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Directed differentiation of human pluripotent stem cells into diverse organ-specific mesenchyme of the digestive and respiratory systems

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Abstract

Development of visceral organs such as the esophagus, lung, liver and stomach are coordinated by reciprocal signaling interactions between the endoderm and adjacent mesoderm cells in the fetal foregut. While the recent successes in recapitulating developmental signaling *in vitro* has enabled the differentiation of human pluripotent stem cells (hPSCs) into various types of organ-specific endodermal epithelium, the generation of organ-specific mesenchyme has received much less attention. This is a major limitation in ongoing efforts to engineer complex human tissue. Here, we describe protocols to differentiate hPSCs into different types of organ-specific mesoderm leveraging signaling networks and molecular markers elucidated from single-cell transcriptomics of mouse foregut organogenesis. Building on established methods, hPSC-derived lateral plate mesoderm (LPM) treated with either retinoic acid (RA) or RA together with a Hedgehog (HH) agonist generates posterior or anterior foregut splanchnic mesoderm respectively (pFG-SpM or aFG-SpM) after 4 days cultures. These are directed into organ-specific mesenchyme lineages by the combinatorial activation or inhibition of WNT, BMP, RA or HH pathways from days

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Author contributions

K.K., L.H., J.M.W., T.T., and A.M.Z. conceived the project. K.K. and A.M.Z wrote the manuscript with support from K.I., M.M., J.M.W., and T.T., K.K., K.I., A.S., C.F.H. performed experiments and K.K. and K.I. analyzed data. A.M.Z supervised the project.

Competing interests

K.K., L.H., M.M. and A.M.Z. have filed patent application on this protocol. K.I., C.F.H., M.M., J.M.W. and T.T. declare no competing interest.

4 to 7 in cultures. By day 7 the cultures are enriched for different types of mesoderm with distinct molecular signatures: 60–90% pure liver septum transversum/mesothelium-like (STM/mesothelium), 70–80% pure liver-like fibroblasts (LF), and populations of ~35% respiratory-like mesoderm (RM), gastric-like mesoderm (GM), or esophageal-like mesoderm (EM). This protocol can be performed by anyone with moderate experience differentiating hPSCs and provides a novel platform to study human mesoderm development and can be used to engineer more complex foregut tissue for disease modeling and regenerative medicine.

Introduction

The development of foregut organs including lungs, esophagus, stomach, pancreas and liver is orchestrated by a reiterative series of growth factors mediated tissue interaction between the embryonic endoderm and the surrounding splanchnic mesoderm^{1, 2}. Much attention has focused on identifying the molecular pathways that instruct the specification of different epithelial lineages of digestive and respiratory organs^{3, 4}. As a result, there have been tremendous advances in replicating developmental signals *in vitro* to direct the differentiation of human pluripotent stem cells (hPSC) into different endodermal lineages^{5–10}. In contrast, much less is known about the development of organ-specific splanchnic mesoderm and to date there are few if any approaches to generate these cell types *in vitro*. Adult visceral organs contain resident fibroblasts, mesothelium, smooth muscle, and in some cases cartilage all derived from the gut tube splanchnic mesoderm. Evidence suggest that in some cases similar cell types in different organs have distinct properties and transcriptional programs^{11–13}, although the developmental origins of this specificity are poorly understood. This is a major limitation towards the goal of engineering human tissue that replicates the cellular complexity of *in vivo* organs— indeed most hPSC-derived foregut organoids reported to date lack the appropriate mesenchyme^{6, 9, 10}. Here, we describe a series of optimized protocols first published by Han et al.¹⁴ to differentiate hPSCs into different types of organ-specific-like mesenchyme progenitors including liver septum transversum/mesothelium-like (STM/mesothelium), liver-like fibroblasts (LF), gastric-like mesoderm (GM), esophageal-like mesoderm (EM) and respiratory-like mesoderm (RM). This novel platform for studying human mesoderm development also has potential applications in disease modeling and regenerative medicine where it can be used to engineer more complex foregut organ tissues.

Development of the protocol:

To develop a protocol for generating organ-specific mesenchyme from hPSCs, we turned to studies of mesoderm development in animal models. In mammals, the mesoderm germ layer is induced by BMP and WNT signals while the endoderm is induced by Nodal/Activin/TGF β (henceforth referred to as TGF β) as the tissues progressively emerge through the primitive streak (PS) during gastrulation^{15, 16}. After gastrulation, the bi-layered sheet of endoderm and lateral plate mesoderm (LPM) folds into a primitive gut tube. In the foregut region, the LPM splits into an outer somatic mesoderm layer next to the ectoderm which gives rise to the forelimbs and body wall, and an inner splanchnic mesoderm (SpM) layer that gives rise to the cardiac mesoderm (CM) and the mesoderm surrounding the presumptive visceral organs of the gut tube^{15, 17–19}.

The endoderm and mesoderm of the fetal gut tube are then progressively patterned along the anterior-posterior and dorsal-ventral axes into organ-specific cell types by reciprocal cell signaling between the tissue layers^{1, 2, 20, 21}. While a lot is known about the combinatorial signals that direct different epithelial lineages, until recently the SpM was less well studied, with exception of cardiac tissue which has received a lot of attention^{3, 22–24}. To address this, we recently carried out a temporally resolved single-cell transcriptomics analysis of the developing mouse foregut identifying a diversity of organ-specific mesenchyme subtypes, which can be identified by distinct gene expression profiles (Supplementary Fig. 1)¹⁴. Furthermore, by examining the expression patterns of ligand-receptor pairs we computationally inferred the signaling network predicted to control the development of these different SpM progenitors. Leveraging this cell signaling roadmap we developed methods to the directed differentiation of hPSCs into different SpM lineages¹⁴.

To develop our protocol, we built on studies by Loh et al.²³ reporting a stepwise method of activating developmental signals while also blocking unwanted lineages to efficiently differentiate hPSCs into PS, LPM and then into CM. This was an excellent starting point since in addition to CM, the LPM also gives rise to different populations of foregut SpM. Our mouse single-cell transcriptomic analysis suggested that these alternative LPM fates were controlled by differential RA and HH signaling¹⁴. This allowed us to establish a protocol where day 1 PS could be directed into three different SpM lineages by day 4: Cardiogenic (CG-SpM), posterior foregut (pFG-SpM), and anterior foregut (aFG-SpM) (Fig. 1)¹⁴. All 3 lineages are induced by a common set of signals; BMP and FGF activation in combination with TGF β inhibition, and WNT inhibition. With no additional factors the cells adopt a CM-SpM fate, whereas addition of RA induces pFG-SpM, and the addition of both RA and HH induces aFG-SpM until day 4. Leveraging predictions from the mouse single-cell roadmap¹⁴, together with empirical testing we then established conditions where different combinatorial activation or inhibition of WNT, BMP, RA or HH pathways from days 4–7 in monolayer culture could further direct, pFG-SpM into liver septum transversum/mesothelium (STM/mesothelium), liver fibroblasts-like (LF) progenitors and gastric-like mesoderm (GM), whereas the aFG-SpM could generate cultures enriched in esophageal-like mesoderm (EM) and respiratory-like mesoderm (RM) lineage, which we were able to identify based on their unique transcriptional signatures¹⁴.

Overview of the Procedure

Figure 1 shows an overview of the protocols in comparison to a published method for generating CM²³. Briefly, the main stages of the Procedure are listed below.

Single-cell passage of hPSCs (day –1; Steps 1–16) and differentiation of hPSCs into Mid. PS (day 0–1; Steps 17–22)

ESCs or iPSCs are differentiated in 2D monolayer on Geltrex coated plates (Steps 1–38). The first step is induction of mid. PS mesendoderm by culturing hPSCs in a combination of Activin A, CHIR (Wnt activator), BMP4, bFGF, and PIK90 (PI3K inhibitor) for 24 hours²³ (Steps 17–22). This results in a downregulation of pluripotency markers *OCT3/4* and *SOX2* and a robust induction of the PS mesendoderm marker (*TBXT*) (Supplementary Fig. 2).

Differentiation of Mid. PS into LPM (days 1–2; Steps 23–27) and LPM into SpM (days 2–4; Steps 28–33)

In the CM protocol described by Loh et al., day 1 PS cells are treated for 24 hours with BMP4, Wnt-C59 (Wnt inhibitor) and A83–01 (TGF β inhibitor) to generate cardiogenic LPM (CG-LPM)²³, followed by culture with BMP4, Wnt-C59, A83–01, and bFGF²³ on days 2–4 to make CG-SpM. To divert the LPM away from CM we add RA to this induction cocktail on days 1 to 4 (Steps 23–33). The addition of only RA generated pFG-SpM, whereas adding both RA and the HH agonist PMA patterned the LPM into aFG-SpM (Fig. 1). While all the SpM subtypes express the mesoderm markers *VIM* and *FOXF1*, only the CG-SpM robustly expresses cardiac mesoderm markers *NKX2–5* and *ISL1* (Fig. 2). Furthermore, both the pFG-SpM and aFG-SpM, but not CG-SpM are enriched in RA-responsive gene such as *HOXA5* and *CYP26A1*, while only aFG-SpM expresses HH-responsive genes such as *GLI1* and *PTCH1* (Table 1 and Fig. 2).

Differentiation of SpM into organ-specific mesoderm (days 4–7; Steps 34–38)

Treatment of pFG-SpM from day 4 to day 7 with RA and BMP4 generates a cell population with characteristics of liver STM/mesothelium, whereas addition of RA, BMP4 and CHIR induces liver fibroblasts-like cells (LF) (Steps 34–38). On the other hand, treatment of pFG-SpM with RA, PMA from days 4–7 with further addition of NOGGIN (BMP antagonist) on day 6 to day 7 generates gastric-like mesoderm (GM) (Fig. 1) (Steps 34–38). The same conditions that generate GM from pFG-SpM directs aFG-SpM into esophageal-like mesenchyme (EM) (Steps 34–38). Finally, the combination of RA, PMA and BMP4 on days 4–7, with the further addition of CHIR on days 6–7 directs the aFG-SpM into cultures enriched for respiratory-like mesoderm (RM) (Fig. 1) (Steps 34–38). The characteristic and purity of each mesenchyme subtype can be determined by the expression of organ-specific marker genes (Table 1 and Fig. 3)¹⁴.

Applications of the method:

Since our approach to generate organ-specific mesoderm recapitulates key steps of organogenesis, one of the most obvious applications is as platform to study the mechanisms of human splanchnic mesenchyme development, which is virtually unexplored. For example, it is possible to use our system to examine the molecular mechanisms by which these organ-specific mesoderm progenitors differentiate into mature mesenchymal cell types. Indeed, we have begun to explore how the RM can be further differentiated into smooth muscle and cartilage typical of the trachea and airway (Fig. 4). Furthermore, co-culturing hPSC-derived mesoderm with hPSC-derived endoderm would provide a simplified reductionist system to investigate the complex epithelial-mesenchymal crosstalk of human foregut organogenesis *in vitro*⁴. Such *in vitro* culture models are particularly well suited to large scale genomic analysis of the gene regulatory networks governing SpM diversification which would be very difficult to study *in vivo*.

