

MIT Open Access Articles

Single-Cell Analysis Reveals that Expression of Nanog Is Biallelic and Equally Variable as that of Other Pluripotency Factors in Mouse ESCs

The MIT Faculty has made this article openly available. **Please share** how this access benefits you. Your story matters.

Citation: Faddah, Dina A., Haoyi Wang, Albert Wu Cheng, Yarden Katz, Yosef Buganim, and Rudolf Jaenisch. "Single-Cell Analysis Reveals That Expression of Nanog Is Biallelic and Equally Variable as That of Other Pluripotency Factors in Mouse ESCs." *Cell Stem Cell* 13, no. 1 (July 2013): 23–29.

As Published: <http://dx.doi.org/10.1016/j.stem.2013.04.019>

Publisher: Elsevier

Persistent URL: <http://hdl.handle.net/1721.1/103900>

Version: Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

Terms of use: Creative Commons Attribution-NonCommercial-NoDerivs License





Published in final edited form as:

Cell Stem Cell. 2013 July 3; 13(1): 23–29. doi:10.1016/j.stem.2013.04.019.

Single-cell analysis reveals that expression of Nanog is biallelic and equally variable as that of other pluripotency factors in mouse embryonic stem cells

Dina A. Faddah^{1,2}, Haoyi Wang¹, Albert Wu Cheng^{1,3}, Yarden Katz^{1,4}, Yosef Buganim¹, and Rudolf Jaenisch^{1,2}

¹Whitehead Institute for Biomedical Research, Cambridge, MA 02142

²Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

³Department of Computation and Systems Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

⁴Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139

Summary

The homeodomain transcription factor Nanog is a central part of the core pluripotency transcriptional network and plays a critical role in embryonic stem (ES) cell self-renewal. Several reports have suggested that Nanog expression is allelically regulated and that transient down-regulation of Nanog in a subset of pluripotent cells predisposes them towards differentiation. Using single-cell gene expression analyses combined with different reporters for the two alleles of Nanog, we show that Nanog is biallelically expressed in ES cells independently of culture condition. We also show that the overall variation in endogenous Nanog expression in ES cells is very similar to that of several other pluripotency markers. Our analysis suggests that reporter-based studies of gene expression in pluripotent cells can be significantly influenced by the gene targeting strategy and genetic background employed.

Embryonic stem cells, derived from the inner cell mass of the embryo, have the ability to divide indefinitely while maintaining the capacity to differentiate into different cell types with core transcription factors being known to regulate the pluripotent state (Jaenisch and Young, 2008; Orkin et al., 2008). Nanog is important for this network but the mechanisms governing Nanog regulation are unclear (Chambers et al., 2003; Mitsui et al., 2003).

Several studies have proposed that Nanog protein expression fluctuates in ES cells suggesting that allelic regulation of the gene itself contributes to this heterogeneity

© 2013 Il Press. All rights reserved.

Correspondence should be addressed to Rudolf Jaenisch jaenisch@wi.mit.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

(Chambers et al., 2007; Kalmar et al., 2009; Macarthur et al., 2012; Miyanari and Torres-Padilla, 2012; Singh et al., 2007; Wray et al., 2010). These allelic fluctuations were seen in medium containing serum/leukemia inhibitory factor (LIF) and to a lesser extent, if at all, in 2i/LIF (inhibition of MAPK and GSK-3) (Silva et al., 2008; Silva et al., 2009; Wray et al., 2010; Ying et al., 2008). It has been suggested that fluctuating levels of Nanog mediate ES cell self-renewal vs. differentiation with low or no Nanog expression thought to render cells susceptible to intrinsic or extrinsic signals inducing differentiation and generating functional heterogeneity within pluripotent cell populations. Recently, it has been shown that Nanog activity is autorepressive and may regulate allelic switching (Fidalgo et al., 2012; Navarro et al., 2012). Surprisingly, Nanog can be deleted in ES cells without affecting their potential to generate chimeras (Chambers et al., 2007).

