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Engineering prevascularized composite cell sheet by light-induced cell sheet technology

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Early vascularization in bone defects has been considered to play a critical role in the bone regeneration process. Although mesenchymal stem cells (MSCs) co-cultured with endothelial cells (ECs) have attracted the most attention in strategies that seek to achieve vascularized bone, it still remains a challenge to achieve a prevascularized construct *in vitro* that can be translated conveniently. Here, we provided a strategy of engineering a transferable prevascularized MSC-EC composite cell sheet to promote the vascular-like network formation for bone tissue engineering. We co-cultured human mesenchymal stem cells (hMSCs) and human umbilical vein endothelial cells (HUVECs) on a distinct light-responsive TiO₂ nanodots film. Various cell ratios of MSC-EC and culture mediums were explored to achieve the optimal angiogenesis capacity. The prevascularized MSC-EC composite cell sheet was then detached as an intact and confluent cell layer by a simple light treatment, and showed high viability and 3D network formation surrounded by mesenchymal stem cells. This light-induced cell sheet technology therefore realized a novel transferable prevascularized MSC-EC composite cell sheet, and will have a profound impact on further strategies for designing in-bone tissue engineering.

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Introduction

The reconstruction of large bone defects using bone tissue engineering remains a major challenge in clinic due to the inadequate vascularization of implanted grafts.¹ Inadequate vascularization, which results in limited delivery of oxygen and nutrients, always leads to the necrosis and failure of implanted constructs. One of the approaches that has increasingly attracted attention to overcome this issue is to generate prevascularized constructs *in vitro* before *in vivo* transplantation.² MSCs are a frequently used type of cells for bone tissue engineering due to their multipotent differentiation ability including osteogenesis. Moreover, ECs that have the ability to organize into vascular structures, which accelerate the anastomosis with the native blood vessels, can be used as part of the strategy.^{3,4} Therefore, co-culture of MSCs and ECs *in vitro* to achieve prevascularized structures will be a promising approach to improve

the success of vascularization in implanted engineered constructs.

Cell sheet engineering, leading to cell recovery in an intact layer of confluent cells, has emerged as a useful strategy in regenerative medicine.⁵ Different from dissociated cells, cell sheets preserve cell junctions and intact extracellular matrix (ECM) that provides signals to control the morphology, structure and function of tissue and organs.⁶ Previous studies have demonstrated the use of cell sheet for the regeneration of myocardium, cornea and periodontal ligaments.⁷⁻⁹ Therefore, we speculate that the layered cell sheet consisting of MSCs and ECs formed *in vitro* might preserve the vascular-like structures and be used directly or in combination with scaffolds for bone tissue engineering. Several studies have investigated the addition of ECs in cell sheets for bone tissue engineering. In most studies, MSCs were seeded on a cell culture dish to form a cell sheet. Then, ECs were seeded on the surface of the MSC cell sheet in 4 days of culture for transplantation.^{10,11} However, few studies have concentrated on the mixed seeding of MSCs and ECs for co-culture to generate prevascularized cell sheet. Since co-cultured cell sheet will make a better combination and cell-cell junction of two kinds of cells, co-culture for prolonged periods might generate a stable and mature vascular network *in vitro* for better vascularization *in vivo*.

Recently, we have developed a novel cell sheet technology based on a light-responsive TiO₂ nanodots film.¹² An intact and confluent cell layer could be detached easily due to the

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generation of distinct surface hydroxyl groups triggered by a simply light treatment. However, these previous efforts have only reported the detachment of mono-cultured cell sheets. The design of co-cultured composite cell sheet as well as engineering the distinct culture conditions, such as cell ratio and media, still need to be explored.

In this study, we aimed to provide a transferable co-cultured MSC-EC composite cell sheet to promote the formation of vascular-like network for bone tissue engineering. We co-cultured hMSCs and HUVECs on light-responsive TiO_2 nanodots film at various cell ratio and media. The vessel-like structures, cell metabolic activity and osteogenic potential of the co-cultured MSC-EC cell sheet were systematic evaluated to identify the optimal prevascularized composite cell sheet with high viability and 3D network formation for bone regeneration.

Materials and methods

Preparation of TiO_2 nanodots film

TiO_2 nanodots film (TN) was prepared on a silicon substrate through phase separation-induced self-assembly as we described previously.¹² Briefly, a precursor sol containing titanium tetrabutoxide (TBOT, Sinopharm Chemical Reagent, CP, >98%), acetylacetone (AcAc, Lingfeng Chemical Reagent, AR, >99%) and polyvinyl pyrrolidone (PVP, K30, Sinopharm Chemical Reagent, AR, >99%), was spin-coated on substrate and allowed for phase-separate after further heat treated at 500 °C. A TiO_2 nanodots film was then obtained as light-responsive culture surface and characterized by scanning electron microscope (Hitachi, Su-70) and transmission electron microscopy (FEI, F-20).

Cell culture

Human mesenchymal stem cells were obtained from iliac crest marrow aspirates collected from healthy donors for iliac crest bone transplantation at The First Affiliated Hospital of College of Medicine, Zhejiang University (China). All the samples were obtained with informed consent. This procedure was performed following approval from the Institutional Review Board of First Affiliated Hospital, College of Medicine, Zhejiang University. The reference number of this study is 2016294. The study was conducted in accordance with Regulations on Research of Medical Science and Technology Involving the Human Body. These regulations are formulated by National Health and Family Planning Commission of the People's Republic of China.

Mononuclear cells (MNCs) were separated according to previous studies.¹³ In brief, hMSCs were isolated by density-gradient centrifugation over Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO, USA) and expanded in DMEM medium (Gibco) supplemented with 10% FBS (Gibco).

Primary human umbilical vein endothelial cells were purchased from ATCC (Rockville, MD, USA). Cells were cultured in VCM medium (ATCC®) that consisted of endothelial cell basal medium and a growth kit of supplements.

