Tankyrase inhibition promotes a stable human naïve pluripotent state with improved functionality

Ludovic Zimmerlin1,2,*, Tea Soon Park1,2,*, Jeffrey S. Huo1,2,*, Karan Verma1,2, Sarshan R. Pather1,2, C. Conover Talbot, Jr.4, Jasmin Agarwal1,2, Diana Steppan1,2, Yang W. Zhang3, Michael Considine3, Hong Guo2, Xiufeng Zhong5, Christian Gutierrez5, Leslie Cope3, M. Valeria Canto-Soler5, Alan D. Friedman2, Stephen B. Baylin3 and Elias T. Zambidis1,2,‡

ABSTRACT

The derivation and maintenance of human pluripotent stem cells (hPSCs) in stable naïve pluripotent states has a wide impact in human developmental biology. However, hPSCs are unstable in classical naïve mouse embryonic stem cell (ESC) WNT and MEK/ERK signal inhibition (2i) culture. We show that a broad repertoire of conventional hESCs and transgene-independent human induced pluripotent stem cell (hiPSC) lines could be reverted to stable human preimplantation inner cell mass (ICM)-like naïve states with only WNT, MEK/ERK, and tankyrase inhibition (LIF-3i). LIF-3i-reverted hPSCs retained normal karyotypes and genomic imprints, and attained defining mouse ESC-like functional features, including high clonal self-renewal, independence from MEK/ERK signaling, dependence on JAK/STAT3 and BMP4 signaling, and naïve-specific transcriptional and epigenetic configurations. Tankyrase inhibition promoted a stable acquisition of a human preimplantation ICM-like ground state via modulation of WNT signaling, and was most efficacious in efficiently reprogrammed conventional hiPSCs. Importantly, naïve reversion of a broad repertoire of conventional hiPSCs reduced lineage-primed gene expression and significantly improved their multilineage differentiation capacities. Stable naïve hPSCs with reduced genetic variability and improved functional pluripotency will have great utility in regenerative medicine and human disease modeling.

KEY WORDS: Differentiation, Ground state, Human embryonic stem cell, Induced pluripotent stem cell, Naïve pluripotency

INTRODUCTION

Although human induced pluripotent stem cells (hiPSCs) share highly similar transcriptional and epigenetic signatures with human embryonic stem cells (hESCs) (Chin et al., 2009; Bock et al., 2011), they demonstrate greater interline multilineage differentiation variability than hESCs (Osafune et al., 2008; Choi et al., 2008; Fung et al., 2009; Hu et al., 2010; Boulting et al., 2011). The discrepancy between highly variable ‘functional pluripotency’ among hiPSC lines, despite similar molecular and phenotypic pluripotency to hESCs, might be partially due to imperfect induction of the somatic donor cell epigenome to a bona fide ESC-like state (Lister et al., 2011; Nishino et al., 2011; Ruiz et al., 2012). Previous studies suggested that reprogramming-associated errors of retention of donor cell-specific epigenetic memory bias the differentiation potency of hiPSCs toward some lineages (Kim et al., 2010b, 2011; Polo et al., 2010). However, other studies did not confirm such correlations, or alternatively suggested that donor-specific genetic variability affecting lineage-primed gene expression might play a more dominant role (Ohi et al., 2011; Hu et al., 2011; Kajiwara et al., 2012; Kyytälä et al., 2016).

Complex determinants may collectively influence the functional pluripotency of both hiPSCs and hESCs. For example, one critical variable impacting the functional pluripotency of conventional hPSCs is their developmental, molecular and epigenetic commonality with ‘primed’ mouse post-implantation epiblast stem cells (mEpiSCs) (Tesar et al., 2007; Brons et al., 2007; Chou et al., 2008; Kojima et al., 2014; Weinberger et al., 2016), which possess a less primitive pluripotency than inner cell mass (ICM)-derived mouse ESC (mESCs). For example, mEpiSCs cannot freely contribute to a blastocyst chimera and are resistant to chemical reversion to ICM-like naïve ‘ground state’ pluripotency with ‘LIF-2i’ (MEK/ERK and GSK3β signal inhibition) (Bremmann et al., 2011; Ying et al., 2008; Marks et al., 2012). Conventional human pluripotent stem cell (hPSCs) rely on self-renewal signaling pathways more similar to those of mEpiSCs than ESCs, and these hPSCs might exist in developmentally primed states that display mEpiSC-like lineage skewing following directed differentiation. Although several hiPSC naïve reversion approaches were recently described, none was maintained with classical MEK/ERK/WNT 2i signaling inhibition alone (Hanna et al., 2010; Chan et al., 2013; Gafni et al., 2013; Takashima et al., 2013; Theunissen et al., 2014; Theunissen et al., 2014; Ware et al., 2014). Thus, although various methods may achieve pluripotent states reminiscent of the human ICM, the determinants required for stable human rewiring to an mESC-like ground state remain undefined and might represent unknown species-specific differences.

The roles of the derivation method and of lineage priming of conventional hiPSCs in the amenability to naive reversion have not been fully evaluated. For example, although human hematopoietic progenitors are more efficiently reprogrammed than fibroblast donors via standard methods (Eminli et al., 2009; Park et al., 2012; Guo et al., 2014), both donor types generated hiPSCs with diminished and lineage-skewed differentiation potencies that were attributed to the retention of donor epigenetic memory (Kim et al., 2011; Hu et al., 2011). By
contrast, hiPSCs reprogrammed efficiently from cord blood-derived CD33^+ CD45^− (PTPRC^−) myeloid progenitors (MPs) (Park et al., 2012) displayed reduced interline variability or differentiation bias (Burridge et al., 2011; Park et al., 2014). These MP-iPSCs generated vascular progenitors (VPs) with less culture senescence, decreased sensitivity to DNA damage, and greater in vivo engraftment potential than MPs generated from standard fibroblast-derived hiPSCs (Park et al., 2014). MP-iPSCs also generated physiologically functional photoreceptors that elicited action potentials in a three-dimensional retinal differentiation system (Zhong et al., 2014).

