Universiteit Leiden
The Netherlands

# Activin A-derived human embryonic stem cells show increased competence to differentiate into primordial germ cell-like cells

Mishra, S.; Taelman, J.; Popovic, M.; Tilleman, L.; Duthoo, E.; Jeught, M. van der; ... ; Heindryckx, B.

**Citation**
Mishra, S., Taelman, J., Popovic, M., Tilleman, L., Duthoo, E., Jeught, M. van der, ... Heindryckx, B. (2021). Activin A-derived human embryonic stem cells show increased competence to differentiate into primordial germ cell-like cells. *Stem Cells*, *39*(5), 551-563. doi:10.1002/stem.3335

Version: Publisher's Version
License: Creative Commons CC BY-NC-ND 4.0 license
Downloaded from: https://hdl.handle.net/1887/3280151

**Note:** To cite this publication please use the final published version (if applicable).

Received: 3 October 2020 | Accepted: 21 December 2020

DOI: 10.1002/stem.3335

EMBRYONIC STEM CELLS/INDUCED PLURIPOTENT STEM CELLS

STEM CELLS

# Activin A-derived human embryonic stem cells show increased competence to differentiate into primordial germ cell-like cells

Swati Mishra[1] | Jasin Taelman[1] | Mina Popovic[1] | Laurentijn Tilleman[2] | Evi Duthoo[1] | Margot van der Jeught[1] | Dieter Deforce[2] | Filip van Nieuwerburgh[2] | Björn Menten[3] | Petra de Sutter[1] | Annekatrien Boel[1] | Susana M. Chuva De Sousa Lopes[1,4] | Björn Heindryckx[1]

[1]Ghent-Fertility and Stem cell Team (G-FAST), Department of Reproductive Medicine, Ghent University Hospital, Ghent, Belgium

[2]Laboratory of Pharmaceutical Biotechnology, Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium

[3]Department of Pediatrics and Medical Genetics, Center for Medical Genetics, Ghent University Hospital, Ghent, Belgium

[4]Department of Anatomy and Embryology, Leiden University Medical Centre, Leiden, The Netherlands

**Correspondence**
Björn Heindryckx, PhD, Ghent-Fertility And Stem cell Team (G-FaST), Department of Reproductive Medicine, Ghent University Hospital, Belgium.
Email: bjorn.heindryckx@ugent.be

Susana M. Chuva De Sousa Lopes, PhD, Department of Anatomy and Embryology, Leiden University Medical Centre, Einthovenweg 20, 2333 ZC, Leiden, The Netherlands.
Email: lopes@lumc.nl

**Funding information**
Agentschap voor Innovatie door Wetenschap en Technologie, Grant/Award Numbers: IWT 131673, IWT 150042; Bijzonder Onderzoeksfonds, Grant/Award Numbers: BOF 01D08114, BOF GOA 2018000504, BOF GOA030-18; Dutch Research Council, Grant/Award Number: VICI-2018-91819642

## Abstract

Protocols for specifying human primordial germ cell-like cells (hPGCLCs) from human embryonic stem cells (hESCs) remain hindered by differences between hESC lines, their derivation methods, and maintenance culture conditions. This poses significant challenges for establishing reproducible in vitro models of human gametogenesis. Here, we investigated the influence of activin A (ActA) during derivation and maintenance on the propensity of hESCs to differentiate into PGCLCs. We show that continuous ActA supplementation during hESC derivation (from blastocyst until the formation of the post-inner cell mass intermediate [PICMI]) and supplementation (from the first passage of the PICMI onwards) is beneficial to differentiate hESCs to PGCLCs subsequently. Moreover, comparing isogenic primed and naïve states prior to differentiation, we showed that conversion of hESCs to the 4i-state improves differentiation to (TNAP [tissue nonspecific alkaline phosphatase]+/PDPN [podoplanin]+) PGCLCs. Those PGCLCs expressed several germ cell markers, including *TFAP2C* (transcription factor AP-2 gamma), *SOX17* (SRY-box transcription factor 17), and *NANOS3* (nanos C2HC-type zinc finger 3), and markers associated with germ cell migration, *CXCR4* (C-X-C motif chemokine receptor 4), *LAMA4* (laminin subunit alpha 4), *ITGA6* (integrin subunit alpha 6), and *CDH4* (cadherin 4), suggesting that the large numbers of PGCLCs obtained may be suitable to differentiate further into more mature germ cells. Finally, hESCs derived in the presence of ActA showed higher competence to differentiate to hPGCLC, in particular if transiently converted to the 4i-state. Our work provides insights into the differences in differentiation propensity of hESCs and delivers an optimized protocol to support efficient human germ cell derivation.

**KEYWORDS**
activin A (ActA), differentiation, human embryonic stem cells (hESCs), pluripotency, primordial germ cell-like cells (PGCLCs)

Susana M. Chuva De Sousa Lopes and Björn Heindryckx contributed equally for this study.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
©2021 The Authors. STEM CELLS published by Wiley Periodicals LLC on behalf of AlphaMed Press 2021



Downloaded from https://academic.oup.com/stmcls/article/39/5/551/6423762 by Universiteit Leiden / LUMC user on 30 June 2022

## 1 | INTRODUCTION

Pluripotency designates the ability of human embryonic stem cells (hESCs) to differentiate into all embryonic lineages, including the germline.[1] In vivo, pluripotency exists as a transient developmental gradient, starting from the fully uncommitted naive state in the blastocyst inner cell mass (ICM) to the more lineage predisposed primed state in post-implantation embryos.[2,3] Mouse embryonic stem cells (mESCs) derived from the ICM reside in the naive state of pluripotency, whereas stem cells derived from the mouse post-implantation epiblast (mEpiSCs) adopt the primed state.[4,5] Notably, ICM-derived canonical hESCs display a primed pluripotent profile, more similar to mEpiSCs.[4,5] Contrary to mESCs, conventional hESCs transition toward primed pluripotency through a post inner cell mass intermediate (PICMI), observed during the establishment of stable hESC lines.[6,7] However, by precisely modulating the culture environment, it is also possible to derive naive hESCs directly from the ICM, or to convert the existing primed hESCs toward the naive state.[8-11] For instance, primed hESCs can be reset to a naive-like pluripotent state through extended culture in naïve human stem cell medium (NHSM) supplemented with human leukemia inhibitory factor (LIF), fibroblast growth factor 2 (FGF2), transforming growth factor beta 1 (TGFβ1) along with inhibitors for mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK), glycogen synthase kinase 3 beta (GSK3β), JUN N-terminal kinase (JNK), and P38 mitogen-activated protein kinase (p38).[9] More recently, the culture of naive hESCs has been achieved through the commercially available RSeT medium, a FGF2- and TGFβ-free formulation of NHSM.[12] The culture conditions of primed hESCs have also been optimized to reduce cellular heterogeneity through inhibition of WNT (wingless-related integration site) signaling.[13,14]

The germline competence of ESCs relies on the state of pluripotency the cells reside in. Accordingly, in the mouse model, primed mEpiSCs fail to differentiate toward mouse primordial germ cell-like cells (PGCLCs) in response to germ cell specification stimuli.[15,16] However, naive mESCs briefly exposed to primed signaling factors, activin A (ActA) and FGF2, generated intermediary germline-competent epiblast-like cells, which could be successfully differentiated to PGCLCs and further matured to form functional gametes and ultimately could contribute to the generation of fertile mice.[15-18] Human PGCLCs (hPGCLCs) can also be generated in vitro from human pluripotent stem cells (hPSCs), both from hESCs and from human induced pluripotent stem cells (hiPSCs).[19-25] Specifically, Irie et al reported the successful differentiation of TNAP (tissue non-specific alkaline phosphatase or ALPL)+/NANOS3 (nanos C2HC-type zinc finger 3) + hPGCLCs starting from germline competent hPSCs maintained in 4i-medium (supplemented with human LIF, FGF2, TGFβ1 along with inhibitors for MEK, GSK3β, JNK, and p38), based on culture conditions adapted from Gafni et al.[9,20] When cultured in germline-differentiation media for 4 days as embryoid bodies (EBs), 4i-hESCs were able to form hPGCLCs that resembled human primordial germ cells (PGCs) at 7 weeks post-conception (WPC) (equivalent to Carnegie stage 18-19), based on their transcriptional signature.[20]

Although hPSCs can be successfully differentiated toward hPGCLCs, different lines exhibit marked differences in lineage differentiation propensity,[26-28] attesting to their inherent cellular heterogeneity and sensitivity to culture conditions. This may pose significant challenges for engineering reproducible multistep in vitro models of human gametogenesis.[29-31] In the context of human germline modelling, it would be highly beneficial to acquire hESC lines with increased germ cell competence. Exogenous supplementation of ActA during hESC derivation has been previously reported to increase the efficiency of hPGCLC differentiation.[32,33] However, it remains unclear at which stage ActA supplementation plays a role during the transition from pluripotency toward hPGCLC fate, particularly in the context of more recent germ cell differentiation protocols.

Here, we have compared the propensity of hESCs derived in both conventional and ActA conditions to form hPGCLCs. For this, we converted hESCs to isogenic primed and naïve-states prior to differentiation and observed that conversion to the 4i-state conferred the highest competence to differentiate to hPGCLCs. Moreover, we derived isogenic hESC lines from a single blastocyst by dividing the PICMI into two halves and culturing them from the PICMI-stage with and without ActA. We observed no difference in hPGCLC yield between these lines, suggesting that increased germline competence is only obtained when ActA is also added in the period from the blastocyst until the formation of the PICMI. As these two cell lines are isogenic, an alternative explanation is that the increased competence to form PGCLCs is due to differences in genetic background and not just due to the use of ActA. Defining key factors involved in human germline development will be essential for unravelling complex molecular transitions between pluripotency and the germ cell fate. Taken together, optimizing hPGCLC differentiation protocols may support the ultimate aim of acquiring in vitro patient-derived gametes in the future.

