Dynamics of temporal OCT4 regulation in human embryonic stem cells

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ABSTRACT Pluripotency is the defining characteristic of human embryonic stem cells (hESCs) allowing them to differentiate into any somatic cell in the human body. For the promising clinical applications of hESCs, improved regulation of pluripotency and differentiation trajectories of colonies is required. The pluripotency transcription factors (PTFs) which regulate pluripotency are inherently stochastic (with small fluctuations impacting cell fate), inherited asymmetrically, and more similar in closely related cells. Here we use available time-lapse experimental data of OCT4 fluorescence intensity to quantify the temporal dynamics of the PTF OCT4 over a cell lifetime. We evaluate the internal self-regulation of OCT4 using the Hurst exponent and autocorrelation analysis, quantify the intra-cellular fluctuations and consider the diffusive nature of OCT4 over time for individual cells and pairs of their descendants. After possible asymmetric splitting, OCT4 abundance fluctuates sub-diffusively, showing anti-persistent self-regulation with a Hurst exponent of 0.38. Auto-correlation analysis shows anti-persistence for five hours or longer is seen in 86% of cells, on average between three and 12 hours into the cell cycle. The OCT4 fluctuations between five minute intervals follow a Laplace distribution, with BMP4 addition provoking smaller changes and tighter self-regulation, particularly in the differentiated fate group. This quantitative framework provides a basis for comparison to other experiments, and the development of mathematical models of pluripotency.

SIGNIFICANCE Increased control of pluripotency is required for the promising clinical applications of human pluripotent stem cells. The pluripotency transcription factors (PTFs) which regulate pluripotency are highly stochastic, with even small changes impacting cell fate. It is therefore necessary to quantify the intra-cellular temporal dynamics of PTFs to deepen our understanding of pluripotency regulation and assist in the development of mathematical models. Here we quantify how the PTF OCT4 behaves temporally over a cell lifetime, filling in the gap between possible asymmetric inheritance and cell division, and quantifying its self-regulation.

INTRODUCTION
Human embryonic stem cells (hESCs) form colonies through repeated mitosis and have the ability to differentiate into all somatic cell types in the human body: the pluripotency property. The pluripotency of hESCs is their defining characteristic, central to their touted applications in drug discovery, regenerative and personalised medicine (1–6). These promising clinical applications of hESCs require great control over colony pluripotency, homogeneity and differentiation trajectories in-vitro (7), yet this remains challenging.

The control and optimisation of pluripotency across colonies is difficult due to the complex inter-regulatory dynamics of pluripotency. At the single-cell level, pluripotency is inherently stochastic. It is suggested that pluripotency is not well defined at the single-cell level but is instead a statistical property of a cell population (8, 9). Cell pluripotency is also affected by many factors: the local environment (10, 11), interactions with neighbours (12, 13), the cell cycle (14) and the substrate (15). On the colony scale, complex collective effects of pluripotency can be seen. In the presence of restrictive geometries, differentiated cells form bands occurring around colony edges (13, 16).

Pluripotency maintenance relies on the inter-regulation of pluripotency transcription factors (PTFs): the genes OCT4, SOX2 and NANOG (17–19). After several divisions, PTF fluctuations lead to the establishment of sub-populations with varying pluripotency (17). The differentiation of a stem cell into a specialised cell is the departure from the pluripotent state led by PTF destabilisation and their interaction with chemical signalling pathways (17, 20, 21). This decision of
a stem cell to either remain pluripotent or to differentiate is known as its fate decision. It is unknown how much cell fate decisions are led by inherited factors versus environmental factors and intra-cellular signalling as even clonal (genetically identical) cells under the same conditions make different fate decisions (22). Colonies exhibit heterogeneous sub-populations of cells with differing levels of PTF expression (17, 20, 23) which suggests a play-off between disruptive single-cell and regulatory community effects (8, 9, 13, 16).

On the intra-cellular level, a narrow range of PTF abundance is necessary for maintained pluripotency (24, 25) and small fluctuations can bias cell fate decisions in the G1 phase of the cell cycle (26). Furthermore, the PTFs are inherited asymmetrically as a cell divides, biasing the fate of the daughter cells and contributing to colony heterogeneity (27–29) with the decision to differentiate largely determined before any differentiation stimulus is added (27). As PTF fluctuations are inherently stochastic (8, 9, 30), it is important to quantify their temporal dynamics and the knock-on effects to cell fate. In this paper we build upon the previously published work of Ref. (27) which considers OCT4 intensity levels and provides experimental data with rich opportunities for further quantitative analysis and mathematical modelling.

Mathematical models are a powerful tool through which to deepen our understanding of the inherent, systematic behaviours of stem cells (31). Recent models have focused on describing pluripotency and cell fate decisions to guide the optimisation and control of pluripotency in-vitro (32) and are informed by recent studies of fluctuations of PTFs throughout colonies (9, 26, 27) and the spatial patterning of differentiation (13, 16). Many models use complex coupled stochastic differential equations to describe PTF fluctuations (33–35) while others use a gene network analysis framework (36, 37) or take a mechanistic approach (38).

Although the dynamics of OCT4 are complex, affected by many genetic factors and closely regulated by the other PTFs (17, 21, 39), here we aim to isolate autonomous properties of OCT4 for pluripotent and differentiated cells to facilitate the development of descriptive mathematical models. This quantification of OCT4 will provide a basis for identifying systematic similarities and differences between PTFs in future experiments, and highlights some key indicators of cell fate. As our quantitative understanding of PTF regulation increases, more complex regulatory properties can be considered to build fundamental models.

