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Decoding pluripotency - genetic screens to interrogate the acquisition, maintenance, and exit of pluripotency

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Abstract

Pluripotent stem cells have the ability to unlimitedly self-renew and differentiate to any somatic cell lineage. A number of systems biology approaches have been used to define this pluripotent state. Complementary to systems level characterization, genetic screens offer a unique avenue to functionally interrogate the pluripotent state and identify the key players in pluripotency acquisition and maintenance, exit of pluripotency, and lineage differentiation. Here we review how genetic screens have helped us decode pluripotency regulation. We will summarize results from RNA interference (RNAi) based screens, discuss recent advances in CRISPR/Cas-based genetic perturbation methods, and how these advances have made it possible to more comprehensively interrogate pluripotency and differentiation through genetic screens. Such investigations will not only provide a better understanding of this unique developmental state, but may enhance our ability to use pluripotent stem cells as an experimental model to study human development and model disease progression. Functional interrogation of pluripotency also provides a valuable roadmap for utilizing genetic perturbation to gain systems understandings of later stages of development and disease etiology.

Graphical Abstract:

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Pluripotent stem cells have the ability to unlimitedly self-renew and differentiate to any somatic cell lineage. Advances in genetic perturbation methods are enabling comprehensive interrogation of pluripotency and differentiation through large-scale genetic screens. Findings from such screening efforts will have broad implications for developmental biology, stem cell and regenerative medicine.

Introduction

Pluripotency describes the potential of a cell to give rise to all cell types in an adult organism. During mammalian development, pluripotent cells exist transiently in early blastocyst embryos before quickly differentiating into the endoderm, mesoderm, and ectoderm lineages. This natural pluripotent state can be maintained *in vitro* in self-renewing embryonic stem cells (ESCs) derived from the inner cell mass (ICM) (Evans & Kaufman, 1981; Martin, 1981; Thomson et al., 1998). Pluripotency can also be experimentally induced by gene overexpression or chemical treatments (Takahashi & Yamanaka, 2016). As first demonstrated by Yamanaka and colleagues in mouse fibroblasts, somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) that closely resemble ESCs (Takahashi & Yamanaka, 2006). These pluripotent stem cells (PSCs) are capable of

unlimited self-renewal while maintaining pluripotency, thus providing an excellent model for studying the transient early developmental stage that these cells represent. As PSCs have the potential to be differentiated to all somatic lineages, understanding the regulation of pluripotency will enhance our ability to utilize PSCs for studying development and disease, and further for generating therapeutic cells for regenerative medicine.

In vitro PSC culture relies on our understanding of the signaling pathways and transcription factor networks required for pluripotency maintenance. Mouse and human PSCs employ different signaling pathways to maintain pluripotency: mouse ESCs (mESCs) can be maintained by Leukemia Inhibitory Factor (LIF) activation of the JAK/STAT pathway and BMP signaling (Chambers & Smith, 2004; A. G. Smith et al., 1988; Williams et al., 1988; Q. L. Ying, Nichols, Chambers, & Smith, 2003), whereas human ESCs (hESCs) are dependent on FGF2 and TGF β signaling pathways and cannot be derived in mESC growth conditions (Amit, Shariki, Margulets, & Itskovitz-Eldor, 2004; Beattie et al., 2005; James, 2005; Thomson et al., 1998; Vallier, 2005; C. Xu et al., 2005; R. H. Xu et al., 2005). The differences in signaling requirements for pluripotency maintenance were thought to be due to intrinsic species differences between mouse and human until the derivation of PSCs from the post-implantation mouse epiblast (mEpiSCs) using FGF and Activin A (a member of the TGF β super-family) which closely resemble the signaling pathways required for hESC maintenance (Brons et al., 2007; Tesar et al., 2007). Pre-implantation derived mESCs, and post-implantation derived mEpiSCs respectively typify the “naïve” and “primed” pluripotent states. As pre-implantation derived hESCs resemble primed mEpiSCs, there has been much interest in identifying signaling pathways that support the establishment and maintenance of naïve hESCs, a state similar to that of mESCs (Chan et al., 2013; H. Chen et al., 2015; Duggal et al., 2015; Gafni et al., 2013; Guo et al., 2016; Hanna et al., 2010; Qin et al., 2016; Takashima et al., 2015; Theunissen et al., 2014; Valamehr et al., 2014). While a number of methods appear to produce naïve cells with varying degrees of similarity to mESCs, no single culture condition thus far supports the maintenance of both human and mouse naïve PSCs, thus the exact nature of human naïve pluripotency remains an open question. Therefore, it is necessary to systematically interrogate the regulation of the pluripotent state in both mouse and human. Identifying the distinct and shared regulatory mechanisms between mouse and human PSCs will inform our understanding of the naïve versus primed pluripotent state in both species.

Three complementary systems biology approaches have been used to define the fundamental mechanism of pluripotency in PSCs. First, transcriptome, epigenome, proteome profiling have been used to capture the signature of pluripotency (Müller, Tarasov, Gundry, & Boheler, 2012). Second, genetic screens are used to identify the key regulatory genes in the pluripotency network and reveal their causative relationship to pluripotency. Third, computational biology approaches are used to model the pluripotency gene regulatory network in order to establish an integrated framework of pluripotency regulation and predict the outcomes of various perturbations (Yachie-Kinoshita et al., 2018). In this review, we will begin with a brief overview of previous omics studies which have described the pluripotent state, and then delve into how genetic screening has enhanced our understanding of the mechanisms underlying stem cell pluripotency regulation, including: pluripotency

maintenance, exit of pluripotency and differentiation, pluripotency state transition, and acquisition of induced pluripotency (Figure 1, Table 1).

