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Application note

Preservation of vascular architecture in long-term vessel organoid cultures using Celvivo ClinoStar™ system

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Summary

- Vessel organoids (VOs) generated from human induced pluripotent stem cells (hiPSCs) serve as a physiological and versatile platform to model and study vascular development and disease.
- Prolonged static cultures of VOs lead to progressive vascular regression.
- Long-term culture of VOs in the ClinoStar incubator and bioreactor system provides active diffusion of nutrients and oxygen, while facilitating waste removal, preserving vascular architecture and promoting vascular remodeling.

Introduction

Human induced pluripotent stem cell (hiPSC)-derived vessel organoids (VOs) are three-dimensional constructs that consist of an extensive organization of robust and functional vascular networks composed of multiple cell types, including endothelial cells (ECs), and mural cells such as pericytes[1,2]. VOs represent a physiologically-relevant and versatile in vitro vascular model system that is suitable for modeling and studying human vascular health and disease[3], as well as drug discovery and testing[4]. More recently, researchers have started exploring the use of VOs to vascularize other organoid types[5–8] with the goal of generating more complex organoid systems. Therefore, there is an increasing interest in utilizing VOs in various aspects of vascular and organoid research.

hiPSC differentiation to VOs is a multi-step process[2,9]. Firstly, embryoid bodies (EBs) are made from hiPSCs. Then, the EBs are directed towards the mesoderm lineage via the activation of BMP and WNT signaling for three days. At day 3, the mesodermal aggregates are subsequently directed towards the vascular lineages using VEGFA and Forskolin, resulting in the formation of vascular progenitors at day 5. These aggregates, consisting of vascular progenitor cells, were then immobilized in an extracellular matrix (ECM) sandwich made of a mixture of Matrigel™ and Collagen I to promote vascular sprouting for 5 days. Pro-angiogenic factors VEGFA and FGF2 were also added to a nutrient-rich culture medium to enable further vascular specification and maturation. At day 10, the vascular sprouts will be excised from the ECM sandwich and transferred into an orbital shaker to allow for excess ECM to be removed and for the vascular sprouts to round up into VOs. After 48h, the VOs are transferred to individual wells of 96-well round-bottom plates, where they are cultured under static conditions for an additional three days. By day 15, the end of the differentiation process, VOs consisting of interconnected vascular networks are formed.

Recent single-cell transcriptomic analyses showed that expression of several EC and mural cell markers is significantly reduced in day 21 VOs compared to day 14 VOs[10], suggesting a loss of vascular cells over time. Static culture conditions may be suboptimal for long-term culture of VOs, as limited diffusion of nutrients and oxygen, coupled with inefficient waste removal, can restrict growth and promote necrotic core formation[11]. Here, we demonstrate that culturing VOs long-term in CelVivo's ClinoReactor™ mini-bioreactor chambers in the ClinoStar incubator provides dynamic microgravity-based rotations[12], increases nutrient and oxygen diffusion while facilitating efficient waste removal, thereby preventing vascular regression and preserving the structure and architecture of vascular networks within VOs.

Materials

- hiPSC-derived VOs[1,2,9]
- StemPro-34™ SFM media (Gibco, 10639011)
- Fetal Bovine Serum (Gibco, A5256701)
- FGF2 (Gemini Bio, 300-112P-010)
- VEGFA-165 (Gemini Bio, 300-827P-500)
- Anti-Adherence Rinsing Solution (Stem Cell Technologies, 07010)
- ClinoReactor™ (CelVivo)
- ClinoStar® (CelVivo)
- 20ml sterile syringe (Fisher Scientific, 14-955-460)
- 16G blunt-end needles (Stem Cell Technologies, 28110)
- 15ml Falcon™ conical tube (Falcon™, 352099)
- 5ml sterile serological pipets (Fisher Scientific, 13-678-11D)
- Ultra-low attachment 96 well round-bottom plate (S-bio, MS-9096UZ)
- Kimwipes™ (Kimberly-Clark Professional™, 34120)

Methods

1. Differentiate hiPSCs to VOs according to published protocols[1,2,9] and obtain day 15 vessel organoids.
2. Prepare blood vessel induction (BVI) media* as follows:

	Components	Working Concentration
1	StemPro-34™ SFM media	-
2	Fetal Bovine Serum	15%
3	FGF2	100ng/ml
4	VEGFA-165	100ng/ml

