Paraxial mesoderm organoids model development of human somites

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Abstract

During the development of the vertebrate embryo, segmented structures called somites are periodically formed from the presomitic mesoderm (PSM), and give rise to the vertebral column. While somite formation has been studied in several animal models, it is less clear how well this process is conserved in humans. Recent progress has made it possible to study aspects of human paraxial mesoderm development such as the human segmentation clock *in vitro* using human pluripotent stem cells (hPSCs), however, somite formation has not been observed in these monolayer cultures. Here, we describe the generation of human paraxial mesoderm (PM) organoids from hPSCs (termed Somitoids), which recapitulate the molecular, morphological and functional features of paraxial mesoderm development, including formation of somite-like structures *in vitro*. Using a quantitative image-based screen, we identify critical parameters such as initial cell number and signaling modulations that reproducibly yielded somite formation in our organoid system. In addition, using single-cell RNA sequencing and 3D imaging, we show that PM organoids both transcriptionally and morphologically resemble their *in vivo* counterparts and can be differentiated into somite derivatives. Our organoid system is reproducible and scalable, allowing for the systematic and quantitative analysis of human spinal cord development and disease *in vitro*.

Introduction

Paraxial mesoderm (PM) development involves the formation of embryonic segments called somites, which are produced sequentially from the presomitic mesoderm (PSM) and arranged periodically along the anterior-posterior (AP) axis of the vertebrate embryo. Somites give rise and contribute to a variety of tissues including skeletal muscle, dermis, cartilage and bone (Chal and Pourquié, 2017). Somite formation is controlled by a conserved molecular oscillator, the segmentation clock (Dequeant et al., 2006; Hubaud and Pourquié, 2014; Oates et al., 2012). Previous efforts have focused on how this oscillator controls somite formation using a variety of model systems such as mouse, zebrafish, and chick, because of ethical and technical limitations of culturing human embryos. Recently, researchers were able to recapitulate paraxial mesoderm development using human and mouse pluripotent stem cells cultured as 2D monolayers (Chu et al., 2019; Diaz-Cuadros et al., 2020; Matsuda et al., 2020). These cells undergo species-characteristic oscillations similar to their *in vivo* counterparts. However, final stages of somite development and vertebra formation was not observed in currently published human cell culture systems (Palla and Blau, 2020), suggesting that certain aspects of *in vivo* development are not recapitulated in these 2D systems. We reasoned that a 3D cell culture system may exhibit all the stages of PM development including morphogenetic processes associated with somite formation.

Here, we describe an organoid system derived from human iPS cells (hiPSCs) (Somitoids), which faithfully recapitulates functional, morphological and molecular features of paraxial mesoderm development, including formation of somite-like structures *in vitro*. To identify the culture conditions that reproducibly yielded somite formation, we developed a quantitative image-based screening platform for individual organoids. Our screening approach identified the optimal parameter values for the culture conditions such as the initial cell number and the concentration of the chemical modulators. We show using single-cell RNA-sequencing (scRNA-seq), immunofluorescence, and qRT-PCR that Somitoids resemble their *in vivo* counterparts both transcriptionally and morphologically and can be differentiated into somite derivatives such as sclerotome *in vitro*.

Our Somitoid system is reproducible and scalable, allowing for systematic and quantitative analysis of paraxial mesoderm development and somite formation to study human spinal cord development and disease in a dish. Finally, our approach can be used to systematically screen organoid cultures for desired phenotypes and reproducibility.

Results

Recently, protocols have been developed to differentiate mouse or human pluripotent stem cells towards paraxial mesoderm using a combination of the WNT agonist CHIR and BMP inhibitor LDN (Chal et al., 2015; Diaz-Cuadros et al., 2020). To adapt the protocol for a 3D model of human somitogenesis, we first optimized the initial conditions of our cultures by generating pluripotent spheroids of defined cell numbers. hiPS cells were allowed to aggregate for 24 hours as suspension cultures in pluripotency media (mTeSR1) in the presence of ROCK inhibitor and Polyvinyl alcohol (PVA) to promote aggregation (Fig 1A). These pluripotent spheroids resemble cavity-stage epiblast embryos as previously described for kidney organoid cultures (Freedman et al., 2015)(Fig 1B and Fig S1A). Next, spheroids were cultured in media containing CHIR and LDN (CL), similar as done for the monolayer cultures (Chal et al., 2015; Diaz-Cuadros et al., 2020) but with CHIR at a higher concentration (10 μM). After 24 hours in CL media, epiblast-stage cells transition to a neuromesodermal (NMp) or primitive streak cell fate, characterized by co-expression of T/BRA and SOX2 (Tzouanacou et al., 2009))(Fig 1B). By 48 hours, cells rapidly downregulate SOX2 and express PSM markers, including TBX6 and MSGN1 (Fig 1B). This PSM state persists from day 2 to day 4 and is also characterized by the expression of segmentation clock genes such as HES7 (Fig 1C). On day 5, organoids showed expression of marker genes associated with somite fate as characterized by qPCR (Fig 1C, S1A). Taken together, the order of activation of marker genes in the paraxial mesoderm organoids followed the expected stages of differentiation observed during PM development.

We observed that following the above protocol resulted in a heterogeneous activation of somite marker genes across cells within the same organoid and across different replicates. To improve reproducibility, we set out to screen for the optimal initial cell number, the concentration of the signaling factors and the timing of their additions

during culture. For our primary screen, we compared organoids with an initial cell number of 500 and 1000 as well as combinations of modulators of candidate signaling pathways that have previously been involved in paraxial mesoderm development and somite formation, including FGF, WNT, BMP and TGF-β (Aulehla et al., 2008; Chal et al., 2015; Hubaud and Pourquié, 2014; Tonegawa et al., 1997; Xi et al., 2017). PSM-stage organoids on day 3 were treated with signaling modulators for 24 and 48 hours, and somite fate and morphogenesis was assessed using PAX3, a somite fate marker, and F-ACTIN, a structural marker of somite formation (Fig 2A). To quantitatively compare conditions, we developed an image analysis pipeline to determine organoid diameter and normalized average Pax3 expression intensity per organoid in an automated manner (Fig 2C,D and Fig S3B). We analyzed three replicates per condition. Strikingly, all organoids that were treated with any combination of FGF or WNT inhibitor reproducibly expressed PAX3 within 24 hours of treatment (Fig 2B,D). However, organoids that were initiated from 500 cells exhibited less heterogeneity in somite marker gene induction and displayed fewer patches of cells which did not express PAX3 compared with organoids that were initiated from 1000 cells (Fig S3A). Additional staining for SOX2, a neural marker, showed that PAX3-negative cells expressed SOX2, suggesting that our paraxial mesoderm organoids derive from neuro-mesodermal progenitors (Fig S3A). In line with these results, F-ACTIN was more localized and resembled rosette-like structures that somites form *in vivo* in organoids initiated from 500 cells compared with organoids made from 1000 cells (Fig 2B). Therefore, we used 500 cells as the initial cell number going forward. Organoids that were treated for 48 hours with signaling pathway modulators showed an overall decrease of PAX3 expression compared with organoids treated for only 24 hours across replicates, indicating that prolonged signaling manipulation does not improve the somite phenotype (Fig 2D). Additionally, organoids initiated from 500 cells and then treated for 48 hrs had a smaller diameter compared with organoids of the same initial cell number that were treated for only 24 hours (Fig 2C). This suggests that long-term inhibition of WNT and/or FGF, known mitogenic signaling pathways, has detrimental effects on proliferation or cell survival. These results indicate that treatment of PSM-stage organoids with pathway modulators for 24 hours is sufficient to induce somite fate.