Another application would be to use the method to model congenital diseases of the LPM *in vitro*. For example, we and other have been investigating the developmental role of the LPM in foregut organogenesis and how disruptions to this process can result in life-threatening birth defects such as esophageal atresia and tracheoesophageal fistulas (EA/

TEF)^{26, 27}. While ongoing patient genome sequencing is rapidly identifying candidate causative mutations, a major challenge is to determine whether these genes act in the endoderm or mesoderm and how they impact development. By using patient-derived iPSCs in our protocols, investigators can determine whether the patient's mutation impact the differentiation of mesoderm or endoderm lineages for a wide array of different congenital anomalies affecting foregut organs.

Finally, our method to generate organ-specific mesoderm can be in tissue engineering approaches to increase the cellular complexity of in vitro-generated organoids. To date most protocols to generate hPSC-derived foregut organoids are primarily epithelial with very little mesenchyme^{6, 9}; the addition of mesoderm from our method could solve this limitation. Indeed, we have recently demonstrated that hPSC-derived SpM from our protocol can be incorporated into both gastric and esophageal organoids resulting in *in vitro* engineered tissue with increase cellular complexity as well as improved growth and maturation of the tissue²⁸.

Comparison with other methods:

There have been a few other reports of protocols that differentiate hPSCs into non-cardiac LPM lineages, such as hepatic or respiratory mesenchyme^{21, 29–31}, but for the most part these are not based on a systematic analysis comparing the development of different mesoderm subtypes as we have done. In Figures 4 and 5, we present a comparison of these other protocols to our methods.

Previous studies have reported the co-development of a small amount of mesenchyme in lung organoids^{33–35}. However, these protocols were optimized to definitive endoderm (DE) epithelium differentiation and the tissue-specific characteristics of the mesenchyme has not been fully assessed. Part of the problem has been our limited understanding of LPM patterning in the foregut. We and others have recently defined how the foregut mesoderm is patterned along the dorsal-ventral axis into *MSC/NKX6-1/WNT4+* dorsal EM that gives rise to esophageal smooth muscle and ventral respiratory mesenchyme (RM) that expresses *TBX4/TBX5/NKX6-1/WNT2* and gives rise to smooth muscle of dorsal trachea and *SOX9+* chondrocytes on the ventral-lateral trachea and large airways¹⁴. In two back-to-back publications, we recently reported similar methods to generate RM: Han et al.¹⁴ which report here and Kishimoto and Morimoto²⁷. Figure 4 shows a direct comparison of these methods.

Both protocols generate RM that express typical markers, but their relative expression levels varied between the protocols. Quantitative RT-PCR (qRT-PCR) analysis revealed that *NKX6-1* and *TBX5* expression was enriched in the cells from our protocol here but that *TBX4* and *WNT2* were higher in the cells generated by the protocol of Kishimoto and Morimoto²⁷ [AU: Here and elsewhere in the paragraph are the changes from “Kishimoto et al. 2020” and “Kishimoto 2020” to Kishimoto and Morimoto²⁷ correct?] (Fig. 4b-d). Immunostaining confirmed that the numbers of NKX6-1+ and TBX5+ cells was greater in our current protocol (NKX6-1+; current protocol 24.8 ± 1.97% and former protocol 13.2±4.28%, TBX5+; current protocol 48.4 ± 6.60% and former protocol 11.2 ± 10.0%). This suggests that the Kishimoto and Morimoto protocol²⁷ generates more

ventral RM, whereas the protocol here produced more medial RM, consistent with *in vivo* expression patterns in the mouse foregut (Fig. 4e)^{14, 21}. The biggest difference between the two protocols is a higher concentration and longer duration of CHIR treatment (WNT activation) in the Kishimoto and Morimoto protocol²⁷, consistent with recent results in mice that differential WNT activity patterns the tracheal mesenchyme^{14, 21, 36}. Even though the two protocols generated slightly different RM character, prolonged culture until day 12 showed that both cultures were similarly competent to differentiate into SOX9-positive chondrocyte progenitors and smooth muscle actin (SMA)-positive smooth muscle cells (Fig. 4f,g) like differentiated tracheal mesenchyme.

In vivo fetal hepatoblasts derived from the ventral foregut endoderm invade the STM (a transient mesoderm population posterior to the heart) to form the liver bud. The STM then gives rise to mesothelium surrounding the liver bud and interstitial liver fibroblasts (LF), which subsequently become hepatic stellate cells (HSCs)³². Several groups have described methods to differentiate hPSCs into mesenchyme similar to that found in the fetal liver²⁹⁻³¹. For example, Takebe et al. reported hPSC-derived liver mesenchyme characteristic of the STM²⁹, while Coll et al. generated PDGFR β + HSC-like cells via a mesothelium intermediate³⁰. All three protocols use BMP and FGF, which are known to play a role in hepatic development (Fig. 5a), however only Takebe uses PDGFBB, but not RA, which is used by Coll and our protocol. Moreover, our LF protocol uses Wnt stimulation while Coll uses palmitic acid. Thus, it is possible that these different protocols generate different types of hepatic mesoderm. We therefore performed side-by-side experiments to identify differences (Fig. 5a and Box 1)^{14, 29, 30}.

qRT-PCR analysis showed that the d7 STM/mesothelium from our protocol expressed most of the known *in vivo* markers of STM (*GATA4/WT1/WNT2/TBX18*) and mesothelium (*LHX2/WT1/UPK1B/UPK3B*) suggesting a mixed cell population of cells transitioning from STM to mesothelium, whereas the LF population from our protocol was rather distinct expressing markers characteristic of fetal LFs and HSCs (*PITX1/KRT19/MSX1/MSX2/TBX5/COL1A1/DES*) (Fig. 5b). In comparison, the STM generated by the Takebe protocol expressed high level of early STM markers (*HAND2/GATA4/TBX18*) but had low expression of mesothelium and fibroblasts markers, suggesting that it represents an earlier developmental stage than the STM-like population generated by our protocol. In contrast, Coll protocol generated cells that express both mesothelium and liver fibroblasts/HSCs markers but not genes typical of early STM suggesting that these are a mixed cell population of slightly later developmental stage. Hierarchical clustering of the qRT-PCR data indicated that our d7 STM/mesothelium had a transcriptional profile more similar to the Takebe STM, whereas our d7 LF was similar to the Coll d12 HSC-like cells (Fig. 5b). Immunostaining also showed that both our STM/mesothelium and the Takebe d9 STM are enriched in GATA4+ cells (this paper: 52.4 \pm 5.47% and Takebe et al.: 73.3 \pm 11.4%) (Fig. 5c), but that only our STM/mesothelium was WT1+ (86.9 \pm 8.37 %) (Fig. 5c). Furthermore, both our LF and Coll d12 HSC-like cultures contained PITX1+ (this paper: 71.5 \pm 13.4% and Coll et al.: 49.2 \pm 5.61%) or KRT19+ cells (this paper: 49.2 \pm 5.61% and Coll et al.: 20.1 \pm 5.87 %) consistent with liver fibroblasts (Fig. 5c,d and Supplementary Fig. 1)¹⁴. Thus, these protocols generate similar liver mesenchyme cell types but appear to be at slightly different

developmental stages or mixed populations, with our approach being able to generate the full spectrum of mesoderm progenitors found in the developing liver bud.

Experimental design:

To generate organ-specific-like mesenchyme, we differentiated hPSCs in a stepwise process mimicking foregut organogenesis *in vivo*¹⁴. In addition we empirically tested culture conditions including duration, concentration and combinations of growth factors and chemical components to optimize differentiation of each mesenchyme population characterized by the expression of tissue-specific makers. Our differentiation protocols have proven to be effective for both male and female human embryonic stem (ES) cells (H1 and H9)²⁵ as well as induced pluripotent stem cells (iPSC72_3)⁶. For each differentiation experiments, we prepared at least three replicates to obtain statistically rigorous results.

Limitations:

Two limitations of our current protocols are differentiation efficiency and heterogeneity of the cell populations. While our protocol generates >90% FOXF1-positive population at day 4 for the CG-SpM, pFG-SpM and aFG-SpM (Fig. 2), later the differentiation becomes less efficient for some lineages: 60–90% WT1+ liver septum transversum/mesothelium (STM/mesothelium), 70–80% PITX1+ liver fibroblasts-like (LF), and ~35% NKX6.1+ respiratory-like mesoderm (RM) (Figs. 3d-e, 4c, 5c, d). Moreover, while qRT-PCR shows that the *BARX1*^{High}/*NKX3.2*^{High} GM, *MSC*^{High} EM and *NKX6-1*^{High}/*TBX5*^{High} RM populations are clearly distinct (Fig. 3c and Table 1), we have not found good antibodies to proteins like BARX1, MSC or NKX3–2 that would allow us to quantify the proportion of cells that express these markers in different populations by immunostaining, despite extensive testing. In the future it will also be important to identify and validate lineage-specific cell-surface proteins that can be used in FACs to easily quantify and purify the different populations. Supplementary Fig. 1c shows the expression of candidate surface protein genes from the published mouse scRNA-seq data, which can also be explored at <https://research.cchmc.org/ZornLab-singlecell/>.

One possible explanation for incomplete differentiation maybe the fact that we only manipulated a handful of pathways (RA, HH, WNT, and BMP). For example, based on mouse scRNA-seq, *in vivo* the EM should express *Nkx6-1* and *Tbx5*, but these are missing in the hPSC-derived esophageal-like cultures. We postulate that additional signals, currently absent from our cultures, might participate in organ-specific mesenchyme differentiation, which include PDGF, TGFβ, FGFs and NOTCH. A further analysis of the mouse signaling roadmap might help identify other candidate pathways to optimize the protocol. In addition, prolonged culture should promote more complete differentiation into smooth muscle, mature mesothelium and tissue resident fibroblasts. It is also possible that the human mesenchyme development is just different than mouse, in which case empirical testing of pathways and an examination of single-cell data from early human fetal samples would be helpful.

Materials

!CAUTION When handling cells and reagents, take the necessary precaution. Use proper personal protective equipment (PPE) at biosafety level (BSL)-2 for tissue culture experiment

and BSL-1 for non-tissue culture experiment. Refer to specific material safety data sheets for additional information.

Biological materials

- Human pluripotent stem cell lines. We used three hPSC lines. WA01-H1 (RRID: CVCL_9771) and WA09-H9 (RRID: CVCL_9773), human embryonic stem cells, were purchased from WiCell (NIH approval number NIHhESC-10-0062). iPSC72_3 (RRID: CVCL_A1BW), human induced pluripotent stem cells, was generated by the CCHMC Pluripotent Stem Cell Facility.

!CAUTION All relevant institutional and governmental regulations for the use of hPSCs must be followed. This work used NIH-approved embryonic stem cell lines and was approved by Embryonic Stem Cell Research Oversight (ESCRO) committee (protocol EIP200117) and CCHMC Biosafety Protocol (protocol IBC2021-0011).