**Significance statement**

Robust differentiation of human primordial germ cell-like cells (hPGCLCs) starting from human embryonic stem cells (hESCs) is currently hampered by the inherent heterogeneous nature of different hESC lines. This article shows that hESC lines derived and cultured in the presence of activin A show high competence to differentiate to PGCLCs, in particular if transiently converted to the 4i-state prior to differentiation. A robust and systematic comparison of isogenic lines was performed and provided an optimized protocol to obtain PGCLCs from hESCs.

## 2 | MATERIALS AND METHODS

### 2.1 | Ethical permissions

Human pre-implantation embryos were donated to research by patients who previously underwent medically assisted reproduction at

Downloaded from https://academic.oup.com/stmcls/article/39/5/551/6423762 by Universiteit Leiden / LUMC user on 30 June 2022

Ghent University Hospital. Embryos were only used in the study following written informed consent. Human ESC derivation was approved by the Institutional Review Board, Ghent University (EC2015/1114) and the Belgian Federal Commission for Research on Embryos in vitro (ADV_060_UZ Gent). The differentiation of germ cells from hESCs was approved by the Institutional Review Board of the Ghent University Hospital, Belgium (EC2019/1595).

The human gonadal material was acquired from elective abortions (without medical indication) and donated for research with written informed consent. Approval for the collection and use of human gonadal material was obtained from the Medical Ethical Committee of the Leiden University Medical Centre (P08.087).

## 2.2 | Derivation of hESCs (via PICMI formation)

Cryopreserved day 5 blastocysts were warmed using the Vitrification Thaw Kit (Irvine Scientific, Santa Ana, CA) as described.[34] Blastocysts were cultured to day 6 in 25 μL drops of culture medium under mineral oil (Irvine Scientific). Good quality embryos were exposed to prewarmed Acidic Tyrode's Solution (Sigma-Aldrich, St. Louis, Missouri), washed and plated whole on center-well IVF (in vitro fertilization)-Falcon culture dishes (Corning, Corning, New York), on a feeder layer of mitomycin-C inactivated mouse embryonic fibroblasts (MEF) in hESC medium (Gibco KnockOut-Dulbecco's Modified Eagle Medium [KO-DMEM] [Thermo Fisher Scientific, Waltham, Massachusetts], 20% Gibco KnockOut-serum replacement [KOSR] [Thermo Fisher Scientific], 1% Gibco Penicillin/Streptomycin (P/S) [Thermo Fisher Scientific], 1% Gibco nonessential amino acids [NEAA] [Thermo Fisher Scientific], 0.4 mM Gibco L-Glutamine [Thermo Fisher Scientific], 0.1 mM Gibco β-mercaptoethanol [Thermo Fisher Scientific], and 4 ng/mL FGF2 [Peprotech, Rocky Hill, New Jersey]). PICMI outgrowths were mechanically excised into two halves. One half was plated and cultured in hESC medium, resulting in line U-19-1, whereas the other half was plated in hESC medium supplemented with 20 ng/mL ActA (R&D Systems, Minneapolis, MI), resulting in line U-19-1-OA2. Cells were cultured at 37°C in hypoxic conditions (5% $O_2$, 6% $CO_2$). For chromosomal profiling, shallow whole-genome sequencing was performed on bulk hESCs as described[14] and WisecondorX was used for CNV calling.[35]

## 2.3 | Culture of hESCs

This study used different hESC lines previously derived at the Ghent University[6,32,33]: two ActA-derived primed hESCs (U-11-4, U-12-3) and two conventional primed hESCs (U-11-2, U-11-60) were cultured on mitomycin-C-inactivated MEFs in hESC medium with and without 20 ng/mL of ActA (R&D Systems), respectively. For chromosomal profiling, shallow whole-genome sequencing was performed on bulk hESCs as described[14] and WisecondorX was used for CNV calling[35] (Figure S1).

Prior to differentiation, hESCs were cultured for six passages in mitomycin-C-inactivated MEFs in different culture media: (1) DhiFI medium (DMEM-F12/Glutamax [Thermo Fisher Scientific], 20% KOSR, 1% P/S, 1% NEAA, 0.1 mM β-mercaptoethanol, 12 ng/mL hFGF2, 2 μM IWP2 [Sigma-Aldrich])[14]; (2) RSeT medium (Stemcell Technologies, Vancouver, Canada)[12]; and (3) 4i medium (KO-DMEM, 20% KOSR, 1% P/S, 1% NEAA, 20 ng/mL recombinant hLIF [Peprotech], 8 ng/mL hFGF2, 1 ng/mL recombinant hTGFβ1 [Peprotech], 3 μM CHIR99021 [Axon Medchem, Groningen, The Netherlands], 1 μM PD0325901 [MedChemExpress, Monmouth Junction, New Jersey], 5 μM SB203580 [Bio-Techne, Minneapolis, Minnesota], 5 μM SP600125 [TOCRIS, Bristol, UK], and 10 μM ROCKi [Enzo Life Sciences, Zandhoven, Belgium]).[20] Cells were cultured at 37°C in hypoxic conditions, refreshed daily, and passaged every 3 to 5 days depending on confluency.

## 2.4 | Differentiation of hESCs toward hPGCLCs-Containing EBs

One day prior to confluency, hESCs were dissociated using either CTK (1 mg/mL Collagenase IV [Life Technologies, Carlsbad, California], 0.25% Trypsin [Life Technologies], 20% KOSR, 1 mM $CaCl_2$ [Sigma-Aldrich] in PBS) (primed and DhiFI) or TrypLE (Thermo Fisher Scientific) (RSeT and 4i), using sterile 3-mm glass beads. Cells were centrifuged at 750 rpm for 5 minutes, followed by resuspension in respective fresh medium supplemented with 10 μM ROCKi. The hESCs were pre-plated on gelatin-coated flasks and incubated for up to 1 hour, allowing selective removal of fast attaching MEFs. EBs were generated as described[20] with some modifications. Briefly, viable cells were counted using a NucleoCounter (ChemoMetec, Allerod, Denmark) and a solution of 65.000 cells/mL was made in differentiation medium (DM) consisting of GK15 (Gibco Glasgow's MEM [Thermo Fisher Scientific], 2 mM L-glutamine, 15% KOSR, 1% P/S, 1% NEAA, 0.1 mM β-mercaptoethanol, 1 mM sodium pyruvate) supplemented with 500 ng/mL recombinant hBMP4 (bone morphogenetic protein 4) (Thermo Fisher Scientific), 100 ng/mL recombinant hSCF (stem cell factor) (Thermo Fisher Scientific), 50 ng/mL recombinant hEGF (epidermal growth factor) (R&D Systems), 1 μg/mL recombinant hLIF, and 10 μM ROCKi. To generate EBs, 100 μL of the cell suspension was added per well to U-bottom low attachment plates (Corning) and centrifuged at 400*g* for 2 minutes. The plates were then incubated at 37°C in hypoxic conditions for 4 days without medium changes. hPGCLC differentiation was performed on each cell line and for each condition in triplicate.

## 2.5 | Immunostaining of hESCs and EBs

EBs and hESCs were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich) for 1 hour (EBs) or 20 minutes (hESCs) at room temperature (RT). Samples were washed in 0.1% bovine serum albumin (BSA) in PBS, permeabilized with 0.5% (EBs) or 0.1% (hESCs) Triton-X100 (Sigma-Aldrich) in PBS for 1 hour (EBs) or 8 minutes (hESCs) and blocked (10% fetal calf serum [FCS, Thermo Fisher Scientific] and

Downloaded from https://academic.oup.com/stmcls/article/39/5/551/6423762 by Universiteit Leiden / LUMC user on 30 June 2022

0.5% BSA in PBS) for 2 hours (EBs) or 1 hour (hESCs). Blocked samples were incubated with primary antibodies (Table S1) overnight at 4°C. Samples were then incubated with secondary antibodies (Table S1) for 2 hours (EBs) or 1 hour (hESCs) at RT. Subsequently, samples were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for 10 minutes and mounted on glass slides in mounting medium containing 2.4% DABCO (Sigma-Aldrich) in glycerol (Novolab, Geraardsbergen, Belgium).

### 2.6 | Image analysis, quantification, and statistical analysis

Samples (EBs and hESCs) were imaged with a 20× objective on a SP8 confocal microscope (Leica, Wetzlar, Germany) using SPX software (Leica). EBs were imaged as Z-stacks (N = 3-7 per EB; each 10 μm) and analyzed using Fiji v2.0.[36] For quantification, cells showing coexpression of POU5F1 (POU class 5 homeobox 1), SOX17 (SRY-box transcription factor 17), PDPN (podoplanin), and DAPI were considered hPGCLCs, using the multipoint tool. Differences between groups were evaluated using one-way ANOVA (Dunnett's multiple comparisons test), with GraphPad Prism software v8.4.1. Moreover, in Fiji, the area of the EBs was measured using the area selection tool to trace the outline of the EBs at maximum projection, followed by measurement calculations using the analyze tool.

### 2.7 | Fluorescence-activated cell sorting (FACS) and statistical analysis

Early PGCs present in human fetal gonads (8 WPC male, 11 WPC female, and 15 WPC female) were used as positive control for FACS. These were isolated in 0.9% NaCl (Fresenius Kabi, Bad Homburg, Germany), dissociated in accutase (Stemcell Technologies) overnight at 4°C followed by 30 minutes at 37°C and cryopreserved in Bambanker (GC Lymphotech, Tokio, Japan). Cryovials were thawed in DMEM with 15% FCS and 10 μM ROCKi and centrifuged at 300x*g* for 5 minutes. The cells were resuspended in GK15 with 10 μM ROCKi, pre-plated on Matrigel (VWR International, Radnor, PA) coated flasks and incubated for 30 minutes at 37°C in hypoxia. The cells remaining in suspension were used for FACS. EBs were dissociated with TrypLE for 20 minutes at 37°C with intermittent pipetting, neutralized DMEM with 15% FCS and used for FACS.