Here we use the experimental data from Ref. (27) of time-lapse fluorescent measurements of the OCT4-mCherry reporter levels in cells in a growing hESC colony to quantify the dynamics of intra-cellular OCT4. In addition to the OCT4 splitting dynamics and fluctuations described in Ref. (27), we describe quantitatively the fluctuations in OCT4 in relation to cell fate and the addition of the differentiation agent BMP4. We quantify the self-regulation of OCT4 through anti-persistence and characterise it within the diffusion framework. This quantitative analysis, along with Ref. (27), provides the basis for developments in mathematical and statistical models of pluripotency.

**MATERIALS AND METHODS**

**Experiment**

The experiment was carried out by Purvis Lab (University of North Carolina, School of Medicine), and published in Ref. (27). The OCT4 levels (mean OCT4-mCherry fluorescence intensity) in a human embryonic stem cell (H9) colony were determined and cells were live-imaged for approximately 70 hours. At 40 hours the differentiation agent BMP4 was added to the cells. The cell IDs, ancestries and positions were extracted along with their OCT4 immuno-fluorescence intensity values (reported in arbitrary fluorescence units a.f.u.). Each cell was classified according to its final fate status as either pluripotent, differentiated or unknown using expression levels of CDX2. Full experimental details are given in Ref. (27). This illuminating study by S. C. Wolff et. al., provides rich opportunities for further quantitative analysis.

**Colony growth summary**

Here we give an introduction to the colony dataset. The colony begins from 30 cells and grows over 68 hours (817 timeframes) to 381 cells, with 1274 cell cycles considered within this time. A differentiation agent BMP4 was added to the cells at 40 hours. In Ref. (27) the cells are categorised according to their final cell fate status as pluripotent, differentiated, or unknown (could not confidently be assigned as pluripotent or differentiated). The number of cells, N, considered in each cell fate category, pre- and post-BMP4 is shown in Table 1.

| N | Pre-BMP4 | Post-BMP4 | All times |
|---|---------|---------|-----------|
| Pluripotent | 96 | 422 | 518 |
| Differentiated | 22 | 111 | 133 |
| Unknown | 112 | 511 | 623 |
| All fates | 230 | 1044 | 1274 |

Table 1: The number of cells, N, in each of the cell fate and pre- and post-BMP4 categories. A post-BMP4 is any cell present at 40 hours or later. There are 1274 cells in total.

Snapshots of the colony at times $T = 0$, $T = 20$, $T = 40$ (the time BMP4 is added) and $T = 68$ (the final recorded time) colour coded by OCT4 intensity are shown in Figure 1. There is clear spatial patterning of cell fates within the colony, with clustering of pluripotent cells in the centre and differentiated cells around the top edge of the colony. Spatial analysis shows that this patterning begins emerging at around $T = 20$ hours (20 hours before BMP4 addition) for differentiated cells, and at around $T = 50$ hours (10 hours post BMP4 addition) for pluripotent cells Ref. [Sirio]. Although here we focus on quantifying the temporal regulation in OCT4,
we must keep in mind that there is a spatial correlation between the cell fates.

For every cell in the colony there is a corresponding time series of the abundance of OCT4 within the cell during its lifetime: \( \text{OCT4}(t_1), \text{OCT4}(t_2), \ldots, \text{OCT4}(t_n) \), where \( t_1 = 0 \) and \( t_n \) are the start and end of the cell cycle for the cell, respectively. The values of \( t_n \) range from 15 minutes to 30 hours across the population. We will use the notation \( t_i \) to describe time-steps in terms of the cell cycle and \( T_i \) for experimental time (between 0 and 68 hours). The OCT4 time series for a cell at the beginning of the experiment and its descendants are shown in Figure 1(e).

An analysis of the number of cells in the colony over time, \( N(T) \), is given in the Supplementary Information (Figure S1). The whole colony follows exponential growth, with a doubling time of \( 16 \pm 0.01 \) hours, as noted in Ref. (27) and consistent with other reports (40, 41). The corresponding doubling times for the different cell fates are \( 17 \pm 0.004 \), \( 21 \pm 0.008 \) and \( 16 \pm 0.01 \) hours for pluripotent, differentiated and unknown cells respectively. As expected, the pluripotent cells proliferate significantly faster than the differentiated cells.

**Dataset**

The original dataset is available from Ref. (27), providing cell IDs, cell ancestries, cell positions, cell fates and mean OCT4-mCherry fluorescence intensities. Cell IDs in this manuscript are consistent with those in the original dataset.

**Quantitative analysis**

The quantitative analysis in both Ref. (27) and this manuscript were performed using MATLAB.

**Averaging and errors**

When average values are given the type of averaging (mean or median) is specified. For means the errors are given in the form \( \pm \) standard deviation (standard error in the mean). For medians the errors represent the lower and upper quartiles or the interquartile range as specified.

**Correlation coefficient**

The correlation between two OCT4 time series is calculated using Pearson’s correlation coefficient. Note that the same conclusion is reached when the Kendall rank correlation coefficient is used.

**De-trending**

We remove trends from the data when it is necessary to analyse fluctuations about any present trend. We used MATLAB’s inbuilt function `detrend` which subtracts the best-fit line from the data.