Co-culture of hMSCs with HUVECs

hMSCs and HUVECs were trypsinized by trypsin-EDTA (Gibco) separately and co-cultured on light sensitive culture surface at different cell ratio in various media. The cell ratio of hMSCs *versus* HUVECs included 100 : 0, 90 : 10, 75 : 25, 50 : 50 and the cell density was 10 000 cells per cm^2 on TiO_2 nanodots surface in 24-well plates. The content of the various media was listed in Table 1. The culture medium was changed every other day. Cell morphology was observed by an inverted light microscopy (Olympus, Tokyo, Japan).

Cell sheet detachment

The detachment of cell sheet from TiO_2 nanodots film was performed using a cold LED UV light. The wavelength of the UV light was 365 nm and the power was set as 2.0 mW cm^{-2} . Cells co-cultured on TiO_2 nanodots film were washed with PBS for three times, followed by UV365 illumination for 20 min. The transmittance power was 1.4 mW cm^{-2} .

Immunofluorescent assay

Immunofluorescence of endothelial cell marker rhodamine labeled UEA I (Vector Laboratories, Burlingame, CA) or CD31 (DakoCytomation, Copenhagen, Denmark) was performed to visualize the vascular-like formation of HUVECs in cell sheet. Briefly, cells were fixed with 4% paraformaldehyde for 15 min after rinsed with PBS. Then, cells were stained with rhodamine UEA I lectin or with primary antibody CD31 followed by an Alexa Fluor®-488 conjugated secondary antibody (Molecular Probes Inc). Nucleus was stained using DAPI (Molecular Probe™, UK). The cell cytoskeleton was labeled red with rhodamine phalloidin after cell sheet detachment (Cytoskeleton, Denver, USA). Finally, the samples were mounted and studied by fluorescence

Table 1 The content of various media for growth of composite cell sheet

Medium name		Content
Mesenchymal stem cells	H	H-DMEM (Gibco), 10% FBS (Gibco)
	L	L-DMEM (Gibco), 10% FBS (Gibco)
growth media	α	α -MEM (Gibco), 10% FBS (Gibco)
Mixed media	H-V	50% H medium and 50% VCM medium (ATCC)
	L-V	50% L medium and 50% VCM medium (ATCC)
	α -V	50% α medium and 50% VCM medium (ATCC)
Osteogenic growth media	H-osteо	H medium and osteogenic factors
	L-osteо	L medium and osteogenic factors
	α -osteо	α medium and osteogenic factors
Osteogenic mixed media	H-V-osteо	H-V medium and osteogenic factors
	L-V-osteо	L-V medium and osteogenic factors
	α -V-osteо	α -V medium and osteogenic factors
Supplements: osteogenic factors	Osteо	50 μM ascorbic acid (Sigma) 100 nM dexamethasone (Sigma) 10 mM β -glycerophosphate (Sigma)



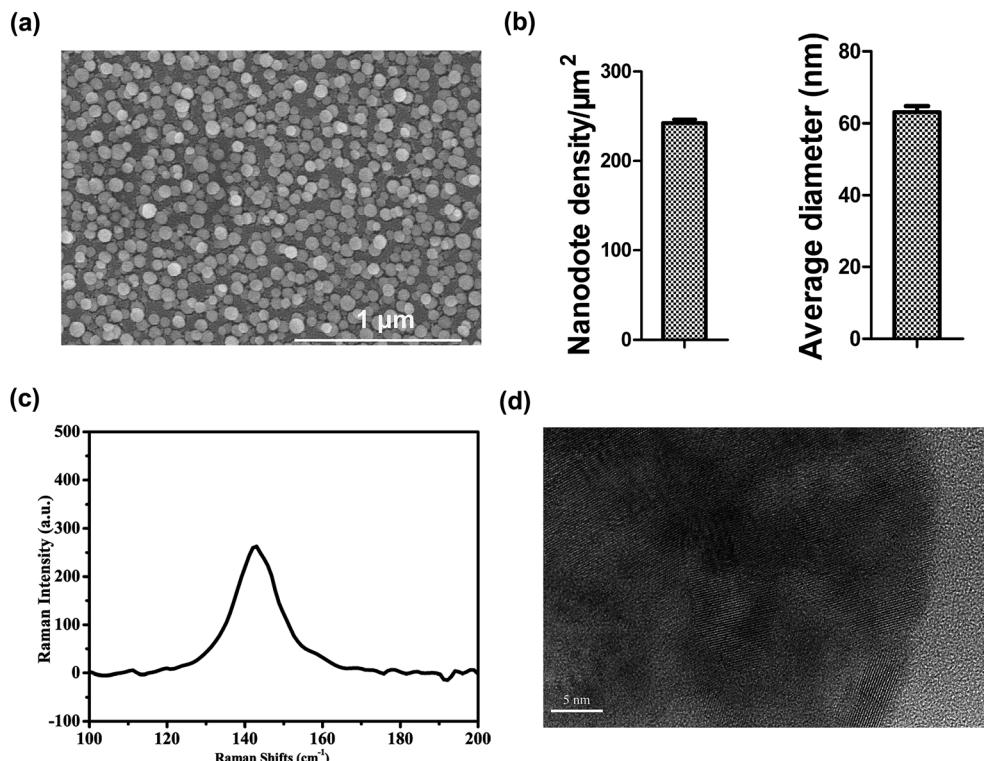


Fig. 1 Characterization of light-responsive TiO_2 nanodots film. (a) SEM image of the TiO_2 nanodots film and statistical analysis of the density and average diameter (b) of the nanodots. (c) The corresponding Raman spectra. (d) High resolution transmission electron microscopy (TEM) image of a typical nanodot.

microscopy (Zeiss, Oberkochen, Germany). The total capillary-like length and the average length of branches were quantified using Image J software (NIH).