For FACS, cells were centrifuged at 300*g* for 5 minutes, washed in Dulbecco's PBS (Sigma-Aldrich), resuspended in 100 μL FACS buffer (3% FCS in PBS with 10 μM ROCKi), containing fluorescent-conjugated antibodies against TNAP and PDPN (Table S1) and incubated for 15 to 20 minutes in the dark at RT. Cells were washed twice in FACS buffer, resuspended in 100 μL of FACS buffer containing 1:100 dilution of 7AAD live/dead exclusion dye (BD Biosciences, San Jose, California) on 35 μm cell-strainer-snap-cap 5 mL FACS-tubes (Corning) and used (analyzed and sorted) using a 100 μm nozzle on a FACS Fusion Cell Sorter (BD Biosciences). The gating strategy is depicted in Figure S2A. To quantify the cells of interest, 10 000 total events were recorded per sample, followed by the removal of doublets. The percentage of TNAP+/PDPN+ (TP+) hPGCLCs was calculated from the live (7AAD-negative) gate. FACS data were analyzed using FlowJo v10. Differences between groups were evaluated using Student's *t* test with GraphPad Prism Software v8.4.1. A *P*-value <.05 was considered statistically significant.

### 2.8 | RNA-sequencing analysis and statistical analysis

Total RNA extraction from FACS-sorted cells and hESCs was performed using RNAEasy Plus Micro kit (Qiagen, Hilden, Germany) and Mini kit (Qiagen), respectively, following manufacturer's instructions and RNA concentration and quality were evaluated using the Quant-it ribogreen RNA-assay (Life Technologies) and the RNA6000 Nanochip (Agilent Technologies, Santa Clara, California), respectively. Library preparation was performed using the QuantSeq 3′ mRNA-Seq Library Prep Kit (Lexogen, Vienna, Austria), using 2 ng of RNA. A high sensitivity DNA chip (Agilent Technologies) was used to control the library size distribution and quality. Sequencing was performed on a high throughput Illumina NextSeq 500, generating 75 bp single reads. The reads were trimmed using cutadapt v1.18,[37] to remove the "QuantSEQ FWD" adaptor sequences, and mapped against *Homo sapiens* GRCh38.89 reference genome using STAR v2.6.0c.[38] RSEM software v1.3.1[39] was used to generate count tables. Log2 [counts per million (cpm) +1] were used for principal component analyses (PCAs), visualized using ggplot2 v3.3.0[40] and heatmaps were generated using pheatmap v1.0.12[41] with Pearson correlation-based clustering. Differential gene expression analysis between groups of samples was performed using edgeR v3.28.1.[42] For each separate analysis, the following steps were performed: (1) normalization using edgeR's standard normalization method, (2) only genes with cpm > 1 in at least four samples were retained, and (3) a general linear model was built, and statistical analysis was preformed using the empirical Bayes quasi-likelihood *F*-test. Genes having a false discovery rate <0.05 and a fold change >1 were considered significantly different. Volcano plots were used visualize pairwise comparisons using ggplot2 v3.3.0.[40]

## 3 | RESULTS

### 3.1 | hESCs converted to 4i-hESCs prior to differentiation produce more hPGCLCs

To investigate the role that the initial pluripotency state of hESCs has on the efficiency to differentiate into hPGCLCs, we compared a conventional primed hESC line (U-11-2) with an ActA-derived primed hESC line (U-11-4), referred to as "primed" and "A2-primed," respectively (Figure 1A). Primed and A2-primed hESCs expressed pluripotency-marker POU5F1 (or OCT4/Octamer-Binding Protein 4) but were negative for both SOX17 and PDPN (Figure 1B). In contrast,

Downloaded from https://academic.oup.com/stmcls/article/39/5/551/6423762 by Universiteit Leiden / LUMC user on 30 June 2022

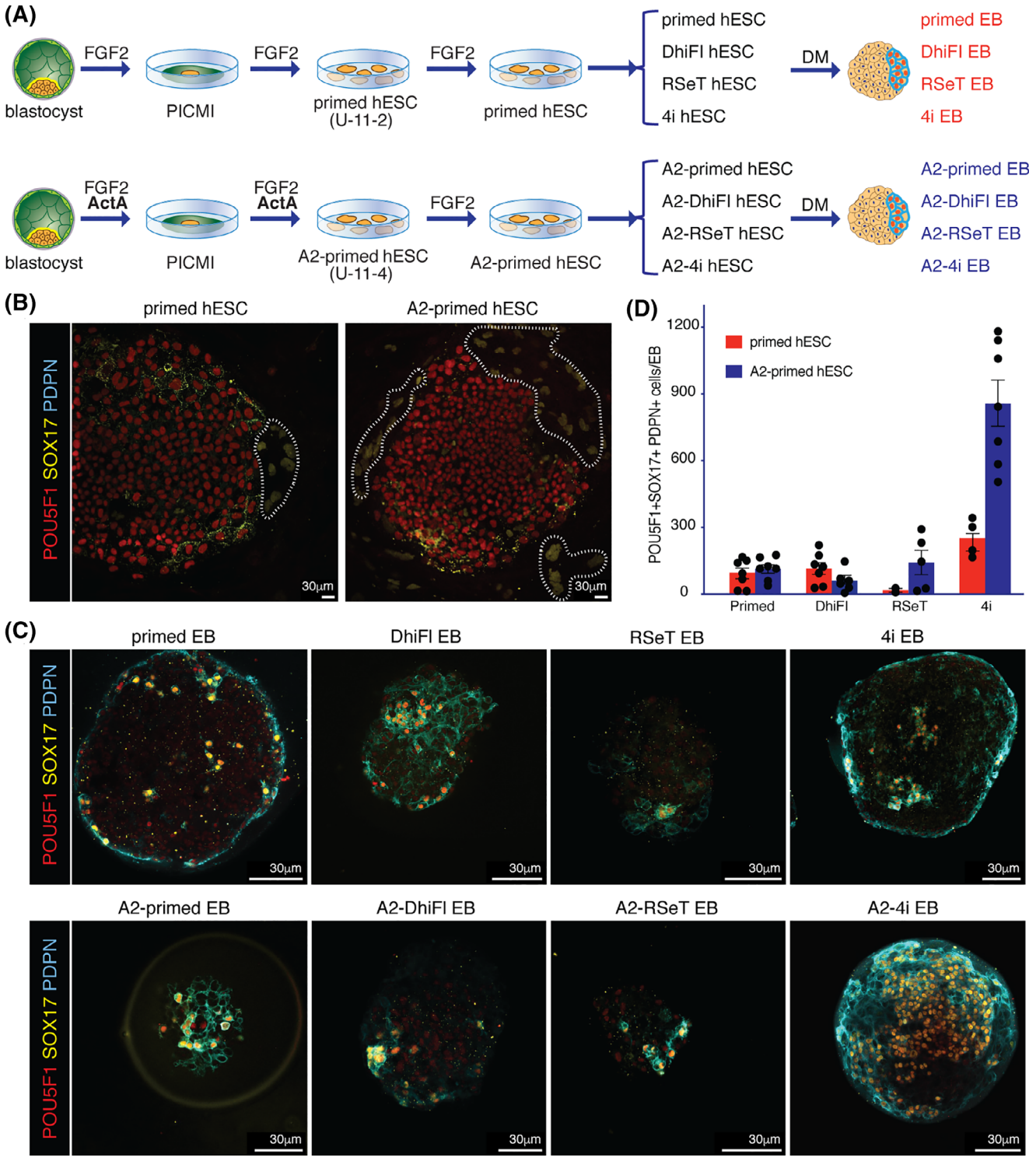


**FIGURE 1** Defining conditions to convert primed human embryonic stem cells (hESCs) to optimize differentiation to human primordial germ cell-like cells (hPGCLCs). A, Schematic protocol of the conversion conditions tested (primed, DhiFI, RSeT, and 4i) in primed and A2-primed hESCs, prior to embryoid body (EB) assay using differentiation medium (DM) to hPGCLC fate. B, Expression of POU5F1 (red), SOX17 (yellow), and podoplanin (PDPN) (cyan) in primed and A2-primed hESC colonies. White dotted lines mark regions of differentiating SOX17+ endodermal cells. C, Single z-plane of day-4 EBs immunostained for POU5F1 (red), SOX17 (yellow), and PDPN (cyan) to identify hPGCLCs. D, Bar chart showing the number of triple POU5F1+SOX17+PDPN+ hPGCLCs per EB in the different conditions tested. Error bars represent the SEM

spontaneously differentiated endodermal cells at the periphery of the hESC colonies were positive for SOX17 and negative for POU5F1 and PDPN (Figure 1B).

Prior to differentiation, the two cell lines (primed and A2-primed) were adapted to three additional pluripotency culture conditions: DhiFI-medium (supplemented with FGF2 and inhibitor of WNT),[14]

Downloaded from https://academic.oup.com/stmcls/article/39/5/551/6423762 by Universiteit Leiden / LUMC user on 30 June 2022

4i-medium (supplemented with LIF, FGF2, TGFβ1, and inhibitors for MEK, GSK3β, JNK, and p38),[20] and commercially available RSeT-medium[12] (Figure 1A; Figure S2B). After at least six passages of adaptation, the different isogenic lines were differentiated to hPGCLCs using an EB-assay.[20] After 4 days, the EBs were analyzed for the presence of hPGCLCs, identified as triple positive POU5F1/SOX17/PDPN cells.[43,44] The triple POU5F1/SOX17/PDPN immunostaining combination was chosen to allow visualization of different cellular compartments (alternating the nuclear markers POU5F1 and SOX17 with the surface marker PDPN) to ensure absence of bleeding-through from microscopy and robustly identify PGCLCs. hPGCLCs were observed in all tested conditions, but with varying efficiencies (Figure 1C,D). Compared to all other conditions, A2-primed hESCs, adapted to 4i-medium prior to differentiation, demonstrated the highest germline competence (one-way ANOVA test, $P = .0023$), showing 857 ± 275 hPGCLCs per EB (Figure 1C,D). To exclude that ActA could influence the size of the EBs and only indirectly affect PGCLC formation, we measured and corrected for the EB area (Figure S2C,D). After