Since murine and human MPs may represent a ‘privileged’ somatic donor type (Park et al., 2012; Guo et al., 2014), we tested the hypothesis that efficient myeloid reprogramming generates an improved primed functional pluripotency with reduced lineage priming and increased amenability to naïve ground state reversion. Here, we demonstrate that effective reprogramming of human CD33^+ CD45^− MPs generates hiPSCs with an improved multilineage differentiation potency that lacks the lineage-priming differentiation bias characteristic of hiPSCs derived via standard reprogramming methods. Moreover, supplementation of classical LIF-2i with only the tankyrase inhibitor XAV939 (LIF-3i) permitted a large repertoire of hiPSCs to efficiently revert to a stable mESC-like naïve state that possessed further improved multilineage functional pluripotency. Interestingly, MP-iPSCs reverted to this stable naïve state more efficiently than hiPSCs derived via less efficient methods.

**RESULTS**

**STAT3-activated MP donors generate hiPSCs with decreased reprogramming-associated genetic variability and high functional pluripotency**

Previous studies demonstrated that stromal-activated (sa) human MPs can be reprogrammed with four (4F-E) or seven (7F-E) functional pluripotency. Interestingly, MP-iPSCs reverted to this stable naïve state more efficiently than hiPSCs derived via less efficient methods.

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mEPSCs possess hierarchies of primed pluripotency with variable propensities for reversion to a naïve ground state, and reversion of EpiSCs via LIF-2i may be limited by residual lineage-primed gene propensities for reversion to a naïve ground state, and reversion of mEpiSCs possess hierarchies of primed pluripotency with variable propensities for reversion to a naïve ground state, and reversion of mESC-like phenotype.

Supplementation of hPSC cultures with the classical two small-molecule MEK/ERK (PD0325901) and GSK3 inhibitors (Ying et al., 2008) was insufficient to stably revert any hPSCs into clonogenic mESC-like lines. To identify novel conditions that stabilize human naïve pluripotency, we screened over 130 LIF-supplemented hPSC culture conditions comprising combinations of more than 15 small molecules known to modulate ESC self-renewal (see strategy for small molecule screening in the supplementary Materials and Methods; Table S3). We initially assayed for conditions that supported stable reversion (>5-10 passages) of conventional hPSC lines to mESC-like dome-shaped morphologies in WNT-MEK/ERK blockade conditions. This screen revealed that supplementing classical LIF-2i with the WNT pathway modulator XAV939 – a tankyrase inhibitor that potentiates axin-mediated cytoplasmic stabilization of activated β-catenin in primed PSCs (Huang et al., 2009; Kim et al., 2013) – uniquely permitted rapid reversion of conventional H9 hESC and multiple transgene-free 4F-E sa-MP-iPSC lines from the same donor (ZPB) that were derived with sa (E29C1, E29C4, E29C6; red) and without sa (E29C10, E29C11, E29C12; purple). Four 7F-E CB-derived sa-MP-iPSC lines were all from the same donor (D003) generated with sa (6.2, 6.13, 19.11) (Fig. S2). *P<0.05, **P<0.005 (one-way ANOVA, P<0.05; fold change >1.5×).

We tested the capacity of our broad repertoire (Table S2, Fig. 1) of conventional, bFGF (FGF2)-dependent, non-integrated hPSCs to revert to a naïve ICM-like state following chemical 2i WNT-MEK/ERK modulation. Supplementation of hPSC cultures with the classical two small-molecule MEK/ERK (PD0325901) and GSK3 (CHIR99021) inhibitors (Ying et al., 2008) was insufficient to stably revert any hPSCs into clonogenic mESC-like lines. To identify novel conditions that stabilize human naïve pluripotency, we screened over 130 LIF-supplemented hPSC culture conditions comprising combinations of more than 15 small molecules known to modulate ESC self-renewal (see strategy for small molecule screening in the supplementary Materials and Methods; Table S3). We initially assayed for conditions that supported stable reversion (>5-10 passages) of conventional hPSC lines to mESC-like dome-shaped morphologies in WNT-MEK/ERK blockade conditions. This screen revealed that supplementing classical LIF-2i with the WNT pathway modulator XAV939 – a tankyrase inhibitor that potentiates axin-mediated cytoplasmic stabilization of activated β-catenin in primed PSCs (Huang et al., 2009; Kim et al., 2013) – uniquely permitted rapid reversion of conventional H9 hESC and multiple transgene-free 4F-E sa-MP-iPSC lines into uniform, compact, dome-shaped, clonogenic colonies with normal karyotypes (Fig. 2A, Table S3).
reverted sa-MP-iPSCs and hESCs revealed high TRA-1-81+SSEA4+ (>95%) surface expression, and increased transcript and protein expression of naïve-specific epiblast factors [e.g. NANOG, E-cadherin (CDH1), NR5A2, STELLA (DPPA3), KLF2, KLF4, KLF5, KLF17, HERV-H and TFCP2L1] (Fig. 2B-D, Fig. S6A-D, Fig. S8C,D). The pluripotencies of multiple LIF-3i-reverted hPSCs were validated by robust tri-lineage teratoma formation in NOG-SCID mice (Fig. 2E, Fig. S6E, Table S3B). Furthermore, conventional female sa-MP-iPSCs with detectable XIST expression and an X-inactivated phenotype expressed significantly lower levels of XIST transcripts following LIF-3i reversion, which is consistent with the bi-allelic X-activation status observed in naïve mESCs (Ying et al., 2008) (Fig. 2B,F). LIF-3i-reverted hPSCs also exhibited decreased levels of HLA-A and HLA-B (Fig. 5E, Fig. S8D). LIF-3i-reverted H9 hESCs and 4F-E sa-MP-iPSCs maintained stable, robust clonal growth proliferation kinetics for at least 30 passages with normal karyotypes in standard mouse embryonic fibroblast (MEF)/hESC conditions (Fig. S7 and Fig. S8B). Phenotypically naïve hPSCs reverted to flattened mEpiSC-like morphologies when transferred back to conventional bFGF hPSC culture.

