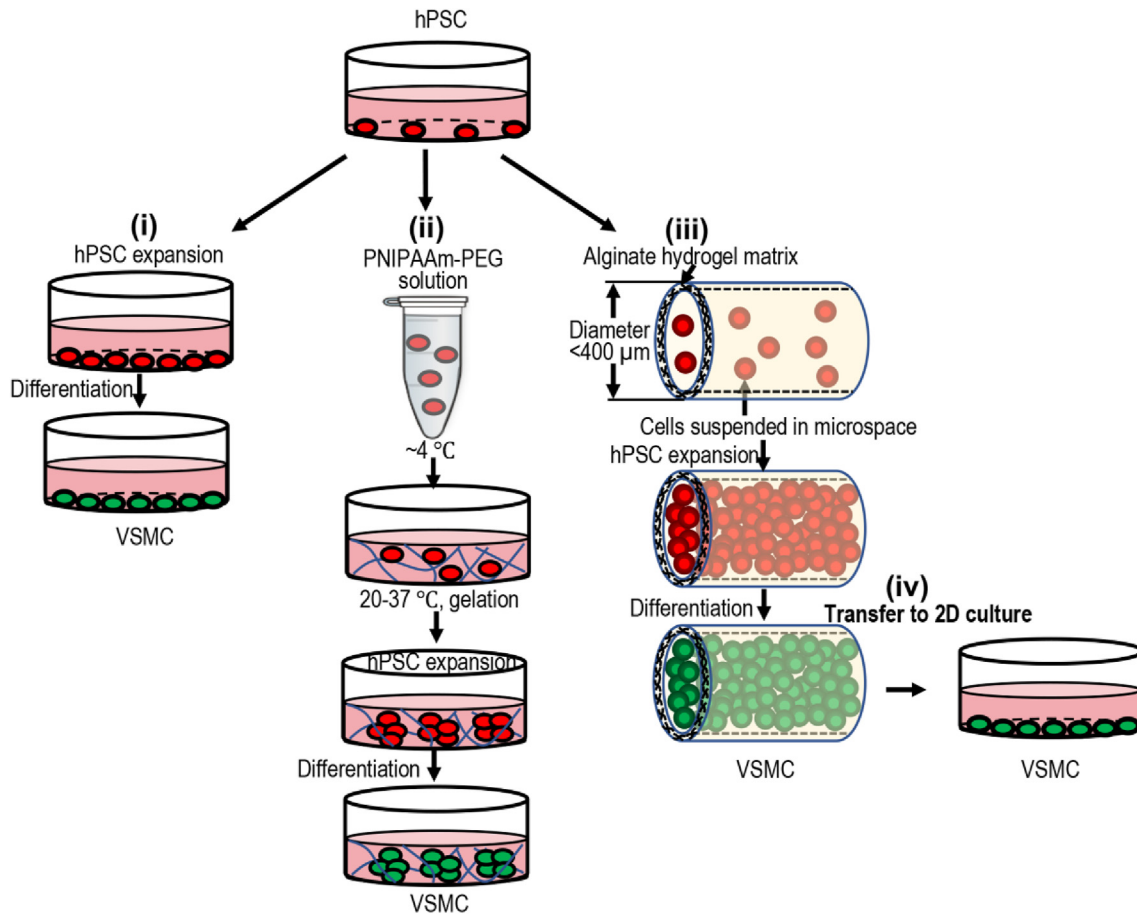
### 2.12. Accession numbers

The accession numbers for the data reported in this paper are GEO: GSE99776 and GSE109683.

## 3. Results

### 3.1. hPSC expansion and differentiation in four culture methods

We first checked and confirmed the quality of H9 hESCs in our previous studies [29,35–38]. Processing and culturing hPSCs in 3D-PNIPAAm-PEG hydrogel [15] and 3D alginate hydrogel fiber [30] are described in our previous publications and Methods (Fig. 1). hPSCs were expanded and differentiated in different culture methods (Fig. 2A and B) and characterized based on our previous report [39]. Live/dead cell staining revealed that initiating hPSCs from different methods were good (Fig. 2C). An efficient and simple protocol of VSMCs differentiation from hPSCs reported by Patsch [20] has been established in 2D monolayer level in 6 days for various biological applications (Fig. 2A). After 5 days differentiation, the typical morphologies of VSMCs derived from 2D and transferring 3D



**Fig. 1. Overview of different culture methods for hPSCs-VSMC differentiation.** (i) Two-dimensional (2D) culture system. (ii) Three dimensional (3D) PNIPAAm-PEG hydrogel culture system. Single hPSCs are mixed with 10% PNIPAAm-PEG solution at low temperature (e.g., 4 °C), which forms an elastic hydrogel at 37 °C. Single hPSCs clonally expand into uniform spheroids in the hydrogel in 5 days, followed by hPSCs differentiation into VSMCs in the hydrogel. (iii) Three-dimensional (3D) alginate hydrogel culture system. hPSC are processed into the alginate hydrogel tubes at low seeding density and expanded for 9 days to fill the tubes. Once the targeted cell number of hPSCs is reached, hPSCs (around day 5) can be differentiated into VSMCs in the alginate hydrogel. (iv) Harvested VSMCs from 3D-alginate hydrogel system were transferred to 2D culture for 6 days.

alginate hydrogel culture to 2D culture were observed, and 3D methods also showed good quality of VSMCs by morphologies observation (Fig. 2D). Immunostaining showed majority of VSMCs were SM22A+ and  $\alpha$ -SMA+ (Fig. 2E and F). About 24-fold,  $2.4 \times 10^7$  cells/mL in 3D-PEG hydrogel, and about 410-fold,  $4.1 \times 10^8$  cells/mL in 3D-Alginate hydrogel was produced on day 10, respectively compared with about 15-fold,  $1.5 \times 10^7$  cells/mL in 2D culture (Fig. 2G and H).