Characterization of pluripotency through omics approaches

Characterization of the pluripotent state by transcriptome profiling

The quest to define the molecular signature of pluripotency began as a search for the common transcriptional program that defines “stemness”. Three groups independently used microarray-based gene expression profiling to identify stemness genes by comparing the gene expression profiles of mESCs, hematopoietic stem cells, neural progenitor cells, and retinal progenitors (Fortunel, 2003; N. B. Ivanova et al., 2002; Ramalho-Santos, Yoon, Matsuzaki, Mulligan, & Melton, 2002). However, these efforts failed to identify a common set of stemness genes. Instead, the results argue that ESCs and tissue-specific stem cells have largely distinct transcriptional signatures. A number of labs have since specifically examined the gene expression profiles of mESCs and hESCs by microarray (Ginis et al., 2004; Wei et al., 2005). The unique gene expression signature of hESCs was also identified by comparison to hESC-derived differentiated cell populations by RNA-seq (Brandenberger et al., 2004). To further establish a common hESC gene expression signature shared among different hESC lines, an international consortium was initiated to compare 59 hESC lines from 17 laboratories worldwide. A common set of genes including *NANOG*, *OCT4*, *TDGF1*, *DNMT3B*, *GABRB3* and *GDF3*, as well as cell surface antigens SSEA3/4, TRA-1–60/81, GCTM2 and GCT343, are expressed in all hESC lines tested by this consortium (Adewumi et al., 2007).

Characterization of the pluripotent state by epigenomic profiling

Using ChIP-on-chip technology, which combines chromatin immunoprecipitation (ChIP) with DNA microarray (chip), a landmark study produced the first draft map of pluripotency transcription factors (Oct4, Nanog, Sox2) binding landscapes in mESCs (Boyer et al., 2005). Due to the technological limitations at the time, only promoter regions were mapped in this study. This limitation was soon overcome by using ChIP-Seq to map genome-wide occupancy of Oct4 and Nanog (Loh et al., 2006). Together, these studies demonstrated that a small number of transcription factors including Oct4, Nanog, and Sox2 co-occupy a large set of genes in PSCs, supporting the hypothesis that the pluripotent transcriptional landscape is maintained by a set of master transcription factors. In recent years, with inputs from the ENCODE and Roadmap Epigenomics projects, we have an increasing understanding of the pluripotent transcriptome and epigenome (including the landscape of transcription factors, histone modifications, DNA methylation, and chromatin accessibility and conformation), as well as datasets from other somatic cell types for comparison (Dunham et al., 2012; Tsankov et al., 2015).

Characterization of the pluripotent state by proteomics

Proteomic approaches such as immunoprecipitation-mass spectrometry (IP-MS) offer additional opportunities to understand the pluripotency network. Experiments mapping protein interaction partners have revealed mini-interactomes among the transcription factors controlling pluripotency such as Oct4, Nanog and Smad2/3, and their interactions with a

variety of histone modification enzymes, chromatin remodelers, and other transcriptional machinery (Bertero et al., 2018; van den Berg et al., 2010; J. Wang et al., 2006). These findings form the basis for studying the regulation of the pluripotent state and its associated epigenome. In one excellent example, Wang and colleagues identified in mESCs 19 Nanog interaction partners including Tet1, which mediate DNA demethylation (Costa et al., 2013). Nanog and Tet1/2 act synergistically to promote reprogramming, suggesting that the interaction of Nanog with Tet1/2 (and possibly other epigenetic regulators) fine-tunes the induction of pluripotency.

Perturbation methods for functional genomics

The aforementioned omics studies have generated rich resources characterizing the molecular signatures of pluripotency. In addition, they also provide the basis for formulating hypotheses regarding the roles of specific regulators of pluripotency, which can then be tested through carefully designed genetic studies. Complementary to the omics approach, forward genetic screening approaches can be used to directly identify genes that are required for pluripotency regulation. Below we review the perturbation methods currently available, and their relevance to screens in PSCs.

Early random mutagenesis for loss-of-function screens

Random mutagenesis by retroviral insertion or chemical mutagen is highly valuable for loss-of-function genetic screens (Acevedo-Arozena et al., 2008; Caspary & Anderson, 2006; Ranzani, Annunziato, Adams, & Montini, 2013). These methods were particularly useful in the pre-genomic era as they do not rely on a reference genome. Many key genes were discovered from forward genetic screens in mice. A retroviral integration screen was performed in mESCs, which led to the identification of *Nodal* as a key regulator of gastrulation and a growth factor in the TGF β signaling pathway (Conlon, Barth, & Robertson, 1991). Chemical mutagenesis screens in mice also uncovered a critical requirement of the cilium, a cellular organelle, in spinal cord development and Hedgehog signal transduction (Huangfu et al., 2003). While powerful, it has been difficult to directly implement random mutagenesis in cell-based screens due to the challenge of target identification.

cDNA over-expression for gain-of-function screens

Gain-of-function screens can also be quite powerful for identifying novel gene functions. For example, the transcription factor *Nanog* was identified from a cDNA over-expression screen based on its ability to sustain mESC self-renewal without LIF dependent JAK/STAT signaling (Chambers et al., 2003). This essential role of *Nanog* in pluripotency maintenance was further supported by an independent study showing that the loss of *Nanog* expression caused mESCs to lose pluripotency and spontaneously differentiate (Mitsui et al., 2003). Although cDNA over-expression experiments are relatively easy to perform, the challenge of constructing large-scale, normalized, full-length cDNA libraries has so far constrained their implementation in large-scale screens (Chong, Ohnishi, Yusa, & Wright, 2018).