Cell metabolic activity

AlamarBlue assay (Invitrogen) was performed to measure cell metabolic activity. After culturing on light sensitive culture

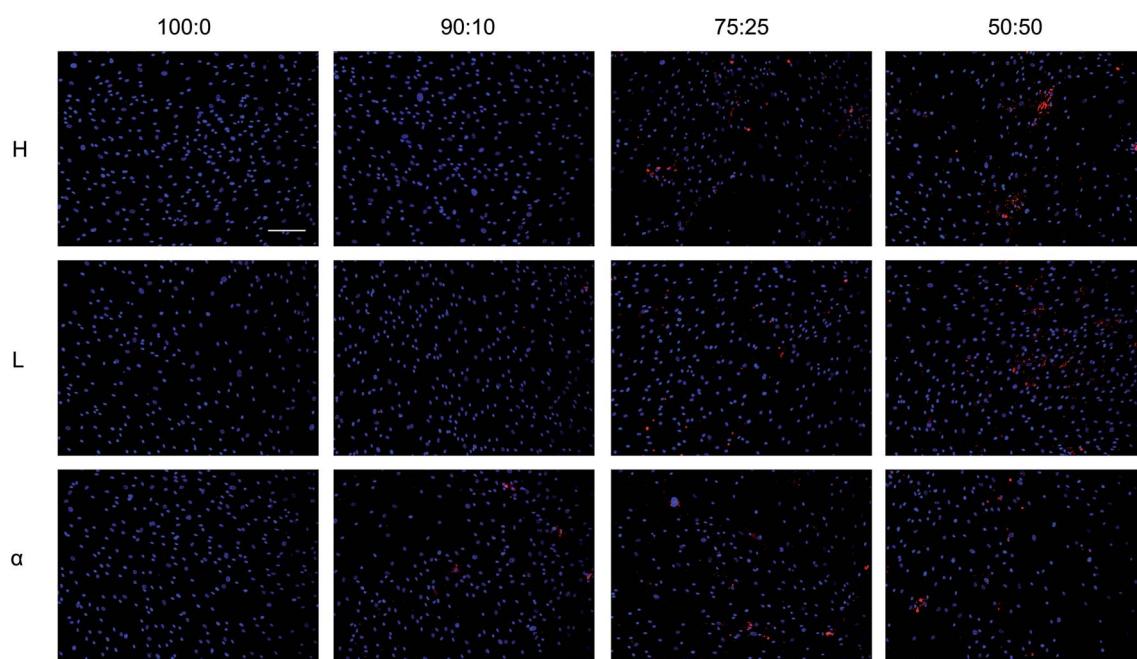


Fig. 2 Effect of pure MSC growth media on HUVECs growth in composite cell sheets. Immunofluorescent assay of UEA I indicated that HUVECs could not survive in pure MSC growth media including H medium, L medium and α medium after 7 days of culture. Scale bars, 200 μm .

surface for 24 h, 48 h and 72 h, the medium in each well was removed and replaced with 500 μ l AlamarBlue solution, which consisted of 50 μ l AlamarBlue and 450 μ l fresh culture medium. Then the plates were incubated for 2 hours in a humidified atmosphere of 5% CO₂ at 37 °C. Finally, 300 μ l AlamarBlue solution derived from each well was aspirated and added to 96-well plates for fluorescence analysis as three duplicates. The resulting fluorescence was evaluated using excitation wavelength of 540 nm and emission wavelength of 590 on a microplate reader SpectraMax i3 (Molecular Devices, Sunnyvale, USA).

Alkaline phosphatase (ALP) activity

ALP LabAssay Kit (Wako, Japan) was used to evaluate the ALP activity, following the manufacturer's instructions. Briefly,

supernatant from different ratio of culture groups were harvested as samples. Standard protein was diluted in series to measure the standard curve. A substrate tablet was dissolved into 5 ml of buffer solution to generate working assay solution. Then, 100 μ l working assay solution was added into 20 μ l sample or standard protein in 96-well plates. Three duplicates of each sample were performed. After incubation at 37 °C for 15 minutes, 80 μ l stop solution was added to stop the reaction. Finally, shaked the plate for 1 minute and measured the absorbance at 405 nm of wavelength using microplate reader SpectraMax i3 (Molecular Devices, Sunnyvale, USA).

Live-dead staining assay

The detached cell sheet viability after UV illumination was assessed using LIVE/DEAD® viability/cytotoxicity kit

