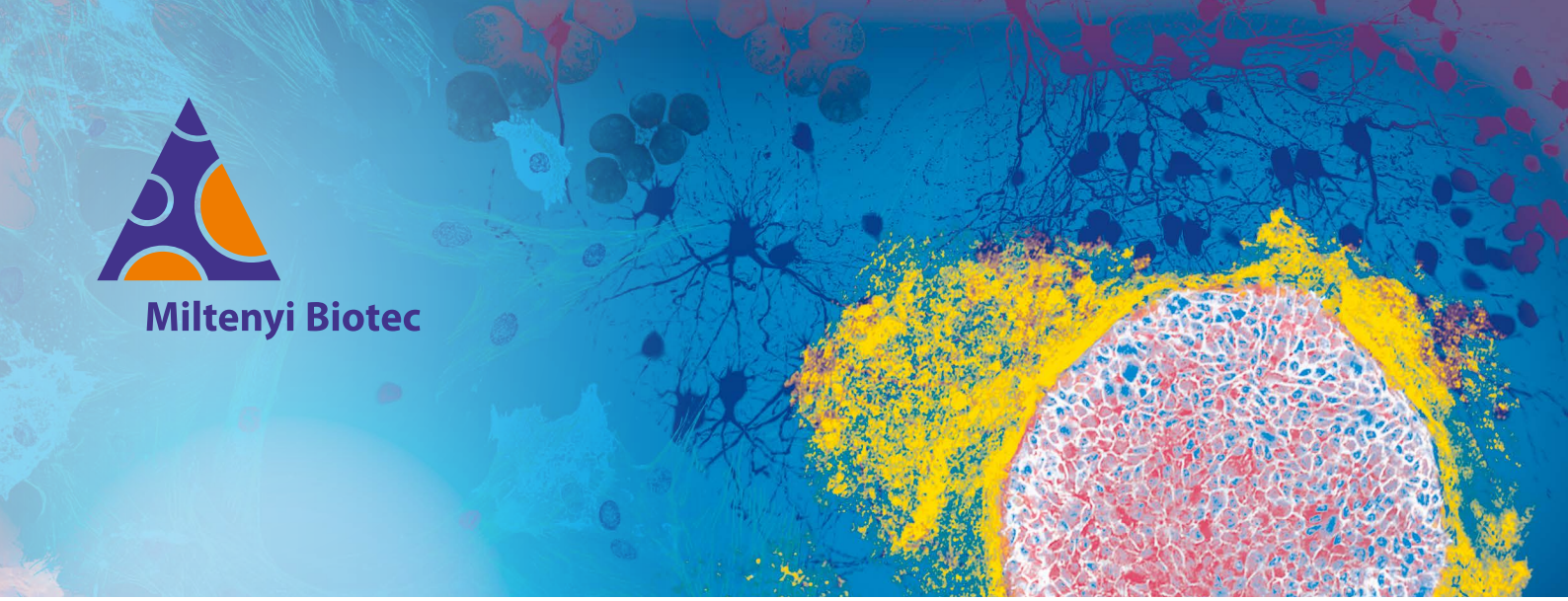

Modeling vascular abnormalities *in vitro* with iPSC-derived blood vessel organoids

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Background

Conventional *in vitro* human disease models rely heavily on the use of primary or immortalized cells. However, these cells have limitations in accurately recapitulating disease pathophysiology and elucidating disease etiology. With the advent of induced pluripotent stem cell (iPSC) technology and sophisticated three-dimensional cell culture systems such as organoids, patient cells can be used to generate physiologically relevant *in vitro* models of disease. Additionally, due to this model's *in vitro* nature, disease progression can now be studied. These model systems improve our understanding of molecular mechanisms underlying disease-relevant phenotypes.

Recently, blood vessel organoids (BVOs) comprising of well-formed vascular networks of endothelial cells (ECs), mural cells such as pericytes, and fibroblasts were generated from human iPSCs.[1–2] The vascular structures in the vessel organoids are functional and possess typical features of the human microvasculature, including a vascular basement membrane enriched with collagen IV.