In this study, we investigated variation in Nanog expression using single-cell analysis in mouse ES cells. To monitor the two alleles of Nanog in single cells using single-molecule-mRNA-FISH (sm-mRNA-FISH) (Buganim et al., 2012; Raj et al., 2008), we generated a V6.5 ES cell line where GFP was inserted immediately downstream of the Nanog coding region with the selectable marker being deleted. Sequences encoding mCherry were inserted by a similar targeting strategy into the second Nanog allele (Figure 1A, **S1A**). In this construct GFP and mCherry dissociate from Nanog by self-cleavage of a 2A peptide and do not alter Nanog function. We quantified transcripts of Nanog, mCherry, and GFP in single Nanog-2A-GFP/Nanog-2A-mCherry ES cells (cells termed NGNC here) by sm-mRNA-FISH and found that all cells expressed mCherry and GFP transcripts (Figure 1B) with the total level of Nanog transcripts in a given cell being approximately equal to the sum of the GFP and mCherry transcripts (Figure 1C). Boxplot analysis revealed GFP expression and mCherry expression to be equal and approximately half that of Nanog expression (Figure 1D). We quantified mCherry⁺/GFP⁺, GFP⁺, and mCherry⁺ cells grown in serum/LIF by flow cytometric analysis and found 96% mCherry⁺/GFP⁺, 0.6% GFP⁺, and 0.1% mCherry⁺ (Figure 1E). Finally, all NGNC cells grown in serum/LIF or 2i/LIF were GFP⁺ and mCherry⁺ by immunostaining (Figure S1B). In summary, our results indicate that both Nanog alleles are expressed in the great majority of cells regardless of culture condition.

To compare the variability of Nanog expression to that of other pluripotency factors, we used sm-mRNA-FISH to quantify transcripts of 9 pluripotency genes (Nanog, Dnmt3b, Utf1, Sox2, Lin28, Sall4, Tet1, Klf2, Fbx15), 1 housekeeping gene (Gapdh), and a known heterogeneously expressed gene (Stella) each in combination with Oct4 in single cells (Figure 1F–1O, **S1C–D**). Out of 899 cells analyzed, we only identified 1% that were Nanog⁻/Oct4⁺ (Figure S1C). Klf2 and Fbx15 were not always co-expressed with Oct4 with 10% of Klf2⁻/Oct4⁺ cells and 14% Fbx15⁻/Oct4⁺ cells (Figure 1N–**S1D**). Figure 1O shows 40% Stella⁻/Oct4⁺ negative cells, a number slightly lower than the 70–80% Stella negative cells identified by immunofluorescence in a previous report (Hayashi et al., 2008). All genes examined had different levels of expression and ranges of expression levels in single cells (Figure 1P). Importantly, Stella had the highest coefficient of variation, while all other genes, including Nanog and Gapdh, had similar coefficients of variation. These data suggest that Nanog is just as variable in gene expression as any other pluripotency factor and even a housekeeping gene, like Gapdh (Figure 1Q). Thus, our data, based upon single cell

expression studies, do not support the concept that Nanog is more heterogeneously expressed than most other pluripotency genes.

Our conclusions about Nanog expression differ from those seen in prior studies, so we investigated potential explanations. The majority of studies characterizing heterogeneity in Nanog expression have used heterozygous loss-of-function knock-in GFP reporters. Specifically, in the Nanog GFP +/- allele generated by (Hatano et al., 2005), the coding sequences were replaced with a GFP-IRES-puro-pA reporter and a selection cassette in the targeted allele (we designate these cells as “NHET” ES cells), whereas the TNGA allele was generated by inserting the eGFP marker at the Nanog AUG codon (Chambers et al., 2007). In a third study a triplicate GFP sequence had been inserted into one and a corresponding mCherry construct into the other Nanog allele resulting in “NGR” ES cells. The GFP and mCherry allele also contained an IRES-Neo or IRES-Hygro selection cassette, respectively (Miyinari and Torres-Padilla, 2012). Both fluorescent proteins dissociate from Nanog by self-cleavage of a 2A peptide and thus were not expected to interfere with Nanog function. Using time-lapse analysis, dynamic fluctuations of Nanog expression were observed in agreement with previous reports (Chambers et al., 2007; Kalmar et al., 2009). In addition, RNA-FISH and allele-specific single cell-RT-PCR found that about 80% of the cells expressed Nanog monoallelically, a fraction that decreased to about 30% when the cells were cultured in 2i/LIF condition.