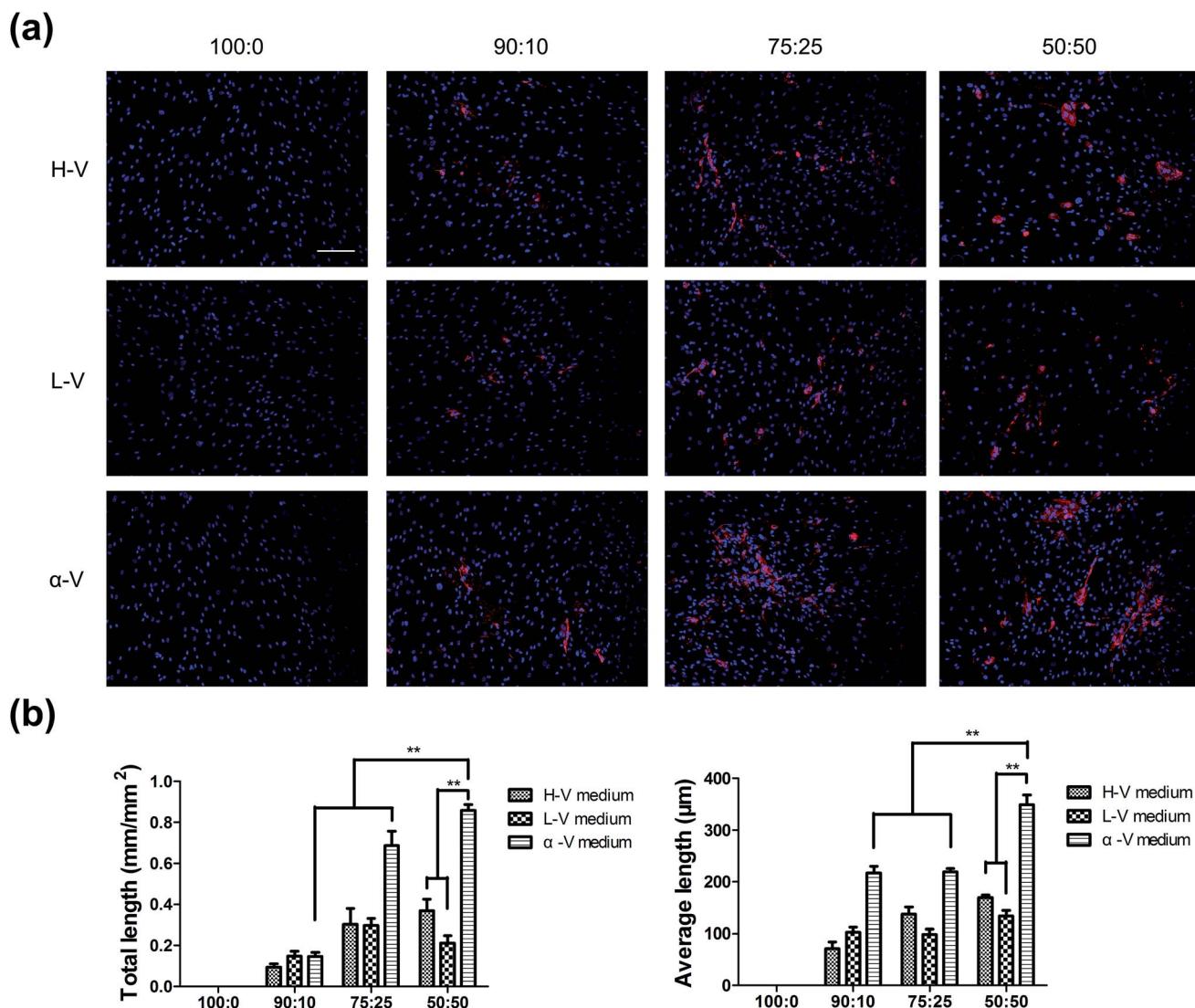


Fig. 3 Effect of mixed media on HUVECs distribution in composite cell sheets. (a) Immunofluorescence indicated that HUVECs distribution increased with endothelial cell ratio in mixed media (50 : 50 > 75 : 25 > 90 : 10). HUVECs in α -V medium appeared as tube-like morphology and displayed more extensive distribution in co-culture than that in H-V and L-V media at the same ratio. (b) Quantitative analysis showed the highest total length and average length of tube-like structures in 50 : 50 group under α -V medium. Results are shown as the average values \pm SD. Significance levels were set at: *: $p < 0.05$, **: $p < 0.01$. Scale bars, 200 μm .



(Invitrogen), in which calcein AM and ethidium homodimer-1 were used to determine the living and dead cells respectively. Briefly, a mixed solution of calcein AM and ethidium homodimer-1 was prepared and added to cell sheets for incubation at room temperature for 30 min. Cell sheets were then studied by a fluorescence microscopy (Zeiss, Oberkochen, Germany).

Statistical analysis

All data were expressed as mean \pm standard deviation (SD) for three independent experiments. One-way analysis of variance (ANOVA) was used to test for significance using the GraphPad Prism Version 5.0 (GraphPad, San Diego, CA, US) and SPSS Statistics 18.0 (IBM, Armonk, NY, US). Differences were considered statistically significant at $p < 0.05$.

Results

Characterization of TiO_2 nanodots film

The TiO_2 nanodots film obtained after 500 °C heating was characterized by scanning electron microscope (SEM) and transmission electron microscopy (TEM). As shown in Fig. 1a, the SEM image showed homogeneous distribution of the TiO_2 nanodots. The density and average diameter of the nanodots were $242 \mu\text{m}^{-2}$ and 63 nm, respectively (Fig. 1b). Furthermore, the Raman spectra was used to analyze the phase of TiO_2 nanodots. As shown in Fig. 1c, the band located at 144 cm^{-1} was indexed to the Raman active modes E_g of anatase TiO_2 . Moreover, the high resolution TEM image of a typical nanodot clearly

showed that the nanodots are polycrystalline (Fig. 1d). These results were consistent with our previous work.^{14,15}

Effects of various culture media and cell ratio on network formation in cell sheets

To achieve the prevascularized cell sheet on light-responsive TiO_2 nanodots film, different cell ratio of HUVECs/hMSCs were cultured in various media to identify the optimal conditions for co-cultures (Table 1). Immunofluorescent assay of UEA I was carried out to characterize the distribution of HUVECs in cell sheets. Specifically, HUVECs were not present within the co-cultures neither in H medium, L medium nor α medium after 7 days of culture (Fig. 2), indicating that HUVECs could not survive in pure growth media of mesenchymal stem cells. In contrast, HUVECs co-cultured in mixed media (H-V medium, L-V medium, α -V medium) dispersedly distributed among hMSCs on day 7 (Fig. 3a). It was notable that the fluorescence of UEA I enhanced with the increase of the cell ratio of HUVECs in co-culture ($50 : 50 > 75 : 25 > 90 : 10$). And HUVECs in α -V medium displayed more extensive distribution in co-culture than that in H-V and L-V media at the same ratio. In the cell ratio of 50 : 50 group under α -V medium, the adjacent ECs gathered together, turned into elongated shape and organized into tube-like structures. Quantitative analysis showed that total length and average length of tube-like structures of cells cultured in 50 : 50 ratio under α -V medium group represented significantly higher than that of other groups (Fig. 3b).