**Line fittings**

Lines of best fit throughout were calculated using a least-
We consider the Laplace distribution, sometimes referred to as the double exponential distribution, using the notation \( \text{Laplace}(\mu, b) \) to distinguish from the usual parameter \( \mu \) in Normal(\( \mu, \sigma^2 \)). The parameters can be estimated using the maximum likelihood estimators \( \hat{\mu} \) and \( \hat{b} \), where \( \hat{\mu} \) is the sample median and \( \hat{b} \) is the mean absolute deviation from the median.

\[
\hat{b} = \frac{1}{N} \sum_{i=1}^{N} |x_i - \hat{\mu}|.
\]

This method of parameter estimation was used in the manuscript to find Laplace distributions to describe the change in OCT4 between time-steps.

**Statistical testing**

To test the null hypothesis that a distribution is Normal, we use both the one-sample Kolmogorov Smirnov and Shapiro-Wilk tests. To test the null hypothesis that two non-parametric distributions are from the same distribution we use the two-sample Kolmogorov Smirnov test.

**The Laplace distribution**

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**Poincaré plots**

Poincaré plots are a method of analysing the self-similarity of a series. A timed signal is plotted against itself after a time delay (here the time delay is 5 min) and its scatter pattern reflects the randomness and variability of the dynamics, thus giving a representation of the correlation between consecutive values of the time series. In the Poincaré plots we colour the values according to their normalised frequency. The data (changes in OCT4) is binned into 100 groups before the relative frequency of that group (a value between 0 and 1) is calculated. The scatter plot can be quantified by fitting an ellipse around the data points and measuring the dispersion along the major SD1 and minor SD2 axes. Further information on Poincaré analysis is given in Refs. (42) and (43).

**The Hurst exponent**

The Hurst exponent, \( H \), is a measure of the long term memory, or the scale of the self-similarity properties of a time series. It is defined as

\[
E \left[ \frac{R(n)}{S(n)} \right] = Cn^H \text{ as } n \to \infty,
\]

with \( R(n) \) the range of the first \( n \) cumulative deviations from the mean and \( S(n) \) their standard deviation. \( E[\cdot] \) denotes the expected value, \( n \) is the number of data points in the time series and \( C \) is a constant.

The quantity \( R/S \) is known as the rescaled range and measures how the apparent variability changes with the length of time considered. For a time series \( X_1, X_2, ..., X_n \), with mean \( \mu \) and variance \( \sigma^2 \), the rescaled range is

\[
R/S = \frac{\max(Z_1, Z_2, ..., Z_t) - \min(Z_1, Z_2, ..., Z_t)}{\sigma \sqrt{N}},
\]

where

\[
Z_t = \sum_{i=1}^{t} X_i - m \text{ for } t = 1, 2, ..., n.
\]

Further details on the Hurst exponent, other methods of estimation and its relation to fractional Brownian motion can be found in Refs. (44–47).

**Autocorrelation analysis**

Autocorrelations were calculated using MATLAB's autocorr function (Econometrics Toolbox). The autocorrelation \( C_i \) of a time series between \( x_t \) and \( x_{t+i} \) for time-lag \( i \) is given as

\[
C_i = \frac{1}{T\sigma} \sum_{t=i}^{T-i} (x_t - \bar{x})(x_{t+i} - \bar{x}),
\]

where \( \sigma \) is the sample variance of the time series. The correlation time is defined as \( \tau = \int_{-\infty}^{\infty} C(t)dt \). The auto-correlations can be described by the function \( C = \cos(2\pi t/\alpha)e^{-t/\beta} \) (48).

**Random walk theory**

We apply the theory of random walks to the OCT4 time series to test if the OCT4 intensity drifts diffusively. Traditionally used for the migration of particles, the mean square displacement (MSD, mean square difference or mean square fluctuation) is calculated as MSD = \( \langle |x(t) - x(0)|^2 \rangle \), where \( x(t) \) and \( x(0) \) are the current (at time \( t \)) and starting positions, and \( \langle \cdot \rangle \) denotes an average over all particles. If the motion is diffusive (Brownian) then the MSD increases linearly with time in the manner MSD = 2 Dt. Sub-diffusion is shown by MSD \( \propto t^\alpha \) with \( \alpha < 1 \) and super-diffusion with \( \alpha > 1 \). Here we consider the one dimensional version, the mean square difference. Further information on the use of random walks in mathematical biology is given in Refs. (49) and (50).
RESULTS
Firstly, we consider the correlation and similarities in OCT4 expression between pairs of descendant (‘sister’) cells, and proceed to characterise how the drift in OCT4 similarity between these cells occurs over cell lifetimes. We analyse the inherent fluctuations in OCT4 expression and quantify its self-regulatory properties using the Hurst exponent, autocorrelation and diffusion analysis. Where appropriate, we consider the pluripotent and differentiated cells, pre- and post-BMP4 groups of cells separately to identify any diagnostic factors.

OCT4 in sister cells
The analysis in Ref. (27) shows that upon cell division the ratio between the OCT4 values of sister cells is centred around a 1:1 distribution, meaning that although asymmetric pluripotency inheritance is seen (for example, 38% of divisions occur in the ratio 5:6 or more extreme), on average sister cells start with similar levels of OCT4. It is also shown that OCT4 levels are more similar in closely related cells, i.e., sister cells and cousins cells show significant similarity when compared with random pairs of cells.

We can consider the strength of the correlation in temporal OCT4 in sister cells over their whole lifetimes by calculating the correlation coefficient, \( \rho \). Before calculating the correlation, each OCT4 time series was de-trended to account for any confounding similarities in sister cells that may be present due to their shared environment. The distribution of \( \rho \) for pluripotent and differentiated, and pre- and post-BMP4 sister cells are shown in Figure 2(a) and 2(b). Here it is necessary to pool pre- and post-BMP4 cells for a cell fate comparison, and vice versa, to keep good statistics. The mean correlations, \( \bar{\rho} \), are given in Table 2 and show moderate positive correlations across all categories.

