Epigenetic resetting of human pluripotency

factor LIF; see Materials and Methods) or MEK inhibitor plus LIF (PDLIF) culture (Fig. S1C). By contrast, the PB-EOS reporter is upregulated during transgene-induced resetting and visible expression is maintained in naïve-like cells (Takashima et al., 2014). These observations suggested that PB-EOS might be subject to reversible epigenetic silencing in primed hPSCs.

Histone deacetylase (HDAC) inhibitors are global epigenetic destabilisers that have been used to facilitate nuclear transfer (Ogura et al., 2013), somatic cell reprogramming (Huangfu et al., 2008) and mouse EpiSC resetting (Ware et al

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Fig. 1. Resetting human pluripotent stem cells (hPSCs) with HDAC inhibitors. (A) Schematic of the chemical resetting protocol. HDACi, HDAC inhibitor. (B) Images of reset S6EOS cells at day 9 in t2iLGα. Red staining is from Go6983. VPA, valproic acid; NaB, sodium butyrate. (C) Flow cytometry analysis of EOS-GFP expression at day 9 of resetting. (D) RT-qPCR analysis of pluripotency markers in S6EOS cells subjected to the resetting culture regime with or without VPA. Error bars indicate s.d. of technical duplicates. (E) Immunostaining for OCT4 and KLF17 during resetting of Shef6 cells. (F) Images of reset S6EOS cultures over the first four passages. (G) RT-qPCR analysis of general and naïve pluripotency markers in various reset cell cultures. Error bars indicate s.d. of technical duplicates. (H) Immunostaining of pluripotency markers in established reset culture, cR-H9EOS. Scale bars: 100m.

day 9, which increased further on passaging into tt2iLGö on MEFs (Fig. 3D, Fig. S2A). After the second passage the majority of colonies displayed domed morphology and readily visible GFP (Fig. 3E). WNT inhibitor-treated H9EOS cultures at passage 2 expressed higher levels of naïve markers and lower GATA6 and GATA3 than parallel cultures reset without WNT inhibition (Fig. 3F). Similarly, S6EOS cells reset using XAV or IWP2 progressed to stable reset cultures expressing naïve markers and minimal levels of brachyury, CDX2 and GATA6 (Fig. S2B). From passage 3, we transferred XAV-treated cells to feeder-free culture in tt2iLGö and Geltrex without XAV. Marker analysis by RT-qPCR confirmed maintained expression of signature naïve pluripotency factors after four passages at similar levels to those in reset cells generated without the use of WNT inhibitors (Fig. 3G).

We also assessed whether vitamin C was required for resetting. For the 3 day period of exposure to VPA we replaced E6 medium, which contains vitamin C, with N2B27 medium with or without addition of vitamin C. Resetting was continued in the presence of XAV as above. After two passages we observed comparable upregulation of EOS-GFP and similar expression of naïve markers with or without exposure to vitamin C (Fig. S2C,D).

Collectively, these findings establish that, following VPA treatment, WNT inhibition can improve the rate and efficiency of conversion to a stable naïve phenotype that can subsequently be propagated robustly in tt2iLGö with or without feeders and without WNT inhibition. The results also indicate that vitamin C supplementation is not required for resetting. Full details of the protocol and cell lines reset are provided in the supplemental Materials and Methods and Table S1.

Global transcriptome profiling

We obtained transcriptome data by RNA sequencing (RNA-seq) of replicate samples of reset cells generated by VPA treatment. We also sequenced the embryo-derived naïve stem cell line HNES1 (Guo et al., 2016) and a parallel culture of HNES1 cells that had been primed by transfer into KSR/FGF for more than ten passages. We added to the analysis published data (see Materials and Methods) from cells reset with inducible transgenes (Takashima et al., 2014), HNES cells cultured in the presence of vitamin C and ROCK inhibitor (Guo et al., 2016), naïve-like cells in 5i/L/A (Ji et al., 2016) and a variety of conventional PSCs from publicly available resources and our own studies. We applied two complementary dimensionality reduction techniques: principal component analysis (PCA) identifies and ranks contributions of maximum variation in the underlying dataset, whereas t-distributed stochastic neighbour embedding (t-SNE) is a probabilistic method that minimises the divergence between pairwise similarities in the constituent data points. Both analyses of global transcriptomes unambiguously discriminate naïve/reset samples from primed PSCs (Fig. 4A,B). In each analysis, cR cells cluster closely together with HNES1 cells that were cultured in parallel. Sample replicates are intermingled despite being from cell lineb di(par)74.9(a)19.3(te)-367.2porvo and cul

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We inspected the expression of transposable elements (TEs) transposcriptome (Friedli and Trono, 2015). A number of TEs are known to be transcriptionally active in early embryos and PSCs, potentially with functional significance. PCA of TE expression separated cR and HNES cells from primed PSCs (Fig. S3D,E).