Random mutagenesis with transposons and screens in haploid cells

Transposons, which are mobile genetic elements, offer an additional tool for inducing random mutagenesis for genetic screens (Kawakami, Largaespada, & Ivics, 2017; Ni, Clark, Fahrenkrug, & Ekker, 2008). Transposon induced mutations, by virtue of their insertion of a DNA fragment can also serve as a tag for easy PCR identification of the mutated locus (O'Donnell, 2018). Inserted DNA often contain “gene-trap” elements, such as an upstream splice acceptor, a promoterless reporter gene and a downstream polyadenylation sequence, that mutate the gene and at the same time also produce a fusion transcript to report the expression of the trapped gene (Hansen et al., 2008; Pettitt et al., 2013, 2017; Skarnes, Auerbach, & Joyner, 1992). Given that transposon mutagenesis occurs at random sites in the genome, it would be impractical to knockout both alleles of a given gene in a diploid cell. Therefore, the generation of haploid mESCs and hESCs have greatly advanced transposons' usefulness in whole-genome genetic screens (Elling et al., 2011; Leeb & Wutz, 2011; W. Li et al., 2012; Pettitt, Tan, & Yusa, 2015; Sagi et al., 2016). It should be noted that transposon insertion can occur preferentially at certain “hot-spots” of the genome, and is more likely to occur in larger genes, creating bias in transposon directed mutagenesis screens (Kawakami et al., 2017).

Targeted gene silencing through RNA interference

RNA interference (RNAi) technology has greatly accelerated the speed of functional genomics studies, as RNAi based genetic screens can be easily integrated with high-throughput sequencing and automation technology to facilitate the process of target identification (Hu & Luo, 2012; Sims et al., 2011). In RNAi, synthetic small interfering RNA (siRNA) or short hairpin RNA (shRNA) are typically used to silence gene expression at the post-transcriptional level by forming double strand RNA with complementary mRNA transcripts. As a result, the double stranded RNA-complex is degraded by the endogenous RNAi machinery and the expression level of mRNA for protein translation is downregulated (Fire et al., 1998). RNAi screens have been widely adopted for cell-based screens to interrogate pluripotency, though it should be noted that the knockdown can be incomplete and off-target activities can make it challenging to verify hits from some RNAi screens.

Targeted gene knockout through CRISPR/Cas9

Unlike RNAi knockdown, CRISPR/Cas9 causes alterations at the pre-transcriptional level. The Cas9 protein is an RNA guided DNA nuclease, repurposed from the prokaryotic immune system, that can be exogenously introduced to eukaryotic cells. A guide RNA (gRNA) can target Cas9 to a 20 bp genomic DNA sequence with a 5'-NGG-3' motif at the end (PAM sequence). The Cas9-gRNA duplex introduces a DNA double-strand break ~3 bp upstream of PAM sequence. This DNA double strand break can be repaired by either non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ can lead to insertion or deletion (indel) mutations that permanently alter the genomic DNA sequences. Frameshift mutations or mutations in sequences corresponding to a key functional domain can be used to interrogate loss-of-function phenotypes (Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013). CRISPR/Cas9 tends to generate stronger loss-of-function phenotypes compared to the RNAi approach, and the target specificity is much improved. There is an

ever-growing list of CRISPR systems (Adli, 2018; Hsu, Lander, & Zhang, 2014), though CRISPR/Cas9 remain the most widely used for knockout screens. It should be noted that the mutations introduced by CRISPR/Cas9 are irreversible. There could also be situations in which a partial loss-of-function is preferable over a complete loss-of-function. For instance, the differentiation function of a gene could be difficult to evaluate through a complete knockout approach if this gene is also essential for PSC self-renewal.

Targeted gene perturbation through CRISPRi and CRISPRa

In addition to CRISPR/Cas9-mediated knockout, engineered nuclease-deactivated Cas9 (dCas9) can be fused with a transcriptional regulatory domain to perturb gene expression without altering the DNA sequence (Pulecio, Verma, Mejía-Ramírez, Huangfu, & Raya, 2017; X. Xu & Qi, 2019). The dCas9-fusion protein and gRNA duplex are recruited to a gene's promoter or enhancer to regulate transcription in a targeted manner. CRISPR-mediated-inactivation (CRISPRi) can be achieved by dCas9 fused with a strong transcriptional repressor domain such as KRAB (Gilbert et al., 2013). dCas9-mediated repression can be tunable and reversible, thus offering a complementary approach to Cas9-mediated knockout.

In parallel to the development of the CRISPRi tools, CRISPR-mediated-activation (CRISPRa) methods are developed through dCas9 fusion with transcriptional activation domain proteins such as VP64, a fusion of 4-tandem copies of the VP16 transactivation domain (Pulecio et al., 2017; X. Xu & Qi, 2019). dCas9 mediated activation of gene expression offers a new method for gain-of-function screens with some unique features. Compared to full-length cDNA libraries, gRNA libraries are much easier to construct, thus making large-scale screens more feasible. The CRISPRa tools support the activation of gene expression from the endogenous locus. However, the levels of gene expression tend to be variable and also typically weaker compared to cDNA over-expression. This could increase the difficulty of CRISPRa screens, as more gRNAs need to be screened to find the ones that support high levels of target gene expression. On the other hand, sometimes the biological outcome is sensitive to the gene dosage, thus the varied gene expression levels achieved by different gRNAs would increase the probability of finding the right match, as shown in a recent screen for genes that promote the induction of naïve pluripotency from mEpiSCs (J. Yang et al., 2019).

Screening strategies

Arrayed and pooled screens

RNAi or CRISPR screens can be conducted in an arrayed or pooled format. Array based screens take advantage of high-throughput robotic automation platforms to perform perturbation and subsequent phenotypic analysis in multi-well plates (e.g. 96- or 384-well plates). In an arrayed screen, cells in each well are matched to a unique perturbation such as those mediated by siRNA, shRNA or gRNA. After a period of cell culture, the cellular phenotypic readouts such as cell proliferation, cell death, morphology, reporter gene expression, or protein subcellular localization can be analyzed by high-throughput imaging

platforms such as luminescence plate reader or high-content automated microscopy (Figure 2).

