Research

Transcription factor heterogeneity and epiblast pluripotency

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Stem cells are defined by the simultaneous possession of the seemingly incongruent properties of self-renewal and multi-lineage differentiation potential. To maintain a stem cell population, these opposing forces must be balanced. Transcription factors that function to direct pluripotent cell identity are not all equally distributed throughout the pluripotent cell population. While Oct4 levels are relatively homogeneous, other transcription factors, such as Nanog, are more heterogeneously expressed. Moreover, Oct4 positive cells fluctuate between states of high Nanog expression associated with a high probability of self-renewal and low Nanog expression associated with an increased propensity to differentiate. As embryonic stem (ES) cells transit to the more developmentally advanced epiblast stem cell (EpiSC) state, the levels of pluripotency transcription factors are modulated. Such modulations are blunted in cells that overexpress Nanog and this may underlie the resistance of Nanog-overexpressing cells to transit to an EpiSC state. Interestingly, increasing the levels of Nanog in EpiSC can facilitate reversion to the ES cell state. Together these observations suggest that Nanog lies close to the top of the hierarchy of pluripotent transcription factor regulation.

**Keywords:** stem cell; self-renewal; pluripotency; transcription factor; heterogeneity

1. INTRODUCTION

Stem cells are defined by the possession of the dual properties of self-renewal and differentiation potential. The cells with the greatest developmental potential are cells that have the ability to contribute to each of the three embryonic germ layers and are known as pluripotent stem cells. Pluripotent stem cells exist in the early embryo and in teratocarcinomas, tumours that spontaneously arise in the testes of specific mouse strains (reviewed by [1,2]).

The first pluripotent cells to be isolated and propagated in vitro, while retaining demonstrable pluripotency, were derived indirectly from teratocarcinomas following their in vivo passage as ascites and are referred to as embryonal carcinoma (EC) cells [3]. EC cells are the undifferentiated cells of teratocarcinomas, and conditions tuned to the in vitro propagation of these cells [4] allowed the subsequent isolation of pluripotent embryonic stem (ES) cells [5,6]. Mouse ES cells are pluripotent stem cells derived directly from the inner cell mass (ICM) of the blastocyst between embryonic day (E)3.5 and E4.5. At E4.5, the blastocyst contains three cell types; the trophoderm, the hypoblast and the epiblast. While the trophoderm and the hypoblast contribute to extraembryonic tissues, the epiblast gives rise to all cell types of the developing embryo proper. This pre-implantation epiblast cell population contains cells that have the ability to differentiate into derivatives of all three somatic lineages [7–9] and the germline [10]. ES cells retain these pluripotent characteristics provided they are maintained in appropriate culture conditions, e.g. in leukaemia inhibitory factor (LIF)/bone morphogenetic protein (BMP). At around E5.5, the embryo implants into the uterus and the epiblast undergoes molecular and cellular changes. However, post-implantation epiblast cells remain pluripotent and can give rise to cell lines in vitro, called epiblast stem cells (EpiSCs) [11,12]. EpiSCs differ from ES cells in that they have a flattened colony morphology, inefficient clonal propagation and rather than requiring LIF/BMP to maintain their pluripotency, require activin/fibroblast growth factor (FGF). EpiSCs are demonstrably pluripotent as they can generate teratocarcinomas containing differentiated cells of each of the somatic germ layers. However, EpiSCs lack the capacity to integrate into a pre-implantation embryo and thus contribute to the soma or germline of chimeric animals. This may suggest that EpiSCs have reduced potency relative to ES cells, or alternatively, that they have features incompatible with incorporation into the blastocyst. The observation that EpiSCs in culture can give rise to primordial germ cells (PGC) indicates that at least some cells in an EpiSC culture have retained germline potential [13]. Here, we discuss how transcription factors regulate the distinct identities and behaviours of these pluripotent cells.

