Stochastic NANOG fluctuations allow mouse embryonic stem cells to explore pluripotency

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ABSTRACT

Heterogeneous expression of the transcription factor NANOG has been linked to the existence of various functional states in pluripotent stem cells. This heterogeneity seems to arise from fluctuations of Nanog expression in individual cells, but a thorough characterization of these fluctuations and their impact on the pluripotent state is still lacking. Here, we have used a novel fluorescent reporter to investigate the temporal dynamics of NANOG expression in mouse embryonic stem cells (mESCs), and to dissect the lineage potential of mESCs at different NANOG states. Our results show that stochastic NANOG fluctuations are widespread in mESCs, with essentially all expressing cells showing fluctuations in NANOG levels, even when cultured in ground-state conditions (2i media). We further show that fluctuations have similar kinetics when mESCs are cultured in standard conditions (serum plus leukemia inhibitory factor) or ground-state conditions, implying that NANOG fluctuations are inherent to the pluripotent state. We have then compared the developmental potential of low-NANOG and high-NANOG mESCs, grown in different conditions, and confirm that mESCs are more susceptible to enter differentiation at the low-NANOG state. Further analysis by gene expression profiling reveals that low-NANOG cells have marked expression of lineage-affiliated genes, with variable profiles according to the signalling environment. By contrast, high-NANOG cells show a more stable expression profile in different environments, with minimal expression of lineage markers. Altogether, our data support a model in which stochastic NANOG fluctuations provide opportunities for mESCs to explore multiple lineage options, modulating their probability to change functional state.

KEY WORDS: Gene expression heterogeneity, Pluripotency, Lineage priming, Nanog, Stem cells

INTRODUCTION

Pluripotent stem cells manifest the unique capacity to commit to any of the lineages that generate the vast diversity of cell types in the body. This capacity relies on a gene regulatory network that must allow access to multiple lineage differentiation programmes, while at the same time ensuring that the pluripotent state is maintained.

The transcription factors (TFs) OCT4 (also known as OCT3/4 or POU5F1), SOX2 and NANOG have been shown to form the core of such network, coordinating the activity of a plethora of other genes that function together to implement the pluripotent state (Young, 2011). However, although NANOG is essential to reach this state in cells of the inner-cell mass of the mouse embryo, pluripotency can be maintained in the absence of NANOG, for instance in mouse embryonic stem cells (mESCs) in which both Nanog alleles have been inactivated (Chambers et al., 2007). This finding suggests that the pluripotency network can operate efficiently with variable amounts of NANOG, also indicated by the consistent observation that mESCs express heterogeneous levels of this protein, as revealed by immunodetection (Singh et al., 2007; Toyooka et al., 2008). The use of fluorescent reporters to monitor NANOG expression in mESCs corroborates the existence of such heterogeneity (Chambers et al., 2007; Miyanari and Torres-Padilla, 2012; Abranches et al., 2013; Filipczyk et al., 2013), and reveals in addition that isolated subpopulations of mESCs with high or low reporter activity re-establish a heterogeneous distribution, when cultured for several days (Chambers et al., 2007; Kalmar et al., 2009; Abranches et al., 2013). This implies that NANOG levels dynamically fluctuate in individual mESCs; however, whether these fluctuations have a functional impact on the pluripotent state is still an open question.

The observation that Nanog−/− mESCs have an increased tendency to spontaneously differentiate (Chambers et al., 2007) led to the hypothesis that the ‘low-NANOG’ state is permissive for differentiation, whereas ‘high-NANOG’ mESCs would be in a pristine state of pluripotency, unresponsive to differentiation cues. This permissiveness of ‘low-NANOG’ mESCs to enter differentiation has been associated with the phenomenon of ‘lineage priming’, during which pluripotent cells display sporadic and reversible expression of lineage-affiliated genes, reflecting their increased predisposition to exit the pluripotent state. Thus, besides being a ‘maker’ of pluripotency, NANOG might be also a ‘marker’ of the mESC differentiation potential. However, little is known on how Nanog and the pluripotency gene regulatory network operate in mESCs to ensure proper access to lineage differentiation programmes. It is therefore important to understand how NANOG heterogenous expression arises in mESCs, and what functional implications this heterogeneity might have for pluripotency and lineage commitment.