In a pooled screen, a lentiviral library expressing shRNA or gRNA is typically used to infect a large pool of cells at a low multiplicity of infection (MOI) to ensure most of the cells only harbor one perturbation cassette (Figure 2). The integrated shRNA or gRNA expression cassette can later be used as a barcode to identify the targeted genes by high-throughput sequencing. There are a number of ways to identify cells with the desired phenotype in a pooled screen. In a positive (enrichment) selection screen, cells with a proliferation or survival advantage (e.g. drug resistance) will be enriched after the genetic perturbation compared to the control group (the non-selected cells). In a negative (drop-out) selection screen, cells with a proliferation or survival defect will be depleted after genetic perturbation and a period of cell culture.

While most pooled screens are based on proliferation and survival/death phenotypes, it is possible to select cells based on marker gene expression as shown in our own genome-wide CRISPR/Cas9 screens that utilized a knockin definitive endoderm reporter in hPSCs for uncovering regulators of endoderm differentiation (Q. V. Li et al., 2019). Fluorescence or magnetic activated cell sorting can be used to enrich cell populations based on the presence or absence of a selective marker using a fluorescence-reporter gene or direct antibody staining for a cell surface or intracellular antigen. Marker positive and negative cells are sorted and sequenced in order to identify the relative enrichment of shRNA or gRNA in each population (Figure 2). Using this strategy, one can identify genes that promote or inhibit a biological process based on the expression of a marker gene.

Comparisons of RNAi and CRISPR technology

One of the major advantages of the CRISPR technology is its ability to generate robust loss-of-function phenotypes. Therefore, CRISPR screens are likely to identify new regulators even when a thorough RNAi screen has been previously conducted for the same phenotype (Boettcher & McManus, 2015; I. Smith et al., 2017). For example, two recent CRISPR screens have identified *Taf5L* and *Taf6L* as robust regulators of mESC pluripotency (M. Li et al., 2018; Seruggia et al., 2019), but these genes were missed in similar screens performed previously using the RNAi approach (Ding et al., 2009; Hu et al., 2009). CRISPR/Cas9 also has considerably fewer off-target effects compared to RNAi (Evers et al., 2016), though the specificity of RNAi has also been improved in recent years (Fellmann & Lowe, 2013; I. Smith et al., 2017). On the other hand, RNAi mediated knockdown can offer some unique advantages as the repression of gene expression is reversible and tunable, which is in contrast to the irreversible CRISPR/Cas9 mediated knockout. Similar to RNAi, CRISPRi is transient and tunable. Together with CRISPRa, it is now possible to achieve a variety of gene expression levels to study complex biological processes that may be sensitive to gene dosage.

In addition to gene dosage effects, the acute consequence of a knockdown experiment may be different from the long-term outcome of a knockout, as the latter could be influenced by secondary effects including compensation by alternate mechanisms (Rossi et al., 2015). For example, *Tbx3* was initially identified as a pluripotency regulator from a RNAi knockdown

screen (N. Ivanova et al., 2006). However, two recent reports have shown that *Tbx3*^{-/-} mESCs can maintain a normal pluripotent phenotype (Russell et al., 2015; Waghray et al., 2015). This apparent discrepancy may be explained by the ability of a subpopulation of *Tbx3* null mESCs to adapt to the loss of *Tbx3*.

Screens to interrogate the maintenance of pluripotency

RNAi screens to interrogate the maintenance of pluripotency

Soon after the initial description of pluripotency using systems genomic approaches, the pluripotency field entered an era of RNAi based functional genomics. Lemischka and colleagues designed a focused shRNA library targeting 70 genes encoding DNA binding proteins that were downregulated after retinoic acid induced differentiation in mESCs (N. Ivanova et al., 2006). They used a competitive cell growth assay as a readout for pluripotency maintenance. shRNA targeted mESCs were labeled with GFP reporter and co-cultured with unlabeled wild-type mESCs. The proliferation phenotype was evaluated based on the percentage of GFP⁺ cells by flow cytometry. This screen identified *Tbx3*, *Esrrb*, *Tcl1*, and *Dapp4* in addition to previously known pluripotency regulator like *Oct4* and *Nanog*. The success of this mini-scale screen has inspired larger scale screening efforts such as an arrayed siRNA screen targeting 1,008 chromatin proteins to identify the genes required for mESC growth (Fazzio, Huff, & Panning, 2008). The knockdown of 68 genes caused varying degrees of phenotypes in mESC viability and morphology. It would be of interest to determine which genes identified from these early screens are required for general cell proliferation and survival, and which genes are specifically required for maintaining the pluripotent state; the latter could be determined by examining the expression of pluripotency genes (e.g. Oct4 and Nanog) and the differentiation potential of the affected mESCs.

In order to interrogate mESC pluripotency beyond cell growth, the Bucholz and Elledge groups carried out two independent genome-wide siRNA screens using transgenic *Oct4-GFP* reporter mESC lines (Ding et al., 2009; Hu et al., 2009). Bucholz and colleagues identified a group of genes in the RNA polymerase II-associated factor 1 complex (Paf1C). Elledge and colleagues identified *Cnot3*, which was later shown to promote the maintenance of pluripotency by driving the deadenylation and degradation of differentiation associated transcripts (Zheng et al., 2016). The results from the two genome-scale RNAi screens (207 hits from Ding et al. and 128 from Hu et al.) have only 13 overlapping genes. While some differences can be explained by the screening strategy, some hits may be false positives, which highlights the need for hit validation. The lack of significant overlap also suggests there were likely false negatives, which is an intrinsic challenge for any genome-wide screen. Therefore, it remains valuable to perform screens using smaller, focused libraries. Such screens require less material and effort, and may offer more precise screening results as exemplified in two focused mESC screens (D. F. Lee et al., 2012; Q. Wang et al., 2017). Lemischka and colleagues performed a shRNA screen for 104 genes involved in the regulation of protein phosphorylation (D. F. Lee et al., 2012). The screen led to the identification of aurora kinase A (Aurka), the depletion of which causes loss of pluripotency and cell differentiation. Further study clarified that Aurka phosphorylates and suppresses Trp53, which was later shown to play a role in promoting mesendoderm differentiation (Q.