Next, we set out to optimize the culture conditions to increase the number of somite-like structures in addition to the expression levels of the somite marker genes. We used 500-cell spheroids as an initial starting point and compared two different inhibitor doses for FGF and WNT in addition to the other pathway modulators applied to PSM-stage organoids on day 3. We characterized the organoids after treating for 24 hours, 48 hours, and 24 hours followed by culture in basal media without any added factor for an additional 24 hours (5 replicates per condition, Fig 3A). In addition to quantifying Pax3 levels (Fig 3C, Fig S4B), we also counted the number of somite-like structures per organoid (Fig 3D, Fig S4A,B). Comparing PAX3 expression levels in our treated organoids, we observed that somite fate can be broadly induced across a range of treatment regimes, concentrations and types of WNT and/or FGF inhibitors (Fig 3C and Fig S4C). However, the number of somite-like structures is not necessarily correlated with average PAX3 expression levels. For example, several conditions (FGFRi/PD173, WNTi/C59, WNT/C59+FGFRi^{lo}, WNTi/XAV) with treatment regimes of 24 hours treatment and 24 hours of culture without added factors were amongst the conditions with highest average PAX3 levels (Fig 3C). However, the number of somite-like structures in these conditions was lower compared with conditions that exhibited lower average PAX3 expression (Fig 3C,D and Fig S4A). This suggests that marker gene expression alone may not be the best predictor when screening for morphologically complex phenotypes such as somite formation.

Surprisingly, organoids which were cultured for an additional 24 hours (day 3 to day 4) in FGF, WNT pathway agonist and BMP inhibitor, considered a treatment control, followed by culture in basal media only for another 24 hours, consistently exhibited the highest number of somite-like structures across all replicates (Fig 3D, Fig S4A,B). This suggests that simply removing FGF, WNT pathway agonist and BMP inhibitor, which maintain cells in a PSM state, is sufficient to reproducibly induce somite fate and morphological formation of somite-like structures (Fig S4A). Computing the variation of somite numbers across the 5 replicates confirmed that this phenotype was highly reproducible (coefficient of variation = 11.1%; Fig S4B) Taken together, by systematically testing critical parameters such as concentration and timing/duration of pathway modulators as well as their combinatorial effects, in addition to initial cell numbers, we

have determined an optimal treatment regime with which we can generate homogenous somite-stage organoids in a highly reproducible manner. These Somitoids show transcriptional and morphological hallmarks of somite formation.

To further characterize the developmental trajectory and transcriptional states of our Somitoid system, we collected 15,558 cells (after post-processing) over the course of the optimized five day differentiation protocol at time-points that capture the key transition steps (day 1, day 2, day 3, day 5) and performed single-cell RNA sequencing (scRNA-seq; Fig 4A). Multiple organoids were used to obtain the required number of cells at each timepoint (see Methods). We first combined all the cells across the time-points and clustered them using the Leiden clustering algorithm. Predominantly, the 4 major clusters corresponded to cells from the 4 different time-points. Therefore, cells at each time-point have transcriptional states that are different compared with cells from the other time-points. In addition, within each time-point, cells exhibit similar transcriptional profiles as indicated by the uniformity of the expression levels of marker genes across individual cells (Fig 4E, Fig S5,S6,S7).

Cells collected on day 1 exhibited gene expression profiles similar to primitive streak or neuro-mesodermal progenitors, expressing genes such as SOX2, T/BRA, MIXL1 and NODAL (Fig 4C,E; Fig S5). Starting on day 2, cells expressed canonical PSM marker genes such as TBX6, MSGN1, WNT3A, RSPO3 and clock genes of the Notch signaling pathway including HES7, LFNG, DLL1 and DLL3 (Fig 4C,E; Fig S6). Day 5 cells expressed somite-marker genes such as TCF15, PAX3, FOXC2 and MEOX2 (Fig 4C,E; Fig S7). Interestingly, a subset of the day 5 cells also expressed somite polarity markers, UNCX and TBX18, which suggests faithful recapitulation of somite patterning in Somitoids (Fig 4C; Fig S7). Furthermore, two of the sub-clusters ('PSM-to-Somite', 'Early Somite'), which are characterized by co-expression of PSM and somite marker genes, were comprised of both day 3 and day 5 cells, indicating that the PSM-to-somite transition is captured in our *in vitro* system (Fig 4A,B). Interestingly, one somite sub-cluster ('Late Somite') was enriched for myogenic genes (MYL4/6/7/9, TROPONIN L1) and sclerotome genes (TWIST1, COL1A1, COL11A1, COL7A1, ACTA2), suggesting that these cells represent more downstream fates of somite-derived cells (Fig 4B; Fig S8A). Finally, we observed the expected sequential activation pattern of the HOX genes in our Somitoid

system starting with HOXA1 on day 1, followed by other cervical and thoracic HOX genes on day 2-3, to HOXD9, a lumbosacral HOX gene, in the somite-stage organoids (day 5; Fig 4D and Fig S8B). Taken together, our scRNA-seq analyses show that our Somitoid system faithfully recapitulates the gene regulatory programs of human paraxial mesoderm development and generates mature somite cells, which express the full repertoire of known marker genes. Furthermore, we did not detect cells of different developmental origins, suggesting that we are generating homogeneous organoids containing only paraxial mesoderm derivatives.

We next assessed whether our *in vitro* derived somites show similar spatial organization and size distribution to their *in vivo* counterparts. To independently confirm some of the somite marker genes that we identified in our scRNA-seq data set, we measured expression levels of TCF15/PARAXIS and PAX3 in day 5 Somitoids using immunostaining (Fig 5A). Day 5 cells co-expressed both somite marker genes throughout the Somitoid. To determine whether *in vitro*-derived somites were similar in size to embryonic segments, we compared them with Carnegie stage 9/10 early somites (Fig 5B). Organoid-derived somites were similar in size to E8.5 mouse embryo somites (diameter range of mouse somites is between 80-140 μm compared with a median size of 94 μm (range 70-113 μm) for organoid-derived somites; (Tam, 1981)) but slightly larger than early stage human somites (Carnegie 9 somites, median 68 μm (range 58-69 μm), Carnegie 10 somites, median 72 μm (range 67-88 μm); Fig 5B). Together, these results suggest that our organoid-derived somites share spatial and molecular features as well as overall size with their *in vivo* counterparts.

Finally, to assess whether Somitoids can give rise to downstream paraxial mesoderm derivatives such as sclerotome, we exposed day 5 somite-stage organoids to Shh agonist and Wnt inhibitor to mimic the signaling environment of ventral somites *in vivo* (Fan et al., 1995; Fan and Tessier-Lavigne, 1994; Loh et al., 2016). After 3 days, the organoids showed a robust induction of canonical sclerotome marker genes such as PAX1, SOX9 and COL2A1 (Fig 5C). These data demonstrate that our *in vitro* induced somites maintain their ability to differentiate further into somitic mesoderm derivatives such as sclerotome.

Discussion

Here, we reported the generation of human paraxial mesoderm organoids from hPSCs that reproduce important features of somitogenesis not previously captured in conventional monolayer differentiation cultures, most notably somite formation. Using a simple suspension culture which does not require manual matrix embedding, we identified optimal differentiation conditions by systematically screening initial cell numbers and modulating the signaling factors. Importantly, our culture conditions are compatible with high-throughput screening approaches. Many established organoid protocols currently have limited applications because they are not reproducible. Therefore, we set out to identify the optimal differentiation conditions that minimized the variability between organoids as quantified by automated image analysis.