In the early mouse embryo [embryonic day (E) 3.5], fluctuations of Nanog expression seem to occur stochastically (Dietrich and Hiiragi, 2008; Ohnishi et al., 2014), and it has been proposed that mESCs in culture do also switch from one Nanog expression state to the other in a stochastic manner, in a process driven by transcriptional noise (Kalmar et al., 2009). This hypothesis led to the development of various mathematical models that incorporate a contribution of stochastic mechanisms in gene expression (Chickarmane et al., 2006; Guantes and Poyatos, 2008; Macarthur...
et al., 2008; Kalmar et al., 2009; Glauche et al., 2010), aimed at explaining how Nanog fluctuations might emerge in mESCs. However, quantitative data to analyse the dynamic Nanog expression and support such models are still scarce in the literature (Kalmar et al., 2009; Miyanari and Torres-Padilla, 2012; Trott et al., 2012; Munoz Descalzo et al., 2013). Other mechanisms to justify the observed NANOG heterogeneity in mESCs have been proposed, coinciding in the view that fluctuations are controlled at the transcriptional level and emerge from particular configurations of the pluripotency gene regulatory network, involving various types of feedback loops (Niwa et al., 2009; Lanner and Rossant, 2010; MacArthur et al., 2012). For instance, transcriptional auto-regression has been proposed to generate fluctuations in Nanog expression (Navarro et al., 2012), whereas other authors propose that these fluctuations result from complex network interactions involving feedback and crosstalk between various signalling pathways and pluripotency TFs (Adachi and Niwa, 2013; Posfai et al., 2014). An intriguing mechanism based on dynamic allele-switching of Nanog in mESCs has also been proposed to underlie its fluctuations (Miyanari and Torres-Padilla, 2012), although recent evidence does not support a contribution of this mechanism to the observed heterogeneity of NANOG expression in mESCs (Faddah et al., 2013; Filipczyk et al., 2013).

Contrary to mESCs maintained in standard culture conditions (serum plus leukemia inhibitory factor/LIF), it has been argued that mESCs cultured in fully defined media containing inhibitors of the fibroblast growth factor (FGF)/mitogen-activated protein kinase pathway and glycogen synthase kinase 3 (GSK3) (2i media; Ying et al., 2008; Nichols et al., 2009) reach a stable and homogeneous state of NANOG expression, unperturbed by intrinsic noise (Silva et al., 2009). This has been defined as a ground state of pluripotency (Silva and Smith, 2008; Nichols and Smith, 2009), in which gene expression heterogeneities would be abolished, and all mESCs would contain homogeneously high levels of NANOG expression (Wray et al., 2010). However, we have previously reported that mESCs grown in 2i conditions do also show heterogeneity in NANOG expression, and contain a minor subpopulation of cells with low NANOG levels (using immunodetection or a Nanog-fluorescent reporter), leaving open the hypothesis that NANOG expression also fluctuates in ground-state mESCs. In this paper, we have analysed by real-time confocal microscopy the dynamic expression of a Nanog:VNP reporter construct in individual mESCs at 15 min intervals (supplementary material Movies 1 and 2), and we focused our analysis on the variations that occur during the interphase of a single cell cycle (around 10–16 h, supplementary material Fig. S1A). In total, 37 mESCs grown in serum/LIF and 49 in 2i/LIF were tracked. The results (Fig. 1A) show that fluctuations in Nanog:VNP expression can be detected in mESCs grown in both conditions, with the major difference being the number of cells that do not display expression of Nanog:VNP at any time point, which is higher in serum/LIF (12/37 cells) than in 2i/LIF (2/49 cells). In either media, all other cells show marked variations in Nanog:VNP over time.