Wang et al., 2017). Aifantis and colleagues focused on the roles of the ubiquitin-proteasome system (UPS) pathway in pluripotency maintenance and performed a siRNA screen targeting 640 genes of UPS pathway in a transgenic *Nanog-GFP* mESC line (Buckley et al., 2012). The screen identified opposing roles of the deubiquitinating enzyme Psm14 and the E3 ligase Fbxw7: Psm14 is essential for mESC self-renewal, whereas Fbxw7 promotes mESC differentiation.

Ng and colleagues reported the first genome-wide siRNA screen in hESCs (Chia et al., 2010). Using a transgenic *OCT4-GFP* line, they identified key proteins and protein complexes in controlling the hESC identity. For instance, the screen discovered a new component of the hESC transcription factor network, PRDM1, which acts through binding to pluripotency genes enhancers. Interestingly, *Prdm14* is required for pluripotency maintenance in naïve mESCs but not in primed mEpiSCs (Ma, Swigut, Valouev, Rada-Iglesias, & Wysocka, 2011; Yamaji et al., 2013). These findings highlight the differences between the mouse and human primed pluripotent states and underscore the necessity of performing such screens in hESCs.

Compared to mESCs, fewer RNAi screens have been conducted using hESCs, which may be due to the longer history of mESCs or the comparative ease of mESC culture (Evans & Kaufman, 1981; Martin, 1981; Thomson et al., 1998; Q.-L. Ying et al., 2008). However, new protocols for naïve and primed culture, some of which are feeder and even-serum free, have made hESCs easier to culture and accelerated hESC growth rates and survival upon single cell passaging (Amit et al., 2004; Braam et al., 2008; G. Chen et al., 2011; Watanabe et al., 2007). The increased ease of manipulation and defined growth conditions will likely facilitate future screening with hPSCs. Indeed, there are already a good number of CRISPR screens performed in hPSCs (Genga et al., 2019; Ihry et al., 2019; Q. V. Li et al., 2019; Mair et al., 2019; Yilmaz, Peretz, Aharony, Sagi, & Benvenisty, 2018), which will be discussed later.

CRISPR screens to interrogate the maintenance of pluripotency

With the emergence of the CRISPR/Cas9 genome editing tools, it is now possible to perform pooled CRISPR screens using CRISPR libraries. For instance, Zhang and colleagues have applied lentiviral CRISPR libraries to identify essential genes in human cells including hPSCs (Shalem, Sanjana, & Zhang, 2015). More recently several groups have conducted screens to define the hESC “essentialome,” genes required for hESC self-renewal (Ihry et al., 2019; Mair et al., 2019; Yilmaz et al., 2018). Benvenisty and colleagues performed a screen in haploid hESCs (Yilmaz et al., 2018). In addition to many essential genes shared by cancer and immortalized cell lines (Blomen et al., 2015; Hart et al., 2015; T. Wang et al., 2015), they discovered 352 hits unique in haploid hESCs. In particular, there was a notable enrichment of genes in the mTOR pathway, suggesting that haploid hESCs are more sensitive to growth regulation by the mTOR pathway compared to somatic cells. In addition, they found genes in the Trp53 pathway, leading them to hypothesize that mTOR could function through Trp53. Moffat and colleagues and Kaykas and colleagues expanded on these findings by performing CRISPR screens on hESCs grown in multiple conditions: comparing hESCs grown on feeders vs. laminin (Mair et al., 2019) and with and without

Rho-associated protein kinase (ROCK) inhibitor (Ihry et al., 2019) to further define gene networks essential for hESC expansion, including those that confer resistance to single-cell passaging. These screens not only help define the gene requirements for the survival and proliferation of hESCs, but also provide the basis for practically improving hESC survival in a variety of culture conditions.

As with RNAi screens, future screens can be improved by integrating pluripotency reporters (such as *OCT4* or *NANOG* reporters) to facilitate the identification of genes required for the maintenance of the pluripotent state independent of cell growth phenotypes. Das and colleagues recently performed one such screen targeting known epigenetic regulators using an *Oct4-GFP* reporter in mESCs (Seruggia et al., 2019). They identified histone-acetyltransferase complex members Taf5L and Taf6L that act largely through the Myc regulatory network to maintain pluripotency.

Screens to interrogate the exit of pluripotency and differentiation

RNAi screens to interrogate the exit of pluripotency and differentiation

Although it is important to untangle the mechanisms of pluripotency maintenance especially for understanding the nature of pluripotency, the natural fate of a pluripotent cell in the developing embryo is not to infinitely self-renew but to differentiate into somatic lineages when exposed to differentiation signaling cues. To study this complex process, ESCs can be cultured in conditions devoid of signaling molecules necessary for ESC self-renewal or stimulated with strong differentiation signals. To identify regulators of the exit of pluripotency, Paddison and colleagues performed a shRNA screen in mESCs targeting 312 chromatin related genes (Schaniel et al., 2009). A transgenic *Nanog-GFP* reporter was used as the readout of mESC pluripotency due to the rapid downregulation of *Nanog* expression after LIF withdrawal and retinoic acid induced neural differentiation. A collection of BRG1-associated factor (BAF) complex (also known as the SWI/SNF complex) genes (*Brg1*, *Baf47*, *Baf155*, and *Baf57*) were identified as repressors of pluripotency exit, as mESCs with these genes downregulated retained a higher level of *Nanog-GFP* expression after retinoic acid treatment compared to wild-type controls. However, other studies have described BAF complex genes (*Brg1*) as promoting pluripotency maintenance and self-renewal (Ho et al., 2009; Kidder, Palmer, & Knott, 2009). siRNA or shRNA knockdown of *Brg1* in mESCs showed typical loss of pluripotency phenotypes (such as downregulation of *Oct4* and *Nanog*, and upregulation of *Gata6*). In addition, siRNA knockdown of *BRG1* in hESCs showed upregulation of mesendodermal lineage genes without immediate downregulation of *OCT4* and *NANOG* (Zhang et al., 2014). The BAF complex is a highly regulated chromatin-remodeling complex that alters DNA-nucleosome structure and controls gene transcription. Each subunit within the BAF complex may have distinct functions depending of the exact composition of BAF complex (Mathur & Roberts, 2018). In order to fully understand the function of each BAF subunit in ESC, a proper knockout and a defined cell culture condition can help better define the function of individual BAF complex genes at different stages of pluripotency and differentiation.

