Direct generation of human naïve induced pluripotent stem cells from somatic cells in microfluidics.

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ABSTRACT

Induced pluripotent stem cells (iPSCs) are generated by expression of transcription factors *OCT4*, *SOX2*, *KLF4* and *cMYC* (*OSKM*) in somatic cells. In contrast to murine naïve iPSCs, conventional human iPSCs are in a more developmentally advanced state - called primed pluripotency. Here we report that human naïve iPSCs (niPSCs) can be generated directly from less than 1000 primary human somatic cells without stable genetic manipulation by delivery of modified messenger RNAs with microfluidics. Expression of *OSKM* in combination with *NANOG* for 12 days generates niPSCs free of transgenes, karyotypically normal, and display transcriptional, epigenetic and metabolic features indicative of the naïve state. Importantly, niPSCs efficiently differentiate into all three germ layers. While niPSCs could also be generated at low frequency under conventional conditions, our microfluidics approach will allow robust and cost-effective production of patient-specific niPSCs for regenerative medicine applications, including disease modelling and drug screening.

INTRODUCTION

Pluripotent stem cells (PSCs) were derived from the early embryos as embryonic stem cells (ESCs)[1,2] and subsequently from somatic cells via transcription factor-mediated reprogramming[3–5]. Murine PSCs are thought to represent a naïve pluripotent state, characterized by expression of the transcription factors Oct4, Sox2, Nanog, Klf4 and Tfcp2l1 in response to the cytokine LIF and inhibition of GSK3 and MEK kinases[6]. Murine naïve PSCs display low levels of repressive epigenetic modification, such as trimethylation of lysine 9 on histone 3 (H3K9me3) or cytosine methylation[7–9] and are metabolically sustained by high levels of oxidative phosphorylation[10,11]. Importantly, murine naïve PSCs efficiently generate cells of all three germ layers.

Conversely, conventional human PSCs, either derived from early embryos[2] or by reprogramming of somatic cells by OSKM[3], resemble a distinct, more advanced developmental stage, called primed pluripotency. Primed PSCs express OCT4, SOX2 and NANOG in response to FGF and TGF-beta signals, display higher levels of repressive epigenetic modifications and are mostly glycolytic[12,13]. Different primed PSC lines display a differentiation bias toward some germ layers[14].

Recently, human naïve PSCs have been generated either by expression of transgenes together with genomic reporter constructs or directly from human embryos[15–17]. The use of human embryos has ethical limitations and obviously does not allow generation of patient-specific naïve PSCs, while conversion of somatic cells to naïve pluripotency with available protocols requires one or more rounds of stable genetic manipulations, which are time-consuming, inefficient, and potentially mutagenic. To overcome such limitations, we derived a strategy for the efficient generation of transgene-free naïve iPSCs directly from somatic cells.

RESULTS

Devising a strategy for transgene-free generation of naïve iPSC colonies

We showed that delivery of modified messenger RNAs (mmRNAs) encoding for *OSKM+LIN28+NANOG* in microfluidics (μF) resulted in rapid and efficient generation of primed iPSCs[18]. We compared the global gene expression profile of primary iPSC colonies generated in μF or under conventional culture conditions (CCC) and observed that several transcription factors associated with naïve pluripotency were upregulated (Fig. 1a-b), suggesting that μF could favour activation of the naïve network. Therefore, we asked whether naïve PSCs could be derived directly from primary somatic cells in μF. To test this hypothesis we transfected BJ human fibroblasts with mmRNAs in μF using our previous setup[18] (Supplementary Fig. 1a) leaving out the primed pluripotency factor *LIN28*[19]. *DPPA5*, a gene highly induced in human naïve cells[20,21], became detectable from day 14 (Supplementary Fig. 1b), despite the use of FGF-2-containing medium commonly used for the generation of primed PSCs (Primed reprogramming medium, PRM). The colonies generated showed the morphology of primed PSCs and lost *DPPA5* expression after passaging. We then tried to sustain the expression of *DPPA5* by providing the correct signalling environment. The cytokine LIF, the GSK3 inhibitor (CHIR99021, CH) and the MEK inhibitor (PD0325901, PD) in N2B27 medium (2iL) are present in several media for murine and human naïve PSC culture[15,16]. Switching media to 2iL at day 14 boosted and maintained *DPPA5* expression for the following 8 days (Fig. 1c), although they were morphologically indistinguishable from colonies kept in PRM (Fig. 1c, bottom panels), and rapidly collapsed upon passaging in 2iL. We reasoned that by day 14 in PRM iPSCs have already lost the competence to reach the naïve state, so we exposed cells to 2iL from the beginning of the reprogramming protocol (Supplementary Fig. 1c), but no iPSC colonies formed and we observed fibroblast senescence, reduced transfection efficiency and failure to execute the mesenchymal to epithelial transition (MET)[22], which normally occurs by day 6 (Supplementary Fig 1c-d).

Based on these observations we devised a two-step protocol, using PRM for the first 6 days to avoid senescence and allow MET and, subsequently, applying 2iL supplemented with 1% knockout serum replacement (KSR) and the PKC inhibitor Gö6983 (2iLGo-KSR) – previously used to enhance and stabilize human naïve pluripotency[20,21,23] (Fig. 1d, top panel). During the first 6 days, we also tested the addition of various inhibitors, and observed the formation of several naïve-like colonies under different combinations, with the most efficient condition being PRM+CH (Fig. 1d, bottom). Under these conditions mmRNA transfection was robust (Supplementary Fig. 1e), MET occurred by day 5 and was followed by appearance of naïve-like colonies from day 9 (Fig. 1e, red arrowheads). Such

colonies expressed the naïve markers TFCP2L1, DPPA3 and DNMT3L (Fig. 1f), the core factor NANOG and were negative for the primed surface maker SSEA4[24]. The protocol appeared robust given that 89% of 213 individual µF channel presented naïve-like colonies (Fig. 1g-h, red) with an efficiency of 0.87% (*i.e.*, 0.87 colonies generated for 100 fibroblasts seeded). Inhibition of rho-associated protein kinase (ROCK) and hypoxic conditions[25,26,36] resulted in generation of colonies in 100% of µF channels, with an increased efficiency of 3.02% (Fig. 1g-h, purple). We conclude that delivery of mmRNAs for *OSKMN* in µF allows rapid and robust generation of naïve PSC colonies from human fibroblasts.

Long-term expansion and characterization of naïve iPSCs

Different media and extracellular matrix proteins have been used for the culture of human naïve cells[15,16]. We transfected cells with *OSKMN* in µF under hypoxia, for 6 days in PRM+CH+Ri, followed by 6 days in different naïve media, such as 4iLA[27] or the commercially available RSeT (Fig. 2a). Naïve colonies, expressing OCT4 and KLF17, formed robustly in all media (Fig. 2b and Supplementary Fig. 1g), with an increase in colony size in RSeT medium (Fig. 2c-d). Coating of µF channels with Fibronectin, Matrigel or Laminin allowed generation of naïve colonies with comparable efficiency (Supplementary Fig. 1g-h), indicating that our protocol can be easily adapted to different culture conditions. Next, we asked whether primary colonies could be expanded for long term. To do so we transferred them from µF to CCC on feeders in the respective media in hypoxia succeeding to expand them over multiple passages (Fig. 2e-f, h). RSeT again appeared the most robust among the 3 media, so it has been used for all further experiments.

Naïve induced pluripotent stem cells (niPSCs), derived either freshly (p0) or after several passages (up to p21), displayed robust expression of naïve pluripotency markers, with strongly reduced expression of differentiation marker T and primed pluripotency markers SSEA4, *OTX2* and *ZIC2*, indicating a stable naïve phenotype (Fig. 2e-h and Supplementary Fig. 2a). We obtained niPSCs starting from 4 other somatic cells (human male foreskin fibroblasts HFF, the female lung fibroblasts WI-38 and IMR-90 and primary skin fibroblasts of an 80-year-old female donor, Fig. 2i, Supplementary Fig. 2b-d and Supplementary Table 1). All niPSC lines expressed naïve markers and expanded robustly over multiple passages retaining naïve morphology and high clonogenicity (Fig. 2e-f, k-j and Supplementary Fig. 3a-b).