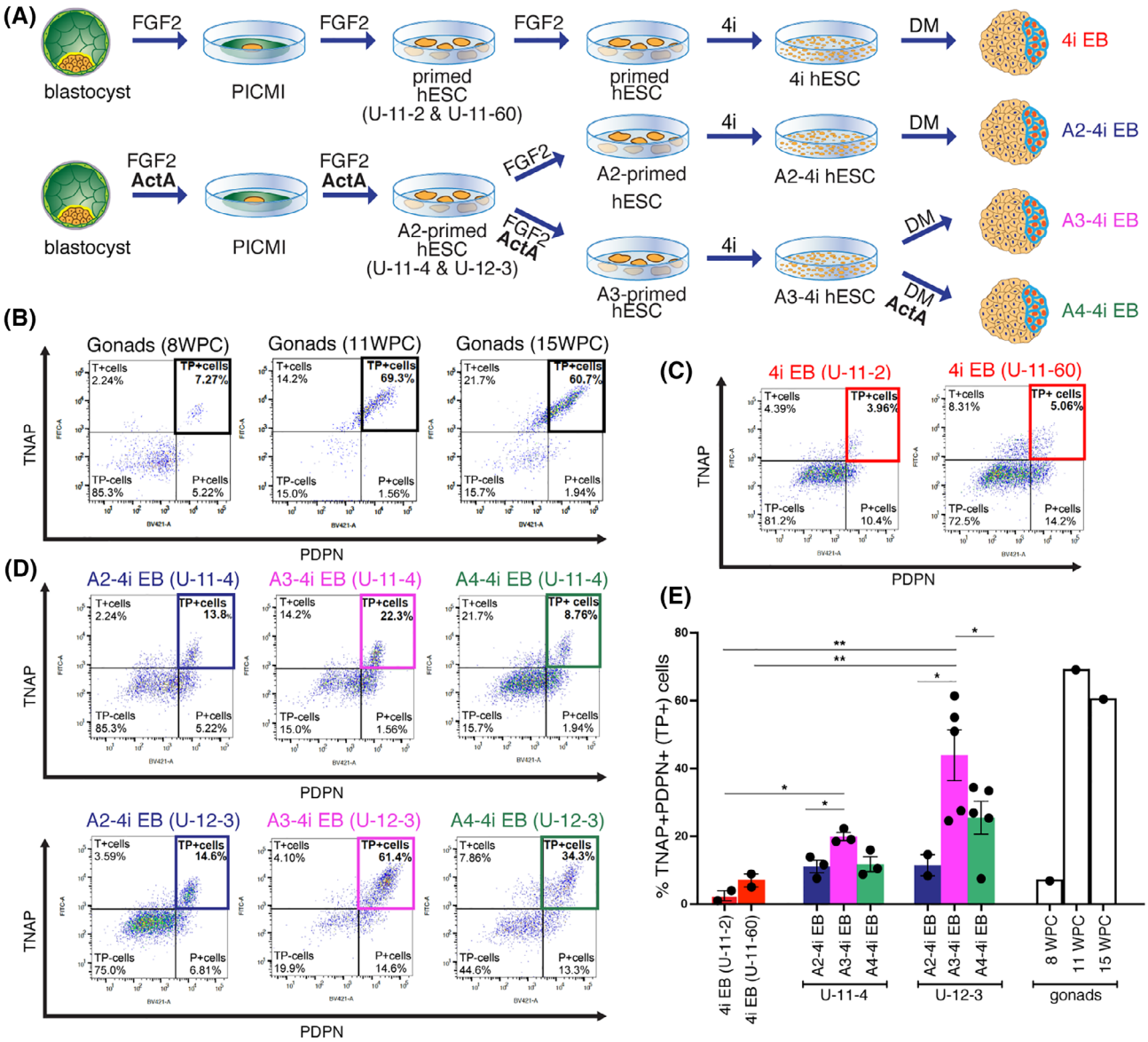


**FIGURE 2** Defining the steps that benefit from activin A (ActA) supplementation to optimize differentiation to human primordial germ cell-like cells (hPGCLCs). A, Schematic protocol used to study the impact of adding ActA (derivation, maintenance, and differentiation) to optimize differentiation to hPGCLCs. B, Fluorescence-activated cell sorting (FACS) plots showing expression of tissue nonspecific alkaline phosphatase (TNAP) and podoplanin (PDPN) used to identity TNAP+/PDPN+ (TP+) human primordial germ cells (hPGCs) in dissociated gonads from 8, 11, 15 weeks post conception (WPC). C, FACS plots showing expression of TNAP and PDPN used to identity (TP+) hPGCLCs in dissociated day-4 embryoid bodies (EBs) obtained from human embryonic stem cells (hESCs) not exposed to ActA. D, FACS plots showing expression of TNAP and PDPN used to identity (TP+) hPGCLCs in dissociated day-4 EBs obtained from different lines and culture conditions. E, Bar plots showing quantification of TP+ cells obtained from dissociated gonads and day-4 EBs from different lines and culture conditions. Asterisks denote statistically significant differences (*$P < .05$; **$P < .01$); error bars denote SEM

Downloaded from https://academic.oup.com/stmcls/article/39/5/551/6423762 by Universiteit Leiden / LUMC user on 30 June 2022

correction for the EB area, A2-primed hESCs, adapted to 4i-medium prior to differentiation, showed the highest proportion of PGCLC per area of EB (Figure S2D).

An additional pre-priming step,[20] following naïve resetting to 4i and RSET, resulted in significantly reduced numbers of hPGCLCs (Student's *t* test, $P = .007$ RSeT and $P = .0004$ 4i) (Figure S2E). Hence, pre-priming was not further used in this study.

## 3.2 | hESCs derived and maintained in ActA are more competent to differentiate to hPGCLCs

Next, we evaluated the effect of derivation and continuous supplementation with ActA (A3-primed hESCs) in the competence to differentiate to hPGCLCs and compared that with A2-primed hESCs (derived, but not continuously maintained in ActA) and conventional primed hESCs (neither derived, nor maintained in ActA) (Figure 2A). All hESC lines were adapted to 4i prior to differentiation and differentiated to hPGCLCs using the EB-assay for 4 days. Thereafter, the EBs were dissociated and hPGCLCs were quantified by FACS using TNAP and PDPN, both known surface markers for hPGCs (Figure 2B-E; Figure S2A). Human fetal gonads containing TNAP+/PDPN+ PGCs were used to setup the FACS-gates (Figure 2B). Using two independent ActA lines (U-11-4 and U-12-3), we demonstrated that hESCs derived in the presence of ActA and continuously maintained in medium supplemented with ActA (A3-primed) showed significantly higher propensity to differentiate to hPGCLCs when compared to isogenic A2-primed and to primed hESCs (Figure 2C-E).

A synergistic cooperation between BMP4 and ActA has been suggested for germline induction in both ex vivo and in vitro models.[33,43] Hence, we also tested the impact of ActA supplementation during the hPGCLC differentiation-step using A3-primed hESCs (Figure 2A). Surprisingly, we observed that the addition of ActA to the DM to form A4-4i EBs resulted in a reduction in the number of hPGCLCs when compared to isogenic A3-4i EBs (Figure 2C-E).

## 3.3 | ActA supplementation during maintenance alone did not increase hPGCLC competence

To determine the optimal time point for ActA supplementation during the hESC derivation process, blastocyst plating was