In order to promote the perivascular differentiation of hMSCs in cell sheet, osteogenic factors were added into the 6 media

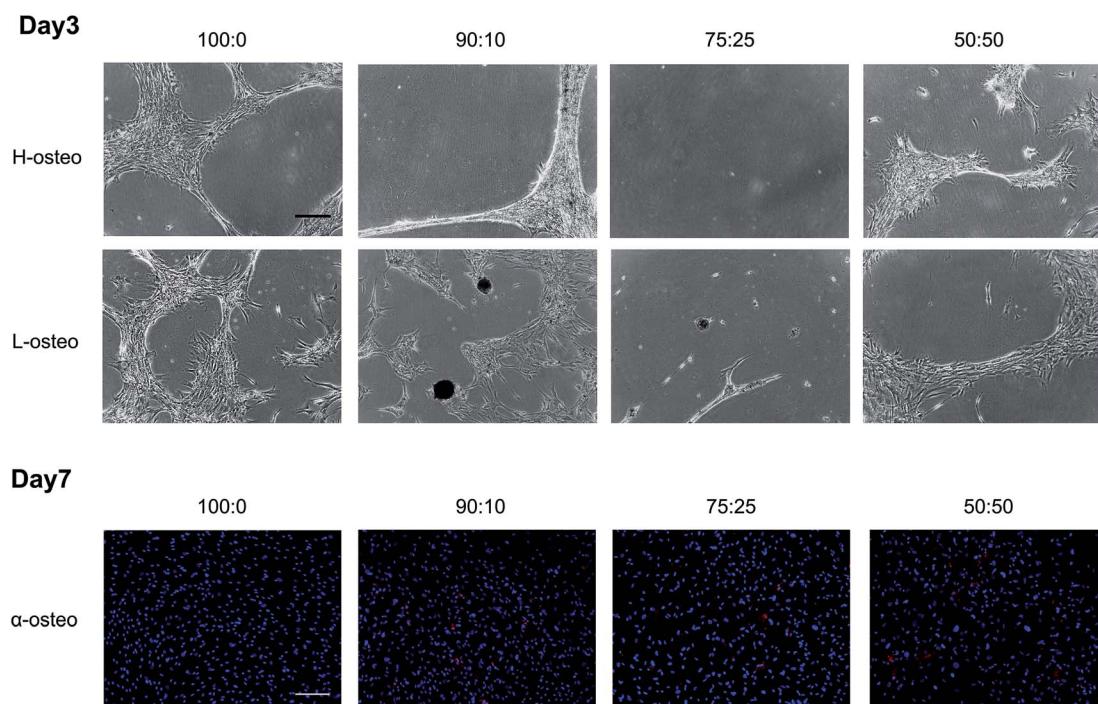


Fig. 4 Effect of osteogenic growth media on HUVECs distribution in composite cell sheets. Cells detached spontaneous from light sensitive culture surface after 3 days of culture in H-osteо medium and L-osteо medium, while cells in α -osteо medium could grow for 7 days but with no HUVECs survival in this medium. Scale bars, 200 μm .