### 3.2. Properties of all VSMCs made in four culture methods

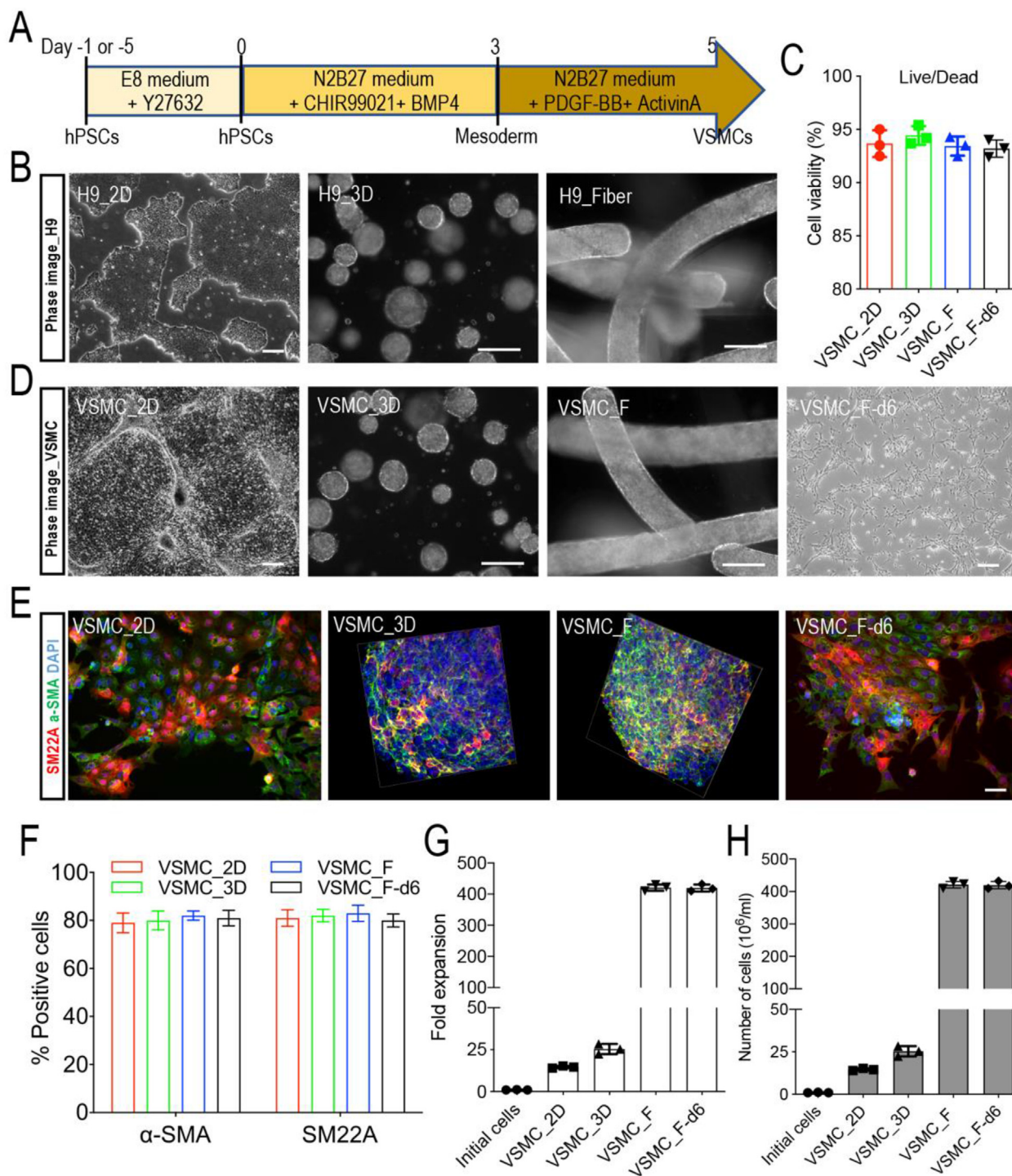
To investigate whether the VSMCs produced from four different culture methods displayed similar properties, we performed *in vitro* and *in vivo* functional assay. The fibronectin deposition assay and Western blot analysis showed 3D methods had higher fibronectin production and expression in response to TGF- $\beta$  (Fig. 3A–C). For the tube formation assays,  $2.0 \times 10^4$  HUVECs per  $\text{cm}^2$  and  $2.0 \times 10^4$  hPSC-VSMCs per  $\text{cm}^2$  were prestained with DiI (red) and DiO (green), respectively, then were co-cultured for 24 h in the incubator. When co-cultured with HUVECs, all groups could form the vascular-like structures, and 3D methods displayed slightly higher mean number of vessels associated VSMCs per 0.5 mm vessel length (Fig. 3D and E). For contractile assay, VSMCs were seeded in N2B27 containing 2 ng/mL Activin-A and 2  $\mu\text{g}/\text{mL}$  at 40,000 cells per  $\text{cm}^2$  on Collagen-coated wells for 48 h. Then the