One critical parameter we identified in our screens was the initial cell number used for aggregation. Our results suggest that if the initial cell number is above a certain threshold then somite fate cannot be induced in a homogeneous manner in our organoid system. This result is in line with previous findings in 3D models such as gastruloids, multi-axial self-organizing aggregates of mouse ES cells, which exhibit a higher degree of variability and multiple elongations when the number of initial cells exceeds a threshold (Beccari et al., 2018; van den Brink et al., 2014). Another important finding of our screens was that simply removing FGF, WNT pathway agonist as well as BMP inhibitor yielded the most reproducible somite-forming organoids. This treatment regime does not necessarily follow from applying prior *in vivo* and *in vitro* knowledge of somitogenesis. Previous protocols have used FGF and WNT inhibitors (Matsuda et al., 2020) or inhibition of all four candidate signaling pathways (FGF, WNT, BMP and TGF-β) to induce somite fate (Loh et al., 2016) in monolayer cultured hiPSCs. While these conditions similarly induced somite fate marker genes in our 3D system, removal of FGF/WNT agonists overall performed better as indicated by larger organoid diameters, higher average PAX3 expression levels, and higher number of somite-like structures. Our results also suggest that focusing on marker gene induction as a phenotypic readout alone is not sufficient to optimize culture conditions of complex organoid models of somitogenesis.

The single-cell RNA-seq analysis of our hPSC-derived Somitoids independently confirmed our immunostaining and qRT-PCR results and showed that all major paraxial

mesodermal cell types are generated, consistent with the cell types observed during PM development. While expression patterns of canonical somitic marker genes seem to be conserved in humans, it will be interesting to perform detailed gene expression analysis to identify putative human-specific genes of somite differentiation. Since our Somitoid system is reproducible, it could serve as a versatile platform to perform functional screens of human-specific or disease-relevant genes using CRISPR/Cas9 or small molecule inhibitor libraries. Somitoids thus provide a powerful *in vitro* system for studying the regulation and dynamics of somitogenesis, including somite formation.

One limitation of this work is that the current protocol does not produce an anterior-posterior axis and therefore does not generate somites in a bilaterally symmetric fashion as in the vertebrate embryo. A similar phenotype was recently reported in mouse gastruloids that were embedded in matrigel to promote self-organization into trunk-like structures (Veenvliet et al., 2020). Chemical modulation of BMP and WNT signaling pathways in matrigel-embedded gastruloids resulted in formation of somites arranged like a bunch of grapes, similar to what we observed in our system. In standard culture conditions, gastruloids recapitulate the axial organization of the embryo, which is missing in our Somitoids (Beccari et al., 2018; Moris et al., 2020). To expand the patterning and morphogenetic potential of our Somitoid system, our approach could be combined with a microfluidics setup to generate spatio-temporally controlled morphogen gradients (Manfrin et al., 2019). In summary, Somitoids provide a scalable, reproducible and easy to manipulate platform to study molecular networks underlying the differentiation of paraxial mesoderm, as well as the morphogenetic processes of somite formation. Furthermore, Somitoids represent a promising *in vitro* system to study congenital diseases that are linked to the human segmentation clock and somite formation, such as congenital scoliosis.

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Author Contributions

C.B. designed, optimized and performed the experiments. C.B. analyzed the experimental data with help from S.G. S.L. assisted with single-cell sample collection and sequencing library preparation. A.R. contributed to an early version of the differentiation protocol. C.B. and S.H. wrote the paper. O.P. and S.H. supervised the project.

Declaration of Interests

The authors declare no competing interests.

Materials and Methods

Human iPS cell culture and 3D differentiation

Human iPS cells were maintained on Matrigel-coated plates (Corning, cat no. 354277) in mTeSR1 media (Stem Cell Technologies, 85870) using maintenance procedures developed by the Allen Institute for Cell Science (https://www.coriell.org/1/AllenCellCollection). NCRM1 iPS cells were obtained from RUCDR Infinite Biologics, and ACTB-GFP (cell line ID: AICS-0016 cl.184) and CTNNB1-GFP (cell line ID: AICS-0058 cl.67) fluorescent reporter iPS cell lines were obtained from the Allen Institute for Cell Science.

For generation of paraxial mesoderm organoids, 500 dissociated iPS cells resuspended in mTeSR1 media containing 10 μM Y-27362 dihydrochloride (ROCKi; Tocris Bioscience, cat. no. 1254) and 0.05% poly-vinyl alcohol (PVA) were dispensed into 96-well U-bottom non-adherent suspension culture plates (Greiner Bio-One, 650185) and allowed to aggregate for 24 hours. To induce paraxial mesoderm differentiation, 24 hour old pluripotent spheroids were subjected to CL media consisting of RHB Basal media (Takara/Clontech, cat. no. Y40000), 5% KSR (Thermo Fisher Scientific, cat. no. 10828028) with 3 μM CHIR99021 (Sigma-Aldrich, cat. no. SML1046), 0.5 μM LDN193189 (Stemgent cat. no. 04-0074), and 5 μM ROCKi for the first 24 hours. Organoids were cultured in CL media without ROCKi from 24-72 hours of differentiation. On day 3 (72-120 hours), CL media was supplemented with 20 ng/ml FGF2 (PeproTech, cat. no. 450-33). On day 4, organoids were cultured in basal media only, without the addition of signaling factors.

To further differentiate Somitoids towards sclerotome fate, day 5 somite-stage organoids were treated with 5 nM of Shh agonist SAG 21k (Tocris, cat. no. 5282) and 1 μM of Wnt inhibitor C59 for 3 days as previously described (Loh et al., 2016). Organoids were subsequently differentiated towards cartilage by culturing them in the presence of 20 ng/ml BMP4 (R&D Systems, cat. no. 314-BP-010) for 6 days.

Small molecule inhibitor screens

For the systematic small molecular inhibitor screen, PM organoids were generated and differentiated until day 3 (PSM) of our protocol. On day 3, media was replaced with fresh media containing combinations of small molecule inhibitors targeting the FGF, WNT, BMP and TGF-β signaling pathways at indicated concentrations. For targeting the WNT pathway, we used C59 (Tocris, cat. no. 5148), XAV939 (Tocris, cat. no. 3748) and CHIR99021 (Sigma-Aldrich, cat. no. SML1046). For inhibiting the FGF pathway we used PD173074 (Sigma-Aldrich, cat. no. P2499). For inhibiting the BMP pathway we used LDN193189 (Stemgent, cat. no. 04-0074). For inhibition of the TGF-β pathway we used A-83-01 (Tocris, cat. no. 2939). Media was changed daily. We analyzed 3 replicates per condition in the primary screen, and 5 replicates per condition in the secondary screen.

Immunostaining

For organoid whole-mount immunostaining, organoids were collected in cold PBS and fixed in 4% paraformaldehyde for 1-2 hours depending on size/stage. Organoids were washed in PBS and PBSFT (PBS, 0.2% Triton X-100, 10% FBS), and blocked in PBSFT+3% normal donkey serum. Primary antibody incubation was performed in the blocking buffer overnight at 4ºC on a rocking platform. After extensive washes in PBSFT, secondary antibody incubation (1:500, all secondary antibodies were raised in donkey) was performed overnight in PBSFT. Organoids were washed first in PBSFT and, for the final washes, were transferred to PBT (PBS, 0.2% Triton X-100, 0.2% BSA), followed by 50% glycerol in PBT and 70% glycerol in PBT prior to mounting. Hoechst (1:2000) was added to the last PBSFT wash. A list of primary antibodies is provided in Table S1.