Some human naïve PSC lines, derived from embryos or from primed PSCs, display chromosomal instability after extensive culture[21,24,28]. Performing DNA staining followed by flow cytometry we observed that for the first 8 passages all lines were diploid. Stable diploid karyotype has been

observed in 6 out of 8 lines for up to 42 passages. Only 2 lines (HPD01 and HPD07) showed a fraction of tetraploid cells after 12 and 23 passages, respectively (Supplementary Fig. 3c). By Fluorescence Activated Cell Sorting (FACS) we isolated the diploid population and cells then maintained a correct karyotype. Correct karyotype has also been confirmed by Q-banding in 5 independent niPSC lines (Fig. 2k and Supplementary Fig. 3d). We conclude that reprogramming in microfluidics does not induce chromosomal abnormalities *per se*, which are instead likely due to extended culture.

A naïve state transcriptome is rapidly acquired during niPSCs generation

We performed transcriptome profiling by RNA-sequencing of niPSCs, primed iPSCs generated in µF (Supplementary Fig. 1c) and human fibroblasts. Unsupervised clustering based on specific markers grouped niPSCs together with other previously described human naïve cells[20,21,27,28], clearly separating them from primed PSCs (Fig. 3a). Similar results were obtained by analysing the whole transcriptome (Fig. 3c and Supplementary Fig. 4a). Naïve iPSCs have been recently generated from human fibroblasts by delivery of *OSKM* via Sendai viruses without µF[29,30]. Depending on the media used, some of those lines grouped together with embryo-derived naïve PSCs and with niPSCs (Fig. 3a, c and Supplementary Fig. 4a-c and 8, see also Discussion).

The presence of a unique set of transposable elements (TE), expressed by cleavage-stage embryos, characterises the human naïve pluripotent state[27]. The TE profile of niPSCs was clearly distinct from those of primed cells and comparable to previously described naïve PSCs (Fig. 3b). Notably, primary colonies ("niPSCs p0" in Fig. 3a-c) collected at day 15 were transcriptionally indistinguishable from established naïve PSCs. We conclude that our reprogramming strategy allows rapid and full acquisition of a human naïve transcriptome.

Next, we asked whether our system would allow the study of the trajectories of iPSCs generation from fibroblasts. As a proof-of-principle, we transfected fibroblasts with mmRNAs for *OSKMN* either under the optimised protocol described in Figure 2a to generate niPSCs, or in PRM to generate primed iPSCs. Gene expression analysis of a panel of naïve- and primed-state markers revealed that the two reprogramming trajectories diverged significantly after day 8 (Fig. 3d-e and Supplementary Fig. 4d). Between day 5 and 8, cells undergo dramatic morphological changes indicative of a MET (see also Fig. 1e). We measured a reduction in mesenchymal markers (Fig. 3f, in grey) and gradual activation of epithelial markers (in orange) for both reprogramming protocols, indicating that MET is a common

early molecular event. We conclude that our reprograming method allows the study of distinct trajectories leading to either primed or naïve pluripotency.

Global reduction in repressive epigenetic modifications in niPSCs

Naïve pluripotency is associated with a reduction in repressive epigenetic modification, such as H3K9me3 or cytosine methylation (5mC)[15,20,24,27,28]. Both markers were found to be reduced by immunostaining in multiple niPSCs compared to primed PSCs (Fig. 4a-b and Supplementary Fig. 5a). Consistent with the reduction in 5mC, we observed decreased levels of DNA methyltransferases *DNMT3A* and *DNMT3B*, a dramatic increase of the catalytically inactive *DNMT3L*, and of the 5mC oxidases *TET1* and *TET2* (Fig. 4c). We determined the pattern of genome methylation by Reduced Representation Bisulfite Sequencing (RRBS) in 5 niPSC lines, 3 fibroblasts (from which they were derived) and an isogenic primed iPSC line (see Supplementary Table 1). The global methylation levels (Fig. 4d) and distributions (Supplementary Fig. 5b) of niPSCs were comparable to those of human blastocysts and other naïve PSCs[20,24,27,31]. Unsupervised clustering of the methylation pattern clearly separated naïve PSCs from somatic cells and primed iPSCs (Fig. 4e). A set of promoters hypomethylated in human blastocysts compared to conventional primed hES cells[31,32] were found highly methylated in somatic and primed pluripotent cells, and hypomethylated in niPSCs - in line with their naïve identity (Fig. 4f).

Analysis of imprinted and X-linked genes status

DNA methylation is crucial for genomic imprinting - the phenomenon which causes the expression of either the maternal or paternal copy of a gene[33]. Pastor and colleagues reported the loss of DNA methylation at imprinted loci in embryo-derived naïve hESCs, therefore we asked whether niPSCs would have the same defects. We analysed 67 bona fide imprinted loci[34], divided in 3 classes (Fig. 5a): "placental" loci, methylated in human oocytes and blastocysts, but not in somatic cells (blood)[35], were hypomethylated in fibroblasts and remained so also after reprogramming. Maternally imprinted loci lost methylation only in niPSCs, as reported[24], while a fraction (5/8) of paternally imprinted loci surprisingly maintained methylation in all niPSCs (Fig. 5a-b). Loss of DNA methylation at the imprinted regions should result in aberrant biallelic expression of the cognate transcripts. We interrogated RNA-seq data taking advantage of annotated Single Nucleotide Polymorphisms (SNPs) in *MEG3* mRNA and found monoallelic expression in somatic cells and biallelic expression in all naive PSCs, in agreement with its reduced methylation (Fig. 5c).

Female naïve pluripotent cells contain two active X chromosomes[36], characterized by low levels of DNA methylation at CG-reach regions called CpG islands (CGIs). CGI methylation on X chromosome in female niPSCs was dramatically reduced (Fig. 5d) relative to female fibroblasts, to levels observed in male cells. Analysis of SNPs on X-linked genes showed biallelic expression of several transcripts in female niPSCs (Fig. 5e). Finally, high *XIST* expression was detected in female niPSCs, compared to male niPSCs and somatic cells (Fig. 5f), as previously reported for female naïve pluripotent cells *in vitro* and *in vivo*[36,37]. These results indicate reactivation of both X chromosomes in female niPSCs.

Naïve iPSCs display high mitochondrial activity

Mitochondrial activity has been reported to be higher in both murine and human naïve cells, compared to their primed counterparts[11,20,38]. We measured the mitochondrial membrane potential by TMRM staining and it was barely detectable in primed cells (Fig. 6a). Conversely, niPSCs displayed robust TMRM staining. Both niPSCs and Reset H9 cells[20] displayed increased mitochondrial transcripts, compared to primed PSCs (Fig. 6b) and to fibroblasts (Fig. 6c), as previously reported in murine PSCs[10]. We conclude that niPSCs display epigenetic and metabolic features consistent with a naïve pluripotent state.

Characterization of the differentiation potential of niPSCs

Recent reports showed that some human naïve PSC lines are lineage biased and fail to differentiate towards mature cell types[39,40], thus limiting their use for disease modelling and developmental studies. We tested the differentiation potential of 3 isogenic niPSC lines (HPD01, HPD03 and HPD04) to gauge the technical variability of our reprogramming system, and included a primed iPSC line (HPD00) as a control. First, we applied 3 different monolayer differentiation protocols and found that all 3 niPSC lines expressed markers of Mesoderm and Endoderm after 6 days of differentiation, while Ectoderm markers were robustly expressed around day 12 (Fig. 7a). As expected, primed iPSCs differentiated faster (Supplementary Fig. 6a, top). Second, we performed embryoid body (EB) differentiation and detected expression of multiple markers of the three germ layers at day 22 in all 3 niPSC lines (Fig. 7b and Supplementary Fig. 6b). Third, after 50 days of EB differentiation (Fig. 7c and Supplementary Fig. 6c), we detected markers of mature cell types comparably in all iPSC lines. Fourth, we directly tested the ability to form mature cell types adapting protocols previously defined for primed PSC. After 15 days of hepatic differentiation protocol[41,42], we observed polygonal shaped hepatocyte-like cells expressing the mature markers CYP3A and HNF4A, while a neuronal differentiation protocol[43] successfully generated $MAP2^+$, $TUJ1^+$, $NeuN^+$ cells (Fig. 7d-e and

Supplementary Fig. 7a-b). Finally, all differentiation assays were repeated with an independent niPSC line (HPD06) derived from HFF fibroblasts (Supplementary Fig. 6-7). In sum, these results demonstrate that niPSCs are pluripotent, respond to differentiation cues and are able to form mature cell types.