2. GENE REGULATORY NETWORKS GOVERNING PLURIPOTENT CELL STATES

Pluripotent stem cell self-renewal efficiency is governed by a gene regulatory network centred around
the triumvirate of transcription factors Oct4, Sox2 and Nanog [14]. Oct4 belongs to the class of transcription factors that possess a bipartite POU domain composed of two DNA-binding domains, a homeodomain (POUH\textsubscript{D}) and a POU-specific domain (POU\textsubscript{S}). Based on studies conducted on Oct4, these domains are considered to be able to bind DNA separately. POU\textsubscript{H\textsubscript{D}} has a much higher affinity for DNA than POU\textsubscript{S}, but together both domains bind DNA cooperatively [15]. Oct4 is expressed in pluripotent cells and is specifically required for cells that become allocated to the interior of the blastocyst to acquire a pluripotent identity [16]. In ES cells, several Oct4 targets have been functionally identified including Oct4 [17], Opn [18], FGF4 [19], Uft1 [20], Fbx15 [21], Nanog [22], Zfp42 (Rex1) [23], Esrrb [24], Lefty1 [25], Cdx2 [26] and Sox2 [27]. In addition, global chromatin localization studies have identified thousands of sites throughout the ES cell epigenome to which Oct4 binds and which are therefore hypothesized to be involved in the control of expression of nearby genes [28–30]. The expression level of Oct4 is decreased to be involved in the control of expression of nearby genes [28–30]. The expression level of Oct4 is decreased to be involved in the control of expression of nearby genes [28–30]. The expression level of Oct4 is decreased to be involved in the control of expression of nearby genes [28–30]. The expression level of Oct4 is decreased to be involved in the control of expression of nearby genes [28–30].

Sox2 is a member of the Sry-related HMG box family of transcription factors that interact with DNA through binding to the minor groove. Sox2 shares many of the same DNA targets as Oct4 [32,33], with many of the characterized target sites being composed of composites of the non-palindromic Oct/Sox recognition sequences. For target genes, where DNA binding and transcriptional activation have been studied, Oct4 and Sox2 bind independently to their recognition sequences, but synergistically activate the enhancers [15,34]. Sox2 and Oct4 bind one another when located on a composite Oct/Sox recognition sequence, and the molecular basis of the cooperative nature of DNA binding by Oct and Sox proteins has been shown to be due to stabilizing interactions between side chains of the alpha helices of the HMG domain of Sox2 and the POU\textsubscript{S} domain of Oct proteins that occur at the interaction surface [15]. Sox2 deletion in the embryo is lethal at the early post-implantation stage. Analysis of the null mutant reveals a role of Sox2 in the maintenance of the epiblast as the only surviving cells at this stage are trophoblast giant cells and extra-embryonic endoderm [35]. Data from Sox2 knockdown by siRNA in ES cells suggest that Sox2 is required to prevent ES cell differentiation into both the trophectoderm and epiblast-derived lineages [36]. Interestingly, however, an inducible genetic deletion of Sox2 in ES cells results in differentiation of ES cells solely into trophodermical cells, similar to the phenotype observed upon deletion of Oct4 from ES cells [37]. The difference between the knockdown and the knock-out phenotypes in ES cells may reflect incomplete knockdown of Sox2 mRNA. Furthermore, the discrepancy between the in vivo and in vitro knock-out phenotypes may be a consequence of long-lived maternal Sox2 stores in the embryo [35]. Following inducible deletion of Sox2 from ES cells, test regulatory sequences containing Oct/Sox DNA recognition sites remain active in differentiated cells, potentially as a result of the continued expression of the Sox2-related proteins Sox4, Sox11 and Sox15, that may substitute for Sox2 in this respect [37]. Interestingly, the trophodermal differentiation of Sox2-deleted ES cells could be rescued not only by Sox2 but also by an Oct4 transgene [37]. Whether overexpression of Sox4, 11 or 14 can similarly compensate for the loss of Sox2 has yet to be reported. Together these results suggest that Sox2 functions in ES cells to stabilize Oct4 expression. This has been hypothesized to occur by modulation of positive (Nr5a2) and negative (Nr2f2) regulators of the Oct4 gene [37]. Further studies will be necessary to determine whether the deletion of Sox2 has subtle effects on Sox2 targets that are not shared by Oct4.