Confocal and time-lapse microscopy

All whole-mount immunostaining images were collected with a Nikon A1R point scanning confocal with spectral detection and resonant scanner on a Nikon Ti-E inverted microscope equipped with a Plan Apo VC 20x objective (NA 0.75). Alexa-488, Alexa-594, Alexa-647 fluorophores coupled to secondary antibodies were excited with the 488 nm, 561 nm, and 647 nm laser lines from a Spectral Applied Research LMM-5 laser merge module with solid state lasers (selected with an AOTF) and collected with a 405/488/561/647 quad dichroic mirror (Chroma). For time-lapse experiments, images were acquired with a Yokagawa CSU-X1 spinning disk confocal on a Nikon Ti inverted

microscope equipped with a Plan Apo 20x objective (NA 0.75) and a Hamamatsu Flash4.0 V3 sCMOS camera. Samples were grown on 6-well glass-bottom multiwell plates with No. 1.5 glass (Cellvis, cat. no. P06-1.5H-N) and mounted in a OkoLab 37°C, 5% CO2 cage microscope incubator warmed to 37°C. Images were collected every 15 min, using an exposure time of 800 ms. At each time-point, 30 z-series optical sections were collected with a step-size of 2 microns. Multiple stage positions were collected using a Prior Proscan II motorized stage. Z-series are displayed as maximum z-projections, and gamma, brightness, and contrast were adjusted (identically for compared image sets) using Fiji/ImageJ (Schindelin et al., 2012)(https://imagej.net/Fiji).

Automated image segmentation and analysis

Automated image analysis, including background de-noising, segmentation and feature extraction was done using ImageJ/Fiji macro language run in batch mode to process the entire screen data set. First, binary masks were generated from the Hoechst (nuclear stain) channel by de-noising the image (Gaussian Blur, sigma=5) followed by applying Li's Minimum Cross Entropy thresholding method (Li and Tam, 1998) and refining binary masks through several rounds of erosion/dilation steps. Next, binary masks were converted to selections and added to the Region of Interest (ROI) Manager. Finally, ROIs were used to perform diameter measurements of organoids. For Pax3 measurements, Hoechst and Pax3 channels were first denoised using a Gaussian blur filter (sigma=10) and then used to create a normalized Pax3 image by dividing the Pax3 channel with the Hoechst channel. Next, ROIs based on Hoechst binary masks were applied to the Pax3 normalized image to extract fluorescence intensity measurements for each z-slice. Finally, mean Pax3 intensity values for each organoid were calculated and compared.

Quantification of organoid and human somite sizes

Carnegie stage 9 and 10 human embryonic somite data was obtained from the Virtual Human Embryo Project (https://www.ehd.org/virtual-human-embryo/). Somite sizes of human embryos were measured using the Ruler Tool on the Virtual Human Embryo website along the medio-lateral and dorso-ventral axis of the embryo. The slice with the largest diameter of each somite was used for measurements. Somite sizes of day 5 organoids were measured along the X and Y axes of the image since, unlike in the embryo, they do not exhibit morphological anisotropies. Somite areas were approximated

by using the two diameter measurements from each somite to calculate the area of the resulting rectangle.

RNA extraction, reverse transcription and qPCR

Organoids were harvested in Trizol (Life Technologies cat. no. 15596-018), followed by precipitation with Chloroform and Ethanol and transfer onto Purelink RNA Micro Kit columns (Thermo Fisher cat. no. 12183016) according to manufacturer's protocol, including on-column DNase treatment. A volume of 22 µl RNase-free water was used for elution and RNA concentration was measured with a Qubit Fluorometer. Typically, between 0.2-1 µg of RNA was reverse transcribed using Superscript III First Strand Synthesis kit (Life Technologies cat. no. 18080-051) and oligo-dT primers to generate cDNA libraries.

For real time quantitative PCR, cDNA was diluted 1:30-1:50 in water and qPCR was performed using the iTaq Universal SYBR Green kit (Bio-Rad cat. no. 1725124). Each gene-specific primer and sample mix was run in triple replicates. Each 10 µl reaction contained 5 µl 2X SYBR Green Master Mix, 0.4 µl of 10 µM primer stock (1:1 mix of forward and reverse primers), and 4.6 µl of diluted cDNA. qPCR plates were run on a Roche LightCycler 480 Real-Time PCR system with the following cycling parameters: initial denaturation step (95°C for 1 minute), 40 cycles of amplification and SYBR green signal detection (denaturation at 95°C for 5 seconds, annealing/extension and plate read at 60°C for 40 seconds), followed by final rounds of gradient annealing from 65ºC to 95ºC to generate dissociation curves. Primer sequences are listed in Table S2. All unpublished primers were validated by checking for specificity (single peak in melting curve) and linearity of amplification (serially diluted cDNA samples). For relative gene expression analysis, the ΔΔCt method was implemented using the R package 'pcr' (https://cran.r-project.org/web/packages/pcr/). PP1A was used as the housekeeping gene in all cases. Target gene expression is expressed as fold change relative to undifferentiated iPS cells.

Preparation of single-cell suspensions for scRNA-seq

Cell dissociation protocols were optimized to achieve single cell suspensions with >90% viable cells and low number of doublets.

Organoids collected at day 1, 2, 3, and 5 of our differentiation protocol were pooled in pre-warmed PBS, transferred to pre-warmed accutase and incubated for 5-7 min at

37ºC. For Day 1 organoid cell suspension, 30 organoids were pooled. For Day 2 organoid cell suspension, 15 organoids were pooled. For Day 3 cell suspension, 8 organoids were pooled. For Day 5 cell suspension, 5 organoids were pooled. Organoids were briefly rinsed in PBS, then transferred to 500 µl PBS/0.05% BSA and carefully triturated to generate a single-cell suspension. Cell suspension was run through a cell strainer (Falcon, cat. no. 352235) and transferred to a 1.5 ml tube. Cells were spun down at 250g for 3 min at 4ºC. Cell pellet was resuspended in 25 µl PBS/0.05% BSA, cell concentration and viability was measured using an automated cell counter, and cell suspension was further diluted as appropriate to reach the optimal range for 10x (700-1200 cells per µl). Cells were subjected to single-cell RNA sequencing (10x Genomics, Chromium Single Cell 3' v3) aiming for the following target cell numbers:

Day 1, 3,000 cells; Day 2, 4,000 cells; Day 3, 5,000 cells; Day 5, 6,000 cells. Estimated actual cell numbers collected were: Day 1, 2,930 cells; Day 2, 4,977; Day 3, 5,968 cells; Day 5, 4,841 cells. Single-cell libraries were generated using standard protocols. Libraries were sequenced together on a NovaSeq 6000 system resulting in 800 million reads.

Analysis of scRNA-seq data

Statistics and plots were generated using R version 4.0.2 "Taking Off Again" and Seurat version 3.0 (Stuart et al., 2019).

QC analysis / processing of scRNA-seq data

Cell Ranger pipeline (10x Genomics, Version 4.0.0) was used to de-multiplex the raw base call files, generate FASTQ files, perform the alignment against the human reference genome (GRCh38 1.2.0), and generate the count matrices.

For the initial QC, we determined the following thresholds for filtering out low-quality cells: UMI counts less than 500, gene counts less than 200, mitochondrial fraction above 0.2 and a complexity score of less than 0.8 (calculated as log10(genes)/log10(UMIs)).

Low-dimensional embedding and clustering

After QC filtering, we normalized our dataset using the sctransform (Hafemeister and Satija, 2019) framework, which is part of the Seurat package. To regress out confounding variation in our dataset, we performed cell cycle scoring and determined mitochondrial mapping percentage using standard workflows. Next, we performed PCA and determined the K-nearest neighbor graph using the first 40 principle components. We then applied

the Leiden clustering algorithm using a parameter range from 0.1-1.0 to determine the best resolution/number of clusters, which reflected biological differences (FindClusters, resolution=0.1-1.0). Clusters were visualized on a UMAP embedding (RunUMAP, dims = 1:40). To determine optimal resolution for clustering and assign cell types for each cluster, we visualized sets of known marker genes for each predicted cell type on UMAP plots. Prior to marker gene identification and final assignments of cluster identities, we also checked additional quality control metrics (UMI count, gene count, mitochondrial gene ratio) to exclude low-quality clusters from downstream analyses. Through iterative analysis we determined Leiden clustering with resolution = 0.8, resulting in 22 clusters, to best capture biological variation of the dataset. Using a combination of quality control metrics and unbiased marker gene identification for each cluster (see below), we excluded seven smaller low-quality clusters (as determined by QC metrics and/or expression of stress signature genes) from further downstream analysis (15 clusters after filtering).