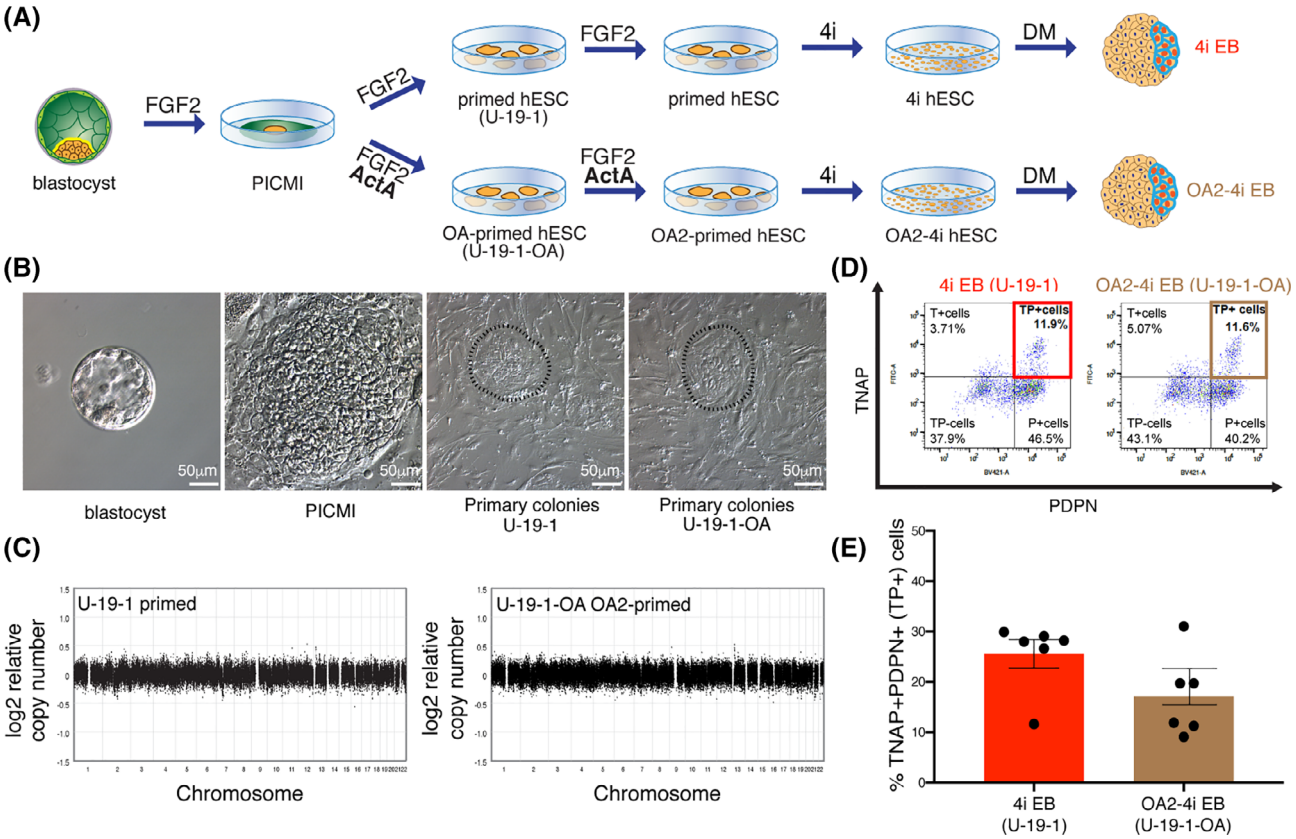


**FIGURE 3** Activin A (ActA) supplementation during maintenance of human embryonic stem cells (hESCs) does not increase competence to human primordial germ cell-like cells (hPGCLCs). A, Schematic protocol used to generate two hESC lines from a single blastocyst by splitting the post-inner cell mass intermediate (PICMI) and maintenance in the presence and absence of ActA. B, Morphology of the blastocyst and primary colonies derived from the split PICMI (encircled by black dotted lines). C, Genomic sequencing of isogenic hESC lines U-19-1 and U-19-1-OA. D, Fluorescence-activated cell sorting (FACS) plots showing expression of tissue nonspecific alkaline phosphatase (TNAP) and podoplanin (PDPN) used to identity (TP+) hPGCLCs in dissociated day-4 EBs obtained from U-19-1 and U-19-1-OA. E, Bar plot showing quantification of TP+ cells obtained from day-4 EBs from U-19-1 and U-19-1-OA. Error bars denote SEM

Downloaded from https://academic.oup.com/stmcls/article/39/5/551/6423762 by Universiteit Leiden / LUMC user on 30 June 2022

performed in hESC medium and ActA was added only after PICMI formation (Figure 3A). Once the PICMI was established, it was mechanically split across two conditions. One half was cultured in standard-hESC medium, whereas the other was plated in hESC-medium supplemented with ActA (Figure 3A,B). This resulted in the derivation of two chromosomally normal (Figure 3C), isogenic hESC lines, U-19-1 (derived in standard primed hESC-conditions) and U-19-1-OA (designating the absence of ActA in the blastocyst-plating step, but supplementation with ActA thereafter). Both lines were assessed for their propensity to differentiate to hPGCLCs, after conversion to 4i medium (Figure 3D,E). Interestingly, no significant difference (Student's *t* test; $P = .12$) was observed in the TNAP+PDPN+ hPGCLC yield between the primed lines maintained with or without ActA, suggesting that supplementation with ActA during the derivation period (during blastocyst plating) is critical for obtaining hESCs with enhanced germline propensity. Alternatively, this could be due to the specific genetic background associated with these specific isogenic hESC lines.

### 3.4 | hESCs derived and maintained in ActA showed increased expression of primitive streak-like markers

Next, we obtained next-generation sequencing data from primed hESCs (U-19-1), OA2-primed hESCs (U-19-1-OA), A2-primed hESCs (U-12-3), A3-primed hESCs (U-12-3), and A3-4i hESCs (U-12-3) as well as from FACS-sorted TNAP+PDPN+ hPGCLCs and the TNAP−PDPN− "somatic" fraction (U-12-3). In the PCA, three main clusters emerged: the hESCs regardless of their culture conditions, the TNAP+PDPN+ hPGCLCs and the TNAP−PDPN− soma (Figure 4A). To further understand the impact of ActA supplementation at different time points during the derivation and maintenance process, we analyzed the different hESC lines separately. Isogenic lines (A2-primed, A3-primed, and A3-4i) from U-12-3 clustered more closely, whereas U-19-1 and U-19-1-OA were quite distinct (Figure 4B). In hierarchical clustering using the expression of selected pluripotency and early differentiation genes to the three germ layers, extracellular matrix (ECM) marker *FN1* (fibronectin 1)

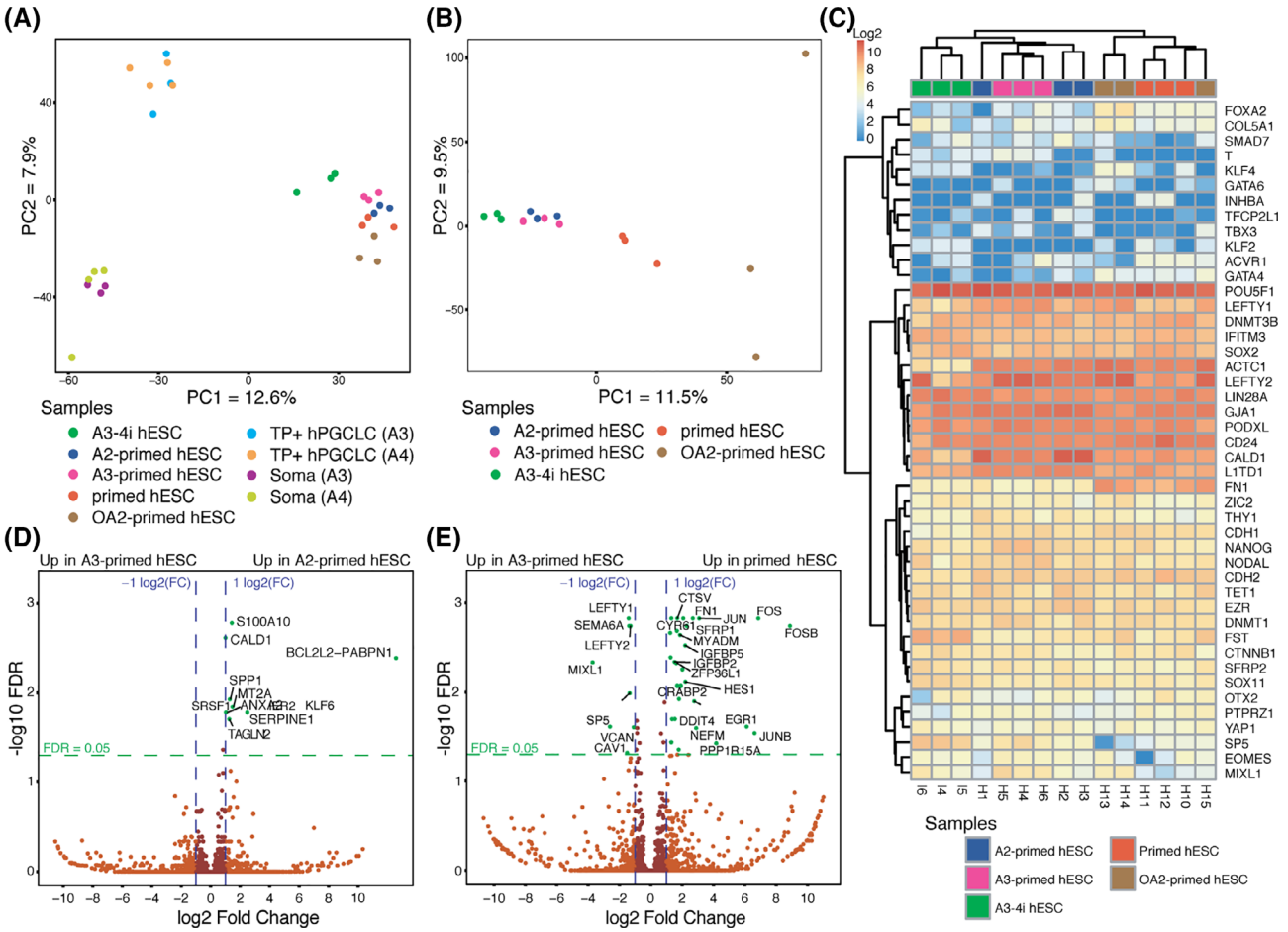


**FIGURE 4** Molecular profile of human embryonic stem cells (hESCs) subjected to supplementation with activin A (ActA) during derivation and/or maintenance. A, Principle component analysis (PCA) showing the distribution of all samples. B, PCA showing the distribution of the different hESCs. C, Heatmap showing hierarchical clustering of the different hESCs using a panel of selected genes. Triplicate samples were I4, I6, I5 for A3-4i hESCs; H1, H2, H3 for A2-primed hESCs; H4, H5, H6 for A3-primed hESCs; H10, H11, H12 for primed hESCs; and H13, H14, H15 for OA2-primed hESCs. D, Volcano plot showing differentially expressed genes (green dots) between A3-primed hESCs and A2-primed hESCs. E, Volcano plot showing differentially expressed genes (green dots) between A3-primed hESCs and primed hESCs

Downloaded from https://academic.oup.com/stmcls/article/39/5/551/6423762 by Universiteit Leiden / LUMC user on 30 June 2022

showed elevated expression in U-19-1 and U-19-1-OA hESCs, compared to the isogenic U-12-3 lines (Figure 4C), perhaps reflecting an earlier passage when compared with U-12-3. As expected, all lines showed high or moderate levels of pluripotency genes such as *POU5F1*,[45,46] *LIN28* (Lin-28 homolog A)[47] and *NANOG* (nanog homeobox),[48] as well as low/absent levels of differentiation markers, such as *FOXA2* (forkhead box A2)[49] and *GATA4* (GATA binding protein 4)[50] (Figure 4C).

