NANOG Amplifies STAT3 Activation and They Synergistically Induce the Naive Pluripotent Program

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Summary

Reprogramming of a differentiated cell back to a naive pluripotent identity is thought to occur by several independent mechanisms. Two such mechanisms include NANOG and activated STAT3 (pSTAT3), known master regulators of naive pluripotency acquisition [1–5]. Here, we investigated the relationship between NANOG and pSTAT3 during the establishment and maintenance of naive pluripotency. Surprisingly, we found that NANOG enhances LIF signal transduction, resulting in elevated pSTAT3. This is mediated, at least in part, by suppression of the expression of the LIF/STAT3 negative regulator SOCS3. We also discovered NANOG to be limiting for the expression of KLF4, a canonical “Yamanaka” reprogramming factor [8] and key pSTAT3 target [2, 7, 8]. KLF4 expression resulted from the codependent and synergistic action of NANOG and pSTAT3 in embryonic stem cells and during initiation of reprogramming. Additionally, within 48 hr, the combined actions of NANOG and pSTAT3 in a reprogramming context resulted in reactivation of genes associated with naive pluripotency. Importantly, we show that NANOG can be bypassed during reprogramming by exogenous provision of its downstream effectors, namely pSTAT3 elevation and KLF4 expression. In conclusion, we propose that mechanisms of reprogramming are linked, rather than independent, and are centered on a small number of genes, including NANOG.

Results

NANOG Amplifies STAT3 Activation

We first investigated the effect of NANOG on STAT3 activation in embryonic stem cells (ESCs), since the pluripotency network is established and functional in this cellular context. Although NANOG and STAT3 are essential for embryonic naive pluripotency establishment [4, 9], they promote but are not required for in vitro ESC maintenance [10–14]. This permits the study of Nanog−/− and Stat3−/− ESCs and suggests differences between network requirements for naive pluripotency establishment versus maintenance.

Active pSTAT3 lies downstream of a LIF-stimulated tyrosine kinase signaling cascade [1] (Figure S1A available online). Wild-type, Nanog−/−, and constitutively NANOG-overexpressing ESCs were harvested from steady-state and LIF-induction cultures. Western blotting revealed that higher NANOG both increased steady-state pSTAT3 levels (Figures 1A and 1B) and enhanced ESC sensitivity to LIF stimulation (Figure 1B).

To explain how NANOG drives pSTAT3 elevation without directly binding the STAT3 protein or gene [17, 18], we examined the effect of NANOG on components of the LIF/STAT3 signaling pathway (Figure S1A). By quantitative RT-PCR (qRT-PCR), no correlation was reliably found between NANOG and transcript levels of the positive signal transducers LIF, Lifr, Gp130, Jak2, and Stat3 (data not shown), despite NANOG binding to Lif, Lifr, and Gp130 gene regulatory sequences in ESCs according to published chromatin immunoprecipitation sequencing data [18]. However, NANOG also binds the Socs3 gene (Figure S1C), which is a negative regulator of STAT3 activation [19, 20]. This prompted our hypothesis that NANOG represses Socs3 transcription.

Since Socs3 transcription is upregulated by pSTAT3 to form a classic negative feedback loop [20, 21], determination of whether NANOG causes Socs3 repression is obfuscated by the effect of pSTAT3 on Socs3. To disentangle the opposing yet interconnected influences of NANOG and pSTAT3 on Socs3 expression, we designed experiments in which the pSTAT3 level is not significantly influenced by NANOG. ESCs can be maintained without exogenous LIF by 2i medium [10], which contains small molecules CHIR99021 (chiron) and PD0325901 (PD03) that inhibit GSK3 and MEK, respectively. In the absence of LIF-stimulated Socs3 activation, the effect of NANOG on Socs3 transcription can be assessed. From qRT-PCR analysis of wild-type, Nanog−/−, and constitutively NANOG-overexpressing ESCs cultured in 2i, a strong negative correlation was evident between Nanog and Socs3 expression levels (Figure 1C). Furthermore, in the absolute absence of pSTAT3 in Stat3−/− ESCs, those constitutively overexpressing NANOG exhibited a lower Socs3 level (Figure 1C).

