Nanog Overcomes Reprogramming Barriers and Induces Pluripotency in Minimal Conditions

Thorold W. Theunissen,1 Anouk L. van Oosten,1 Gonzalo Castelo-Branco,2 John Hall,1,3 Austin Smith,1 and José C.R. Silva1,4
1Wellcome Trust Centre for Stem Cell Research and Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QR, UK
2Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, Tennis Court Road, Cambridge CB2 1QH, UK
3Current address: Cancer Research UK Paterson Institute, The University of Manchester, Manchester M20 4BX, UK
4Present address: Cancer Research UK Paterson Institute, The University of Manchester, Manchester M20 4BX, UK

Summary

Induced pluripotency requires the expression of defined factors and culture conditions that support the self-renewal of embryonic stem (ES) cells [1]. Small molecule inhibition of MAP kinase (MEK) and glycogen synthase kinase 3 (GSK3) with LIF (2i/LIF) provides an optimal culture environment for mouse ES cells [2] and promotes transition to naive pluripotency in partially reprogrammed (pre-IPS) cells [3]. Here we show that 2i/LIF treatment in clonal lines of pre-IPS cells results in the activation of endogenous Nanog and rapid downregulation of retroviral Oct4 expression. Nanog enables somatic cell reprogramming in serum-free medium supplemented with LIF, a culture condition which does not support induced pluripotency or the self-renewal of ES cells, and is sufficient to reprogram epiblast-derived stem cells to naive pluripotency in serum-free medium alone. Nanog also enhances reprogramming in cooperation with kinase inhibition or 5-aza-cytidine, a small molecule inhibitor of DNA methylation. These results highlight the capacity of Nanog to overcome multiple barriers to reprogramming and reveal a synergy between Nanog and chemical inhibitors that promote reprogramming. We conclude that Nanog induces pluripotency in minimal conditions. This provides a strategy for imposing naive pluripotency in mammalian cells independently of species-specific culture requirements.

Results and Discussion

Investigating the Response to Kinase Inhibition in Clonal Lines of Pre-IPS Cells

Pre-IPS cells have successfully acquired a proliferative capacity but have not yet attained the transcriptional and epigenetic hallmarks of naive pluripotency [3–5]. To establish clonal lines of pre-IPS cells, we first infected mouse embryonic fibroblasts (MEFs) and neural stem (NS) cells with retroviral transgenes. We then picked and expanded individual pre-IPS cell colonies in serum/LIF conditions. Transfer and passaging in serum-free 2i/LIF medium generated a culture of IPS cells with uniform Oct4-GFP reporter activity (Figure 1A) and the capacity to contribute to adult mice (see Figure S1A available online). Weak activity of the Oct4 reporter was detected in <2% of pre-IPS cells in serum/LIF conditions (Figure 1A). Individual GFP events in pre-IPS cells, however, were significantly less intense than in IPS cells obtained from the same clones in 2i/LIF. To clarify the identity of the subset of pre-IPS cells with weak Oct4-GFP reporter activity, we performed serial purification of GFP-positive pre-IPS cells to obtain sufficient amounts of pure material for transcriptional and epigenetic characterization (Figure S1B). Retroviral transgene expression was maintained in GFP-positive pre-IPS cells, but fully silenced in 2i-IPS cells derived from the same clonal lines (Figure S1C). GFP-positive pre-IPS cells expressed Fgf4 and Nr0b1, which are recurrently detected in partially reprogrammed cells [3, 4]. However, other markers of authentic pluripotency such as Nanog and Rex1 remained undetectable in these cells. The Nanog promoter region was methylated in a pure sample of GFP-positive pre-IPS cells, but completely demethylated in 2i-IPS cells (Figure S1D). These results demonstrate that weak Oct4-GFP activity in clonal lines of pre-IPS cells in serum/LIF is not a sign of complete reprogramming. Consequently, 2i treatment does not select for expansion of an already resident pluripotent subpopulation, but actively induces conversion to pluripotency in pre-IPS cells.

