Design principles of pluripotency

Pluripotency is the capacity of individual cells to initiate all lineages of the mature organism in a flexible manner directed by signals in the embryo or cell culture environment. It is the keystone of mammalian development and of embryonic stem cell (ES) biology. A pluripotent cell has no predetermined programme; it is a blank slate. What are the design principles of this unrestricted cell state? Genetic and cell biological studies point to transcription factor command rather than epigenetic governance (Niwa, 2007; Silva & Smith, 2008). Persuasive support for this view comes from the remarkable discovery of Shinya Yamanaka that pluripotency can be recreated from somatic cells through transcription factor induced reprogramming (Takahashi & Yamanaka, 2006). So how is this unique status acquired, how can it be maintained, and how is the exit path(s) to committed lineage progenitors routed? These are some of the questions that my laboratory is addressing. What follows here is a personal perception of the underlying design principles of pluripotency and lineage determination.

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Mammalian embryos develop from a small group of 10–20 pluripotent cells that form a few days after fertilization. The pluripotent state is established under the direction of two transcriptional organizers, Oct4 and Nanog (Niwa, 2007). It is no coincidence that both of these factors were originally identified based on their expression in pluripotent cells and germ cells, and absence from somatic cells. Without either factor, cells destined to become pluripotent in the blastocyst never acquire the ability to generate embryonic lineages (Mitsui et al, 2003; Nichols et al, 1998; Silva et al, 2009).

Oct4 also drives the developmental progression towards lineage commitment by inducing expression of fibroblast growth factor 4 (Fgf4). This factor acts back on pluripotent cells to initiate specification (Fig 1). Yet cells can be maintained in the naïve state if embryogenesis is halted prior to implantation, a process known as diapause. This is a natural facultative response in female rodents when suckling. During diapause persistence of the epiblast becomes dependent on the glycoprotein130 (gp130) cytokine pathway (Nichols et al, 2001). This pathway proved the key to sustaining pluripotency ex vivo. The epiblast is present only transiently in early development but can be immortalized in culture in the form of ES cells (Evans & Kaufman, 1981; Martin, 1981). ES cells are obtained and propagated in vitro using the gp130 cytokine leukaemia inhibitory factor (LIF), whose action is mediated by the transcription factor Stat3 (Niwa et al, 1998). ES cells can be expanded indefinitely in an uncommitted state; this process of replication without commitment is called self-renewal in the language of stem cell biology (Smith, 2006).

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Extended maintenance of pluripotency therefore involves an additional module in the gene regulatory network. How Stat3 integrates with the core circuitry is an outstanding challenge in the field. One important element, however, is the induction of another transcription factor, the Kruppel-like factor 4 (Klf4) (Niwa et al, 2009). Klf4 is also one of the quartet of transcription factors identified by Takahashi and Yamanaka (2006) that can collectively reprogram somatic cells to pluripotency. The others are Oct4, its partner Sox2, and the proto-oncogene c-myc. Subsequent studies have shown that c-myc accelerates the reprogramming process but is not essential (Nakagawa et al, 2008). Thus the critical factors for producing induced pluripotent stem (iPS) cells are also central to sustaining pluripotency in ES cells. Molecular reprogramming can be considered in two discrete phases. Initially, deconstruction of the somatic cell gene regulatory network and epigenome produces an intermediate or partially reprogrammed state (Silva et al, 2008; Sridharan et al, 2009). In appropriate circumstances some of these dedifferentiated intermediates may progress to acquire pluripotency (Yamanaka, 2009). The entire process is suggested to occur through a series of stochastic transitions (Jaenisch & Young, 2008), but this view may simply reflect limitations of current methodologies and our ignorance of the molecular mechanisms at play.

Somewhat surprisingly, Nanog is not one of the primary inducing factors for iPS cells, even though it has been shown to promote pluripotency in hybrids generated by fusion of somatic cells with ES cells (Silva et al, 2006). Significantly, however, partially reprogrammed cells express little or no Nanog and it is up-regulated during the transition to pluripotency (Sridharan et al, 2009). In fact, Nanog is probably essential to obtain fully reprogrammed iPS cells, but this requirement may be fulfilled by activa-
tion of the endogenous gene (Okita et al., 2007; Silva et al., 2009).

**ES cell populations may similarly exist in a metastable equilibrium.**

ES cells are traditionally cultured in the presence of both LIF and serum or serum derivatives. In these conditions several genes including Nanog are expressed in a dynamic and heterogeneous fashion (Silva & Smith, 2008). The indication that ES cells may fluctuate through a range of transcriptional states is reminiscent of the multi-lineage priming phenomenon first described for haematopoietic progenitor cells (Hu et al., 1997). Such priming or ‘preview’ is proposed to provide a bias towards and opportunity for selective or instructive lineage commitment (Enver et al., 1998). ES cell populations may similarly exist in a metastable equilibrium in which a fraction of the cells are transiently potentiated for commitment (Enver et al., 1998). Consistent with this idea, some level of ‘spontaneous’ differentiation is generally evident in ES cell cultures. A central question is whether stochastic noise in a transcriptionally permissive state can be sufficient to establish a lineage specification programme (Chang et al., 2008; Huang, 2009), or is simply a foundation that requires coordination, amplification and selection by extrinsic stimuli.