To compare the dynamic range of these fluctuations in the two media, we calculated a ‘fluctuation index’ (Fln) for each cell, defined as the amplitude between the maximum and minimum fluorescence levels detected along an interphase. When the distribution of Fln values is plotted for cells showing fluctuations (Fig. 1B), no significant differences can be detected between mESCs cultured in serum/LIF and 2i/LIF, implying that the amplitude of Nanog:VNP fluctuations is independent of the signalling environment. To evaluate if these fluctuations also occur with similar pace in the two media, we calculated the rates of fluorescence increase and decrease (per hour) for all cells, and plotted these values in a histogram (Fig. 1C). The graph shows a peak around zero for mESCs in serum/LIF (both for negative and positive rates), denoting those cells that show no Nanog:VNP fluctuations. A second peak contains fluctuating mESCs, and no significant difference in the positive or negative rates of Nanog:VNP variation can be detected when these cells are cultured in serum/LIF and 2i/LIF, indicating that fluctuation rates of Nanog:VNP in mESCs are also independent of culture conditions.

Analysis of fluctuation paces (Table 1) reveals in addition that Nanog:VNP levels can change very fast in mESCs, either increasing or decreasing. As a result, mESCs can transit between high and low Nanog:VNP states in periods as short as 4 h, implying that NANOG fluctuations can occur in the range of hours and not days, as has been previously suggested (Macarthur et al., 2008; Kalmar et al., 2009). Although statistically significant differences are not observed between serum/LIF and 2i/LIF conditions, consistently higher rates of Nanog:VNP variation are observed in 2i/LIF (Table 1).
We have also plotted Nanog:VNP levels when mESCs enter mitosis (Fig. 1D), aiming to establish possible correlations with the cell cycle. Our findings reveal that mESCs can initiate mitosis with a large range of Nanog:VNP levels, with a similar distribution to that observed for all measured time points along the cell cycle (compare histograms in Fig. 1D and 1E), thereby indicating that entry into mitosis is not correlated with NANOG expression. The main difference between mESCs grown in serum/LIF or 2i/LIF conditions is the higher number of mESCs that enter mitosis without Nanog:VNP expression in serum/LIF (asterisk in Fig. 1D), reflecting the higher percentage of non-expressing cells in these conditions. Average values for Nanog:VNP fluorescence at the time of mitosis tend to be higher in 2i/LIF conditions, although not statistically different.

Finally, we have analysed Nanog:VNP levels in sister cells along a single cell cycle, starting when they emerge from mitosis, both in serum/LIF and 2i/LIF conditions (supplementary material Fig. S4). Using the empirical cumulative distribution function and Kolmogorov–Smirnov (K–S) test, our analysis indicates that sister cells considerably diverge in their Nanog:VNP profiles after mitosis, with only two pairs (out of 16) showing significant similarity in their Nanog:VNP profiles along the whole cell cycle. These data support the hypothesis that NANOG fluctuations in mESCs are regulated in a stochastic manner (Kalmar et al., 2009; MacArthur and Lemischka, 2013). Altogether, our analysis of Nanog:VNP dynamic expression indicates that NANOG fluctuations are a cell-autonomous property of pluripotent mESCs that occur with very similar dynamics in different signalling environments, underlying the observed heterogeneity in NANOG expression in mESCs.

### Nanog transcription is a noisy process in mESCs

We next asked whether the heterogeneous NANOG expression is accompanied by similar variability in Nanog mRNA expression, at
show that a small percentage (around 5%) of mESCs in 2i/LIF do not detect nascent transcripts. In these experiments, the authors show with the percentage of E14tg2a and Nd mESCs in which we detect serum/LIF conditions (around 25%). These results are in agreement with evidence (Hansen and van Oudenaarden, 2013) of transcriptional bursts in mESCs, which reveals that its transcription in mESCs is pulsatile and occurs in bursts. This is supported by recently published data (Abranches et al., 2013), implying that each mESC spends a considerable fraction of time with Nanog mRNA production in mESCs is associated with an underlying heterogeneity at the level of Nanog mRNA transcription.