cells were stained with 2.5  $\mu\text{M}$  Fluo-4 AM at 37 °C for 1 h, followed by 100  $\mu\text{M}$  carbachol treatment. Hydrogel-based groups had better contraction in response to carbachol treatment using ImageJ analysis function (Fig. 3F–H). Calcium imaging by measuring relative fluorescent change of the same cell using ImageJ analysis function revealed that the carbachol-induced a temporary increase in intracellular calcium levels (Fig. 3I). For Matrigel plug assay, the Matrigel mixture (300  $\mu\text{L}$ ) containing HUVECs and hPSC-VSMCs was engrafted subcutaneously into the dorsal flank of the immunodeficient mouse for 14 days. The results revealed that hydrogel-based groups showed better vascular like structure in the matrix (Fig. 3J and K).

### 3.3. Transcriptomic analysis of hPSCs-VSMCs from different methods

To investigate differential expression genes (DEGs), the whole transcriptome analysis by sequencing the mRNAs was performed including the undifferentiated H9s, VSMC\_2D, VSMC\_3D, VSMC\_F, and VSMC\_F-d6 (3 biological replicates for each group). Hierarchical clustering heat map showed all produced VSMCs clustered closely and were very different from the undifferentiated H9s (Fig. 4A). Principle components analysis (PCA) showed slightly DEGs among VSMC\_2D, VSMC\_F and VSMC\_F-d6, while VSMC\_3D displayed different gene expression at PC2 components compared