![Figure 2: The correlation, \( \rho \), between temporal OCT4 in sister cells where both sisters cells were (a) pluripotent (red unfilled circles) and differentiated (green diamonds) and (b) cells pre-BMP4 (blue filled circles) and post-BMP4 (orange unfilled squares). OCT4 values for all sister pairs (c) at the start and (d) end of their cell cycles. The lines of best fit (orange solid lines) with standard errors in predicting a future observation (dashed lines) are (c) OCT1 = (1 ± 0.003)OCT2 with \( R^2 = 0.98 \) and (d) OCT1 = (0.97 ± 0.02)OCT2 with \( R^2 = 0.78 \).](image)

|          | Pre-BMP4 | Post-BMP4 | All times |
|----------|----------|-----------|-----------|
| Pluri.   | -        | -         | 0.5 ± 0.3(0.02) |
| Diff.    | -        | -         | 0.5 ± 0.2(0.04) |
| All fates| 0.3 ± 0.2(0.03) | 0.5 ± 0.3(0.01) | 0.5 ± 0.3(0.01) |

Table 2: The mean correlation, \( \bar{\rho} \), ± the standard deviation (standard error) between pairs of sister cells.

There is no difference between cells of different fates, both with \( \bar{\rho} = 0.5 \) (±0.2 and ±0.3 for pluripotent and differentiated cells, respectively). The Kolmogorov Smirnov test provides no evidence at the 95% confidence level to reject the null hypothesis that the distributions of \( \rho \) for pluripotent and differentiated cells, Figure 2(a), are the same. Sister cells pre-BMP4 show a weaker correlation than those post-BMP4, with \( \bar{\rho} = 0.3 \pm 0.2 \) and \( \bar{\rho} = 0.5 \pm 0.3 \), respectively. The Kolmogorov Smirnov test provides evidence at the 95% confidence level to reject the null hypothesis that the two distributions, Figure 2(b), are the same. This suggests that BMP4 treatment exacerbates the similarities in sister cell OCT4 expression. These results quantify the regulation between closely related cells and further illustrate that this regulation is systematic and importantly, still present when confounding external trends are removed.

We can also quantify how this correlation between sister cells drifts throughout their lifetimes. The initial and final OCT4 values for all sister cells are shown in Figure 2(c) and 2(d). The initial values follow a very close relationship (as also shown by the OCT4 ratio splitting distribution in Ref. (27)), with a correlation of \( \rho = 0.99 \) and the trend line \( OCT_1 = (1 ± 0.003)OCT_2 \). (Note that the labelling of cell 1 and cell 2 is entirely arbitrary.) By the end of their respective lifetimes, the distribution spreads, with a correlation of \( \rho = 0.78 \) and a line of best fit \( OCT_1 = (0.97 ± 0.02)OCT_2 \).

In the next section we will consider the behaviour of OCT4 from the initial point of possible asymmetric inheritance to the final time before mitosis at the end of the cell lifetime and characterise how this drift of similarity in sister cells occurs.
Temporal OCT4 dynamics

In this section we quantify the temporal behaviour of OCT4 dynamics on the cellular level over the course of a cell lifetime. We consider the variability between discrete time-steps and quantify the self-regulatory behaviour of OCT4 using several methods.

OCT4 distribution

To get an overall view of the OCT4 expression levels, the distributions of all measured OCT4 values for pluripotent and differentiated cells, pre- and post-BMP4 can be considered, shown in Figure 3. Pre-BMP4, the differentiated cells show a skewed distribution, with an increased preference towards lower OCT4 expressions than the pluripotent cells. This is fitting with the fact that the analysis in Ref. (27) suggests the decision to differentiate is largely pre-determined before the addition of the differentiation stimulus. Post-BMP4, both pluripotent and differentiated cells also show a reduction in their OCT4 expression, with the effect seen more strongly in the differentiated cells. It is expected that the BMP4 causes a reduction in OCT4 in the differentiated cells, but it is interesting the same effect (to a lesser extent) is also present in the cells which remain pluripotent. The average (median) OCT4 expression levels, $\mu$, are shown in Table 3.

Variability between time-steps

Even small fluctuations in PTF abundance impact cell fate (26) with both high and low PTF values resulting in differentiation (24, 25). Mathematical models of PTF fluctuation will allow for the description of pluripotency over discrete time-steps, fitting for time-lapse experiments such as the one considered here (27). Quantifying these integral fluctuations is therefore necessary. First, we will consider the change in the intra-cellular OCT4 abundance between the five minute time intervals, $t_1, t_2, \ldots, t_n$, as $\Delta$OCT4=$\text{OCT4}(t_i) - \text{OCT4}(t_{i-1})$. Note the consideration of the five minute intervals allows for good statistics with 90% of cells having greater than 50 data points. It is likely that a large proportion of these individual fluctuations will be due to experimental noise, but considering all of these values together reveals the average behaviour.

The distributions of $\Delta$OCT4 for pluripotent and differentiated cells, pre- and post-BMP4 are shown in Figure 4. For both fates across all times, the change in OCT4 is centred around zero (although the individual values range from -1300 to 1200). This means that, on average, the change in OCT4 is isotropic for both pluripotent and differentiated cells. There is no preference for the abundance to increase or decrease in a time-step, the fluctuations are symmetric overall. Interestingly, although symmetric, the distributions are not Normal (confirmed by the Kolmogorov-Smirnov and Shapiro-Wilk tests at the 95% confidence level) due to a narrower and steeper peak, shown in Figure 4. A Laplace distribution, Laplace($\mu^\dagger, b$), better fits the experimental data in all cases, with the parameters given in Table 4.

