Transcription Factor Network in Embryonic Stem Cells: Heterogeneity under the Stringency

Yoko Nakai-Futatsugi and Hitoshi Niwa*

Laboratory for Pluripotent Stem Cell Studies, RIKEN Center for Developmental Biology;
Received October 30, 2012

Leukemia inhibitory factor (LIF) signaling regulates transcription factors to maintain the self-renewability and pluripotency of embryonic stem (ES) cells. Recently, we have proposed a network model that consists of transcription factors such as, Klf4, Sox2, Tbx3, Nanog, and Oct3/4, which form a parallel pathway downstream from LIF signaling (Nature, 460, 2009, Niwa et al.). In this parallel pathway, the transcription factors maintain the pluripotency of ES cells through mutual balance with some degree of redundancy and compensation. While self-renewability and pluripotency are maintained well under such seemingly stringent regulation, studies of single cells revealed heterogeneity among individual ES cells. This heterogeneity may underlie the mechanism that allows ES cells to exit self-renewal and enter into differentiation to exert pluripotency. Here we focus on recent studies on the heterogeneity of ES cells and discuss their inherent metastability.

Key words embryonic stem cell; transcription factor network; metastability

1. INTRODUCTION

During mouse development, pluripotency is established in the inner cell mass (ICM) of the blastocyst around embryonic day 3.0 (E3.0) (Fig. 1). Pluripotency is the capacity of an individual cell to give rise to all other cell types of the body and the germ line, which is an emergent property realized in the ICM. Pluripotency persists until the ICM develops into the primitive ectoderm (PrEc) by E5.5 (Fig. 1). Embryonic stem (ES) cells are the cells captured from the ICM during this short time period (24h in vivo), which continue to multiply while remaining pluripotent indefinitely under certain culture conditions,1,2 The factor that maintains the cells in this “forever-young” state was identified3 and determined to be identical to the cytokine, leukemia inhibitory factor (LIF).4,5) LIF signaling is mediated via the signaling molecule Stat36) and regulates the pluripotency transcription factor network.7) This regulation maintains the core transcription factor Oct3/4 at a certain level, which apparently makes the population ‘homogeneous’ with indefinite self-renewability and pluripotency. Then

Fig. 1. Pluripotent Lineages in the Mouse Embryo

The authors declare no conflict of interest.

*To whom correspondence should be addressed. e-mail: niwa@cdb.riken.jp © 2013 The Pharmaceutical Society of Japan
how do the cells differentiate by exiting this well-maintained condition? Detailed studies on single cells showed that ES cells are heterogeneous and fluctuating even under the same culture conditions, which supposedly makes them continually poised for differentiation in response to external signals.

2. TRANSCRIPTION FACTOR NETWORK SAFE-GUARDING PLURIPOTENCY

In pluripotent cells of the ICM, transcription factors such as Oct3/4, Sox2, and Nanog are specifically expressed, while in its counterpart, the trophoectoderm, another transcription factor, Cdx2, is specifically expressed. The transcription factors that govern the pluripotency become downregulated during differentiation, while other transcription factors such as Gata6 (a primitive endoderm (PrE) marker) become upregulated (Fig. 1). Thus transcription factors are key elements that define the undifferentiated state of the ICM and its derivatives, ES cells. Among them, Oct3/4 and Sox2 are considered to be core pluripotency transcription factors: Oct3/4 is essential to establish pluripotent cells in the ICM and in the absence of Oct3/4 the cells of the ICM eventually differentiate into the trophoblast lineage; and Sox2 cooperatively acts with Oct3/4. How LIF signaling is connected to these core factors was depicted from one aspect by a gain-of-function study in the absence of LIF: exogenous expression of Klf4 maintained Sox2 (but not Nanog or Tbx3) in the absence of LIF, and this pathway was significantly suppressed by the inhibition of Stat3 signaling. Likewise, exogenous expression of Tbx3 maintained Nanog (but not Klf4 or Sox2) in the absence of LIF, and this pathway was suppressed by the inhibition of phosphatidylinositol-3-OH kinase (PI3K)-Akt signaling and maintained by the constitutive activation of PI3K-Akt. In addition, the inhibition of mitogen-activated protein kinase (MAPK) significantly upregulated both Tbx3 and Nanog, which indicates that the Tbx3-Nanog pathway is positively and negatively regulated by PI3K-Akt and MAPK, respectively. Regardless of the artificial alteration in each factor, however, the level of Oct3/4 was always maintained.