This allows for the study of vascular implications in various human diseases in a more physiologically relevant model with the goal of accelerating therapeutic discovery. This application note showcases the use of human iPSC-derived BVOs as a viable, human-specific model to investigate vascular abnormalities associated with developmental diseases.

Herein, we differentiated iPSCs obtained from a patient with a congenital lung disease called alveolar capillary dysplasia with misalignment of pulmonary veins (ACD/MPV) (ACD iPSCs), caused by forkhead box F1 (FOXF1) haploinsufficiency, into BVOs (ACD BVOs).

We found that ACD BVOs exhibit distinct vascular abnormalities such as poorly developed vessel networks and reduced numbers of ECs and pericytes.[3] Lastly, we show that BVOs also serve as a physiologically relevant platform to perform therapeutic testing.

Materials and methods

Miltenyi Biotec products	Order no.
StemMACS iPS-Brew XF, human	130-104-368
MACS SmartStrainers (30 µm)	130-098-458
FcR Blocking Reagent, human	130-059-901
StemMACS CHIR99021	130-103-926
Human BMP-4, research grade	130-111-168
StemMACS Forskolin	130-117-341
Human VEGF (165) IS, research grade	130-109-383
Human FGF-2, research grade	130-093-837
MACS BSA Stock Solution	130-091-376
CD144 (VE-Cadherin) MicroBeads, human	130-097-857
LS Columns	130-042-401
QuadroMACS Separator	130-090-976
StemMACS Y27632	130-103-922

Expansion and culture of human iPSCs
Human iPSC lines were maintained under feeder-free conditions on Cultrex®-coated 6-well plates using StemMACS™ iPS-Brew XF, human. When the cells reached 80% confluency, they were passaged into freshly coated wells at a 1:12 ratio as small clumps using 0.5 mM EDTA. These cells were then cultured in StemMACS iPS-Brew XF, human, supplemented with StemMACS Y27632.

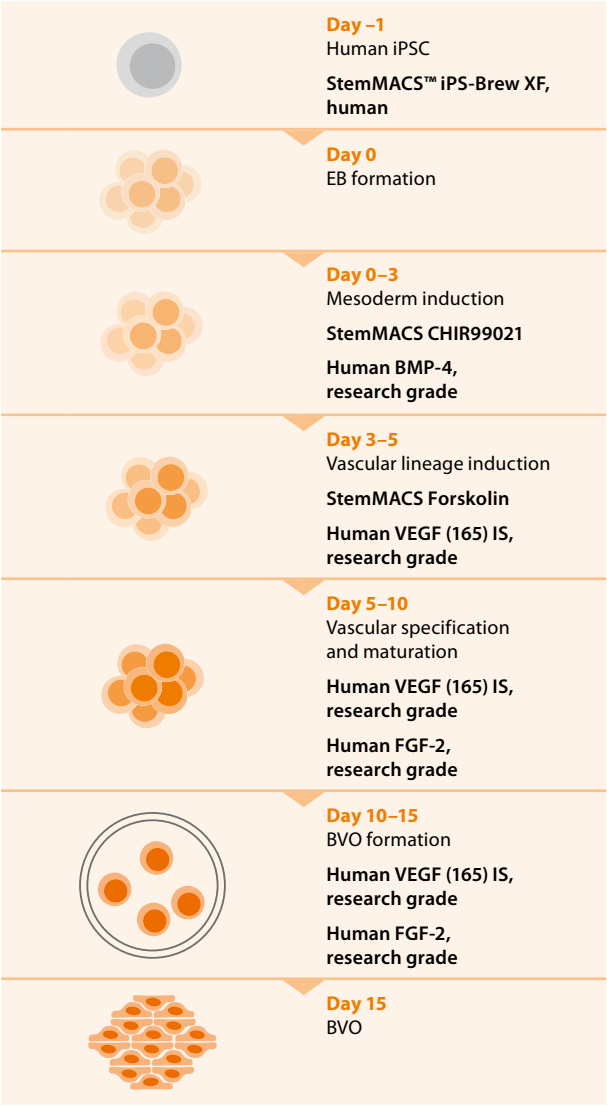

Figure 1: Protocol used to generate BVOs from human iPSCs. Schematic representation of products from Miltenyi Biotec used in each protocol step.