Table 3: The median OCT4 values, with lower and upper quartiles given as OCT4 [lower quartile upper quartile].

| OCT4 | Pre-BMP4 | Post-BMP4 | All times |
|------|----------|-----------|-----------|
| Pluri. | 1500 [1280 1730] | 1090 [930 1260] | 1160 [980 1380] |
| Diff. | 1100 [960 1290] | 720 [450 990] | 840 [510 1070] |
| All fates | 1420 [1190 1670] | 1050 [850 1230] | 1110 [900 1320] |

Table 4: The parameters from the Laplace($\mu^\dagger, b$) fittings to the $\Delta$OCT4 distributions shown in Figure 4.

| Laplace($\mu^\dagger, b$) | Pre-BMP4 | Post-BMP4 | All times |
|-------------------------|----------|-----------|-----------|
| Pluri. | $(-0.7, 52.1)$ | $(-0.3, 34.7)$ | $(-0.3, 38.5)$ |
| Diff. | $(-0.7, 45.6)$ | $(-2.6, 23.3)$ | $(-2.4, 28.1)$ |
| All fates | $(-0.8, 50.8)$ | $(-0.9, 32.4)$ | $(-2.4, 28.1)$ |

Post-BMP4 addition, the distributions for both cell fates become significantly narrower, with the parameter $b$ showing a reduction of 49% for differentiated cells, and 33% for the pluripotent cells. There is also a subtle skew in the differentiated cells towards negative values of $\Delta$OCT4 which is consistent with the fact that the OCT4 levels overall decrease after the BMP4 addition. The narrowing of the distributions show a preference to smaller changes in OCT4 in all cell fates provoked by the differentiation agent. This could be
Figure 4: Distributions of the change in OCT4 between the five minute time frames ($\Delta$OCT4) for pluripotent cells (a) pre-BMP4 and (b) post-BMP4, and differentiated cells (c) pre-BMP4 and (d) post-BMP4. Solid lines show the Laplace distribution fittings, Laplace($\mu^\dagger$, $b$), with the parameters (a) $\mu^\dagger = -0.7$ and $b = 52.1$, (b) $\mu^\dagger = -0.3$ and $b = 34.7$, (c) $\mu^\dagger = -0.7$ and $b = 45.6$ and (d) $\mu^\dagger = -2.6$ and $b = 23.3$. Dashed lines show the Normal distribution fittings.

Driven by induced selectivity caused by the BMP4 addition (i.e., the BMP4 causes a systematic change, preferencing smaller $\Delta$OCT4 values), or it could suggest some collective self-regulation (8). Further experiments are needed to investigate if this is a collective behaviour effect, considering the effect of colony size. It is expected, since the differentiated cells are most affected by the BMP4, that this group show the biggest reduction in variation and therefore the strongest regulation in their OCT4 values.

We can also consider the self-similarity of the OCT4 series using Poincaré plots (42, 43). For each cell, its OCT4 time series can be plotted against itself with one time-step delay, i.e., OCT4($t_i$) against OCT4($t_i + 1$), shown in Figure 5.

By assessing qualitatively the shape formed by the return map, we observe changes in the distribution of points between pluripotent and differentiated cells, pre- and post-BMP4. Even pre-BMP4 addition, the differentiated cells show less variation compared to the pluripotent cells, with the addition of BMP4 exacerbating this effect. Quantitatively these results can be described by fitting an ellipse to the shape formed by the data plots and measuring the dispersion along the major SD1 and minor SD2 axes, given in Table 5.

Figure 5: Poincaré plots for the OCT4 signal for pluripotent cells (a) pre-BMP4 and (b) post-BMP4, and differentiated cells (c) pre-BMP4 and (d) post-BMP4. The colour bar shows the normalised relative frequency of the points.

| SD1, SD2 | Pre-BMP4 | Post-BMP4 | All times |
|----------|----------|-----------|-----------|
| Pluri.   | 64, 1430 | 51, 1150  | 54, 1320  |
| Diff.    | 54, 1070 | 33, 990   | 38, 1090  |
| All fates| 62, 1440 | 48, 1170  | 51, 1330  |

Table 5: Quantitative results for the Poincaré analysis ellipse fittings. The major axis (SD1) and minor axis (SD2) from fitting ellipses to the plots shown in Figure 5.

This information quantifies step changes in OCT4 for mathematical models, suggesting the use of the Laplace distribution to simulate variation and shows that the addition of BMP4 provokes tighter self-regulation across both cell fates. It also highlights that even between small time increments such as these, the fluctuations post BMP4 should be considered separately for cells of different fates, not only in terms of their average, as expected, but also their variability. Note that this allows us to capture the nature of the variation in OCT4 only and further aspects of the behaviour need to be considered to fully describe the OCT4 regulation over time.
OCT4 self-regulation

To investigate the self-regulation and internal memory of OCT4 during a cell cycle, we consider three related approaches, the Hurst exponent, the autocorrelation function and diffusion analysis.