Identification of differentially expressed genes

Marker genes for every cluster were identified by a two-sided Wilcoxon rank-sum test comparing cells from each cluster to all other cells in the combined dataset. Genes were considered differentially expressed if the log2 fold-change average expression in the cluster is equal or greater 0.25 relative to the average expression in all other clusters combined, and the adjusted P-value <0.05. Multiple comparison correction was performed using the Bonferroni method. Identified marker genes for the top 20 differentially expressed transcripts are listed in Figure S8A. The full list of differentially expressed genes, ranked by adjusted P-values and associated fold-changes are provided in Supplementary Table 1.

Supplemental Tables

Table S1: Antibodies for immunostaining

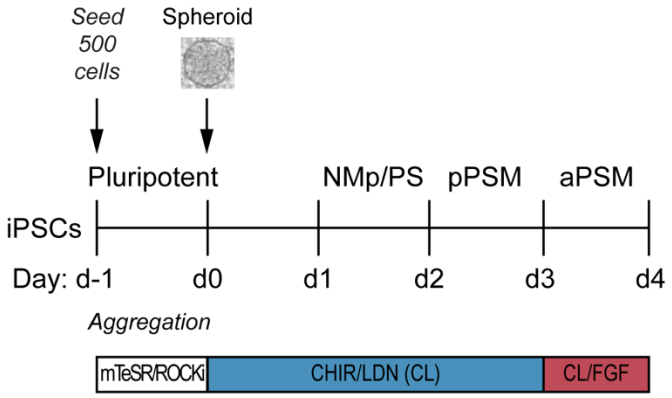
Antibody	Company	Catalog Number	Dilution
CDH2/NCADHERIN	Abcam	ab18203	1:400
PAX3	DSHB	Pax3-c	1:250
SOX2	R&D Systems	AF2018	1:200
T/BRACHYURY	Abcam	ab209665	1:200
TBX6	Abcam	ab38883	1:300
TCF15/PARAXIS	Abcam	ab204045	1:50

Table S2: Primer sequences for quantitative PCR

Gene	Forward Primer	Reverse Primer	Reference
COL2A1	CCAGATGACCTTCCTACGCC	TTCAGGGCAGTGTACGTGAAC	Loh et al.
FOXC1	TATACTGCGGGTTGGAAAGG	TCGCCTTCCTTCTTGTTATTG	This study
FOXC2	CCTCCTGGTATCTCAACCACA1	GAGGGTCGAGTTCTCAATCCC	Loh et al.
HES7	CTCCCTTGCGTCTAGGATTG	CTGAGGGTGGGAGACAGAAG	Diaz-Cuadros et al.
MEOX1	TCTGAGCGCCAGGTCAAAG	CTGAACTTGGAGAGGCTGTGG	This study
MEOX2	GTGGCGGCTACAAGGTCATC	CTGGCGCGGAACATAAACA	Loh et al.
MSGN1	CTGGGACTGGAAGGACAGG	ACAGCTGGACAGGGAGAAGA	Diaz-Cuadros et al.
PAX1	CGCTATGGAGCAGACGTATGGCGA	AATGCGCAAGCGGATGGCGTTG	Loh et al.
PAX3	AGGAACTGGAGCGTGCTTT	CAGCTTGCTTCCTCCATCTT	This study
PAX9	TGGTTATGTTGCTGGACATGGGTG	GGAAGCCGTGACAGAATGACTACCT	Loh et al.
PP1A	TCTGCCCACCTTAACAGACC	AATTCACGCAGAAGGAACCA	This study
SOX9	CGTCAACGGCTCCAGCAAGAACAA	GCCGCTTCTCGCTCTCGTTCAGAAGT	Loh et al.
T/BRA	TGCTTCCCTGAGACCCAGTT	GATCACTTCTTTCCTTTGCATCAAG	Loh et al.
TBX18	CATTCTGGCGACCATCACTAC	ATTCCCAGTACCTTGGAGCAA	This study
TBX6	CTACTCGGCTGCATTTCTGG	GGTTCAGTACATGGGTTTGGA	This study
TCF15	GAGCTGAGGAGAGTCCCGT	TGTGCCTCTCTCTAGGTCCA	This study
TWIST1	CTGCAGCACCGGCACCGTTT	CCCAACGGCTGGACGCACAC	Loh et al.
UNCX	CTATCCCGACGTGTTCATGC	GAACTCGGGACTCGACCAG	Loh et al.

Fig 1, Budjan et al.

A



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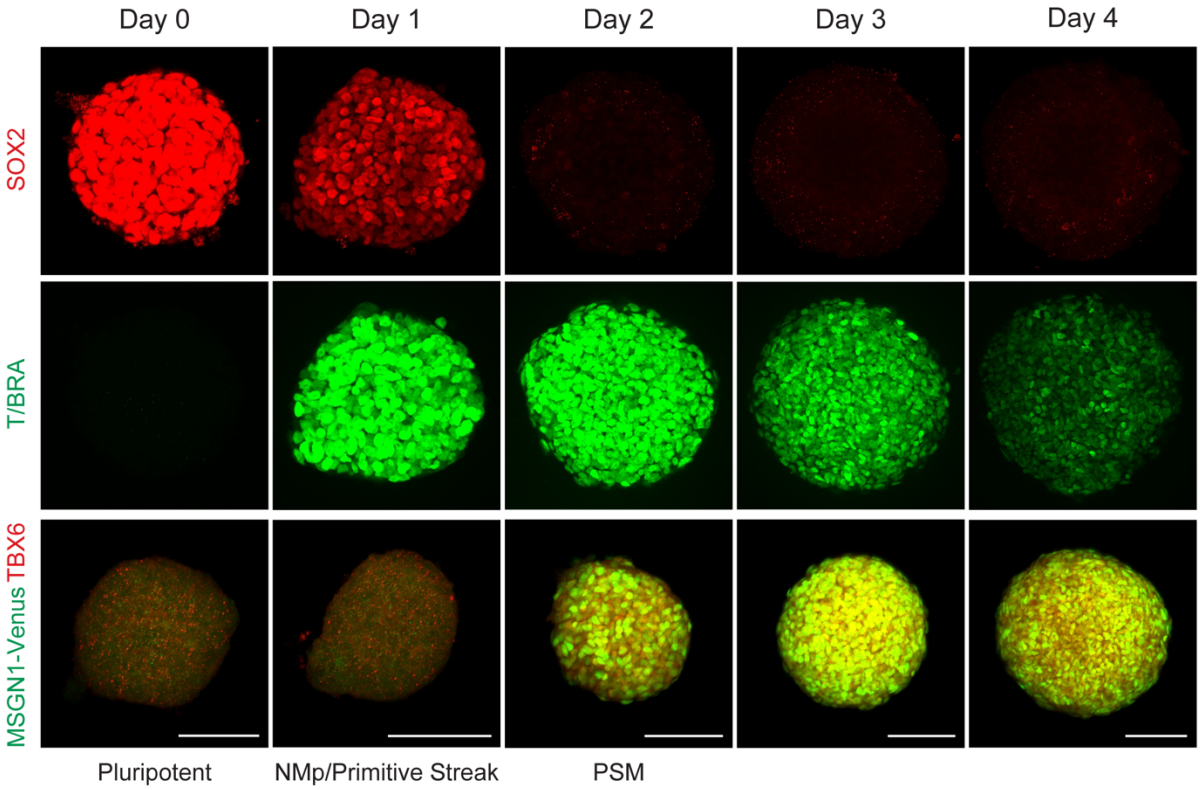