To identify differences that could explain the higher competence of A3-primed hESCs (derived and maintained in ActA) to differentiate into hPGCLCs compared to primed hESCs (neither derived nor maintained in ActA) and A2-primed hESCs (derived, but not maintained in ActA), we performed a pairwise-differential gene expression analysis of the different undifferentiated hESCs (Figure 4D,E). A3-primed and A2-primed hESCs were rather similar (Figure 4D; Table S2). When compared to primed hESCs, A3-primed hESCs showed increased expression of ActA-pathway components *LEFTY1* (left-right determination factor 1) and *LEFTY2* (left-right determination factor 2)[51] as well as upregulation of *MIXL1* (mix paired-like homeobox) and *SP5* (Sp5 transcription factor), both associated with primitive streak formation and lineage priming[21,23,52] (Figure 4E; Table S3). The A3-primed hESCs showed downregulation of *SFRP1* (secreted frizzled related protein 1), an antagonist of the WNT pathway[53] and a decrease in expression of AP-1 transcription factors such as *FOS* (FBJ murine osteosarcoma viral oncogene homolog), *FOSB* (FBJ murine osteosarcoma viral oncogene homolog B), and *JUN*

(V-Jun avian sarcoma virus 17 oncogene homolog) and Kruppel-like factor *KLF6*, associated with regulation of cell growth, proliferation, and cell cycle control.[54] The AP-1 transcription factors are SMAD (Sma- and Mad-related proteins) responsive and play an important role in transcriptional regulation activities of the ActA/TGFβ pathway.[55,56] The A3-primed hESCs thus expressed relatively higher levels of mesendodermal-like and enhanced WNT-signaling transcripts compared to conventional primed hESCs.

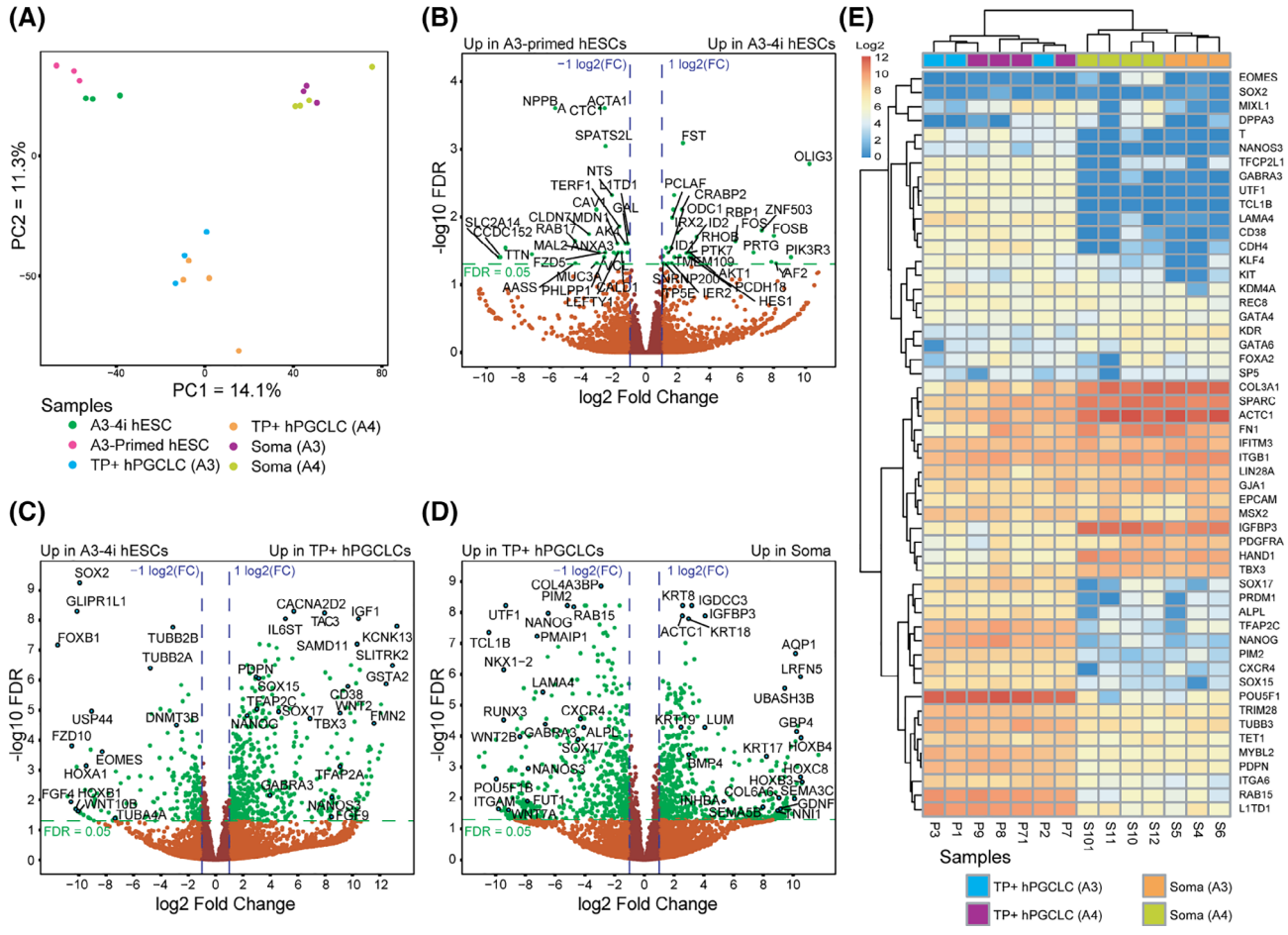


**FIGURE 5** Molecular transition from A3-primed to A3-4i to human primordial germ cell-like cells (hPGCLCs). A, Principle component analysis (PCA) showing the distribution of A3-primed human embryonic stem cells (hESCs), A3-4i hESCs along with the TNAP (tissue nonspecific alkaline phosphatase)+/PDPN (podoplanin)+ (TP+) hPGCLCs and TNAP−/PDPN− soma isolated from A3-4i-EBs (A3) and A4-4i-EBs (A4). B, Volcano plot showing differentially expressed genes (green dots) between A3-primed hESCs and A3-4i hESCs. C, Volcano plot showing differentially expressed genes (green dots) between A3-4i hESCs and TP+ hPGCLCs (A3+A4). D, Volcano plot showing differentially expressed genes (green dots) between TP+ hPGCLCs (A3+A4) and TP− soma (A3+A4). E, Heatmap showing hierarchical clustering of TP+ hPGCLCs (A3+A4) and TP− soma (A3+A4) using a panel of selected genes. Triplicate samples were P1, P2, P3 for TP+ hPGCLCs (A3); P7, P8, P9 for TP+ hPGCLCs (A4); S4, S5, S6 for soma A3; and S10, S101, S11, S12 for soma A4

Downloaded from https://academic.oup.com/stmcls/article/39/5/551/6423762 by Universiteit Leiden / LUMC user on 30 June 2022

### 3.5 | Molecular transition from A3-primed hESCs to 4i-hESCs to hPGCLCs

We characterized the transition from the primed to the 4i-state, which we and others[20,22] showed to be important to induce competence to differentiate to (TNAP+PDPN+) hPGCLCs (Figure 5A). Differential analysis between the A3-primed hESCs and the A3-4i hESCs showed upregulation of markers involved in the retinoic acid signaling pathway during this transition. These included *CRABP2* (cellular retinoic acid binding protein 2), *ZNF503* (zinc finger protein 503), *IRX2* (iroquois homeobox 2), known coactivators of the ActA/TGFβ pathway[57] as well as of BMP-targets *ID1* (inhibitor of DNA binding 1) and *ID2* (inhibitor of DNA binding 2) (Figure 5B; Table S4). ID proteins have been shown to guide early cell fate decisions and proliferation in response to SMAD4-dependent TGFβ and BMP signaling.[58]

The differential expression between A3-4i hESCs and differentiated hPGCLCs (A3+A4) (692 genes) revealed, as expected, expression of *SOX2* (SRY-box transcription factor 2) and *EOMES* (eomesodermin) in the 4i-state, as well as several tubulins (*TUBB2B*/tubulin beta 2B, *TUBB2A*/tubulin beta 2A, *TUBA4A*/tubulin alpha 4A), WNT signaling (*WNT10B* and *FZD10*/frizzled class receptor 10) and *DNMT3B*/DNA methyltransferase 3 beta (Figure 5C; Table S5). In contrast, specific markers of early germ cells emerged in the hPGCLCs, such as *CD38* (cluster of differentiation 38), *SOX17*, *SOX15*, *PDPN*, *TFAP2A*, *TFAP2C*, and *GABRA3* (gamma-aminobutyric acid type A receptor subunit alpha 3) (Figure 5C; Table S5). In addition, hPGCLCs (A3+A4) showed a distinct gene expression profile from the somatic cells, present in the same EBs (Figure 5A,D,E). A total of 1052 differentially expressed genes (DEGs) were observed between hPGCLCs and somatic cells (Figure 5D; Table S6). In agreement with previous studies profiling human early PGCs[59] and hPGCLCs,[20] hPGCLCs showed upregulation of pluripotency genes (*POU5F1*, *NANOG*), several naive genes (*KLF4*, *TCL1B*/T cell leukemia-lymphoma 1B, and *TFCP2L1*/transcription factor CP2 like 1), but show the characteristic absence of *SOX2* (Figure 5C-E). Moreover, hPGCLCs expressed multiple early germ cell genes, such as *CD38*, *NANOS3*, *PRDM1*/positive regulatory domain I-binding factor 1, *TFAP2C*, *SOX17*, *TET1*/ten-eleven translocation 1 gene protein, suggesting the acquisition of a *bona fide* early germ cell fate. Although markers associated with a later germ cell fate, such as *DDX4* (DEAD [Asp-Glu-Ala-Asp] box polypeptide 4) or *DAZL* (deleted in azoospermia like), were absent in our dataset, markers associated with germ cell migration, such as *ITGA6* (integrin subunit alpha 6), *CXCR4* (C-X-C motif chemokine receptor 4), *CDH4* (cadherin 4), and *LAMA4* (laminin subunit alpha 4)[21,22,60] were upregulated in hPGCLCs. Having optimized the protocol to differentiate large numbers of hPGCLCs efficiently will facilitate the optimization of the next steps of human gametogenesis in vitro, into more mature germ cells.