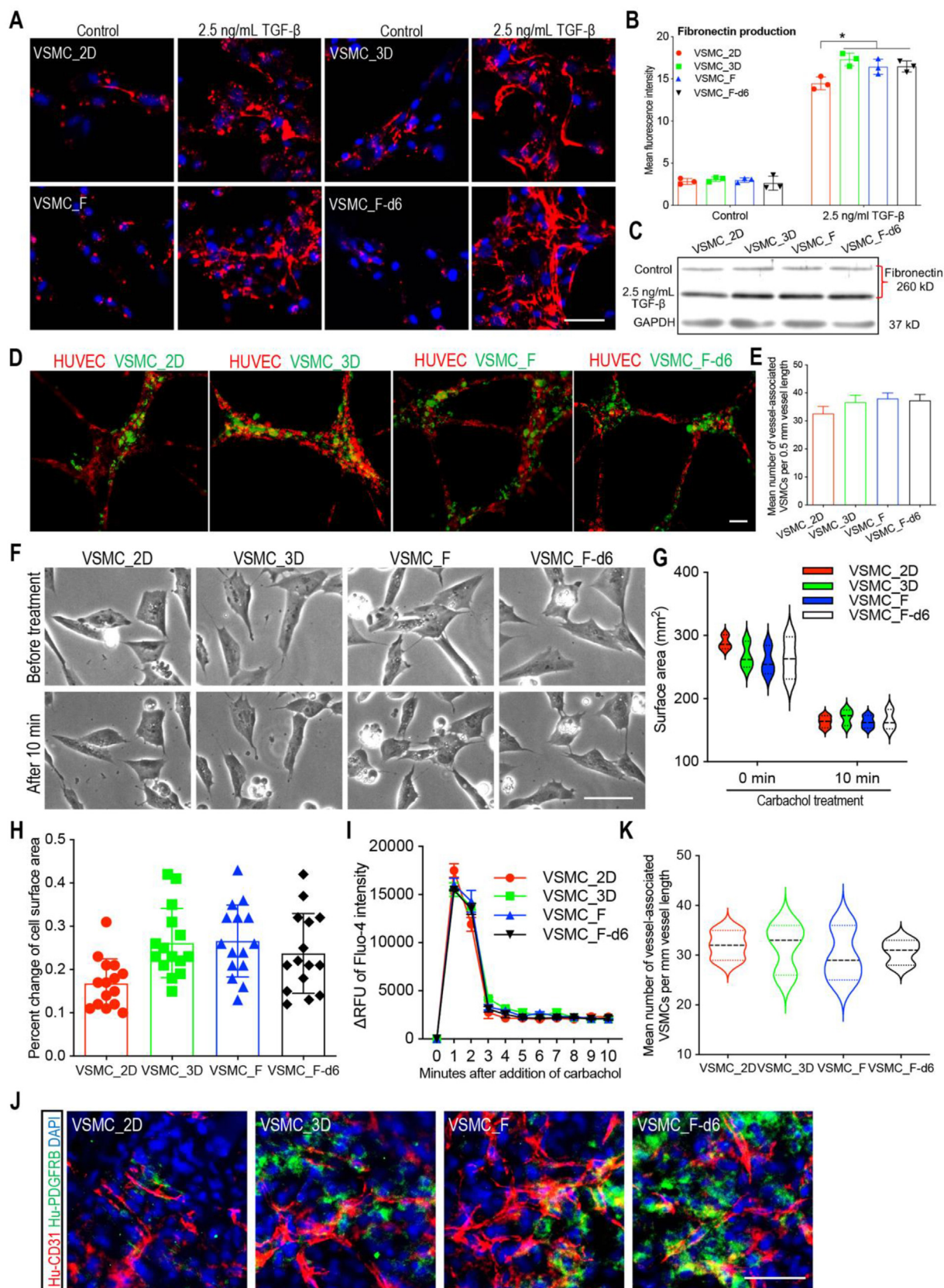




**Fig. 2. The comparison of hPSCs derived VSMCs in different culture systems** (A) Schematic illustration of the VSMC differentiation protocol (B) Phase images of hPSC (H9) in different culture systems. Scale bar, 200 μm (C) Live/dead staining of harvested cells from 2D, 3D-PEG and 3D-Alginate culture system (n = 3) (D) Phase images of hPSC-VSMCs on day 5 in different culture systems. Scale bar, 200 μm (E) Immunostaining analysis of VSMC markers SM22A and α-SMA on day 5 cells. Scale bar, 50 μm (F) Statistical analysis of differentiation efficiency for VSMC markers SM22A and α-SMA on day 5 cells (n = 3) (G, H) When seeded at 1.0 × 10<sup>6</sup> cells/mL, ~15-, ~24- and ~410-fold expansion, yielding ~1.5 × 10<sup>7</sup> VSMCs/mL, 2.4 × 10<sup>7</sup> VSMCs/mL PEG hydrogel and ~4.1 × 10<sup>8</sup> VSMCs/mL alginate hydrogel are produced in 2D, 3D-PEG and 3D-Alginate culture system, respectively. Data are presented as mean ± SD of three independent replicates (n = 3). \*\*p < 0.01, \*\*\*\*p < 0.0001.

to other VSMCs, but very different from H9s (Fig. 4B). The correlation coefficients between VSMC\_3D/VSMC\_2D, VSMC\_F/VSMC\_2D, VSMC\_F-d6/VSMC\_2D were >0.79, >0.78 and > 0.81 respectively, while among all groups, both VSMC\_F and VSMC\_F-d6 showed higher correlation coefficients, indicating they were

derived from 3D-alginate hydrogel method (Fig. 4C). Gene Ontology (GO) term showed that both VSMC\_3D/VSMC\_2D and VSMC\_F-d6/VSMC\_2D are enriched in vasculature development, blood vessel development, cardiovascular system development, angiogenesis, while VSMC\_F/VSMC\_2D are enriched in



**Fig. 3. Properties of hPSC-VSMCs made in different culture systems (A)** Immunostaining of fibronectin production of VSMC\_2D, VSMC\_3D, VSMC\_F and VSMC\_F-d6 after 24 h of 2.5 ng/mL TGF- $\beta$  treatment **(B)** Quantification of produced fibronectin. Data are represented as mean  $\pm$  SD (n = 3). Scale bar, 50  $\mu$ m **(C)** Western blot analysis of produced fibronectin. GAPDH was used as the control **(D, E)** Co-culture of VSMCs and HUVECs and statistical analysis of number of VSMCs attached to the vessels **(E)**. Scale bar, 50  $\mu$ m **(F–H)** Phase images **(F)**, surface area **(G)** and percent change of cell surface area (n = 15) **(H)** of VSMC\_2D, VSMC\_3D, VSMC\_F and VSMC\_F-d6 in response to carbachol treatment. Data are represented as mean  $\pm$  SD. Scale bar, 50  $\mu$ m **(I)** The relative fluorescence unit ( $\Delta$ RFU) of Fluo-4 loaded VSMCs over 10 min after adding carbachol. Data are represented as mean  $\pm$  SD (n = 3) **(J, K)**.



extracellular matrix organization, muscle tissue development, muscle organ development, endothelium development (Fig. 4D).