NANOG fluctuations create ‘primed’ states without lineage bias

Fluctuations in NANOG levels have been suggested to create the opportunity for mESCs to explore their multi-lineage differentiation potential in response to environmental signals (Silva and Smith, 2008), with the low-NANOG state correlating with a higher propensity to enter differentiation (Chambers et al., 2007; Kalmar et al., 2009). Low-NANOG mESCs have been proposed to be in a ‘primed’ state associated with a particular configuration of the pluripotency network, in which low-level transcription of lineage-affiliated genes is allowed, anticipating the transition into differentiation. Although there are various reports supporting this hypothesis, a detailed evaluation of the NANOG states that result from the observed fluctuations in mESCs has never been described. We therefore used the Nd reporter to assess the differentiation potential of the low- and high-NANOG states, in both serum/LIF and 2i/LIF media, and complemented this analysis with a detailed molecular characterization of these states in serum/LIF, 2i/LIF and BMP4/LIF media.

Clonogenic assays were used to evaluate the potential of Nd mESCs according to their levels of Nanog:VNP expression. Cells with low (VNPL) and high (VNPH) levels of Nanog:VNP were sorted by fluorescence-activated cell sorting (FACS) (Fig. 3A) and plated at clonal density (60 cells/cm²) in either serum/LIF or 2i/LIF, followed by culture for 6 days, after which colonies were stained for

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**Fig. 2. Nanog heterogeneity at the mRNA level, in different culture conditions (serum/LIF and 2i/LIF).** (A) Representative field from Nd mESCs grown in serum/LIF, showing individual mRNA ‘spots’ in different cells. Quantifications are depicted in C. (B) Same as A for Nd cells grown in 2i/LIF. (C) Histograms showing the distribution of Nanog mRNA molecules per cell, for Nd and E14tg2a mESCs. The number of analysed cells in each condition is shown in brackets. Population mean, Fano factor (defined as the ratio of the variance to the mean) (Raj and van Oudenaarden, 2009) and coefficient of variation (CV, defined as the ratio of the standard deviation to the mean) are also shown for each cell population. For both conditions, the calculated Fano factor and CV values are similar between Nd and E14tg2a mESC lines. The Fano factor allows an estimation of noise strength and is much higher than predicted for a normal Poissonian distribution (equal to 1). Insets show respective boxplots, with median values depicted as solid black lines within the box, and mean values as full black circles. The edges of the box indicate the 25th and 75th percentiles and the whiskers indicate the range of non-outlier data points. Outliers are plotted individually (open circles). (D) Same as C for Nd mESCs grown in 2i/LIF medium. Scale bars: 10 µm.
VNPH cells, independently of the culture media (Fig. 3B,C). In undifferentiated mESCs, VNPL, VNPH were collected for posterior analysis. (B) Number and type of colonies mESC subpopulation (All, VNPL, VNPH) initially grown in serum/LIF (600 cells per well of a six-well dish) and colony types analysed after 6 days. (C) Same as B for cells initially grown in 2i/LIF media (600 cells per well of a six-well dish) and colony types analysed after 6 days.

This global analysis reveals that subpopulations of mESCs with high Nanog:VNP-expression produce functionally heterogeneous subsets of mESCs, with low-NANOG mESCs being more susceptible to enter differentiation, whereas mESC with high NANOG expression are in a more pristine pluripotent state.

At the molecular level, recent work (MacArthur et al., 2012) showed that transient Nanog knockdown (for 36 h) in mESCs leads to increased expression of several lineage-affiliated genes, providing further support for the current hypothesis that the low-NANOG state is permissive for lineage priming. To explore this hypothesis, we took advantage of the Nd reporter line and generated a comprehensive gene expression profiling of FACS-purified subsets of mESCs with either VNPL or VNPH levels, employing a microfluidic qPCR approach (Fluidigm BioMark system). Purified subpopulations were collected from mESCs grown in three different conditions [serum/LIF, 2i/LIF and BMP4/LIF (Ying et al., 2003)], in which distinct proportions of low- and high-Nanog:VNP-expressing cells are present (Fig. 3A, supplementary material Fig. S6A). In addition, mESCs initially grown in serum/LIF were differentiated for 48 h in serum only (no LIF), and were analysed as a ‘differentiation control’ (‘Diff’). A panel of 48 genes was profiled, including diagnostic markers for pluripotency and for lineage commitment.

