Journal of Biological Engineering

RESEARCH

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Serum-free endothelial cell culture medium for vascular smooth muscle cells sheet formation

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Abstract

Background Cell sheet technology has been identified as a promising approach for the construction of tissue-engineered vascular grafts (TEVGs). However, concerns regarding immunogenicity and ethical issues, which are raised by the use of fetal bovine serum (FBS) in traditional culture systems, limit its potential for clinical translation. Serum-free medium (SFM) has emerged as a safer and more controllable alternative, but further validation is required to determine its effectiveness and superiority in generating high-quality cell sheets.

Methods This study systematically compared cell sheets generated under SFM and 10% FBS culture conditions in terms of structure, cellular phenotype, and functional properties. The expression levels of α-SMA and SM22, markers of vascular smooth muscle cells(VSMCs), were evaluated using immunofluorescence staining, qRT-PCR, and Western blot analysis to assess cellular phenotype. Histological staining and mechanical testing were employed to compare the morphology and mechanical properties of the cell sheets, while extracellular matrix (ECM) deposition and biochemical characteristics were also analyzed.

Results Under SFM conditions, cells exhibited significantly higher α-SMA and SM22 expression levels (qRT-PCR showed a 1.8-fold and 2-fold increase, respectively; $^{****}p < 0.0001$) with clearer cytoskeletal arrangement. Cell sheets formed in SFM displayed comparable area(ns, $p > 0.05$), thickness($^{**}p < 0.01$), and mechanical properties to those cultured in 10% FBS, while ECM deposition was significantly enhanced (collagen content increased by approximately 40%, $^{**}p < 0.01$). Furthermore, histological analysis revealed that cell sheets generated under SFM conditions were more compact and uniform, exhibiting superior structural organization.

Conclusion SFM facilitates the generation of cell sheets that exhibit structural and functional properties analogous to those cultured in FBS. Additionally, SFM promotes cellular phenotype transition and ECM deposition. Consequently, SFM provides a safer, more controllable, and clinically translatable solution for cell sheet construction.

Keywords Serum free, Cell sheet, Vascular smooth muscle cells, Extracellular matrix, Tissue-engineered vascular grafts

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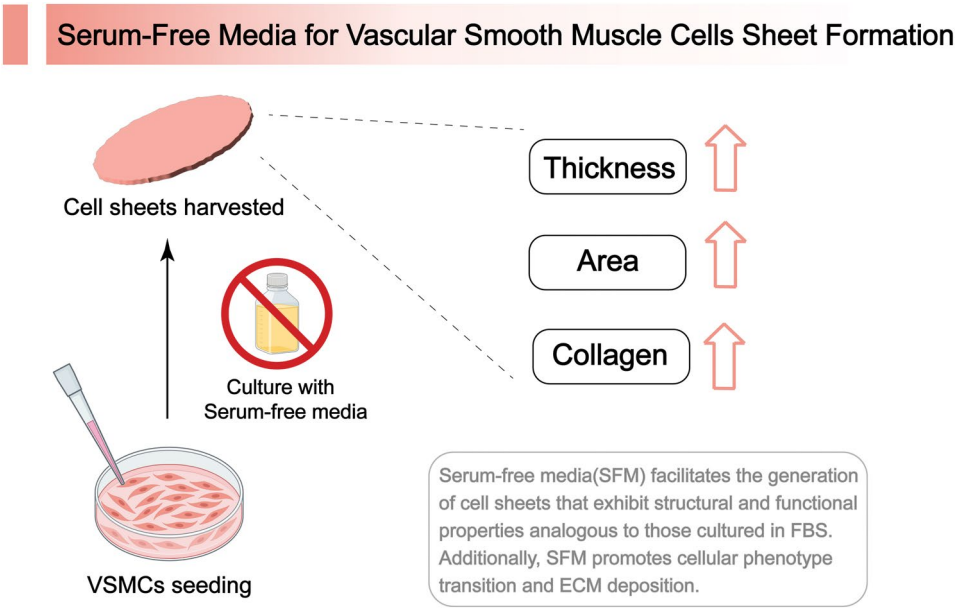
Graphical Abstract



Introduction

Cell sheet technology has been demonstrated to be an effective approach for constructing tissue-engineered vascular grafts (TEVGs). The extracellular matrix(ECM), which is characterized by a stable structure, is obtained through this method and provides crucial support for successful TEVGs construction [1–3]. The formation of cell sheets necessitates the cultivation of densely packed cells. Fetal bovine serum(FBS) has been a traditional component in cell culture, facilitating proliferation and ECM deposition [4, 5]. However, FBS contains undefined components and can lead to immunogenicity, pathogen contamination, and ethical concerns, which impose significant limitations on its use [4, 6, 7]. Consequently, there has been a significant focus on the development of alternative culture conditions, such as serum-free medium (SFM), to facilitate the efficient and clinically applicable preparation of cell sheets for TEVGs.

Recent studies have explored the potential of serum-free culture systems in cell cultivation. For example, it has been reported that serum-free conditions can modulate fibroblast behavior, enhancing ECM deposition while reducing adverse inflammatory responses [8]. Furthermore, it was demonstrated by Werkmann et al. that SFM promotes more controllable and reproducible cellular phenotypes, thereby reducing batch-to-batch variability associated with FBS [9]. Despite these advantages, transitioning from FBS to SFM remains challenging, as the removal of serum often weakens cell proliferation, survival, and ECM production, all of which are crucial for cell sheet formation.

To address these challenges, serum-free formulations have been optimized for specific cell types and applications. Vascular smooth muscle cells (VSMCs) are commonly utilized in TEVGs due to their critical role in vascular mechanics and ECM production [2, 10]. Studies have shown that SFM can induce a more contractile and aligned phenotype in VSMCs. In addition, key markers of VSMCs, such as α-SMA and SM22, are typically upregulated under serum-free conditions, further supporting the notion that SFM promotes the ideal cellular phenotype [11–13]. However, the specificity of SFM for different cell types remains a significant challenge, as different applications require distinct formulations. Currently, no SFM has been specifically developed for the cultivation of VSMCs and their cell sheets. Whether SFM can satisfy the growth requirements of VSMCs and generate cell sheets with mechanical and biological properties comparable or superior to those produced in FBS is still an open question.

Cell sheet technology preserves the ECM secreted by VSMCs, offering a scaffold-free tissue engineering strategy. This study validated the ability of SFM to facilitate cell sheet formation, supporting its potential application in TEVGs [14, 15]. The serum-free medium used in this study is an endothelial cell culture medium (C140JV, BasalMedia, Shanghai Yuanpei Biotechnology). The aim of this study is to evaluate the potential of SFM in generating functional cell sheets. By comparing the structural, biochemical, and functional characteristics of cell sheets formed under SFM and 10% FBS conditions, the study investigates whether SFM can serve as a feasible

alternative to traditional serum-based culture systems. The ultimate goal of this research is to provide new insights into the development of safer and more clinically translatable cell sheet technologies, thus advancing the field of vascular tissue engineering.