A Venn diagram showed that 157 genes and 3897 genes were significantly upregulated and downregulated in all VSMCs, respectively; and 165 upregulated genes and 665 downregulated genes in VSMC\_2D, VSMC\_F, and VSMC\_F-d6 samples; 123 upregulated genes and 256 downregulated genes in VSMC\_3D, VSMC\_F, and VSMC\_F-d6 samples; 53 upregulated genes and 201 downregulated genes in VSMC\_2D, VSMC\_3D, and VSMC\_F samples; 21 upregulated genes and 258 downregulated genes in VSMC\_2D, VSMC\_3D, and VSMC\_F-d6 samples (Fig. 5A and B), which drove us to perform differential gene expression analysis. Detailed gene expression of the RNA-Seq showed the following differences in VSMC\_2D, VSMC\_3D, VSMC\_F, and VSMC\_F-d6 according to regulation of VSMC adhesion, migration, proliferation and cytoskeletal arrangement [40]: (a) For collagens gene expression: COL3A1, COL1A2, COL24A1, COL12A1, COL25A1, COL22A1, COL16A1, COL15A1, COL14A1 were higher expression in VSMC\_F group compared to other two groups, indicating VSMC\_F may have great tensile strength; For laminins gene expression: LAMA1, LAMA4, LAMB4 and LAMA3 were co-downregulated among VSMC\_3D, VSMC\_F, and VSMC\_F-d6, but LAMB3, LAMC2 were only higher expression in VSMC\_3D, LAMC3 was only higher expression in VSMC\_F-d6, indicating VSMC\_3D may hold better adhesive capability (Fig. 5D); For integrins expression [40], ITGA10 and ITGA11 were co-upregulated among VSMC\_3D, VSMC\_F, and VSMC\_F-d6, while ITGA2 was higher expressed in VSMC\_3D and VSMC\_F, ITGA1 was higher expressed in VSMC\_F and VSMC\_F-d6, and ITGB3 was only higher expressed in VSMC\_3D and downregulated in VSMC\_F-d6 (Fig. 5E). For proteases gene expression: MMP9, MMP1, MMP16 were co-upregulated among VSMC\_3D, VSMC\_F, and VSMC\_F-d6; TIMP2, MMP19, TIMP3, MMP11, MMP17 were co-downregulated among VSMC\_3D, VSMC\_F, and VSMC\_F-d6; and other ECM components: DCN, THBS2, VWF, THSD1, NTN1, TNC were co-upregulated among VSMC\_3D, VSMC\_F, and VSMC\_F-d6, but EFEMP1, FBLN5 were higher expression in VSMC\_3D and VSMC\_F, and FN1 was higher expression in VSMC\_F, and VSMC\_F-d6 (Fig. 5F and G); (b) For glycolysis gene expression, ALDOA, ENO1, ENO2, HK2, PGK1, TPI1 were only higher expression in VSMC\_3D; PGAM2 was co-upregulated among VSMC\_3D, VSMC\_F, and VSMC\_F-d6, indicating that VSMC\_3D may display higher glycolysis activity (Fig. 5H); (c) For angiogenesis associated gene expression, EMCN, IGF1, DLL4, LMO2, NOTCH4, FGF10, SOX18, MMP9, NPR1, SOX17, PTGS2, IL1B, VEGFB were co-upregulated among VSMC\_3D, VSMC\_F, and VSMC\_F-d6; IL6, NOS3, PDGFA, TGFA were co-downregulated among VSMC\_3D, VSMC\_F, indicating their may have similar angiogenesis capability (Fig. 5I).