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Fig. 3. WNT inhibition stabilises resetting. (A) Alkaline phosphatase staining of H9-NK2 colonies at first and second passage after DOX withdrawal and transfer into t2iLGo alone or plus L-proline (L-Pro) or the tankyrase inhibitor XAV939 (XAV). (B) RT-qPCR analysis of marker expression in H9-NK2 cells at passage 2, treated as in A. KSR/FGF reference sample is a conventional S6EOS culture. (C) Resetting protocol with WNT inhibitors. (D) (Top) Flow analysis of resetting H9EOS cells cultured in the presence or absence of WNT inhibitors. (Bottom) Flow analysis after two passages (a further 8 days) in tt2iLGowith WNT inhibitors on MEFs. (E) cR-H9EOS colonies in tt2iLGowith XAV or the WNT pathway inhibitor IWP2 after two passages on MEFs. (F) Marker analysis by RT-qPCR for cR-H9EOS cells at passage 2 cultured in tt2iLGowith and without WNT inhibitors. (G) Marker analysis by RT-qPCR of cR-H9EOS cultures generated with or without XAV and transferred into tt2iLGoon Geltrex (without XAV) for four passages. Error bars on PCR plots indicate s.d. of technical duplicates. Scale bars: 200 m in A; 100 m in E.

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Fig. 4. Transcriptome analysis of reset PSCs. (A) Principal component analysis (PCA) of whole-transcriptome RNA-seq data from the indicated cell lines. (B) t-SNE analysis of RNA-seq data. (C) Heatmap of differentially expressed genes between chemically reset (cR) and embryo-derived HNES cells (naïve) compared with conventional hPSCs (primed). Genes unregulated in naïve cells are shown, ranked by $\log_2$ fold-change (FC). Values displayed correspond to the average expression level in each sample group scaled by the mean expression of each gene. (D) Heatmap showing expression of all transposon families that are differentially expressed ($\log_2$ FC>1.5, P<0.05). (E) Comparative expression of pluripotency markers in human embryo cells (Blakeley et al., 2015; Yan et al., 2013), HNES cells, cR cells, conventional primed PSCs, NHSM cultures and purported expanded potency (EPS) cells. Data shown reflect mean expression levels from cell lines and biological replicates belonging to each sample group, and single cells from indicated embryo stages. Published datasets used are identified in the Materials and Methods.

expression of several of these key markers. A set of genes

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Fig. 5. Methylome analysis of reset PSCs. (A) Bean plots showing the global distribution of CpG methylation levels from pooled replicates of the indicated samples compared with human ICM data (Guo, 2014). Reset samples are from independent derivations without or with addition of XAV. Methylation was quantitated over 20 kb genomic tiles. Note that KCL37 and HNES1 are male and H9 and Shef6 are female. (B) Scatter plots of CpG methylation percentages over tiles spanning 20 kb on chromosome 7 and chromosome X, comparing parental Shef6EOS (in KSR/FGF) with cR-S6EOS. (C) Scatter plots of CpG methylation over promoters (−900 to +100), for parental and cR-S6EOS cells. Promoters with >40% gain in CpG methylation in reset cells are highlighted in red. (D) CpG methylation levels of a subset of promoters highlighted (red) in C in the indicated samples. (E) Averaged CpG methylation of known DMRs of imprinted maternal and paternal genes. Sperm and oocyte data are from Okae et al. (2014); ICM from Guo et al. (2014); H9 and H9-NK2 from Takashima et al. (2014). (F) Scatter plot showing the change in expression ($\log_2$ FC) against the difference in promoter methylation for reset (averaged over cR-H9EOS and cR-S6EOS) versus parental Shef6EOS. (G) Scatter plots for prominent differentially expressed transposon families showing the change in expression ($\log_2$FC) versus the difference in methylation for all loci.

29 days we detected expression of neuronal markers by RT-qPCR (Fig. 6D). Many cells with neurite-like processes were immunopositive for MAP2 and NEUN (RBFOX3) (Fig. 6E). By 40 days, markers of maturing neurons were apparent: vesicular glutamate transporter (vGlut2; SLC17A6), the post-synaptic protein SNAP25 and the presynaptic protein bassoon (Fig. 6F).