Like other homeodomain proteins, Nanog has been shown to adopt a typical three helix structure within the DNA-binding domain and binds the DNA sequence TAAT [38]. Gel shift assays using DNA duplexes in which all possible base identities in the 2 base pairs 3’ of this core motif were tested for binding to Nanog identified the sequence TAATGG as the sequence with the highest DNA-binding affinity [38]. Residues within the contact helix are known to influence the sequence specificity of DNA binding and this result is consistent with an effect of a glutamine residue at position 50 within the homeodomain upon DNA-binding specificity [39]. It was, therefore, somewhat unexpected that global localization studies would indicate that Nanog binds to many sites in chromatin in close proximity to Oct/Sox sites. Indeed, a DNA discovery programme revealed that at sites of Nanog binding, the most commonly occurring sequence was not TAAT, but rather a composite Oct/Sox motif [30]. Presently, the molecular explanation for this is unknown. However, protein–protein interactions are likely to play a key role here. Nanog has been documented to interact with a range of partner proteins (reviewed by [40]) and at least some of these interactions are likely to be critical in determining the function of Nanog in transcriptional regulation. In vivo analysis of Nanog-null embryos indicates that Nanog is essential for the specification of the pluripotent epiblast similar to the requirement for Oct4 [41,42]. At E3.5, Nanog-null embryos have a similar number of Oct4-positive ICM cells as Nanog\textsuperscript{+/+} or Nanog\textsuperscript{−/−} embryos. However, by E4.5 the number of Oct4-positive ICM cells is markedly reduced in Nanog-null embryos, and the persistent ICM cells have reduced Oct4 expression [42]. In female E4.5 Nanog-null embryos, the X chromosome appears to remain in an inactive state [42], consistent with a requirement for Nanog in controlling X chromosome reactivation [43]. Interestingly, there is no increased expression of the hypoblast-specific genes, indicating that the hypoblast does not form in the absence of Nanog [42]. It has been hypothesized that the lack of
the hypoblast in Nanog mutants is due to the fact that epiblast cells are necessary for the paracrine support of the hypoblast compartment [44].

Once the epiblast has been specified, Nanog mRNA is downregulated around the peri-implantation stage [45]. Following implantation, Nanog mRNA is subsequently detected in the epiblast [46]. These observations are in contrast to the continued expression of Oct4 and Sox2 during implantation and suggest that Nanog may have a brake function that must be removed in order for the changes in pluripotent phenotype that occur at implantation to proceed.

3. TRANSCRIPTION FACTOR OVEREXPRESSION CAN MODULATE DIFFERENTIATION PROPENSITY

One of the most striking features of Nanog is that when overexpressed in ES cells, Nanog can confer cytokine independent self-renewal. Indeed based on this phenotype, the gene was named after the mythological Celtic Land-of-the-Ever-Young, where visiting mortals remain unaged [47]. Nanog, therefore, has the ability to modulate the activity of the pluripotency gene regulatory network. Relative to wild-type cells, Nanog-overexpressing cells have a greater probability of self-renewal [45]. In contrast, ES cells from which Nanog has been deleted can continue to self-renew, albeit with reduced efficiency [48]. The strong, direct correlation between Nanog expression and self-renewal efficiency in a range of mutant ES cells argues powerfully for an interesting and fundamental regulatory role for Nanog in ES cell self-renewal [48]. Moreover, the importance of Nanog to the pluripotency network has been underscored by the demonstration that Nanog is required during the process of somatic cell reprogramming [49] in order for intermediate cells to transit to a fully reprogrammed or ‘naive’ pluripotent state [42]. This susceptibility of ES populations to have their self-renewal/differentiation behaviour radically altered through the modulation of a single transcription factor, like Nanog, may be a general feature of the regulation of stem cell populations (figure 1).

In this respect, it is interesting that a small group of transcription factors do have a strong influence on the self-renewal phenotype of ES cells. In addition to Nanog, factors such as Klf2, Klf4, Tbx3 and Esrrb have been reported to confer LIF-independent self-renewal of transfected ES cells [24,45,50,51]. In the case of Nanog and Klf2, overexpression can also confer self-renewal in the absence of BMP [50,52]. This result suggests that forced expression of factors at the cusp of the pluripotency hierarchy can artificially sustain the network in the absence of extrinsic signalling.