Methods

Cell isolation and culture

VSMCs were isolated from the thoracic aorta of neonatal calves. Neonatal bovine VSMCs were selected for their accessibility and low cost, aiming to provide a viable cell source for the scalable production of TEVGs, with their suitability substantiated by experimental results. After anesthesia and euthanasia of the calves, the thoracic aorta was aseptically removed. After washing with PBS, the medial layer of the aorta was taken and placed onto culture dishes. The cells were cultured in DMEM/F12 medium(Gibco, USA) containing 10% FBS(Gibco, USA) at 37 °C in a 5% CO2 incubator. The medium was changed every 3 days, and after 21 days, cells migrated out from the tissue explants. The harvested cells were considered to be P0 generation. All cells used in this study were from passages 4–7.

The serum-free medium used in this study was an endothelial cell complete medium (C140JV, BasalMedia), purchased from BasalMedia (Shanghai, China). C140JV is a serum-free medium designed for endothelial cells. It contains insulin-transferrin-selenium(ITS) and human platelet lysate (HPL). Among them, insulin supports the proliferation of VSMCs and the synthesis of the extracellular matrix (ECM), and HPL provides a variety of growth factors, the specific components remain to be further analyzed.

Cell proliferation assay

Cell proliferation was assessed using the CCK-8 assay (Dojindo, Japan). VSMCs were seeded in 96-well plates at a density of 2,000 cells per well. According to the manufacturer's instructions, CCK-8 reagent was added on days 0, 1, 3, 5, and 7, followed by incubation at 37 °C in the dark for 4 h. Absorbance was measured at 450 nm, and the relative proliferation rate of the cells was calculated.

EdU incorporation assays (Beyotime, China) were performed to assess cell proliferation. VSMCs were seeded in 12-well plates at a density of 40,000 cells per well. After 2 days of culture, the cells were incubated with EdU-containing medium for 2 h in the dark. Following fixation, permeabilization, and staining according to the manufacturer's instructions, images were captured using a fluorescence microscope (Zeiss, Germany).

Hydroxyproline detection

Hydroxyproline content was measured using a hydroxyproline assay kit (Solarbio, China) according to the manufacturer's instructions. Briefly, 100 μL of 6 M HCl was added to the samples and incubated at 110 °C for 4 h. The pH was adjusted to 6–8 with 10 mM NaOH, and the final volume was adjusted to 200 μL. The detection solution was added, and absorbance was measured at 560 nm to calculate the hydroxyproline content.

Collagen content(μg) = Hydroxyproline content(μg) * 100/13.5 [16, 17].

Immunofluorescence

Cells were seeded at a density of 10,000 cells per well in a 12-well plate and cultured for 3 days. After incubation, the cells were washed with cold PBS and fixed with 4% paraformaldehyde for 15 min. Permeabilization was performed using 0.25% Triton X-100 (Regent, China), followed by blocking with 5% BSA (biofroxx, China) at room temperature for 1 h. The cells were then incubated overnight at 4 °C with primary antibodies against collagen I (1:200, 14695-1-AP), collagen III (1:300, 22734-1-AP), α-SMA (1:300, 14395-1-AP), and SM22 (1:300, 10493-1-AP), all purchased from Proteintech (USA). Afterward, the cells were incubated with DyLight 594 (Abbkine, USA) or DyLight 488 (Abbkine, USA) secondary antibodies at room temperature for 1 h. Finally, the samples were stained with an anti-fade solution containing 4′,6-diamidino-2-phenylindole (DAPI, Beyotime, China). Images were captured using a confocal microscope (Zeiss LSM980, Germany).

Western blot

Cells were lysed using RIPA buffer (Biocharp, Canada) and mixed with sample buffer (Invitrogen, USA) and antioxidants (Invitrogen). Protein samples were subjected to electrophoresis using a 10% pre-cast gel (Biovigen, China) in a Bio-Rad electrophoresis system. Proteins were then transferred to a PVDF membrane (Merck Millipore, Germany) and blocked with non-fat milk. The membrane was incubated overnight with primary antibodies against α-SMA (1:20,000, 14395-1-AP, Proteintech, USA) and SM22 (1:5,000, 10493-1-AP, Proteintech, USA), followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (1:20,000, Abbkine, China). Tubulin (1:10,000, 66031-1-Ig, Proteintech, USA) was used as the loading control.

qRT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. cDNA was synthesized from total RNA using the PrimeScript RT reagent kit (TaKaRa, Japan). Quantitative real-time PCR (qRT-PCR) was performed using Hieff qPCR SYBR Green Master Mix (YEASEN, China) and the qTOWER3G Real-Time PCR System (Jena, Germany). The following cycling conditions were used: 95°C for 5

minutes, 95°C for 10 seconds, and 60°C for 30 seconds for 40 cycles. β-actin was used as the internal control for quantitative mRNA. The sequence of primers used is as follows: α-SMA(F: 5'-AAGTACTCTGTCTGGATTGG TG-3'; R: 5'- GTGTCTTAGAAACATTTGCGGT-3'), SM22(5'-ATGTTCCAGACCGTTGACCT-3'; 5'-CCTC TTATGCTCCTGGGCTT-3'), β-actin(5'-TTTGATGAC GATGAGCTGCG-3'; 5'-AGCGCAAGTACTCTGTGTG G-3').

Glucose consumption and lactate production

Glucose Consumption: Culture medium supernatant was collected, and glucose consumption was measured according to the manufacturer's instructions (Nanjing Jianchen Bioengineering Institute, China). A 2.5 μL sample was mixed with 250 μL working solution and incubated in the dark for 10 min. Absorbance was measured at 505 nm, and glucose consumption was calculated.

Lactate Production: Lactate production was measured following the manufacturer's instructions (Nanjing Jianchen Bioengineering Institute, China). The sample was mixed with the working solution and color reagent, incubated in the dark at 37 °C for 10 min, and then the termination solution was added. Absorbance was measured at 530 nm.

LDH assay

Cell lysates were collected and the supernatant was used for the LDH assay according to the manufacturer's instructions (Abbkine, USA). The sample was mixed with the working solution and incubated in the dark at 37 °C for 30 min. Absorbance was measured at 450 nm.

NAD+/NADH measurement

Cells were lysed using NAD+/NADH extraction buffer (Beyotime, China), and part of the supernatant was mixed with the working solution and incubated at 37 °C in the dark for 10 min. Absorbance was measured at 450 nm to determine the total NAD + and NADH content. The remaining supernatant was incubated at 60 °C for 30 min to decompose NAD+, and the incubation and absorbance measurement steps were repeated to determine the NADH content.