Differentiation of human iPSCs into BVOs

The iPSCs were differentiated into BVOs using a previously published protocol[1] (fig. 1). When the iPSCs reached approximately 80% confluency, they were dissociated into single cells using 0.5 mM EDTA. The single-cell suspension was passed through 30 µm MACS® SmartStrainers to remove unwanted cell clumps.

Following this, 1.2×10^6 cells were added to AggreWell™ 400 Microwell Plates to form uniformly sized embryoid bodies (EBs). The next day, the EBs were transferred into low-attachment 6-well plates with differentiation media (1:1 DMEM/F-12: neurobasal media, glutamine, non-essential amino acids, 2-Mercaptoethanol) containing 12 µM StemMACS CHIR99021 to activate Wnt signaling and 30 ng/mL Human BMP-4, research grade, to induce mesoderm differentiation. After day 3, aggregates were transferred into fresh differentiation media containing 100 ng/mL Human VEGF (165) IS, research grade, and 2 µM StemMACS Forskolin (cAMP activator) to drive vascular lineage induction.

On day 5, aggregates were embedded in a collagen 1-Matrigel sandwich to which blood vessel induction (BVI) media (StemPro™-34 SFM, glutamine) containing 100 ng/mL Human VEGF (165) IS, research grade, and Human FGF-2, research grade, were added. Vessel sprouting was initiated at this step, and on day 10, the embedded aggregates and vessel sprouts were micro-dissected and transferred into low attachment round-bottom, 96-well plates for BVO formation. Fresh BVI media were added to the organoids once every 2 days. On day 15, spherical BVOs with smooth edges formed (fig. 2).

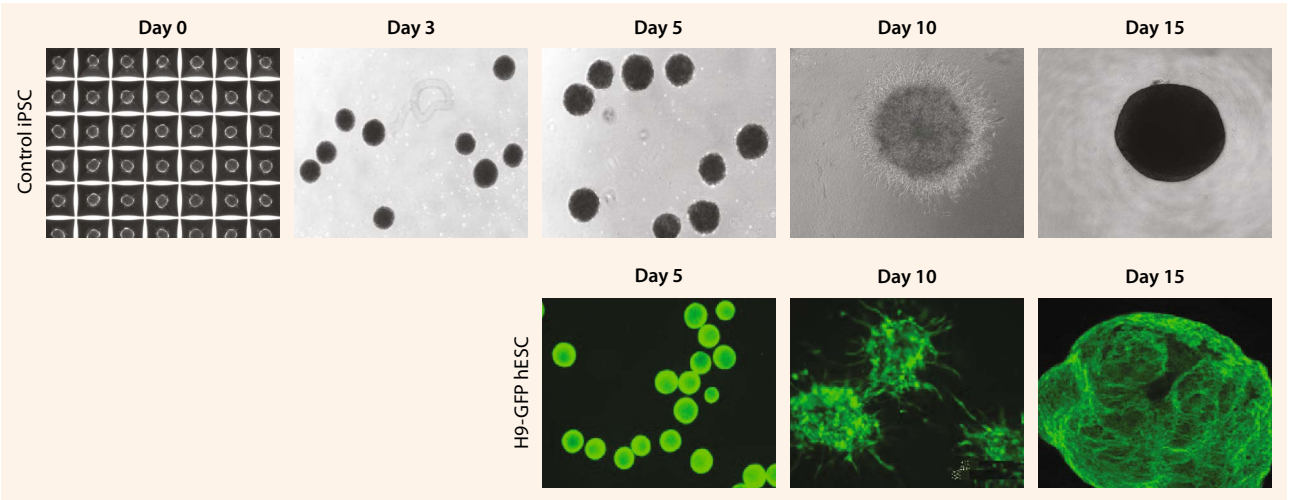

Figure 2: Morphology of BVOs at various stages of differentiation. Brightfield and fluorescence images depicting morphological changes at specific time points throughout differentiation.