## 4 | DISCUSSION

Derivation of hPGCLCs from hESCs provides a useful premise to understand human germline specification which can be widely studied if reproduced as a scalable laboratory model.[61-63] In our study, we demonstrated that hESCs derived and maintained in medium with ActA and converted to the 4i-state prior to differentiation showed a propensity to generate a high numbers of hPGCLCs. The propensity to differentiate efficiently to hPGCLCs was the highest in hESCs that were exposed to ActA during the derivation period (during blastocyst plating). ActA signaling is prevalent in preimplantation embryos and along with insulin like growth factor 1 (IGF1) aids derivation and culture of hESC lines, which can remain pluripotent in culture without MEFs and FGF2,[64,65] suggesting a strong and long-lasting priming effect. Previous studies detailing transcriptional changes during conventional hESC derivation have shown that although hESCs were in the primed state of pluripotency, the ICM upregulates markers for naïve pluripotency, but the intermediate PICMI showed both naïve and primed markers along with markers of the early germline.[6,66] Our data suggest that the propensity to generate hPGCLCs may be acquired via ActA-priming, during a "formative" interval following the exit from naive pluripotency, but preceding the establishment of the primed state.[3]

ActA/Nodal signaling occurs through the transcriptional regulatory effect of the phosphorylated SMAD2/3-SMAD4 complex, which translocates to the cell nucleus to maintain pluripotency in undifferentiated hESCs.[67,68] However, this complex also regulates the expression of mesendodermal genes.[4] Interestingly, these early differentiation genes seem to have bivalent histone marks (both activating and silencing marks), which ensure rapid cell fate choices during differentiation.[67,69] In addition, although some degree of mesendodermal transcriptional expression may be detected, their proteins seemed absent in undifferentiated hESCs,[67] indicating multiple levels of regulation. Therefore, derivation and continued culture in ActA may allow a rapid switch of states resulting in an increase in the number of cells primed for the hPGCLC fate.

The upregulation of germline-competency in human pluripotent stem cells is associated with the expression of primitive streak-like markers such as *MIXL1*, *SP5*, *RUNX1* (runt-related transcription factor 1), *EOMES*, and *T* (or TBXT/ T-box transcription factor T), either through transient differentiation toward intermediate incipient mesoderm-like cells (iMeLC)[21,23] or, surprisingly, through conversion to the 4i-state[20,26] prior to directed differentiation. We have observed that A3-primed hESCs carried over the expression of primitive streak-like markers when converted to the 4i-state, suggesting a translated advantage of germline competence added to the 4i pluripotency. Using isogenic hESCs lines allowed us to systematically compare the degree of germline-competency and provide optimized conditions to potentiate the derivation of hPGCLCs.

The aim of this study was to provide a robust and efficient method to differentiate hPGCLCs, with a transcriptome similar to that of human early PGCs.[20,59,61,70] The obtained hPGCLCs not only expressed high levels of early PGC markers, but also expressed markers of migratory PGCs, such as *ITGA6*, *CXCR4*, *CDH4*, and *LAMA4*.[21,22,60] This may have been supported by the upregulation in the EB-somatic cells of ECM (*COL3A1*/collagen type III alpha 1 chain) and ECM-related (*SPARC*/secreted protein acidic and cysteine rich)

Downloaded from https://academic.oup.com/stmcls/article/39/5/551/6423762 by Universiteit Leiden / LUMC user on 30 June 2022

genes that are also expressed in gonadal supporting cells.[71] In this regard, it will be interesting to further investigate the maturation potential of the obtained hPGCLCs through long-term coculture with mouse gonadal somatic cells[25] to facilitate meiotic entry and eventually generate functional gametes.

## 5 | CONCLUSIONS/SUMMARY

We report that hESC lines both derived and continuously cultured in ActA and transiently converted to the 4i-state (naïve resetting) show high competence to differentiate to hPGCLCs, when compared to isogenic hESCs that were derived but not further cultured in ActA or hESCs not previously exposed to ActA. In their primed state, these ActA-hESC lines showed elevated expression of pluripotency genes, and of primitive streak/mesendodermal-like genes and enhanced WNT signaling. Interestingly, the expression of these particular genes remained unchanged during conversion to the 4i-state. The molecular signature of the differentiated (TNAP+PDPN+) hPGCLCs resembled those of early human PGCs and included genes associated with germ cell migration, suggesting they may be suitable for further differentiation. Our work contributes to understanding the difference in differentiation propensity of (isogenic) hESCs and provides an optimized protocol to support efficient human early germ cell differentiation.

### ACKNOWLEDGMENTS

We would like to acknowledge the patients of the Department of Reproductive Medicine, Ghent University Hospital for donating embryos for this study, as well as the Gynaikon Clinic, in Rotterdam for the efforts to collect the human fetal gonadal material used in this study. In addition, we thank the Ghent University Centre for Advanced Light Microscopy for use of their facilities; K. Szymanska for technical support with confocal imaging; S. Vermaut and I. Van Colen for the support with flow cytometry; V. Thys for supplying the MEFs; and the staff of NXTGNT sequencing facility at Ghent University, S. De Keulenaer, E. De Meester, and S. Decraene. This research was conducted through collaboration with the Bimetra biobank, a high-quality biorepository for the Ghent University Hospital and Ghent University. We thank the Strategic Basic Research (SBO) formerly known as Innovation by Science and Technology in Flanders (IWT 150042 awarded to Dieter Deforce and Petra de Sutter; IWT 131673 awarded to Jasin Taelman), the Dutch Research Council (VICI-2018-91819642 awarded to Susana M. Chuva De Sousa Lopes) and the Bijzonder Onderzoeksfonds (BOF 01D08114 awarded to Mina Popovic; BOF GOA030-18 and BOF GOA 2018000504 awarded to Dieter Deforce and Petra de Sutter).

### CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

### AUTHOR CONTRIBUTIONS

S.M.: study design, performed experiments, analyzed data; J.T., M.P., L.T., E.D., M.V.D.J.: performed experiments, analyzed data; D.D., F.V.N., B.M., P.D.S., A.B., B.H., S.M.C.D.S.L.: study design, analyzed data. All authors wrote the manuscript and accepted the final version.

### DATA AVAILABILITY STATEMENT

RNA-sequencing data are deposited in Gene Expression Omnibus (GEO) with accession number GSE155749 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE155749).

### ORCID

*Swati Mishra* https://orcid.org/0000-0002-6123-8712
*Filip van Nieuwerburgh* https://orcid.org/0000-0001-8815-5485
*Annekatrien Boel* https://orcid.org/0000-0003-3256-7020
*Susana M. Chuva De Sousa Lopes* https://orcid.org/0000-0003-3866-2803

### REFERENCES

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. *Science*. 1998;282(5391):1145-1147.
2. Cahan P, Daley GQ. Origins and implications of pluripotent stem cell variability and heterogeneity. *Nat Rev Mol Cell Biol*. 2013;14(6):357-368.
3. Smith A. Formative pluripotency: the executive phase in a developmental continuum. *Development*. 2017;144(3):365-373.
4. Brons IG, Smithers LE, Trotter MW, et al. Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature*. 2007;448(7150):191-195.
5. Tesar PJ, Chenoweth JG, Brook FA, et al. New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature*. 2007;448(7150):196-199.
6. O'Leary T, Heindryckx B, Lierman S, et al. Tracking the progression of the human inner cell mass during embryonic stem cell derivation. *Nat Biotechnol*. 2012;30(3):278-282.
7. Van der Jeught M, O'Leary T, Duggal G, et al. The post-inner cell mass intermediate: implications for stem cell biology and assisted reproductive technology. *Hum Reprod Update*. 2015;21(5):616-626.
8. Duggal G, Warrier S, Ghimire S, et al. Alternative routes to induce naive pluripotency in human embryonic stem cells. *Stem Cells*. 2015;33(9):2686-2698.
9. Gafni O, Weinberger L, Mansour AA, et al. Derivation of novel human ground state naive pluripotent stem cells. *Nature*. 2013;504(7479):282-286.
10. Guo G, von Meyenn F, Santos F, et al. Naive pluripotent stem cells derived directly from isolated cells of the human inner cell mass. *Stem Cell Rep*. 2016;6(4):437-446.
11. Warrier S, Van der Jeught M, Duggal G, et al. Direct comparison of distinct naive pluripotent states in human embryonic stem cells. *Nat Commun*. 2017;8:15055.
12. Collier AJ, Rugg-Gunn PJ. Identifying human naive pluripotent stem cells - evaluating state-specific reporter lines and cell-surface markers. *Bioessays*. 2018;40(5):e1700239.
13. Kurek D, Neagu A, Tastemel M, et al. Endogenous WNT signals mediate BMP-induced and spontaneous differentiation of epiblast stem cells and human embryonic stem cells. *Stem Cell Rep*. 2015;4(1):114-128.
14. Taelman J, Popovic M, Bialecka M, et al. WNT inhibition and increased FGF signaling promotes derivation of less heterogeneous primed human embryonic stem cells, compatible with differentiation. *Stem Cells Dev*. 2019;28(9):579-592.
15. Hayashi K, Ogushi S, Kurimoto K, Shimamoto S, Ohta H, Saitou M. Offspring from oocytes derived from in vitro primordial germ cell-like cells in mice. *Science*. 2012;338(6109):971-975.