In an effort to reconcile our data with the published Nanog expression patterns we used sm-mRNA-FISH to measure Nanog, Oct4, and GFP expression in V6.5 ES cells targeted with an identical vector as previously described (NHET ES cells (Hatano et al. 2005)) or by using the published targeted E14Tg2a ES cells (TNGA: (Chambers et al., 2007)) (Figure S1E). Confirming the published data, this analysis revealed that the majority of NHET ES cells cultured in serum/LIF or 2i/LIF were GFP- (79% and 69%, respectively) (Figure 2A). However, the great majority of the GFP- cells grown in 2i/LIF (98%) and 100% of GFP- cells in serum/LIF expressed Nanog RNA. Similarly, most TNGA GFP- ES cells cultured in serum/LIF condition were Nanog+ (Figure 2B). These data, summarized in Figure 2C, indicate that GFP+ and GFP- NHET and TNGA ES cells expressed Nanog and Oct4 mRNA at comparable levels. Cultivation of NHET cells in 2i/LIF substantially increased the number of Nanog transcripts in NHET but not in TNGA cells. Quantification of GFP+ and GFP- fractions in both cells lines cultured in serum/LIF by flow cytometry was consistent with the sm-mRNA-FISH analysis (Figure 2D). Immunostaining of each cell line revealed that both the GFP+ and GFP- cells expressed Nanog and Oct4 protein (Figure 2E and **S1F**). In both TNGA and NHET cell lines we found GFP-, GFP+, and ‘speckled’ colonies containing both GFP+ and GFP- cells (Figure 2E and **S1F**). We also found that GFP- cells can give rise to GFP+ cells and GFP+ can generate GFP- cells within 1 or 2 passages (Figure S1G), consistent with previous reports (Chambers et al., 2007).

To monitor the non-targeted allele of NHET ES cells, mCherry was inserted immediately downstream of the Nanog coding region (using Nanog-2A-mCherry construct). We found the NHET GFP- cells to be mCherry+, further supporting that the other allele of Nanog is active in the GFP- cells (Figure S1H). Importantly, western blotting was performed on protein derived from the GFP+ and GFP- fractions of NHET and TNGA and confirmed that

GFP expression did not reflect Nanog protein expression (Figure S1I–J), a result different from published data (Chambers et al., 2007). In summary, these observations demonstrate (a) that only a fraction of NHET and TNGA cells express GFP in agreement with previous reports (Chambers et al., 2007), (b) that the NHET and TNGA GFP[−] cells also express Nanog, (c) that 2i/LIF affects Nanog, Oct4, and GFP expression differently in TNGA and NHET ES cells and (d) that the GFP reporter targeting strategies that disrupt one allele may not be a faithful indicator of endogenous Nanog expression.

To compare the GFP⁺ and GFP[−] cells in terms of their pluripotent state, we analyzed the transcriptional profiles of NHET GFP⁺ and GFP[−] cells by single-cell gene expression quantitative RT-PCR using Fluidigm Biomark (Buganim et al., 2012) (Figure 2F–H). The genes tested in this analysis included ES cell-associated chromatin remodeling genes and modification enzymes, ES cell cell-cycle regulator genes, pluripotency markers, MEF markers, and genes active in signal transduction pathways important for ES cell maintenance and differentiation (see list of genes in legend). Expression of all of the genes analyzed showed similar distributions of expression levels in single GFP⁺ and GFP[−] cells, supporting the notion that GFP⁺ and GFP[−] ES cells have a very similar expression profile (Figure 2F). In agreement with this conclusion, hierarchical clustering (Figure 2G) and principal component analysis (Figure 2H) did not separate the GFP positive and negative cells. Only 3% of GFP[−] cells were separated from the majority of cells, and these likely represent differentiating cells as they differed in cell cycle regulators and some pluripotency markers. We conclude that the GFP⁺ and GFP[−] cells have very similar gene expression profiles, suggesting that they are equivalent in terms of their pluripotency status.

To test whether haploinsufficiency of Nanog was responsible for the large proportion of GFP[−]/Nanog⁺ cells in NHET and TNGA ES cells we overexpressed Nanog (Figure S1K–L). NHET and TNGA ES cells were infected with M2rtTA and tetO-Nanog-2A-Blue Fluorescent Protein (BFP). Dox was added to the cells and high BFP⁺/GFP⁺ cells were sorted onto feeder MEFs. Equal numbers of cells from single BFP⁺/GFP⁺ colonies were plated in the presence and absence of dox and analyzed for GFP and BFP. In three lines from both TNGA and NHET backgrounds none exhibited an increase in GFP⁺ cells upon Nanog overexpression (Figure S1K–L). The presence of GFP[−]/BFP⁺ cells and the observation that over-expression of Nanog did not increase the fraction of GFP⁺ cells (Figure S1K–L) is consistent with previous reports (Fidalgo et al., 2012; Navarro et al., 2012).