Cell sheet cultivation and harvesting

Cells were seeded at a density of $15 \times 10\text{^}4/cm^2$ in standard culture dishes (Sorfa, China) and temperature-sensitive culture dishes (Thermo Fisher, USA), with the addition of 50 mg/mL ascorbic acid. In the standard culture dishes, ITS (insulin-transferrin-selenium) was also added. The culture medium was changed every 2 days, and cells were cultured for 7 days. Images were captured prior to harvesting using a phase contrast microscope (model, country). Afterward, the TRCD was placed at 20 °C for 30 min to allow the cell sheet to detach automatically. The cell sheets were then harvested by gently scraping the edge of the culture dish with a pipette. The area of the harvested cell sheets was quantified using Image J software.

Histological analysis

The harvested cell sheets were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned into 4 μm thick slices. Hematoxylin and eosin (H&E) staining and Masson's trichrome staining were performed using conventional methods. Images were captured using a fluorescence microscope (NIKON, Japan).

Live/dead staining assay

Cell viability of the harvested cell sheets was assessed using the Calcein/PI cell viability and cytotoxicity detection kit (Beyotime, China). The harvested cell sheets were washed with PBS (Gibco, USA) and incubated with the prepared working solution at 37 °C in the dark for 30 min. Images were then captured using a confocal microscope (Zeiss LSM980, Germany).

Young's modulus measurement

The young's modulus of the cell membrane patches was measured using an atomic force microscope (AFM) test (Bruker, Germany). Firstly, the probe was calibrated with a standard sample. A silicon nitride probe with a conical tip (MLCT-E, Bruker) was used, which had an elastic coefficient of 0.1 N/m and a resonance frequency of 38 kHz. In the Peak-Force tapping mode, 5 regions were randomly selected for testing in each sample, and each region was composed of a 256×256 point grid. The collected force curves were fitted and calculated by the software NanoScope to obtain the average Young's modulus of the selected regions.

Statistical analysis

Data analysis was performed using GraphPad Prism software (version 9.5.1, USA). All results represent at least three independent experiments. Data are expressed as SD ± SEM. For comparisons between two experimental groups, an unpaired t-test was used. For comparisons involving multiple groups, one-way analysis of variance (ANOVA) was applied. All experiments repeat for thrice($n = 3$). A p-value ≤ 0.05 was considered statistically significant. The following symbols represent significance levels: $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$, $^{****}p \le 0.0001$. p-values > 0.05 were considered not statistically significant (ns).

Result

SFM promotes cell sheet formation

To evaluate the effect of SFM on cell sheet formation, P5 VSMCs were cultured under 10% FBS and SFM conditions for 7 days, and cell sheets were harvested. The macroscopic structure, microscopic structure, and area characteristics of the cell sheets were observed. The harvested cell sheets under both conditions appeared intact, displaying a uniform, thin-film structure, and their diameters were comparable (Fig. 1a). Further morphological examination under an optical microscope (Fig. 1b) revealed that the cells in both 10% FBS and SFM conditions were evenly and tightly arranged, with no significant differences, indicating that SFM maintained the cell density and arrangement in the cell sheets. Further histological analysis of the cell sheets was conducted using H&E and Masson staining. The results showed that the tissue structure of the cell sheets was uniform and compact, with an even distribution of cells under both culture conditions (Fig. 1c). Masson staining indicated no significant differences in the ECM density between the two groups, while the cell sheets cultured in SFM were thicker (Fig. 1d). The measurement of the cell sheet area further supported these observations, with the cell sheets under SFM showing a slightly larger area than those under 10% FBS, although the difference was not statistically significant (Fig. 1e, n.s, $p > 0.05$). Meanwhile, the cell sheet formed in the SFM was significantly thicker