A similar shRNA screen was performed in a transgenic *Nanog-GFP* mESC line (Gingold et al., 2014) to identify factors involved with regulating *Nanog* expression during exit of

pluripotency induced by LIF withdraw and retinoic acid treatment. This screen was unique in the ability to identify both positive and negative regulators of the exit of pluripotency from a single experiment relying on the turnover rate of *Nanog-GFP*. Using the normal rate of *Nanog-GFP* downregulation in response to retinoic acid treatment as a baseline, Wang and colleagues showed that shRNA knockdown of mesenchymal transcription factor *Snai1* led to an accelerated downregulation of *Nanog* expression in response to retinoic acid treatment (Gingold et al., 2014). Interestingly however, shRNA knockdown of a related gene *Snai2* (also known as *Slug*) led to a delayed loss of *Nanog* gene expression during retinoic acid-induced exit of pluripotency. The important role of *Snai1* in inhibiting pluripotency exit is consistent with the finding that *Snai1* null mice are embryonic lethal with gastrulation defects (D. E. Smith, Franco del Amo, & Gridley, 1992). However, the role of *Snai2* in promoting pluripotency exit is not reflected *in vivo*, as *Snai2* null mice are viable and fertile (Jiang, Lan, Norton, Sundberg, & Gridley, 1998). This discrepancy between these *in vivo* and *in vitro* findings may be due to the transient nature of pluripotency *in vivo*, or potential compensatory mechanisms that overcome the downregulation of *Snai2* *in vivo* but not *in vitro*.

hESC maintenance is dependent on FGF2 and TGF β signaling, which is distinct from the JAK/STAT and BMP signaling that maintains mESC pluripotency. The difference in signaling requirements suggests that the routes of pluripotency exit and lineage specification may also be different between mouse and human development. Ng and colleagues performed a siRNA screen in a transgenic *NANOG-GFP* hESC reporter line to investigate different routes of exit of pluripotency using five conditions to perturb hESC signaling (withdrawal of FGF2/TGF β , TGF β inhibition, MEK inhibition, PI3K inhibition, and retinoic acid treatment) (Gonzales et al., 2015). These screens revealed a number of context-dependent pathways during exit of pluripotency, including negative regulators of signaling pathway such as *TGIF* in the TGF β inhibition condition and *DUSP6* in the MEK inhibition condition. As TGF β inhibitor and MEK inhibitor treatment are strong inducers for the dissolution of pluripotency, negative regulators of these pathways were easily identified from the screen. Genes involved with DNA replication during S phase or G2/M transition also stood out in multiple contexts. Depletion of DNA replication genes or G2 phase progression genes, or treatment with S/G2 phase cell cycle inhibitors induced hESCs to extend S and G2 phases and delay the exit of pluripotency after the withdrawal of FGF2 and TGF β signaling. In turn, extension of S and G2 phases activates ATM/ATR mediated checkpoint signaling pathway to upregulate TRP53 activity and TGF β signaling, which consequently delayed the dissolution of pluripotency. By examining the exit of pluripotency in multiple contexts, this study demonstrated the role of S/G2 cell cycle progression in the regulation of pluripotency and differentiation.

Transposon and CRISPR screens to interrogate the exit of pluripotency and differentiation

In order to identify genes influencing the dissolution of pluripotency that may have been overlooked in previous screens due to insufficient suppression by RNAi, Smith and colleagues employed transposon mutagenesis in haploid mESCs containing the naïve pluripotency reporter *Rex1-GFP* (Leeb, Dietmann, Paramor, Niwa, & Smith, 2014). They removed the pluripotency sustaining 2i inhibitors and LIF for 7 to 10 days, flow sorted or

clonally picked cells based on *Rex1-GFP* expression, and then expanded these cells by replating them in 2i/LIF. Importantly, given the random nature of transposon directed mutagenesis, Smith and colleagues repeated their screen 5 times to inform significance of hits. They found a large number of genes known to promote mESC differentiation as well as hits not previously identified in RNAi screens. One such hit was *Prkci*, which was missed in previous RNAi screens likely due to insufficient knockdown, which underscores the utility of using a knockout approach to uncover genes missed by previous RNAi screens.

In a genome-wide CRISPR screen Yusa and colleagues interrogated the dissolution of pluripotency in mESCs cultured in Serum/LIF and the neural-basal medium NDiff227, which approximates the early stages of neural differentiation (they did not however include growth factors or small molecule inhibitors used in directed neuroectoderm differentiation protocols) (M. Li et al., 2018). By sorting cells based on *Rex1-GFP* expression, they identified genes that impeded or accelerated the exit from naïve pluripotency. As expected, they observed a positive correlation between these screening conditions: hits that increased the GFP+ fraction in Serum/LIF conditions showed higher retention of GFP under differentiation conditions. On the other hand, many hits that increased the GFP+ fraction under differentiation conditions did not influence *Rex1-GFP* expression or heterogeneity in the Serum/LIF condition. These findings suggest that there are distinct and overlapping gene networks controlling the dissolution and the maintenance of pluripotency (M. Li et al., 2018).