It seemed possible that the different Nanog expression patterns in NGNC cells vs. TNGA and NHET cells were a result of the gene targeting strategy used, which in the latter two cell lines resulted in a Nanog null allele and may have disturbed normal Nanog regulation. To directly test if gene targeting of *Nanog* was responsible for “GFP fluctuations” of Nanog expression, we targeted V6.5 (C57Bl/6 × 129) cells, the background of NHET, and E14Tg2a (129/Ola) cells, the background of TNGA, with our Nanog-2A-GFP vector (Figure S2A). We found that all V6.5 and E14Tg2a Nanog-2A-GFP cells expressed GFP and Nanog by sm-mRNA FISH, immunostaining and flow cytometry and that GFP expression faithfully reflected Nanog expression with GFP expression (48 transcripts/cell) approximately half of Nanog expression (112 transcripts/cell) in single cells (Figures 2I–L, S2B, S2C). To assay

for pluripotency of TNGA and NHET GFP⁺ and GFP⁻ cells and our V6.5 + Nanog-2A-GFP cells, we sorted 150 of the lowest GFP⁻ cells and 150 of the highest GFP⁺ cells from TNGA and NHET and counted the number of undifferentiated colonies at one week after plating. We also sorted 150 of the lowest GFP⁺ cells and 150 of the highest GFP⁺ cells from our V6.5 + Nanog-2A-GFP line. The low GFP⁺ cells are prone to differentiation generating only 16 undifferentiated colonies as compared to 44 from the high GFP⁺ cells. TNGA and NHET GFP⁺ and GFP⁻ cells gave rise to approximately the same number of undifferentiated colonies, further supporting that the cells are in equivalent states of pluripotency (Figure S2D). V6.5 + Nanog-2A-GFP ES cells were induced to differentiate by treatment with retinoic acid for 48 hours and, as expected, all GFP was lost (Figure S2E). Similarly to NHET and TNGA, a Nanog-GFP human ES cell reporter line generated by inserting GFP into the 5' untranslated region of the Nanog gene upstream of the Nanog start codon (ATG) yielded many GFP⁻, ES cell-like cells, suggesting similar regulation of Nanog expression in humans (Fischer et al., 2010) (Figure S2F).

The targeting strategy for NGR cells (Miyaniari and Torres-Padilla, 2012) did not disrupt the coding sequences of the Nanog alleles but nevertheless showed monoallelic expression in a significant fraction of the cells. We considered two possibilities to explain the difference between these results and ours. First, the targeting of the Nanog alleles in NGR cells involved the insertion of a ~4kb transgene containing a selectable marker in addition to three repeats of the GFP or mCherry coding sequences into the 3' UTR, resulting in a ~4kb insert compared to our construct that comprised only ~700bp with the selection cassette removed. It is possible that the larger insert disrupted Nanog regulation of the NGR alleles. We tested whether deletion of the selectable marker affected expression of the inserted transgene and, using sm-mRNA-FISH to measure Nanog expression, found that deletion of the selectable marker reduced the proportion of GFP negative cells from ~20% to 0, suggesting that the size of the genetic construct used may influence the results for this type of reporter (compare Figure 2J with S2G). We also noticed that Miyaniari et al. used C57BL/6 x cas (BC1) ES cells and C57BL/6 (BD10) ES cells, while we used C57BL/6 × 129 (V6.5) ES cells. To examine if genetic background could affect Nanog and Oct4 expression heterogeneity, we measured Nanog and Oct4 expression in single ES cells from different genetic backgrounds cultured in serum/LIF and 2i/LIF using sm-mRNA-FISH (Figure S2H and 2C (contains V6.5 and E14Tg2a data)). Out of 1113 single cells analyzed from the 6 ES cell lines, we only found 3 cells with no Nanog transcripts, consistent with our previous data in Figure S1C. However, we also found that V6.5 had fewer low Nanog-expressing cells (0%) as compared to V26.2 (C57BL/6) (9%) and ESC1 (C57BL/6 x cas) (13%) in serum/LIF condition (Figure S2I). Importantly, these low expressing Nanog cells were not differentiated and had high expression of Oct4 (~150 transcripts). Thus, genetic background does appear to influence the pattern of Nanog expression.

Filipczyk et al., in this issue of *Cell Stem Cell*, generated ES cells that carried different fluorescent reporters in both alleles of Nanog, similar to the construct described in Figure 1A (Filipczyk et al., 2013). In agreement with our results (Figure 1B–E) they observed that most cells expressed both reporters, although with greater variability in expression level that may in part be a result of their use of a larger size insert.