We also subjected cR-S6EOS cells to a protocol for inducing primordial germ cell-like cells (PGCLCs). Cells were transferred from t2iLGö into TGF and FGF for 5 days, followed by exposure to germ cell-inductive cytokines (I et al., 2015; von Meyenn et al., 2016). Cells co-express tissue non-specific alkaline phosphatase and EOS-G suggestive of germ cell identity, were isolated by flow cytome on day 9. Analysis of this double-positive population by RT-qP showed upregulated expression of a panel of primordial g cell markers (Fig. S7G). These data indicate that germ

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Fig. 6. Differentiation of reset PSCs. (A) RT-qPCR analysis of lineage specification markers after induction of re-primed cR lines.'Non' indicates non-induced; Ecto, neuroectoderm; DE, definitive endoderm; LPM, lateral plate mesoderm. (B) Immunostaining for lineage specification markers. (C) Summary of flow cytometric quantification of neuroectodermal, mesodermal and endodermal lineage specification. (D) RT-qPCR assays for pan-neuronal markers after 29 days differentiation from re-primed cR-S6EOS cells. (E) Immunostaining for neuronal markers MAP2 and NEUN after 29 days. (F) Immunostaining for neuronal maturation markers after 40 days. Arrowheads (middle) highlight expected punctate clusters of SNAP25; arrows (right) indicate a non-stained cell to show antibody specificity. Error bars in PCR plots are s.d. of technical duplicates. Scale bars: 100 µm in B,E; 10 µm in F.

specification may be induced from chemically reset cells, as also shown for reset cells generated by transgene expression (Ware et al., 2014; Meyenn et al., 2016).

X chromosome activity

Female naïve cells are expected to have two active X chromosomes in human, as in mouse. Unlike in mouse, however, XIST is

expressed from one or both active X chromosomes in human ICM cells (Okamoto et al., 2011; Petropoulos et al., 2016; Vallot et al., 2017) as well as from the inactive X in differentiated cells. Primed female hPSCs usually feature an inactive X, although this has frequently lost *XIST* expression, a process referred to as erosion (Mekhoubad et al., 2012; Silva et al., 2008). X chromosomes in female cR-S6EOS cells show more marked loss of methylation than autosomes (Fig. S6C), suggestive of reactivation (Takashima et al., 2014). We employed RNA FISH to assess nascent transcription from X chromosomes at the single-cell level. In parental S6EOS and H9EOS cells the presence of two X chromosomes was confirmed by RNA FISH for *XACT* (Fig. S8A), which is transcribed from both active and eroded X chromosomes (Patel et al., 2017; Vallot et al., 2017). No *XIST* signal was evident in either cell line but we detected monoallelic transcription of *HUWE1*, an X-linked gene typically

XIST from an active X chromosome in a subset of reset cells resembles the pattern of the human pre-implantation embryo. Upon re-priming, monoallelic expression of X-linked genes is restored in many cells. Significantly, although no XIST was observed in the original primed cells, an XIST signal is detected in re-primed cells on a silenced X chromosome. Resetting and subsequent differentiation thus offer a system to characterise X-chromosome regulation in human, which appears to diverge substantially from the mouse paradigm (Okamoto et al., 2011).

In summary, this study provides the requisite technical protocols and resources to facilitate routine generation and study of candidate human naïve PSCs. Moreover, feeder-free culture simplifies the propagation of reset cells. Nonetheless, further refinements are desirable to enhance the quality and robustness of naïve hPSCs, including preserving imprints and maximising long-term karyotype

MATERIALS AND METHODS

Conventional hPSC culture

Primed hPSCs were routinely maintained on irradiated mouse embryonic fibroblast (MEF) feeder cells in KSR/FGF medium: DMEM/F-12 (Sigma-Aldrich, D6421) supplemented with 10 ng/ml FGF2 (prepared in-house), 20% KnockOut Serum Replacement (KSR) (Thermo Fisher Scientific), 100 mM 2-mercaptoethanol (2ME) (Sigma-Aldrich, M7522), 1×MEM non-essential amino acids (NEAA) (Thermo Fisher Scientific, 11140050) and 2 mM L-glutamine (Thermo Fisher Scientific, 25030024). Cells were passaged as clusters by detachment with dispase (Sigma-Aldrich, 11097113001). To establish PB-EOS stable transfectants, 1 μg/ml puromycin was applied for two passages (10 days) to transfected cells on Matrigel (Roche). Some PSC lines were propagated without feeders on Geltrex (growth factor-reduced, Thermo Fisher, A1413302) in E8 medium [made in-house according to Chen et al. (2011)].