The above screens all used retinoic acid treatment and/or withdrawal of self-renewing signals as a general strategy to induce the exit of pluripotency and differentiation, but they do not fully recapitulate the signaling process by which cells exit pluripotency *in vivo*. To better appreciate the orchestration of pluripotency exit and lineage-specific differentiation, it would be necessary to conduct screens in lineage-specific differentiation conditions, as differentiation of definitive endoderm, mesoderm, and ectoderm require activation/inhibition of distinct signaling pathways (Keller, 2005; Murry & Keller, 2008). Examining the dissolution of pluripotency in the context of current directed differentiation protocols for ectoderm, endoderm, and mesoderm may allow us to better understand the processes by which pluripotency is dissolved during early embryonic development in each embryonic lineages. In this context, we recently conducted a genome-wide CRISPR screen to identify genes that regulate the differentiation of hPSCs to definitive endoderm (Q. V. Li et al., 2019). This screen identified known DE differentiation genes in the Nodal (*ACVR1B*, *SMAD2*, *SMAD4*) and Wnt signaling pathways (*CTNNB1*) and endoderm transcription factors (*EOMES*, *FOXH1*, *MIXL1*). In addition, five genes in the JNK signaling pathway (*MEKK1*, *MKK7*, *MKK4*, *JNK1* and *JUN*) were found to inhibit DE differentiation. Interestingly, the AP-1 transcription factor JUN does not act through directly inhibiting the endoderm enhancers. Instead JUN co-occupies ESC enhancers with OCT4, NANOG and SMAD2/3, and specifically inhibits the exit from the pluripotent state, and safeguards pluripotency from precocious DE differentiation. While this screen focused on the regulation of endoderm differentiation, comparison to screens specifically probing the regulation of the pluripotency network in the same differentiation conditions could in future identify distinct pathways that influence the dissolution of pluripotency versus the entrance into lineage differentiation. Furthermore, comparison between screens interrogating the differentiation of

ESCs to other lineages, such as primordial germ cells (Hackett et al., 2018), could further distinguish factors that regulate pluripotency dissolution in general versus those that act in a lineage-specific manner.

Screens to interrogate alternative pluripotent states

Transition between different pluripotent states

It has become increasingly clear that pluripotency encompasses more than a single developmental state. mESCs, derived from the pre-implantation mouse epiblast, and mEpiSCs, derived from the post-implantation mouse epiblast, respectively typify two pluripotent states termed naïve and primed. These two pluripotent states have distinct transcriptional networks, epigenomic states, and signaling requirements (Nichols & Smith, 2009). While naïve mESCs can be maintained in a self-renewing state by the LIF and BMP pathways, primed mEpiSCs, which represent a later developmental time point, are dependent on FGF and Activin (Brons et al., 2007; Tesar et al., 2007). Naïve mESCs can be further stabilized and homogenized with the 2i/LIF protocol using ERK and GSK inhibitors together with LIF to generate a “ground-state” of naïve pluripotency (Q.-L. Ying et al., 2008). Recent work has posited yet another state of pluripotency, “formative pluripotency”, as a transitional state between naïve and primed (Kalkan et al., 2017; A. Smith, 2017). It is unclear whether naïve cells must necessarily pass through the formative or primed state in the process of lineage differentiation. The discovery of multiple distinct pluripotent states suggests that pluripotency exists on a continuum in development and that other distinct states of stable pluripotency may yet be discovered.

hESCs resemble primed mEpiSCs more than naïve mESCs, as they too require FGF and TGF β /Activin signaling and cannot be derived in mESC growth conditions (Thomson et al., 1998). As hESCs are more readily derived from very late human pre-implantation blastocysts (A. E. Chen et al., 2009), it is possible that the cells of this late ICM are similar to the post-implantation epiblast of the mouse, particularly given the comparatively delayed human implantation schedule (Rossant, 2015). There is great interest in the generation of naïve hESCs, which may offer a model of an earlier developmental time point than primed cells. The 2i/LIF protocol forms the basis for attempts to recapitulate the mouse naïve protocol with hESCs due to the stability and homogeneity of the ground state of naïve pluripotency. Indeed the 2i/LIF protocol has enabled the generation of ESC lines from previously non-permissive mouse strains (Q.-L. Ying et al., 2008) and even the rat (Buehr et al., 2008; P. Li et al., 2008). However, the 2i/LIF cocktail alone cannot achieve a naïve human ground-state solely dependent on intrinsic signaling, and requires the addition of growth factors such as FGF2 and TGF β (Chan et al., 2013; Duggal et al., 2015; Gafni et al., 2013; Theunissen et al., 2014; Ware et al., 2014) or overexpression of transgenes such as *OCT4*, *SOX2*, and *KLF4* (Hanna et al., 2010), *NANOG* and *KLF4* (Takashima et al., 2015), activated *STAT3* (H. Chen et al., 2015), or *YAP* (Qin et al., 2016). Each of these protocols may represent a different route to establish naïve pluripotency, or a unifying protocol optimal for the achievement of human ground-state naïve pluripotency is yet to be discovered. Given the intrinsic differences between the mouse and human, some features of naïve pluripotency identified in mouse may not apply to human cells. For example, high

levels of MEK inhibition lead to genomic instability in naïve hESCs but not in naïve mESCs (Di Stefano et al., 2018).