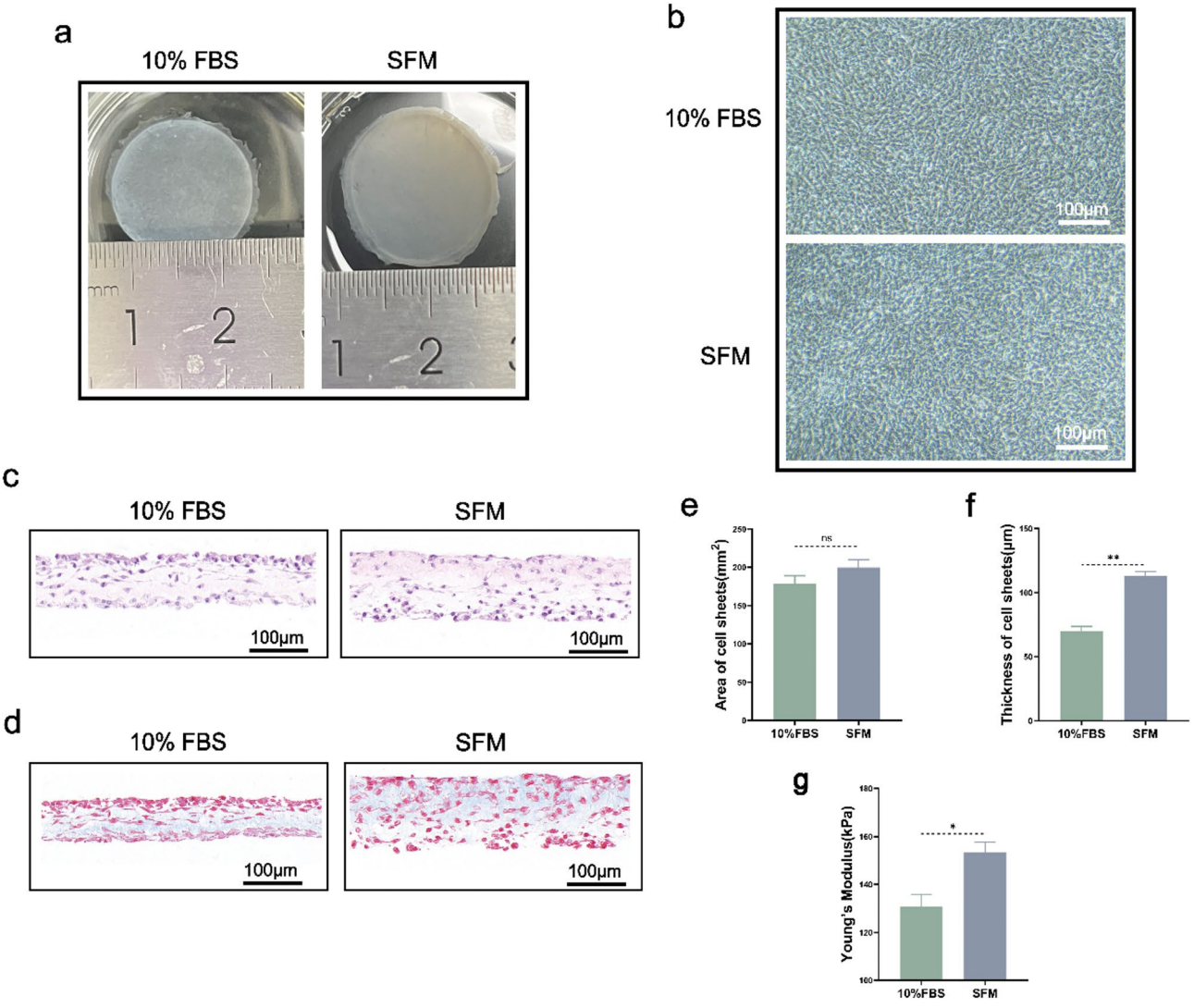


Fig. 1 Serum-free medium promotes cell sheet formation. After 7 days of culture with different medium, the appearance of the cell sheets (**a**) and cell morphology under optical microscope (**b**) were observed. Histological staining results of the cell sheets were performed using Masson's trichrome (**c**) and Sirius Red staining (**d**). The area of the cell sheets harvested from both culture medium (**e**) shows that the cell sheets formed in SFM were slightly larger than those formed in 10% FBS, but no significant difference was observed (ns, $p > 0.05$). (**f**) The thickness of cell sheets in the SFM group was significantly higher than that in the 10% FBS group(**$p \le 0.01$). (**g**) The storage modulus of cell sheets in the SFM group was also higher(*$p \le 0.05$)

than that in the 10% FBS group (Fig. 1f, $^{**}p \le 0.01$). Correspondingly, the Young's modulus of the cell sheet in the SFM was also higher than that in the 10% FBS group (Fig. 1g, [153.27 ± 9.89 kPa] vs. [130.884 ± 11.22 kPa], $^{*}p \le 0.05$). These results suggest that under serum-free conditions, cells can maintain good cell sheet formation ability, and the macroscopic and microscopic structures of the sheets are similar to those formed in traditional 10% FBS medium.

SFM maintains cell phenotype

Immunofluorescence staining results showed that compared to the 10% FBS culture condition, the fluorescence signals of α-SMA and SM22 were significantly enhanced in cells cultured in SFM, suggesting an upregulation of these markers under serum-free conditions, with a clearer arrangement of the cytoskeleton (Fig. 2a and b, $^{**}p \le 0.01$, $^{***}p \le 0.001$). qRT-PCR results further confirmed this, showing that the mRNA expression levels of α-SMA and SM22 were significantly increased by

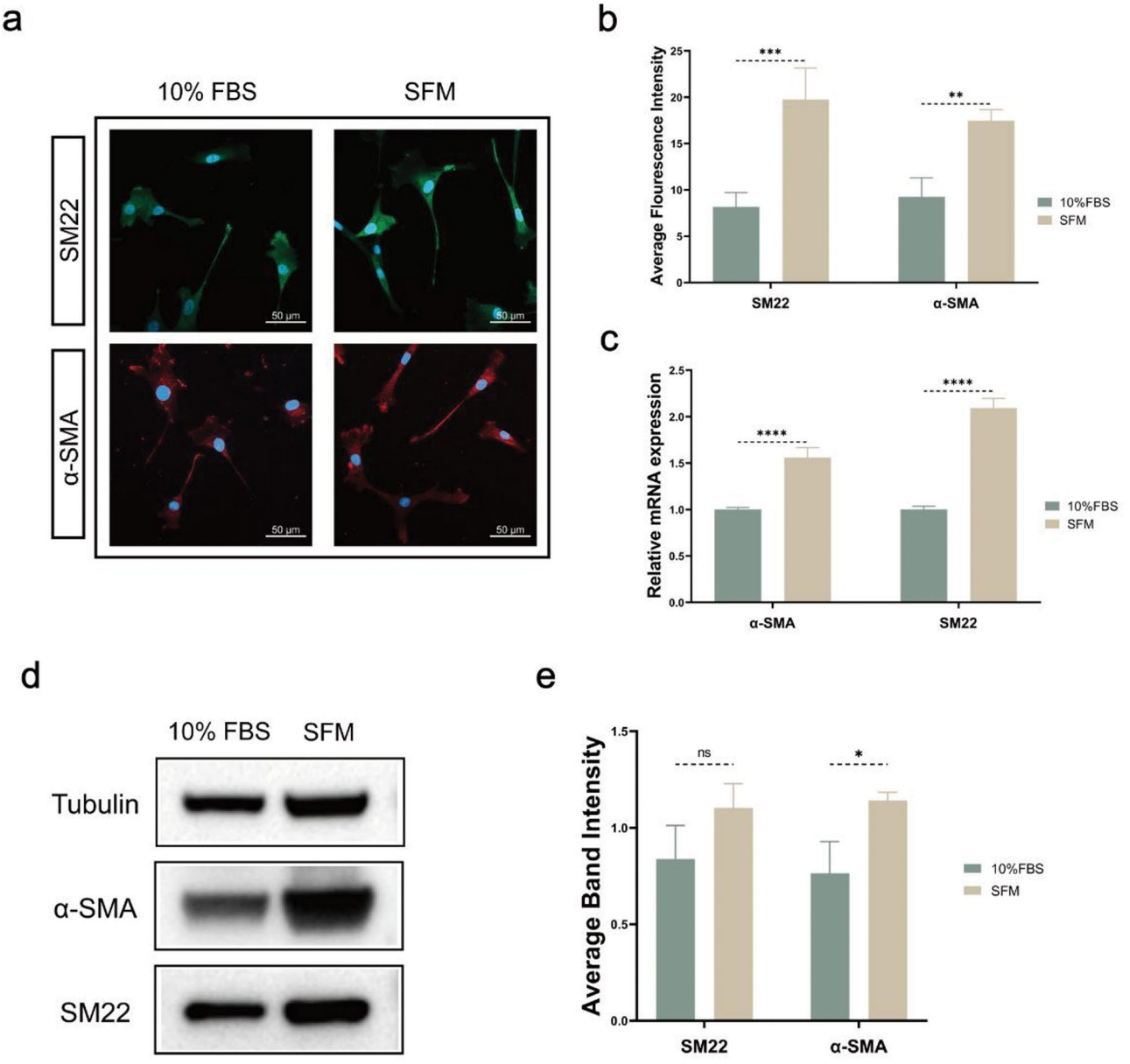


Fig. 2 Serum-free medium maintains cellular phenotype. After culturing VSMCs for 3 days in SFM and 10% FBS, the expression of cellular phenotype markers (SM22, α-SMA) was assessed. (**a**, **b**) Immunofluorescence results showed stronger fluorescence in the SFM group($^{**}p \le 0.01$, $^{***}p \le 0.001$). (**b**) qRT-PCR results also indicated that the gene expression levels of the markers were significantly higher in the SFM group ($^{****}p \le 0.0001$). (**c**, **d**) Western blot results showed a significant increase in α-SMA protein expression ($^{*}p \le 0.05$), while SM22 expression was slightly higher but not statistically significant (ns, $p > 0.05$)

approximately 1.8-fold and 2-fold, respectively, under SFM conditions, with statistical significance (Fig. 2c, $^{****}p \leq 0.0001$). Western blot analysis demonstrated that the protein expression levels of α-SMA and SM22 were significantly higher in the SFM group compared to the 10% FBS group, with a particularly notable change in α-SMA expression. Semi-quantitative analysis of the Western blot bands further showed that α-SMA protein expression was significantly higher under SFM conditions compared to 10% FBS ($^{*}p \leq 0.05$), while the increase in SM22 expression was slight and not statistically significant (Fig. 2d, e and n.s, $p > 0.05$). These results collectively suggest that serum-free conditions can induce the upregulation of α-SMA and SM22 expression, potentially promoting the associated cellular phenotype transition.