**LIF-3i reversion induces LIF-JAK/STAT3 signaling, BMP4 responsiveness and augmentation of activated β-catenin in both nuclear and cytoplasmic compartments**

LIF-3i-reverted hPSCs did not require supplementation with primed PSC growth factors (e.g. bFGF, activin A, TGFβ) or apoptosis inhibitor cocktails to maintain long-term viability and robust proliferation. Furthermore, western blotting and chemical inhibition assays demonstrated that naïve-reverted hPSCs adopted authentic mESC-like signaling pathways that included increased active nuclear STAT3 phosphorylation, dependence on JAK/STAT3, LIF/gp130, CREB and PI3K signaling, and independence from FGF and MEK/ERK signaling (Fig. 3A,B). Notably, although
LIF withdrawal reduced proliferation of naïve hiPSCs after three passages (12 days) by ~50%, supplementation with bFGF (or TGFβ) did not exert further proliferative effects on viability of SSEA4+ TRA-1-81+ cells. Supplementation of LIF-2i with XAV939 resulted in elevated axin levels with an apparent stabilization and augmented expression of the activated isoform of non-phosphorylated β-catenin in both cytoplasmic and nuclear compartments of hPSCs (Fig. 3A-C,E). Interestingly, conventional sa-MP-iPSCs already possessed higher basal nuclear and cytoplasmic β-catenin activities than other hPSCs. One distinctive effect of LIF-3i reversion of sa-MP-iPSCs was a potent mESC-like BMP4 proliferative responsiveness (~5-fold) of naïve SSEA4+ TRA-1-81+ cells, with concordant susceptibility to BMP4 inhibition (dorsomorphin) (Fig. 3B).

A broad repertoire of conventional hPSC lines stably reverts to naïve morphologies in LIF-3i

We evaluated 23 independent non-integrated, conventional, primed hPSC lines for their capacity to tolerate stable, clonogenic self-renewal of SSEA4+ TRA-1-81+ cells for at least ten passages in LIF-3i. (Fig. S8A,B, Table S3B). Long-term stability of colonies with undifferentiated dome-shaped morphologies for >10-20 passages via direct LIF-3i reversion alone was most reproducible for sa-MP-iPSCs and select hESC lines (e.g. H9). However, brief adaptation (one passage) in LIF-3i plus two additional molecules, namely forskolin and purmorphamine (LIF-5i), increased the initial survival of enzyme-digested hPSC single cells, and facilitated a broader repertoire of hPSCs to tolerate subsequent stable clonal self-renewal in LIF-3i alone (Fig. S7A-C and Fig. S8A,B, Table S3B). This initial LIF-5i modification permitted a wide repertoire of ~16 conventional hPSC lines to revert with long-term stability in LIF-3i alone.

LIF-3i-reverted hPSCs increase expression of core pluripotency circuits and acquire mESC-like transcriptional and epigenetic features

mESCs possess molecular signatures, clearly distinct from those of EpisCs and hPSCs, that are characterized by more open, derepressed chromatin configurations, decreased global CpG DNA methylation, and a transcriptome reflective of the naïve preimplantation epiblast (Marks et al., 2012; Leitch et al., 2013). To ascertain if preimplantation epiblast-like states were achieved in LIF-3i-reverted hPSCs, we investigated the expression of core pluripotency circuits and the acquisition of mESC-like transcriptional and epigenetic features.
LIF-3i-reverted hPSCs, we evaluated whole-genome transcriptional and epigenomic signatures of 12 independent LIF-3i-reverted lines and their isogenic conventional counterparts [i.e. six sa-MP-iPSCs, three hESCs and three fibro-iPSCs before and after LIF-3i reversion at early post-reversion passages (p5)] (Figs 4 and 5, Fig. S8). These studies established that LIF3i-reverted hPSCs acquired signatures distinct from conventional primed states, with robust upregulation of genes associated with both the preimplantation human epiblast and mESC ground state. Cross-species whole-genome hierarchical clustering using mEpiSCs, mESCs and LIF-2i-reverted mESCs as controls revealed distinct transcriptional landscapes. (A) Genome-wide cross-species hierarchical clustering. Shown is a dendrogram of expression microarrays of mESC [serum/LIF; naïve (LIF-2i)], primed mEpiSC, and isogenic hPSC samples from this study before (hPSC primed) and after 5 passages in LIF-3i (hPSC naïve). Human PSC lines (n=12) included: three hESC lines H9, H7 and ES03 (gold); six sa-MP-iPSC lines E5C3, E5C1, E17C6, LZ6+2, LZ6+10 and 6.2 (red); and three fibro-iPSC lines 7ta, C1.2 and C2 (green). (B) (Top) CpG methylation. Box plot shows beta values of genome-wide autosomal differentially methylated region (DMR) CpG probes from Infinium methylation arrays ([16,282 of 473,864 autosomal probes significantly (P<0.05) differentially methylated (SD>0.15); see supplementary Materials and Methods for further details] in the same isogenic primed (−) versus LIF-3i-reverted (+) hPSC samples used for the microarrays above. Gold, hESCs (n=3); red, sa-MP-iPSCs (n=6); green, fibro-iPSCs (n=3). ***P<0.001 (paired two-way t-test). (Bottom) Global 5MC and 5hMC levels from dot blot immunoassays (relative to primed) for representative LIF-3i-reverted hPSCs. Genomic DNA samples were collected before (−) and after (+) LIF-3i reversion from H9 (gold), E5C3 (red) and C1.2 (green). (C) Activities of proximal enhancer (PE) and distal enhancer (DE) elements of the human OCT4 promoter in primed (bFGF) versus LIF-3i-reverted E5C3. Shown are relative firefly luciferase activities following normalization with Renilla luciferase and negative control basal activities ±s.d. (n=3). *P<0.05 (paired t-test). (D) Stable BAC reporter transgenic OCT4 PE/DE mutant lines. (Top) Cytometry plots of representative LIF-3i-reverted C2 hiPSC subclones (n=3) stably transfected with full-length OCT4-GFP-2A-PURO PE/DE sequences (control), mutant ΔPE-OCT4-GFP-PURO constructs, or non-transfected (no construct) controls. (Bottom) Percentage GFP* cells among naïve cultures of individual hiPSC subclones (n=3) expressing control or mutant ΔPE sequences. (E) Pluripotency circuits in LIF-3i-reverted hPSCs. (Top) Mean beta values of core module-specific CpG DMRs in primed (−) versus LIF-3i-reverted (+) hPSC; (bottom) corresponding log2 mean subtracted normalized expression of core module genes (Table S1) of the same independent hPSC samples (identical to those used above for expression microarrays). Gold, hESCs (n=3); red, sa-MP-iPSCs (n=6); green, fibro-iPSCs (n=3). (F) Pluripotency gene-specific promoter CpG methylation. Heatmap-dendrogram clustering and box plots of mean beta values of ESC module gene-specific CpG DMRs [P<0.001 (paired two-way t-test)] of LIF-3i (+) versus primed (−) hPSCs. Samples are the same 12 hPSC lines in each category, as described above. Percentages represent reduction of median beta value following LIF-3i reversions.