The above results are worth considering with respect to the differences between pluripotent ES cells and EpiSCs. Despite expressing Oct4, Sox2 and Nanog, differences in the core transcription factor circuitry and growth factor responsiveness referred to earlier indicate that ES and EpiSC are two distinct pluripotent states. It is possible to harness this differential growth factor responsiveness to convert ES cells into EpiSCs simply by switching the media composition and passaging [53]. During this transition key regulators of ES cell identity, such as Klf2, Klf4, Esrrb, Nr5a2, Tbx3, Rex1 and Nanog are downregulated (figure 2b).

Interestingly, different patterns and rates of decline can be discerned. Klf4 drops precipitously at day 1, Esrrb by day 2 and both remain low. By contrast, Rex1 and Klf2 decrease by approximately 50 per cent between days 2 and 3 and remain at a similar level for the next 24 h. A final pattern is exemplified by Nr5a2, which takes longer to be completely downregulated, but is undetectable in EpiSCs. It has been shown that Klf4 overexpression does not block differentiation of ES cells into EpiSCs [53]. On the other hand, forced expression of Nanog in ES cells decreases the propensity of these cells to transit to the EpiSC state (figure 3). EpiSC cells that overexpress different levels of Nanog were tested for their ability to make EpiSCs. We found that there was negative correlation between the Nanog levels and the ability of ES cells to commit to an EpiSC state. Two independent ES cell lines that overexpress Nanog were cultured in EpiSC media for eight passages and failed to downregulate ES cell-specific pluripotency factors, including Rex 1, Klf4 and Esrrb (figure 3a). Moreover, in contrast to controls, ES cell lines overexpressing Nanog resist upregulation of differentiation markers Foxa2, P-brachyury and Fgf5 (figure 3a). Since EpiSCs differentiate when exposed to ES cell media [53], this can be used as an assay for testing the commitment of cells to an EpiSC state. Unlike controls, Nanog-overexpressing cells formed ES cell colonies when returned to ES cell media, even after prolonged cultured in EpiSC conditions (figure 3b). This suggests that forced expression of Nanog in ES cells can block their full transition into an EpiSC state. It is possible that ES cells with higher levels of constitutively expressed Nanog are more resistant to differentiation.

Figure 1. Model depicting the stem cell paradox. The cells can either self-renew or differentiate. Populations of stem cells maintain a balance between these opposing forces. This balance is influenced by environmental cues, as well as intrinsic fate regulators. Here, the effect of modulating the expression of the transcription factor Nanog is depicted. In cells that overexpress Nanog, population behaviour is skewed towards self-renewal. In contrast, in Nanog-null cells, the balance is tipped in the opposite direction towards differentiation.
cues because they lack a primed undifferentiated compartment. Therefore, Nanog levels may affect the probability of an ES cell to exit the naive pluripotent state (figure 3c). We hypothesize that, similar to the in vivo dynamics, the in vitro differentiation of ES cells into EpiSCs requires Nanog downregulation. Whether this requires complete shutdown in Nanog expression is an interesting question for future study.

Figure 2. Transition from naive to primed pluripotency. (a) The developmental changes that occur between the nascent epiblast found in the blastocyst and the post-implantation epiblast can be recapitulated in vitro by changing growth factor supplements to the medium. (b) Gene expression analysis associated with the developmental restriction of pluripotency. Quantitative real-time polymerase chain reaction (qPCR) showing the changes that occur to transcription factor mRNAs when ES cells differentiate into EpiSCs. ES cells were plated on conventional media (LIF/FCS). Twenty-four hours later, the media was switched to activin/FGF. qPCR analyses were determined relative to TATA box-binding protein (TBP) and normalized to the ES cell level of 1.0. Yellow bars, day 1; red bars, day 2; blue bars, day 3; maroon bars, day 4; green bars, EpiSCs.