Taking those results together, we developed a model in which Oct3/4 is protected by two independent parallel pathways (Fig. 2A). The most upstream signals of this parallel pathway, Stat3 and PI3K-Akt, are both activated by LIF. However, the withdrawal of LIF does not cause immediate collapse of the network (Fig. 2B). Upon the withdrawal of LIF, Oct3/4 did not show significant reduction at least for 24 h. Although Tbx3 and Klf4 showed a rather rapid response, they were not completely depleted within 24 h but continued to decrease by day 4. This duration appears rather long for the dephosphorylation of signaling molecules and translocation/ degradation of transcription factors after the withdrawal of LIF. One explanation for this may be the involvement of positive feedback from Oct3/4, Sox2, or Nanog to the entire network. Although the Stat3-Klf4-Sox2 pathway was illustrated at one side of the parallel pathway, constitutive activation of Stat3 did not exclusively activate Klf4 and Sox2, but instead in long-term (3-week) culture it brought all factors to normal levels in the absence of LIF. This suggests that there is also crosstalk among the factors, which may activate or suppress each other when one becomes too low or too high, respectively.

In this model, the mutual regulation of transcription factors maintains the core factor Oct3/4 at a certain level. The expression level of Oct3/4 is crucial for maintaining ES cells in an undifferentiated state, as a slight increase in its expression level leads to differentiation into the PrE and mesoderm, while a slight decrease in its expression level leads to differentiation into the trophoectoderm. The existence of a parallel pathway with a feedback loop and crosstalk may explain how the expression level of Oct3/4 is maintained within such a critical range.

3. MONITORING THE NETWORK STATUS WITH THE INDICATOR REX1

As mentioned above, regulating the Oct3/4 level is crucial for ES cells to maintain their pluripotency. Although the level of each transcription factor likely increases and decreases easily, the alterations are compensated for by a mutual balance in the network. How can this ‘balance’ (rather than single components) in the network be monitored?
Rex1 (also known as Zfp42) is a transcription factor that is strongly expressed in the ICM but downregulated in the epiblast thus is commonly used as a marker of pluripotency.[15] Although it makes no contribution to the maintenance of pluripotency,[13] its promoter activity sensitively reflects the expression level of Oct3/4 in vitro: when Oct3/4 is either upregulated OR downregulated, the promoter activity of Rex1 is turned off[14] (Fig. 3). Thus the insertion of GFP under the Rex1-promoter (Rex1-GFP) allows monitoring of the network status at the single-cell level, and indeed the dynamics indicated by Rex1 was revealed for the first time.[14]

4. HETEROGENEITY OF REX1

ES cells are derived from the ICM and were thought to have a homogeneous population in culture. However, when ES cells expressing Rex1-GFP were monitored closely, there were at least two different populations: Rex1+/Oct3/4− cells; and Rex1−/Oct3/4− cells.[14] As Rex1 is exclusively expressed in the ICM in vivo, these could represent an ICM-like and -unlike population, respectively. Pluripotent cells in the ICM differentiate into the epiblast and then into the PrE (Fig. 1). Rex1-negative cells appear to have a more differentiated PrE-like character as they express PrE markers such as Fgfl5, T, and Eomes at a higher level compared with Rex1-positive cells and express undifferentiated markers such as Klf4, Tbx3, Nanog, and Esrrb at a lower level.[14] Interestingly, these two conditions were found to be interconvertible: when either Rex1-positive or -negative cells were isolated, both eventually gave rise to a mixed population of Rex1-positive and -negative cells again.[14]

As reasoned earlier (Fig. 3), in Rex1-positive cells the transcription factor network should be well balanced with Oct3/4 at a firmly stable level, while in Rex1-negative cells it should be slightly unbalanced. The heterogeneity and interconvertibility of Rex1-expression in ES cells can be interpreted as fluctuations in the network providing an opportunity for differentiation but also allowing a return to a balanced condition.