Fig. 4. Transcriptional and epigenetic profiling of LIF-3i-reverted hPSCs. (A) Genome-wide cross-species hierarchical clustering. Shown is a dendrogram of expression microarrays of mESC [serum/LIF; naïve (LIF-2i)], primed mEpiSC, and isogenic hPSC samples from this study before (hPSC primed) and after 5 passages in LIF-3i (hPSC naïve). Human PSC lines (n=12) included: three hESC lines H9, H7 and ES03 (gold); six sa-MP-iPSC lines E5C3, E5C1, E17C6, LZ6+2, LZ6+10 and 6.2 (red); and three fibro-iPSC lines 7ta, C1.2 and C2 (green). (B) (Top) CpG methylation. Box plot shows beta values of genome-wide autosomal differentially methylated region (DMR) CpG probes from Infinium methylation arrays ([16,282 of 473,864 autosomal probes significantly (P<0.05) differentially methylated (SD>0.15); see supplementary Materials and Methods for further details] in the same isogenic primed (−) versus LIF-3i-reverted (+) hPSC samples used for the microarrays above. Gold, hESCs (n=3); red, sa-MP-iPSCs (n=6); green, fibro-iPSCs (n=3). ***P<0.001 (paired two-way t-test). (Bottom) Global 5MC and 5hMC levels from dot blot immunoassays (relative to primed) for representative LIF-3i-reverted hPSCs. Genomic DNA samples were collected before (−) and after (+) LIF-3i reversion from H9 (gold), E5C3 (red) and C1.2 (green). (C) Activities of proximal enhancer (PE) and distal enhancer (DE) elements of the human OCT4 promoter in primed (bFGF) versus LIF-3i-reverted E5C3. Shown are relative firefly luciferase activities following normalization with Renilla luciferase and negative control basal activities ±s.d. (n=3). *P<0.05 (paired t-test). (D) Stable BAC reporter transgenic OCT4 PE/DE mutant lines. (Top) Cytometry plots of representative LIF-3i-reverted C2 hiPSC subclones (n=3) stably transfected with full-length OCT4-GFP-2A-PURO PE/DE sequences (control), mutant ΔPE-OCT4-GFP-PURO constructs, or non-transfected (no construct) controls. (Bottom) Percentage GFP* cells among naïve cultures of individual hiPSC subclones (n=3) expressing control or mutant ΔPE sequences. (E) Pluripotency circuits in LIF-3i-reverted hPSCs. (Top) Mean beta values of core module-specific CpG DMRs in primed (−) versus LIF-3i-reverted (+) hPSC; (bottom) corresponding log2 mean subtracted normalized expression of core module genes (Table S1) of the same independent hPSC samples (identical to those used above for expression microarrays). Gold, hESCs (n=3); red, sa-MP-iPSCs (n=6); green, fibro-iPSCs (n=3). (F) Pluripotency gene-specific promoter CpG methylation. Heatmap-dendrogram clustering and box plots of mean beta values of ESC module gene-specific CpG DMRs [P<0.001 (paired two-way t-test)] of LIF-3i (+) versus primed (−) hPSCs. Samples are the same 12 hPSC lines in each category, as described above. Percentages represent reduction of median beta value following LIF-3i reversions.
reverted hPSCs possess similar and significantly decreased (exon, gene body, and 3′UTR) CpG sites per gene region distributed across the promoter, 5′ of CpG islands of 99% of RefSeq genes, with an average of 17 methylation sites at single nucleotide resolution, and covers 96% counterparts. This methodology interrogates more than 485,000 these 12 LIF-3i-reverted lines and their isogenic conventional performed Infinium CpG DNA 450K methylation array analysis of and LIF-2i-reverted naïve mESCs. Alternatively clustered distinctly alongside serum-grown mESCs mEpiSCs, whereas the same isogenic LIF3i-reverted hPSC lines (Fig. 4A) revealed that conventional hPSCs clustered with counterparts. Human morula/blastocyst PCA in C is clustered on a module of the most differentially expressed genes in E4-E5 human pluripotent epiblast (Petrooulos et al., 2016) (see Table S1). Z, this study and includes the n=12 independent hPSC lines in Fig. 4A; H, Hanna et al., 2010; G, Gafni et al., 2013; M, Takashima et al., 2014; S, Theunissen et al., 2014; V, Vassena et al., 2011. (E) Comparison of differentially expressed (P≤0.05, FC≥1.5) naïve-specific and lineage-primed transcripts in naïve hPSCs derived in this work or other labs. FC: normalized ratios of naïve/primed expression microarray signal intensities. Ratios are of LIF-3i-reverted versus primed hPSC samples (n=12 hPSCs, as above) versus samples of those published as indicated. To characterize the epigenetic status of naïve-reverted hPSCs, we performed Infinium CpG DNA 450K methylation array analysis of these 12 LIF-3i-reverted lines and their isogenic conventional counterparts. This methodology interrogates more than 485,000 methylation sites at single nucleotide resolution, and covers 96% of CpG islands of 99% of RefSeq genes, with an average of 17 CpG sites per gene region distributed across the promoter, 5′UTR, first exon, gene body, and 3′UTR. These studies revealed that all LIF-3i-reverted hPSCs possess similar and significantly decreased (P<0.001) epigenome-wide CpG DNA methylation at differentially methylated regions (DMRs) compared with their primed hPSC sources (Fig. 4B, top panel). Additionally, quantification of dot blot immunosays of global CpG 5-methylcytosine isomers (5mC versus 5mC) revealed reduced global 5mC activities and increased ratios of 5mC/5mC CpG DNA methylation, which was most evident for sa-MP-iPSC ESC3 (Fig. 4B, bottom panel). Taken together, these data were consistent with a more epigenetically open configuration, and a potential role for TET-mediated CpG DNA demethylation activities in sustaining naïve pluripotency in LIF-3i-reverted hPSCs (Leitch et al., 2013). Interestingly, in contrast to previous reports that naïve reversion results in loss of CpG methylation at known imprinted genomic sites (Pastor et al., 2016), allele-specific analysis of over 1400 known imprinted CpG sites in these 12 independent isogenic hPSC lines (before and after LIF-3i reversion) revealed stability of methylation imprints established in conventional hPSCs, with no systematic loss of imprinted methylation patterns resulting from LIF-3i culture (Table S4A). To query for naïve-specific epigenetic functionality, we assayed for the activation of the proximal (PE) and distal (DE) enhancers of the OCT4 (POU5F1) promoter in LIF-3i-reverted versus primed hPSCs. Using both transient luciferase reporter assays and stable transgenic genomic DE/PE sequence mutant reporter hPSC lines (Gafni et al., 2013), we demonstrated that LIF-3i reversion potentiated naïve ESC-like activation of the DE of the OCT4 promoter, whereas primed hPSCs displayed preferential mEpiSC-like PE OCT4 activity (Fig. 4C,D). Finally, to probe the status of pluripotency circuits in naïve-reverted hPSCs, we conducted modular GSEA and bioinformatics
analysis of expression and methylation arrays for key pluripotency-associated stem cell circuits (e.g. the ESC module and SOX2-NANOG-OCT4-regulated core module; Table S1) in these 12 lines, before and after isogenic LIF-3i-reversion. These studies revealed that LIF-3i reversion significantly rewired both core and ESC module genes in naïve hPSCs, with (~50%) decreases in gene promoter CpG DNA methylation and corresponding increases in gene expression of these pluripotency circuits (Fig. 4E,F).