!CAUTION Cell lines should be regularly checked to ensure that they are authentic and not infected with mycoplasma.

Reagents

! CAUTION When handling reagents, take the necessary precautions. Use proper personal protective equipment for BSL-2 for tissue culture work and BSL-1 for non-tissue culture work. Refer to specific reagent material safety data sheets for additional information if unfamiliar with the reagents.

Growth medium and supplements

CRITICAL We have not tested growth medium and supplements from other vendors.

- mTeSR1 medium (StemCell Technologies, cat. no. 85850)
- DMEM/F12 medium (Thermo Fisher Scientific, cat. no. 11330-032)
- Advanced DMEM-F12 (Invitrogen, cat. no. 12634-010)
- Matrigel (basement membrane matrix; Corning, cat. no. 354234)
- Geltrex (LDEV-Free hESC-qualified Reduced Growth Factor Basement Membrane Matrix, Thermo Fisher Scientific, cat. no. A14133-02)
- B27 supplement (50×, without vitamin A; Thermo Fisher Scientific, cat. no. 12587-010)
- Penicillin-streptomycin (100×; Thermo Fisher Scientific, cat. no. 15140-122)
- N2 supplement (100×; Thermo Fisher Scientific, cat. no. 17502-048)
- HEPES buffer (1M; Thermo Fisher Scientific, cat. no. 15630-080)
- Glutamax (100×, Thermo Fisher Scientific, cat. no. 35050061)

Enzymes and growth factors

CRITICAL We have not tested enzymes and growth factors from other vendors.

- Accutase (Sigma Aldrich, cat. no. A6964)
- Dispase (Thermo Fisher Scientific, cat. no. A34181)
- Activin A (Cell Guidance Systems, cat. no. GFH6)
- Bone morphogenetic protein 4 (BMP4; R&D Systems, cat. no. 314-BP-050)
- Basic fibroblast growth factor (bFGF; R&D Systems, cat. no. 233-FB)
- CHIR99021 (CHIR; Tocris, cat. no. 4423)
- Thiazovivin (Tocris, cat. no. 3845/10)
- PIK90 (EMD Millipore, cat. no. 528117-5MG)

CRITICAL As PIK90 is photosensitive, wrap stock tubes and plates in foil and turn the biosafety cabinet light off to prevent light exposure.

- 2-phospho-L-ascorbic acid trisodium salt (Sigma, cat. no. 49752)
- XAV-939 (Selleckchem.com, cat. no. S1180)
- A83-01 (Tocris, cat. no. 2939)

CRITICAL As A83-01 is photosensitive, wrap stock tubes and plates in foil and turn the biosafety cabinet light off to prevent light exposure.

- Wnt-C59 (Cellagen, cat. no. C7641-10)
- Retinoic acid (RA; Sigma, cat. no. R2625)

CRITICAL As RA is photosensitive, wrap stock tubes and plates in foil and turn the biosafety cabinet light off to prevent light exposure.

- Purmorphamine (PMA; TOCRIS, cat. no. 4551)
- Noggin (R&D systems, cat. no. 6057-NG-100)

Immunostaining reagents

- Paraformaldehyde (PFA; Thermo Fisher Scientific, cat. no. T353-500)
- Normal donkey serum (Jackson ImmunoResearch Laboratories, Inc, cat. no. 017-000-121)
- Tween-20 (Fisher Scientific, cat. no. BP337-100)
- Triton-X100 (Sigma-Aldrich, cat. no. X100)
- Phosphate buffer solution (PBS; Sigma, cat. no. T8787)
- Fluoromount-G fluorescent mounting medium (Southern Biotech, cat. no. 0100-01)
- Antibodies (Table 2)

Equipment

- Portable Pipet-Aid XP pipette controller (Drummond, cat. no. 4-000-101)

- Pipetman single-channel pipettes (2, 20, 200, and 1,000 μ l; Gilson, cat. no. F144801, cat. no. F123615, cat. no. F123601, and cat. no. F123602)
- Horizontal clean bench (Labconco, cat. no. 3600004)
- Labgard class II type A2 biological safety cabinet (NuAire, cat. no. NU-425–400)
- –80°C Freezer (Thermo Scientific, cat. no. UXF700086D)
- Forma Steri-Cycle i160 CO₂ incubator (Thermo Scientific, cat. no. 51030533)
- Stereomicroscope (Leica, cat. no. S8APO)
- Inverted microscope (Nikon, TMS model)
- Nunclon delta surface tissue culture dish (6 well; Nunc, cat. no. 140675)
- Nunclon delta surface tissue culture dish (12 well; Nunc, cat. no. 150628)
- Nunclon delta surface tissue culture dish (24 well; Nunc, cat. no. 142475)
- Serological pipettes (5 and 10 ml; Falcon, cat. nos. 357543 and 357551)
- Sterilized filter pipette tips (10, 20, 200 and 1250 μ l (VWR, cat. nos. 76322–528, 76322–134, 76322–150, and 76322–156)
- Millipore 0.22- μ m conical sterilization tubes (Millipore, cat. no. SCGP00525)
- 1.5-ml Low adhesion microcentrifuge tubes (USA scientific, cat. no. 1415–2600)
- Cell culture coverslips (sterile plastic; Thermanox; Thermo Scientific, cat. no. 174969)
- Microscope slides (Superfrost; Fisher Scientific, cat. no. 12–550-133)
- Microscope cover glass (Fisher Scientific, cat. no. 12–545-M)
- Hydrophobic pen (ImmeEdge pen; Vector Laboratories, cat. no. H-4000)
- Nikon A1 single-photon confocal microscope with NIS-Elements Advanced Research imaging software (Nikon, http://www.microscope.healthcare.nikon.com/en_AOM/products/software/nis-elements)
- GraphPad Prism 9.1.2 (GraphPad Software, <http://www.graphpad.com/>)

Reagent setup

Dispase—Resuspend dispase in DMEM/F12 at a final concentration of 1mg ml⁻¹. After sterilization with filter-sterilization tube, make 10-ml aliquots and store at –20 °C for up to 6 months.

Accutase—Thaw vial on ice or at 4 °C. Make 5-ml aliquots and store at –20 °C.

Aliquots of hESC-qualified Matrigel for hPSCs maintenance.—Thaw a bottle of hESC-qualified Matrigel on ice or at 4 °C. Pre-chill sterile microcentrifuge tubes on ice. Make 270–350 µl of aliquots and store at –80 °C for up to 6 months.

CRITICAL Matrigel has the property to solidify at room temperature (X–Y °C)[AU: **Please define room temperature here by replacing ‘X–Y °C’ with the appropriate range**]. Matrigel should be aliquoted quickly on ice or at 4 °C.

Aliquots of Geltrex for mesoderm differentiation from hPSCs.—Thaw a bottle of Geltrex on ice or at 4 °C. Pre-chill sterile microcentrifuge tubes on ice. Make 1-ml aliquots and store at –80 °C for up to 6 months.

CRITICAL Geltrex has the property to solidify at room temperature. Geltrex should be aliquoted quickly on ice or at 4 °C.

Matrigel coating plates for hPSC maintenance—Coat six-well plate with hESC-qualified Matrigel according to the manufacturer’s procedure. The dilution is calculated for individual lot based on protein concentration. Thaw Matrigel aliquot on ice and dilute 270–350 µl of the aliquot in 25 ml of ice-cold DMEM/F12 medium to coat four six-well plates. 1 ml per well is required. The plates wrapped in Parafilm can be kept for 2 weeks in 4 °C.

Geltrex coating plates for mesoderm differentiation from hPSCs.—Coat 12-well plates or coverslips placed in 24-well plates with 1% (vol/vol) Geltrex diluted in DMEM/F12 according to the manufacturer’s procedure. Either 1 ml per well in a 12-well plate or 0.5 ml per well in a 24-well plate is required. The plates wrapped in Parafilm can be kept for 2 weeks at 4 °C.

Reconstitution of growth factor and small molecules—Reconstitute growth factors and small molecules as summarized in Table 3. Reconstitution was largely based on manufacturers’ instruction. Divide growth factors into aliquots and store at –80 °C for up to 6 months. Thaw growth factors on ice before use and keep at 4 °C for 2 weeks. We do not re-freeze growth factors once thawed. Divide small molecules into aliquots and store at –20 °C for up to 6 months. Avoid repeating freeze–thaw cycles.

Gut medium

Supplement advanced DMEM/F12 medium with 1x B27 without vitamin A, 1x N2, 15 mM HEPES, 1x GlutaMAX, 100 unit ml^{–1} penicillin–streptomycin. Gut medium can be stored at 4 °C for 2 weeks.

Middle primitive streak induction medium (days 0–1)

Add growth factors and small molecules (30 ng ml^{–1} Activin A, 6 µM CHIR, 40 ng ml^{–1} BMP4, 20 ng ml^{–1} bFGF, 100 nM PIK90) to gut medium. The medium, including growth factors and small molecules, should be freshly prepared each day.

Lateral plate mesoderm induction medium (days 1–2)

For CG-LPM induction, add growth factors and small molecules (1 μ M A83–01, 30 ng ml⁻¹ BMP4, and 1 μ M Wnt-C59) to gut medium. The medium, including growth factors and small molecules, should be freshly prepared each day. For pFG-LPM induction, add growth factors and small molecules (1 μ M A83–01, 30 ng ml⁻¹ BMP4, 1 μ M Wnt-C59, and 2 μ M RA) to gut medium. The medium, including growth factors and small molecules, should be freshly prepared each day. For aFG-LPM induction, add growth factors and small molecules (1 μ M A83–01, 30 ng ml⁻¹ BMP4, 1 μ M Wnt-C59, 2 μ M RA, and 1 μ M PMA) to gut medium. The medium, including growth factors and small molecules, should be freshly prepared each day.

Splanchnic mesoderm induction medium (days 2–4)

For CG-SpM induction, add growth factors and small molecules (1 μ M A83–01, 30ng ml⁻¹ BMP4, 1 μ M Wnt-C59, and 20 ng ml⁻¹ bFGF) to gut medium. The medium, including growth factors and small molecules, should be freshly prepared each day. For pFG-SpM induction, add growth factors and small molecules (1 μ M A83–01, 30 ng ml⁻¹ BMP4, 1 μ M Wnt-C59, 20 ng ml⁻¹ bFGF, and 2 μ M RA) to gut medium. The medium, including growth factors and small molecules, should be freshly prepared each day. For aFG-SpM induction, add growth factors and small molecules (1 μ M A83–01, 30 ng ml⁻¹ BMP4, 1 μ M Wnt-C59, 20 ng ml⁻¹ bFGF, 2 μ M RA, and 1 μ M PMA) to gut medium. The medium, including growth factors and small molecules, should be freshly prepared each day.