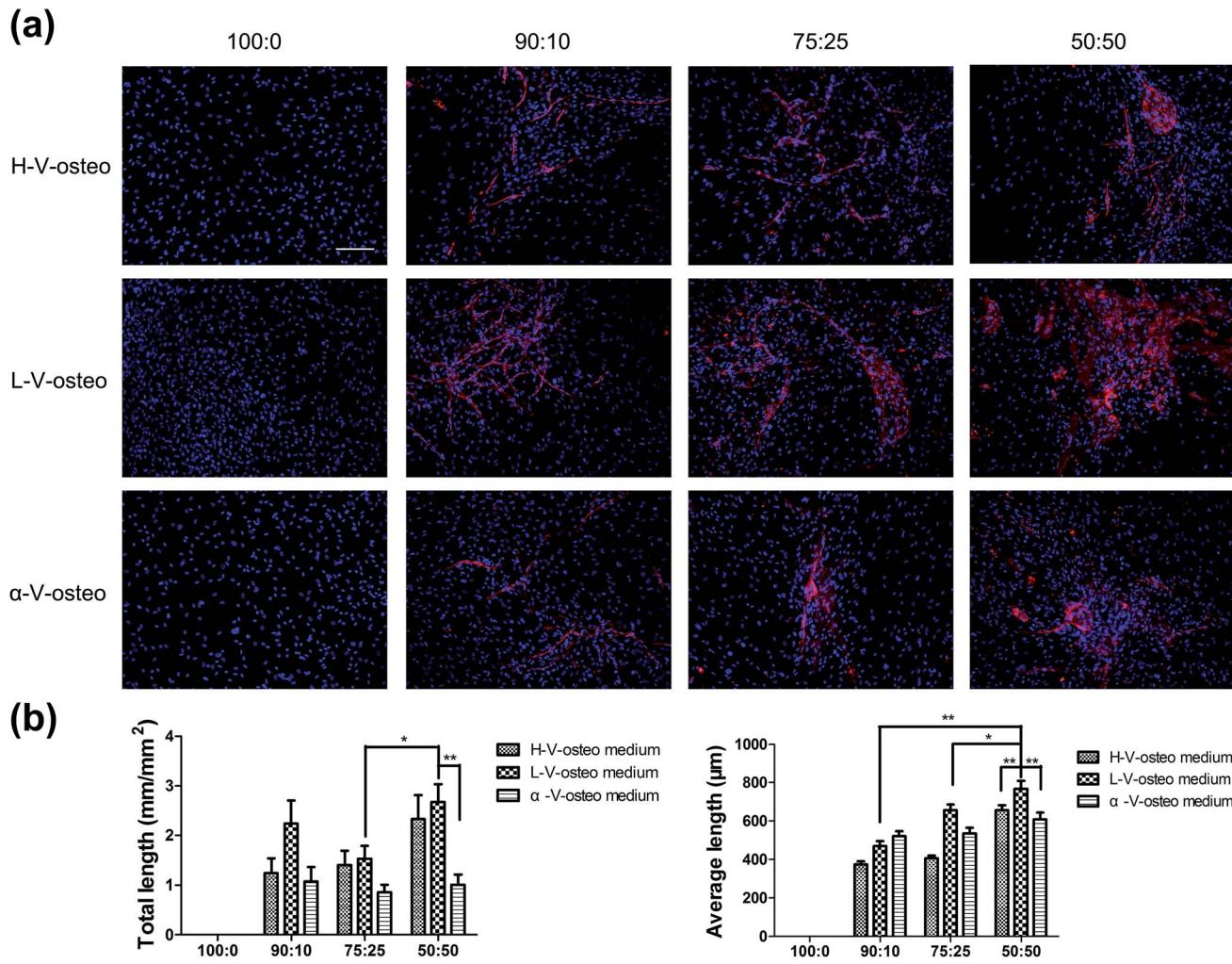


Fig. 5 Effect of mixed osteogenic media on network formation in composite cell sheets. (a) HUVECs in cell sheet proliferated dramatically after addition of osteogenic factors and rearranged into vascular-like network especially in L-V-osteoblast medium. The network formation in 50 : 50 group under L-V-osteoblast medium appeared into interconnected and branched morphology with large diameter compared with that in other groups. (b) Quantitative analysis of vascular-like network showed an increase of both total network length and average branch length in 50 : 50 group under L-V-osteoblast medium. Results are shown as the average values \pm SD. Significance levels were set at: *: $p < 0.05$, **: $p < 0.01$. Scale bars, 200 μm .

above for 7 days of co-culture. Interestingly, after 3 days of culture in H-osteoblast medium and L-osteoblast medium, a number of cells detached spontaneously from light-responsive TiO_2 nanodots film, while cells cultured in α -osteoblast medium could grow for 7 days but with no HUVECs (Fig. 4). In contrast, under osteogenic mixed media, HUVECs proliferated dramatically after the stimulation of osteogenic factors, especially in L-V-osteoblast medium (Fig. 5a). Meanwhile, in L-V-osteoblast medium, HUVECs in 50 : 50 group proliferated obviously and numerous ECs organized into vascular-like network with large diameter, whereas cells in 75 : 25 group displayed in thick tube-like structure with fewer branches. However, HUVECs in 90 : 10 group were observed to form predominantly of a single elongated cell layer and organize into capillary-like structures of much smaller diameters (Fig. 5a). Furthermore, quantitative analysis of vascular-like network also showed that both total network length and average branch length were up-regulated in 50 : 50 group under L-V-osteoblast medium compared with other groups (Fig. 5b).

Screen of the optimal culture system for prevascularized composite cell sheet

Based on the analysis above, we proposed that L-V-osteoblast medium might be the optimal medium for the construction of prevascularized cell sheet. To further confirm the hypothesis and identify the optimal cell ratio, different cell ratio of hMSCs and HUVECs were co-cultured in L-V-osteoblast medium on light-responsive TiO_2 nanodots film. After 7 days of culture, immunofluorescence staining of CD31 was performed to visualize the network formation among different groups. As shown in Fig. 6a, HUVECs in 50 : 50 group interconnected and rearranged into more extensive vascular-like network with thick branches compared to that of cells in 75 : 25 group. Nevertheless, HUVECs co-cultured in 90 : 10 group appeared to be elongated and cord-like structure with few branches.

Cell metabolic activity of cell sheet was then assessed by AlamarBlue assay among different ratio of groups at selective

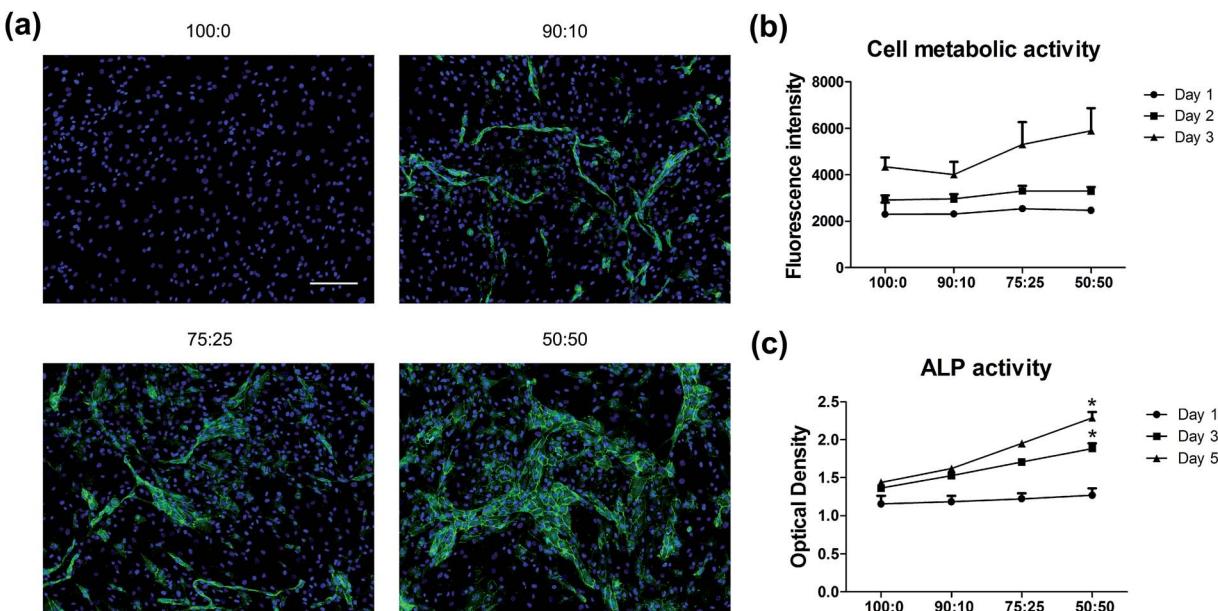


Fig. 6 Screen of the optimal prevascularized composite cell sheet among different cell ratio. (a) Immunofluorescence of CD31 revealed that HUVECs in 50 : 50 group organized into more extensive vascular-like network with thick and more branches than the other three groups. (b) 50 : 50 group displayed significantly higher metabolic activity than the other three groups at the same time point. (c) ALP activity up-regulated with the increased ratio of HUVECs in cell sheet and exhibited highest in 50 : 50 group. Results are shown as the average values \pm SD. Significance levels were set at: *: $p < 0.05$. Scale bars, 200 μ m.

time point. As shown in Fig. 6b, metabolic activity of cells sheet increased from day 1 to day 3, and 50 : 50 group displayed higher metabolic activity than the other two groups at the same time point (75 : 25, 90 : 10), while the 90 : 10 group displayed the lowest metabolic activity. These data indicated the highest cell proliferation and viability in 50 : 50 group.