**LIF-3i induces human preimplantation epiblast-specific genes and increases expression of naïve-specific STAT3 and WNT transcriptional targets**

We next aimed to determine the transcriptional correlation of LIF-3i-reverted hPSCs to human preimplantation epiblasts and to naïve hPSCs derived by other methods. We conducted comparative meta-analyses with published expression data from human embryonic day (E)3-E7 ICM epiblast cells (Petrooulos et al., 2016), as well as naïve hPSCs from several laboratories.

We found that the most differentially expressed (P<0.05) transcripts in LIF-3i-reverted hPSCs were also among the highest-ranked overexpressed genes in E3-E6 human morula and epiblast ICM cells (e.g. DNM3L, NODAL, GDF3, IFITM1, LEFT2, WNT7) (Petrooulos et al., 2016) or, alternatively, were known core regulators of naïve mESC pluripotency (e.g. NANOG, STELLA, KLF2, NR5A2) (Fig. 5, Fig. S8C-E). Many of these human epiblast-specific genes were overexpressed in LIF-3i-reverted hPSCs are direct downstream targets of activated STAT3 signaling that are known to enhance mESC self-renewal and inhibit meso-endothelial differentiation (e.g. STAT3, FOS, SALL3, KLF2, KLF4, MYCN, IFITM1, EOMES) (Table S4B) (Bourillot et al., 2009). By contrast, the expression of lineage-primed developmental pathways (Fig. 5B) and genes (e.g. COL1A1, DKK3, TGFBI, SOX3, SOX8, SOX9) (Fig. 5A,E, Fig. S8D) were significantly underexpressed in LIF-3i-reverted hPSCs relative to their primed states. To further determine over-represented pathways acquired in LIF-3i-reverted hiPSCs, we conducted genome-wide GSEA of the microarray data from LIF-3i-reverted hiPSCs versus their isogenic conventional counterparts using curated pathway databases. This analysis revealed that, relative to conventional hPSCs, LIF-3i-reverted hiPSCs possess significantly higher transcriptional activities, increased chromatin remodeling, increased telomere function, increased expression of targets of WNT activation, and decreased expression of pathways associated with lineage-specific differentiation (FDR<0.05, P<0.01; Fig. 5B).

We next compared the whole-genome transcriptional signatures of LIF-3i-reverted hPSCs with both human ICM-derived epiblasts and mESCs by PCA normalized to published human blastocyst/morula, and with mESCs/hiPSC-LIF-2i data sets as benchmark controls (Fig. 5C,D) (Bao et al., 2009; Vassena et al., 2011). We employed modular bioinformatics using the most differentially expressed genes in E4-E5 human epiblast cells (Petrooulos et al., 2016), and revealed that the patterns of gene expression in LIF-3i-reverted hPSCs cluster closely with human morula/blastocyst epiblast cells. Additional PCA demonstrated that LIF-3i-reverted hPSCs were not only transcriptionally similar to mESCs, but also naïve-reverted hPSCs derived by others with alternate methods.