Liver septum transversum induction medium (days 4–7)

Add growth factors (2 μ M RA and 30 ng ml⁻¹ BMP4) to gut medium. The medium, including growth factors, should be freshly prepared each day.

Liver fibroblast induction medium (days 4–7)

Add growth factors and a small molecule (2 μ M RA, 30ng ml⁻¹ BMP4, and 6 μ M CHIR) to gut medium. The medium, including growth factors, should be freshly prepared each day.

Respiratory mesenchyme induction medium (days 4–6)

Add growth factors and a small molecule (2 μ M RA, 30ng ml⁻¹ BMP4, and 2 μ M PMA) to gut medium. The medium, including growth factors, should be freshly prepared each day.

Respiratory mesenchyme induction medium (days 6–7)

Add growth factors and a small molecule (2 μ M RA, 30ng ml⁻¹ BMP4, 2 μ M PMA, and 1 μ M CHIR) to gut medium. The medium, including growth factors, should be freshly prepared each day.

Esophageal and gastric mesenchyme induction medium (days 4–6)

Add growth factors and a small molecule (2 μ M RA and 2 μ M PMA) to gut medium. The medium, including growth factors, should be freshly prepared each day.

Esophageal and gastric mesenchyme induction medium (days 6–7)

Add growth factors and a small molecule (2 μ M RA, 2 μ M PMA and 200 ng ml⁻¹ Noggin) to gut medium. The medium, including growth factors, should be freshly prepared each day.

PBST

Add 0.5% (vol/vol) Triton-X100 to PBS. PBST can be stored at room temperature for 6 months.

Normal donkey serum (NDS)

Rehydrate with 10 ml of sterile H₂O. Divide into 1-ml aliquots and store at -80 °C for 12 months. Avoid repeated freeze-thaw cycles.

Immunostaining blocking buffer

Add 5% (vol/vol) NDS to PBS. The diluted NDS-PBS can be stored at 4 °C for up to 2 weeks but make fresh blocking buffer before use and keep on ice.

Human pluripotent stem cell lines

Maintain hPSCs in feeder-free condition on hESC-qualified Matrigel coated six-well plates at 37°C in a 5% CO₂ tissue incubator. Feed cells with 2 ml/well mTeSR1 medium every day. hPSCs will grow as colonies and reach to 70–90% confluency after 4 days in culture. These cells should be dispase-passaged as cell clumps, commonly at a well/well ratio of 1:6, into hESC-qualified Matrigel coated six-well dishes (see Matrigel coating plates for hPSCs maintenance section above) every 4 days for maintenance. Briefly, wash cells with 2ml/well DMEM/F-12. Incubate the cells with dispase for 6 minutes at 37°C in a 5% CO₂ tissue incubator. Aspirate dispase and then, wash the cells with 2 ml/well DMEM/F12 twice. Add 3.2ml/well mTeSR1 medium and scrape cells. Pipette the cell cluster four or five times. Add 1.5 ml/well mTeSR1 to hESC-qualified Matrigel coated 6-well dishes. Add 0.5ml/well cell suspension. Incubate the cells 37°C in a 5% CO₂ tissue incubator. Cells should be observed every day and differentiated cells removed by scratching with a pulled-glass Pasteur pipette.

Procedure

CRITICAL All tissue culture steps in the protocol should take place in a sterile environment and be performed with aseptic technique to prevent culture contamination.

Single-cell passage of hPSCs for mesoderm differentiation (day -1) •Timing 24 hours (30–45 minutes for plating cells)

1. Pre-coat 12-well plates with Geltrex as described in Reagent setup.
2. Pre-warm mTeSR and DMEM/F12 medium at 37 °C. The total volume of medium depends on the numbers of wells: mTeSR1 (2ml/well in 6-well plate) and DMEM/F12 (4.4ml/well in 6-well plate).
3. Take a six-well plate of undifferentiated hPSC colonies that are 70–90% confluent (see Reagent setup). Make a note of the confluency as this will determine the subsequent dilution ratio (Step 13). Remove spontaneously

differentiated cells by scratching with a pulled -glass Pasteur pipette. Aspirate mTeSR1 from wells.

4. Wash wells with 2ml of DMEM/F12 medium. Aspirate DMEM/F12.
5. Add 0.6ml Accutase to each well to dissociate hPSC colonies to single cells.
6. Incubate at 37°C for 5 min. Observe under a microscope to confirm dissociation to single cells.

? Troubleshooting

7. Add 2.4ml pre-warmed DMEM/F12 supplemented with 1µM Thiazovivin to dilute Accutase.
8. Triturate hPSC colonies by gentle pipetting to break down any undissociated colonies.
9. Centrifuge the cells at 1,300 x g for 3minutes at room temperature.
10. Meanwhile, supplement prewarmed mTeSR1 with 1µM Thiazovivin.
11. Aspirate media from tube.

CRITICAL STEP Do not aspirate the cells at the bottom of the tube.

12. Resuspend cells in prewarmed mTeSR1 supplemented with 1µM Thiazovivin.
13. Further diluted the suspended cells in mTeSR1 supplemented with 1µM Thiazovivin. The dilution ratio is determined by the original confluency of the cell in 6-well plate (as noted in Step 3). For wells at 90% confluency, cells are diluted at the ratio of 1:20 (resulting in an approximate plating density of $1.0\text{--}2.0\times 10^5$ cells ml^{-1}). For wells at 70% confluency, the cells are diluted at 1:16 (approximate density of $1.0\text{--}2.0\times 10^5$ cells ml^{-1}).
14. Remove the Geltrex from prepared Geltrex coated 12-well or 24-well plate (from Step 1). Plate 1ml or 0.5ml cell suspension in a well of 12-well plate or 24-well plate, respectively.
15. Gently rock the plates by hand to ensure that single cells are evenly distributed in the wells and place the 12-well or 24-well plate in a tissue culture incubator.
16. Incubate cells overnight in an incubator with 5% CO₂ at 37°C.

Differentiation of hPSCs into middle primitive streak (days 0–1) •Timing 24 hours (15–30 minutes for changing medium)

17. Observe the 12-well or 24-well plate with hPSCs under an inverted microscope. The monolayer of hPSCs should be 30–40% confluent (Fig. 2a). At this time point assay some cells by immunostaining and qRT-PCR:
 - Collect cells on coverslips with forceps to check the pluripotency of hPSCs by immunostaining for OCT3/4 and SOX2. The protocol for immunostaining is described in Box 1.

- Aspirate the media, harvest RNA from cells and analyze by qRT-PCR for pluripotency markers *OCT3/4* and *SOX2* (Table 4)¹⁴.
- For rest of the cells proceed to Step 18 to continue differentiation to middle primitive streak.

? Troubleshooting

18. Pre-warm middle primitive streak induction media and DMEM/F12 media at 37 °C. The total volume of medium depends on the numbers of wells: 1ml/well in 12-well plate.
19. Aspirate media from wells.
20. Wash wells with DMEM/F12 media pre-warmed at 37 °C (1 ml/well in a 12-well plate or 0.5 ml/well in a 24-well plate). Aspirate DMEM/F12 media.
21. Add middle primitive streak induction media pre-warmed at 37 °C (1 ml/well in a 12-well plate or 0.5 ml/well in a 24-well plate).
22. Incubate cells overnight in an incubator with 5% CO₂ at 37 °C.

CRITICAL STEP Because PIK90 is photosensitive, wrap stock tubes and plates in foil and turn the biosafety cabinet light off to prevent light exposure.

Differentiation of Mid. PS into LPM (days 1–2) •Timing 24 hours (15–30 minutes for changing medium)

23. The following day, observe the 12-well or 24-well plate containing middle primitive streak under an inverted microscope. The cells should reach 40–50% confluency (Fig. 2a). Floating dead cells and debris can be observed. At this time point assay some cells by immunostaining and qRT-PCR:
 - Collect cells on coverslips with forceps to check the efficiency of mid PS induction by immunostaining for TBXT. The protocol for immunostaining is described in Box 1.
 - Aspirate the media, harvest RNA from cells and analyze by qRT-PCR for Mid. PS and LPM markers (Table 4)¹⁴.
 - For rest of the cells proceed to Step 24 to continue differentiation of Mid.PS into LPM.
24. Pre-warm lateral plate mesoderm induction media and DMEM/F12 media at 37 °C. The total volume of medium depends on the numbers of wells: lateral plate mesoderm induction media (1ml/well in 12-well plate) and DMEM/F12 media (1ml/well in 12-well plate).
25. Aspirate the media. Wash cells with pre-warmed DMEM/F12 media (1 ml/well in a 12-well plate or 0.5 ml/well in a 24-well plate). Aspirate DMEM/F12 media.
26. Add pre-warmed lateral plate mesoderm induction media (1 ml/well in a 12-well plate or 0.5 ml/well in a 24-well plate).

27. Incubate cells overnight in an incubator with 5% CO₂ at 37 °C.

CRITICAL STEP Because RA and A83-01 are photosensitive, wrap stock tubes and plates in foil and turn the biosafety cabinet light off to prevent light exposure.

Differentiation of LPM into SpM (day2 – 4) •Timing 48 hours (15–30 minutes for changing medium)

28. After 24 hours, observe the 12-well or 24-well plate containing LPM under an inverted microscope. The monolayer of cells should be 80–100% confluent (Fig. 2a).
29. Pre-warm splanchnic mesoderm induction media at 37 °C. The total volume of medium depends on the numbers of wells: 1 ml/well in 12-well plate.
30. Aspirate the media. Add pre-warmed splanchnic mesoderm induction media (1 ml/well in a 12-well plate or 0.5 ml/well in a 24-well plate). It is not necessary to wash cells with DMEM/F-12 media.

CRITICAL STEP Because RA and A83-01 are photosensitive, wrap stock tubes and plates in foil and turn off the biosafety cabinet light to prevent light exposure.

31. Incubate cells overnight in an incubator with 5% CO₂ at 37 °C.
32. After 24 hours, aspirate media and add fresh pre-warmed splanchnic mesoderm induction media (1 ml/well in 12-well plate or 0.5ml/well in 24-well plate).
33. Incubate cells overnight in an incubator with 5% CO₂ at 37 °C.

Differentiation of SpM into organ-specific mesoderm (days 4–7) •Timing 72 hours (15–30 minutes for changing medium)

34. After 24 hours incubation, observe the cells under inverted microscope. Cells should reach 100% confluency (Fig. 2a). At this time point assay some cells by immunostaining and qRT-PCR:
- Collect cells on coverslips with forceps to check the efficiency of SpM induction by immunostaining for FOXF1 and ISL1. The protocol for immunostaining is described in Box 1.
 - Aspirate the media, harvest RNA from cells and analyze by qRT-PCR for CG-SpM, aFG-SpM and pFG-SpM markers (Table 4)¹⁴.
 - For rest of the cells proceed to Step 35 to continue differentiation of SpM into organ-specific mesoderm.