The confined microfluidic environment promotes establishment of naïve pluripotency

Initial attempts to generate niPSCs using mmRNA under CCC failed. We reinvestigated this with our optimized protocol (Fig. 8a) comparing μF and CCC. Generation of niPSCs in μF was robust (100% of channels contained colonies, with an efficiency of 3.7%±1.0 and 3.1%±0.4 from BJ and HFF-1, respectively). Conversely, in CCC only 25% of wells contained naïve-like colonies, with efficiency of 0.2% and 0.04%. Interestingly, a mixture of colonies displaying primed and naïve morphology emerged in CCC (Fig. 8b, blue and red circles) – a phenomenon never observed in μF. These results strongly indicate that the confined microenvironment promotes generation of naïve iPSCs over primed iPSCs, and that the culture conditions used (i.e. RSeT medium with feeder cells) allow propagation of different pluripotent cell types (see Supplementary Fig. 8). By adding the FGF receptor inhibitor PD173074, we selectively eliminated primed-like iPSCs (Fig. 8c, right) and obtained a stable niPSC line (HPD05) transcriptionally comparable to other naïve iPSCs and clearly distinct from primed PSCs (Fig. 8d-e and 3a-c).

To directly test if the confined microenvironment promotes reprogramming to the naïve state, we generated microfluidics chips with channels of different heights, inside which we then reprogrammed BJ fibroblasts. At the height of 200 μm, used in all other experiments, we obtained colonies at the high efficiency and all colonies expressed both KLF17 and POU5F1 (Fig. 8f and Supplementary Fig. 7c). Increasing the height caused a reduction in both the number of colonies and in the percentage of double positive colonies, indicating the presence of either primed or partially reprogrammed colonies. Finally, decreasing the channel height to 100 μm caused a reduction in colony number, likely due to rapid exhaustion of the culture medium. We conclude that reprogramming to naïve pluripotency is enhanced by an optimal spatial confinement.

Finally, we asked whether μF could also promote conversion of primed PSCs to naïve pluripotency. We generated primed H9 hESCs expressing NANOG and KLF2 (NK2) in DOX-inducible manner, as previously reported[20,21]. Exposure to 2iL+DOX led to naïve colonies formation, with a 5-fold increase in μF compared to CCC (Fig. 8g). In contrast, no naive colonies have been observed under conditions promoting primed pluripotency (FGF/KSR), even in μF. We conclude that a confined

environment promotes activation of the naïve pluripotency network, regardless of the starting cell type, but only in conditions supporting naïve pluripotency.

DISCUSSION

Microfluidics allows manipulation of cells in a confined environment, where autocrine and paracrine factors are more concentrated[18,44]. The use of μF renders the generation of naïve iPSCs robust and efficient, reducing the costs and number of somatic cells required by several orders of magnitude. The efficiency of reprogramming in our system (3-5%) is substantially higher than the efficiency of other non-integrating methods for generation of conventional human iPSCs[45]. We speculate that cells undergoing reprogramming release extracellular factors supporting survival or cell fate transition. Identification of such factors could help improve the efficiency of reprogramming protocols under conventional conditions.

Previous studies reported the conversion of human somatic cells to naïve pluripotency[20,21,23], but they either relied on secondary systems, viral vectors or required two rounds of reprogramming (*OSKM* followed by *NK2*) via an intermediate primed state. Our results show that it is possible to directly convert somatic cells to naïve pluripotency by transient transfection of *OSKMN* mmRNAs. Whilst *KLF2* has previously been used to convert primed PSCs to naïve pluripotency[20,21,27], it has not been included in our reprogramming cocktail, perhaps reminiscent of the functional redundancy between *KLF4* and *KLF2* as observed in murine cells[46]. Correspondingly, *KLF4* expression has recently been used to convert human primed PSCs to naïve pluripotency[29].

While this manuscript was under revision, two studies reported the generation of naïve iPSCs from somatic cells by expression of *OSKM* using Sendai viruses[29,30]. In those studies, iPSCs generated in t2iLGöY or 5iLAF consistently displayed a transcriptome similar to embryo-derived naïve PSCs and niPSCs (Fig. 3a, c and Supplementary Fig. 4a-c), with robust expression of naïve markers such as KLF17, TFCP2L1 and DNMT3L. Conversely, the use of RSeT medium led to variable results: in one study[29], iPSCs generated in RSeT were transcriptionally similar to primed iPSCs (Fig. 3a, c), while in the other study[30], iPSC lines displayed either a naïve or an intermediate phenotype, with heterogeneous expression of KLF17[30] (Supplementary Fig. 4b-c). In contrast, we used *OSKMN* mmRNAs in μF in RSeT and obtained niPSCs homogeneously expressing several naive markers (Fig. 2g-h, Supplementary Fig. 2a-b) and displaying transcriptomes similar to other embryo-derived naïve PSCs (Fig. 3a-c, Supplementary Fig. 4a). These disparate outcomes with RSeT medium may arise

due to other methodological parameters, such as Sendai viruses vs mmRNAs, *OKSM* vs *OKSMN*, conventional conditions vs µF.

We compared RSeT, 2iLGo and 4iLA media during naïve iPSC generation in µF (Fig. 2b, Supplementary Fig. 1g) and observed formation of KLF17^{+}/POU5F1^{+} iPSC colonies with similar efficiency, suggesting that the confined environment promotes acquisition of naïve pluripotency and that this effect is dominant over media composition. Indeed, all 3 media allow robust expansion of established niPSCs (Supplementary Fig. 8). To directly test the effect of the confined environment we increased the height of the microfluidic channels (Fig. 8f, Supplementary Fig. 7c), or performed reprogramming under conventional conditions rather than µF, and observed that only in an optimally confined environment could we obtain a homogenous population of naïve colonies at high efficiency (Fig. 8a-c). Interestingly, in both a higher channel and conventional conditions we obtained a mixed population of primed- and naïve-like colonies in RSeT (Fig. 8b), resembling the results reported by Kilens and colleagues[30]. Thus, we would argue that the confined environment is instructive for induction of naïve pluripotency, and that RSeT is a more permissive medium, which allows the formation and expansion of a range of different pluripotent phenotypes under conventional conditions.

A recent study investigated the acquisition of naïve pluripotency with a secondary system of *in vitro*-differentiated fibroblasts bearing a DOX-inducible *OSKM* cassette[47]. A naïve transcriptome was observed after 20 days of reprogramming and transgene-independent iPSCs were obtained at day 24. In contrast, our method appears more rapid given that by day 15 naïve iPSC colonies showed a naïve transcriptome (Fig. 3a-c, Supplementary Fig. 4a) and homogeneous protein expression of naïve factors (Fig. 1f, Fig. 2g, Supplementary Fig. 2a-b), and could be readily expanded without further mmRNA delivery. Since generation of primed iPSCs from fibroblasts with mmRNA was also faster than with Sendai virus[22,45], we suggest that here rapid and efficient generation of naïve iPSCs is attributable to the combination of mmRNA and microfluidic technologies. It will be interesting to exploit our system to study, at high resolution, the trajectories followed by somatic cells towards naïve pluripotency. As a proof-of-principle, we performed bulk analyses on fibroblasts reprogrammed to either naïve or primed pluripotency and observed a common initial phase followed by two divergent trajectories (Fig. 3 d-e and 8h).

Chromosomal abnormalities in some naïve PSCs have been reported[24,27,28]. Only 2 lines out of 8 displayed a tetraploid fraction of cells after long-term expansion, which could be eliminated by

FACS. We conclude that our method allows the generation of karyotypically normal naïve PSCs, possibly thanks to the use of mmRNAs, which have been shown to cause the lowest number of chromosomal abnormalities compared to other non-integrating methods[45]. We argue that the prolonged expansion in culture, together with some yet unknown factors, might negatively influence genomic stability.

In conclusion, we showed that the confined microfluidics environment strongly enhances direct conversion of somatic cell to naïve pluripotency. With our technology, transgene-free niPSCs could be efficiently generated from patients, and their high clonogenic capacity will allow efficient gene editing. Conventional PSC lines display strong differentiation biases[14,48] and retain some somatic epigenetic features[49]. We provided evidence of prompt differentiation capacity, yet it is still an open question whether naïve PSCs could represent a better system for the generation of differentiated cell types for therapeutic purpose or for disease modelling. Furthermore, naïve PSCs represent a good model system to study early development, as exemplified by a recent study of X-chromosome inactivation dynamics[36]. Future studies comparing the epigenetic profile and differentiation potential of multiple patient-specific niPSCs and conventional primed iPSCs will reveal the full potential of human niPSCs in regenerative medicine, disease modelling and developmental studies.