SFM promotes VSMCs proliferation

To evaluate the impact of culture conditions on cell proliferation and survival, several experimental analyses were performed under 10% FBS and SFM conditions. EdU staining (Fig. 3a) revealed that cells under both conditions exhibited strong proliferative capacity, with green fluorescent signals (EdU-positive cells) distributed similarly in both groups, indicating that serum-free conditions did not significantly affect cell proliferation. CCK-8 assay results (Fig. 3b and c) showed that the OD450 values gradually increased over time under both culture conditions, indicating that cell proliferation increased with time. However, at days 1, 3, and 5, the relative cell proliferation rate in the SFM group was slightly higher than that in the 10% FBS group, although no statistically significant difference was observed(ns, $p > 0.05$). Calcein/PI double staining assay (Fig. 3d) further evaluated

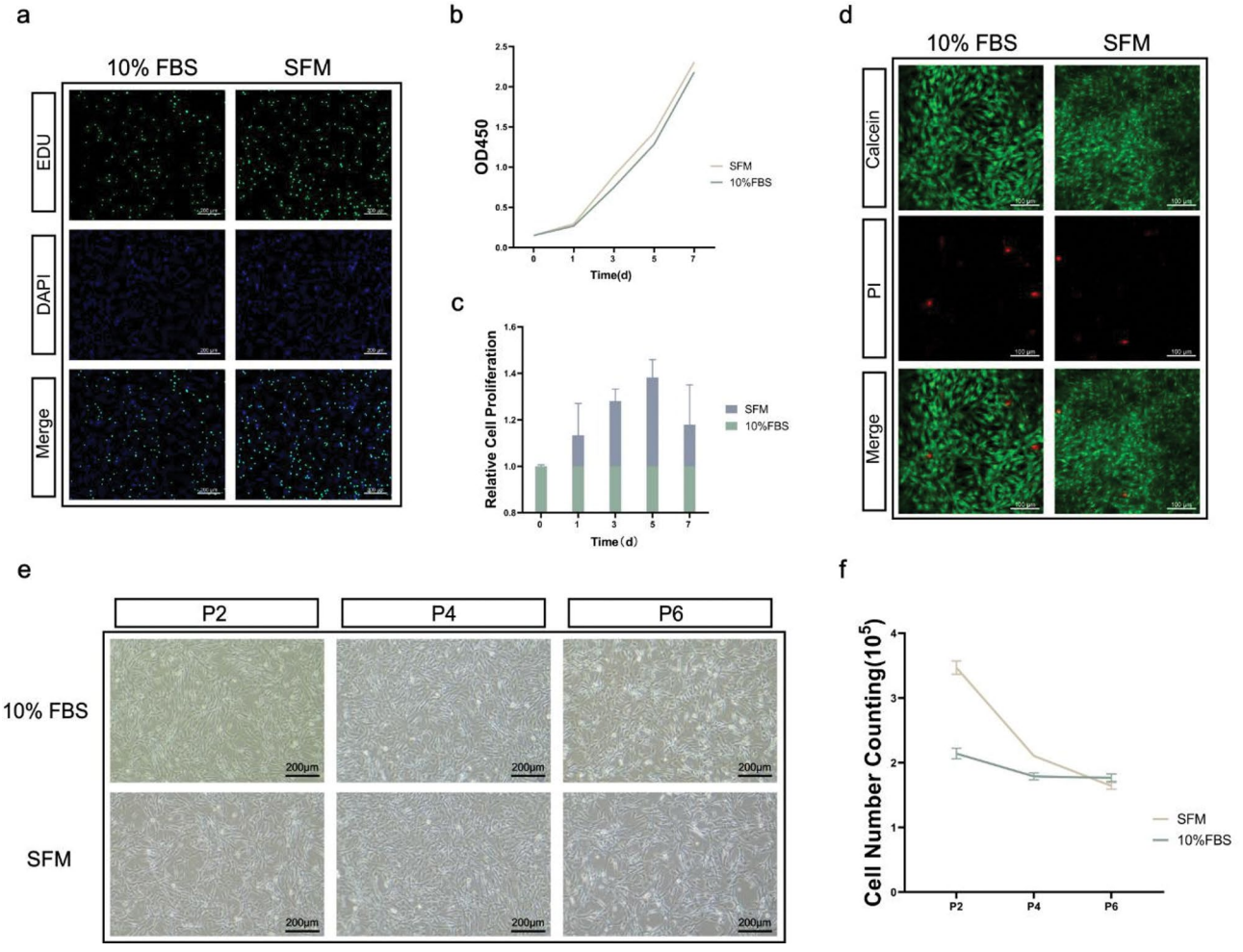


Fig. 3 Serum-free medium promotes VSMC proliferation. (**a**) EdU staining was used to observe cell proliferation activity under both culture conditions; EdU-positive cells (green) indicate proliferating cells. (**b**, **c**) Cell proliferation was assessed at different time points (0, 1, 3, 5, 7 days) by CCK-8 assay. The proliferation activity of cells in the SFM group was not lower than that in the FBS group at any time point. (**d**) Cell survival was assessed using Calcein/PI double staining, with Calcein (green) indicating live cells and PI (red) indicating dead cells. The morphological changes of cells at different passages (P2, P4, P6) (**e**) and the change in cell number at different passages (**f**) were also evaluated. Cells in the SFM group were more affected by the passage number than those in the FBS group

cell viability and apoptosis. The results showed that the proportion of live cells labeled with Calcein was high in both 10% FBS and SFM conditions, while the proportion of apoptotic cells stained with PI was low, indicating that both conditions were able to maintain a high survival rate. Passage results (Fig. 3e and f) demonstrated that while cell numbers decreased more rapidly with increasing passage number under SFM conditions, comparable numbers to the 10% FBS group were maintained until passage 6.These results suggest that the serum-free medium slightly promotes cell proliferation but generally reaches the same level as the 10% FBS complete medium.