In summary, we have found using single-cell analysis that Nanog is biallelically expressed in mouse ES cells and that the degree of variation in expression level is very similar to that of many other pluripotency factors. We do not see evidence of a distinct subpopulation of cells with low Nanog expression, although it is possible that such a population exists in some circumstances. Our analysis of a range of Nanog-GFP reporters suggests that disruption of one of the two alleles or insertion of a large downstream cassette may disturb normal transcriptional control and thus not give a faithful reflection of endogenous Nanog expression. More broadly, our findings also suggest that these issues are important to take into account when designing reporter constructs to monitor other factors, in the pluripotency network and beyond.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Austin Smith for sharing TNGA cells, Bryce Carey, Meelad Dawlaty, Frank Soldner, and Ameera Salama for helpful discussions, Sandy Klemm for discussions and Stella FISH probe, Thor Theunissen for help with sorting, and Alexander van Oudenaarden for facilities. D.A.F. is a Vertex Scholar and was supported by a NSF Graduate Research Fellowship and Jerome and Florence Brill Graduate Student Fellowship. A.W.C was supported by a Croucher and Ludwig Research Fellowship. Y.B. was supported by a NIH Kirschstein NRSA (1 F32 GM099153-01A1). R.J. is an adviser to Stemgent and a cofounder of Fate Therapeutics. This work was supported by NIH grants HD 045022 and R37CA084198 to R.J.

References

- Buganim Y, Faddah DA, Cheng AW, Itskovich E, Markoulaki S, Ganz K, Klemm SL, van Oudenaarden A, Jaenisch R. Single-Cell Expression Analyses during Cellular Reprogramming Reveal an Early Stochastic and a Late Hierarchic Phase. *Cell*. 2012; 150:1209–1222. [PubMed: 22980981]
- Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell*. 2003; 113:643–655. [PubMed: 12787505]
- Chambers I, Silva J, Colby D, Nichols J, Nijmeijer B, Robertson M, Vrana J, Jones K, Grotewold L, Smith A. Nanog safeguards pluripotency and mediates germline development. *Nature*. 2007; 450:1230–1234. [PubMed: 18097409]
- Fidalgo M, Faiola F, Pereira CF, Ding J, Saunders A, Gingold J, Schaniel C, Lemischka IR, Silva JC, Wang J. Zfp281 mediates Nanog autorepression through recruitment of the NuRD complex and inhibits somatic cell reprogramming. *Proc Natl Acad Sci U S A*. 2012; 109:16202–16207. [PubMed: 22988117]
- Filipczyk A, Gkatzis K, Fu J, Hoppe P, Lickert H, Anastasiadis K, Schroeder T. Biallelic expression of Nanog protein in mouse embryonic stem cells. *Cell Stem Cell*. 2013
- Fischer Y, Ganic E, Ameri J, Xian X, Johannesson M, Semb H. NANOG reporter cell lines generated by gene targeting in human embryonic stem cells. *PLoS One*. 2010; 5:e12533. [PubMed: 20824089]
- Hatano SY, Tada M, Kimura H, Yamaguchi S, Kono T, Nakano T, Suemori H, Nakatsuji N, Tada T. Pluripotential competence of cells associated with Nanog activity. *Mech Dev*. 2005; 122:67–79. [PubMed: 15582778]
- Hayashi K, Lopes SM, Tang F, Surani MA. Dynamic equilibrium and heterogeneity of mouse pluripotent stem cells with distinct functional and epigenetic states. *Cell Stem Cell*. 2008; 3:391–401. [PubMed: 18940731]
- Jaenisch R, Young R. Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. *Cell*. 2008; 132:567–582. [PubMed: 18295576]

