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Brief Report

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

Dina A. Faddah,[1,2] Haoyi Wang,[1] Albert Wu Cheng,[1,3] Yarden Katz,[1,4] Yosef Buganim,[1] and Rudolf Jaenisch[1,2,*]
[1]Whitehead Institute for Biomedical Research, Cambridge, MA 02142, USA
[2]Department of Biology
[3]Department of Computational and Systems Biology
[4]Department of Brain and Cognitive Sciences
Massachusetts Institute of Technology, Cambridge, MA 02139, USA
*Correspondence: jaenisch@wi.mit.edu
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SUMMARY

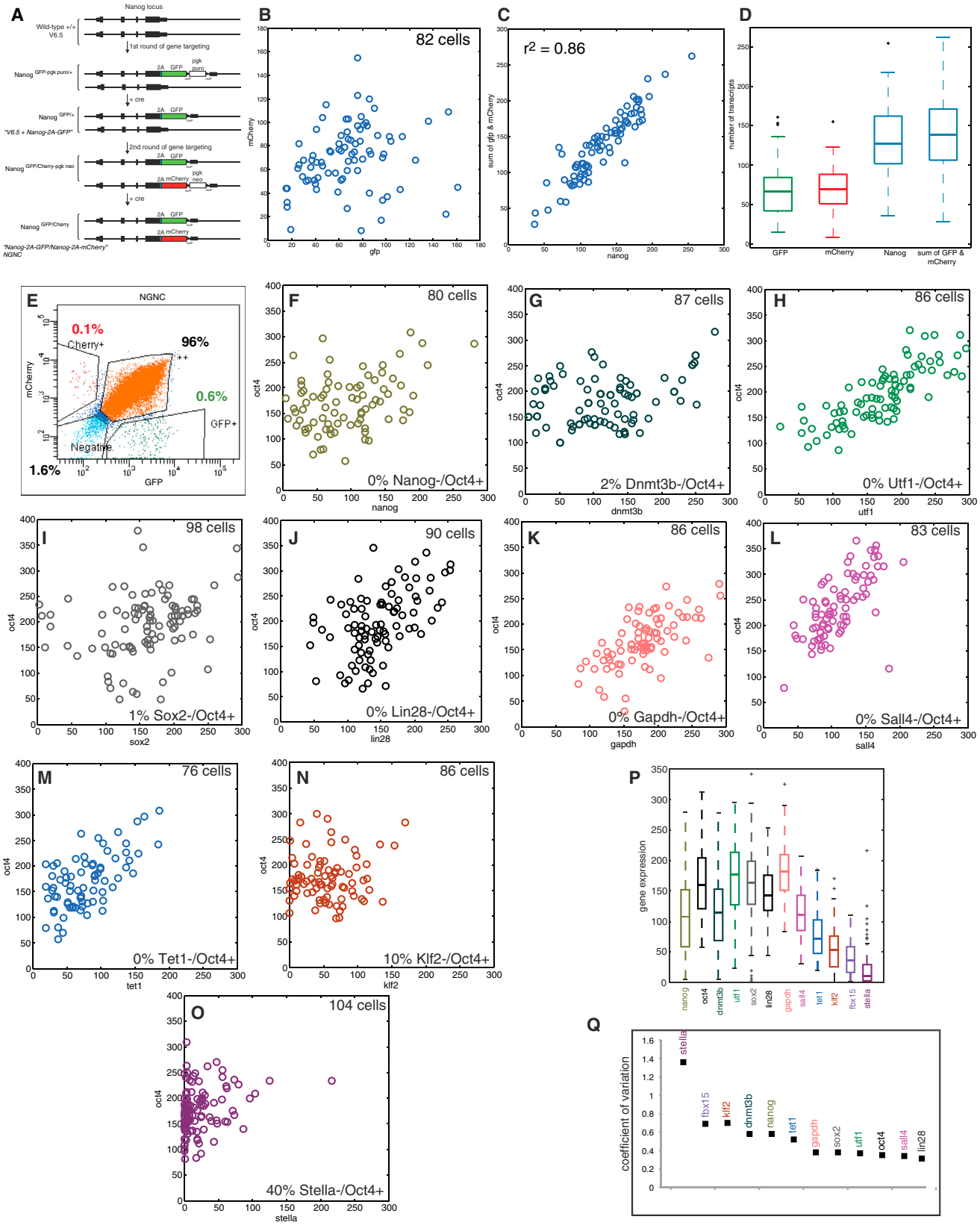
The homeodomain transcription factor Nanog is a central part of the core pluripotency transcriptional network and plays a critical role in embryonic stem cell (ESC) self-renewal. Several reports have suggested that Nanog expression is allelically regulated and that transient downregulation of Nanog in a subset of pluripotent cells predisposes them toward 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 ESCs independently of culture condition. We also show that the overall variation in endogenous Nanog expression in ESCs 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.