SFM promotes ECM secretion

To assess the impact of different culture conditions on collagen synthesis and expression, the levels of collagen I (COL1) and collagen III (COL3) in cells cultured under 10% FBS and SFM conditions were systematically analyzed. Immunofluorescence staining (Fig. 4a) showed that COL1 and COL3 were expressed in both groups, with significantly higher fluorescence intensity in the SFM group(Fig. 4d, $*p \le 0.05$, $**p \le 0.01$), indicating that serum-free conditions may promote the expression of these two key collagen proteins. At the protein level, Western blot analysis of COL1 and COL3 expression (Fig. 4b) further confirmed the findings from immunofluorescence staining. Quantitative analysis (Fig. 4c) revealed that COL1 and COL3 protein expression was significantly higher in the SFM group compared to the 10% FBS group ($*p \le 0.05$). Additionally, at the gene expression level, qRT-PCR analysis (Fig. 4e) showed that the mRNA expression of COL1A1 and COL3A1 was significantly upregulated under SFM conditions ($****p \le 0.0001$ and $***p \le 0.001$), suggesting that serum-free culture

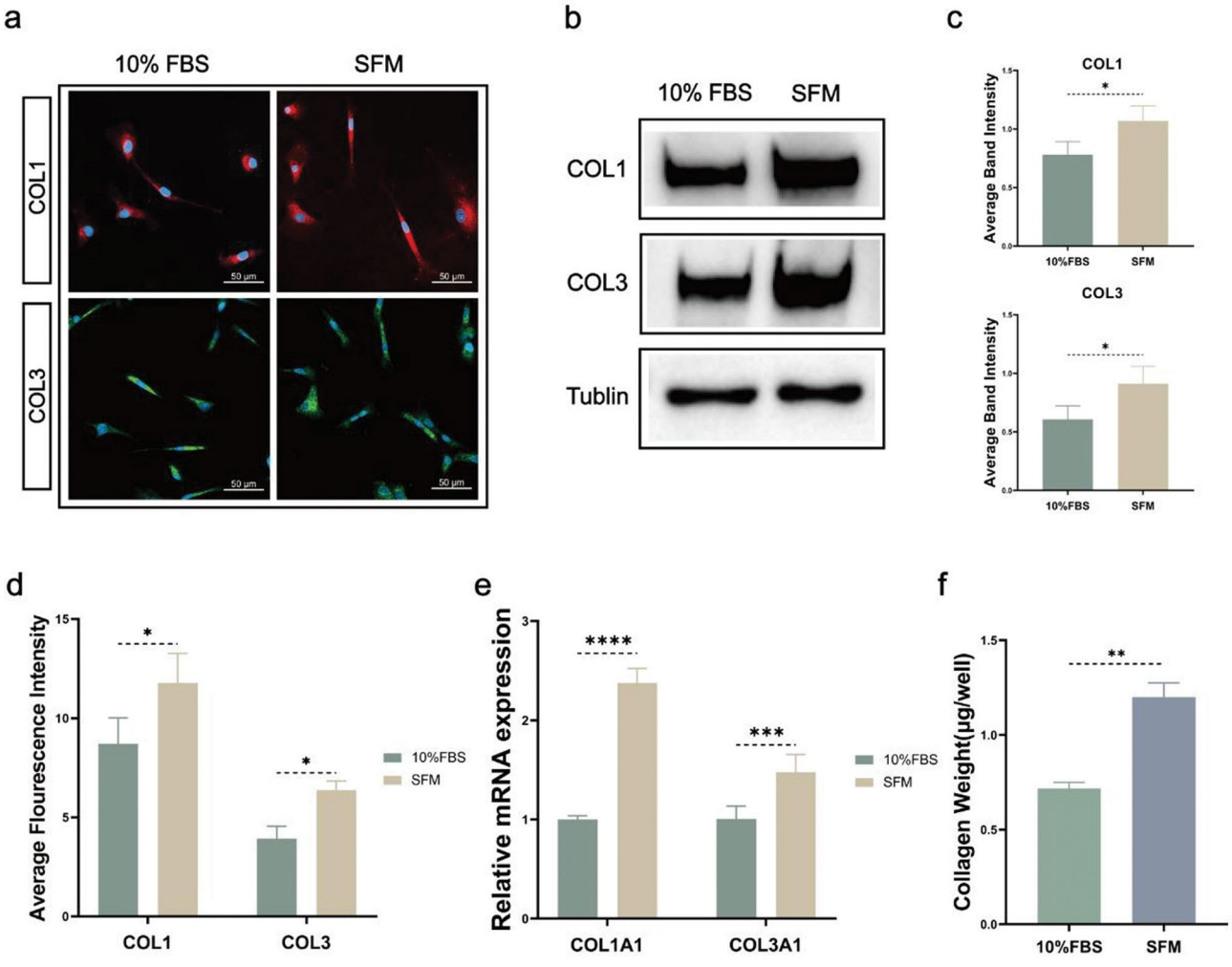


Fig. 4 Serum-free medium promotes ECM secretion. (**a**, **b**, **c**, **d**) Immunofluorescence and Western blot analysis of collagen I and collagen III protein expression, with tubulin as the internal control. Both collagen proteins were expressed at higher levels in the SFM group ($*p \le 0.05$). (**d**) qRT-PCR results indicated that the gene expression of both collagen proteins was increased in the SFM group ($****p \le 0.0001$, $***p \le 0.001$). (**e**) Hydroxyproline quantification showed that the total collagen content was higher in the SFM group

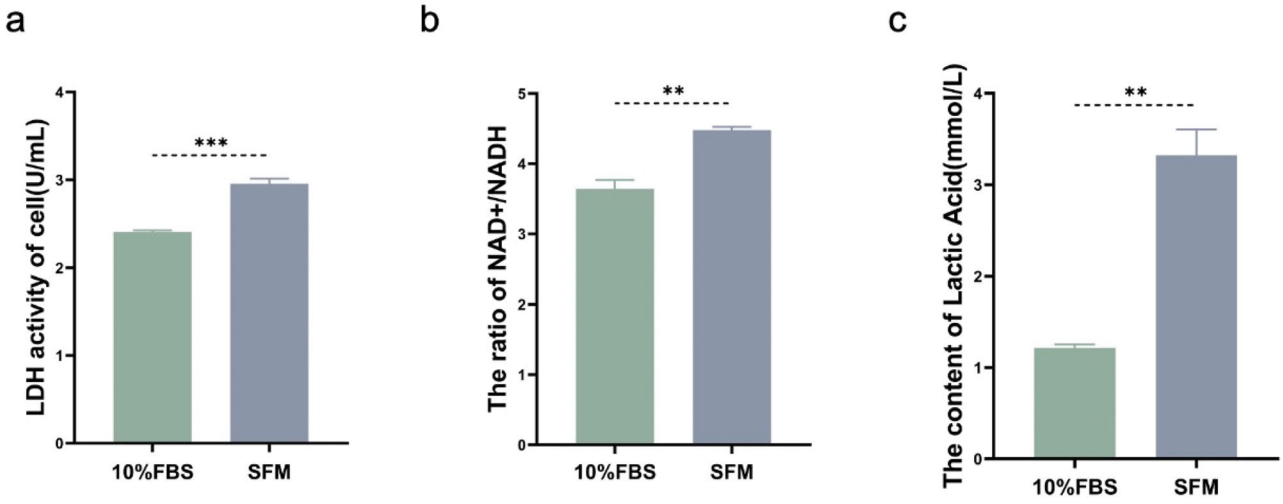


Fig. 5 Serum-free medium promotes cellular glycolytic metabolism. (**a**) LDH activity analysis of the 10% FBS and SFM groups. (**b**) NAD+/NADH ratio analysis of the 10% FBS and SFM groups. (**c**) Lactate concentration analysis in the culture medium of the 10% FBS and SFM groups (**$p \le 0.01$, ***$p \le 0.001$)