A pre-condition for addressing this issue is a neutral culture environment. Fortunately, this can now be achieved by: (i) elimination of the autocrine Fgf4 signal, either genetically or using small molecule inhibitors; and (ii) partial inhibition of the multifunctional enzyme glycogen synthase kinase-3 (Gsk3), which is a negative regulator both of canonical Wnt signalling and of a broad range of metabolic, biosynthetic and transcriptional activities (Frame & Cohen, 2001). These inhibitor conditions are known as 3i or 2i, depending on whether both an Fgf receptor (Fgfr) inhibitor and a mitogen-activated protein kinase (extra-cellular signal regulated kinase) kinase (Mek) inhibitor or just a Mek inhibitor are used along with Gsk3 inhibition (Ying et al., 2008). It should be noted, however, that even in 3i/2i culture, ES cells invariably respond positively to LIF/Stat3. Strikingly, in 3i/2i, heterogeneity in Nanog protein expression is reduced (Silva et al., 2009). While global expression profiling is needed to assess whether there is a general homogenization of the transcriptome, ES cells anchored by Fgfr and Gsk3 inhibition show no ‘spontaneous’ differentiation, indicating that they are not functionally challenged by intrinsic noise. We have proposed that this represents the ground state for pluripotent cells (Nichols & Smith, 2009; Ying et al., 2008) (Fig 1).

To exit from pluripotency, the ground state must be perturbed. We speculate that Fgf signalling through the Mek/Erk1/2 mitogen-activated protein kinases first induces a metastable state (Fig 1). Nanog can play a decisive role at this juncture. ES cells that constitutively overexpress Nanog are highly resistant to differentiation and can be maintained without 3i/2i or LIF (Chambers et al., 2003; Ying et al., 2003a). Conversely, ES cells lacking Nanog are highly susceptible to differentiation (Chambers et al., 2007). Nanog may therefore constrain the induced noise either directly and/or by boosting the activity of the Oct4 network. The latter possibility is suggested by genome location analyses, which indicate that Nanog co-occupies multiple gene regulatory regions with Oct4 and Sox2 (Chambers & Tomlinson, 2009). However, in the presence of Fgf signalling, endogenous Nanog is subject to fluctuation and is low or absent from many ES cells. Such cells may be permissive for

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**Figure 1. From ground state to lineage commitment.** Two models of progression from unrestricted pluripotency to differentiated soma and germ cells.

**A.** The metastable transition state gives rise to specified cells that are lineage-biased but not committed. Multi-lineage priming in the metastable state may be a permissive condition for lineage specification. The specified progenitors may be induced to switch lineage in exceptional conditions such as teratomas produced by ectopic transplantation.

**B.** The metastable transition state generates cells with ‘actual’ pluripotency that then give rise to lineage-committed progeny. Multi-lineage priming may be ongoing in the state of actual pluripotency. In this model the apparent bias in the differentiation behaviour of EpiSCs and related human ES cells may be attributed to inadequate in vitro induction protocols, rather than intrinsic specification.
progression to commitment (Silva & Smith, 2008).

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Surprisingly, the primary committed product during ES cell differentiation is not characterized. It is generally assumed that this is a cell corresponding to the post-implantation epiblast of the mouse egg cylinder. However, it is not known whether that tissue consists of equivalent cells. Indeed, this may be rather unlikely, given the bombardment with inductive signals underway in the embryo after implantation. While there is good evidence that the fate of post-implantation epiblast cells can be altered by heterotopic transplantation, they may be specified towards particular lineages. What is clear is that egg cylinder stage epiblast is morphologically, transcriptionally and epigenetically distinct from the epiblast of the late blastocyst and from ES cells (Nichols & Smith, 2009). Post-implantation epiblasts cultured in the presence of Fgf and activin give rise to cells termed epiblast stem cells (EpiSCs) (Brons et al, 2007; Tesar et al, 2007). These cells are dependent on Fgf/Erk signalling for maintenance, in complete distinction from mouse ES cells. ES cells can differentiate into EpiSCs but EpiSCs can only revert to ES cells by introduction of reprogramming factors (Guo et al, 2009). A notable feature of EpiSCs is the expression of genes associated with germ layer specification. We have suggested that post-implantation epiblast and EpiSCs may be considered a state of ‘primed’ pluripotency (Nichols & Smith, 2009). This descriptor serves to discriminate from the naïve ground state of pre-implantation epiblast and ES cells. It also indicates the possibility of a category of pluripotency that at the single cell level may have inherent lineage bias even though not committed (Fig 1). Cells in this state may be subject to lineage switching at some frequency in response to powerful inductive signals in the embryo or during teratoma formation. Alternatively, post-implantation epiblast and EpiSCs may be unbiased. In this scenario the ground state becomes a predecessor to ‘actual’ pluripotency (Fig 1). Such ‘actual’ pluripotency might be characterized by continuous metastability but without the option for reversion to the ground state. Interestingly, the relationship between quiescent haematopoietic stem cells and multi-lineage progenitors may share some similar features (Enver & Greaves, 1998).

Single cell analyses and clonally expanded populations may give some insight into possible diversity between EpiSCs. However, this will be confounded if the culture conditions in Fgf and activin select for, or instruct, a particular lineage bias. Therefore, distinguishing between the two models is likely to require well-defined systems for committing ES cells or EpiSCs en masse to individual lineages. Lineage bias could then be quantified and related to mapping of gene expression trajectories and epigenome modifications. Currently, reliable single lineage commitment is available only for the neuroectoderm and even in that case it is handicapped by asynchrony and persistence of undifferentiated cells (Lowell et al, 2006; Ying et al, 2003b). However, one great advantage of ES cells and EpiSCs is the relative ease with which they can be expanded, genetically manipulated, live imaged and biochemically interrogated. Consequently their secrets will increasingly be revealed.

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