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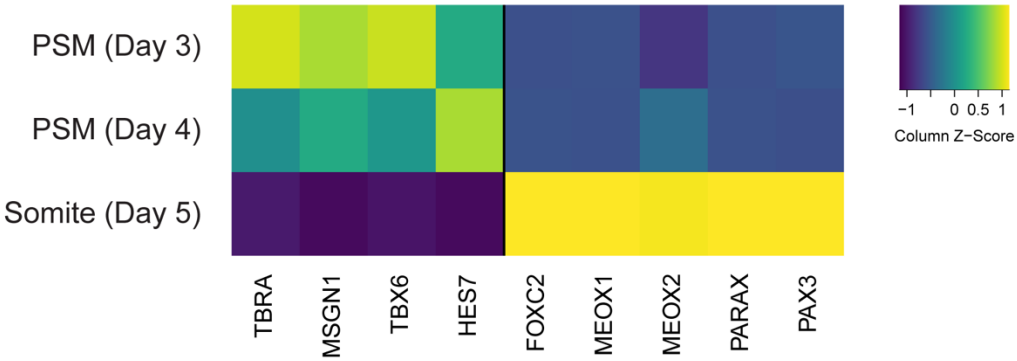


Figure 1. **Human PSC-derived paraxial mesoderm organoids turn on marker genes associated with paraxial mesoderm differentiation.**

(A) Schematic overview of paraxial mesoderm (PM) organoid differentiation protocol from human pluripotent stem cells (PSCs). Human PSCs aggregated and formed spheroids for 24 hours prior to differentiation. For differentiation, spheroids were exposed to Wnt agonist (CHIR) and BMP inhibitor (LDN) for 72 hours. On day 3, FGF2 was added to the media in addition to CHIR and LDN.

(B) Immunofluorescence analysis of cell fate-specific marker genes shows progressive differentiation towards PSM fate (top and middle row). Organoids derived from human iPS cells harboring an MSGN1-Venus reporter express TBX6 at the same time as the reporter is activated (bottom row). Scale bar represents 100 μm.

(C) qRT-PCR analysis of PSM and Somite markers reveals PSM-to-somite transition from day 4 to day 5. Relative gene expression levels are shown as Z-scores, expressed as fold-change relative to undifferentiated iPS cells (see Methods).

Fig 2, Budjan et al.

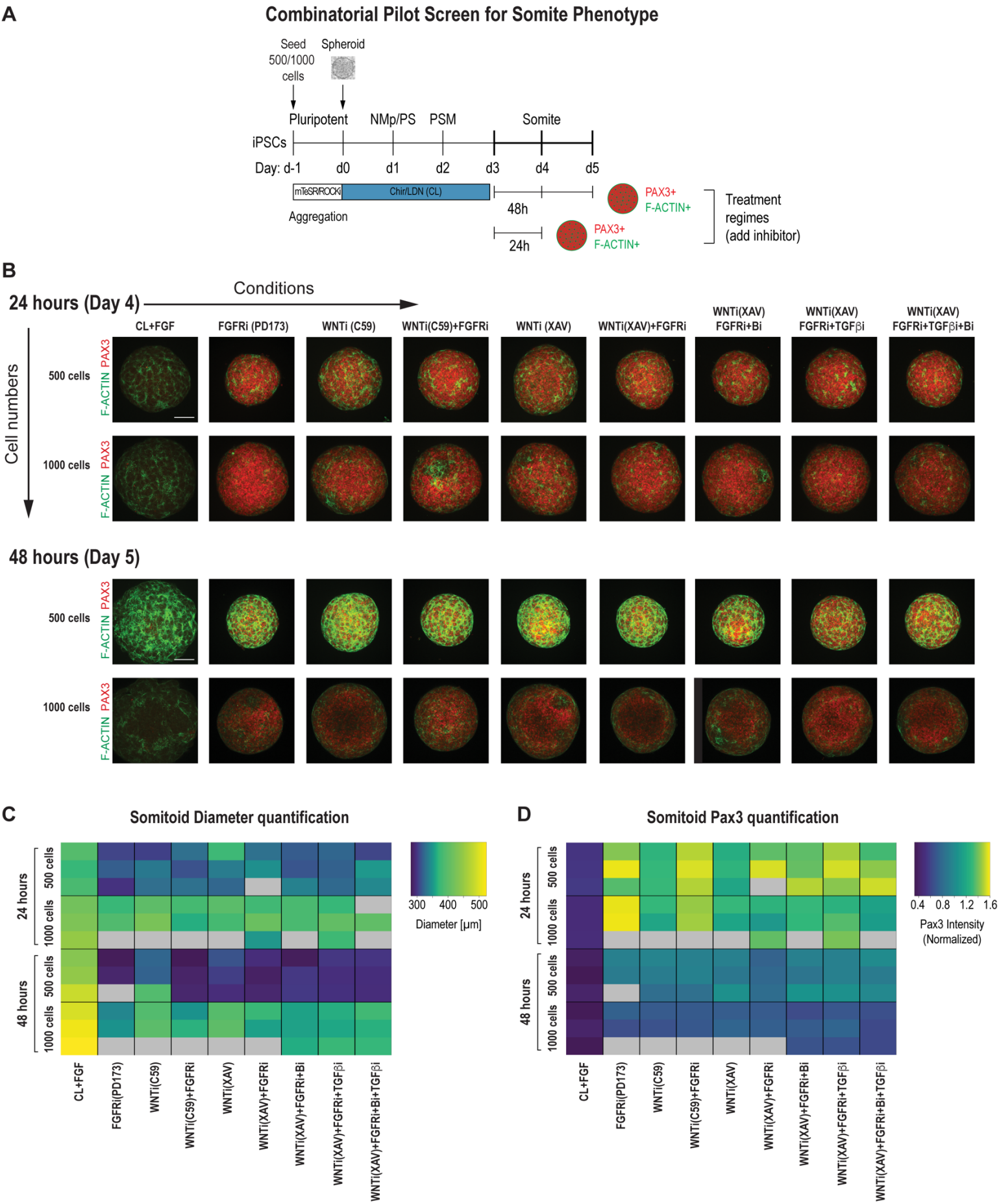


Figure 2. Pilot screen to optimize differentiation conditions for somite phenotype in PM organoids reveals optimal initial number of cells and duration of treatment.

(A) Schematic overview of systematic screen in PM organoids (Somitoids). PSM-stage organoids were treated on day 3 for 24 hours or 48 hours with signaling agonists/antagonists. Treated organoids were cultured in basal media with inhibitors as indicated. Control organoids were maintained in CL media with FGF added.

(B) Representative immunofluorescent images of day 4 and day 5 organoids after treatment for 24 hours or 48 hours, respectively, stained for somite marker PAX3 and F-ACTIN to visualize rosette-like somite structures. Organoids generated from 1000 cells generally show a more diffuse F-ACTIN pattern compared to organoids made from 500 cells, which exhibit bright foci, consistent with somite formation. Confocal images are shown as maximum intensity z-projections. Scale bar represents 100 μm. Small molecule inhibitors used are indicated in brackets. FGFRi: FGF receptor inhibitor (PD173074). WNTi: Wnt inhibitor (C59 or XAV939). Bi: BMP inhibitor (LDN). TGF-βi: TGF-β inhibitor (A-83-01).

(C) Automated quantification of organoid diameter for each organoid/replicate treated as indicated (see Methods for details). Grey boxes indicate missing data points. Organoids initiated from 500 cells show a decreased diameter when treated for 48 hours compared with 24 hours.

(D) Automated quantification of normalized average PAX3 intensity for each organoid/replicate treated as indicated. Grey boxes indicate missing data points. Organoids initiated from both 500 and 1000 cells show higher average normalized PAX3 levels when treated for 24 hours compared with 48 hours.