**BVI media can be stored at 4°c for up to 2 weeks*

3. Transfer up to 30 VOs into a 15ml conical tube and allow organoids to settle by gravitation. Aspirate and remove supernatant and replace it with 5ml of fresh BVI media.
4. Hydrate ClinoReactor™ according to manufacturer's protocol at least 24h ahead of time.
5. Add 2ml of Anti-Adherence Rinsing Solution into the ClinoReactor™ chamber and swirl 2-3 times to ensure that the chambers are well-coated with the solution.
6. Aspirate and remove the Anti-Adherence Rinsing Solution from the chamber.
7. Add 5ml of the StemPro-34™ SFM media into the chamber and swirl 2-3 times to ensure that the chambers are well-coated with the media.
8. Aspirate and remove the StemPro-34™ SFM media from the chamber.
9. Add 5ml of fresh BVI media into the chamber.
10. Add 5ml of the VOs and BVI mixture into the chamber using a 5ml serological pipette. Fill chamber completely with BVI media.
11. Gently tap ClinoReactor™ to remove air bubbles before sealing the chamber with the top plug.
12. Carefully aspirate and remove any overflowed media.
13. Wipe the area around the top plug dry with a Kimwipe.
14. Place ClinoReactor™ into the ClinoStar® that has been adjusted to 2% O_2, 5% CO_2, and 37°*c.* Axle speed should be set to 15-20 RPM*, depending on the number of organoids in the chamber.
    **Adjust axle speed to maintain a well-dispersed organoid suspension while preventing collisions with each other or the chamber walls.*
15. Replace with fresh BVI media every 3 days and transfer VOs into a new ClinoReactor™ (prepared based on steps 4-8) every 10 days.
16. To remove VOs from the remove the ClinoReactor™, open the Petri dish lid using the lifter tool provided. Using a 5ml serological pipet (or a 1000ml wide-bore tip), transfer the organoids into a new bioreactor for continued culture or to a tube for downstream assays.

Results & discussion

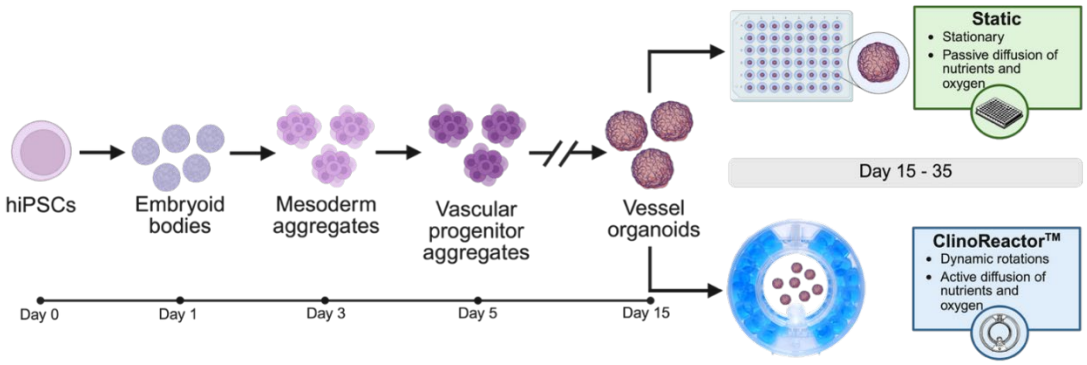


Figure 1: Schematic diagram illustrating the experimental design

Prolonged static VO cultures lead to vascular regression

During VO differentiation, organoids are transferred into individual wells of 96 round-bottom well-plates at day 12 and maintained under static conditions for an additional 3 days[9] (**Fig. 1**). At day 15, dense and spherical-shaped VOs (**Fig. 2A**) that consist of vascular networks made up of CD31-expressing ECs and PDGFRβ-expressing pericytes are formed (**Fig. 2B**). To assess the effects of prolonged static culture on the vascular structures, the VOs were further maintained in these static conditions up to day 35 (**Fig. 1**). Whole-organoid immunostaining performed at both days 25 and 35 revealed an apparent loss of vascular networks over time, as illustrated by progressively reduced numbers of endothelial cells (CD31) and pericytes (PDGFRβ) (**Fig. 2B** and **2C**). This suggests that static culture conditions are unable to support prolonged culture and maintenance of the vascular networks in VOs.

Vos cultured in ClinoReactor™ showed preserved vascular networks and enhanced vascular remodeling

To determine the effects of enhanced diffusion of nutrients and oxygen, coupled with improved waste removal, provided by ClinoReactor™ on vascular structures, day 12 VOs were cultured in the system through to day 35 (**Fig. 1**). Notably, VOs cultured in ClinoReactor™ exhibited more uniform shape and size compared to those cultured in static conditions (**Fig. 2A**). Whole-organoid immunostaining performed at both days 25 and 35 showed reduced vascular regression compared to VOs cultured in static conditions (**Fig. 2B** and **2C**). Interestingly, the vascular networks within VOs cultured in ClinoReactor™ showed a mix of large and small vessel structures, suggesting some degree of vascular remodeling. By providing a constant circulation of media through dynamic rotations, the ClinoReactor™ improved diffusion of nutrients and oxygen while enabling waste removal, likely through enhancing interstitial flow throughout the VOs, thereby improving the survival of the vascular cell populations.