### 3.4. Marker gene expression of VSMC derived from different methods

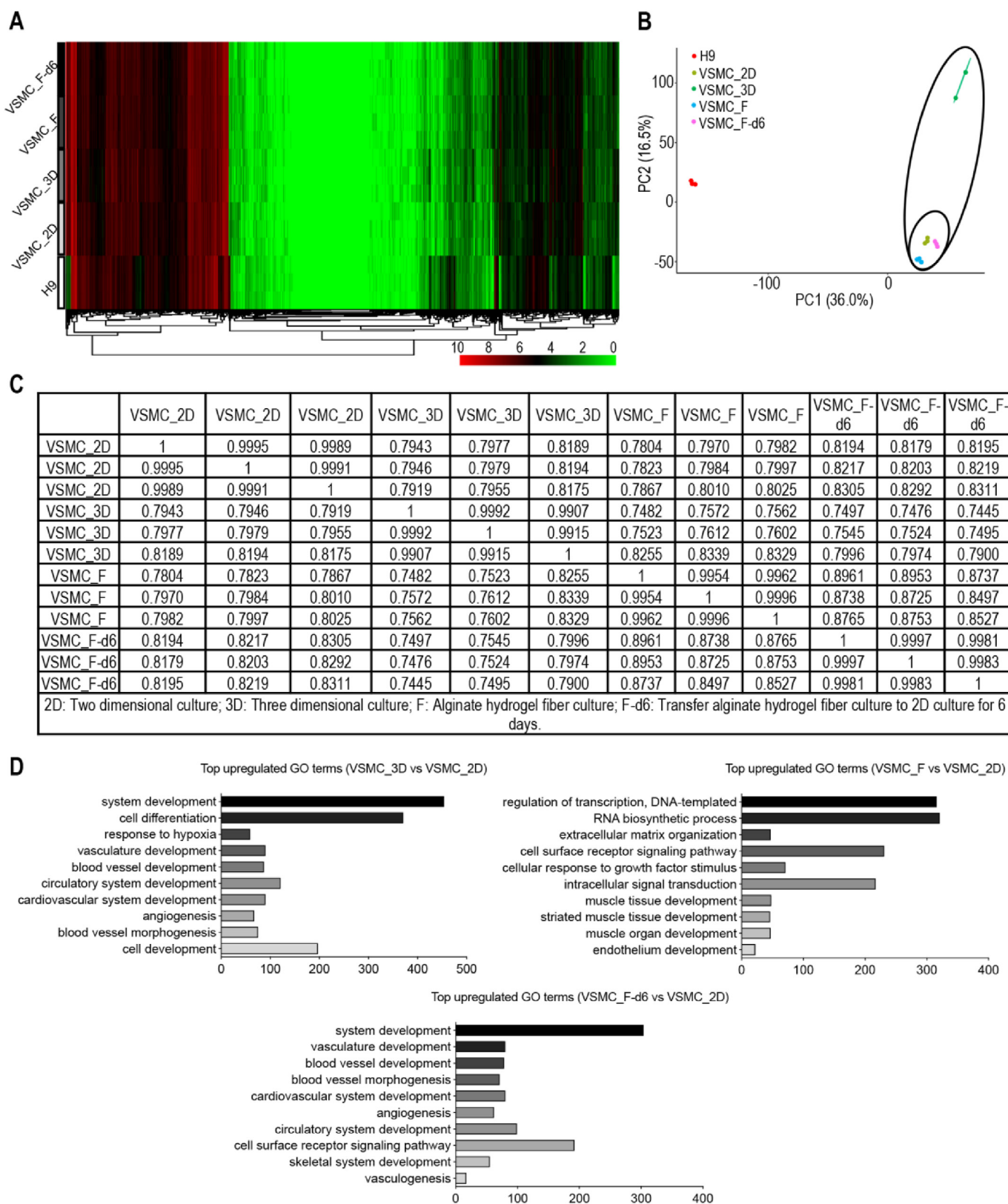
We also compared the differences of cell cycle, cell apoptosis and cell differentiation at transcriptional levels between VSMC\_3D, VSMC\_F and VSMC\_F-d6. The significantly co-upregulated genes BCL2, CDKN2A, CDKN2B, and the opposite gene expression E2F1, ARUKB, CDC25C which higher expression in VSMC\_F to promote cell proliferation were shown in cell cycle (Fig. 6A). The significantly upregulated genes BCL2, BNIP3, TNFRSF10A, and TNF were shown in cell apoptosis in VSMC\_F group, but CASP3 as executors of apoptosis was not obviously expressed in cell apoptosis (Fig. 6B). In cell differentiation, BMP2, BMP4, BMP5, FGF1 and OSCIN1 was