Fig 3, Budjan et al.

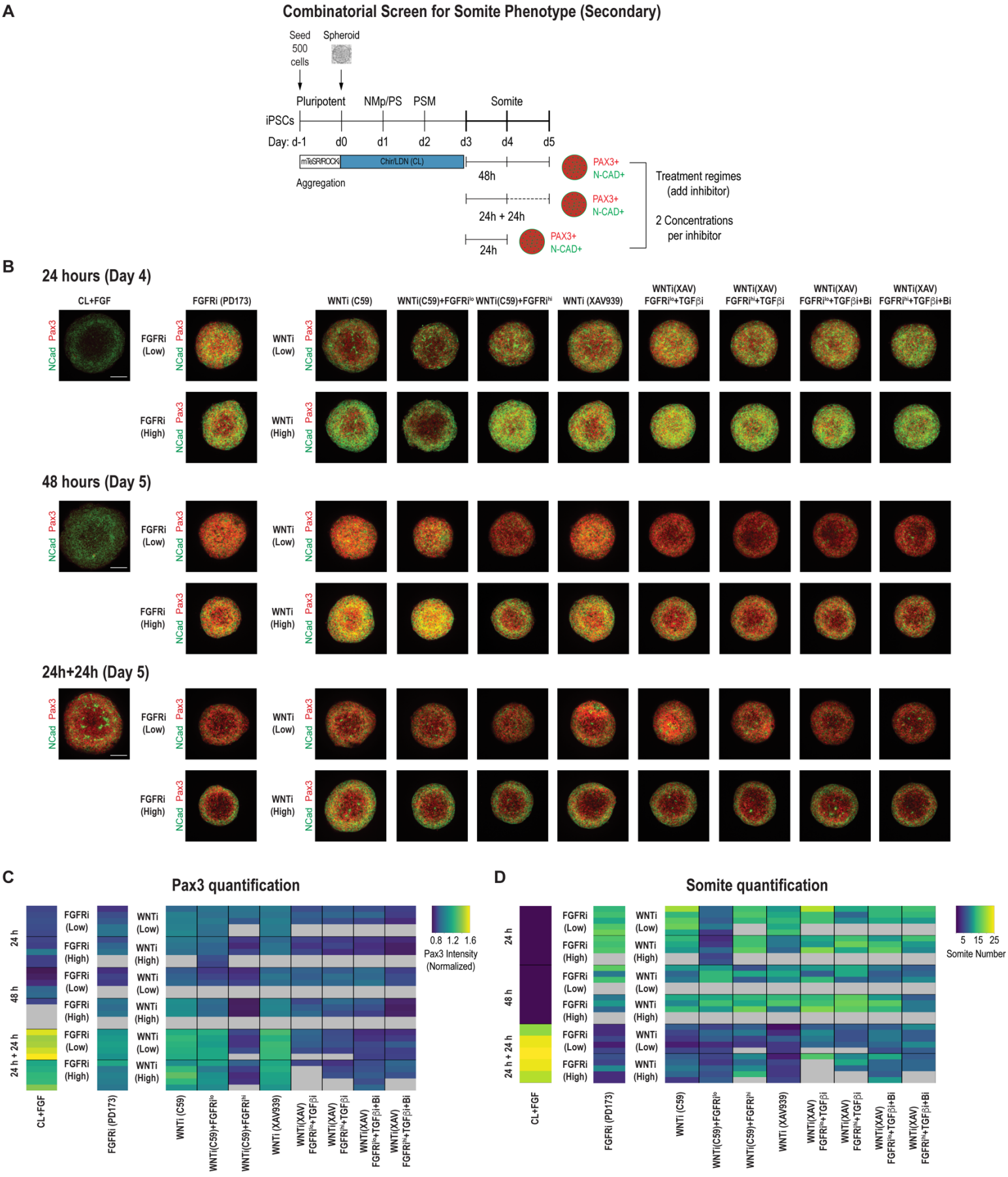


Figure 3. **Secondary screen of PM organoids identifies optimal differentiation protocol for somite formation.**

(A) Schematic overview of secondary screen in PM organoids. PSM-stage organoids were treated on day 3 for 24 hours followed by measurement, 24 hours of treatment followed by 24 hour culture in basal media (no added factors) and then measured (24h+24h), and 48 hours of treatment followed by measurement. Treated organoids were cultured in basal media with inhibitors as indicated. WNT and FGF inhibitors were tested at two different concentrations. Control organoids were maintained in CL media with FGF added.

(B) Representative immunofluorescent images of day 4 and day 5 organoids stained for somite markers PAX3 and NCAD to visualize rosette-like somite structures. Confocal images are shown as maximum intensity z-projections. Scale bar represents 100 μm. Small molecule inhibitors used are indicated in brackets. FGFRi: FGF receptor inhibitor (PD173074). WNTi: Wnt inhibitor (C59 or XAV939). Bi: BMP inhibitor (LDN). TGF-βi: TGF-β inhibitor (A-83-01).

(C) Automated quantification of normalized average PAX3 intensity for each organoid/replicate treated as indicated (see Methods for details). Grey boxes indicate missing data points. Several inhibitor combinations with a treatment regime of 24 hour treatment followed by 24 hour cultured in basal media show highest average PAX3 levels.

(D) Quantification of the number of somite-like structures for each condition. Each row represents one organoid replicate. Grey boxes indicate missing data points. Organoids which were maintained in CL media with added FGF for 24 hours followed by culture in basal media for 24 hours reproducibly exhibit the highest number of somites per organoid.

Fig 4, Budjan et al.