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Contributions

S.G. and M.P. performed reprogramming, isolation, characterization and differentiation of niPSCs. I.Z. helped in establishing conditions for expansion of naïve PSCs and characterizing niPSCs. O.G. helped with microfluidics experiments. P.M. and C.R. performed bioinformatic analyses. D.C., M.J.Z. and G.M. designed the RRBS experiment; M.M. and M.J.Z. analysed the RRBS data. S.G., M.P. and G.M. designed experiments. N.E. and G.M. supervised the study and wrote the manuscript. S.G. and M.P edited the manuscript.

Data Availability

RNA-Seq data of this study are deposited in Sequence Read Archive (SRA) with BioProject accession number PRJNA381757. We reported accession numbers of other published datasets in each figure plotting RNA-Seq data. Source data of all repeats are provided in Supplementary Table 2.

Competing financial interests

N.E. is inventor of a patent application describing the reprogramming and differentiation processes in microfluidics, application number PD2013A000220.

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Graziano Martello (graziano.martello@unipd.it).

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ONLINE METHODS

Cell culture. Naïve human embryonic stem cells (Reset H9 described in Takashima et al. 2014, generated by transient expression of NANOG+KLF2 and kindly provided by Austin Smith's lab) were cultured on mitotically-inactivated mouse embryonic fibroblasts (MEF, DR4 ATCC) in 2iLGo medium prepared as follow: 1% N2 and 2% B27 supplements in DMEM-F12:Neurobasal (N2B27 medium, all Thermo scientific) were supplemented with 1 μM PD0325901 (PD, Axon Medchem), 1 μM CHIR99021 (CH, Axon Medchem), 10 ng ml^{-1} human LIF (produced in house), 1-2 μM Gö6983 (Go, Axon Medchem). It was particularly critical to titrate the concentration of each batch of Gö6983 in order to minimize cell stress due to accumulation of the inhibitor and maximize expression of naïve markers. Naïve human iPSCs (niPSCs) were cultured in various naïve-supporting media reported on MEF feeders: 4iLA medium prepared as follow: N2B27 supplemented with 1 μM PD, 1 μM WH-4-023 (Axon Medchem), 0.5 μM SB590885 (Axon Medchem), 10 μM Ri, 10 ng ml^{-1} LIF and 20 ng ml^{-1} Activin-A; the FGF-free medium RSeT (05970, StemCell technologies) prepared as manufacturer instruction: basal medium supplemented with 5X, 500X, and 1000X supplements. niPSC colonies were passaged every 4-6 days as follows. Cells were washed with phosphate buffer without Ca^{2+}/Mg^{2+} (PBS) and incubated with 300 μl TripLE select (Thermo Scientific) per 12-well plate for 3 min at room temperature. N2B27 medium (700 μl) was added to inhibit dissociation. Clusters (3-5 cells) were obtained by pipetting twice the entire volume. Cell suspensions were centrifuged at 300g for 4 min, resuspended in naïve medium with 10 μM Y27632 Rho-associated kinase (ROCK) inhibitor (Ri, Axon Medchem) and seeded on ~300 MEF per mm^2. ROCK inhibitor was used only for 24 h after passaging. BJ (passage 12) and HFF-1 (passage 18) human foreskin fibroblasts (ATCC), WI-38 and IMR-90 female lung fibroblasts (ATCC), and primary skin fibroblasts of an 80-year-old female donor were cultured in DMEM with 10% fetal bovine serum (FBS, Sigma-Aldrich) before reprogramming. Somatic cells were cultured in normoxia (21% O_2, 5% CO_2, 37 ºC), pluripotent stem cells were cultured in hypoxia (5% O_2, 5% CO_2, 37 ºC) with daily medium changes. All cell lines were mycoplasma-negative (Mycoalert, Lonza). Low passage and karyotipically normal Primed human ES cells H9 (WA09) were obtained from and used under authorisation from WiCell Research Institute. Primed human ES cells were cultured on Matrigel-coated (Corning) conventional plates in mTeSR1 (StermCell Technologies). Alternatively, for the reprogramming of primed human ES cells to naïve (see below), primed cells were cultured on MEF feeders in DMEM-F12 supplemented with 20% KSR (Gibco) and 10 ng ml^{-1} bFGF (produced in house). The primed iPSCs generated in this study (HPD00) were cultured on Matrigel in FGF2- and TGFβ-containing expansion medium mTeSR1.

Microfluidic chips production. Microfluidics chips were produced as previously reported[18]. Briefly, Sylgard 184 (Dow Corning) was cured on a 200-µm-thick patterned SU-2100 photoresist (MicroChem) in order to obtain a single polydimethylsiloxane (PDMS) mold with multiple independent channels. The PDMS mold was punched and sealed on a 75 × 25 mm microscope glass slide (Thermo Scientific) by plasma treatment (Harrick). Channels were rinsed with isopropanol and distilled water to check proper flow before autoclaving. The height of each channel is 200 µm and the area is 27 mm^2. Each channel holds, considering the inlet, the outlet and the culture channel itself, 10 µl of medium (see Supplementary Fig. 1a).

Reprogramming. Naïve human iPSCs (niPSCs) or primed iPSCs were generated via mmRNA-mediated reprogramming of somatic cells using the same microfluidic platform reported in our previous work[18,44] (Supplementary Fig. 1a). Previous studies showed that mmRNA transfection leads to transient production of the proteins of interest, with a peak of production between 12 and 24 h, and complete clearance at 42 h after transfection[22]. Thus, to guarantee stable protein expression we transfected cells daily. Moreover, for analysis of primary colonies by immunofluorescence, qPCR or RNA sequencing we waited at least 3 days since the last round of transfection - to allow full clearance of the mmRNAs. Microfluidic channels were coated with 25 µg ml^{-1} fibronectin (Sigma-Aldrich) for 30 min at room temperature. Somatic cells were seeded on day -1 of reprogramming at 25 cell mm^{-2} for feeder-free reprogramming in DMEM/10% FBS. Such seeding density allowed robust proliferation and survival of somatic cells during reprogramming. Of note, a density of 10 cell mm^{-2}, optimal for generation of primed iPSCs[18], did not allow generation of niPSCs. On day 0, 2 hours before the first mmRNA transfection, the medium was switched to a medium optimised from mRNA transfection and previously used for derivation of primed PSCs (StemMACS ReproBREW XF, Miltenyi biotec). We refer to this medium as Primed Reprogramming Medium (PRM). When indicated, we added 5 µM Ri and 1 µM CH to PRM. In order to maximize the delivery of transgenes due to a slightly lower transfection efficiency in PRM+CH, we extended the usual transfection period[18], from 4 h to 9 h. Cells were transfected daily at 9 am, and fresh PRM was given daily at 6 pm. On day 6, medium was changed with a naïve-supporting medium, and the same regime of transfection and fresh medium change was maintained until day 12. From day 0 to 12, media were supplemented with 200 ng mL^{-1} B18R (eBioscience) in order to suppress single-strand-RNA-induced immune response mediated by type I interferons. Transfections using a *OSKMN+nGFP* mmRNA mix (*POU5F1*, *SOX2*, *KLF4*, *c-MYC*, *NANOG*, *nuclear GFP*, provided by S. Wild, M. Jurk from Miltenyi biotec) were started on day 0 and daily repeated for 12 d. A mix without *nGFP* mmRNA

was used from day 5 to perform any staining at the end of reprogramming. We used an incremental dosage of mmRNAs during the first three daily transfections, with 50%, 75%, 100% mmRNA amount of subsequent transfections at 0.28 ng mm^{-2}. The transfection mix was prepared according to the StemMACS mRNA transfection kit (Miltenyi biotec): 100 ng μl^{-1} mmRNA mix of *OSKMNG*, with stoichiometry 3:1:1:1:1:1, was diluted in transfection buffer (TB) by mixing 10 µl of mmRNA mix with 30 µl of TB. Transfection reagent (TR) was diluted separately in TB by mixing 3 µl of TR with 37 µl of TB. The two solutions were mixed (final volume=80 µl) and incubated for 20 min. For each microfluidic channel, we diluted 1.2 µl of transfection solution in 8.8 µl of medium (either PRM or naïve medium) and added to the cells (corresponding to 0.28 ng mm^{-2}). In well, the transfection solution was added dropwise before gently rocking the plate. For each well of 12-well plate we used 46.3 µl of transfection solution in 0.8 ml of medium, which corresponds to 1.5 ng mm^{-2}.