**LIF-3i reversion reduces lineage-primed gene expression and improves multilineage differentiation potency for a broad repertoire of conventional hiPSCs**

Since core pluripotency circuits were expressed at higher levels in LIF-3i-reverted hPSCs (Fig. 4E,F) with concomitant dramatic reduction in lineage-primed gene expression, including the broad network of lineage-specifying targets of the Polycomb PRC2 circuitry (Fig. 6A), we tested the hypothesis that naïve reversion improves multilineage functional pluripotency. We differentiated seven representative isogenic 4F-E sa-μP-iPSC and 7F-E fibro-iPSC lines (before and after LIF-3i reversion) to multiple representative derivatives of all three germ layers (Fig. 6B, Fig. S9). These studies revealed that LIF-3i reversion of multiple independently derived hiPSCs significantly improved their differentiation efficiency to endodermal (e.g. FOXA2 and CXCR4, SOX17), ectodermal neural progenitor (e.g. SOX1+ nestin+; PAX6+ nestin+), and mesodermal vascular-pericytic [e.g. CD31+ CD146 (MCAM)+; KDR+ CD73 (NT5E)+] progenitor populations. In multiple differentiation protocols, LIF-3i-reverted hPSC lines differentiated more efficiently, with less interline variability, and in some cases with more rapid kinetics (e.g. to neural ectoderm) than their conventional states. Altogether, these data suggested that LIF-3i reversion produced a more homogenous PSC population, with reduced lineage-primed gene variability and increased functional pluripotency (Fig. 7A).

**DISCUSSION**

Stable reversion to a naïve epiblast-like ground pluripotent state may improve the functional utility of conventional hPSCs. Here, we comprehensively evaluated how the variables of derivation method, lineage priming, and baseline-primed functional pluripotency influence the stability of subsequent reversion to a human naïve epiblast-like pluripotent state. Our studies revealed that stable, long-term reversion to an mESC-like state could be achieved from a wide spectrum of Episc-like lineage-primed hPSC states via supplementation of classical LIF-2i with only a tankyrase inhibitor (LIF-3i). LIF-3i-reverted hiPSCs were highly proliferative, generated well-differentiated tri-lineage teratomas, possessed normal karyotypes and stable genomic CpG methylation imprints within a globally more hypomethylated genome that was highly transcriptional, and could be stably passaged as undifferentiated, clonal SSEA4+ TRA-1-81+ dome-shaped colonies for at least 30 passages.

Although human chimera generation and germ line contribution is the most stringent measure of naïve pluripotency and could not be tested here, LIF-3i-reverted hPSCs possessed most of the accepted characteristics of mESCs that we tested. These characteristics included high clonal proliferation rates, MEK/ERK independence, bFGF signaling unresponsiveness, STAT3 phosphorylation and signaling, JAK/STAT3 and BMP4 signal dependence, increased naïve-specific transcript expression (e.g. STELLA, NR5A2), upregulation of core pluripotency networks with concomitant decrease in lineage-primed gene circuits, whole-genome transcriptomic clustering with both human preimplantation epiblasts and mESCs, dominant distal OCT4 enhancer usage, global DNA CpG hypomethylation with increased 5hmC/5MC ratios, X-chromosome activation, decreased class I MHC, increased E-cadherin expression, and augmented expression of cytoplasmic and nuclear activated β-catenin. Importantly, LIF-3i-reverted hPSCs had significantly reduced lineage-primed gene expression and improved multilineage differentiation potency relative to their primed states. The derivation of naïve hPSC lines with improved functional pluripotency has broad impact for optimizing future hiPSC-based cellular therapies.

Although efficiently reprogrammed sa-MP-iPSC and select hESC lines demonstrated increased stability to LIF-3i reversion, transient supplementation of LIF-3i with forskolin and purmorphamime (LIF-5i) allowed the reversion of a broader
repertoire (>16 independent hPSC lines) of variably lineage-primed hPSC lines. Interestingly, sa-MP-iPSCs already possess an improved functional pluripotency, with reduced lineage-primed differentiation skewing at baseline. Previous studies noted that STAT3 signaling was rate limiting for donor cell reprogramming completion (Yang et al., 2010; van Oosten et al., 2012). Thus, we hypothesize that the sustained STAT3 activation of ‘privileged’ CD33+ CD45+ sa-MP donors might play a crucial role not only in their efficient reprogramming, but also in facilitating the acquisition of a high-quality primed pluripotency with reduced lineage priming.