- Kalmar T, Lim C, Hayward P, Munoz-Descalzo S, Nichols J, Garcia-Ojalvo J, Martinez Arias A. Regulated fluctuations in nanog expression mediate cell fate decisions in embryonic stem cells. *PLoS Biol.* 2009; 7:e1000149. [PubMed: 19582141]
- Macarthur BD, Sevilla A, Lenz M, Muller FJ, Schuldt BM, Schuppert AA, Ridden SJ, Stumpf PS, Fidalgo M, Ma'ayan A, et al. Nanog-dependent feedback loops regulate murine embryonic stem cell heterogeneity. *Nat Cell Biol.* 2012
- Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell.* 2003; 113:631–642. [PubMed: 12787504]
- Miyazari Y, Torres-Padilla ME. Control of ground-state pluripotency by allelic regulation of Nanog. *Nature.* 2012; 483:470–473. [PubMed: 22327294]
- Navarro P, Festuccia N, Colby D, Gagliardi A, Mullin NP, Zhang W, Karwacki-Neisius V, Osorno R, Kelly D, Robertson M, et al. OCT4/SOX2-independent Nanog autorepression modulates heterogeneous Nanog gene expression in mouse ES cells. *EMBO J.* 2012; 31:4547–4562. [PubMed: 23178592]
- Orkin SH, Wang J, Kim J, Chu J, Rao S, Theunissen TW, Shen X, Levasseur DN. The transcriptional network controlling pluripotency in ES cells. *Cold Spring Harb Symp Quant Biol.* 2008; 73:195–202. [PubMed: 19478325]
- Raj A, van den Bogaard P, Rifkin SA, van Oudenaarden A, Tyagi S. Imaging individual mRNA molecules using multiple singly labeled probes. *Nat Methods.* 2008; 5:877–879. [PubMed: 18806792]
- Silva J, Barrandon O, Nichols J, Kawaguchi J, Theunissen TW, Smith A. Promotion of reprogramming to ground state pluripotency by signal inhibition. *PLoS Biol.* 2008; 6:e253. [PubMed: 18942890]
- Silva J, Nichols J, Theunissen TW, Guo G, van Oosten AL, Barrandon O, Wray J, Yamanaka S, Chambers I, Smith A. Nanog is the gateway to the pluripotent ground state. *Cell.* 2009; 138:722–737. [PubMed: 19703398]
- Singh AM, Hamazaki T, Hankowski KE, Terada N. A heterogeneous expression pattern for Nanog in embryonic stem cells. *Stem Cells.* 2007; 25:2534–2542. [PubMed: 17615266]
- Wray J, Kalkan T, Smith AG. The ground state of pluripotency. *Biochem Soc Trans.* 2010; 38:1027–1032. [PubMed: 20658998]
- Ying QL, Wray J, Nichols J, Batlle-Morera L, Doble B, Woodgett J, Cohen P, Smith A. The ground state of embryonic stem cell self-renewal. *Nature.* 2008; 453:519–523. [PubMed: 18497825]

Highlights

- Single-cell analysis reveals Nanog is biallelically expressed in mESCs.
- Variation in Nanog expression is similar to that of other pluripotency markers.
- Heterozygous loss of function knock-in reporters do not reflect Nanog expression.
- Genetic background can influence the range of Nanog expression.