Reprogramming was performed in hypoxia if not stated otherwise. Primed-like colonies present after reprograming under CCC (Fig. 8) were selectively removed by addition of 1 µM PD173074 (PD17).

Reprogramming of fibroblasts towards primed-state was achieved using the protocol described above, keeping PRM until day 12. Importantly, to allow a direct comparison of the reprogramming trajectories towards primed and naïve pluripotency, we used the same seeding density of 25 cell mm^{-2} and the same OSKMN mmRNA cocktail, and not the conditions used in Luni et al (i.e., 10 cell mm^{-2} and OSKMN+LIN28).

When we tested the effect of different microfluidics channel heights (Fig. 8f), the same mmRNA amount per cell was daily delivered, independently of the microfluidic configuration (100 to 1000 µm culture channel heights). For each configuration, mmRNA dosage was progressively increased as in standard protocol (200 µm height) to accommodate cell proliferation.

Reprogramming of primed H9 to naïve was performed as in Takashima et al. 2014[20]. Primed H9 cultured on Matrigel-coated plates in mTeSR1 were cotransfected with piggyBac (PB) constructs carrying doxycycline-inducible *NANOG* (0.5 µg) and *KLF2* (0.5 µg) coupled with Venus fluorescent protein, a PB construct carrying a constitutive Tet3G (1 µg) and a pBase plasmid (2 µg) using the FuGENE Transfection Reagent (Promega, ratio 3.5:1). After 2 days, 10 µg ml^{-1} Zeocyn (InvivoGen) was applied. After two weeks of selection, cells were conditioned on MEF feeders in FGF/KSR, then dissociated as single cells with TrypLE and replated on day -2 in presence of ROCK inhibitor for 24 hours in microfluidics and CCC at a density of 4 cells mm^{-2}. Medium was changed daily in CCC and twice a day in microfluidics (9 am and 6 pm). On day -1, cells were incubated with FGF/KSR supplemented with doxycycline 1 µg ml^{-1} (DOX, Thermo Scientific). From day 0, medium was switched to 2iL with DOX. On day 14, cells were fixed and stained with anti-TFCP2L1 antibody. FGF/KSR + DOX was used as control both in CCC and microfluidics.

In vitro differentiation. For germ layer differentiation, niPSCs were seeded on 1% Matrigel-coated coverslips with 100 MEF mm^{-2} and cultured in RSeT medium for 2 days. Germ layer-specific media were used thereafter with daily medium changes; ectoderm medium, for 3 days: KO DMEM (Thermo Scientific), 15% KSR, 1% non-essential aminoacids (NEAA), 1% L-glutamine, 0.1 µM LDN193189 (Miltenyi biotec) and 20 ng ml^{-1} hFGF2 (Peprotech); for the following 9 days hFGF2 was replaced with 10 µM SB431542 (Miltenyi biotec); mesoderm medium, for 6 days: RPMI with 2% B27 (Thermo Scientific), 20 ng ml^{-1} hFGF2 (Peprotech), 50 ng ml^{-1} hBMP4 (R&D), 3 µM CH (only first 2 d); endoderm medium, for 6 days: RPMI with 2% B27, 100 ng ml^{-1} Activin-A (Peprotech), 3 µM CH (only first 2 d). Embryoid bodies (EB): niPSC colonies were mechanically scratched with a tip and transferred in ultra-low adhesive wells (Corning) in the presence of DMEM, 20% FBS, 200 mM L-glutamine, 1% NEAA, 0.1 mM 2-mercaptoethanol[20]. Medium was changed every other day for 15 days before plating EBs on 1% Matrigel-coated glass plates (Labtek). After 5 days, adherent and spread cells were fixed for immunostaining.

Neuronal differentiation. Neuronal differentiation protocol was adapted from literature[43]. Naïve iPSCs were seeded as single cells at high density (530 cell mm^{-2}) on Matrigel-coated plates. Cells were cultured 2 days in RSeT. On day 0, cells were cultured in N2B27, 1% NEAA, 200 ng ml^{-1} L-Ascorbic Acid (Neural Medium, NM) supplemented with 20 ng ml^{-1} bFGF and 0.1 µM LDN193189. Medium was refreshed daily. On day 3, medium was switched to NM supplemented with 0.1 µM LDN193189 and 10 µM SB431542. On days 4-9, the same medium was supplemented with 1 µM all-trans Retinoic Acid (RA, Sigma Aldrich) and 1 µM SAG (Calbiochem) and was refreshed daily. On days 10-15, medium was switched to NM supplemented with 5 µM DAPT (Sigma Aldrich) and 4 µM SU-5402 (Sigma Aldrich), 1 µM RA and 1 µM SAG, and was refreshed daily. On day 16, cells were dissociated with TrypLE for 10 minutes; 2 volumes of NM were added to inhibit dissociation. Cells were seeded on Matrigel-coated glass coverslips in well and cultured in maturation medium based on NM supplemented with 20 ng ml^{-1} BDNF, 10 ng ml^{-1} GDNF, 10 ng ml^{-1} CNTF (PeproTech), and 10 µM ROCK inhibitor for the first 24 hours. Fresh maturation medium was provided daily up to day 22 when cells were fixed for immunostaining.

Hepatic differentiation. Hepatic differentiation protocol was adapted from[41,42]. Briefly, niPSCs were seeded as single cells (20 cell mm^{-2}) on Matrigel-coated plates with sparse MEF (50 cell mm^{-2}). Cells were cultured for 2 days in RSeT and medium. On day 0, cells were cultured in RPMI, 2% B27, 3 µM CH. Same medium with the addition of 100 ng ml-1 Activin-A was refreshed on day 1-2. On day

3-8, medium was switched to KO-DMEM with 20% KSR, 2 mM L-glutamine, 1% NEAA, 0.1 mM 2-mercaptoethanol, 1% DMSO, and changed every other day. On day 9-15, cells were cultured in maturation medium based on L15 basal medium, 8% FBS, 8% tryptose phosphate broth (Sigma), 10 μM hydrocortisone (Sigma), 1 μM insulin, 2 mM L-glutamine, 10 ng mL^{-1} HGF (Peprotech), 20 ng ml^{-1} Oncostatin-M (Peprotech). Fresh maturation medium was provided every other day.

Immunofluorescence and stainings. Immunofluorescence analysis was performed either on 1% Matrigel-coated glass coverslips in wells or *in situ* in microfluidic channels with the same protocol. Cells were fixed in 4% (w/v) paraformaldehyde (Sigma-Aldrich) in PBS for 10 min and blocked in 5% horse serum with 0.3% (v/v) Triton-X-100 (Sigma-Aldrich) for 1 h. Blocking buffer:PBS 1:2 was used to dilute primary antibodies (Supplementary Table 3). For staining with 5-methylcytosine (5mC), fixed samples were permeabilized with 0.5% Triton X-100 for 1 h and treated with 2 N HCl for 30 min at room temperature to denature DNA. Samples were neutralized with PBS before blocking and antibody incubation. Alexa488, Alexa568, or Alexa647-conjugated anti-rabbit, anti-mouse, or anti-goat secondary antibodies raised in donkey (A-21206, A10042, A-21202, A-31571, A10037, A-11057, A-11055, Thermo Scientific) were incubated at room temperature for 45 min at 1:500. Nuclei were stained with either Hoechst 33342 (Thermo Scientific) or DAPI (Sigma-Aldrich). For mitochondrial staining, cells were incubated with 1:50000 TMRM and 1:20000 MitoTracker (T668, M7514, Thermo Scientific) for 30 min in culture medium at 37 ºC and washed twice in PBS before image acquisition. EdU staining. Cells were exposed to an EdU pulse of 1 h before fixation in formaldehyde for 15 min. Samples were processed with Click-iT EdU Alexa Fluor 488 Imaging kit and counterstained with Hoechst nuclear dye (all Thermo Scientific). Fluorescence images were acquired through a Leica SP5 II confocal system or a Leica 6000B epifluorescence microscope.
For AP staining, cells were fixed with a citrate-acetone-formaldehyde solution and stained using an alkaline phosphatase kit (Sigma-Aldrich). Plates were scanned using an Epson scanner and scored manually.