Fig. 6. Multilineage differentiation of isogenic primed versus LIF-3i-reverted hiPSC lines. (A) Differential expression of lineage-primed genes in the Polycomb (PRC2) circuit (ANOVA, \( P < 0.001 \); Table S1) in seven hiPSC lines before (−) and after (+) LIF-3i reversion. Shown are heatmaps and associated log2 mean subtracted expression of PRC2 module genes of the LIF-3i-reverted versus isogenic-primed hiPSCs used in the differentiation studies below. Red, 4F-E sa-MP-iPSCs (n=4): circle, E5C3; square, E5C1; and triangle, E17C6 (or LZ6+10 for neural differentiations). Green, fibro-iPSCs (n=3): circle, C1.2; square, C2; and triangle, 7ta. *\( P < 0.05 \) (paired t-tests). (B) Definitive endoderm differentiations (FOXA2+, CXCR4+ SOX17+) of isogenic LIF-3i-reverted versus primed hPSCs. Neural differentiations. (C,D) Kinetics of SOX1+ nestin+ and PAX6+ nestin+ neural progenitors in the same primed versus LIF-3i-reverted isogenic sa-MP-PSC (n=3) and fibro-hiPSC (n=3) lines described above. *\( P < 0.05 \), **\( P < 0.01 \) (paired t-tests). (E) Confocal microscopy of CDr3+ dye-binding neural progenitor rosettes (Yun et al., 2012). Neural rosettes were evaluated following passage of day 7 neural-induced LIF-3i-reverted (+) versus isogenic primed (−) E5C1 hiPSCs. Scale bars: 100 µm. (F) Isogenic vascular-endothelial hEB differentiations. Flow cytometry kinetics of CD31+ CD146+ (left) and KDR+ CD73+ (right) VP populations of the same isogenic sa-MP-iPSC lines as above (n=3). Error bars indicate s.e.m.
Recently, hPSC naïve reversion approaches have variably required the imposition of transgenic core factor overexpression, complex anti-apoptosis cocktails (e.g. ROCK, BRAF, SRC or JNK inhibition) to sustain survival/proliferation, HDAC inhibition to reset global epigenetic barriers, and/or EpiSC-specific growth factor reinforcement (e.g. bFGF, activin, TGFβ or BMP inhibition) (Table S5). These studies suggest that hPSCs might be generally ‘non-permissive’ to classical mESC 2i WNT and MEK/ERK pathway reversion, or that human and murine naïve states might be fundamentally non-equivalent. Although our comparative bioinformatics meta-analyses suggested a common pathway between other reversion methods and ours, we demonstrated that a stable human naïve epiblast-like state could be maintained in conventional hPSCs via LIF-2i and only a tankyrase inhibitor.

The mechanism of stabilizing human naïve pluripotency by tankyrase inhibition currently remains unclear and potentially complex, but is likely to involve a synergy with GSK3β inhibition (CHIR99021) to further augment WNT signaling (Fig. 7B). Canonical WNT signaling is determined by a post-translational balance between activated non-phosphorylated β-catenin and its phosphorylation by its destruction complex (axin/APC/GSK3β). Inhibition of the protein kinase GSK3β impedes β-catenin destruction, and allows its non-phosphorylated form to reach the nucleus where both pluripotency- and differentiation-associated factors are targeted (Ye et al., 2012; Buehr et al., 2008; Sato et al., 2004). Distinct mechanisms of reinforcement of pluripotency by GSK3β inhibition include TCF repression (Wray et al., 2011) and direct targeting of NANOG, OCT4, KLF4, ESRRB and CBP (CREBBP) (Takao et al., 2007; Tam et al., 2008; Kelly et al., 2011; Evans et al., 2010; Martello et al., 2012). WNT activation was previously shown to be not only rate limiting for naïve reversion of mEpiSCs, but also facilitated mESC derivation from mouse strains (Faunes et al., 2013). Additionally, both nuclear (transcriptional) and cytoplasmic (non-transcriptional) activities of β-catenin have been linked to stabilizing mouse naïve pluripotency via interactions with E-cadherin and cytoplasmic OCT4 and NANOG (Marucci et al., 2014). Furthermore, although 2i sufficiently stabilized naïve reversion of mEpiSCs, some mouse strains required reinforcement of KLF4, c-MYC or β-catenin activities (Hanna et al., 2010; Ye et al., 2012).

XAV939, an inhibitor of the poly-ADP-ribosylating enzymes tankyrase 1 and 2 (TNKS and TNKS2; also known as PARP5A/B, ARTDF5/6), was originally identified for its capacity to stimulate β-catenin degradation via stabilizing axin. The sole use of XAV939 in cancer cells inhibited WNT signaling (Huang et al., 2009) by increasing axin expression, stabilizing the axin-catenin complex, and increasing cytoplasmic retention of β-catenin (Kim et al., 2013; Schmitz et al., 2013). Our hPSC studies herein demonstrated that XAV939 similarly synergizes with CHIR99021 in naïve conditions (i.e. in the absence of MEK/ERK signal; PD0325901) to paradoxically stabilize and augment the expression of activated β-catenin in both nuclear and cytoplasmic compartments.

We speculate that additional, potentially complex activities of tankyrase beyond WNT signaling may further support stabilization of a human naïve ground state (Fig. 7B). These mechanisms might include the promotion of genomic integrity via telomere recombination/elongation and stability of the non-homologous end-joining (NHEJ) protein DNA-PKc (Dregalla et al., 2010;
subsequently passaged with LIF-3i alone, and routinely evaluated for additional pre-treatment for one passage with forskolin and purmorphamine a series of hPSC lines to stable, clonal cultures. Other hPSC lines required of hPSCs in classical 2i (CHIR99021 and PD0325901). A combination of Methods, Table S3) identified XAV939 for permitting long-term survival described in the supplementary Materials and Methods.

performed by the JHU Cytogenetics Core Facility. RNA-FISH for Time PCR System (Life Technologies) for expression of western blotting see the supplementary Materials and Methods. Conditions Biosciences) antibodies. For details of flow cytometry and associated Biosciences), SSEA4 (R&D Systems) and TRA-1-60/TRA-1-81 (BD Morphological changes were photomicrographed using a Nikon Eclipse TE-
hPSC cultures were passaged every 3-4 days on fresh irradiated MEFs. subsequent passages. Exposure (24-48 h) to small molecules prior to initial •-catenin (ABC clone 8E7, 1:100; 05-665, Millipore), β-catenin (total) (1:50; M3539, DAKO) and E-cadherin (1:25; M3612, DAKO). Slides were washed twice in Wash Buffer (DAKO), incubated for 1 h with either Alexa 488-conjugated highly cross-adsorbed goat anti-mouse secondary antibody (1:200; A11029, Life Technologies) or Alexa 488-conjugated highly cross-adsorbed goat anti-mouse primary antibodies (1:200; A11034, Life Technologies). Alternatively, second immunostains were performed using directly conjugated anti-human SSEA4 NL493 (1:50; SC023 Kit, R&D Systems), TRA-1-81-Alexa 488 (1:10; 560174, BD Biosciences) or anti-TRA-1-60-Dylight 488 (1:100; 09-0068, Stemgent). Nuclear staining was performed with DAPI (10 µg/ml; Life Technologies). Labtek chambers adsorbed goat anti-rabbit secondary antibody (1:200; A11034, Life Technologies) or Alexa 488-conjugated highly cross-adsorbed goat anti-mouse secondary antibody (1:200; A11029, Life Technologies) or Alexa 488-conjugated highly cross-adsorbed goat anti-rabbit secondary antibody (1:200; A11034, Life Technologies). Alternatively, second immunostains were performed using directly conjugated anti-human SSEA4 NL493 (1:50; SC023 Kit, R&D Systems), TRA-1-81-Alexa 488 (1:10; 560174, BD Biosciences) or anti-TRA-1-60-Dylight 488 (1:100; 09-0068, Stemgent). Nuclear staining was performed with DAPI (10 µg/ml; Life Technologies). Labtek chambers were separated and mounted with Prolong Gold Anti-Fade Reagent (Life Technologies), and observed and photographed with a Zeiss LSM 510 Meta confocal microscope. Universal negative control for mouse and rabbit primary antibodies (DAKO) were used.