To further explore the osteogenic differentiation of composite cell sheet, ALP activity evaluation was carried out on day 1, day 3 and day 5. It was notable in Fig. 6c that ALP activity was up-regulated with the increased ratio of HUVECs, showing that co-cultures displayed higher ALP activity than the monoculture of hMSCs. It was found that cell ratio in 50 : 50 group achieved the highest ALP activity among all groups.

Characterization of optimal light-induced cell sheet after detachment

After identifying the best culture conditions for prevascularized composite cell sheet, we further characterized the properties of the optimal cell sheet after detachment. As shown in Fig. 7a, the detached cell sheet exhibited as an intact and translucent membrane. Live-dead staining assay revealed that almost all cells survived with good viability in cell sheet after detachment (Fig. 7b), indicating that there was no significant difference of cell growth before and after light triggering. Meanwhile, immunofluorescence indicated that HUVECs organized into 3D networks and were surrounded by numerous hMSCs in horizontal and vertical directions (Fig. 7c). These results demonstrated the successful harvest of a viable and prevascularized composite cell sheet using our light-induced cell sheet technology.

Discussion

Bone formation is a complex process involving calcified matrix deposition and vascular formation, while insufficient vascularization inside the engineered construct has been a significant factor hampers its implanted survival. Therefore, rapid and sufficient formation of vascular network is a big challenge in bone regeneration, especially in large bone reconstruction. One way to overcome this challenge is to generate prevascularized constructs using osteogenic and angiogenic cells by mimicking the natural environment *in vivo*.¹⁶ The main objective of the present study was to create a prevascularized cell sheet combining mesenchymal stem cells and endothelial cells based on cell sheet technology.

Recently, we have reported the development of a novel light-induced cell sheet technology based on a light-sensitive TiO₂ nanodots film.¹² This TiO₂ nanodots film was prepared through phase separation induced self-assembly, with the dot diameter ranged from 30 to 110 nm. The nanodots obtained were polycrystalline (Fig. 1c and d). During cell cultivation, proteins from the culture medium adsorbed rapidly on the surface of TiO₂ nanodots and then mediated the adhesion of cells. After UV illuminated, the hydroxyl groups on the surface of TiO₂ nanodots were increasing, which would stimulate the protein to change its conformation and release from the TiO₂ nanodots, making the confluent cell layer detached from the TN film.¹² As a strategy to fabricate prevascularized construct, one of its significances falls on the determination of the ideal *in vitro* culture conditions that facilitate the robust vascular-like networks formation. In the present study, we generated