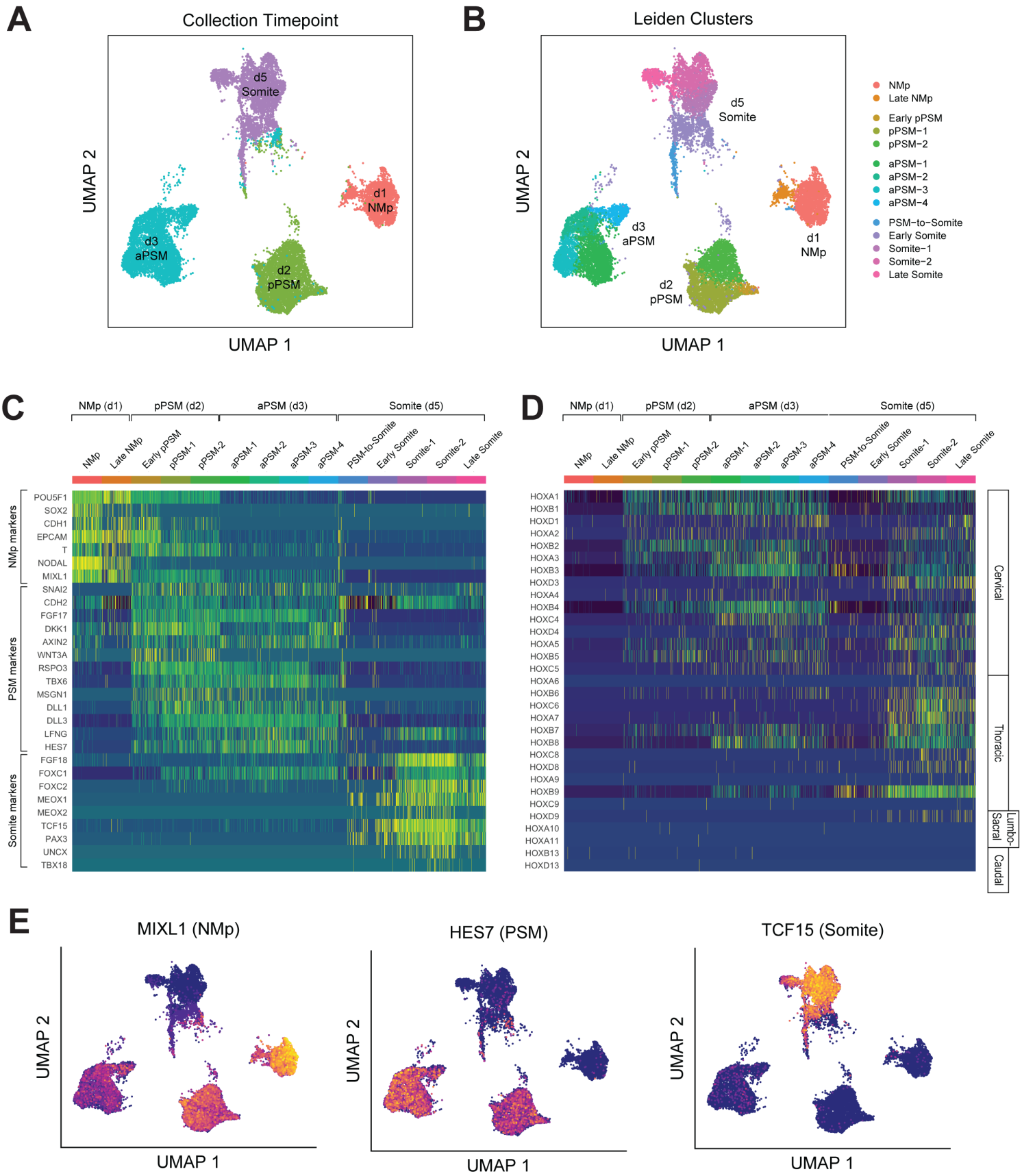


Figure 4. Single-cell RNA sequencing analysis of PM organoids (Somitoids) reveals differentiation trajectory from NMp-like cells to somite-stage paraxial mesoderm.

(A) UMAP (Uniform Manifold Approximation and Projection) of single-cell transcriptomes of differentiating human PM organoids, colored by collection time point (15,558 cells).

(B) UMAP of human PM organoids, colored by assigned Leiden cluster identity based on marker gene expression profile (see Methods).

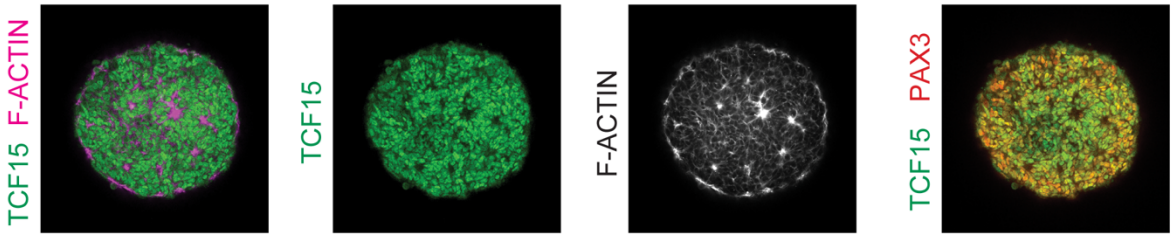
(C) Heat map of selected marker genes of paraxial mesoderm differentiation. Collection time point and Leiden cluster identities are indicated. Marker genes are grouped based on primary associated cell fate as indicated.

(D) Heat map of single-cell HOX gene expression levels. Cells are grouped by Leiden cluster identity. Hox genes are ordered by position, with anatomical positions of HOX paralogues indicated on the right.

(E) UMAP plots overlaid with normalized transcript counts of representative cell fate marker genes.

Fig 5, Budjan et al.

A



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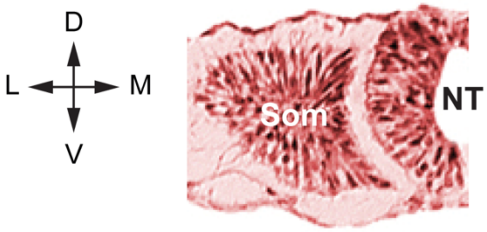
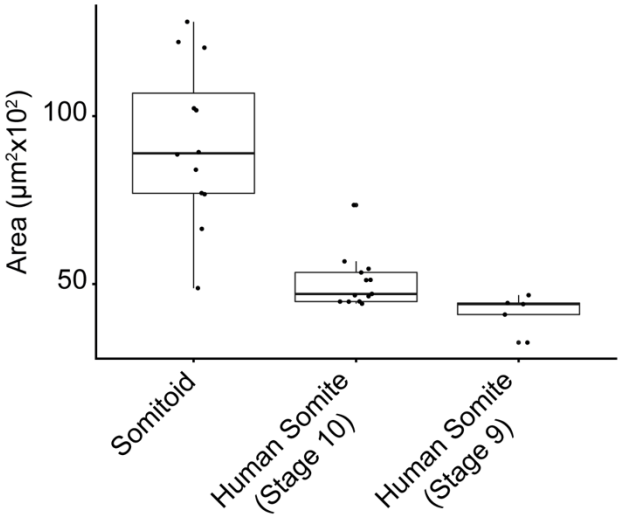


Carnegie Stage 10



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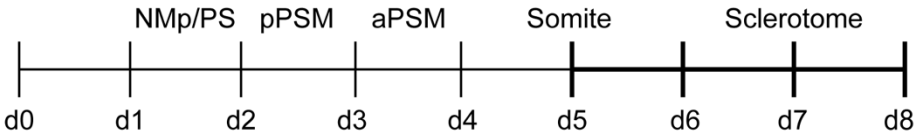

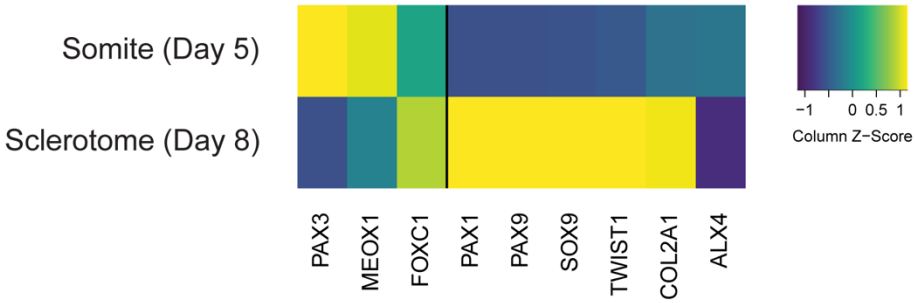

Figure 5. **Somitoids express known somite-stage specific marker genes and can differentiate to sclerotome fate.**

(A) Immunofluorescence analysis of day 5 Somitoids reveals co-expression of somite markers TCF15/PARAXIS and PAX3 and polarized rosette-like structures as indicated by F-ACTIN localization, suggesting that somite-like structures resemble *in vivo* counterparts in both a molecular and morphological manner.

(B) Quantification of somite sizes in day 5 human Somitoids and human embryos (Carnegie stage 9/10) reveals that the range of *in vitro* somites sizes observed contains the size of somites observed *in vivo* (see Methods section), although on average the area of *in vitro* somites were larger compared with their *in vivo* counterparts. Boxes indicate interquartile range. End of whiskers indicate minimum and maximum. Points indicate individual somites. Central lines represent the median. Carnegie embryo data were obtained from the Virtual Human Embryo Project (https://www.ehd.org/virtual-human-embryo).

(C) Sclerotome differentiation of Somitoids. Day 5 Somitoids were exposed to SHH agonist and WNT inhibitors to induce sclerotome differentiation as indicated. qPCR analysis of somite and sclerotome markers reveals induction of sclerotome markers on Day 8. Relative gene expression levels are shown as Z-scores, expressed as fold-change relative to undifferentiated iPS cells (see Methods).

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