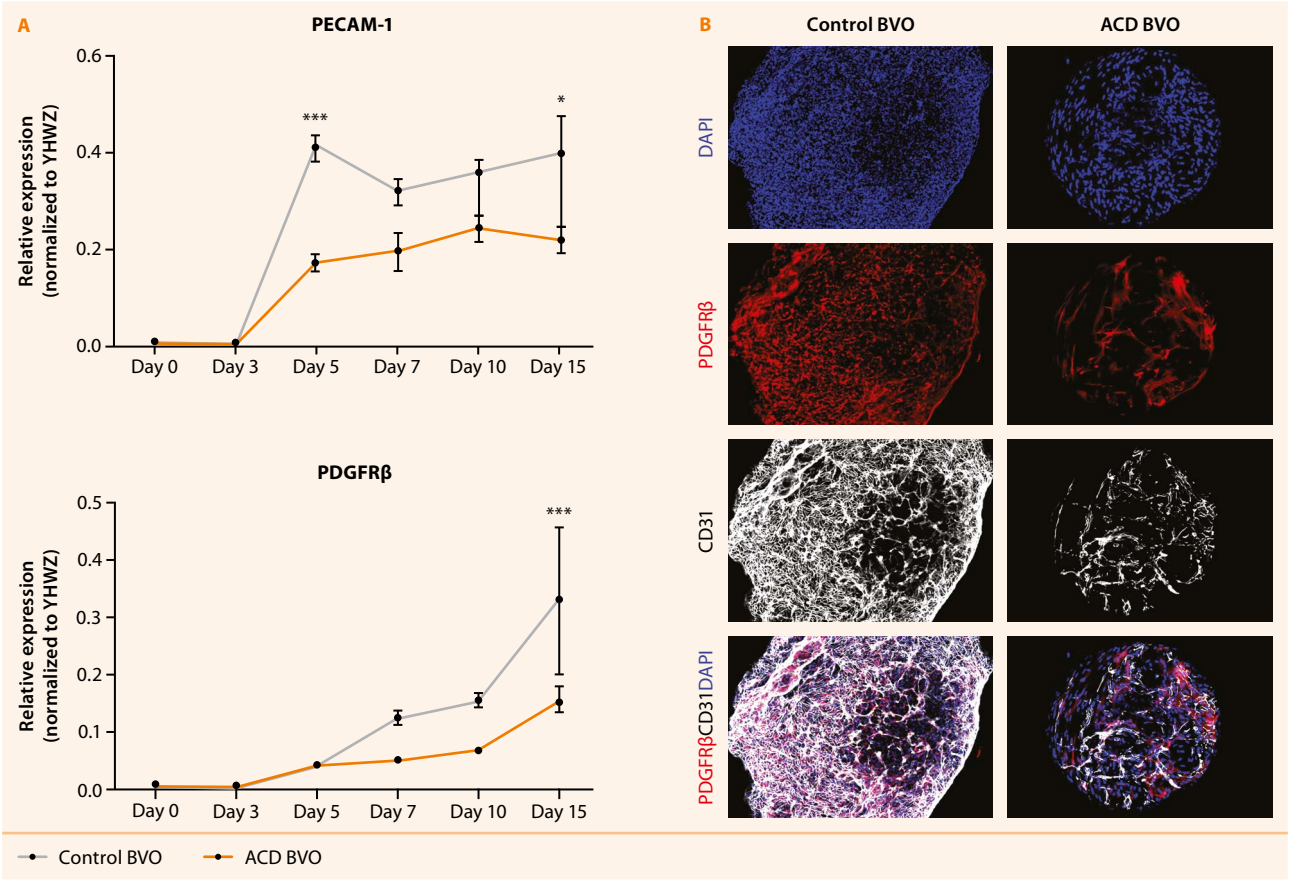

Figure 3: ACD BVOs exhibit severe vascular abnormalities. mRNA expression profile of endothelial marker PECAM-1 and pericyte marker PDGFRβ throughout BVO differentiation. Data is presented as the mean ± SD; *p<0.05, **p<0.01, ***p<0.001 (A). Whole-mount immunofluorescence staining of CD31 (grey), PDGFRβ (red), and DAPI (blue) in day 15 BVOs (B).

Treatment of BVOs with TanFe

To the BVI media, 10μM TanFe was added during day 5–15 of BVO differentiation, with fresh media change every 2 days.