? Troubleshooting

35. Pre-warm organ-specific mesoderm induction media and DMEM/F12 media at 37 °C. The total volume of medium depends on the numbers of wells: 1 ml/well in 12-well plate.
36. Aspirate the media. Wash cells with pre-warmed DMEM/F12 media (1 ml/well in a 12-well plate or 0.5 ml/well in a 24-well plate). Aspirate DMEM/F12 media.

37. Add pre-warmed organ mesoderm (liver STM/LF/GM/RM/EM) induction media (1 ml/well in a 12-well plate or 0.5 ml/well in a 24-well plate).

CRITICAL STEP Because RA is photosensitive, stock tubes and plates can be wrapped in foil and turn the biosafety cabinet light off to prevent light exposure.

38. Refresh the media every day. On day 7, cells are analyzed by immunostaining and qRT-PCR:
- Collect cells on coverslips with forceps to check the efficiency of organ-specific mesoderm differentiation by immunostaining for WT1, PITX1, FOXF1, NKX6.1, TBX5. The protocol for immunostaining is described in Box 1. Fixed cells can be stored, at least less than 6 months, in PBS at 4 °C.
 - Aspirate the media, harvest RNA from cells and analyze by qRT-PCR for organ-specific markers (Table 4)¹⁴. Cell lysate can be stored at –80 °C for up to than 6 months, prior to qRT-PCR.

? Troubleshooting

Timing—Steps 1–16, Single-cell passage of hPSCs for mesoderm differentiation (days –1 to 0): 24 hours (30–45 minutes for plating cells)

Steps 17–22, Differentiation of hPSCs into mid PS (days 0–1): 24 hours (15–30 minutes for changing medium)

Step 17, Immunostaining and qRT-PCR for pluripotency markers (Box 1): 24 hours.

Steps 23–27, Differentiation of mid PS into LPM subtypes (days 1–2): 24 hours (15–30 minutes for changing medium)

Step 23, Immunostaining and qRT-PCR for Mid.PS markers (Box 1): 24 hours.

Steps 28–33, Differentiation of LPM into SpM subtypes (days 2–4): 48 h (15–30 min for changing medium)

Steps 34–38, Differentiation of SpM into organ-specific mesoderm (days 4–7): 72 hours (15–30 minutes for changing medium)

Step 34, Immunostaining and qRT-PCR for SpM markers (Box 1): 24 hours.

Step 38, immunostaining and qRT-PCR for lineage-specific markers (Box 1): 24 hours.

Troubleshooting

Troubleshooting advice can be found in Table 5.

Anticipated results

The protocol starts from 30–40% confluency of hPSCs at day 0. The cells grow to 100% confluency and differentiate into SpM subtypes by day 4 (Fig. 2a). qRT-PCR

and immunostaining analyses confirm that day 0 hPSCs express the pluripotent markers OCT3/4 and SOX2 (Supplementary Fig. 2a and b). At day1, OCT3/4 and SOX2 expression is extinguished and >90% cells express the mid PS mesendoderm marker TBXT (Supplementary Fig. 2c and d). By day 4 of the culture all three SpM subtypes robustly express VIM and ~90% of the cells are FOXF1+, while definitive endoderm (DE) markers FOXA2 and CDH1⁸ are almost undetectable (Fig. 2b-f, and Supplementary Fig. 2e). In the absence of RA or HH activation the protocol generates CG-SpM at day 4 that expresses high levels of cardiac markers *NKX2-5* and *ISL1* (Fig. 2d, f). RA treatment results in a shift from cardiac to visceral FG-SpM with the expression of RA-responsive genes such as *HOXA5*, *CYP26A1*, in both the pFG-SpM and aFG-SpM (Fig. 5d). Activation of HH together with RA induces aFG-SpM, and expression of the HH-responsive genes *GLI1* and *PTCH1*. (Fig. 2d). Optimization experiments not shown here indicate that the pFG-SpM is more predisposed to develop into posterior hepatic and gastric lineages whereas the aFG-SpM is predisposed to more anterior RM and EM. These results are consistent with our previous finding that HH signaling is less activated in liver mesenchyme compared with the RM and EM¹⁴.

By day 7 the cultures remain confluent monolayers with the STM/mesothelium, GM, RM and EM cells having a polygonal shape and honeycomb arrangement while LF displays an elongated fibroblastic morphology (Fig. 3a, b). Most day 7 cells of each lineage are positive for VIM, but negative for the DE marker FOXA2, and neuroectoderm marker SOX2 (Supplementary Fig. 3). The different cell population can be identified by a unique set of molecular markers inferred from single-cell transcriptomics of the mouse foregut¹⁴, a subset of which are presented in Supplementary Figure S1. Activation of RA/BMP signaling in pFG-SpM promotes robust expression of liver STM/mesothelium specific-genes including *WT1* and *UPK1B*, while RA/BMP/WNT induces a LF fate characterized by co-expression of *PITX1*, *MSX2* and *TBX5* (Fig. 3c)¹⁴. By contrast, addition of the HH agonist PMA to the d4–7 induction cocktails promotes gastric, respiratory and esophageal fate while suppressing liver fate. pFG-SpM or aFG-SpM treated with RA and HH agonist followed by NOGGIN on days 6–7 displays a *BARX1/NKX3-2+* gastric or *MSC/NKX3-2+* esophageal identity, respectively (Fig. 3c)¹⁴. Finally, aFG-SpM treated with RA, BMP4 and HH agonist followed by d6–7 WNT activation differentiated into RM characterized by the co-expression of *NKX6-1*, *TBX5*, *TBX4* and *WNT2* (Fig. 3c and 4b)¹⁴. The lack of marker gene expression from other lineages is also important (e.g., lack of *MSC* in STM, LF, GM and RM).

When possible, immunostaining should be used to quantify the efficiency of differentiation. We expect 60–90% WT1+ liver STM/mesothelium cells and 70–80% PITX1+ LF cells, while the efficiency of TBX5/NKX6-1+ RM differentiation is 20–40% (Fig. 3d, e)¹⁴. Immunostaining also shows the differentiation specificity where each cell population should not express other lineage markers, indicate that for the most part these are not mixed cell populations. As mentioned in the “limitations” section we have not yet identified robust antibody markers to quantify the GM and EM populations. This will be important going forward as will the validation of robust cell-surface markers that can be used in FACs to more easily quantify the differentiation efficiency and further optimize the protocol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

The main data discussed in this protocol were generated as part of the studies published in the supporting primary research papers¹⁴. The raw datasets of qRT-PCR and immunostaining have been deposited in the online open access figshare repository (10.6084/m9.figshare.19723189). The published mouse foregut scRNA-seq data used to infer the signaling roadmap is available from the Gene Expression Omnibus (GEO) database under accession code: GSE136689. The scRNA-seq data can also be explored at <https://research.cchmc.org/ZornLab-singlecell/>.

APPENDIX

Appendix

Related links

Key references using this protocol

- Han, L. et al. *Nat. Commun.* **11**, 4158 (2020): <https://doi.org/10.1038/s41467-020-17968-x>
- Eicher, A. K. et al. *Cell Stem Cell* **29**, 36–51.e6 (2022): <https://doi.org/10.1016/j.stem.2021.10.010>

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Box1 Immunostaining •Timing 24 h

1. Transfer coverslip from 24-well plate to new 24-well plate.
2. Add 4% (vol/vol) paraformaldehyde (PFA) and fix the cells for 30 minutes at room temperature.
3. Remove PFA and wash the cells on coverslip three times with 0.5 ml PBS.
4. Add 0.5% (vol/vol) Triton X-100 in PBS and permeabilize the cells for 10 minutes at room temperature.
5. Aspirate Triton X-100 in PBS and wash the cells on coverslip three times with 0.5 ml PBS.
6. Add 0.3ml of immunostaining blocking buffer (5% Normal Donkey Serum (NDS) in PBS) and block the cells for 1 hour at room temperature.
7. Transfer identically treated coverslips from 24-well plate to a 10-cm dish.
8. Add 50µl primary antibody diluted in immunostaining blocking buffer to the cells on coverslip and incubate the cells overnight at 4°C (Table 2).
9. Transfer coverslip from 10-cm dish to new 24well plate.
10. Wash the cells three times with 0.5 ml PBS.
11. Add 0.3ml secondary antibody and DAPI in immunostaining blocking buffer to the cells on coverslip and incubate the cells for 1 hour at room temperature.
12. Wash the cells three times with 0.5 ml PBS.
13. Remove coverslip and mount on a microscopic slide with Fluoromount-G.
14. Visualize the cells under a confocal microscope.