significantly upregulated in VSMC\_F (Fig. 6C). Finally, we confirmed some interesting genes expression by quantitative RT-PCR. We use qRT-PCR to quantitatively analyze several genes including  $\alpha$ -SMA, SM22A, Calponin, VEGFA, VEGFB, VEGFC, FN and COL4A. VSMC\_2D and VSMC\_F-d6 had higher expression of  $\alpha$ -SMA, SM22A and Calponin, and VSMC\_3D and VSMC\_F showed higher expression of Smoothelin (SMTN). VSMC\_3D and VSMC\_F had higher expression of VEGFA, VEGFC, FN and COL4A6 compared to VSMC\_2D and VSMC\_F-d6 (Fig. 6D). These results suggested that 3D conditions held relatively high proliferative capacity and functional phenotype, implicating their suitable for large-scale cell production.

## 4. Discussion

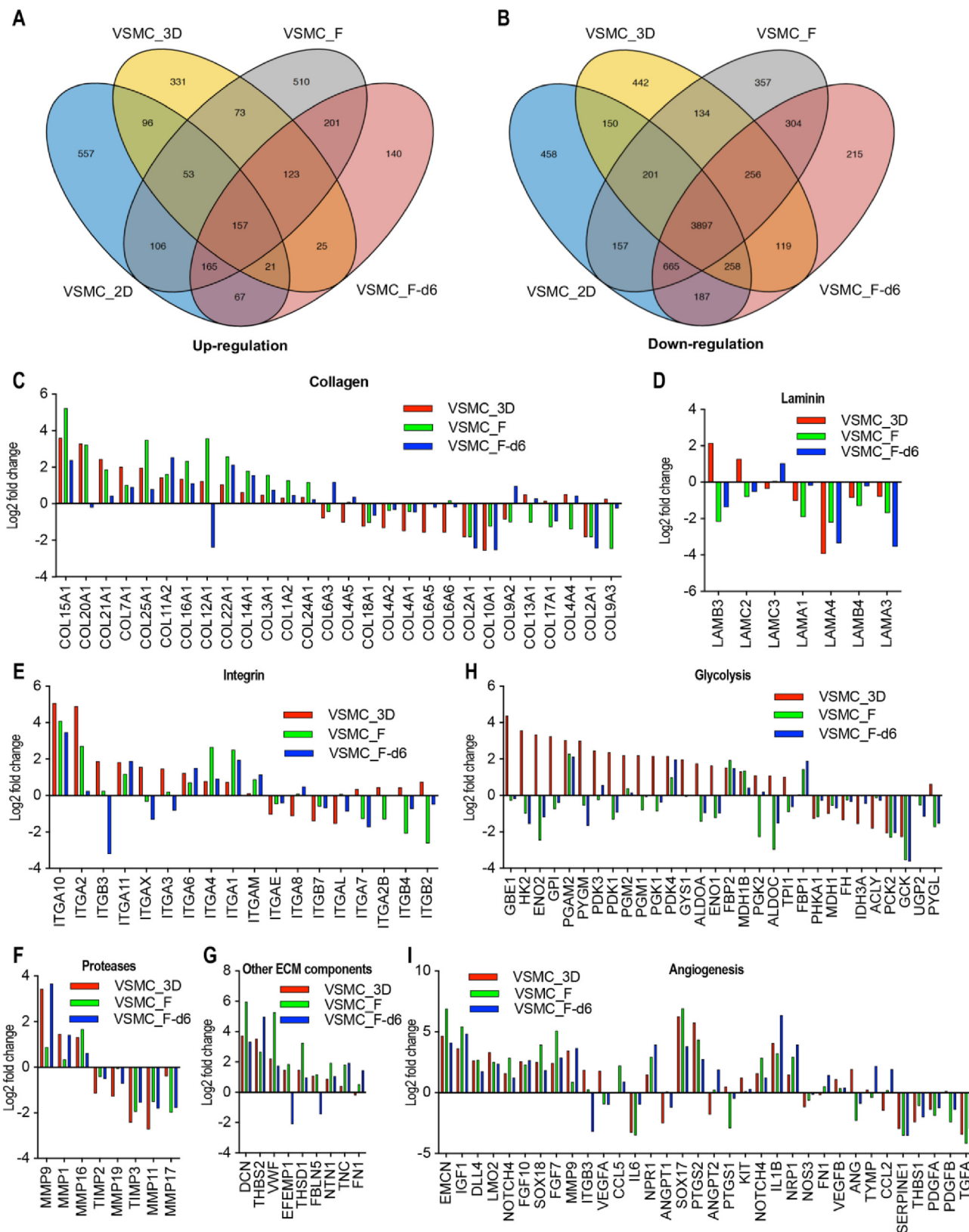
VSMCs are mainly obtained either in human tissues or *in vitro* culture. However, obtaining large numbers of primary VSMCs from human tissues are very challenging due to the ethical concerns. In addition, *in vitro* 2D culture is not suitable for some specific cell types because of proliferative limitation and phenotypic change, and small scale production due to its time consuming [27]. 3D suspension culturing leads to big cell agglomerates, leading to low cell production, cell death and spontaneous differentiation [27]. Thus, developing novel culture methods for large scale production of hPSCs and derivatives for biomedical applications are needed. In this study, we presented four different culture methods for hPSCs (Fig. 1) and their derivatives based on our previous reports [29,35–38], and systematically evaluated their functional properties and the whole transcriptomic changes. We found hPSC-VSMCs produced from four culture methods had similar contractile phenotypes and the potential of vasculature formation (Fig. 3). The transcriptomic analysis showed the hydrogel-based culture methods displayed more upregulated genes in vasculature development, blood vessel development and angiogenesis, extracellular matrix compared with 2D culture (Fig. 4). Unlike our previous studies, here we systematically compare the all aspects of different culture methods, including cell yield (Fig. 2), differentiation efficiency (Fig. 2), functions (Fig. 3), gene expression (Figs. 4–6), and to determine which is more suitable for industrial production in future.