The results (Fig. 4) confirm that VNPL mESCs have increased expression of several lineage-affiliated genes, in all culture conditions, when compared with VNPH mESCs. By contrast, pluripotency genes show lower variations in expression between VNPL and VNPH mESCs, with the lowest differences being observed in 2i/LIF conditions (Fig. 4A). We note that the pluripotency genes showing higher variations in VNPL cells (Klf4, Esrrb, Zfp42) are those known to be heterogeneously expressed in mESCs. Their expression is thus likely to correlate stronger with Nanog fluctuations. However, these variations are expected to have little impact on the overall activity of the pluripotency network (MacArthur et al., 2012), and are not expected to affect the pluripotent status of VNPL and VNPH mESCs.

By comparing the changes in expression of all lineage-affiliated genes between the VNPL and VNPH stages, in the three culture conditions (Fig. 4B-D), marked variations in the expression of particular genes can be observed (for instance, Pax3, Pdgfra, Gata6 and Mx1), but a coherent pattern in lineage priming cannot be detected. In fact, the various lineage-specific genes show uncorrelated variations in all three culture conditions, making it difficult to establish whether priming for particular lineages is occurring in different culture media. We therefore applied principal component analysis (PCA) to perform a global and unbiased evaluation of the expression profiling data for each subpopulation, independently of single-gene incoherent variations and of the correctness of selected lineage markers (Fig. 5). This global analysis reveals that subpopulations of mESCs with high Nanog:VNP cluster together in the multidimensional transcript profile space, independently of culture conditions, indicating that they are more similar at the molecular level than the corresponding VNPL subpopulations (Fig. 5A). These, by contrast, occupy unique profile spaces, with VNPL subpopulations from each culture condition showing more unrelated expression profiles between them than the corresponding VNPH subpopulations. This finding reflects both the higher variability and higher expression of lineage markers in VNPL mESCs. Altogether, our analysis offers further support to the hypothesis that the low-NANOG state is more permissive for lineage priming and, furthermore, indicates that this priming is strongly modulated by the signalling environment in which mESCs are maintained. However, PCA analysis of individual gene expression
profiles across the various subpopulations from different media (Fig. 5B) does not show any preferential gene clustering along specific lineages, with only two discernible profile spaces: a more constrained space occupied by pluripotency genes, and a more scattered space where lineage-affiliated genes are distributed. This suggests that priming at the low-NANOG state is not biased for specific lineages, and that modulation by the signalling environment of mESCs does not impose definitive constraints on the initial steps of lineage specification.

**DISCUSSION**

Heterogeneity in NANOG expression is a hallmark of mESCs maintained in standard culture conditions (serum/LIF), and has been attributed to the existence of dynamic fluctuations in its expression in individual mESCs. It has also been speculated that such fluctuations might be an important property of pluripotent stem cells, creating windows of opportunity during which these cells can initiate lineage commitment (Silva and Smith, 2008). However, NANOG fluctuations in mESCs have not been characterized in detail, and the lineage potential of mESCs with variable levels of fluctuating NANOG remains to be fully assessed.

In this paper, we have used a novel fluorescent reporter to appropriately monitor the dynamic expression of NANOG in mESCs cultured in various conditions. We also took advantage of this reporter to obtain purified populations of mESCs with low or high NANOG levels, and evaluated the differentiation potential of these cells. Altogether, our data suggest that NANOG fluctuations are an intrinsic property of the pluripotent state, providing a potential mechanism for mESCs to explore the available differentiation options before definitive lineage commitment.

**NANOG fluctuations are an inherent feature of pluripotent mESCs**

Confirming our previous report (Abranches et al., 2013) that a subpopulation of low-NANOG mESCs is present in 2i/LIF culture conditions (see also Morgani et al., 2013), our time-lapse analysis of...
Nanog:VNP levels in mESCs reveals that NANOG fluctuations do also occur in 2i/LIF conditions, and with very similar dynamics to those observed in serum/LIF. Although the percentage of mESCs with NANOG expression is higher in 2i/LIF, the levels are not static and all expressing cells show Nanog:VNP fluctuations. Thus, in all tested culture conditions, fluctuations in NANOG levels seem to be an inherent feature of pluripotent mESCs.