Fixing, cryo-sectioning, and immunofluorescence staining of BVOs

The BVOs were fixed in 4% PFA overnight at 4 °C. Fixed BVOs were washed 3 times in DPBS and transferred into 30% sucrose overnight at 4 °C for cryopreservation. Then, fixed BVOs were embedded in OCT and sectioned at 7 μm. The sections were permeabilized with 0.5% Triton-X for 15 min at room temperature (RT) before incubation, with blocking buffer containing 2% donkey serum in DPBS. The sections were then incubated overnight at 4 °C with primary antibodies diluted in blocking buffer.

The next day, the sections were washed 3 times with DPBS and incubated with diluted Alexa Fluor-conjugated secondary antibodies for 1 h at RT. Nuclei were stained with DAPI. The sections were washed with DPBS prior to imaging. Similarly, ECs were fixed in 4% PFA for 1 h and permeabilized with 0.5% Triton-X for 15 min at RT before immunofluorescence staining and imaging.

Whole-mount immunofluorescence staining of BVOs

The PFA-fixed BVOs were first blocked using a blocking buffer for 4 h on a shaker at RT. Following this, they were incubated with diluted primary antibodies overnight at 4 °C on a shaker. The next day, BVOs were washed 3 times with DPBS with 0.05% Tween®-20 (DPBS-T) and incubated with diluted secondary antibodies and DAPI for 5 h on a shaker at RT. Prior to imaging, BVOs were washed 3 times with DPBS-T and incubated in 88% glycerol overnight at 4 °C.

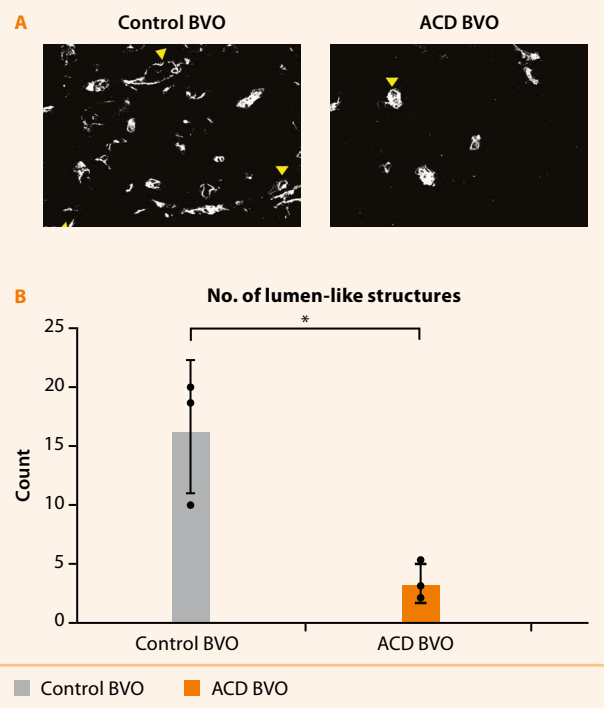

Figure 4: ACD BVOs develop less lumen-like structures. Immunofluorescence staining of CD31 (grey) on sections of day 15 BVOs. Lumen-like structures within BVOs are indicated by the yellow arrows (A). Quantification of the number of lumen-like structures in the immuno-stained BVO sections (n=3). Data is presented as the mean ± SD; *p<0.05, **p<0.01, ***p<0.001 (B).

BVO dissociation into single cells

The BVOs were washed once with DPBS and transferred into a 5 mL Eppendorf Tube®. Supernatant was removed and the BVOs were minced with surgical scissors. An enzymatic cocktail containing 5 mg/mL Dispase® and 0.5 mg/mL Liberase™ TH was diluted in DPBS and added to the BVO mince. The BVO-enzyme mixture was incubated at 37 °C for 30 min. After the first 5 min, 60 U/mL of DNase1 was added and the mixture was gently mixed using a wide bore tip.