To investigate the transcriptional response to 2i/LIF treatment in pre-IPS cells, we plated pre-IPS cells at clonal density on a feeder layer in serum/LIF until emergence of macroscopic colonies, at which point medium was switched to 2i/LIF. Oct4-GFP reporter activity was monitored at daily time points and samples were collected for gene expression analysis. Surprisingly, weak Oct4-GFP reporter activity initially disappeared completely upon switch to 2i/LIF (Figure 1B). This further indicates that sporadic Oct4-GFP reporter activity in serum/LIF reflects transient activation of the Oct4 promoter and is not a sign of pluripotency (Figures S1C and S1D). By day 6 of 2i/LIF treatment, stable Oct4-GFP reporter activity began to appear in multiple colonies. The proportion of GFP-positive cells increased to approximately 30% by day 10. 2i treatment was accompanied by a degree of cell death, which became apparent 3 days after the medium switch (Figure 1B). Phospho-Erk (p-Erk) signal was completely extinguished within 24 hr of inhibitor treatment (Figure 1C). Fgf4 and Nr0b1 expression was initially downregulated upon switch to 2i/LIF, but reappeared during later time points together with other pluripotency markers (Figure 1D). In contrast, we observed a 30-fold upregulation of Nanog expression within 48 hr after switching to 2i/LIF (Figure 1E). Expression of Nanog increased steadily in subsequent time points. Unlike the initial reduction seen in pre-IPS cells, the same transcripts changed little in ES cells transferred from serum/LIF to 2i/LIF (Figures S1E–S1G). This confirms that the observed expression patterns are specific to pre-IPS cells as they reprogram.

In agreement with a recent study [6], we found that total expression of Oct4 and Klf4 was considerably higher in retrovirally derived pre-IPS cells than in ES cells (Figure S1H). However, we observed a reduction in retroviral transgene expression upon 2i/LIF treatment in pre-IPS cells (Figure 1F). Oct4 protein expression was 4 to 5-fold higher in pre-IPS cells compared with IPS cells and was significantly downregulated in pre-IPS cells 2 days after switching to 2i/LIF (Figures 1G and
Figure 1. Characterization of the Response to Kinase Inhibition in Clonal Lines of Pre-iPS Cells

(A) Top: phase and Oct4-GFP images of MEF-OKMS clone 1 and NS-OKM clone 1 pre-iPS cells cultured on a MEF feeder layer in serum/LIF conditions, and iPS cells derived in 2i/LIF from the same clonal lines. Bottom: flow cytometry analysis indicates the proportion of cells with Oct4-GFP reporter activity. OKMS and OKM refer to combinations of retroviral Oct4, Klf4, c-Myc, and Sox2 transgenes.

(B) Experimental system for assessing transcriptional dynamics in clonal lines of pre-iPS cells during switch from serum/LIF to 2i/LIF conditions. Flow cytometry diagrams indicate the proportion of cells positive for the Oct4-GFP reporter transgene, and the proportion of live cells at daily time points during 2i/LIF treatment of pre-iPS cells (MEF-OKMS clone 1). Inlaid percentages in cell viability charts indicate the proportion of DAPI-negative (live) cells at each time point.

(C) Western blot analysis for p-Erk1/2 and total Erk1/2 protein expression in pre-iPS cells cultured for 1 day in 2i/LIF medium.

(D) Time course qRT-PCR analysis of endogenous pluripotency genes Fgf4, Nr0b1, and Rex1 during switch from serum/LIF (d0) to 2i/LIF conditions in pre-iPS cells. Error bars indicate the range of fold change relative to the day 10 sample.

(E) Time course qRT-PCR analysis of Nanog expression after switching pre-iPS cells from serum/LIF to 2i/LIF conditions. Relative expression is shown on a logarithmic scale. Error bars indicate the range of fold change relative to the day 0 sample.

(F) Time course qRT-PCR analysis of retroviral transgene expression during switch from serum/LIF (d0) to 2i/LIF conditions in pre-iPS cells. Error bars indicate the range of fold change relative to the day 0 sample.

(G) Western blot analysis for Oct4 protein expression in pre-iPS cells cultured in serum/LIF or for 2 days in 2i/LIF and 2i-iPS cells.

(H) Infra-red quantification of Oct4 protein intensity relative to α-tubulin in the samples shown in (G). Error bars indicate SD from analysis of two gels.