We noticed also that mESCs grown in 2i/LIF show increased Nanog:VNP levels, when compared with serum/LIF (Fig. 3A). As our results indicate that fluctuations have similar dynamics in either media, this raises the question of how mESCs reach higher Nanog:VNP levels in 2i/LIF. This can be explained by the fact that mESCs grown in 2i/LIF have higher levels of Nanog and Vnp mRNAs (Fig. 2 and data not shown), which must result in increased levels of VNP and NANOG per cell. As fluctuation rates are calculated as relative values, these are independent of the absolute number of reporter molecules per cell; hence, two individual mESCs can show similar fluctuation rates while having different VNP concentrations. In addition, our analysis points to a small increase in fluctuation rates of mESCs in 2i/LIF, together with a higher frequency of fluctuating cells. Altogether, these differences must underlie the observed higher levels of Nanog:VNP in 2i/LIF mESCs, when compared with serum/LIF conditions.

We further show that transitions from low- to high-NANOG expression (and vice versa) are much faster (in the range of hours) than previously predicted from population reversibility experiments that used long-lived GFP reporters (Chambers et al., 2007; Kalmar et al., 2009), occurring within the interphase of a single cell cycle. This finding challenges the hypothesis that NANOG heterogeneity in mESCs results from slow and global transcriptome fluctuations, over long timescales (one week or more) (Huang, 2009), and highlights the highly dynamic nature of Nanog expression.

Our analysis shows also that mESCs can enter mitosis with very different levels of Nanog:VNP expression, and that sister cells that emerge from cytokinesis with similar levels might then diverge during the ensuing interphase. Thus, despite the finding that NANOG can regulate expression of cell cycle-associated genes (Choi et al., 2012), our work reveals that NANOG fluctuations are not correlated with the cell cycle. In addition, mESCs do not seem to retain a ‘memory’ of NANOG expression during successive cell divisions, highlighting the intrinsic stochasticity of NANOG fluctuations. Also, no periodicity in Nanog:VNP expression can be detected within a cell cycle. Altogether, these findings offer strong evidence for the stochastic nature of NANOG fluctuations in pluripotent mESCs, independently of the culture conditions. Actually, our finding that fluctuations are widespread in 2i/LIF conditions implies that NANOG fluctuations are not dependent on autocrine ERK signalling mediated by FGF4, as previously proposed (Silva et al., 2008; Nichols et al., 2009; Yamanaka et al., 2010; Herberg et al., 2014), although ERK signalling might modulate some dynamical aspects of these fluctuations.

Our analysis of Nanog mRNA distribution, using smFISH in Nd and E14tg2a mESCs, offers further evidence for the stochastic nature of Nanog expression, revealing that NANOG heterogeneity is accompanied by an underlying variability at the level of mRNA transcription. In addition, the observed non-Poissonian Nanog mRNA distributions hint at a burst-like stochastic production of Nanog transcripts, even when mESCs are at the pristine ‘ground state’ (2i/LIF conditions). This is in agreement with recently published data, which show that Nanog burst-like transcription in mESCs may occur from both alleles in a non-coordinated manner (Hansen and van Oudenaarden, 2013; Deng et al., 2014). Strikingly, Nanog transcription from each allele seems to be even less correlated in mESCs grown in 2i/LIF conditions than in serum/LIF conditions (as shown in figure 2C of Hansen and van Oudenaarden, 2013), further demonstrating the stochastic nature of Nanog expression in pluripotent mESCs.

A marked heterogeneity in Nanog mRNA expression has also been reported in a recent paper (Faddah et al., 2013), in a different set of mESC lines. However, the authors also report a significant heterogeneity in mRNA expression for the pluripotency genes Oct4 and Sox2, although the coefficient of variation is about half of that reported for Nanog mRNA. This finding is in agreement with the hypothesis that mRNA transcription in mESCs is a noisy process, probably as a result of the uniquely permissive chromatin environment found in mESCs (Gaspar-Maia et al., 2011). However, as both SOX2 and OCT4 proteins are more stable than NANOG in mESCs, the observed transcriptional noise is buffered at...
the protein level for SOX2 and OCT4, leading to almost homogenous expression.