The mixture was resuspended every 5 min until a single-cell suspension was achieved. The digestion reaction was quenched with cold KnockOut™ DMEM/F-12 supplemented with 10% FBS and spun down for 5 min at 300×g in a precooled centrifuge. The cell pellet was then resuspended in cold DPBS supplemented with 2% FBS and strained using a 30 µm MACS SmartStrainer. Single cells were washed once more with DPBS with 2% FBS and the cell pellet was resuspended in DPBS with 2% FBS.

Isolation of ECs from BVOs using magnetic-cell sorting

Single cells obtained from the BVOs were resuspended in a buffer containing DPBS, 0.5% MACS BSA Stock Solution and 2 mM EDTA, and then counted. The cells were spun down for 5 min at 300×g. The cell pellet was resuspended in 60 µL of buffer and 20 µL of FcR Blocking Reagent per 10^7 cells. The cells were then incubated with 20 µL of CD144 MicroBeads, human. The CD144-labeled cells were isolated using LS Columns and the QuadroMACS™ Separator, according to the protocol.

Isolated cells that were confirmed to express endothelial marker CD31, were cultured onto 0.2% gelatin-coated wells, and maintained in EGM™ Endothelial Cell Growth Medium-2 BulletKit™.

Results

ACD BVOs exhibit severe vascular abnormalities

The ACD BVOs expressed reduced mRNA levels of endothelial marker PECAM-1 and pericyte marker PDGFRβ throughout differentiation compared to the control (fig. 3a) suggesting aberrant vascular development. On day 15 of differentiation, the number of ECs (CD31) and pericytes (PDGFRβ) in ACD BVOs were reduced compared to the control BVOs (fig. 3b).
The number of lumen-like structures was also significantly reduced in ACD BVOs compared to the controls (fig. 4).

Collectively, the ACD BVOs exhibited vascular maldevelopment leading to a loss of vessel networks, recapitulating the loss of capillaries observed in the lungs of ACD/MPV patients.