(I) Time course flow cytometry analysis of changes in Oct4-GFP reporter activity during switch of pre-iPS cells plated in serum/LIF to serum-free 2i/LIF or serum-free medium supplemented with LIF, 2i, the GSK3 inhibitor (CHIR99021) and LIF or the MEK inhibitor (PD0325901) and LIF. See also Figure S1.
1H; Figure S1J). This reduction in Oct4 expression was a combina-
natorial effect of the kinase inhibitors rather than serum deple-
tion (Figures S1J–S1L). A modest increase in Oct4 expression is
known to induce a differentiation program in ES cells [7]. We
investigated the effect of further increases in Oct4 expression
by stably transfecting a tamoxifen-inducible Oct4 vector in ES
cells (Figure S1M) [8]. Induction of Oct4-ires-GFP occurred
rapidly and by 48 hr ~80% of cells were GFP-positive in two
independent clones (Figure S1N). Oct4 overexpression induced
an acute reduction in Nanog and Sox2 expression, which preceded
modest induction of the differentiation markers Brachyury and Gata6 (Figures S1O and S1P). We infer that the levels of Oct4 observed in pre-iPS cells present an
impediment to pluripotency gene expression.

We asked which component in 2i/LIF medium was respon-
sible for the induction of pluripotency in pre-iPS cells. Switch-
ing to serum-free medium with LIF alone did not give rise to
stable Oct4-GFP reporter activity (Figure 1I). Treatment with
2i in absence of LIF slowed the appearance of Oct4-GFP reporter activity compared with the 2i/LIF control induction, but reprogramming efficiency was still robust. A small number (0.1%) of strong Oct4-GFP events emerged after treating pre-
iPS cells with the GSK3 inhibitor and LIF. Application of the
MEK inhibitor and LIF, however, induced robust Oct4 reporter
activity in up to 43% of cells by day 12. This was also the most
selective culture medium we tested, with less than 40% cell
viability after just 4 days of treatment. These data indicate
that MEK inhibition is the main reprogramming cue in 2i and
also exerts selection against pre-iPS cells.

Nanog Enhances Reprogramming in Cooperation
with 2i or Inhibition of DNA Methylation
Nanog was activated early in response to 2i/LIF treatment (Fig-
ure 1E) and occupies a central position in the transcriptional
network regulating pluripotency [9, 10]. Using a loss-of-func-
tion approach we previously demonstrated that Nanog is
necessary for the formation of embryonic and induced pluripo-
tency [11]. In addition, Nanog was reported to accelerate
reprogramming in a study using inducible lentiviral transgen-
es [12]. Here, we asked whether forced expression of Nanog
might be sufficient to overcome the reprogramming block in
pre-iPS cells. We made use of PiggyBac (PB) transposition
[13] to introduce a transgene driving Nanog expression under
control of a CAG promoter (PB-Nanog) in a clonal line of MEF-
derived pre-iPS cells. Control transfectants expressing an
empty vector transgene (PB-Empty) were generated in
parallel. Expression of Nanog in stable transfectants
expanded in serum/LIF was 1.5-fold higher compared with
iPS cells. Forced expression of Nanog did not result in activa-
tion of the pluripotency marker Rex1 or downregulation of
destinative expression (Figure 2A). Lack of Oct4-GFP reporter
activity further demonstrates that Nanog could not overcome
the block to full reprogramming in presence of serum (Fig-
ure 2B). Oct4 promoter methylation persisted in PB-Nanog
pre-iPS cells (Figure 2C). In contrast, the percentage of meth-
ylated CpG sites in the Oct4 distal enhancer was reduced from
38% in PB-Empty pre-iPS cells to 8% in PB-Nanog pre-iPS
cells (Figure 2C). The distal enhancer is responsible for driving
Oct4 expression in preimplantation embryos and ES cells [14]
and contains the CR4 element, a critical binding site of Nanog
in ES cells [15]. Chromatin immunoprecipitation analysis sug-
gested that reduced CpG methylation might correlate with low
level Nanog occupancy of the CR4 element in PB-Nanog pre-
iPS cells (Figure 2D). However, Nanog occupancy was signifi-
cantly higher in iPS cells.