Downloaded from https://academic.oup.com/stmcls/article/39/5/551/6423762 by Universiteit Leiden / LUMC user on 30 June 2022

16. Hayashi K, Ohta H, Kurimoto K, Aramaki S, Saitou M. Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. *Cell*. 2011;146(4):519-532.
17. Hikabe O, Hamazaki N, Nagamatsu G, et al. Reconstitution in vitro of the entire cycle of the mouse female germ line. *Nature*. 2016; 539(7628):299-303.
18. Zhou Q, Wang M, Yuan Y, et al. Complete meiosis from embryonic stem cell-derived germ cells in vitro. *Cell Stem Cell*. 2016;18(3): 330-340.
19. Chen D, Sun N, Hou L, et al. Human primordial germ cells are specified from lineage-primed progenitors. *Cell Rep*. 2019;29(13): 4568-4582.e4565.
20. Irie N, Weinberger L, Tang WW, et al. SOX17 is a critical specifier of human primordial germ cell fate. *Cell*. 2015;160(1–2):253-268.
21. Kojima Y, Sasaki K, Yokobayashi S, et al. Evolutionarily distinctive transcriptional and signaling programs drive human germ cell lineage specification from pluripotent stem cells. *Cell Stem Cell*. 2017;21(4): 517-532.e515.
22. Mitsunaga S, Odajima J, Yawata S, et al. Relevance of iPSC-derived human PGC-like cells at the surface of embryoid bodies to prechemotaxis migrating PGCs. *Proc Natl Acad Sci USA*. 2017; 114(46):E9913-E9922.
23. Sasaki K, Yokobayashi S, Nakamura T, et al. Robust in vitro induction of human germ cell fate from pluripotent stem cells. *Cell Stem Cell*. 2015;17(2):178-194.
24. Sugawa F, Arauzo-Bravo MJ, Yoon J, et al. Human primordial germ cell commitment in vitro associates with a unique PRDM14 expression profile. *EMBO J*. 2015;34(8):1009-1024.
25. Yamashiro C, Sasaki K, Yabuta Y, et al. Generation of human oogonia from induced pluripotent stem cells in vitro. *Science*. 2018;362(6412): 356-360.
26. Chen D, Liu W, Lukianchikov A, et al. Germline competency of human embryonic stem cells depends on eomesodermin. *Biol Reprod*. 2017; 97(6):850-861.
27. Osafune K, Caron L, Borowiak M, et al. Marked differences in differentiation propensity among human embryonic stem cell lines. *Nat Biotechnol*. 2008;26(3):313-315.
28. Yokobayashi S, Okita K, Nakagawa M, et al. Clonal variation of human induced pluripotent stem cells for induction into the germ cell fate. *Biol Reprod*. 2017;96(6):1154-1166.
29. Chuva de Sousa Lopes SM, Roelen BAJ. Current status of in vitro differentiation of stem cells into gametes. *Anim Reprod*. 2015;12(1): 46-51.
30. Ohta H, Kurimoto K, Okamoto I, et al. In vitro expansion of mouse primordial germ cell-like cells recapitulates an epigenetic blank slate. *EMBO J*. 2017;36(13):1888-1907.
31. Saitou M, Miyauchi H. Gametogenesis from pluripotent stem cells. *Cell Stem Cell*. 2016;18(6):721-735.
32. Duggal G, Heindryckx B, Warrier S, et al. Influence of activin A supplementation during human embryonic stem cell derivation on germ cell differentiation potential. *Stem Cells Dev*. 2013;22(23):3141-3155.
33. Duggal G, Heindryckx B, Warrier S, et al. Exogenous supplementation of activin A enhances germ cell differentiation of human embryonic stem cells. *Mol Hum Reprod*. 2015;21(5):410-423.
34. Van Landuyt L, Verpoest W, Verheyen G, et al. Closed blastocyst vitrification of biopsied embryos: evaluation of 100 consecutive warming cycles. *Hum Reprod*. 2011;26(2):316-322.
35. Raman L, Dheedene A, De Smet M, et al. WisecondorX: improved copy number detection for routine shallow whole-genome sequencing. *Nucleic Acids Res*. 2019;47(4):1605-1614.
36. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods*. 2012;9(7): 676-682.
37. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J*. 2011;17(1):10-12.
38. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2013;29(1):15-21.
39. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*. 2011;12:323.
40. Wickham H. *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer-Verlag; 2016.
41. Kolde R. pheatmap: Pretty Heatmaps. In: package R; . 2019.
42. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2010;26(1):139-140.
43. Popovic M, Bialecka M, Gomes Fernandes M, et al. Human blastocyst outgrowths recapitulate primordial germ cell specification events. *Mol Hum Reprod*. 2019;25(9):519-526.
44. Vertesy A, Arindrarto W, Roost MS, et al. Parental haplotype-specific single-cell transcriptomics reveal incomplete epigenetic reprogramming in human female germ cells. *Nat Commun*. 2018;9(1):1873.
45. Nichols J, Zevnik B, Anastassiadis K, et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell*. 1998;95(3):379-391.
46. Scholer HR, Hatzopoulos AK, Balling R, et al. A family of octamer-specific proteins present during mouse embryogenesis: evidence for germline-specific expression of an Oct factor. *EMBO J*. 1989;8(9): 2543-2550.
47. Zhang J, Ratanasirintrawoot S, Chandrasekaran S, et al. LIN28 regulates stem cell metabolism and conversion to primed Pluripotency. *Cell Stem Cell*. 2016;19(1):66-80.
48. Mitsui K, Tokuzawa Y, Itoh H, et al. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell*. 2003;113(5):631-642.
49. Howard L, Mackenzie RM, Pchelintsev NA, et al. Profiling of transcriptional and epigenetic changes during directed endothelial differentiation of human embryonic stem cells identifies FOXA2 as a marker of early mesoderm commitment. *Stem Cell Res Ther*. 2013;4(2):36.
50. Heikinheimo M, Scandrett JM, Wilson DB. Localization of transcription factor GATA-4 to regions of the mouse embryo involved in cardiac development. *Dev Biol*. 1994;164(2):361-373.
51. Juan H, Hamada H. Roles of nodal-lefty regulatory loops in embryonic patterning of vertebrates. *Genes Cells*. 2001;6(11):923-930.
52. Huggins IJ, Bos T, Gaylord O, et al. The WNT target SP5 negatively regulates WNT transcriptional programs in human pluripotent stem cells. *Nat Commun*. 2017;8(1):1034.
53. Bovolenta P, Esteve P, Ruiz JM, Cisneros E, Lopez-Rios J. Beyond Wnt inhibition: new functions of secreted Frizzled-related proteins in development and disease. *J Cell Sci*. 2008;121(Pt 6):737-746.
54. Garces de Los Fayos Alonso I, Liang HC, Turner SD, et al. The role of activator protein-1 (AP-1) family members in CD30-positive lymphomas. *Cancers (Basel)*. 2018;10(4):93–114.
55. Verrecchia F, Vindevoghel L, Lechleider RJ, Uitto J, Roberts AB, Mauviel A. Smad3/AP-1 interactions control transcriptional responses to TGF-beta in a promoter-specific manner. *Oncogene*. 2001;20(26):3332-3340.
56. Zhang Y, Feng XH, Derynck R. Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-beta-induced transcription. *Nature*. 1998;394(6696):909-913.
57. Pendaries V, Verrecchia F, Michel S, Mauviel A. Retinoic acid receptors interfere with the TGF-beta/Smad signaling pathway in a ligand-specific manner. *Oncogene*. 2003;22(50):8212-8220.
58. Kowanetz M, Valcourt U, Bergstrom R, et al. Id2 and Id3 define the potency of cell proliferation and differentiation responses to transforming growth factor beta and bone morphogenetic protein. *Mol Cell Biol*. 2004;24(10):4241-4254.
59. Li L, Dong J, Yan L, et al. Single-cell RNA-Seq analysis maps development of human germline cells and gonadal niche interactions. *Cell Stem Cell*. 2017;20(6):858-873.e854.

Downloaded from https://academic.oup.com/stmcls/article/39/5/551/6423762 by Universiteit Leiden / LUMC user on 30 June 2022

60. Gomes Fernandes M, Bialecka M, Salvatori DCF, Chuva de Sousa Lopes SM. Characterization of migratory primordial germ cells in the aorta-gonad-mesonephros of a 4.5-week-old human embryo: a toolbox to evaluate in vitro early gametogenesis. *Mol Hum Reprod*. 2018; 24(5):233-243.
61. Li L, Yang R, Yin C, Kee K. Studying human reproductive biology through single-cell analysis and in vitro differentiation of stem cells into germ cell-like cells. *Hum Reprod Update*. 2020;26(5): 670-688.
62. Mishra S, Kacin E, Stamatiadis P, et al. The role of the reprogramming method and pluripotency state in gamete differentiation from patient-specific human pluripotent stem cells. *Mol Hum Reprod*. 2018; 24(4):173-184.
63. Taelman J, Mishra S, Van der Jeught M, et al. Stem cell-derived spermatozoa. In: Horcajadas JA, Gosálvez J, eds. *Reproductomics*. Cambridge, MA: Academic Press; 2018.
64. Blakeley P, Fogarty NM, del Valle I, et al. Defining the three cell lineages of the human blastocyst by single-cell RNA-seq. *Development*. 2015;142(18):3151-3165.
65. Wamaitha SE, Grybel KJ, Alanis-Lobato G, et al. IGF1-mediated human embryonic stem cell self-renewal recapitulates the embryonic niche. *Nat Commun*. 2020;11(1):764.
66. Warrier S, Taelman J, Tilleman L, et al. Transcriptional landscape changes during human embryonic stem cell derivation. *Mol Hum Reprod*. 2018;24(11):543-555.
67. Pauklin S, Vallier L. Activin/nodal signalling in stem cells. *Development*. 2015;142(4):607-619.
68. Vallier L, Mendjan S, Brown S, et al. Activin/nodal signalling maintains pluripotency by controlling Nanog expression. *Development*. 2009; 136(8):1339-1349.
69. Pan G, Tian S, Nie J, et al. Whole-genome analysis of histone H3 lysine 4 and lysine 27 methylation in human embryonic stem cells. *Cell Stem Cell*. 2007;1(3):299-312.
70. Tang WW, Kobayashi T, Irie N, et al. Specification and epigenetic programming of the human germ line. *Nat Rev Genet*. 2016;17(10): 585-600.
71. Piprek RP, Kolasa M, Podkowa D, Kloc M, Kubiak JZ. Transcriptional profiling validates involvement of extracellular matrix and proteinases genes in mouse gonad development. *Mech Dev*. 2018;149:9-19.

## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Mishra S, Taelman J, Popovic M, et al. Activin A-derived human embryonic stem cells show increased competence to differentiate into primordial germ cell-like cells. *Stem Cells*. 2021;39:551–563. https://doi.org/10.1002/stem.3335

Downloaded from https://academic.oup.com/stmcls/article/39/5/551/6423762 by Universiteit Leiden / LUMC user on 30 June 2022