The Hurst exponent  The Hurst exponent, $0 < H < 1$ is a measure of the long term memory of a time series. If a series is Brownian, $H = 0.5$, then the fluctuations are isotropically random, with the variable just as likely to increase as decrease at each time-step. If the series is persistent, $H > 0.5$, then at each time-step the series is more likely to fluctuate in the same direction as the previous step, i.e., if in the last time-step there was an increase, it is more likely there will be another increase during the next time-step. For anti-persistence, $H < 0.5$, the series is less likely to fluctuate in the same direction as the previous step.

The Hurst exponent was calculated for all cells which live longer than 50 time frames (4.16 hours). The distributions of all $H$ values for pluripotent and differentiated cells, pre- and post-BMP4 are shown in Figure 6. The average Hurst exponents, $\bar{H}$, are given in Table 6 for each group. In all cases, the Hurst exponents are less than 0.5, showing moderate anti-persistence. This shows the self-regulation of OCT4 on the intra-cellular scale, if the OCT4 value has just increased, it is more likely to next decrease, and vice versa. This is the case across each cell fate group. Although the means are within errors of one another, the Kolmogorov Smirnov tests reject the null hypothesis that the distributions of $H$ for pluripotent and differentiated pre-BMP4 cells are the same at the 95% level. There is no significant difference in $H$ before and after the BMP4 addition for both cell fates (confirmed by the Kolmogorov Smirnov test at the 95% level) suggesting this aspect of the self-regulatory behaviour is inherent within the cells and unchanged by the differentiation stimulus. This quantification via the Hurst exponent is directly transferable to use in fractional Brownian motion modelling methods (44–47).

|       | Pre-BMP4         | Post-BMP4        | All times       |
|-------|------------------|------------------|-----------------|
| Pluri.| 0.37 (0.08 0.008)| 0.37 (0.09 0.004)| 0.37 (0.09 0.004)|
| Diff. | 0.42 (0.08 0.02) | 0.39 (0.09 0.009)| 0.40 (0.09 0.008)|
| All fates | 0.38 (0.08 0.007)| 0.38 (0.09 0.004)| 0.38 (0.09 0.004)|

Table 6: The mean Hurst exponent $\bar{H}$ with (standard deviation, standard error) for all cell categories.

Autocorrelation  The anti-persistence can be further explored by considering the autocorrelation of the time series. The autocorrelation is the correlation of a time series with itself at increasing time lags, hence $-1 \leq C \leq 1$ where $C = 0$ signifies no correlation, $C < 0$ a negative correlation (corresponding to anti-persistence) and $C > 0$ a positive correlation.
Figure 7: Typical autocorrelations showing (a) a period of anti-persistence before settling at zero correlation (seen in 51% of cells), (b) two periods of anti-persistence followed by persistence (seen in 28% of cells) and (c) a period of anti-persistence followed by a period of persistence (seen in 14% of cells). The panels (d)-(f) show the OCT4 variation in time for these cells respectively. The average behaviour is similar to that in (a).

The first time anti-persistence occurs, $t_{AP}$, can be extracted for each individual cell. The distribution of $t_{AP}$ for cells with at least one hour anti-persistence is shown in the Supplementary Information, Figure S2 and reveals the critical cell cycle time in which it first occurs. In all cells with anti-persistence, it has begun by 8 hours into the cell cycle (just over half a cell cycle $^{(27)}$), suggesting that before they reach the latter halves of their lifetimes the internal self-regulation of OCT4 begins. This could be due to the memory effects or the down-regulation of the PTF which occurs prior to mitosis $^{(51, 52)}$.

The periodic nature and decay of the autocorrelation can be captured by the function $C = \cos(2\pi t / a)e^{-t/b}$ $^{(48)}$ (note that this periodicity in the autocorrelation does not necessarily imply periodicity in the time series). These fittings are shown in the Supplementary Information, Figure S3, for 25 random cells in the colony. This quantifies the temporal, periodic decay in the autocorrelation, with the parameter $a$ representing the time-scale of the periodicity, and $b$ the time-scale of the decay (the correlation decay time). Histograms of $a$ and $b$ for all 1274 cells are shown in Figure 8. Both distributions are skewed, with medians of 11.7 h and 3.0 h, and 90th percentiles of 30 h and 7 h for $a$ and $b$ respectively. This quantifies the characteristic time-scale of the periodicity and the correlation decay time as less than 7 hours in 90% of cases. For consistency, the parameters split by cell fate pre- and post-BMP4 are given in the Supplementary Information, Figure S4 and S5.

The correlation time is defined as $\tau = \int_{-\infty}^{\infty} C(t)dt$, with a mean correlation time across all cells of $\tau = 0 \pm 0.002$ h. The distribution of all correlation times is shown in the Supplementary Information, Figure S3.

We can identify the average behaviour by considering all autocorrelations for all cells. The mean (and standard deviation) and median (and interquartile range) autocorrelations $\bar{C}$ for all cells is shown in Figure 8(c). Notably the mean and median are comfortably within errors of each other and the autocorrelation is robust to the chosen averaging method. The average autocorrelation decreases to zero at around three hours, followed by a period of negative autocorrelations indicative of anti-persistent behaviour between approximately three and 12 hours. By 13 hours, the average autocorrelation settles at zero, showing no internal memory past this time. These observations are robust to cell fate and the equivalent autocorrelations for pluripotent and differentiated cells are shown in the Supplementary Information, Figure S6. This shows that during a cell cycle, there is long-term memory in the OCT4 expression up to around 12 hours, but the nature of the effect differs over this time with initial persistence being replaced by anti-persistence. Notably, the mean autocorre-
Diffusion analysis A further method of quantifying the internal regulation of OCT4 is to consider the diffusive behaviour of the time series. The theory of diffusivity and random walks is widely used across many biological applications, including stem cells and so it is important to quantify the OCT4 behaviour within this framework (49, 50, 53–56).