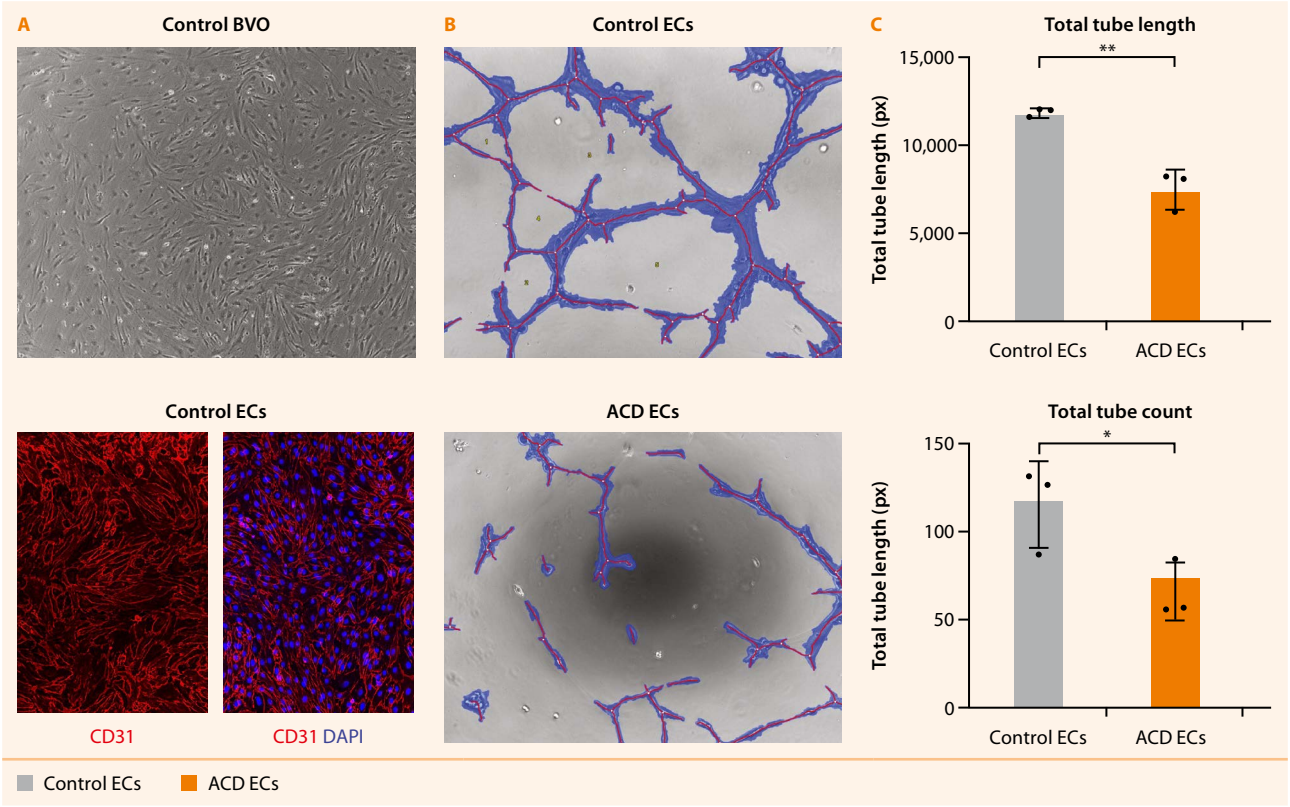

Figure 5: ECs were isolated from BVOs via magnetic-cell sorting. Morphology of CD144⁺ cells 24 h after magnetic-cell sorting (A). Representative image of tubes formed by control and ACD ECs (B). Quantification of total tube length and total tube count from control and ACD ECs (n=3). Data is presented as the mean ± SD; *p<0.05, **p<0.01, ***p<0.001 (C).

ECs isolated from diseased BVOs exhibit endothelial dysfunction

CD144-expressing ECs can be isolated from BVOs via magnetic-cell sorting, yielding a pure population of ECs that express endothelial marker CD31 (fig. 5a). The ECs that were isolated from ACD BVOs had reduced tube formation capacity compared to ECs isolated from control BVOs (fig. 5b and 5c), clearly illustrating EC dysfunction.

TanFe improves angiogenesis in ACD BVOs

A nitrile compound, known as transcellular activator of nuclear FOXF1 expression (TanFe), was recently discovered to increase FOXF1 protein levels *in vivo*. Mouse models of ACD/MPV treated with TanFe exhibited increases in lung angiogenesis and improved overall survival.[3] We found that ACD BVOs treated with TanFe (fig. 6), throughout day 5–15 of differentiation, showed an improved vascular network formation and an increased number of lumen-like structures (fig. 7).[3]
This indicates that TanFe is able to promote angiogenesis in ACD BVOs, thereby increasing endothelial density and well-formed vessels within BVOs.

