findings are consistent with previous reports that identified human miR-200c as a direct repressor of BMI1, limiting the expansion and tumorigenicity of breast cancer cells (Shimono et al., 2009). Importantly, the effects of miR-22 on the expression of miR-200c and Bmi1 are mediated through a direct interaction of miR-22 with TET mRNAs and can be reproduced in a line of immortalized mammary epithelial cells by shRNA-mediated knockdown of TET2 and TET3. These observations provide fundamental mechanistic insights into developmental biology in that they explain how different arms of the molecular machinery that shapes the epigenetic identity of stem cells work together in an integrated system to control the capacity to self-renew. Members of the TET family act as initiators of DNA demethylation while Bmi1, a member of the *Polycomb* repressor complex 1 (PRC1), regulates chromatin remodeling through specific histone modifications such as ubiquitination of lysine-119 of histone-2A. Both systems oversee the coordinated regulation of multiple gene expression programs during differentiation. Learning how these epigenetic pathways interact is a fundamental step toward understanding how even relatively subtle genetic manipulations (e.g. the constitutive expression of one miRNA) can "ripple" into profound perturbations of stem cell homeostasis and cause cancer.

In our opinion, however, the most compelling finding that emerges from the aggregate work of Song and collaborators is that chromatin-remodeling systems with opposing effects on cell identity (self-renewal versus differentiation) appear to directly antagonize each other through opposing sets of miRNAs (e.g. miR-22 versus miR-200c). A series of theoretical questions thus arises. If chromatin-remodeling systems directly antagonize each other as part of a dynamic equilibrium between self-renewal and differentiation, what tilts the balance toward one fate or the other? Under physiological conditions, what makes changes in stem cell identity (i.e., differentiation) irreversible? The answer to these questions lies in a more advanced, systems-level understanding of these molecular circuitries and in a deeper characterization of their positive and negative feedback loops. For example, are members of the *Polycomb* family able to regulate miR-22 expression? If so, do they positively affect miR-22 expression, thus "locking" the stem cell identity in a self-reinforcing loop, or do they suppress it, thus "limiting" the stem cell identity in a cell-autonomous manner? The challenge for the future will be to develop new experimental approaches, and mathematical algorithms, to model the integrated action of these complex relationships and their impact on cell fate (Sahoo, 2012).

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Nanog Heterogeneity: Tilting at Windmills?

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Fluctuating expression of transcription factors in embryonic stem cells is an alluring observation, but, as outlined by two articles in this issue, appearances can be misleading.

Mouse embryonic stem cells (ESCs) closely approximate pluripotent embryo founder cells resident in the blastocyst. However, it is important to keep in mind that ESC propagation is a cell culture phenomenon. ESCs may be liberated from constraints imposed by the developmental program in vivo, but they are also subject to stimuli and conditions that do not occur in the embryo. Depending on the specific culture setting, ESCs exhibit different morphology, gene expression, epigenetic features, and self-renewal efficiency (Wray et al., 2010). Notably, ESCs on a feeder layer present as homogenous clusters of small, tightly packed cells, whereas without feeders and in the presence of leukemia inhibitory factor (LIF), ESCs are flattened and exhibit heterogeneous morphologies. A suite of transcription factors is expressed in a mosaic

fashion in such feeder-free serum and LIF cultures (Marks et al., 2012). Nonetheless almost all cells express the essential pluripotency determinants Oct4 and Sox2, and at the population level ESCs cultured in serum and LIF can reliably form chimeras and give germline transmission.