3D PNIPAAm PEG hydrogel [33] and alginate hydrogel [38] are optimized to expand hPSCs and differentiate hPSCs into VSMCs based on our previous studies [29,35–38]. In this paper, we compared hPSC-VSMCs derived from four different culture methods. Why we used 3D PNIPAAm-PEG hydrogel for comparison with alginate hydrogel fiber in this study due to the fact that they both offer a number of promising features for GMP-compatible, large-scale culture. In particular, they are synthetic and defined, biocompatible, and enable cell harvest or passaging by simply changing temperature or EDTA treatment. They have the potential to translate into clinical application. And, the high culture efficiency of hydrogel-based culture methods are very attractive for large-scale and high-quality of cell production, especially for 3D alginate hydrogel fiber due to different dimension of microfiber and flat hydrogels. The flat hydrogel was greatly thicker than the microfibers, resulting in limited diffusion of oxygen and nutrients toward the thick hydrogels. The limited diffusion might lead to slow expansion of VSMCs. Although, it seems that further 2D culture after 3D differentiation has some beneficial effects, the process of this method is relatively complicated and expression of VSMC marker genes were not higher than 3D alginate hydrogel fiber. In addition, this method is not suitable for large-scale production in

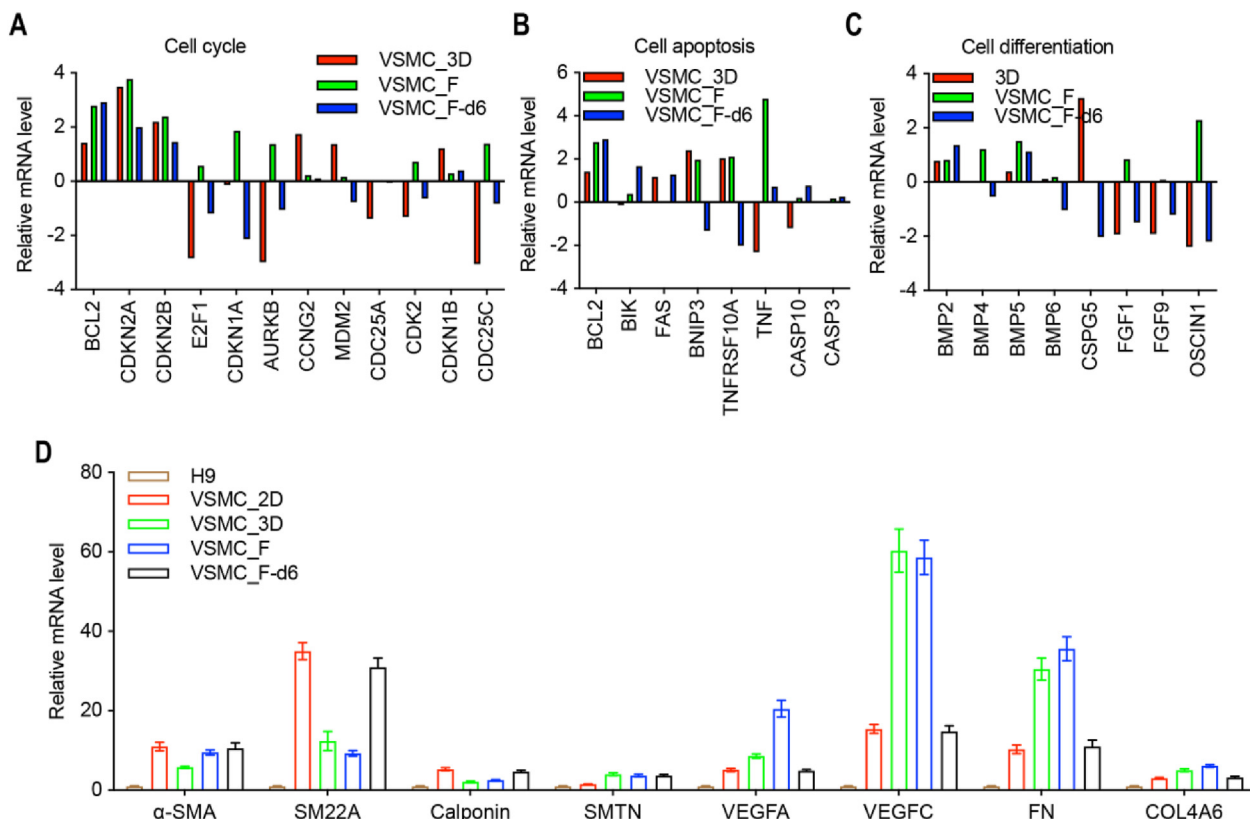


**Fig. 4. Whole transcriptome analysis VSMC\_F, VSMC\_F-d6, VSMC\_3D, VSMC\_2D derived from H9s (A, B)** Global heat map of expressed genes and Principal Component Analysis (PCA) of all VSMCs. Three biological replicates are used for each sample (n = 3) **(C)** The global gene expression correlation coefficients of all VSMC\_F, VSMC\_F-d6, VSMC\_3D, VSMC\_2D **(D)** Top 10 upregulated GO terms in VSMC\_3D, VSMC\_F and VSMC\_F-d6 group compared with VSMC\_2D, respectively.





**Fig. 5.** Differential gene expression analysis among all VSMCs derived from H9s (A, B) Venn diagram showing the up-regulated and down-regulated gene counts in VSMC\_2D, VSMC\_3D, VSMC\_F and VSMC\_F-d6 groups (C–G) Log<sub>2</sub> (expression level in VSMC\_3D, VSMC\_F and VSMC\_F-d6/expression level in VSMC\_2D) of extracellular matrix genes (H, I) Log<sub>2</sub> (expression level in VSMC\_3D, VSMC\_F and VSMC\_F-d6/expression level in VSMC\_2D) of genes related to glycolysis (H), and angiogenesis (I). In Fig. 5C–I, VSMC\_2D was as a control group.



**Fig. 6.** Differential gene expression analysis between hydrogel-based VSMCs derived from H9s (A–C) Log<sub>2</sub> (expression level in VSMC\_3D, VSMC\_F and VSMC\_F-d6/expression level in VSMC\_2D) of cell cycle (A), cell apoptosis(B) and cell differentiation (C). VSMC\_2D was as a control group (D) qRT-PCR analyses of VSMC\_2D, VSMC\_3D, VSMC\_F and VSMC\_F-d6 for synthetic VSMC markers  $\alpha$ -SMA, SM22A and Calponin, and contractile VSMC marker SMTN, and other genes related to VSMCs including growth factors VEGFA and VEGFC, and ECM genes FN and COL4A. Data are represented as mean  $\pm$  SD of three biological replicates (n = 3). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001.

industrial production. High quality cells have irreplaceable advantages in cell therapy, tissue engineering. The hydrogel-based culture methods eliminate large agglomeration and shear force. hPSC derived VSMCs produced in alginate hydrogel fiber ( $\sim 4.1 \times 10^8$  cells/mL) is about 200 times of cell yield in 3D suspension culturing and  $\sim 20$  times in 3D-PEG hydrogel culture system [15,30]. In addition, alginates are non-toxic and clinical-grade materials, affordable and available [41]; mechanically and chemically stable, transparent, and alginate fibers provide very friendly microenvironment to prevents cells agglomeration. In addition, previous studies reported that high cell density affected the stemness genes expression [42] and cell behavior [43] due to over aggregation of cells resulting in apoptosis/necrosis and uncontrolled differentiation in the core of cell aggregates. In our study, we demonstrated that 3D culture methods, especially for 3D alginate hydrogel, limited the over aggregation by controlling the size, which not only efficiently transport the nutrients and oxygen, but also lead to high culture efficiency. It is the innovation of cell culture technology. Therefore, this study provided the direct evidence for alginate hydrogel fiber used in industrial production.

## 5. Conclusion

In summary, we systematically compared hPSC-VSMCs generated from four different culture methods, and demonstrated that alginate hydrogel-based culture method will become a versatile culture system for hPSCs and their derivatives in various biological applications and industrial production.

## Author contributions

Conceptualization, Yuguo Lei, Zhanqi Wang and Haishuang Lin; Data curation, Qing Liu, Hongyu Gu and Xuesheng Wu; Formal analysis, Hongyu Gu, Yuxia Ge, and Xuesheng Wu; Funding acquisition, Zhanqi Wang, Qing Liu and Fuxing Zuo; Methodology, Yuxia Ge and Zhen Liu; Project administration, Qing Liu and Hongyu Gu; Software, Qian Du; Writing – original draft, Zhanqi Wang and Haishuang Lin; Writing – review & editing, Haishuang Lin, Zhen Liu and Fuxing Zuo. All authors have read and agreed to the published version of the manuscript.

## Data availability

The authors declare that all data supporting the results in this study are available within the paper.

## Declaration of competing interest

The authors declare no competing financial interest.

## Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 82000449), Basic-Clinical Research Cooperation Fund of Capital Medical University (No. 16JL90) and CAMS Innovation Fund for Medical Science (CIFMS) (2020-I2M-C&T-B-071).

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