After the asymmetric division of OCT4 there is a short period of increased fluctuations (27). Here we consider each OCT4 time series from half an hour after cell division to allow for this. Each cell has an initial OCT4 value at the start of its lifetime, denoted OCT0. The mean square difference of OCT4 over time, MSD(t), can be calculated as \( \langle (\text{OCT4}(t) - \text{OCT4}_0)^2 \rangle \), where \( \langle \rangle \) denotes the average across all cells in the group considered. The MSD for pluripotent and differentiated cells, pre- and post-BMP4 between 0 and 12 hours is shown in Figure 9. For pluripotent cells, both pre- and post-BMP4, the distinct sub-diffusive behaviour of the MSD is visible, with MSD = \( \beta t^\alpha \), \( \alpha < 1 \). The parameters \( \alpha \) and \( \beta \) are shown in Table 7. The differentiated cells do not follow a power law relationship, pre- or post-BMP4, but the limiting of the MSD can still be seen from around 2 hours pre-BMP4 and from 9 hours post-BMP4.

|               | Pre-BMP4 | Post-BMP4 |
|---------------|----------|-----------|
| \( \alpha \)  | 0.59 ± 0.03 | 0.54 ± 0.03 |
| \( \beta \)   | 42000 ± 2700| 35000 ± 2300|

Table 7: Parameters for MSD = \( \beta t^\alpha \) fittings for pluripotent cells.

This sub-diffusivity is consistent with the anti-persistence illustrated by the Hurst exponent and autocorrelation. For mathematical modelling purposes, this drift in OCT4 values can be considered between sister cells with the diffusion framework. We have shown that, on average, the intra-cellular OCT4 abundance behaves in a sub-diffusive manner throughout a cell lifetime. This has a knock-on effect for the relationship between sister cell OCT4 which is presented in the Supplementary Information (Figure S7-9).

This further quantifies the self-regulatory behaviour of OCT4 within the diffusion framework, a fundamental starting point for many mathematical models. The anti-persistence of OCT4 suggests possibilities for mathematical modelling methods to capture the internal regulation of pluripotency, including fractional Brownian motion and correlated random walk theory.
Non-invasive diagnostic tools
We have quantified the behaviour of OCT4 using a variety of mathematical techniques, some of which could provide a non-invasive diagnostic tool to identify pluripotent and differentiated cells. To illustrate this, we will use the unknown cells (unable to be classified as either pluripotent or differentiated) and compare their time series parameters to the pluripotent and differentiated cells.

Firstly, the distribution of all OCT4 values for the unknown cells lies between that for the pluripotent and differentiated cells, shown in Figure 10(a). This is unsurprising as these cells had an OCT4 expression (along with a CDX2 expression) that did not correspond to either cell fate. Having measured the OCT4 time series for enough time steps to get a distribution of OCT4, comparison could be made to identify whether it bests corresponds to that of the pluripotent or the differentiated cell fates, even before any differentiation stimulus is added. However, we still have a large portion of cells which fall in the middle of the two categories (the unknown cells), unable to be confidently classified. The distributions for OCT4 post-BMP4 are similar (shown in the Supplementary Information, Figure S10) with the unknown cells also showing a reduction in their expression, and the distribution lying between the pluripotent and differentiated groups.

There is also a significant difference in the ΔOCT4 distributions post-BMP4, shown in Figure 10(b) for the unknown cells. The distribution fits a Laplace distribution with the parameters $\mu^* = -1.8$ and $b = 27$, both between their pluripotent and differentiated counterparts. The distributions pre-BMP4 aren’t different enough to distinguish between the fates, shown in the Supplementary Information Figure S10.

There is no distinguishable difference between the cell fates using an autocorrelation analysis or the Hurst exponent (with $H$ within errors for all cell fates, including the unknown cells). However, the MSD plots to identify sub-diffusion show a significant difference between the fates, with the MSD for pluripotent cells well described by a power law relationship. The MSD for the unknown cells in shown in Figure 10(c). Pre-BMP4, the MSD shows significant sub-diffusivity in the first 4 hours, but can not be described by a power law, unlike for pluripotent cells. The MSD also shows more similarities with the differentiated cells post-BMP4, with a more linear MSD which levels off at around 11 hours.

These results show that overall, the unknown cells behaviour lies between that of the pluripotent and differentiated cell fates. The distinct differences between cell fates, seen in the OCT4 distributions, the change in OCT4 post-BMP4 and the sub-diffusive nature of the MSD could provide non-invasive diagnostic tools to identify cell fates.
DISCUSSION

Promising clinical applications of hESCs require tight control over the pluripotency of hESC colonies. It has been shown that even small PTF fluctuations can bias cell fate decisions and that PTFs are inherited asymmetrically upon cell division (26–29). It is therefore necessary to quantify the dynamics of key PTFs to further our understanding of how pluripotency is regulated and assist in the development of mathematical modelling. Rigorous quantification also provides the basis for experimental comparisons, and the identification of systematic and universal behaviours. Here we have used a published data set from Ref. (27) to analyse and quantify the dynamics of the pluripotency transcription factor OCT4.

The colony considered here grows exponentially, with changing proportions of pluripotent, differentiated and unknown cells. Snapshots of the colony show some spatial patterning of the OCT4 abundance (Figure 1), with higher levels of expression of OCT4 visible in cells clustered in the colony centre. A spatial analysis of the colony can be found in Ref. [Sirio]. Here we have focused on the quantification of the temporal dynamics of OCT4.