Since the Oct4 promoter remained hypermethylated in PB-
Nanog pre-iPS cells, we asked whether chemical inhibition of
DNA methylation could promote reprogramming to pluripo-
tency. Indeed, treatment with the DNA methyltransferase
inhibitor 5-aza-cytidine (AZA) for 10 days generated a signifi-
cant proportion of cells with stable Oct4-GFP reporter activity
in serum/LIF (Figure 2E). A small number of GFP-positive cells
also emerged upon AZA treatment in PB-Empty or wild-type
pre-iPS cells (Figure 2E; Figures S2A and S2B). Purification
and subcloning of these GFP-positive cells generated a line
of homogenous iPS cells capable of contribution to chimeric
mice (Figures S2C–S2E). This confirms a previous report that
global inhibition of DNA methylation promotes direct reprog-
gramming [4]. The de novo methyltransferase Dnmt3a was ex-
pressed in pre-iPS cells throughout 2i/LIF induction, while
Dnmt3b expression was initially reduced and then upregulated
(Figure S2F). Immunofluorescence analysis for Dnmt3b during
2i/LIF treatment showed weak cytoplasmic staining in pre-iPS
cells and a stronger nuclear signal in iPS cells positive for both
Oct4-GFP and Nanog protein (Figure S2G). This pattern shows
resemblance to primordial germ cells where Dnmt3b is
excluded from the nucleus at the time of DNA demethylation
and epigenetic reprogramming [16]. The significant increase
in efficiency of AZA-induced reprogramming in the PB-Nanog
background reveals a synergy between Nanog and inhibition
of global DNA methylation. We also examined reprogramming
kinetics upon the application of 2i/LIF medium in PB-Nanog
and PB-Empty pre-iPS cells. When transferred to 2i/LIF
Oct4-GFP activity appeared earlier and the proportion of posi-
tive cells was more than 10-fold higher in the constitutive
Nanog background (Figure 2E).

These results show that Nanog cooperates with distinct
small molecules to enhance the efficiency of direct reprogram-
mimg. These pathways also intersect since endogenous Nanog
was activated in response to 2i/LIF treatment in pre-iPS cells
(Figure 1E), and suppression of p-Erk signaling results in
increased Nanog expression in ES cells [17, 18]. Further gains
in reprogramming yield may be obtained by integrating other
chemical strategies that complement or reinforce the effects
of these inhibitors. TGF-beta inhibition was reported to induce
endogenous Nanog expression in partially reprogrammed
cells but did not affect the kinase targets of the 2i cocktail
[19]. Vitamin C treatment also promoted reprogramming in a
MEK-independent manner and resulted in demethylation of the
Nanog promoter [20]. This suggests that chemicals that
promote or enhance the efficiency of reprogramming may
converge on common transcriptional targets.

Nanog Promotes Somatic Cell Reprogramming
in Serum-Free Medium with LIF
We then considered whether constitutive expression of Nanog
might be sufficient to promote transition to pluripotency in
serum-free conditions. No stable Oct4-GFP reporter activity
was observed after switching PB-Nanog pre-iPS cells to
serum-free medium alone. However, the Oct4-GFP reporter
was robustly induced in presence of LIF (Figures 3A and 3B).
PB-Empty pre-iPS cells did not give rise to stable GFP-ex-
pressing cells in either condition. GFP-positive cells that
emerged in the constitutive Nanog background expressed
Rex1, Klf4, and endogenous Oct4 and fully silenced retroviral
transgenes (Figure 3C). To confirm this result in a different
somatic origin, we introduced a PB-Nanog transgene in adult
NS cells prior to retroviral infection (Figure S3A). Medium was switched after 5 days to serum-free medium with LIF or serum-free medium alone. Stable Oct4-GFP activity emerged in multiple colonies in serum-free medium with LIF within 7 days (Figure S3B). These GFP-positive cells had a pluripotent gene expression profile (Figure S3C). Since constitutive Nanog expression is likely to interfere with embryonic development, we opted to assess developmental potential 52 hr after morula aggregation, which corresponds to a late blastocyst stage. PB-Nanog iPS cells derived in serum-free medium with LIF readily colonized the epiblast and maintained homogeneous Oct4 reporter activity (Figure 3D; Figure S3D). In contrast, postimplantation epiblast-derived stem cells (EpiSCs) incorporated in the epiblast but Oct4-GFP reporter activity was greatly reduced 52 hr after aggregation.

These results demonstrate that constitutive expression of Nanog promotes transition to pluripotency in serum-free medium with LIF. The requirement of LIF for Nanog-induced reprogramming provides evidence that kinase inhibition has additional targets, since 2i was sufficient to convert pre-iPS cells to pluripotency in absence of LIF (Figure 1I). p-Erk levels were sustained in serum-free medium with LIF (Figure 3E). We also found that high Oct4 levels were unaffected after switching pre-iPS cells to serum-free medium or AZA (3 μM) in presence of serum/LIF. See also Figure S2.