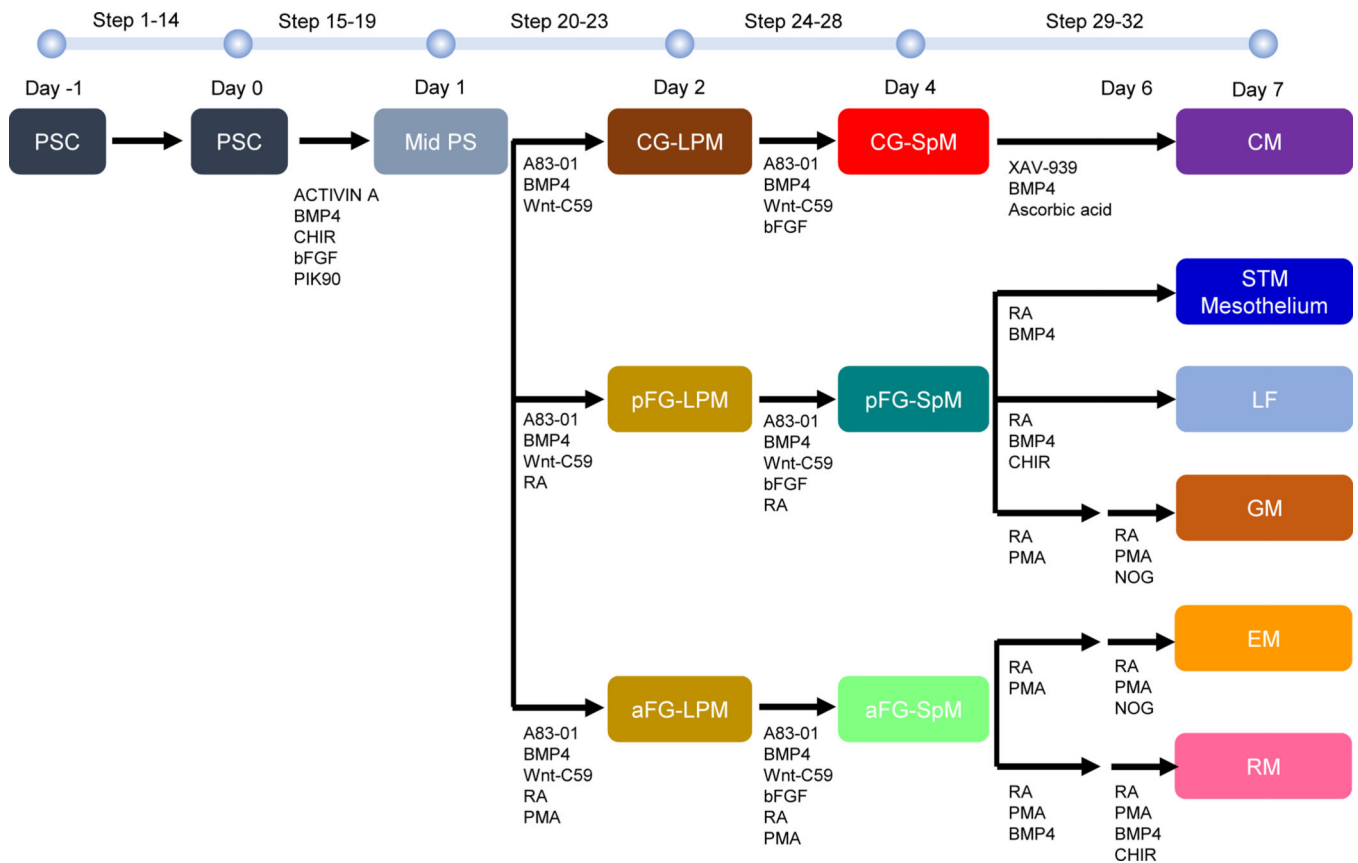


Fig. 1. Schematic diagram of protocols for generating organ-specific mesenchyme from hPSCs
 The protocols were carried out by sequentially changing the gut media with growth factors and chemical components according to the stepwise process of organogenesis. On day -1, wells are coated with 1% Geltrex in DMEM/F-12. Then, 70–90% confluent cells are gently dissociated with Accutase, before cells are suspended in mTeSR1 supplemented with Thiazovivin. Cells are then split (1:16–20) on Geltrex coated wells. On day 0, after washing cells with DMEM/F-12, cells are fed with middle primitive streak induction media. On day 1, after washing cells with DMEM/F-12, cells are fed with lateral plate mesoderm induction media. On days 2 and 3, cells are fed with either cardiogenic splanchnic mesoderm induction mesoderm or anterior foregut splanchnic mesoderm induction mesoderm or posterior foregut splanchnic mesoderm induction mesoderm. On day 4, after washing cells with DMEM/F-12, cells are fed with organ-specific mesoderm induction media. On days 5 and 6, cells are fed with organ-specific mesoderm induction media. On day 7, cells are harvested for analyses. CG-LPM; cardiogenic-lateral plate mesoderm, CM; cardiac mesoderm, EM; esophageal-like mesoderm, GM; gastric-like mesoderm, aFG-LPM; anterior foregut SpM, aFG-SpM; anterior foregut SpM, LF; liver fibroblast-like, LPM; lateral plate mesoderm, Mid. PS; middle region of primitive streak, pFG-LPM; posterior foregut LPM, pFG-SpM; posterior foregut SpM, RM, respiratory-like mesoderm, PSC; pluripotent stem cell, SpM; splanchnic mesoderm, STM; septum transversum.

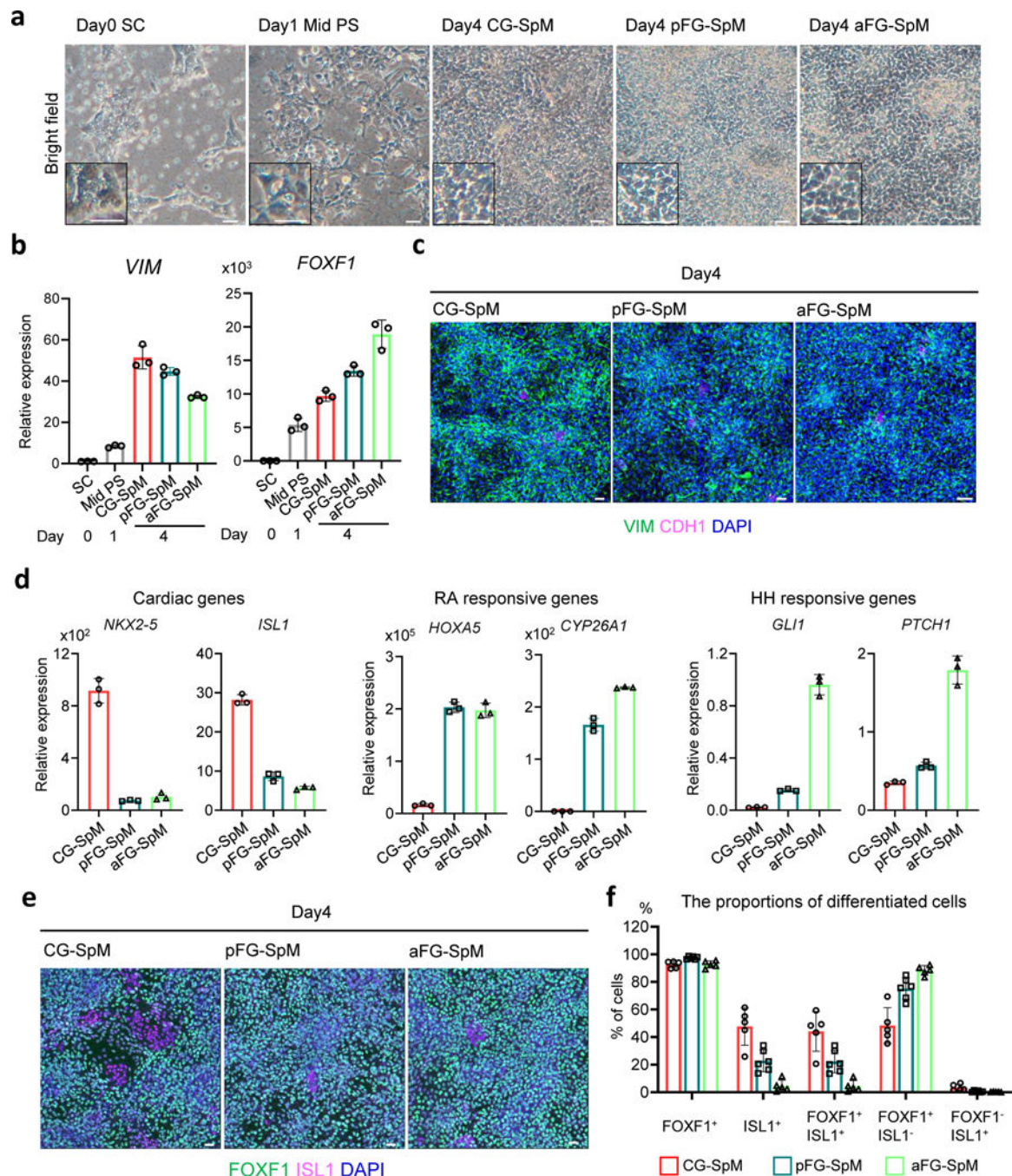


Fig. 2. hPSC differentiation to splanchnic mesoderm subtypes

a, Bright field images of differentiating cells from hPSCs from day0 to day4. **b**, Relative mRNA expression of *VIM* and *FOXF1* by qRT-PCR from day0 to day4. Each bar indicates the average from the 3 independent wells with standard deviation. **c**, Immunostaining for *VIM* (green), a pan mesoderm marker, and *CDH1* (magenta), an epithelial marker at day 4. The images are maximum intensity projection of confocal stacks. **d**, Relative mRNA expression of cardiac genes (*ISL1* and *NKX2-5*), RA-responsive genes (*HOXA5* and *CYP26A1*) and HH-responsive genes (*GLI1* and *PTCH1*) by qRT-PCR at day 4.

Each bar indicates the average from the 3 independent wells with standard deviation. **e**, Immunostaining for FOXF1 (*green*), a splanchnic marker, and ISL1 (*magenta*), a cardiac mesoderm marker. The images are maximum intensity projection of confocal stacks. **f**, The average % proportion of differentiated cells positive for the indicated marker by immunocytochemistry. The numbers of marker positive cells were divided by the total number of DAPI positive cells. Mean with S.D. n=3 independent fields at day4. CG-SpM; cardiogenic splanchnic mesoderm, aFG-SpM; anterior foregut SpM, Mid. PS; middle region of primitive streak, pFG-SpM; posterior foregut SpM, SC; stem cell. Each column indicates the average from the 3 independent fields with standard deviation. Scale bar; 50µm

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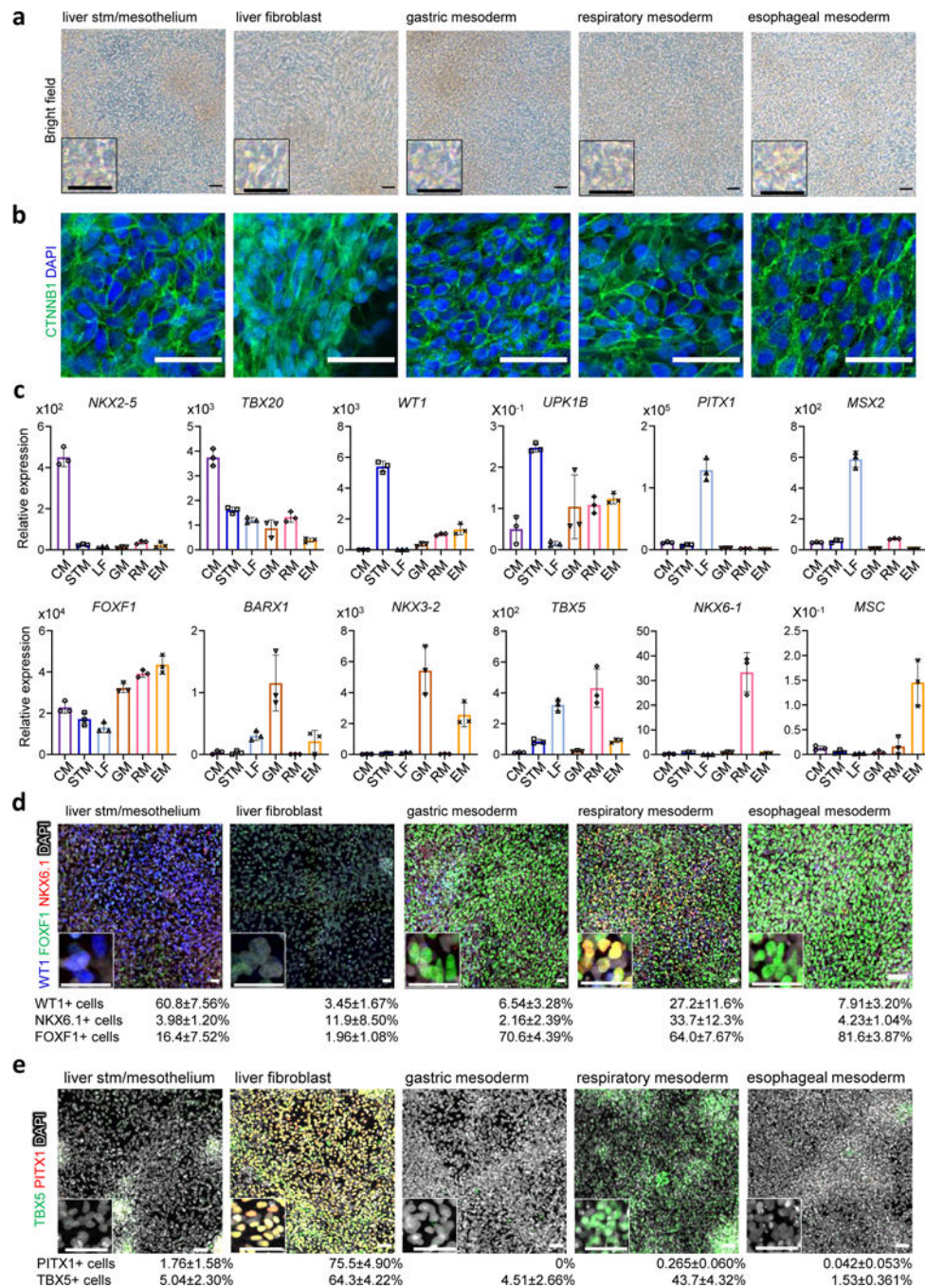