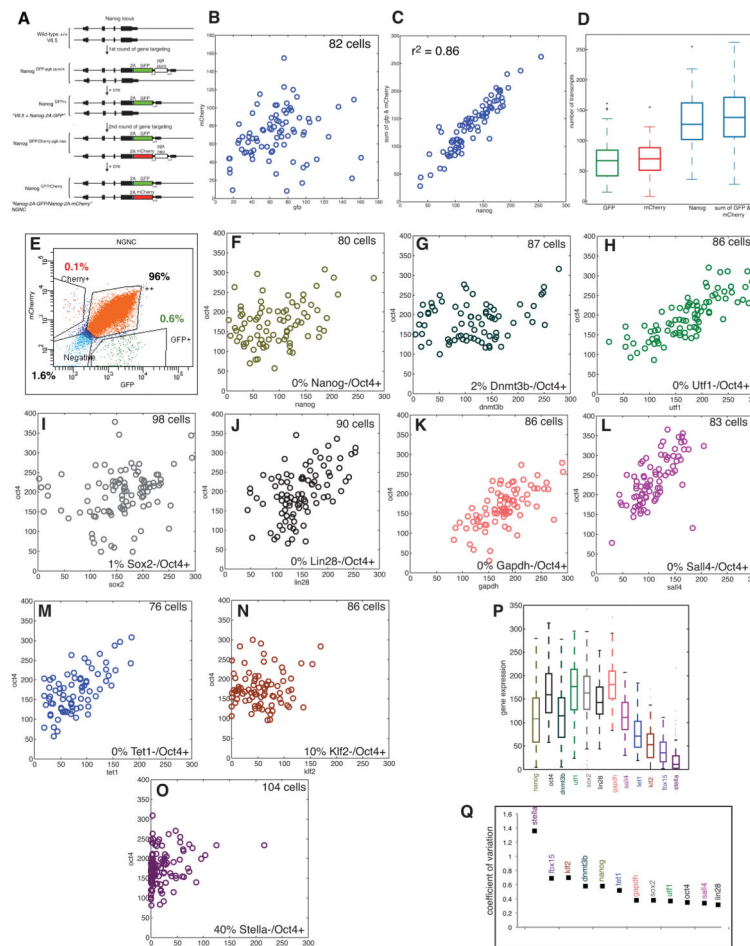


Figure 1. Nanog is biallelically expressed in ES cells and equally variable as that of other pluripotency factors

(A) Schematic of the NGNC reporter targeting. Four rounds of gene targeting were performed: (1) V6.5 ES cells targeted with Nanog-2A-GFP floxed pgk puro (2) Cre excision of the floxed pgk puro (3) Nanog-2A-GFP ES cells targeted with Nanog-2A-mCherry pgk neo (4) Cre excision of the floxed pgk neo (B) sm-mRNA-FISH of mCherry vs. GFP expression in single NGNC ES cells cultured with serum/LIF, 82 cells analyzed. (C) sm-mRNA-FISH of sum of mCherry and GFP vs. Nanog expression in single NGNC ES cells cultured with serum/LIF. (D) Box plot of GFP (green), mCherry (red), Nanog (Blue), and sum of GFP and mCherry (blue) transcripts in single cells, quantified by sm-mRNA-FISH. On each box, the central mark is the median, the edges of the box are the 25th and 75th percentiles, the whiskers extend to the most extreme datapoints not considered to be not outliers, and the outliers (+) are plotted individually. Points are drawn as outliers if they are larger than $Q3+W*(Q3-Q1)$ or smaller than $Q1-W*(Q3-Q1)$. (E) Flow cytometric analysis of NGNC ES cells in serum/LIF. (F–O) sm-mRNA-FISH of Oct4 vs. Nanog (F), Dnmt3b (G), Utf1 (H), Sox2 (I), Lin28 (J), Gapdh (K), Sall4(L), Tet1 (M), Klf2(N), and Stella (O) expression in single V6.5 ES cells cultured with serum/LIF. (P) Box plot of transcripts in single cells, quantified by sm-mRNA-FISH, of the genes in (F–O). On each box, the central mark is the median, the edges of the box are the 25th and 75th percentiles, the whiskers

extend to the most extreme datapoints not considered to be not outliers, and the outliers (+) are plotted individually. Points are drawn as outliers if they are larger than $Q3+W*(Q3-Q1)$ or smaller than $Q1-W*(Q3-Q1)$. (Q) Coefficient of variation of the genes in (F-O). See also Figure S1A and S1B.