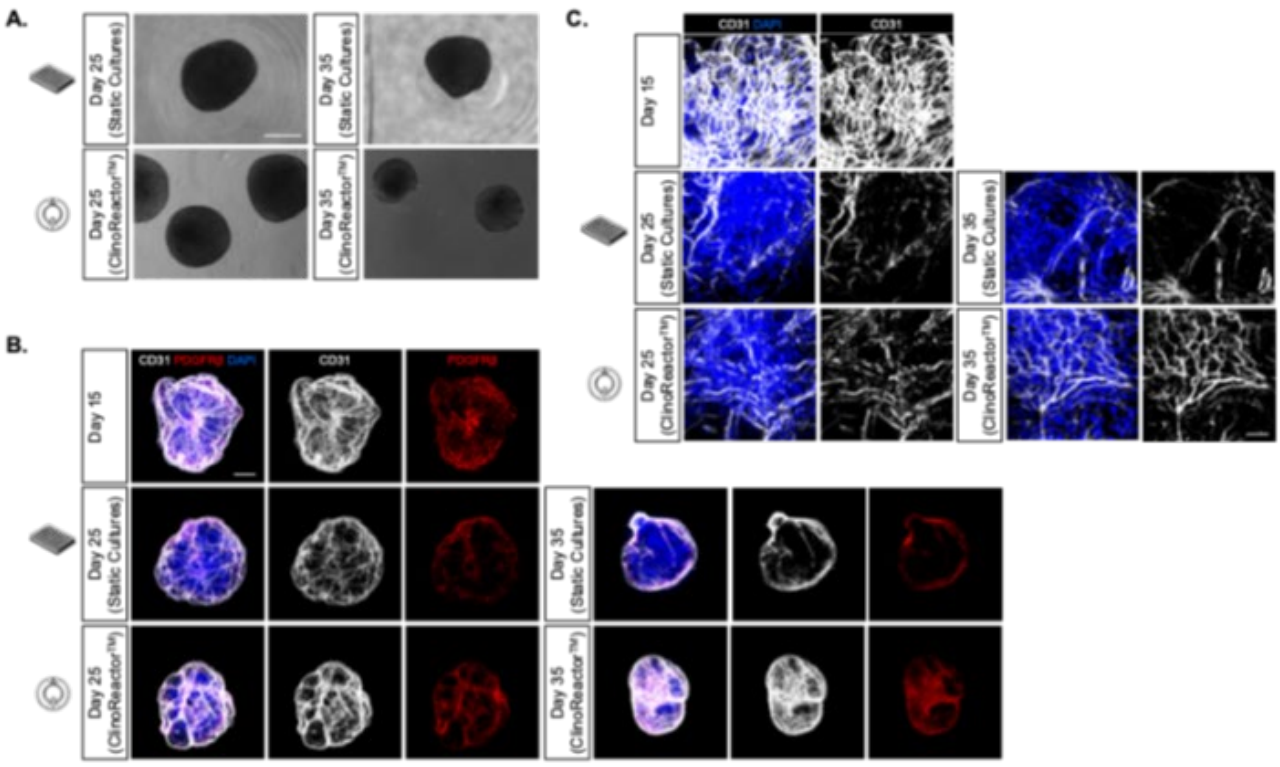


Figure 2: Vascular networks in prolonged cultures of Vos

(A) Brightfield images of day 25 and day 35 VOs. Scale bar=500um. (B) Representative whole-organoid immunofluorescence images (10x) showing CD31 (white), PDGFRβ (red) in VOs. Nuclei are counterstained with DAPI (blue). Scale bar represents 100um. (C) Representative whole-organoid immunofluorescence images (40x) showing CD31 (white) in VOs. Nuclei are counterstained with DAPI (blue). Scale bar represents 100um.

Conclusion & Future Directions

In conclusion, prolonged VO cultures under static conditions result in significant vascular regression over time. Instead, the ClinoStar system enhances nutrient and oxygen diffusion through continual microgravity-simulating rotations while maintaining a low shear-stress environment, thereby providing a viable approach to maintain the structure and overall architecture of vascular networks in long-term cultured VOs, while supporting vascular remodeling. Further studies will be needed to evaluate the transcriptomic profiles of these VOs. Comparisons to published human fetal and adult datasets will help determine the maturity and identity of the vascular cell populations within these VOs. Additionally, functional studies would also be necessary to assess vascular functionality. Overall, the ClinoStar system provides an easy, reproducible, and scalable approach to extending VO cultures while preserving vascular networks, thus enabling broader downstream translational applications.

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