Fig. 3. Characterization of organ-specific-like mesoderm at day7

a, Bright field images of differentiated STM/Methothelium-like, liver-like fibroblasts, gastric-like mesoderm, respiratory-like mesoderm, and esophageal-like mesoderm at day7. **b**, Immunostaining for CTNNB1 (*green*). The images are maximum intensity projection of confocal stacks. **c**, Relative mRNA expression of cardiac gene (*NKX2-5* and *TBX20*), liver septum transversum gene (*WT1*), liver mesothelial gene (*UPK1B*), liver fibroblast gene (*PITX1*, *MSX2* and *TBX5*), gastric mesoderm gene (*FOXF1*, *BARX1* and *NKX3-2*), respiratory mesoderm gene (*FOXF1*, *NKX6-1* and *TBX5*) and esophageal mesoderm gene

(*FOXF1* and *MSC*) by qRT-PCR. Each bar indicates the average from the 3 independent wells with standard deviation. **d**, Immunostaining for *FOXF1* (*green*), *NKX6.1* (*red*), and *WT1* (*blue*). **e**, Immunostaining for *PITX1* (*red*) and *TBX5* (*green*). The images in **d** and **e** are maximum intensity projection of confocal stacks with the average % proportion of differentiated cells positive for the indicated marker by immunocytochemistry also given. The numbers of marker positive cells were divided by the total number of DAPI positive cells. Mean with S.D. $n = 3$ independent fields. Scale bar, 50 μm

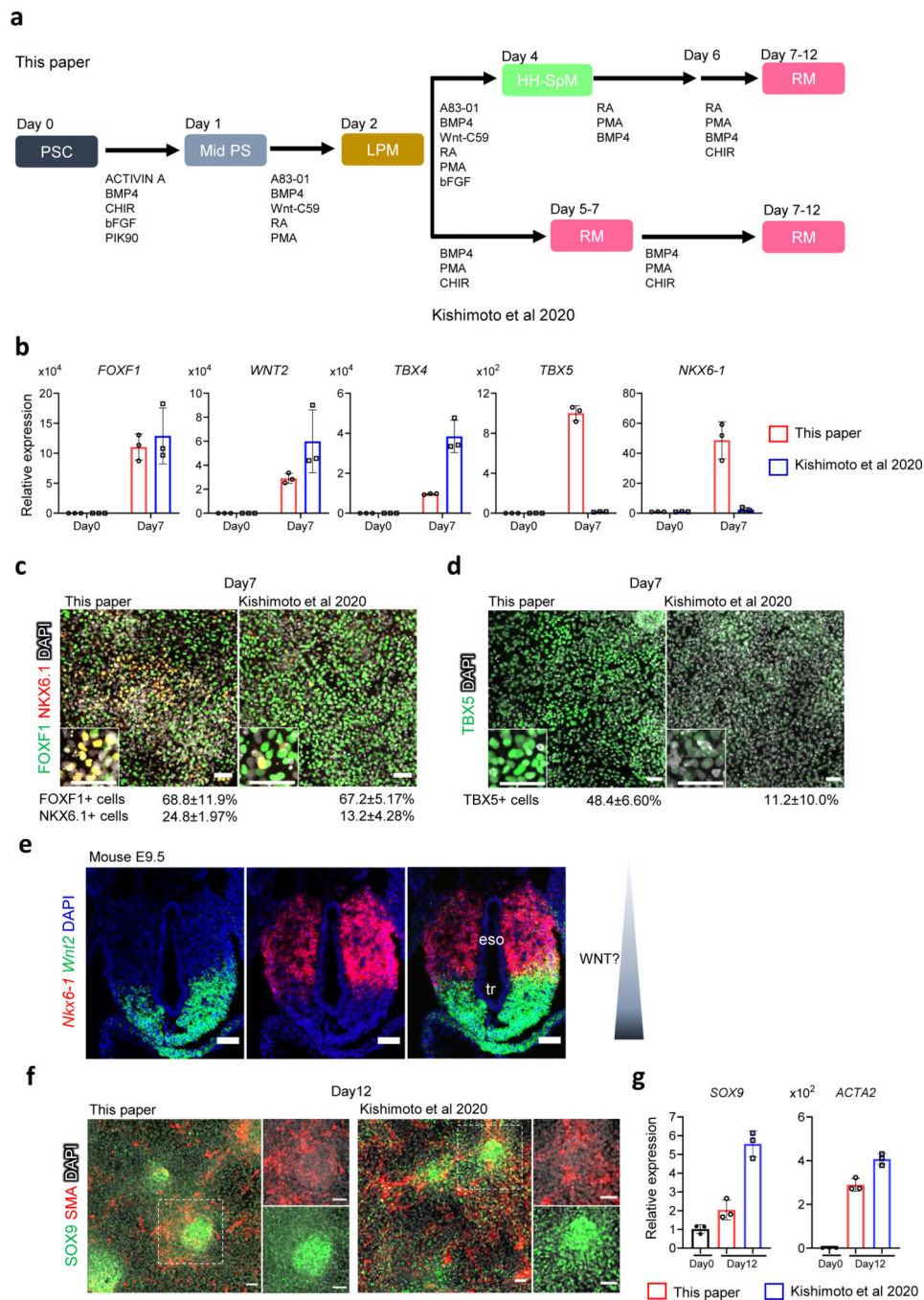


Fig. 4. Comparison of the protocols for generating trachea/lung-like mesoderm

a, Overview of the protocols. Two protocols (this paper and Kishimoto and Morimoto²⁷) were compared. **b**, Relative mRNA expression of trachea/lung mesoderm genes (*FOXF1*, *NKX6-1*, *TBX5*, *TBX4*, and *WNT2*) by qRT-PCR. Each column indicates the average from the 3 independent wells with standard deviation. **c**, Immunostaining for FOXF1 (green) and NKX6.1 (red) at day7. **d**, Immunostaining for TBX5 (green) at day7. The images in **c** and **d** are maximum intensity projection of confocal stacks with the average % proportion of differentiated cells positive for the indicated marker by immunocytochemistry also given.

The numbers of marker positive cells were divided by the total number of DAPI positive cells. Mean with S.D. n=3 independent fields. **e**, Maximum intensity projection of confocal RNAscope for *Wnt2* (*green*), *Nkx6-1* (*red*) in mouse embryo at E9.5¹⁴, indicating the proposed WNT activity gradient. **f**, Maximum intensity projection of confocal stacks from *SOX9* (*green*) and *SMA* (*red*) immunostaining at day12. **g**, Relative mRNA expression of chondrocyte progenitor gene, *SOX9* and smooth muscle gene *ACTA2* by qRT-PCR. Each column indicates the average from the 3 independent wells with standard deviation. Scale bars, 50 μ m

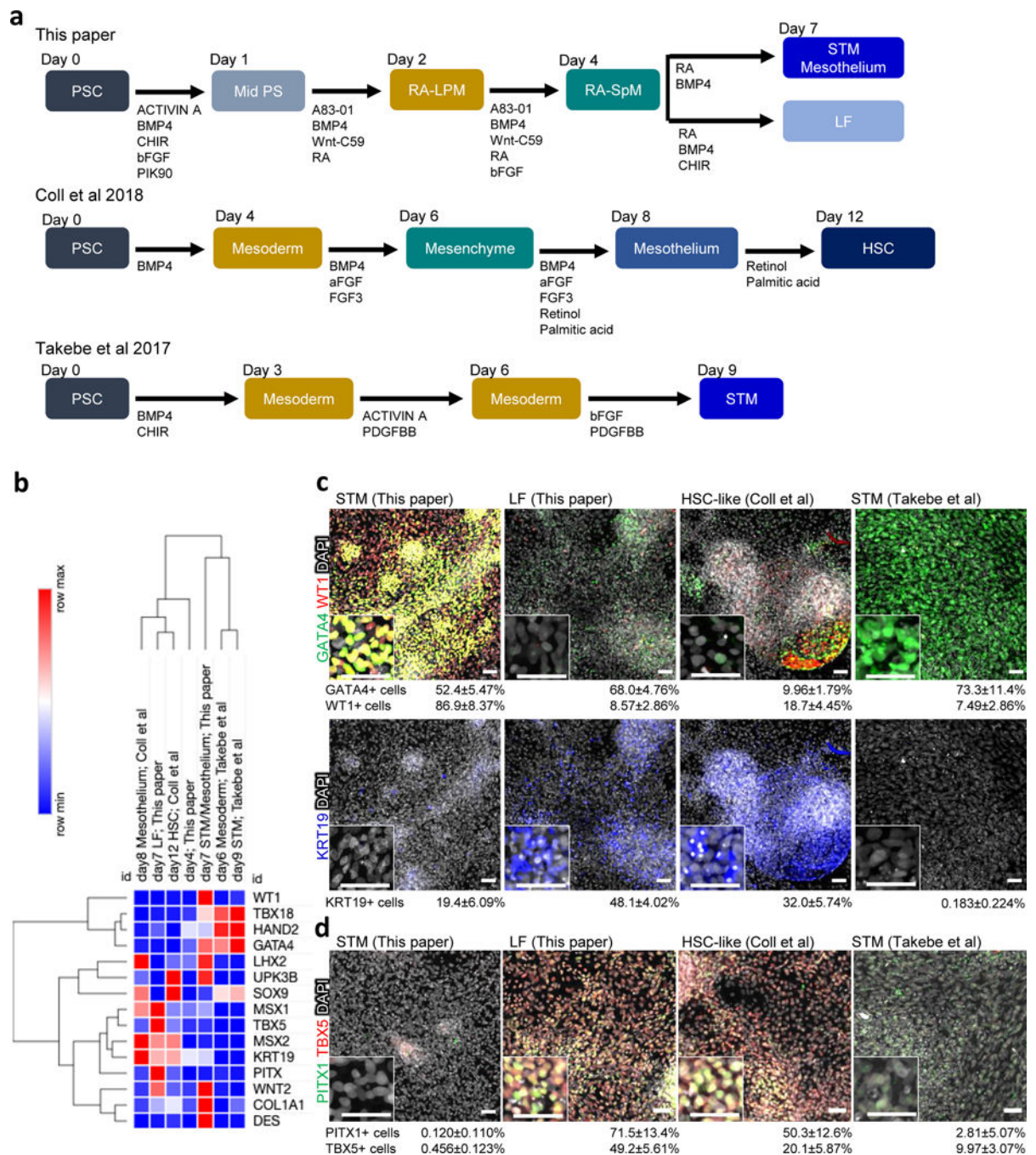


Fig. 5. Comparison of the protocols for generating liver-like mesoderm

a, Overview comparing three protocols (this paper, Coll et al.³⁰ and Takebe et al.²⁹). **b**, Heat map based on relative mRNA expression of liver mesenchyme gene expression from qRT-PCR. Each row indicates the average from the three independent wells. **c**, Immunostaining for GATA4 (green), WT1 (red), and KRT19 (blue). **d**, Immunostaining for PITX1 (green) and TBX5 (red). The images in **c** and **d** are maximum intensity projection of confocal stacks with the average % proportion of differentiated cells positive for the indicated marker by immunocytochemistry also given. The numbers of marker positive cells were divided by

the total number of DAPI positive cells. Mean with S.D. n=3 independent fields. Scale bar; 50µm