Time-lapse experiments such as the one considered here (27) provide a wealth of opportunities for the quantification of temporal PFT regulation which can be compared to, and enhance, current biological knowledge. For example, a sharp decline in OCT4 levels occurring before cell division is noted in Ref. (27), in keeping with the transcription factor down regulation known to occur before mitosis (51,52). This phenomena can be quantified, with the decrease in OCT4 beginning, on average, 35 minutes (0.58 hours) before cell division, lasting for 15 minutes (0.25 hours), and showing a reduction of 22% from the interphase OCT4 expression. The OCT4 expression levels recover as mitosis occurs and the cycle repeats for the cell’s descendants consistent with experimental results showing OCT4 resets on re-entry to the G1 phase (57, 58). This is shown for all cells before BMP4 addition in the Supplementary Information, Figure S11.

Ref. (27) reveals that sister cells show more closely related OCT4 values than pairs of random cells. Here we take this a step further by quantifying their temporal dynamics in relation to one another. Taking into account any common trends which may affect both cells due to their shared environment, the sister cells before BMP4 show a moderate correlation with each other with a correlation coefficient of 0.5. This is reduced to a slight correlation for pairs that exist after the BMP4 addition (0.3). The fact that these correlations still occur after de-trending further highlights the inherent similarities between related cells. We then consider the OCT4 behaviour over cell lifetimes to explore the manner in which this drift in similarity occurs. The behaviour is summarised in the schematic in Figure 11.

Stochastic fluctuations in OCT4 have been shown to bias cell fate (26) with evidence of asymmetric noise leading to noise-mediated cell plasticity (30). Here we see the change in

Figure 10: (a) The distribution of all OCT4 values for unknown cells pre-BMP4. (b) The distribution of all changes in OCT4, ΔOCT4, for all unknown cells post-BMP4. In both cases the corresponding distributions for pluripotent and differentiated cells as a red solid line and green dashed line, respectively. (c) The MSD for unknown cells pre-BMP4 (blue solid line) and post-BMP4 (orange dashed line) with standard error error bars.
OCT4 between each 5 minute time interval is isotropic, with an average of zero. A natural assumption in model development would be to simulate this symmetric time-step change in OCT4 with a Normal distribution, however the distribution of all these changes best fits a Laplace distribution. Further experimental data is needed to confirm this is a robustly appropriate choice, elucidate the parameters for other experimental conditions and investigate how this is affected by cell-cell interactions. Note that this allows us to capture the nature of the variation in OCT4 only and further aspects of the behaviour need to be considered to fully describe the OCT4 regulation over time.

Although this shows that overall, positive changes in OCT4 are just as likely to occur as negative ones, it does not reveal anything about the temporal nature of these fluctuations and hence any correlation properties which may be evident over time (for example, all the positive changes in OCT4 could come one after the other, followed by all the negative changes, it doesn’t mean that a positive change is necessarily followed by a negative change). There is also a difference in these fluctuations after the differentiation agent, with the addition of BMP4 provoking tighter self-regulation across all cell fates. Further experiments are needed to investigate whether this self-regulation is a collective behaviour effect.

A significant finding of this analysis is the quantification of the self-regulatory properties of OCT4 within cells. An autocorrelation analysis, along with the calculation of the Hurst exponent of 0.38 shows significant anti-persistence, in keeping with the regulation of PTFs (17, 21, 37). Throughout the colony growth, anti-persistence of at least five hours is seen in 86% of cells (with no significant difference between the cell fates, suggesting OCT4 regulation is quantitatively comparable in pluripotent and differentiated cells), and on average occurs between 3 and 12 hours into a cell’s lifetime. This is further illustrated by considering the behaviour of the cells in the diffusion framework, with cells across all fates showing significant sub-diffusivity. For pluripotent cells, the sub-diffusivity can be described by a power law relationship. The sub-diffusivity analysis allows another characterisation of the self-regulation of OCT4 expression and provides a quantitative starting point for the mathematical modelling of OCT4 time series. The results open up techniques such as fractional Brownian motion, where a random time series with a certain Hurst exponent can be simulated, and correlated random walk theory.

The experiment in Ref. (27) has led to a rich analysis, allowing us to establish the language through which to quantitatively compare this experiment to others. In general, this highlights the need for further temporal experimental data on OCT4 and other transcription factors. These quantitative analyses provide the basis for the identification of systematic behaviours, the comparison to future experimental data and the basis for the mathematical modelling of pluripotency.

CONCLUSION

The quantification of temporal fluctuations in PTFs is essential for their experimental comparison and the development of mathematical models of pluripotency. We have further developed the experimental analysis provided in Ref. (27) to quantify the self-regulation of OCT4 over a cell lifetime. Cells begin with a possible asymmetric inheritance of OCT4 (27) and over their lifetimes their OCT4 abundance fluctuates sub-diffusively. We have quantified this sub-diffusivity and the internal self-regulation of OCT4 using the Hurst exponent and auto-correlation analysis. The addition of a differentiation agent provokes tighter self-regulation of pluripotency across all cell fates, but particularly differentiated cells.
AUTHOR CONTRIBUTIONS

See Ref. (27) for author contributions for the original experiment. L.E.W. and S.O.F. analysed the data and prepared the figures. L.E.W., S.O.F., I.N., M.L., N.G.P. and A.S. wrote the manuscript.

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**SUPPLEMENTARY MATERIAL**

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