Flow cytometry and cell sorting. Single cells in suspension were obtained by incubating samples for 5 min with TrypLE. For DNA-content analysis by flow cytometry, cells pellets fixed in cold 70% ethanol were resuspended in PBS, incubated with 50 μg ml^{-1} propidium iodide, and processed with FACSCanto™ II (BD). For cell sorting based on DNA-content, cells were resuspended in naïve medium with 10 μM ROCK inhibitor and 5 μM Vybrant DyeCycle Ruby live stain (Thermo Scientific) for 30 min before sorting. Sorted samples were cultured on new MEFs with 10 μM Ri for 24 h. See Supplementary Figure 3c for gating strategy.

Karyotype. Cells were incubated with 0.06 μg ml^{-1} KaryoMAX (Thermo Scientific) in culture medium for 6 h at 37 °C. niPSCs were isolated with ReLeSR (Stemcell technologies) and resuspended in PBS before centrifugation. Pellet was resuspended in pre-warmed 75 mM KCl for 10 min at 37 °C. After centrifugation, pellet was gently resuspended in 1 ml of freshly-prepared fixative (3:1 methanol:acetic acid). This step was repeated twice. Q-banded fixed samples were analysed by Research & Innovation S.p.A. (Padova, Italy).

Quantitative PCR. Total RNA was isolated using Total RNA Purification Kit (Norgen Biotek), and complementary DNA (cDNA) was made from 500ng using M-MLV Reverse Transcriptase (Invitrogen) and dN6 primers. For real-time PCR SYBR Green Master mix (Bioline. Cat. BIO-94020) was used. Primers are detailed in Supplementary Table 4. Three technical replicates were performed for all quantitative PCR. GAPDH was used as endogenous control to normalize expression.

RNA-Seq. niPSCs were preferentially isolated from MEFs with ReLeSR. Briefly, cells were washed with PBS, incubated with ReLeSR for 60 s, and left with a film of liquid for 7 min. N2B27 medium was added and pipetted 4-5 times to detach colonies. Total RNA was isolated as above and sequenced with an Illumina NexSeq500, in 75bp pair-end format. Libraries were prepared following standard protocols from Illumina using TruSeq Stranded mRNA Library Kit. We produced a total of ~249M reads from the 13 samples (3 fibroblasts, 1 primed hiPSC and 9 naïve cell lines; 19M reads per sample on average). Expression levels for all the genes from ENSEMBL 87 were quantified using RSEM 1.3.0[50] with STAR 2.5.2b[51] (human genome GRCh38.p7). We built the genome index for STAR alignment using "rsem-prepare-reference" with options '--star-sjdboverhang' set to mean read length minus 1 according to STAR guidelines. Alignment and quantification were performed with default parameters using stranded pair-end mode. Gene expression level quantification of 21 samples collected from public available datasets has been done as described for the in-house samples. The RSEM parameters were set according to the library design (mean reads length, stranded/non-stranded, paired/single end, see Supplementary Table 5). We generated the final expression matrix excluding the genes that did not have more than 10 raw counts in at least 3 out of 34 samples. After this filter, we obtained the expression of 20936 genes.

All RNA-seq statistical analyses were carried out in R environment (version 3.4.3) with Bioconductor 3.6. We computed the differentially expressed genes (DEG) among the 3 groups (primed, naïve, fibroblast) using edgeR [52] (function call "fit = glmQLFit(counts, design=~group); glmTreat(fit, coef=n, lfc=2)" where 'n' is one of the contrasts). A gene was considered a DEG when the absolute

value of log2 fold change was higher than 2 and the adjusted p-value was smaller than 0.05 (p.adjust function, Benjamini-Hochberg method). Principal component analysis (PCA) was performed using log2-normalized pseudo-counts (defined as count plus 1) with prcomp function with default parameters using DEGs. Heatmaps were made using the log2-normalized pseudo-counts (unless stated otherwise) with pheatmap function from pheatmap R package (version 1.0.8, distance used 'euclidean', 'ward' linkage, scale='row') on DEGs or selected markers. The raw counts were normalized using *betweenLaneNormalization* function with upper quantile method (EDAseq R package)[53].

For the comparison between our RNA-Seq dataset and the DGE-Seq dataset from Kilens et al. presented in Supplementary Fig. 4c we first obtained expression values in UMI Per Million (UPM) from Supplementary Data 1 of Kilens et al., while our dataset was transformed into Transcripts per Million (TPM). Both datasets were quantile normalized and z-scored by row separately, as described in Kilens et al. We merged the two datasets by gene id to obtain a 14,944 genes merged dataset. PCA was computed using 4606 genes obtained intersecting the merged dataset and the DEGs used for the RNA-Seq comparison in the PCA shown in Fig. 3c.

Transposon analysis. Transposons coordinates were downloaded from UCSC repeat masker track (hg38). We filtered out those transposons that overlap any genomic features annotated in ENSMBL 87. We aligned the raw reads using bowtie2 sensitive and end-to-end mode on the human genome GRCh38.p7. Transposon expression was quantified using bamtools multicov (v2.26.0).

We analysed transposons with at least 20 cpm (count per million) in at least one sample. Differentially expressed transposons were identified using edgeR R package. A transposon was considered differentially expressed when the p-value was lower or equal to 0.05 and the log2 fold change greater or equal to 2. Heatmap was made using the log2-normalized pseudo-counts scaled by row means with pheatmap R package as describe for RNA-seq data.

SNP analysis for imprinting and X re-activation. We extracted, from the alignments, the reads with a minimum quality of 30 (samtools view –q 30 align.bam align-f.bam). Using GATK haplotypeCaller (genotyping_mode: DISCOVERY, minReadsPerAlignmentStart: 5, max_alternate_alleles: 1, stand_conf_call: 1 and filter_reads_with_N_cigar) we generated allele counts over the SNPs of dbSNP human version b149. Non bi-allelic variants as well as In-DELs were filtered out. Moreover, we considered only SNPs with at least 10 reads in at least one sample. A SNP was considered heterozygous when the ratio of the counts between minor and major alleles was greater than 0.2 and the minor allele has at least 5 reads. Minor allele is the allele with fewer counts.

The loss of imprinting (LOI) of a gene was quantified as the number of SNP that shows heterozygosity in each gene. We called a LOI when at least 2 SNPs show heterozygosity in a gene. We tested LOI on the genes associated to the imprinted regions defined for the methylation analysis.
X re-activation (Xa) was quantified selecting the heterozygous SNP from X-cromosome genes and by plotting the minor/total allele count ratios of heterozygous SNP (defined as above). We excluded the pseudo-autosomals regions (PAR1: chrX:10,000-2,781,479 and chrY:10,000-2,781,479; PAR2: chrX:155,701,383-156,030,895 and chrY:56,887,902-57,217,415) from the analysis.

Methylation analysis. RRBS libraries were produced using the Ovation RRBS Methyl-Seq System (NuGEN) starting from 100ng of genomic DNA extracted with the Quick-DNA Plus kit (Zymo) - according to manufacturer's specifications. Libraries were sequenced on a NextSeq 500 (Illumina) using a single-end 75-cycle high-output flow cell. Sequence reads were first trimmed using Trim Galore software[54] (version 0.4.1) to remove adapter sequences and low-quality end bases and, then, trimmed with a custom python script provided by NuGEN Technical Support (version 1.11) to remove any read that did not contain a MspI site signature (YGG) at the 5'-end. Reads alignment on hg19 reference sequence and methylation calling were thus performed with bismark[55] (version 0.18.1). Coordinates of DMRs were obtained from literature[32,35]. Subsequently, average DNA methylation levels and total coverage for each DMR regions were determined for all Bismarck processed RRBS data files using R and the methylkit package[56]. To that end, RRBS data files were processed with the methRead function and the bismarkCoverage parameter set. Next, the regionCounts function was used to determine the number of methylated and unmethylated C's in each of the DMRs in each sample. These values were then combined using the unite function with the min.per.group paramater set to 1. Finally, we only retained those regions that were covered by at least 5 reads and computed a coverage weighted average methylation level for each region across all the CpGs that were covered. These values were then plotted in Fig. 4d-f and listed in Supplemenary Table 2 along with the genomic coordinates of each genomic region on hg19.