Derived from the inner-cell mass of the embryo, embryonic stem cells (ESCs) have the ability to divide indefinitely while maintaining the capacity to differentiate into different cell types, and core transcription factors are 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 ESCs, suggesting that allelic regulation of the gene itself contributes to this heterogeneity (Chambers et al., 2007; Kalmar et al., 2009; Karwacki-Neisius, 2013; 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 and leukemia inhibitory factor (LIF) and, to a lesser extent, if at all, in 2i and 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 ESC self-renewal versus differentiation, and low or no Nanog expression is 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 ESCs 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 ESCs. To monitor the two alleles of Nanog in single cells with single-molecule messenger RNA fluorescence in situ hybridization (sm-mRNA-FISH) (Buganim et al., 2012; Raj et al., 2008), we generated a V6.5 ESC line where GFP was inserted immediately downstream of the Nanog-coding region, the selectable marker being deleted. Using a similar targeting strategy, we inserted sequences encoding mCherry into the second Nanog allele (Figure 1A, Figure S1A available online). In this construct, GFP and mCherry dissociate from Nanog by the 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 (NGNC) ESCs by sm-mRNA-FISH and found that all cells expressed mCherry and GFP transcripts (Figure 1B), the total level of Nanog transcripts in a given cell being approximately equal to the sum of the GFP and mCherry transcripts (Figure 1C). Box plot analysis revealed that GFP and mCherry expression levels were equal and approximately half that of Nanog expression (Figure 1D). We quantified mCherry+/GFP+, GFP+, and mCherry+ cells grown in serum and 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 and LIF or 2i and 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.

In order to compare the variability of Nanog expression to that of other pluripotency factors, we used sm-mRNA-FISH to quantify transcripts of nine pluripotency genes (Nanog, Dnmt3b, Utf1, Sox2, Lin28, Sall4, Tet1, Klf2, and Fbx15), one housekeeping gene (Gapdh), and a known heterogeneously expressed gene (Stella), each in combination with Oct4 in single cells (Figures 1F–1O and S1C–S1D). Out of 899 cells analyzed, we only identified 1% that were Nanog−/Oct4+ (Figure S1C). Klf2 and Fbx15 were not always coexpressed with Oct4 with 10% of



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Klf2−/Oct4+ cells and 14% Fbx15−/Oct4+ cells (Figures 1N and S1D). Figure 1O shows 40% Stella−/Oct4+ negative cells, a number slightly lower than the 70%–80% Stella− cells identified by immunofluorescence in a previous report (Hayashi et al., 2008). All genes examined had different levels of expression, and their expression levels ranged in single cells (Figure 1P). Importantly, Stella had the highest coefficient of variation, whereas 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, such as 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; therefore, we investigated potential explanations. The majority of studies characterizing heterogeneity in Nanog expression have used heterozygous loss-of-function knockin GFP reporters. Specifically, in the Nanog GFP+/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 NHET ESCs), 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 was inserted into the other Nanog allele, resulting in NGR ESCs. The GFP and mCherry allele also contained an IRES-Neo or IRES-Hygro selection cassette, respectively (Miyanari 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, they observed dynamic fluctuations of Nanog expression 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 and 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 ESCs targeted with an identical vector as previously described (NHET ESCs; Hatano et al., 2005) or by using the published targeted E14Tg2a ESCs (TNGA; Chambers et al., 2007) (Figure S1E).

To assess the influence of culture condition, we compared gene expression in NHET ESCs that were grown under three different conditions: (1) on feeders in serum and LIF, (2) on feeders in 2i and LIF and no serum, and (3) on gelatin (no feeders) in 2i and LIF and no serum. Although growth in serum and LIF (condition 1) resulted in a lower number of Nanog transcripts in comparison to growth on feeders in 2i and LIF and no serum (condition 2, Figure 2C), we found that the culture conditions (2) and (3) did not significantly affect the level of Nanog (between 140 and 145 transcripts), of Oct4 (between 190 and 205 transcripts), and of GFP (between 175 and 180 transcripts). In the following experiments, we only used cells grown on feeders that were either cultured in serum and LIF or in 2i and LIF and no serum.