The analysis of different NANOG reporter cell lines performed by Faddah et al. (2013) also raised important questions about their reliability, which seems to be affected by the insertion of reporter cassettes, and emphasizes the need for designing adequate reporter constructs. In the case of the Nd reporter mESC line used in our studies, we show in this paper that it reproduces the variability in Nanog mRNA distributions found in the parental E14tg2a line. Together with the previous demonstration (Abranches et al., 2013) that NANOG heterogeneity is also similar in Nd and E14tg2a mESCs, these findings validate the Nd line as an adequate NANOG reporter, without the pitfalls found for other reporter lines due to interference with endogenous Nanog expression and/or use long-lived fluorescent reporters.

Functional relevance of NANOG fluctuations in the pluripotent state

Our evaluation of the lineage potential of mESCs with fluctuating levels of Nanog:VNP confirms that the low-NANOG state is molecularly and functionally distinct from the high-NANOG state. Actually, low-NANOG mESCs consistently show less clonogenic capacity than cells with high-NANOG, independently of the cell culture environment, and are also more predisposed to originate differentiated cell colonies. In addition, gene expression profiling of low- and high-NANOG mESCs reveals that lineage-priming at the low-NANOG state is a generalized phenomenon, as shown by the marked increase in expression of known lineage-affiliated regulators of all germ layers, including trophoblast. This increase occurs in any of the three different culture conditions used in our studies, although there is no obvious trend in the specific lineages that low-NANOG mESCs seem to be primed: in each of the tested conditions, the combination of lineage-affiliated genes with increased expression is qualitatively and quantitatively different, suggesting that lineage commitment does not occur through fixed and hierarchically organized pathways. Our results are more compatible with stochastic models of lineage decision (Pina et al., 2012; Teles et al., 2013), in which the initial events that bias cells to specific lineages are not predetermined, and mESCs can follow multiple trajectories into commitment, exploring the whole pluripotent decision space. These initial exploratory events are still revertible and occur when mESCs reach low NANOG levels; when fluctuating NANOG levels increase and mESCs move to a high-NANOG state, active lineage-specific genes are silenced and cells return to a naive state. This model of an initially stochastic commitment is also supported by the single-cell profiling analysis of Nanog mRNA distributions found in the parental E14tg2a line. Together with the previous demonstration (Abranches et al., 2013) that NANOG heterogeneity is also similar in Nd and E14tg2a mESCs, these findings validate the Nd line as an adequate NANOG reporter, without the pitfalls found for other reporter lines due to interference with endogenous Nanog expression and/or use long-lived fluorescent reporters.

The kinetics of Nanog:VNP for mESCs grown in serum/LIF or 2i/LIF are shown in supplementary material Figs S2 and S3, respectively. Histograms deduced using the global data of all measured time points and all analysed cells are shown in supplementary material Fig. S1B. For each analysed cell during interphase, the amplitude of Nanog:VNP fluctuations was also calculated and represented in the form of a ‘fluctuation index’ (FIh). The rates of fluorescence increase (gain, +) or decrease (loss, −) were calculated for each time interval (15 min), and histograms were plotted for the obtained values in arbitrary units of fluorescence (A.U.F.) per hour (Fig. 1C). Finally, the kinetics of eight mother cells and their respective progeny were analysed for mESCs grown in either serum/LIF or 2i/LIF (supplementary material Fig. S4).

Single-molecule fluorescent in situ hybridization (smFISH)

smFISH using Stellaris™ FISH probes (Biosearch Technologies) (Raj et al., 2008) was performed for mESCs grown in serum/LIF and in 2i/LIF conditions. TMR-labelled Nanog probes (supplementary material Table S1) were designed, prepared and used as published in Raj et al. (2008). Cells were imaged within 24 to 48 h on an inverted fluorescence Zeiss Axiovert 200M microscope, using a 100×1.4 oil-immersion objective and a cooled CCD camera (Roper Scientific CoolSnap HQ CCD), and data were processed using MATLAB (Raj et al., 2008).

Flow cytometry (FC) analysis

Live cell FC analysis and sorting experiments were performed as described in Abranches et al. (2013), on a FACS Calibur cytometer (Becton Dickinson) or a FACS Aria cell sorter (Becton Dickinson). For sorting, VNP− and VNP+ Nd mESC populations were collected and processed for clonal assays or RNA extraction. Representative histogram of FACS-sorted Nd subpopulations, grown in serum/LIF, 2i/LIF or BMP4/LIF are shown in Fig. 3A and supplementary material Fig. S6A. Bulk populations of mESCs (All) and ‘Diff’ samples were also collected from the sorter and analysed.