Heterogeneity in transcription factor expression is commonly observed by immunostaining and thus reflects protein levels. In some cases, knockin of a fluorescent reporter (FP) has been used to infer transcriptional regulation. Nanog is studied frequently because it plays key roles in establishment of pluripotency, self-renewal, and reprogramming. *Nanog* reporters are expressed heterogeneously in ESCs cultured in serum and LIF without feeders. Furthermore, they indicate that a fraction of cells can revert from Nanog low to Nanog high states (Chambers et al., 2007). Similar observations for *Rex1* and *Stella* reporters have led to the proposition that ESCs experience dynamic heterogeneity and that such metastability may be an essential component of pluripotent identity (Hayashi et al., 2008; Toyooka et al., 2008). However, if inductive signaling through the fibroblast growth factor/mitogen-activated protein kinase pathway is blocked and activity of glycogen synthase kinase 3 is inhibited with two small molecules (2i), ESCs are highly homogenous yet fully pluripotent even in the absence of feeders (Wray et al., 2010). Heterogeneity and fluctuation are therefore culture-induced perturbations and their relevance to potency or fate choice is questionable. Nonetheless, these phenomena continue to attract interest. To add fuel to this debate, it has recently been suggested that monoallelic expression may underlie Nanog heterogeneity (Miyanari and Torres-Padilla, 2012). This inference is based primarily on localization of nascent transcription sites by RNA FISH, although the authors also claim that it is reflected in the alternating expression of fluorescent reporters.

Contrary to these previous findings, in this issue Faddah et al. (2013) now describe a failure to detect significant heterogeneous expression using new *Nanog:FP* knockin reporters and single-molecule mRNA FISH. These authors ascribe previous results to artifacts of endogenous gene disruption. Indeed, the authors show some differences between reporters—a useful reminder that a knockin cannot blithely be assumed to recapitulate all aspects of normal regulation. Remarkably, however, Faddah et al. did not examine ESCs without feeders in serum and LIF, and therefore cannot draw conclusions pertinent to the circumstance in which heterogeneity has been documented. It would be intriguing if their reporter remained homogeneously expressed in these conditions, unlike Nanog protein. In a second report, Filipczyk et al. (2013) create functional Nanog-FP fusion proteins and generate reporters that are anticipated to mirror normal Nanog protein distribution. These authors do employ feeder-free culture and observe heterogeneity in serum and LIF compared to relative homogeneity in 2i. The interesting feature of this report is that in both conditions they find a high correlation between reporters expressed from either allele, as also seen by Faddah et al. (2013). This finding therefore challenges the idea that there is significant monoallelic expression of Nanog and points to sporadic transcriptional bursting as an alternative explanation for the previous FISH results. Why the burst interval should be longer for Nanog than other pluripotency factors expressed at similar mRNA levels is unknown.

Leaving aside disputes over construct design, the real issue is whether Nanog heterogeneity in ESCs under certain conditions has biological meaning. Without feeders or 2i, ESCs in serum and LIF show variegated expression not only of Nanog but also of several other pluripotency transcription factors. These factors, such as Klf4, Esrrb, and Rex1, are typically downregulated at the onset of ESC differentiation, during implantation in the embryo, and in cultured postimplantation epiblast stem cells (EpiSCs) (Nichols and Smith, 2012). This observation, along with the readily detected upregulation of early differentiation markers, suggests that feeder-free ESCs in serum and LIF comprise both self-renewing stem cells and a spectrum of cells in transition toward differentiation (Marks et al., 2012). The conflicting stimuli provided by serum may promote disorder, while the potent activity of LIF as both a self-renewal and a reprogramming signal (Yang et al., 2010) may induce reversion during transition. However, it should be noted that a substantial proportion of Nanog low cells are destined for differentiation and shedding from the culture during passaging (Chambers et al., 2007). Thus, the ESC heterogeneity that has been documented may well be primarily a consequence of a disordered signaling environment created by a specific set of in vitro conditions. This culture specificity raises questions about overall functional significance. Nanog expression in the very early embryo appears to fluctuate stochastically. However, that form of heterogeneity precedes emergence of the pluripotent epiblast, in which Nanog expression is consolidated and from which ESCs are actually derived (Nichols and Smith, 2012). Importantly, there is currently no evidence that either fluctuating expression of pluripotency factors or state reversion occurs during epiblast progression and lineage commitment in vivo. Instead, the variation that many have observed may simply be a culture epiphenomenon: an attractive playbox for experimentalists and modellers, but with questionable relevance for the way in which pluripotent cells really make fate decisions.

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