may promote collagen synthesis by regulating transcriptional levels. Furthermore, total collagen production by the cells was measured using the hydroxyproline assay (Fig. 4f), and the results showed that the collagen content in the SFM group was significantly higher than in the 10% FBS group (**$p \le 0.01$). This finding is consistent with the protein and gene expression results, indicating that serum-free conditions promote collagen production. In summary, the experimental results demonstrate that serum-free culture conditions significantly enhance the expression of collagen I and III, as well as the total collagen production, suggesting their potential in promoting ECM formation and tissue regeneration.

SFM promotes cellular glycolytic metabolism

To investigate the impact of culture conditions on cellular metabolic activity, lactate dehydrogenase (LDH) activity, NAD+/NADH ratio, and lactate concentration were measured under 10% FBS and SFM conditions. The results showed that LDH activity was significantly higher under SFM conditions compared to 10% FBS (***$p \le 0.001$), indicating that serum-free culture may enhance cellular metabolic activity (Fig. 5a). Additionally, the NAD+/NADH ratio was also significantly elevated under SFM conditions (**$p \le 0.01$), suggesting that serum-free conditions may promote oxidative metabolism (Fig. 5b). Moreover, lactate concentration was found to be significantly higher in the SFM group than in the 10% FBS group (**$p \le 0.01$), indicating that serum-free conditions may influence cellular metabolism by enhancing the glycolytic pathway (Fig. 5c). Overall, these results demonstrate that cellular metabolic activity is enhanced under serum-free culture conditions.

Discussion

This study systematically evaluated the cellular phenotype, proliferation, collagen synthesis, metabolic characteristics, cell sheet formation, and other aspects of VSMCs cultured under 10% FBS and SFM conditions, aiming to explore the feasibility of obtaining cell sheets using serum-free culture medium. The results demonstrate that serum-free culture not only supports the basic functions of VSMCs but also significantly affects cellular metabolic reprogramming and collagen expression. This provides a new perspective for serum-free cell culture and its application in cell sheet technology.

Cell sheets are an effective means of constructing tissue-engineered vascular grafts and have significant implications for the development of TEVGs. The results of this study show that cell sheets formed under SFM conditions are similar in morphology and area to those formed under 10% FBS conditions. H&E and Masson staining further indicate that serum-free culture did not affect the structural integrity of the cell sheets (Fig. 1). SFM not only promoted the compact structure and collagen deposition of cell sheets but also enhanced their mechanical support capacity by increasing the elastic modulus (Fig. 1g), thereby providing more favorable material properties for the construction of TEVGs. This finding is consistent with previous studies, which have reported that serum-free culture systems can support the formation of corneal epithelial cell sheets, showing excellent functionality in transplantation applications [18, 19]. The results of this study further demonstrate that serum-free culture conditions provide structural support for cell sheets, which not only meet the requirements for in vitro culture but also facilitate future clinical translation applications. Meanwhile, despite the comparable ECM densities of the two types of cell sheets, the cell sheets in the

SFM group were thicker(Fig. 1f). This is likely related to the increase in both cell number and cell volume, rather than changes in the unit density of the ECM. In the future, this mechanism can be further validated through three - dimensional imaging or quantitative analysis.

This study shows that under SFM conditions, the expression of α-SMA and SM22 at both the protein and gene levels is significantly higher than that under 10% FBS conditions. Notably, the protein expression of α-SMA shows a particularly significant change. This suggests that serum-free conditions can promote the upregulation of α-SMA and SM22, which may drive cellular phenotype transition (Fig. 2). Previous studies support this finding. Hinz et al. noted that the upregulation of α-SMA is closely associated with the differentiation and enhanced contractility of myofibroblasts, and its expression is typically regulated by the TGF-β/Smad pathway [13]. Other studies have pointed out that SM22 plays a crucial role in cytoskeletal remodeling and cellular mechanical adaptation, which may contribute to the enhanced formation of stress fibers under SFM conditions [20, 21]. Furthermore, previous studies suggested that the expression of SM22 may be influenced by post-transcriptional regulatory mechanisms, which aligns with the observation in this study, where the protein level of SM22 changed minimally, but its mRNA level was significantly upregulated [22]. Combining previous studies with the results of this experiment, it can be speculated that under SFM conditions, cells may activate specific signaling pathways by removing inhibitory factors from serum, thereby promoting the upregulation of α-SMA and SM22 expression. This process not only enhances the stability of the cytoskeleton but also potentially provides a new approach for tissue repair and the cultivation of functionalized cells.

The results showed that the proliferative capacity of cells under SFM conditions was comparable to that under 10% FBS, with no significant differences observed in both EdU staining and CCK-8 assays (Fig. 3a, b and c). This is consistent with previous studies, which have demonstrated that serum-free culture, under optimized conditions, can maintain cell proliferation potential while avoiding the uncertain effects of potential animal-derived components in serum on cell growth [23, 24]. Regarding cell survival, Calcein/PI double staining results indicated that the cell survival rate was slightly higher in the SFM group than in the 10% FBS group (Fig. 3d). This may be related to the reduction of pro-inflammatory factors in SFM which could be present as impurities in traditional serum, thereby reducing cellular stress responses [25, 26]. Moreover, passage results showed that although serum-free culture maintained higher cell proliferation in lower passage numbers, the proliferation rate declined more rapidly with increasing passage number compared to FBS-cultured VSMCs (Fig. 3e and f), which is also consistent with prior studies [27, 28]. Therefore, it can be concluded that the SFM can maintain high proliferative capacity for at least six passages of VSMCs and serves as a good alternative to FBS. Within the identical cultivation duration, the marginal advantage in the cell proliferation rate of the serum - free medium (SFM) group accumulates progressively. This cumulative effect culminates in a remarkable augmentation of the cell count in the second - generation cultures. The outcome of this study implies that SFM may possess a greater efficacy in sustaining the continuous proliferation process of VSMCs. In the subsequent research, proliferation kinetic analysis can be employed as a means to further validate this inference.