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Table 1

Gene expression profiles of differentiating mesoderm

Cell type	Gene expression
d4 CG-SpM	NKX2-5 ^{High} , ISL1 ^{High} , HOXA5 ^{Low} , CYP26A1 ^{Low} , GLI1 ^{Low} , PTCH1 ^{Low}
d4 pFG-SpM	NKX2-5 ^{Low} , ISL1 ^{Low} , HOXA5 ^{High} , CYP26A1 ^{High} , GLI1 ^{Low} , PTCH1 ^{Low}
d4 aFG-SpM	NKX2-5 ^{Low} , ISL1 ^{Low} , HOXA5 ^{High} , CYP26A1 ^{High} , GLI1 ^{High} , PTCH1 ^{High}
d7 Cardiac mesoderm (CM)	NKX2-5 ⁺ , TBX20 ⁺ , ACTC1 ⁺
d7 Liver STM/mesothelium	WT1 ⁺ , TBX18 ⁺ , LHX2 ⁺ , GATA4 ⁺ , UPK1B ⁺ , UPK3B ⁺
d7 Liver fibroblast (LF)	PITX1 ⁺ , MSX1 ⁺ , MSX2 ⁺ , TBX5 ⁺ , WNT2 ⁺
d7 Gastric mesoderm (GM)	BARX1 ⁺ , NKX3-2 ⁺ , FOXF1 ⁺
d7 Respiratory mesoderm (RM)	TBX5 ⁺ , NKX6-1 ⁺ , WNT2 ⁺ , FOXF1 ⁺
d7 Esophageal mesoderm (EM)	NKX6-1 ⁺ , MSC ⁺ , WNT4 ⁺ , FOXF1 ⁺

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Table 2

Antibodies

Antibody	Company	Cat. no.	Species	Dilution	RRID
CDH1	Cell Signaling Technologies	#3195	Rabbit	1:400	AB_2291471
FOXF1	R&D Systems	AF4798	Goat	1:500	AB_2105588
GATA4	Santacruz	sc-1237	Goat	1:200	AB_2108747
ISL1	Abcam	ab109517	Rabbit	1:200	AB_10866454
KRT19	eBioscience	14-9898-82	Mouse	1:50	AB_10598673
NKX6.1	Developmental Studies Hybridoma Bank	F55A12	Mouse	1:40	AB_532379
OCT3/4	Santacruz	sc-5279	Mouse	1:100	AB_628051
PITX1	ThermoFisher Scientific	PA5-52600	Rabbit	1:100	AB_2645587
SMA	Sigma	C6198	Mouse	1:500	AB_476856
SOX2	R&D Systems	AF2018	Goat	1:200	AB_355110
SOX9	Abcam	AB5535	Rabbit	1:500	AB_2239761
TBX5	Santacruz	sc-17865	Goat	1:200	AB_2200830
TBXT	Cell Signaling Technologies	#81694	Rabbit	1:200	AB_2799983
VIM	Santacruz	sc-7557	Goat	1:200	AB_793998
WT1	Abcam	ab89901	Rabbit	1:200	AB_2043201

Table 3

Reconstitution of growth factors and small molecules

Growth factors and small molecules	Solvent	Stock concentration	Long-term storage
A83-01 *	DMSO	10 mM	-20 °C
Activin A	1XPBS, 0.1% BSA	100 µg ml ⁻¹	-80 °C
bFGF	1XPBS, 0.1% BSA	100 µg ml ⁻¹	-80 °C
BMP4	1XPBS, 0.1% BSA, 4mM HCl	50 µg ml ⁻¹	-80 °C
CHIR99021	DMSO	6 mM	-20 °C
Noggin	1XPBS, 0.1% BSA	200 µg ml ⁻¹	-80 °C
PMA	DMSO	20 mM	-20 °C
PIK90 *	DMSO	0.5 mM	-20 °C
RA *	DMSO	20 mM	-80 °C
Wnt-C59	DMSO	10 mM	-20 °C
XAV-939	DMSO	2 mM	-20 °C

* A83-01, PIK90 and RA are photosensitive. Wrap aliquots in foil to avoid light.

Table 4

Primers

Gene	Forward primer	Reverse primer
<i>BARX1</i>	CCAGTGGGAACCTGAACACC	CTGAAGTTCGGCGTGCAG
<i>COL1A1</i>	GACGAAGACATCCCACCAATCA	GGACTCGTCACAGATCACGTC
<i>CYP26A1</i>	GCTGCCTCTCTAACCTGCAC	TGCTTTAGTGCCTGCATGTC
<i>DES</i>	AATGACCGCTTCGCCAACTA	GGTTAGTGAGCACCTCCACC
<i>FOXA2</i>	GGGAGCGGTGAAGATGGA	TCATGTTGCTCACGGAGGAGTA
<i>FOXF1</i>	AGCAGCCGTATCTGCACCAGAA	CTCCTTTCGGTCACACATGCTG
<i>GATA4</i>	TAGCCCCACAGTTGACACAC	GTCCTGCACAGCCTGCC
<i>GLI1</i>	GGGATGATCCACATCCTCAGTC	CTGGAGCAGCCCCCAGT
<i>HAND2</i>	CACCTTCTGAATTCGCCAC	TCTCTTTGACCCCTTTGAG
<i>HOXA5</i>	CGCCCAACCCAGATCTAC	CGGGCCGCCTATGTTGT
<i>ISL1</i>	AGATTATATCAGGTTGTACGGGATCA	ACACAGCGGAAACTCGAT
<i>KRT19</i>	CACCAGCCGGACTGAAGAAT	GCAGGTCAGTAACCTCGGAC
<i>LHX2</i>	TCGGGACTTGGTTTATCACCT	GCAAGCGGCAGTAGACCAG
<i>MSC</i>	TATGAGAACGGCTACGTGCAC	AGTCCGATTTAAGCGGTGGTT
<i>MSX1</i>	CTCCGCAAACAAGACGAAC	GGCGGTTCTGGAACCATATCT
<i>MSX2</i>	CGCCAAGACATATGAGCCCT	GTTCTGCCTCCTGCAGTCTT
<i>NKX2-5</i>	TGGAGAAGACAGAGCCGACAA	ATAGACCTGCGCCTGCGAGAA
<i>NKX3-2</i>	CAACACCGTCGTCCTCG	CCGCTTCCAAAGACCTAGAG
<i>NKX6-1</i>	ATGACAGAGAGTCAGGTCAAGG	CTCCGAGTCTGCTTCTTCTT
<i>OCT3/4</i>	TGAGAGGCAACCTGGAGAATT	TTTCTTTCCCTAGCTCCTCCC
<i>PITX1</i>	TTCTTGCTGGGTCGTCT	TCGTCTGACACGGAGCTG
<i>PTCH1</i>	CCACAGAAGCGCTCCTACA	CTGTAATTTGCCCCCTTCC
<i>SOX2</i>	GCTTAGCCTCGTCGATGAAC	AACCCCAAGATGCACAACCTC
<i>SOX9</i>	GTAATCCGGGTGGTCTTCT	GTACCCGCACTTGACAAC
<i>TBX4</i>	TGATCATCACTAAGGCTGGCAG	ACAGAACTTGTAGCGATGGTCAT
<i>TBX5</i>	ACAAAGTGAAGGTGACGGCCTTA	ATCTGTGATCGTCGGCAGGTACAA
<i>TBX18</i>	TTAACCTTGTCCTGCTGAGT	GTAATGGGCTTTGGCCTTTGCACT
<i>TBX20</i>	GGCGACGGAGAACAATCAA	CTGGGCACAGGACGACTTC
<i>TBXT</i>	CAGTGGCAGTCTCAGGTTAAGAAGGA	CGTACTGCAGGTGTGAGCAA
<i>UPK1B</i>	TGGAAGCAACGAACAGTTGA	CTACCGTGTGCCAGAAA
<i>UPK3B</i>	ATACCGTCTGGCTCGTGGTG	CAGGGGCAGCGTCATGTAGT
<i>VIM</i>	ATTCCACTTTGCGTTCAAGG	CTTCAGAGAGAGGAAGCCGA
<i>WNT2</i>	CTGTATCAGGGACCGAGAGG	CCCACAGCATGACTTCAC
<i>WT1</i>	ATAGGCCAGGCATGTGTATGTGT	AGTTGCCTGGCAGAACTACATCCT

Table 5

Troubleshooting table

Step	Problem	Possible reason	Solution
6	Not enough dissociation of cells Not enough agitation of cells	Cell density is too high Tap side of the plate.	Start experiment with a lower density of hPSCs.
17	Low cell density	Low concentration of plated cells Low attachment of the plated cells	Check plating cell density. Check thiazovivin concentration and expiry date. Check protein concentration and expiry data of Geltrex.
34	Poor differentiation of hPSCs to SpM	Poor growth of the cells Cell density is too high	Check the quality of hPSCs. New line of hPSCs should be thawed. Check plated cell density and cell confluency at each process. Check cell distribution in the wells. Cells should be evenly distributed.
	Poor induction of pFG-SpM and aFG-SpM	Cell density is too high Cells should be evenly distributed. RA might be degraded by light	Check plated cell density and cell confluency at each process. Check cell distribution in the wells.
38	Cells lift off the plate	Cell density is too high	Use fresh and photo-protected RA. Check plated cell density and cell confluency at each process. Check cell distribution in the wells. Cells should be evenly distributed.
	Poor differentiation of hPSCs to organ-specific mesoderm	Cell density is too high	Check plated cell density and cell confluency at each process. Check cell distribution in the wells. Cells should be evenly distributed.