Confirming the published data, this analysis revealed that the majority of NHET ESCs cultured in serum and LIF or 2i and LIF were GFP− (79% and 69%, respectively) (Figure 2A). However, the great majority of the GFP− cells grown in 2i and LIF (98%) and 100% of GFP− cells in serum and LIF expressed Nanog RNA. Similarly, most TNGA GFP− ESCs cultured in serum and LIF condition were Nanog+ (Figure 2B). These data, summarized in Figure 2C, indicate that GFP+ and GFP− NHET and TNGA ESCs expressed Nanog and Oct4 mRNA at comparable levels. Cultivation of NHET cells in 2i and 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 and 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 (Figures 2E and S1F). In both NHET and TNGA cell lines, we found GFP−, GFP+, and "speckled" colonies containing both GFP+ and GFP− cells (Figures 2E and S1F). We also found that GFP− cells can give rise to GFP+ cells, and GFP+ can generate GFP− cells within one or two passages (Figure S1G), which is consistent with previous reports (Chambers et al., 2007).

To monitor the nontargeted allele of NHET ESCs, we inserted mCherry immediately downstream of the Nanog coding region (using a Nanog-2A-mCherry construct). We found the NHET GFP− cells to be mCherry+, further supporting the notion that the other allele of Nanog is active in the GFP− cells (Figure S1H).

Figure 1. Nanog Is Biallelically Expressed in ESCs and Equally Variable as the Expression of Other Pluripotency Factors

(A) A schematic of NGNC reporter targeting. We performed two rounds of gene targeting: (1) V6.5 ESCs targeted with Nanog-2A-GFP floxed pgk puro, (2) Cre excision of the floxed pgk puro, (3) Nanog-2A-GFP ESCs targeted with Nanog-2A-mCherry pgk neo, and (4) Cre excision of the floxed pgk neo.

(B) sm-mRNA-FISH analysis of mCherry versus GFP expression in single NGNC ESCs cultured with serum and LIF. A total of 82 cells were analyzed.

(C) sm-mRNA-FISH analysis of the sum of mCherry and GFP versus Nanog expression in single NGNC ESCs cultured with serum and LIF.

(D) A box plot of GFP (green), mCherry (red), Nanog (blue), and the 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 data points not considered to be not outliers, and the outliers (+) are plotted individually. Points are drawn as outliers if they are larger than $Q3 + W \times (Q3 - Q1)$ or smaller than $Q1 - W \times (Q3 - Q1)$.

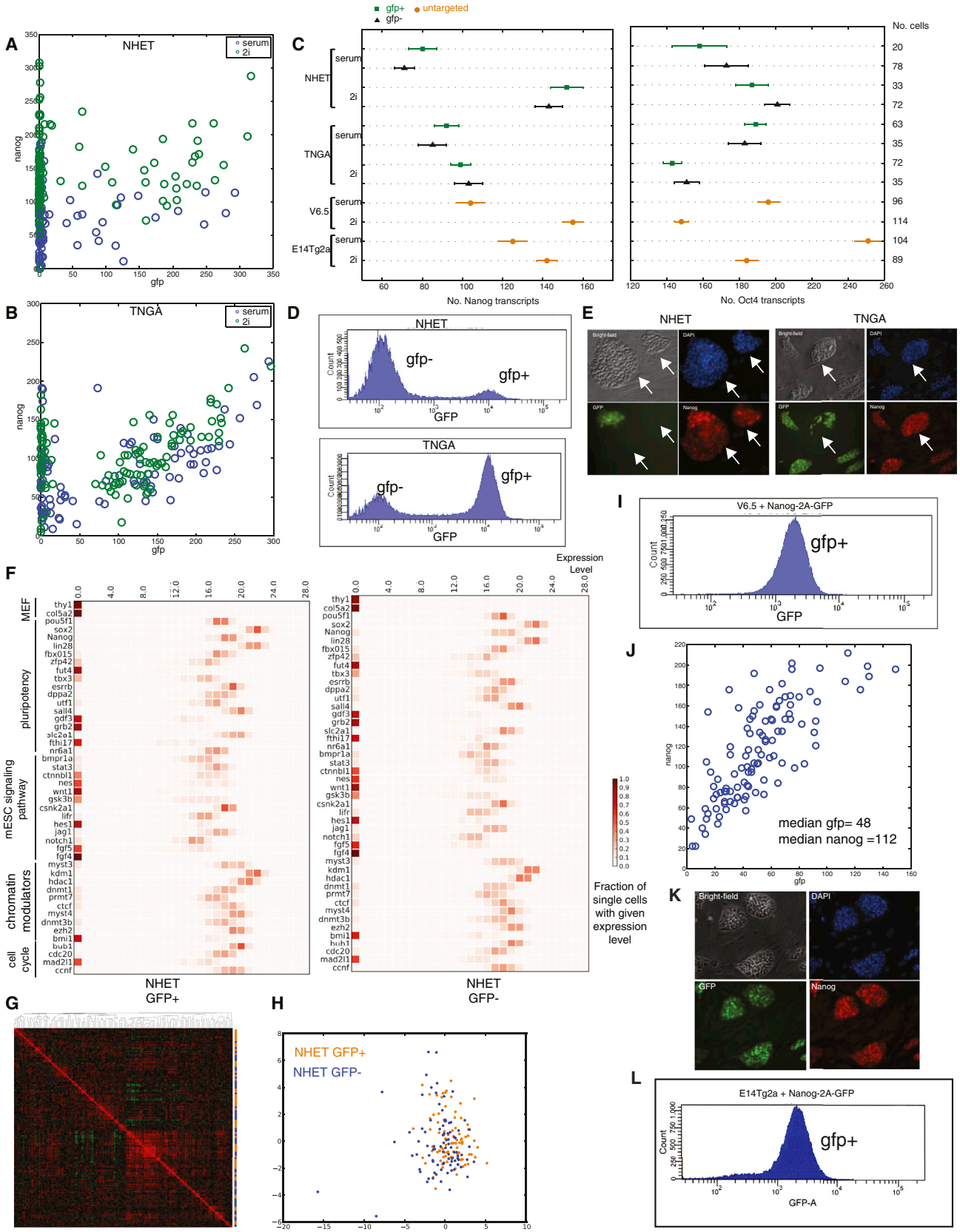
(E) Flow cytometric analysis of NGNC ESCs in serum and LIF.