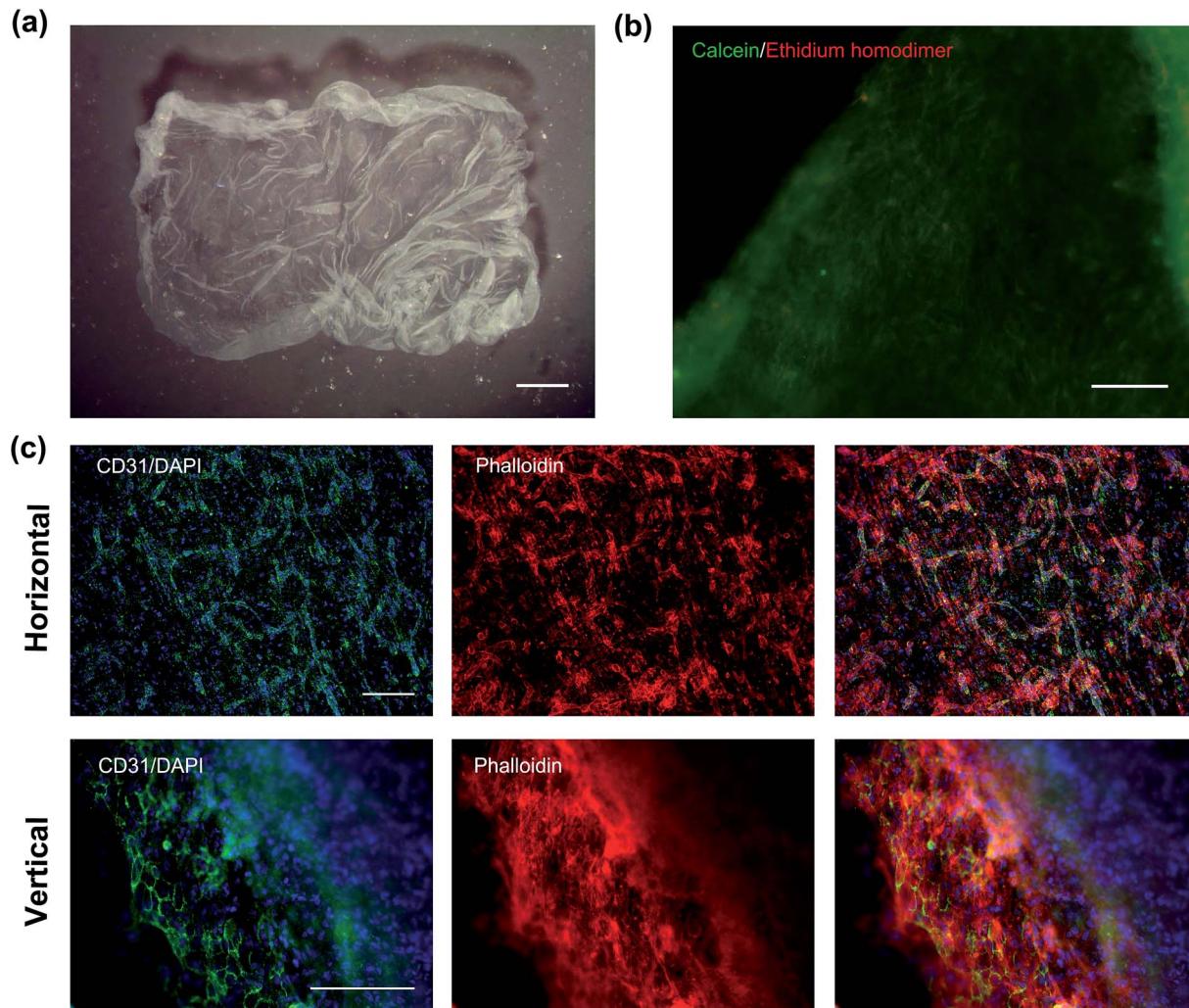


Fig. 7 Characterization of optimal light-induced cell sheet after detachment. (a) Macroscopic observation of the optimal prevascularized composite cell sheet after light-induced detachment. Scale bars, 1 mm. (b) Live–dead staining assay revealed that almost all cells survived in cell sheet after detachment. Live cells were stained with calcein (green), and dead cells were stained with ethidium homodimer (red). Scale bars, 200 μ m. (c) Immunofluorescence of CD31 (green) and phalloidin (red) showed that HUVECs organized into 3D networks and were surrounded by numerous hMSCs in horizontal and vertical directions. Scale bars, 200 μ m.

various MSC–EC composite cell sheets in various culture media and cell ratio. We found that HUVECs could survive in mixed media (H–V medium, L–V medium, α –V medium) and formed into tube like structures (Fig. 3), but couldn't survive in pure MSCs growth media (H medium, L medium, α medium) in cell sheets (Fig. 2). These results suggested that some growth factors contained in mixed media might promote the growth of HUVECs in cell sheets. However, HUVECs co-cultured in osteogenic mixed media proliferated dramatically and organized into prevascularized cell sheets (Fig. 5 and 6a). The formation of vascular-like network might be stimulated by the osteogenic factors, since several studies have also described the role of osteogenic medium on vascularization.^{17,18}

After identifying the best culture medium for the vascular-like network formation, we then focused on the optimal cell ratio of hMSCs and HUVECs in the composite cell sheet. As different cell ratios exerted different effects on angiogenesis and

osteogenesis in co-culture system, results have been conflicting. Some studies demonstrated that low percentages of HUVECs stimulated the formation of the vascular network and up-regulated the expression of alkaline phosphatase.^{18,19} Nevertheless, others showed that 50% ECs combined with 50% MSCs resulted in the highest proliferation and ALP activity in culture plates.^{20,21} In this study, we evaluated the cell metabolic activity and ALP activity in cell sheets using different cell ratio, and found 50 : 50 group presented the highest cell proliferation and osteogenic ability. Moreover, HUVECs in 50 : 50 group organized into more extensive vascular-like network with thick branches than other groups during cell sheet culture (Fig. 5 and 6a). These networks with long vascular-like structures and thick branches would minimize the distance between host ECs and capillaries, and thereby accelerating functional anastomosis after transplantation. These results implied that L–V–osteogenic medium combined with 50 : 50 cell ratio were the optimal

culture conditions for the construction of prevascularized composite cell sheet.

The coincidence of more pronounced networks formation and higher ALP activity was found in 50 : 50 group suggested the close connection between the two processes of angiogenesis and osteogenesis. Actually, during bone development, MSCs and ECs interact with each other to promote bone generation.^{22,23} Previous studies have also demonstrated that MSCs promoted the proliferation and angiogenic potential of ECs.^{24,25} Meanwhile, ECs could also facilitate the osteogenic differentiation of MSCs by release of the growth factors.^{26,27} It was also reported that ECs stimulated human osteoprogenitor cells differentiation through a specific gap junction connexin 43 in direct contact.²⁸ Therefore, the direct contact or the soluble factors secreted by HUVECs organized into vascular-like networks might accelerate the osteogenesis of hMSCs in cell sheets.

Engineered prevascularized 3D constructs using prevascularized MSC-EC composite cell sheets by stacking several cell sheets together or combined with biomaterial scaffold is a promising strategy in bone regeneration. In contrast to the addition of suspension of ECs to MSC cell sheet, the co-cultured composite cell sheet could generate more stable and pronounced 3D vascular-like network *in vitro*. Meanwhile, the prevascularized network would stimulate a rapid and effective anastomosis to the host vasculature in the early transplanted period.²⁹ Further researches will be carried out to investigate the vascularization and bone formation effect of the prevascularized composite cell sheet *in vivo*.

In summary, we demonstrated a strategy of engineering a transferable prevascularized MSC-EC composite cell sheet to promote the vascular-like network formation for bone tissue engineering by using a light-induced cell sheet technology. Immunofluorescence elucidated that variety of vessel like structures could form under various culture conditions, such as cell ratio and media. It was found that L-V-osteо medium facilitated the robust formation of vascular-like network, especially in the cell ratio of 50/50. Furthermore, we demonstrated that cell ratio 50/50 of MSC/EC was the optimal condition for proliferation and osteogenic differentiation of composite cell sheet, based on cell metabolic activity and ALP activity assay results. In addition, the prevascularized composite cell sheet could be detached as an intact and confluent cell layer that appeared 3D network formation surrounded by mesenchymal stem cells. The prevascularized MSC-EC composite cell sheets can be engineered into 3D construct through multiple layers stacking or combined with scaffold, indicating its great potential in further bone regeneration.

Conflict of interest

The authors declare that they have no conflict of interest.

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