5. HETEROGENEITY OF OTHER LINEAGE-SPECIFIC MOLECULES IN ES CELLS

Nanog is a homeodomain protein expressed in pluripotent cells, and its downregulation is an early marker of differentiation. However, a study on Nanog-null ES cells showed that it is dispensable for the self-renewal of ES cells, although still required for germline development in vivo.[19] The ICM at E3.5 shows a random “salt-and-pepper” pattern of Nanog and Gata6 expression,[16] including overlapping expression of both in a single cell,[17] which afterward segregate into the epiblast and the PrE (Fig. 1) by E4.5.[16,17] ES cells in normal culture conditions also consistently show homogeneous expression of Nanog,[15,18] and ES cells with low levels of Nanog predominantly express the PrE marker Gata6.[15] As in the heterogeneity of Rex1, Nanog-high and -low conditions are reversible.[15,18]

Tbx3 and Klf4 also show heterogeneous expression by immunostaining.[7] Although their expression is not always synchronized,[7] both show a close correlation with Rex1-GFP expression.[14] This strongly suggests that, as in Rex1-positive/-negative cells, both Tbx3-positive/-negative and Klf4-positive/-negative conditions should be reversible.

Stella is a definitive marker of the germ cell lineage but is first observed in preimplantation embryos. Stella also shows heterogeneous expression in ES cells, and Stella-positive cells are more like ICM cells, while Stella-negative cells are more like epiblast cells.[19] Stella-positive and -negative conditions are also interconvertible, and observations showed that the transition from Stella-positive to -negative condition is faster than the opposite transition, which was assumed to be because although the conversion from Stella-positive to -negative condition follows an inherent developmental process from the ICM toward the epiblast, the reciprocal conversion is contrary to the normal developmental program in vivo.[19]

Platelet endothelial cell adhesion molecule (PECAM)-1 and stage-specific embryonic antigen (SSEA)-1 are known cells surface markers of ES cells, however the expression of these molecules in normal ES cell cultures were also found to be heterogeneous.[20] PECAM1+/SSEA1−, PECAM1+/SSEA1+, and PECAM1−/SSEA1− populations were found, and each population was interconvertible. When injected into mouse blastocysts, PECAM1-positive cells predominantly contributed to the epiblast, while PECAM1-negative cell derivatives localized in the PrE and the trophoectoderm.[20] The characteristics of PECAM1-positive cells were similar to those of Rex1-positive cells described above.[14]

Hex is an early endodermal marker but is already expressed at a low level in SSEA1-positive ES cells heterogeneously.[21] Although the expression level is very low in ES cells, when Hex-negative and -positive cells were isolated, the former contributed to chimeras but the latter instead contributed to the extraembryonic endoderm (visceral and parietal endoderm; Fig. 1).[22] This heterogeneity is also interconvertible in vitro.[21]

The zinc finger and SCAN domain containing 4 (Zscan4) shows a different pattern of heterogeneity in ES cells. Unlike the heterogeneities mentioned above which phenotypically show differentiation biases, Zscan4 actively makes ES cells “young.” Zscan4 is predominantly expressed in two-cell embryos (Zscan4d) and in ES cells (Zscan4c). In ES cells, it is expressed transiently in 5% of the population at all times. It stabilizes the genome and, surprisingly, elongates the telomere, which should be at least one of the mechanisms that support ES cells in the ability to defy senescence and undergo more than 250 doublings without crisis or transformation.[23] It is expressed heterogeneously but randomly, so although only 5% of the population is returning to the young condition at one time, in total the whole population is maintained homogeneously.
6. MECHANISMS OF HETEROGENEITY