STAT3 can be specifically activated by GCSF stimulation of the GY118F receptor transgene [22–24] (Figure S1A). When GCSF/GY118F are used in the absence of LIF, Socs3 downregulation by NANOG should have little or no effect on pSTAT3 levels; GY118F is insensitive to SOCS3 repression, and nearly all STAT3 activation will be attributable to GY118F rather than LIFR-GP130. In addition, we generated a doxycycline (dox)-inducible Nanog transgene (iNANOG) (Figure S1D). Dox induction of NANOG expression rapidly led to enriched NANOG binding at the Socs3 gene in ESCs, consistent with direct transcriptional regulation (Figure S1C). In Nanog−/− ESCs containing both iNANOG and GY118F, NANOG expression and STAT3 activation were induced separately and in combination in 2i. Induction of NANOG alone caused Socs3 repression, while Socs3 induction in response to STAT3 activation was reduced by 60% when NANOG was also induced (Figure 1D). This suggests that Socs3 repression is a mechanism by which NANOG...
augments LIF signal transduction in ESCs, resulting in higher levels of active pSTAT3. The same trends were observed in Nanog^2/2/NANOG^+GY118F postimplantation epiblast stem cells (EpiSCs) in standard FGF2+ActivinA conditions (Figure 1D), demonstrating that NANOG-mediated Socs3 repression is not restricted to ESCs and may be of functional relevance during NANOG-driven reprogramming.

NANOG and pSTAT3 Synergistically Upregulate KLF4

To further explore our newfound mechanistic link between NANOG and LIF/STAT3 signaling, we investigated the effect of NANOG on expression of LIF/STAT3 targets in ESCs. In the steady-state presence of LIF, we found strong positive correlation between levels of NANOG and KLF4, a canonical pSTAT3 target [2, 7, 8] (Figures 1A and S1B). Interestingly, in response to LIF stimulation, Klf4 upregulation required NANOG to be present, while NANOG overexpression cooperated with LIF to substantially increase the rate and levels of Klf4 induction (Figures 2A and 2B). In agreement with previous work [8], modest upregulation of NANOG was found in response to LIF (Figures 1B and S2A).

The correlation between NANOG and Klf4 was abrogated in the absence of pSTAT3 in Stat3^+/+ ESCs without LIF and in Stat3^2/2 ESCs (Figure 2C), showing that NANOG-driven...
Klf4 upregulation was pSTAT3 dependent. However, the effect of NANOG on Klf4 transcription was not solely attributable to pSTAT3 elevation, since STAT3 hyperactivation in the absence of NANOG could not rescue Klf4 expression (Figure 2C).

The relationship between NANOG, pSTAT3, and Klf4 expression was further dissected using Nanog<sup>−/−</sup> iNANOG+GY118F ESCs in 2i without LIF. Together, induction of NANOG expression and STAT3 activation elicited Klf4 upregulation in a synergistic manner compared to induction of either factor alone (Figure 2D). This synergistic action of NANOG and pSTAT3 is specific to Klf4: other pluripotency factors did not respond in this striking manner, including NANOG-target Esrrb [25] and pSTAT3-target Klf5 [7, 8] (Figure S2B).

Since NANOG and pSTAT3 both bind the Klf4 enhancer (Figure S2C), it is likely that they regulate Klf4 transcription directly. The Klf4 enhancer lies around 60 kb downstream of the Klf4 transcription start site and was recently identified as an archetypal “super-enhancer” [26]. We found novel noncoding RNA to be expressed from the Klf4 enhancer in ESCs, and we termed it K4eRNA (Figure S2C). Given that K4eRNA expression positively correlates with Klf4, responding to NANOG and LIF/STAT3 in the same synergistic manner (Figures 2A, 2C, and 2D), we hypothesize that K4eRNA is a cis activator of Klf4 transcription.

NANOG and pSTAT3 Induce Rapid and Efficient Reactivation of Naïve Genes

EpiSC reprogramming requires reversion from primed to naive pluripotency and thus provides an excellent system in which to study naïve pluripotency acquisition. Conversion of EpiSCs does not occur simply in naïve-state culture conditions, but can be driven by a minimum of one factor [27]. It is known that NANOG overexpression and STAT3 hyperactivation together increase EpiSC reprogramming efficiency in a synergistic manner [1]. Since we have mechanistically linked NANOG and pSTAT3 in ESCs where the pluripotency network is fully operational, we turned to EpiSCs to study the role of these connected mechanisms during naïve pluripotency establishment.
Combined pSTAT3+KLF4 Bypass NANOG in Reprogramming

Figure 3. NANOG and pSTAT3 Induce Rapid and Efficient Reactivation of Naive Genes

(A) qRT-PCR analysis of Nanog-/- and Nanog+/F (Nanog+/Flox) EpiSCs derived from Nanog-/- and Nanog+/Flox littermate embryos, cultured in FGF2+ActivinA (F+A), compared to wild-type ESCs in 2i+LIF. Lack of Nanog expression in Nanog-/- EpiSCs confirmed the null genotype, while their expression of Oct4 and Fgf5 but not Rex1 or Klf4 confirmed their EpiSC identity. Gene expression was measured relative to Gapdh and normalized to the highest value. Data shown are the mean of three technical replicates and are from one of two representative experiments. Error bars indicate ±SD.