Figure 3f demonstrates that SFM significantly increased cell numbers at P2 compared to the 10% FBS group, indicating an early proliferation advantage that may provide a larger cell reservoir for cell sheet formation. Although Fig. 1b microscopic images (P5) show similar densities between groups, likely due to high confluence masking numerical differences, the thicker cell sheets in the SFM group (Fig. 1d) and higher elastic modulus (Fig. 1g) indicate structural optimization potentially driven by the cumulative effect of proliferation. Future studies could quantitatively assess the impact of passage number on cell sheet quality to further elucidate SFM's mechanisms.

Collagen is a major component of the ECM and one of the most important elements for maintaining vascular mechanical properties. It plays a crucial role in both cell function maintenance and tissue repair. This study found that SFM significantly promoted the synthesis of collagen I and III, with immunofluorescence, Western blot, and qRT-PCR results all indicating that the expression levels were significantly higher in the SFM group than in the 10% FBS group (Fig. 4). This finding is in line with previous research, which has reported that serum-free culture can enhance ECM synthesis by regulating gene expression and metabolic pathways [29, 30]. Specifically, the TGF-β signaling pathway may play a key role in this process, as it has been shown to be a critical pathway in regulating collagen synthesis, and serum-free conditions may enhance its expression by activating this pathway [31]. The increased collagen synthesis further supports the potential application of SFM in cell sheet culture. Adequate ECM is essential for maintaining vascular structure and mechanical properties in tissue-engineered blood vessels, making the acquisition of sufficient collagen during the cell sheet culture phase crucial for engineered vascular development. The immunofluorescence images in Fig. 4a primarily illustrate the intracellular expression of COL1 and COL3. Due to the short culture duration and low seeding density, no significant ECM deposition was observed in the background. Notable ECM production was confirmed through hydroxyproline assay and histological analysis in 7-day cell sheet cultures

(Figs. 1 and 4e). For the same reasons, elastin expression was not detected, potentially due to the SFM composition failing to induce elastin synthesis [16, 32, 33]. Future studies could further characterize ECM deposition by extending culture duration, adjust culture conditions or optimizing staining conditions.

Research has demonstrated that the SFM significantly enhanced the expression of SM22 and α - SMA (Fig. 2), cell proliferation (Fig. 3), and collagen production (Fig. 4). This is likely achieved through insulin in ITS promoting cell proliferation and protein synthesis, as well as PDGF and TGF - β in HPL supporting cell phenotype maintenance and ECM generation [11]. In the future, cytokine - specific inhibition experiments can be conducted to further clarify the underlying mechanism of action.

Collagen deposition was validated through hydroxyproline assay (Fig. 4e) and Masson's trichrome staining (Fig. 1d), with increased elastic modulus (Fig. 1g) indicating improved mechanical properties. However, the alignment of collagen fibers has not been directly characterized using imaging techniques such as second-harmonic generation (SHG) or polarized light microscopy [34–36], limiting a comprehensive understanding of the mechanical property origins. Future studies will employ imaging technologies to further validate collagen fiber orientation and organization, elucidating SFM's specific contributions to the mechanical characteristics of cell sheets.

The significant increase in LDH activity, NAD+/NADH ratio, and lactate levels suggests that the metabolic pathways of cells under SFM conditions have been adjusted, with a marked enhancement of glycolytic activity (Fig. 5). The activation of glycolysis under serum-free conditions may represent a strategy by which cells adapt to nutrient-poor environments by rapidly generating energy to maintain function and survival. Previous studies by our group have indicated that during the early stages of TEVGs formation, VSMCs in the rapid proliferation phase on PGA scaffolds tend to adopt an aerobic glycolysis metabolic mode to rapidly generate energy to meet cellular energy demands [11, 37]. Similar metabolic reprogramming phenomena have been reported in tumor cells and hypoxic environments, suggesting that glycolysis may be a key mechanism for cells to meet energy demands [38, 39]. Therefore, we propose that under serum-free conditions, the increased energy demand for rapid cell proliferation drives cells to favor glycolytic pathways.

In summary, this study has validated the effectiveness of SFM in promoting cell sheet formation and VSMCs culture. A significant advantage of serum-free culture is the avoidance of potential immunogenicity and pathogen contamination inherent in traditional serum, making it more suitable for clinical applications. Additionally, SFM typically contain defined components, making them easier to standardize for production, thereby improving experimental reproducibility and data reliability [9, 40]. This study confirmed that SFM not only maintains basic cell functions and metabolic activity but also enhances collagen synthesis, thereby promoting cell sheet formation. These results provide a data-driven foundation for the application of SFM in cell sheet acquisition and tissue-engineered vascular formation.

Although this study demonstrates the superiority of serum-free culture in several aspects, the underlying molecular mechanisms still require further investigation. For instance, multi-omics analyses (transcriptomics, metabolomics, and proteomics) could be employed to comprehensively analyze cell behavior under serum-free conditions. While, this study focused on COL1 and COL3 to assess the impact of SFM on major collagen deposition in VSMC cell sheets; however, alterations in other collagen types and ECM components have not yet been investigated. Notably, differences in protein expression between SFM and FBS conditions may influence ECM composition. Future research will broaden the analytical scope to more comprehensively characterize and analyze ECM composition. Furthermore, additional in vivo studies and the development of fully serum-free culture conditions from cell isolation are essential to assess the efficacy and safety of serum-free culture systems in practical transplantation applications.

Acknowledgements
We thank the member of Zhanyi Lin's research group at Ji Hua Laboratory for their insightful discussion.

Author contributions
J.Y.: Conceptualization, Methodology, Validation, Formal analysis, Investigation. XH.S.: Data Curation, Visualization, Writing - Original DraftJH.J.: Methodology, Formal analysis, Visualization, Conceptualization. JD.L.: Methodology. JR.L.: Methodology. ZY.L.: Conceptualization, Writing-Review & Editing, Supervision, Funding acquisition, Resources, Project administration.Jing Yang and Xuheng Sun are co-first authors.All authors reviewed the manuscript.

Funding
This research was funded by JIHUA Laboratory of Foshan city, Guangdong province(X210111TD210), the authors send their sincere appreciation for it.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics and consent to participate
This study have been approved by the Ethics Committee of the Guangdong General Hospital(KY2023-192-01). Consent to Participate declaration: not applicable.

Consent for publication
All authors have read and approved the final manuscript. We confirm that the content of the manuscript has not been published or submitted for publication elsewhere. We also confirm that the manuscript has been approved by all relevant parties and that all necessary permissions have been obtained for the publication of any material that is not original to this manuscript.

Competing interests
The authors declare no competing interests.

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Received: 27 January 2025 / Accepted: 15 May 2025
Published online: 28 May 2025

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