Which mechanisms allow ES cells to show such transcriptional heterogeneity? A recent study has shown the involvement of the nucleosome remodeling and deacetylation (NuRD) complex in ES cell heterogeneity. Generally, the NuRD complex functions as a transcriptional repressor. In ES cells, it is required for differentiation by silencing the pluripotency-associated genes. It binds proximally to the transcription start sites of pluripotency-associated genes such as Klf4, Klf5, Rex1, Tbx3, Esrrb, and Zfp57 even in the undifferentiated state, albeit it has an inhibitory function. In mutant ES cells lacking NuRD activity, heterogeneous expression of pluripotency-associated factors such as Klf4 becomes homogeneous, and their overall expression is elevated. Thus NuRD silences the genes in ES cells in an approximate balance with putative activators, which may be one of the mechanisms of silencing developmental genes in ES cells incompletely while still maintaining them poised for activation.

Another transcriptional repressor, Hes1, also shows heterogeneous expression in ES cells with an oscillatory period of 3–5 h. It regulates the cell fate via repression of Notch signaling. ES cells expressing low and high levels of Hes1 are prone to differentiate into neural and mesodermal cells, respectively, and Hes1-null ES cells display less heterogeneity in both the differentiation timing and fate choice.

Not only the transcriptional regulators but also the structure of our network model itself can drive heterogeneity. If the balance of the network is maintained by feedback and crosstalk among the transcription factors, there should be a time delay before the balance is adjusted after an alteration in one of the factors. Thus this delay may appear as heterogeneity in terms of expression levels of individual transcription factors composing the network. If there is an applicable mathematical model, the time delay may allow us to predict the network structure, or vice versa.

Whether ES cells are inherently metastable is still under discussion. Smith's group showed that ES cells form a homogeneous population when MAPK and glycogen synthase kinase 3 (GSK3) are blocked: in a defined medium with a MAPK inhibitor and a GSK3 inhibitor (so-called 2i-culture), ES cells are not only homogeneous but are also able to self-renew without LIF and serum. This may suggest that ES cells are inherently homogeneous when the external signaling from MAPK that induces differentiation is removed. However, whether ES cells are indeed shielded from all external signals (to achieve the so-called "ground state") in the presence of these two inhibitors is controversial, as the GSK3 inhibitor added here consequently becomes a Wnt-signaling activator (because Wnt-signaling is blocked by GSK3, and Wnt-signaling is involved in many important cell biological activities), and also as it has recently been shown that the inhibition of GSK3 activates the pluripotency factor Esrrb via repression of the transcriptional repressor Tcf3.

7. CONCLUSION

ES cells are the cells captured during the short time period when the pluripotent cells of the ICM differentiate into the early epiblast. They are able to self-renew indefinitely, contrary to the fate of the ICM that is programmed to undergo differentiation within 24h. To acquire this self-renewability, some genes change their expression profile during the transformation from ICM to ES cells: the expression of differentiation factors such as GATA6 and Nr5a2 is decreased and that of self-renewing factors such as Tcf15 and Nodal is increased. Nevertheless, they inherit the pluripotency of their origin. The heterogeneity of ES cells may represent chaotic expression of lineage-specific molecules during a brief time window in the ICM. Interestingly, all the heterogeneities described here are interconvertible. This may be interpreted as, although the chaotic expression of the factors during immediate-early...
differentiation is only for a limited period of time in vitro, ES cells are trapped in the steady state and thus they continue fluctuating in vitro indefinitely.

While maintaining the self-renewability of ES cells is one area of interest, determining the mechanism of their exit from self-renewal and entry into differentiation is another. When ES cells are in a homogeneous population under culture conditions such as 2i-culture, they do not differentiate. And, as described here, the heterogeneity of lineage-specific factors accompanies biases in the competency of ES cells to differentiate. Thus fluctuations of self-renewing regulators could be responsible for making ES cells always poised to undergo differentiation.

We do not consider our proposed network model (Fig. 2A) as a master model that covers the whole transcription factor network. Rather it represents one of the ways to dissect the entire network. Still, based on its stringent but flexible parallel pathway, we can interpret both the safeguard of self-renewability and the adjustable heterogeneity that continuously provide an opportunity for exerting pluripotency in response to external signals.

REFERENCES