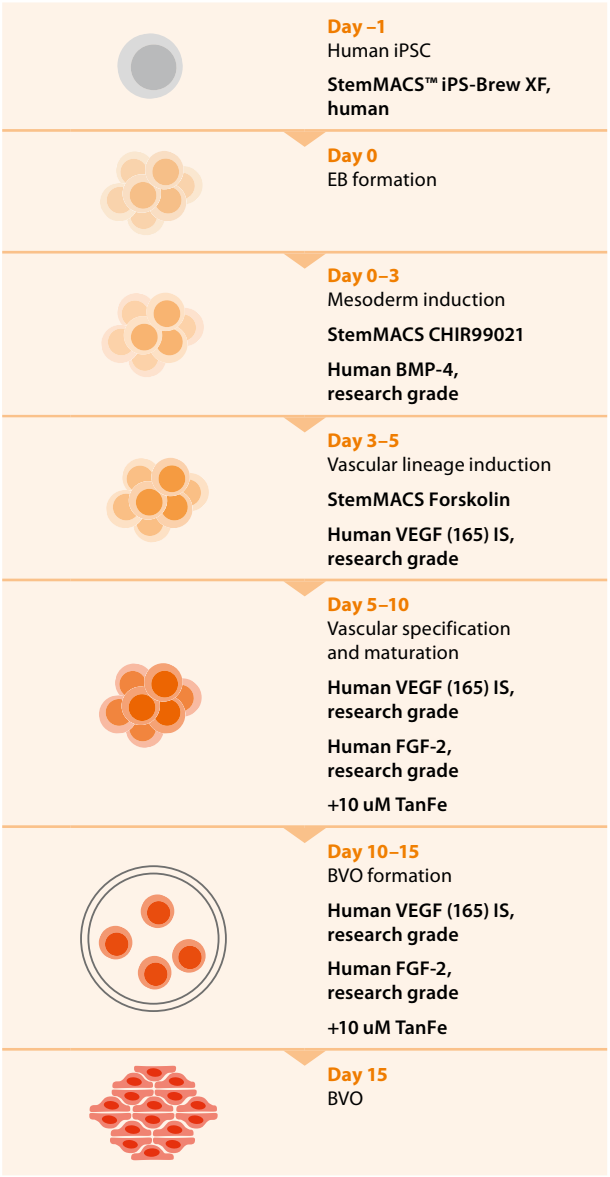

Figure 6: Protocol used for treatment of ACD BVOs and TanFe. Schematic representation of products from Miltenyi Biotec used in each protocol step for the treatment of TanFe from day 5–15 of BVO differentiation.

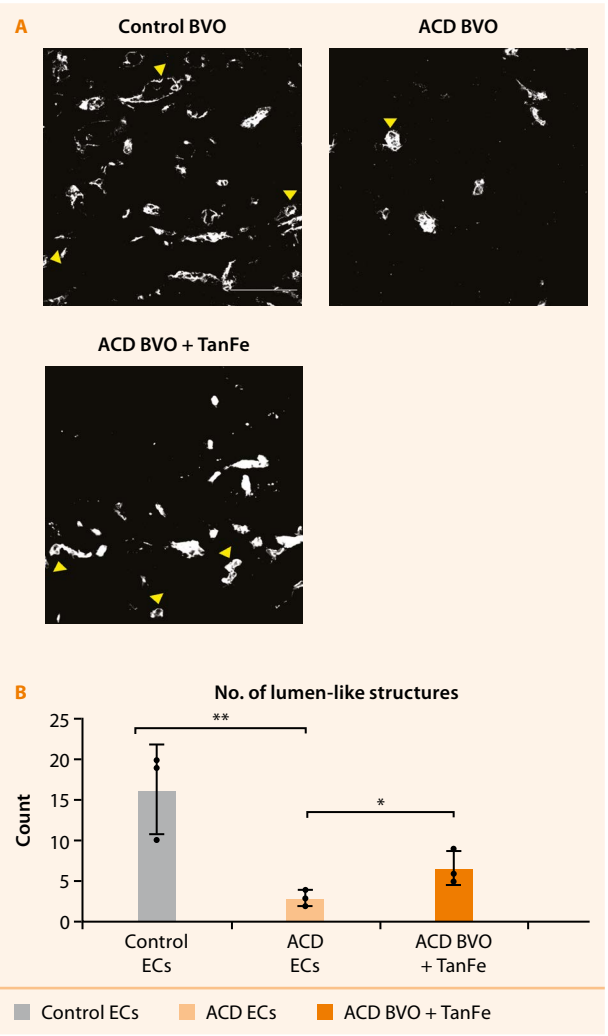

Figure 7: ACD BVOs treated with TanFe showed an increase in lumen-like structures. Immunofluorescence staining of day 15 BVO sections with CD31 (grey) (A). Quantification of the number of lumen-like structures in the immuno-stained BVO sections (n=3). Data is presented as the mean ± SD; *p<0.05, **p<0.01, ***p<0.001 (B).

Conclusion

Here, we present a workflow for the differentiation of human iPSCs to disease-relevant BVOs and EC isolation using:

- StemMACS iPS-Brew XF, human, for human iPSC maintenance.
- StemMACS small molecules and cytokines for BVO-directed differentiation.
- CD144 (VE-Cadherin) MicroBeads, human, with LS Columns and the QuadroMACS Separator for magnetic isolation of ECs from BVOs.

Human iPSC-derived BVOs serve as viable human models to study vascular abnormalities associated with congenital diseases such as ACD/MPV. We show that:

- The vascular abnormality phenotypes observed in ACD/MPV patients are represented in ACD BVOs.
- ACD BVOs exhibit enhanced angiogenesis and vessel formation with TanFe treatment, making it a potential therapeutic candidate for the treatment of ACD/MPV.

References

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