Naïve cell culture

Chemically reset and embryo-derived (HNES) naïve stem cells were propagated in N2B27 (see the supplementary Materials and Methods) supplemented with t2iLGö [1 μM CHIR99021 (CH), 1 μM PDO325901 (PD), 10 ng/ml human LIF and 2 μM Gö6983] with or without ROCK inhibitor (Y-27632) on irradiated MEF feeders. Where indicated as tt2iLGö, CH was used at 0.3 μM. For feeder-

imaged using an Imager M1 microscope (Zeiss) and AxioVision software. ImageJ was used for collapsing z-stacks, merging different channels, and adjusting brightness and contrast to remove background. A minimum of 100 nuclei were scored for each sample. Cells that appeared to have more than two X chromosomes were excluded.

Transposable elements

RepeatMasker annotations for the human reference genome were obtained from the UCSC Table Browser. To calculate repeat expression, adapter-trimmed RNA-seq reads were mapped to the reference genome using bowtie (Langmead and Salzberg, 2012) with parameters `–M1 –v2 –best –strata`, i.e. two mismatches were allowed, and one alignment location was randomly selected for reads that multiply align to the reference genome. Read counts for repeat regions and Ensembl transcripts were calculated by featureCounts, normalised by the total number of RNA-seq reads that mapped to protein-coding gene regions. Differential expression of repeat copies across samples was evaluated by the R Bioconductor DESeq package (Anders and Huber, 2010).

Embryoid body differentiation

Embryoid body formation and outgrowth were performed in DMEM/F12 supplemented with 15% fetal calf serum (FCS), 2 mM L-glutamine. 1 mM sodium pyruvate, 1× non-essential amino acids and 0.1 mM 2ME as described (Guo et al., 2016). Alternatively, reset cells were aggregated in t2iLIF medium with ROCK inhibitor in PrimeSurface 96V cell plates (Sumitomo Bakelite MS-9096V) then plated after 3 days on Geltrex (Thermo Fisher Scientific, 12063569) for outgrowth in serum-containing medium. Outgrowths were fixed with 4% paraformaldehyde for 10 min at room temperature for immunostaining.

Adherent differentiation

Except where specified, reset cells were 're-primed' before initiating differentiation. Cells were plated on Geltrex in t2iLGö and after 48 h the medium was changed to E8. Cultures were maintained in E8, passaging at confluence. Lineage-specific differentiation was initiated between 25 and 44 days.

Definitive endoderm was induced according to Loh et al. (2014). Cells were cultured in CDM2 medium (in-house according to Loh et al., 2014) supplemented with 100 ng/ml activin A (produced in-house), 100 nM PI-103 (Bio-Techne, 2930), 3 µM CHIR99021, 10 ng/ml FGF2, 3 ng/ml BMP4 (Peprotech) for 1 day. For the next 2 days the following supplements were applied: 100 ng/ml activin A, 100 nM PI-103, 20 ng/ml FGF2, 250 nM LDN193189.

For lateral mesoderm induction (Loh et al., 2016), cells were treated with CDM2 supplemented with 30 ng/ml activin A, 40 ng/ml BMP4 (Miltenyi Biotech, 130-098-788), 6 µM CHIR99021, 20 ng/ml FGF2, 100 nM PI-103 for 1 day, then with 1 µM A8301, 30 ng/ml BMP4 and 10 µM XAV939 (Sigma-Aldrich).

For neural differentiation via dual SMAD inhibition (Chambers et al., 2009), cells were treated with N2B27 medium supplemented with 500 nM LDN193189 (Axon, 1509) and 1 µM A 83-01 (Bio-Techne, 2939) for 10 days, then passaged to plates coated with poly-L-ornithine and laminin and further cultured in N2B27 without supplements.

Flow cytometry

Flow analysis was carried out on a Fortessa instrument (BD Biosciences). Cell sorting was performed using a MoFlo high-speed instrument (Beckman Coulter).

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Competing interests

G.G. and A.Sm. are inventors on a patent filing by the University of Cambridge relating to human naïve pluripotent stem cells. W.R. is a consultant to, and shareholder in, Cambridge Epigenetix.

Author contributions

Conceptualization: G.G., F.v.M., A.Sm.; Methodology: G.G., F.v.M., M.R., J.C., D.978o

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