Image analysis. Fiji 1.0 (ImageJ2) was used for image analysis. The size of niPSC colonies (Fig. 2d) was measured by delimiting a colony area and calculating the equivalent diameter as (area/π)^0.5 (Shape filter plugin function). Fluorescence intensity (H3K9me3 or 5mC) across nuclei (Fig. 4a-b and Supplementary Fig. 5a) was measured with the Plot Profile function. For each cell line we selected representative fields and 6 nuclei were randomly chosen, as in Takashima et al. 2014[20].

Statistics and Reproducibility. For statistical analyses, normality assumption was tested with D'Agostino-Pearson normality test in Prism (Graphpad) and Kruskal-Wallis test was used with non-normal datasets. Sample size calculation was not performed. Sample size was chosen based on standards in the field and is indicated in figure legends and in Supplementary Table 2, which reports values for all technical replicates of each dataset in this study. All key experiments were repeated independently at least two times and, when possible, using different cells lines (e.g. reprogramming of 5 different somatic cell types or differentiation of 4 different niPSC lines). For reprogramming experiments under conventional condition we performed at least two independent experiments, while for microfluidic experiments we performed up to 8 independent experiments, with 5 to 60 technical replicates. All differentiation experiments were performed at least in two biologically independent experiments, using between 2 and 4 different niPSCs lines.

Figure Legends

Figure 1. Devising a strategy for transgene-free generation of naïve-like iPSCs. (**a**) Primary colonies of primed iPSCs generated in well (conventional culture conditions, CCC) or microfluidics (μF) with modified messenger RNAs (mmRNAs) underwent differential expression analysis, revealing (**b**) increased expression of naïve-associated markers in μF compared to CCC. Mean and s.e.m., *n*=4 biologically independent samples. Data from Luni et al., 2015. (**c**) OSKMN mmRNAs transfection in presence of Primed Reprogramming Medium (PRM) led to formation of primed hiPSC colonies at day 14 (middle, left panel), expressing DPPA5 (black bar of bottom panel, see also Supplementary Fig. 1b). Exposure of primary colonies to 2iL transiently upregulated DPPA5 expression (grey bars), but did not allow formation of naïve-like iPSCs. *GAPDH* served as a loading control. *n*=1 biologically independent experiment. (**d**) Evaluation of the effect of different inhibitors during the first 6 days in PRM. CH promotes colony formation and allows efficient transfection (Supplementary Fig. 1d). *n*=2 biologically independent experiments, indicated as red and purple dots. (**e**) Fibroblasts reprogramming using PRM+CH for 6 days followed by 2iLGo-KSR. Fibroblasts convert from a spindle-like (day 1) to an epithelial-like morphology (day 5-6). Cells become progressively compact (day 7) and small colonies emerge from day 9 (arrowheads). Representative pictures of 8 independent experiments. (**f**) Primary colonies emerged at day 12 and were cultured for 3 days without mmRNAs to allow their complete clearance. Immunostaining reveals expression of naïve markers and absence of SSEA4. Photos from 1 representative experiment. (**g-h**) ROCK inhibitor (Ri) and Hypoxia (Hyp) increased both the success rate and efficiency of reprogramming. (**g**) Each bar indicates the success rate of each independent experiment, calculated as the percentage of technical replicates (numbers above bars) containing naïve-like colonies. (**h**) Efficiency of generation of naïve-like colonies. See also Supplementary Fig. 1f. Scale bars 200 μm (c, e), 10 μm (f). All statistical tests performed are defined in Methods. In b, c, d, and h bars indicate means, dots indicate technical replicates, whose values are provided in Supplementary Table 2.

Figure 2. Optimized generation and stabilization of the naïve phenotype. (**a**) Optimised naïve reprogramming setup. (**b**) Effect of 3 different naïve media on generation of naïve-like colonies. 2iLGo-KSR+Ri (modified from Takashima et al., 2014), 4iLA[27] and RSeT were applied at day 6. Primary colonies were quantified at day 12 either by their compact morphology (green dots) or by the expression of KLF17 and POU5F1/OCT4 (red dots) after immunostaining. *n*=2 biological experiments indicated in two different shades of red or green. (**c**) Morphology of representative colonies obtained with different naïve media. (**d**) Quantification of colony size by measure of the

equivalent diameter (see Methods). Analysis of >30 colonies from 5 technical replicates of 1 representative experiment. (**e**) Gene-expression analysis by qPCR of niPSCs, primed PSCs and human naïve Reset H9 cells. Expression relative to Primed H9 ES cells was calculated. *GAPDH* served as loading control. *n*=1 biologically experiment. (**f**) The primed pluripotency markers *OTX2* and *ZIC2* are down regulated in niPSCs. Expression relative to HPD00 Primed iPSCs. *GAPDH* served as loading control. *n*=1 biologically experiment. (**g**) Immunofluorescence staining of entire microfluidic channels containing primary colonies at day 12. See Supplementary Fig. 2a. *n*=2 biological experiments. (**h**) Naïve associated markers are expressed after extensive culture (p14) without detectable expression of primed- or differentiation-associated markers. *n*=2 biological experiments. (**i**) efficiency of niPSC generation from different somatic cells. *n*=3 biologically independent experiments for HFF-1 indicated in different shades of green; *n*=1 biologically independent experiment for WI38, IMR90 and Fibroblasts XX 80 y.o. (**k**) Clonal assay performed by plating 2000 cells in a well of a 12 well plate with feeder cells. HPD01 niPSCs in RSeT display high clonogenic capacity, comparable to Reset H9 cells in 2iLGo[20]. See Supplementary Fig. 3b for results obtained in 5 other niPSCs. *n*=2 biologically independent experiments (**l**) Left: Q-banding showing normal karyotype in HPD01 line at passage 19. Similar results obtained in 7/7 metaphases. See also Supplementary Fig. 3d. Scale bars, 50 µm (c, h), 1 mm (g, j). In b, d, e, f and i bars indicate means, dots indicate technical replicates, whose values are provided in Supplementary Table 2.

Figure 3. A naïve state transcriptome is rapidly acquired during niPSCs generation

(**a**) Heatmap of unsupervised hierarchical clustering based on markers highly expressed in human naïve ESCs and human embryos, (e.g. *TFCP2L1*, *KLF17*, *DPPA5* and *KLF4*)[20,21,28,57], or specifically expressed in primed PSCs (e.g. *ZIC2*, *ZIC5*, *SOX11*)[21] or in fibroblasts (e.g. *VIM*, *FN1* and *CD44*)[58–60]somatic and pluripotency associated markers. The naïve (red) and primed (blue) iPSCs generated in this study cluster together with previously characterised PSCs. Values displayed correspond to the gene expression level (normalized log2 pseudo-counts) scaled by the row mean. (**b**) Heatmap of unsupervised clustering based on Transposable Elements expression[27] in primed and naïve PSCs. Values displayed are transposon expression level (normalized log2 pseudo-counts) scaled by row mean. (**c**) Principal components analysis (PCA) of RNA-sequencing samples. Inset shows naïve PSC lines derived in this study. As an internal control, we sequenced the transcriptome from Reset H9[20] cells cultured in parallel to niPSCs. See also Supplementary Fig. 4a for heatmap of unsupervised clustering based on transcriptome. (**d-f**) Gene-expression analysis by qPCR of reprogramming trajectories in microfluidics. Fibroblasts were plated at the same density and transfected with

OSKMN either in PRM (blue) to generate primed iPSCs, or in conditions allowing niPSCs formation (red), as in Fig. 2a. Samples were collected at Day 1, 5, 8, 10 and 12 and the expression of pluripotency markers was analysed. *n*=2 biologically independent experiments. See also Supplementary Fig. 4d. **d,** PCA analysis shows that reprogramming towards primed and naïve pluripotency clearly diverge at day 8. **e,** Expression of the indicated markers during reprogramming to either naïve or primed pluripotency (red and blue, respectively). Expression relative to the highest value was calculated. GAPDH served as loading control. **f**, Reduced expression of the Mesenchymal markers (grey) and gradual increase in Epithelial (orange) markers at day 5 and 8 or reprogramming. Expression relative to the highest value was calculated. GAPDH served as loading control. Bars indicate means, dots indicate technical replicates, whose values are provided in Supplementary Table 2.