Characterization of naïve hPSCs
A description of the generation of a repertoire of episomal hiPSC lines for the functional and molecular characterization of the reprogrammed state is provided in Table S2 and in the supplementary Materials and Methods, along with a description of how hESCs and hiPSCs were differentiated into the various mesodermal, ectodermal and endodermal lineages.

Cellularreversion screens with small molecules and cytokines
We tested >130 combinations of 23 small molecules/cytokines known to regulate ESC self-renewal. Permutations were tested using standard bFGF-supplemented KOSR-based hESC medium on irradiated MEF feeders without bFGF (Table S3). Modifications included N2B27 supplement (Life Technologies) and incubation in 5% O2. Molecules included bFGF (10 ng/ml; Peprotech, 100-18B), human LIF (hLIF) (20 ng/ml; Sigma, L5283; Cell Signaling, 8911LC; or Peprotech, 300-05), PD0325901 (1 µM; Stemgent, 040006; Sigma, PZ0162), CHIR99021 (3 µM; Stemgent, 04-0004; Tocris Bioscience, 4423), XAV939 (4 µM; Sigma, X3004), SB431542 (2 µM; Stemgent, 04-0010), forskolin (10 µM; Stemgent, 04-0025), ACTH peptide (1-24 (10 µM; American Peptide, 10-1-21), 2′,5′-dideoxyadenosine (500 µM; Sigma, D7408), AICA-riboside (100 µM; EMD Millipore, 123040), 8-(4-chlorophenylthio)-2′-O-methyladenosine 3′,5′-cyclic monophosphate monosodium hydrate (100 µM; Sigma, C8988), 3-isobutyryl-1-methylxanthine (100 µM; Sigma, L5879), BayK8644 (1 µM; Stemgent, 04-0013), DLPC (100 µM; Tocris Bioscience, 4378), purmorphamine (2 µM; Stemgent, 04-0009), AM580 (10 nM; Sigma, AM580), CCL2 (200 ng/ml; 571406, Biologend), SCF (10 ng/ml; Peprotech), JGF1 (10 ng/ml; Peprotech), IL6 (10 ng/ml; Peprotech), BMP4 (10 ng/ml; Peprotech) and thiazovivin (2 µM; Stemgent, 04-0017).

Initial testing was performed using conventional hPSCs (hESC-H9, sa-

Methods, Table S3) identified XAV939 for permitting long-term survival of conventional hPSCs. Naïve-reverted hPSC cultures were passaged every 3-4 days on fresh irradiated MEFs. Morphological changes were photomicrographed using a Nikon Eclipse TE-2000 inverted microscope, DS-F1 camera and NIS-Elements software.

Cultures were assayed by flow cytometry using anti-SSEA1 (BD Biosciences), SSEA4 (R&D Systems) and TRA-1-60/TRA-1-81 (BD Biosciences) antibodies. For details of flow cytometry and associated western blotting see the supplementary Materials and Methods. Conditions that supported stable expansion of hiPSC lines for >3-5 passages were further evaluated by qRT-PCR using TaqMan assays on a ViA7 Real-Time PCR System (Life Technologies) for expression of OCT4, NANOG, ZFP42, KLF2, NR5A2 and STELLA. For details of qRT-PCR and primers see the supplementary Materials and Methods. Karyotype analysis was performed by the JHU Cytogenetics Core Facility. RNA-FISH for XIST is described in the supplementary Materials and Methods.

Extensive small-molecule screening (supplementary Materials and Methods, Table S3) identified XAV939 for permitting long-term survival of hPSCs in classical 2i (CHIR99021 and PD0325901). A combination of CHIR99021, PD0325901 and XAV939 was sufficient for directly reverting a series of hiPSC lines to stable, clonal cultures. Other hiPSC lines required additional pre-treatment for one passage with forskolin and purmorphamine along with LIF-3i (LIF-5i) to enhance initial clonal viability. Cultures were subsequently passaged with LIF-3i alone, and routinely evaluated for expression of SSEA1/SSEA4, TRA-1-60/TRA-1-81 by flow cytometry every two to five passages thereafter.

Characterization of naïve hPSCs
A description of the generation of a repertoire of episomal hiPSC lines for the functional and molecular characterization of the reprogrammed
Bioinformatics analysis
Details of bioinformatics analyses, including gene expression and CpG DNA methylation microarrays and transcriptome analysis, are provided in the supplementary Materials and Methods.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
All authors designed, performed and interpreted experiments. In addition, J.S.H., D.S. and M.V.C.-S. contributed to the manuscript. E.T.Z. provided administrative and financial support, and edited/approved the final manuscript.

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Data availability
Microarray data are available at Gene Expression Omnibus under accession number GSE44430: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE44430.

Supplementary information
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