**Figure 2.** Nanog Enhances Reprogramming in Synergy with 2i or Inhibition of DNA Methylation

(A) A piggyBac (PB) transgene was used to generate stable Nanog expressing cells in a clonal line of pre-iPS cells (MEF-OKMS clone 1). qRT-PCR analysis comparing expression of Nanog, Rex1, retroviral (r) Oct4 and r-Klf4 in PB-Nanog and PB-Empty pre-iPS cells expanded in serum/LIF. Error bars indicate the range of fold change relative to the sample with highest expression. 

(B) Flow cytometry analysis indicates the proportion of cells with Oct4-GFP reporter activity in both transgenic backgrounds in serum/LIF. The percentage of methylated CpG sites is indicated above each methylation panel.

(C) Bisulfite sequencing analysis of DNA methylation in the Oct4 distal enhancer and promoter in PB-Empty and PB-Nanog pre-iPS cells and 2i-iPS cells. Results of two independent experiments are shown. iPS cell occupancy was not measured in experiment A. Occupancy is plotted as fold enrichment over IgG after normalization to the input, and error bars represent standard deviation of the technical replicates of the qPCR for each experiment.

(D) Flow cytometry analysis comparing Oct4-GFP reporter activity after treatment of PB-Nanog and PB-Empty pre-iPS cells with 2i/LIF in serum-free medium or AZA (3 μM) in presence of serum/LIF. See also Figure S2.
lack of alkaline phosphatase activity (Figures S3E and S3F). In contrast, self-renewal and expression of pluripotency genes were maintained upon coexpression of Oct4 and Nanog transgenes (Figures S3E–S3G). This indicates that Nanog safeguards establishment and maintenance of pluripotency against the effects of high levels of Oct4.

**Nanog Is Sufficient to Reprogram Epiblast-Derived Stem Cells to Naive Pluripotency**

Finally, we asked whether Nanog is sufficient to mediate reprogramming in absence of both exogenous self-renewing factors and other reprogramming transgenes (Figures S3E–S3G). This indicates that Nanog safeguards establishment and maintenance of pluripotency against the effects of high levels of Oct4.

In this study, we investigated limiting components during the final stages of direct reprogramming. We found that Nanog has the capacity to overcome p-Erk signaling and high levels of Oct4 and enable reprogramming in minimal conditions. This result is significant in light of recent interest to generate a naive pluripotent state in cells of non-rodent origin, including human [25–27]. Conventional human ES cell culture conditions induce differentiation in naive pluripotent mouse cells [13]. By identifying factors such as Nanog that enable reprogramming in minimal conditions, it may be possible to bypass species-specific culture requirements and establish naive pluripotency in other mammalian species.
Experimental Procedures

Pre-iPS cells were obtained by retroviral infection of NS cells or MEFs with pMXs-based retroviral reprogramming factors [1, 3]. Cultures were changed into ES cell medium (serum/LIF) at day 3 posttransduction. For further expansion, pre-iPS cells were replated onto feeders at day 5 in serum/LIF. Pre-iPS and NS cells were transfected using nucleofection (Amaxa) with 1 μg of PB-flox-Nanog-Pgk-Hygro plus 2 μg PBase expression vector, pCAGPBase [11]. For time course real-time PCR analysis of iPS cell induction, pre-iPS cells were plated in serum/LIF at clonal density on a STO or...
DsRed-expressing fibroblast feeder layer in 10 cm (3000 pre-iPS cells plated) or 6W (600 pre-iPS cells plated) format. Medium was switched to 2i/LIF when colonies reached macroscopic colony density after 6–8 days. Samples were collected daily for RNA extraction, either directly from whole pellets or after flow cytometric elimination of DsRed-expressing feeders. By time pre-iPS cell colonies had reached macroscopic density, feeders comprised no more than 2% of the complete culture. PB-Nanog iPS cells were at a proliferative disadvantage compared with pre-iPS cells in serum-free medium with LIF, requiring flow cytometric purification of cells positive for the Oct4-GFP reporter.

Supplemental Information

Supplemental Information includes three figures, Supplemental Experimental Procedures, and one table and can be found with this article online at doi:10.1016/j.cub.2010.11.074.

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