Figure 3. The effect of altering Nanog expression on the transition of ES cells to EpiSCs. ES cells overexpressing Nanog five-fold (green bars, 5× Nanog; EF4 cells [45]), or twofold (orange bars, 2× Nanog; RCN cells [48]) and a control line with wild-type Nanog levels (black bars, wild-type ES cells; Oct4GiP [54]) were switched into EpiSC media and cultured for eight passages. (a) Nanog-overexpression prevents downregulation of ES cell-specific transcription factors. qPCR showing mRNA levels of ES cell and EpiSC markers. qPCR analyses were determined relative to TBP and normalized to the ES cell level of 1.0. (b) Cell lines that overexpress Nanog retain the ability to self-renew in stringent ES cell culture conditions; after eight passages in EpiSC culture, cells were replanted in ES cell media (supplemented with LIF and 2i [55]) and colonies scored for expression of the ES cell marker alkaline phosphatase (AP). (c) The level of Nanog influences the ability of ES cells to exit the ES cell-specific attractor. This is represented by the depth of the basin in which the cells are located. ES cells with higher Nanog levels can buffer differentiation with greater efficiency and display a bias towards self-renewal.
4. CELLULAR HETEROGENEITY

Several groups have shown that under self-renewing conditions, mouse ES cells exhibit considerable heterogeneity in the levels of expression of self-renewal factors such as Nanog [48] and other pluripotency markers like Pecam1 [56,57], Stella [58,59] and Rex1 [60]. One could imagine that cellular heterogeneity reflects an irreversible developmental progression. However, sorting of single cells and monitoring their subsequent behaviour in culture have established that such heterogeneous states are reversible. Thus, Nanog negative cells can revert to a state of Nanog positivity associated with an enhanced self-renewal capacity [48]. Simultaneous expression of two or more lineage associated transcription factors in an undifferentiated stem or progenitor cell is known as lineage priming [61]. A number of fate determining transcription factors have been reported to be expressed at low level in a fraction of the undifferentiated ES cells, including T-brachyury [62], Sox17 [63] and Hex [64]. Such lineage priming could be advantageous as it may facilitate prompt differentiation along specific lineages when the correct cue arises. An important unanswered question is what the degree of overlap is between these distinct heterogeneous populations. Within this is a subsidiary question concerning hierarchies: does heterogeneity in any one transcription factor drive a heterogeneous pattern in others, does heterogeneity in transcription factor expression vary independently or is the system governed by more stochastic principles. Irrespective of this, these observations suggest that cells within the pluripotent state may fluctuate between different substates. The fact that the relative abundance of these substrates can be influenced by environmental cues is illustrated by the fact that Stella expression becomes pronounced only on co-culture with fibroblasts [59]. Interestingly, Erk inhibition reduces the Nanog-negative population both in vitro and in vivo. During pre-implantation development specification of the compartments that make up the epiblast is at least partially dependent on FGF signaling. An inverse correlation between FGF4 and FGF receptor 2 has been detected by single cell quantitative real-time polymerase chain reaction (qPCR) [65]. FGF4 transcription is directly activated by Oct4 and Sox2 [66]. Whereas Oct4 is expressed throughout the epiblast. Sox2 expression persists in the neurectoderm long after E7.5 [35,71]. Oct4 expression is maintained until E8.5, whereas Nanog is expressed until E7.5. (A. Tsakaridis, V. Wilson, R. Osorno & I. Chambers, unpublished data). Pluripotent cells have been reported to be lost around the time of germ layer inversion [72]. This conclusion was reached using a teratocarcinoma forming assay in which the tissue was transplanted under the kidney capsule of a recipient animal and assessed after several weeks for the presence of differentiated derivatives of all three germ layers. Whether the ability to give rise to pluripotent EpiSC lines is lost at the same time has yet to be determined. Furthermore, it will be interesting to determine the precise developmental time at which pluripotency is lost and whether pluripotency becomes extinguished uniformly throughout the epiblast.