(F–O) sm-mRNA-FISH of Oct4 versus 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 ESCs cultured with serum and LIF.

(P) A box plot of transcript number (gene expression) 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 data points not considered to be not outliers, and the outliers (+) are plotted individually. Points are drawn as outliers if they are larger than $Q3 + W \times (Q3 - Q1)$ or smaller than $Q1 - W \times (Q3 - Q1)$.

(Q) The coefficient of variation of the genes shown in (F–O).

See also Figures S1A and S1B.



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Western blotting was performed on protein derived from the GFP+ and GFP− fractions of NHET and TNGA, and, importantly, these experiments confirmed that GFP expression did not reflect Nanog protein expression (Figures S1I–S1J)—a result different from previously published data (Chambers et al., 2007). In summary, these observations demonstrate that (1) only a fraction of NHET and TNGA cells express GFP, which is in agreement with previous reports (Chambers et al., 2007), (2) the NHET and TNGA GFP− cells also express Nanog, (3) 2i and LIF affects Nanog, Oct4, and GFP expression differently in TNGA and NHET ESCs, and (4) 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) (Figures 2F–2H). The genes tested in this analysis included ESC-associated chromatin remodeling genes and modification enzymes, ESC-cycle regulator genes, pluripotency markers, mouse embryonic fibroblast (MEF) markers, and genes active in signal transduction pathways important for ESC maintenance and differentiation (see the list of genes in the Figure 2F 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− ESCs 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+ and GFP− cells. Only 3% of GFP− cells were separated from the majority of cells, and these most likely represent differentiating cells, given that 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 the haploinsufficiency of Nanog was responsible for the large proportion of GFP−/Nanog+ cells in NHET and TNGA ESCs, we overexpressed Nanog (Figures S1K–S1L). NHET and TNGA ESCs 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. We confirmed overexpression of Nanog by sm-mRNA-FISH. In three lines from both NHET and TNGA backgrounds, none exhibited an increase in GFP+ cells upon Nanog overexpression (Figures S1K–S1L). The fact that there is a presence of GFP−/BFP+ cells and the observation that the overexpression of Nanog did not increase the fraction of GFP+ cells (Figure S1K–S1L) are consistent with previous reports (Fidalgo et al., 2012; Navarro et al., 2012).

It seemed possible that the different Nanog expression patterns in NGNC cells versus NHET and TNGA 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 whether gene targeting *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). Using sm-mRNA-FISH, immunostaining, and flow cytometry, we found that all V6.5 and E14Tg2a Nanog-2A-GFP cells expressed GFP and Nanog and that GFP expression faithfully reflected Nanog expression, GFP expression (48 transcripts per cell) being approximately half that of Nanog (112 transcripts per cell) in single cells (Figures 2I–2L, S2B, and S2C). To assay for the 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 1 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, only generating 16 undifferentiated colonies in comparison to the 44 colonies

Figure 2. Nanog Heterozygous Loss-of-Function Knockin Reporters Do Not Reflect Nanog Expression

(A and B) sm-mRNA-FISH of Nanog versus GFP expression in single NHET ESCs (A) and TNGA ESCs (B) cultured in serum and LIF (blue) and 2i and LIF (green) conditions. A total of 102 NHET serum, 105 NHET 2i, 98 TNGA serum, and 107 TNGA 2i cells were 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 ESCs and V6.5 and E14Tg2a (untargeted ESCs) cultured in serum and LIF (serum) and 2i and LIF (2i) conditions. Error bars represent SEM.