(B) Representative phase and Nanog-GFP images of the Nanog-/- EpiSCs confirmed the null genotype, while their expression of Oct4 and Fgf5 but not Rex1 or Klf4 confirmed their EpiSC identity. Gene expression was measured relative to Gapdh and normalized to the highest value. Data shown are the mean of three technical replicates and are from one of two representative experiments. Error bars indicate ±SD.

See also Figure S3.

The iNANOG+GY118F system provides a powerful platform for the quantitative dissection of NANOG and pSTAT3 mechanisms, as they can be induced separately and in combination within a single cell line. We generated Nanog-/- background EpiSCs (Figure 3A, 3B, and S3A–S3C) to eliminate confounding endogenous Nanog expression, and maintained EpiSC FGF2+ActivinA culture conditions so that putative reprogramming kinetics could be ascribed exclusively to transgene induction. Strikingly, Nanog and pSTAT3 codependently reactivated Klf4 and K4eRNA in this distinct cellular and environmental context (Figure 3C). This demonstrates that their effect on Klf4 is not an ESC-specific phenomenon and may be of functional relevance for Nanog/pSTAT3-driven reprogramming. We observed activation of naive pluripotency marker Rex1 at 48 hr (Figure 3C). Since Rex1 did not respond to Nanog/pSTAT3 in this manner in ESCs (Figure S2B), we believe Rex1 induction in EpiSCs to be indirect, indicating identity changes toward iPSCs within the population. Interestingly, Rex1 activation positively correlates with Klf4 expression.

Known NANOG-target Esrrb [25] responded to dox induction of Nanog expression in EpiSCs (Figure 3C). Similarly, known pSTAT3 targets Klf5 [7, 8] and Tcfp2/1 [15, 28] were upregulated after GCSF induction of STAT3 activation (Figure 3C). However, the synergistic response of Klf4 to NANOG and pSTAT3 remains unique. In total, NANOG and pSTAT3 rapidly reactivated many key components of the naive pluripotency network to near ESC level, shedding light on their ability to drive fast and efficient reprogramming. This is even more remarkable when taking into account that the assay used EpiSC culture conditions instead of conditions promoting reprogramming or ESC self-renewal.

Combined pSTAT3 and KLF4 Bypass NANOG in Reprogramming

Although NANOG is dispensable for pluripotency maintenance [11], it is required for establishment of the pluripotent epiblast during preimplantation embryonic development [4]. Correspondingly, NANOG is essential for naive pluripotency establishment during conventional in vitro reprogramming experiments [4]. Rescue of Nanog-/- reprogramming thus provides a means of functionally testing proposed downstream mechanisms of NANOG.

We have described two new NANOG mechanisms: pSTAT3 elevation by SOCS3 repression and KLF4 upregulation in cooperation with pSTAT3. Therefore, we investigated the ability of pSTAT3 and KLF4 to rescue reprogramming of Nanog-/- EpiSCs. We also tested ESRRB, since it has previously been reported as a NANOG downstream target able to bypass NANOG in reprogramming of ESC-derived EpiSCs [25]. Expression of GFP and igeo under the control of endogenous Nanog promoters provide visual and selective reporters in our system, since EpiSCs cannot survive long-term in the reprogramming culture conditions (Figure 4A).
Forced expression of NANOG, ESRRB, KLF4 or pSTAT3 individually can drive reprogramming of \( \text{Nanog}^{2/-} \) EpiSCs in 2i+LIF conditions \([1, 4, 25, 27]\). We verified the ability of our \( \text{Nanog}^{2/-} \) EpiSCs to generate iPSCs in 2i+LIF when rescued by NANOG expression and confirmed that STAT3 activation in conjunction with NANOG expression drives rapid reprogramming at high efficiency (Figure 4B). However, in the absence of NANOG, individual and combined overexpression...
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of ESSRB, KLF4, and pSTAT3 could not rescue reprogramming in 2i+LIF (Figure 4B).

In parallel, we tried to rescue Nanog−/− reprogramming with individual and combined overexpression of ESSRB, KLF4, and pSTAT3 in chiron+LIF (Figure 4C) and in serum+LIF (data not shown). To our surprise, we found that ESSRB overexpression was unable to drive Nanog−/− EpiSC reprogramming in any condition. We speculate that this is due to differences in reprogramming propensity between our EpiSCs derived from Nanog+/− embryos and ESC-derived secondary EpiSC systems [25]. Individually, KLF4 and pSTAT3 also failed to yield iPSCs. Although they initially generated GFP+ colonies in chiron+LIF (Figure 4C), these lacked the capacity to self-renew after passaging into 2i+LIF, suggesting that reprogramming was incomplete.