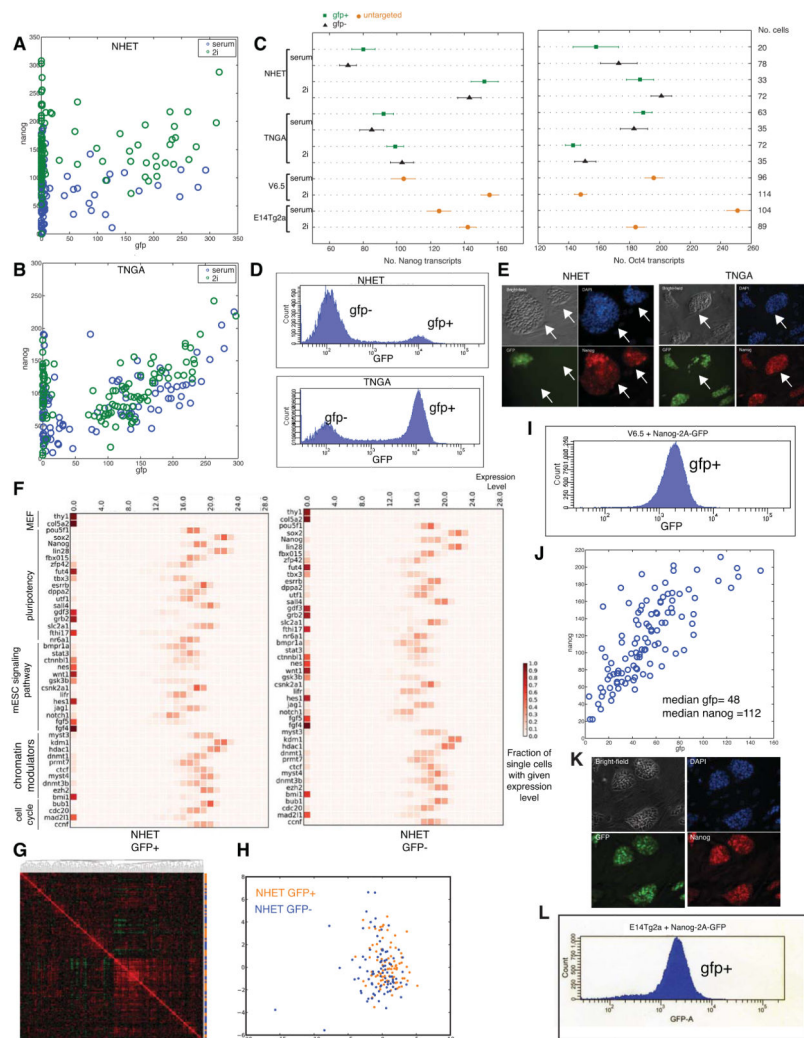


Figure 2. Nanog heterozygous loss of function knock-in reporters do not reflect Nanog expression
 sm-mRNA-FISH of Nanog vs. GFP expression in single (A) NHET ES cells and (B) TNGA ES cells cultured in serum/LIF (blue) and 2i/LIF (green) condition. (NHET serum-102, NHET 2i-105, TNGA serum-98, TNGA 2i-107 cells analyzed). (C) Plot of the median number of Nanog (left) and Oct4 (right) transcripts, quantified by sm-mRNA-FISH, in GFP+ (square, green) and GFP- (triangle, black) fractions of NHET and TNGA ES cells and V6.5 and E14Tg2a (untargeted ES cells) cultured in serum/LIF (serum) and 2i/LIF (2i) condition. Error bars represented standard error of the mean. (D) Flow cytometric analysis of GFP in NHET ES cells (top) and TNGA ES cells (bottom). (E) Representative bright-field image (upper left), DAPI (upper right), and immunostaining of GFP protein (bottom left) and Nanog protein (bottom right) of NHET (left) and TNGA (right) ES cells cultured in serum/LIF. White arrows indicate GFP-/Nanog+ cells. (F) Heatmap of gene expression values of single NHET GFP+ (left) and GFP- (right) ES cells. Fraction of single-cells with an expression level (top number) is indicated by color of the box (see key on right). The genes tested in this analysis included ES cell-associated chromatin remodeling genes and modification enzymes (Myst3, Kdm1, Hdac1, Dnmt1, Prmt7, Ctf, Myst4, Dnmt3b, Ezh2,

Bmi1), ES cell cell-cycle regulator genes (Bub1, Cdc20, Mad211, Ccnf), pluripotency markers (Oct4, Sox2, Nanog, Lin28, Fbxo15, Zfp42, Fut4, Tbx3, Esrrb, Dppa2, Utf1, Sall4, Gdf3, Grb2, Slc2a1, Fthi17, Nr6a1), MEF markers (Thy1 and Col5a2), and genes active in signal transduction pathways important for ES cell maintenance and differentiation (Bmpr1a, Stat3, Ctnnb1, Nes, Wnt1, Gsk3b, Csnk2a1, Lifr, Hes1, Jag1, Notch1, Fgf5, Fgf4). (G) Hierarchical clustering of single NHET GFP⁺ and GFP⁻ ES cells. Bar on right displays GFP⁺ (orange dot) and GFP⁻ cells (blue dot). (H) Principal component (PC) projections of single NHET GFP⁺ (orange) and GFP⁻ (blue) ES cells, colored by their sample identification. (I) Flow cytometric analysis of GFP in V6.5 + Nanog-2A-GFP ES cells cultured with serum/LIF. (J) sm-mRNA-FISH of Nanog vs. GFP expression in single V6.5 + Nanog-2A-GFP ES cells (pgk puro looped out) cultured with serum/LIF, 107 cells analyzed. (K) Representative bright-field image (upper left), DAPI (upper right), and immunostaining of GFP protein (bottom left) and Nanog protein (bottom right) of V6.5 + Nanog-2A-GFP ES cells cultured with serum/LIF (L) Flow cytometric analysis of GFP in E14Tg2a + Nanog-2A-GFP (pgk puro looped out) ES cells cultured with serum/LIF. See also Figures S1 and S2.