(D) Flow cytometric analysis of GFP in NHET ESCs (top) and TNGA ESCs (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) ESCs cultured in serum and LIF. White arrows indicate GFP−/Nanog+ cells.

(F) A heatmap of gene expression values of single NHET GFP+ (left) and GFP− (right) ESCs. The fraction of single cells with an expression level (top number) is indicated by the color of the box (see key on right). The genes tested in this analysis included ESC-associated chromatin remodeling genes and modification enzymes (Myst3, Kdm1, Hdac1, Dnmt1, Prmt7, Ctcf, Myst4, Dnmt3b, Ezh2, and Bmi1), ESC-cycle regulator genes (Bub1, Cdc20, Mad2l1, and Ccnf), pluripotency markers (Oct4, Sox2, Nanog, Lin28, Fbxo15, Zfp42, Fut4, Tbx3, Esrrb, Dppa2, Utf1, Sall4, Gdf3, Grb2, Slc2a1, Fthi17, and Nr6a1), MEF markers (Thy1 and Col5a2), and genes active in signal transduction pathways important for ESC maintenance and differentiation (Bmpr1a, Stat3, Ctnnbl1, Nes, Wnt1, Gsk3b, Csnk2a1, Lifr, Hes1, Jag1, Notch1, Fgf5, and Fgf4).

(G) Hierarchical clustering of single NHET GFP+ and GFP− ESCs. The 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) ESCs colored by their sample identification.

(I) Flow cytometric analysis of GFP in V6.5 + Nanog-2A-GFP ESCs cultured with serum and LIF.

(J) sm-mRNA-FISH of Nanog versus GFP expression in single V6.5 + Nanog-2A-GFP ESCs (pgk puro looped out) cultured with serum and LIF. A total of 107 cells were 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 ESCs cultured with serum and LIF.

(L) Flow cytometric analysis of GFP in E14Tg2a + Nanog-2A-GFP (pgk puro looped out) ESCs cultured with serum and LIF.

See also Figures S1 and S2.

generated 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 ESCs were induced in order to differentiate by treatment with retinoic acid for 48 hr, and, as expected, all GFP was lost (Figure S2E). Similarly to NHET and TNGA, a Nanog-GFP human ESC 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−, ESC-like cells, suggesting a similar regulation of Nanog expression in humans (Fischer et al., 2010) (Figure S2F).

The targeting strategy for NGR cells (Miyanari 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 ~4 kb transgene containing a selectable marker in addition to three repeats of the GFP or mCherry coding sequences into the 3′ untranslated region, resulting in a ~4 kb insert in comparison our construct that comprised only ~700 bp with the selection cassette removed. It is possible that the larger insert disrupted Nanog regulation of the NGR alleles. We tested whether the deletion of the selectable marker affected the expression of the inserted transgene and, using sm-mRNA-FISH to measure Nanog and GFP expression, found that the deletion of the selectable marker reduced the proportion of GFP− 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 to Figure S2G). We also noticed that Miyanari and Torres-Padilla, 2012 used C57BL/6 × cas (BC1) ESCs and C57BL/6 (BD10) ESCs, whereas we used C57BL/6 × 129 (V6.5) ESCs. To examine whether genetic background could affect Nanog and Oct4 expression heterogeneity, we measured Nanog and Oct4 expression in single ESCs from different genetic backgrounds cultured in serum and LIF and 2i and LIF with sm-mRNA-FISH (Figures S2H and 2C [both contain V6.5 and E14Tg2a data]). Out of 1,113 single cells analyzed from the six ESC lines, we only found three 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%) in comparison to V26.2 (C57BL/6) (9%) and ESC1 (C57BL/6 × cas) (13%) in serum and LIF condition (Figure S2I). Importantly, these low Nanog+ cells were not differentiated and highly expressed Oct4 (~150 transcripts per cell). Thus, genetic background does appear to influence the pattern of Nanog expression.

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

In summary, using single-cell analysis, we have found that Nanog is biallelically expressed in mouse ESCs 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 the disruption of one of the two alleles or the 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, both in the pluripotency network and beyond.

SUPPLEMENTAL INFORMATION

Supplemental Information contains Supplemental Experimental Procedures and two figures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.04.019.

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