In combination, pSTAT3+ESSRB and pSTAT3+KLF4 yielded Nanog−/− GFP+ iPSCs in chiron+LIF conditions (Figure 4C). Reprogramming occurred with the highest rate and efficiency with pSTAT3+KLF4. Although Nanog−/− iPSCs could not be established in 2i+LIF, once naive pluripotency was established in chiron+LIF, Nanog−/− iPSCs could be passaged indefinitely in 2i+LIF, consistent with known discrepancies between NANOG requirement in pluripotency establishment versus maintenance. After passaging in 2i+LIF, the gene expression profile and observed chimeric competence of Nanog−/− iPSCs formally demonstrated their reacquisation of a naive pluripotent program (Figures 4D and 4E). This highlights pSTAT3 activation as a key functional mechanism acting downstream of NANOG, which, in conjunction with overexpression of either NANOG-target KLF4 or ESRRB, can efficiently rescue Nanog−/− reprogramming. It is of interest to note that KLF4 but not NANOG overexpression can drive EpiSC reprogramming in the presence of JAK inhibitor [1], further supporting the placement of NANOG upstream and KLF4 downstream of STAT3 activation during reprogramming.

NANOG is dispensable for the initial formation of reprogramming intermediates (pre-iPSCs) from somatic cells, but is essential for pre-iPSCs to transit to naive pluripotency in 2i+LIF [4] (confirmed in Figures S4A and S4B). Since Nanog−/− EpiSCs were able to reprogram in chiron+LIF but not 2i+LIF, we tested whether Nanog−/− pre-iPSCs could also reprogram in alternative conditions. From these, we successfully obtained Nanog−/− iPSCs in chiron+LIF and KSR+LIF conditions, albeit with low speed and efficiency (Figures S4C–S4E). Microarray analysis revealed that pre-iPSC-derived and EpiSC-derived Nanog−/− iPSCs clustered closely with both wild-type and Nanog−/− ESCs, demonstrating reprogramming to a naive pluripotent identity (Figure S4F). It should be noted that retroviral KLF4 and exogenous LIF provided the reprogramming impetus for these pre-iPSCs, again implicating pSTAT3 and KLF4 in the rescue of Nanog−/− reprogramming.

Discussion

We connect NANOG with the activation of STAT3, two major mechanisms for the establishment and maintenance of naive pluripotency. Our finding that NANOG modulates signal transduction of extracellular cues adds a new dimension to the interplay between external environment and nuclear control networks to instate and reinforce cellular identity. We also provide mechanistic insight into the process of induced pluripotency by showing that expression of KLF4, a canonical “Yamanaka” factor, results from codependent and synergistic action between NANOG and pSTAT3. Interestingly, the only remaining factor to be used by all reprogramming protocols is LIF and consequently STAT3 activation, now linked to NANOG.

The role of NANOG is thus to build a naive pluripotent transcriptional network by concurrently inducing the expression of ESSRB, enhancing LIF/STAT3 signal transduction, and inducing KLF4 expression in cooperation with pSTAT3 (Figure S4G). Ultimately, combinations of these factors allow in vitro bypassing of NANOG for the establishment of a naive pluripotent cell state. However, the observed slower kinetics and reduced efficiency of Nanog−/− somatic cell reprogramming imply the existence of further mechanisms by which NANOG operates. These may include additional downstream effectors of reprogramming and the activities of NANOG cofactors such as TET1/2 [29]. In this light, it is interesting to note that bypass of NANOG in reprogramming was enhanced by KSR medium (Figure S4C), which contains ascorbic acid, a powerful coactivator of dioxygenases such as the jumonji histone demethylases and TETs [30–34]. Additionally, it will be of future interest to ascertain why 2i conditions are detrimental to Nanog−/− reprogramming.

Successful induction of naive pluripotency can be achieved by the combined actions of different culture environments with different sets of transgenes. This has led to the notion that iPSCs can be generated by different, independent reprogramming mechanisms acting in an additive, linear manner. In contrast to this, our newfound cooperative relationship between NANOG and STAT3 activity raises the possibility of an integrated reprogramming mechanism. Therefore, we propose that allegedly independent mechanisms of naive pluripotency induction may instead be linked and centered on a small group of genes including NANOG.

Accession Numbers

The Gene Expression Omnibus accession number for the microarray data reported in this paper is GSE53529.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.12.040.

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