Figure 4. Global reduction in repressive epigenetic modifications in niPSCs. (**a-b**) niPSCs show significant lower histone H3K9me3 (**a**) and DNA (**b**) methylation compared to primed PSCs by immunofluorescence staining. Left: representative images. Right: staining intensity quantification by image analysis of 6 randomly selected nuclei. See Supplementary Fig. 5 and Methods. (**c**) Expression of known regulators of DNA methylation in primed and naïve PSCs measured by RNA-seq. (**d**) Average genome-wide CG methylation levels measured by RRBS in somatic (grey), primed (blue) and naïve (red) iPSCs. Reset H9 cells[20] were also included as control. See Supplementary Fig. 5b. (**e**) Unsupervised hierarchical clustering of the genome-wide methylation pattern separates niPSCs from somatic and primed cells. (**f**) Violin plots showing DNA methylation levels on 52 promoters identified[20,32] as hypomethylated in pre-implantation blastocysts relative to primed hESCs. Box plots show medians and first and third quartiles. Violins span the interval between the maximal and minimal value of each sample. See also Supplementary Fig. 5b. Scale bars (a-b) 10 μm.

Figure 5. Analysis of imprinted and X-linked genes status. (**a**) Methylation levels at imprinted loci. Maternal, or Paternal, loci are those where the maternal, or the paternal, alleles are methylated[35]. Placental loci are characterised by transient methylation of the maternal allele in oocytes and blastocysts that is then lost in somatic cells. Each dot indicates the average methylation level at an imprinted locus. Black bars indicated the mean methylation levels of each class of loci. Somatic and primed cells show robust methylation at Maternal and Paternal loci, while niPSCs retain only partial methylation at Paternal loci. *n*=1 biologically independent experiment. See Supplementary Table 2.

(**b**) Methylation patterns at the human *IGF2* and *MEG3* loci. The y axis indicates the level of methylation. Imprinted regions are highlighted in light blue. (**c**) SNPs analysis of the *MEG3* transcript reveals monoallelic expression in BJ fibroblasts and biallelic expression in 3 isogenic niPSC lines. (**d**) Violin plots show methylation levels at CpG island on X chromosome and at chromosome 19. Methylation on Chr. 19 is reduced to the same extent in male and female niPSCs, compared to fibroblasts. Methylation on Chr. X is very low in both male cells and female niPSC, compared to female fibroblasts. Violins span the interval between the maximal and minimal value of each sample. *n*=1 biologically independent sample. (**e**) SNPs analysis of X-linked genes. Top: histograms showing the fraction of SNPs expression. Female fibroblasts show monoallelic, while female niPSCs show biallelic expression. Bottom: vertical bars indicate the position of the SNPs analysed on the X chromosome. (**f**) Expression levels measured by RNAseq of *XIST*. Female niPSCs express high levels of *XIST*. The Y-linked gene *USP9Y* is shown as a control.

Figure 6. Enhanced mitochondrial activity in niPSCs.

(**a**) TMRM fluorescent accumulation in niPSCs shows a significant increase in mitochondrial activity compared to primed PSCs. Left: representative images of PSC colonies. MitoTracker stains all mitochondria, while TMRM signal is dependent on mitochondrial membrane potential. *n*=5 technical replicates from 1 representative biological experiment. (**b**) Genes encoded by the mitochondrial DNA are upregulated in naïve PSCs compared to primed PSCs. Gene-expression analysis by qPCR of the indicated PSCs lines for 3 transcripts of the mitochondrial genome. Expression relative to Primed H9 ES cells was calculated. GAPDH served as loading control. Bars indicate means, dots indicate technical replicates. n=2 biologically independent experiments showing comparable results. (**c**) Analysis by RNAseq of the indicated mitochondrial transcripts in fibroblasts and niPSCs. Expression was normalised to BJ fibroblasts. Scale bars (a, left) 50 μm, (a, right) 25 μm. See Supplementary Table 2 for source data.

Figure 7. Characterization of the differentiation potential of niPSCs

(**a**) Monolayer differentiation of 3 isogenic niPSC lines (HPD01, HPD03 and HPD04). Markers of Mesoderm and Endoderm were detected in all lines at day 6 of differentiation, while Neurectoderm markers were detected at day 12. *n*=4 biologically independent experiments. See Supplementary Fig. 6a. (**b**) niPSCs form embryoid bodies (EBs). After 15 days of culture in suspension, EBs were plated on Matrigel to allow the spreading of differentiated cells for 7 days. Immunostaining confirms expression of markers of the 3 germ layers. *n*=4 biologically independent experiments. See Supplementary Fig. 6b. (**c**) Starting from BJ human fibroblasts we generated isogenic primed (HPD00) or naïve PSCs (HPD01/3/4) using mmRNAs in µF. Expression of markers of the three germ layers was measured by qPCR before and after 50 days of EB differentiation. Early, mid and late markers are shown in different shades of colours. Robust and comparable induction of differentiation markers was observed in niPSCs and primed iPSCs. For each preparation, we pooled at least 15 EBs that were >0.5mm in size. See also Supplementary Fig. 6c. Data are expressed as log2 fold change relative to the highest value. GAPDH served as loading control. *n*=2 independent experiments. (**d**) *In vitro* hepatic differentiation of niPSCs generated mature polygonal hepatocyte-like cells expressing the specific markers HNF4A and CYP3A. *n*=2 biologically independent experiments conducted in 2 niPSC lines. See also Supplementary Fig. 7. (**e**) *In vitro* differentiation of niPSCs in mature neurons. At day 22 of differentiation, we observed cells positive for the pan-neuronal markers TUJ1, NEUN and MAP2. We should stress that the efficiency of hepatic and neural differentiation was higher in HPD01 and HPD06, indicating the need for protocols optimised for naïve PSCs. n= 2 biologically independent experiments. See also Supplementary Fig. 7. Scale bars: 50 µm (a, b, e, immunofluorescence in d). 100 µm bright field in (d).

Figure 8. Comparison of naïve iPSCs generation under microfluidics and conventional culture conditions. (**a**) Comparison of efficiency of our optimised protocol in microfluidics vs conventional culture conditions (CCC). The number of naïve colonies generated for 100 cells seeded is shown. *n*=2 biological independent experiments shown in different shades of green. (**b**) At day 12 in well, a mixed population of naïve- and primed-like colonies emerged (red and blue circles). *n*=2 biologically independent experiments. (**c**) Clearance of primed-like colonies after culture in presence of RSeT + FGF receptor inhibitor PD173074 for 6 passages. Colonies with primed morphology (blue arrowheads) were still present at p4, together with naïve-like colonies (red arrowheads). *n*=2 biologically independent experiments. (**d**) niPSCs generated in CCC (HPD05) show an expression profile comparable to microfluidic-derived niPSCs and previously derived naïve PSCs. Quantification by qPCR relative to Primed H9 hESCs. GAPDH served as a loading control. *n*=1 biological experiment. (**e**) The transcriptomes of isogenic niPSCs generated in CCC and µF are highly similar (Pearson Correlation coefficient R=0.980, see also Fig. 3c), and distinct from primed iPSCs. Naïve- (red) and primed-specific genes (blue) are indicated. *n*=1 biological experiment. (**f**) BJ fibroblasts were reprogrammed with our optimised protocol in µF channel of different heights. At day 12, immunostaining for KLF17 and POU5F1 was conducted and colonies were scored. In red double positive colonies are shown, in green the colonies identified by their naïve-like morphology. The percentages of double positive colonies out of total colonies at different heights is indicated. *n*=2 biological independent experiments shown in different shades of red and green. See also Supplementary Fig. 7c. (**g**) Conversion of primed H9 into naïve pluripotent colonies in both µF and CCC using DOX-inducible *NANOG-KLF2* transgenes in presence of 2iL[20], or conditions supporting primed pluripotency (FGF/KSR). After 14 days colonies were stained for the naïve-specific marker TFCP2L1 and counted. *n*=2 biological independent experiments. (**h**) Model of human somatic cell reprogramming routes. Scale bars 100 µm (b-c, g), 1 mm (b, tiling). In a, d, f and g bars indicate means, dots indicate technical replicates, whose values are provided in Supplementary Table 2.