The reversion of EpiSCs into an ES-like pluripotent state has been reported by the overexpression of several factors: Klf4, Nanog, Klf2, Nr5a2 and Stat3 [42,50,53,73,74]. It is interesting that EpiSCs respond poorly to LIF stimulation [74], suggesting that components of the signalling pathway such as the LIF receptor and Stat3, even if they are present in these cells, are attenuated in function. This is consistent with the fact that the ectopic introduction of a Jak/Stat3 activating receptor (GY118F) or Stat3 overexpression can reprogram EpiSCs to naive pluripotency [74]. This data suggest that Stat3 activation correlates with the ability of cells to contribute to chimeras following blastocyst injection. In support of this, Klf4, a known target of Stat3 in ES cells, can revert EpiSCs to chimera-forming competency. In fact, the combination of Stat3 activation and ectopic Klf4 expression increases the efficiency of cell reversion from EpiSCs to ES cells [74]. This suggests that Stat3 activation could reach saturation, but activation of downstream targets can amplify its effects. The ability of Nr5a2 to revert EpiSCs to an ES cell state is interesting given the observation that Nr5a2 can replace Oct4 during reprogramming of fibroblasts to pluripotency [75]. Nr5a2 can bind to the Oct4 promoter in ES cells [37] and has been shown to be necessary for the maintenance of Oct4 expression in the post-implantation epiblast [76]. These considerations suggest that Nr5a2 may substitute for Oct4 during reprogramming because Nr5a2 activates Oct4 expression. It will, therefore, be interesting to test whether Oct4-null population that is susceptible to exit the ES cell-specific basin of attraction (figure 3C [70]). We hypothesize that the functional differences between Nanog-expressing and Nanog-non-expressing compartments are brought about through the action of Nanog on Nanog-sensitive target genes. It is the equilibrium between ‘pristine’ and ‘poised’ states that allows the population to respond to differentiation cues without becoming completely depleted of pristine cells.

5. ALTERNATIVE POSSIBLE DIRECTIONS FOR POST-IMPLANTATION PLURIPOTENT CELLS

Upon implantation, the epiblast is transformed from an unstructured cell mass into a columnar epithelium. The core transcription factors Oct4 [54], Sox2 [35,71] and Nanog [46] are all expressed in the post-implantation epiblast. Sox2 expression persists in the neuroectoderm long after E7.5 [35,71]. Oct4 expression is maintained until E8.5, whereas Nanog is expressed until E7.5. (A. Tsakaridis, V. Wilson, R. Osorno & I. Chambers, unpublished data). Pluripotent cells have been reported to be lost around the time of germ layer inversion [72]. This conclusion was reached using a teratocarcinoma forming assay in which the tissue was transplanted under the kidney capsule of a recipient animal and assessed after several weeks for the presence of differentiated derivatives of all three germ layers. Whether the ability to give rise to pluripotent EpiSC lines is lost at the same time has yet to be determined. Furthermore, it will be interesting to determine the precise developmental time at which pluripotency is lost and whether pluripotency becomes extinguished uniformly throughout the epiblast.

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fibroblasts are unable to be reprogrammed by Nr5a2. Nanog and Sox2 are heterogenous in EpiSCs (data not shown), but the overall protein levels are lower than those observed in ES cells [77]. Therefore, the levels of Nanog and Sox2 may be critical for specification of cell fate in EpiSCs. In human ES cells, Nanog overexpression can confer Activin/Nodal independent self-renewal [78]. One may predict that Nanog overexpression in EpiSCs would allow FGF/Activin independent self-renewal. When combined with a medium switch, Nanog overexpression in EpiSCs restores the pristine pluripotent state [42], indicating that the level of Nanog during reprogramming is critical. Nanog, thereby, modulates pluripotency network activity to facilitate entry into an ES cell-specific basin of attraction.

6. CONCLUDING REMARKS

Alterations in transcription factor levels can have knock-on effects on the composition of gene regulatory networks, which may induce changes in cell identity. By examining the details of how such changes occur, both during developmental progression and in the reverse direction during cellular reprogramming, we can expect to achieve a greater understanding of the molecular mechanisms underlying orderly differentiation.

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