Clonal assays

To assess the clonogenic potential of different mESC subpopulations (All, VNPL and VNPH), grown in serum/LIF or 2i/LIF, cells were plated at a

MATERIALS AND METHODS

Maintenance and differentiation of mESCs

The mESC lines used for this study were Nd (Abranches et al., 2013) and E14tg2a (a kind gift from Austin Smith’s lab, University of Cambridge, UK). Nd mESCs were derived from E14tg2a mESCs and are a BAC-transgenic line for VNP-tagged Nanog gene (Abranches et al., 2013). mESCs were grown as described in Abranches et al. (2013). Different mESC culture media were used: Glasgow minimum essential medium (Invitrogen) supplemented with ES-qualified serum (Invitrogen) and LIF (serum/LIF conditions); 2i (iStem medium, Stem Cells Inc.) (Ying et al., 2008) supplemented with LIF (2i/LIF conditions); and ESGRO Complete Clonal Grade medium (Millipore Inc.) (Ying et al., 2003) supplemented with LIF (BMP4/LIF conditions). Additionally, mESCs were grown in Glasgow minimum essential medium supplemented with serum but in the absence of LIF for 48 h, as a ‘differentiation control’ (‘Diff’).

Single-cell live imaging

Cells were plated on poly-L-ornithine- (Sigma) and laminin- (Sigma) coated dishes and time-lapse images were acquired with an Andor spinning disk confocal microscope. A z-stack of seven images was acquired for each chosen field with slice intervals of 3.5 μm. The time interval between image acquisitions was set to 15 min and the duration of the experiment ranged from 18 to 39 h. Fluorescence quantification was performed using ImageJ software and subsequent mathematical analyses were carried out using R packages. The kinetics of Nanog:VNP for mESCs grown in serum/LIF or 2i/LIF are shown in supplementary material Figs S2 and S3, respectively. Histograms deduced using the global data of all measured time points and all analysed cells are shown in supplementary material Fig. S1B. For each analysed cell during interphase, the amplitude of Nanog:VNP fluctuations was also calculated and represented in the form of a ‘fluctuation index’ (FIh). The rates of fluorescence increase (gain, +) or decrease (loss, −) were calculated for each time interval (15 min), and histograms were plotted for the obtained values in arbitrary units of fluorescence (A.U.F.) per hour (Fig. 1C). Finally, the kinetics of eight mother cells and respective progeny were analysed for mESCs grown in either serum/LIF or 2i/LIF (supplementary material Fig. S4).
concentration of 60 cells/cm² in six-well dishes. After 6 days in culture, cells were fixed, stained with AP and manually counted for the number of undifferentiated (AP positive), mixed and differentiated colonies (AP negative) (supplementary material Fig. S5).

**RNA extraction and Fluidigm analysis**

Total RNA was extracted from 10⁶ mESCs of each analysed subpopulation using the High Pure RNA Isolation kit (Roche Diagnostics), with the inclusion of DNAseI treatment. The first strand cDNA was synthesized from 0.5 µg of total RNA using SuperscriptIII Reverse Transcriptase (Invitrogen) and random hexamers. The absence of contaminating genomic DNA was confirmed by PCR amplification from RT negative samples. Converted RNA was subjected to preamplification PCR reaction to increase the number of template molecules. 

Mathematical analysis was performed using R, which provided a set of principal component values for each sample and gene. The first two with reliable expression values.

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**Competing interests**

The authors declare no competing financial interests.

**Author contributions**

E.A. and D.H. conceived and designed the experiments. E.A., A.M.V.G. and M.M. contributed reagents/materials/analysis tools. E.A. and D.H. wrote the paper.

E.A. and D.H. conceived and designed the experiments. E.A., A.M.V.G. and M.M. contributed reagents/materials/analysis tools. E.A. and D.H. wrote the paper.

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**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.108910/-/DC1

**References**