RNAi screens to interrogate naïve and primed pluripotency

As of yet, no genetic screens have been performed to address the exit from naïve pluripotency or the developmental transition between the naïve and primed pluripotent state in human. Therefore, we discuss RNAi (here) and CRISPR (next subsection) screens performed to date on naïve versus primed pluripotency in mouse. A number of genetic screens have investigated the exit of naïve pluripotency in mice by taking advantage of the 2i/LIF protocol's ability to achieve the ground-state naïve pluripotency. Sharrocks and colleagues performed a genome-wide RNAi screen for the exit from naïve pluripotency utilizing mESCs with a naïve pluripotency reporter *Rex1-GFP* (Yang et al., 2012). They identified over 400 genes involved in the exit of naïve pluripotency by screening for delay in *Rex1-GFP* loss after 2i withdrawal. They also performed a secondary screen testing for a delay in *Rex1-GFP* loss in response to removal of only one of the 2i (either ERK or GSK inhibitor), allowing for the categorization of hits into mediators and regulators of the MAPK/ERK or GSK pathways. Smith and colleagues also performed an siRNA screen in mESCs for genes that resist pluripotency exit after 2i withdrawal (Betschinger et al., 2013). The tumor-suppressor Folliculin was found to be involved in the dissolution of naïve pluripotency by restricting nuclear localization of the transcription factor Tfe3, which in turn regulates pluripotent transcription factor Esrrb. Compellingly, they found that Tfe3 translocated to the cytoplasm upon mESC differentiation, and became cytoplasmic in the primed post-implantation state both *in vitro* (in mEpiSCs) and *in vivo* (in e5.5 epiblast cells). Thus the genetic screen performed on *in vitro* mESCs revealed a previously unknown developmental regulation specific to the transition between naïve and primed pluripotency.

To distinguish regulators specific to naïve and primed pluripotency, Hanna and colleagues conducted a mini-RNAi screen comparing *Oct4-GFP* downregulation in naïve mESCs and primed mEpiSCs with a small siRNA library of genes previously implicated in the regulation of naïve pluripotency (Geula et al., 2015). They were able to categorize genes into subsets: those that were important to the maintenance of both naïve and primed pluripotency, such as *Sox2*, *Oct4*, and *Wrd5*, previously shown to interact with Oct4 (Ang et al., 2011), and those that were specific to the regulation of the maintenance of pluripotency in only the naïve or primed context. Knockdowns that destabilized *Oct4-GFP* in primed mEpiSCs but not naïve mESCs included the epigenetic repressors *Dnmt1* and Polycomb-complex members *Eed* and *Suz12* as well as members of the N6-methyladenosine (m6A) mRNA methylating complex *Mettl3* and *Mettl14*. *Mettl3*^{-/-} mESCs failed to exit naïve pluripotency in culture, nor could they form teratomas. These findings are consistent with mouse knockout phenotypes: *Mettl3*^{-/-} embryos are grossly normal at E3.5, and become deformed and fail to downregulate pluripotency factors post-implantation (E5.5–7.5) (Geula et al., 2015) METTL3 has subsequently been shown to be necessary for the differentiation of hESCs specifically to neuroectoderm (Bertero et al., 2018).

CRISPR screening that informs the naïve to primed transition

It is worth noting that the RNAi screens described above did not directly assay the exit of the naïve state into the primed state of pluripotency. Rather, they interrogated the dissolution of naïve or primed pluripotency. It is unclear whether naïve pluripotent cells must pass through the primed pluripotent state in order to differentiate, therefore the dissolution of naïve pluripotency is an imperfect proxy for the primed state. To directly interrogate the transition from the naïve to the primed pluripotent state would require reporter assays that also examine the establishment of the latter. For instance, *OCT4* expression in the naïve or primed pluripotent state in mouse and human is driven by the distal or proximal enhancer, respectively (Gafni et al., 2013; Tesar et al., 2007; Yeom et al., 1996). This trait may be utilized to evaluate the naïve to primed pluripotency transition.

A recent CRISPR screen by Surani and colleagues offers a powerful system for interrogating the transition between the naïve and primed pluripotent state using a multi-reporter system of *Stella-GFP* and *Esg1-tdTomato*. While *Stella* is expressed in the mouse pre-implantation epiblast it is not expressed post-implantation except in primordial germ cells (PGCs), whereas *Esg1* maintains high expression post-implantation until the dissolution of the pluripotent state. Using this “traffic light” system, naïve pluripotent stem cells are double positive (yellow), post-implantation epiblast cells are *Esg1-tdTomato* positive (red), and differentiated PGCs are *Stella-GFP* positive (green) (Hackett et al., 2018). Assaying for loss of *Stella* expression by flow cytometry, Surani and colleagues identified a large number of hits that failed to downregulate naïve pluripotency, as well as 21 candidate genes with potential intrinsic roles in the dissolution of naïve pluripotency including *Tcf3*, *Zfp281*, *Dnmt1*, and *Rest* which all have a known role in developmental dissolution of pluripotency and promotion of early differentiation (Fidalgo et al., 2011; Jackson et al., 2004; Martello et al., 2012; Yamada, Aoki, Kunisada, & Hara, 2010). Mutation of novel candidates *Zmym2*, *FoxP1*, and *Uchl5* caused impaired Epiblast-like cell (EpiLC) formation, *Stella* was not down regulated and primed markers *Fgf5*, *Dnmt3b*, and *Otx2* were not upregulated despite prolonged culture in FGF and Activin containing mEpiSC medium (Hackett et al., 2018). Similarly, while Yusa and colleagues performed the screens on mESCs using the naïve pluripotency reporter Rex1-GFP (and without a specific primed or formative pluripotency reporter), they were able to show that knockout of these MTORC1 regulators *Nprl2* and *Tsc2* caused an upregulation in formative pluripotent markers or naïve pluripotent markers respectively (M. Li et al., 2018).

In addition to the transition between the naïve and primed pluripotent state, comparisons have been made between genes necessary for the maintenance of pluripotency in Serum/LIF versus 2i/LIF conditions (Hackett et al., 2018; M. Li et al., 2018). Some genes, such as *Sall4*, are essential for survival and proliferation in Serum/LIF but not in 2i/LIF, whereas the mutation of some other genes such as *Tfcp2l1*, *Nprl2*, *Nprl3*, *Depdc5*, had a much more profound effect on proliferation and survival in 2i/LIF than in Serum/LIF (M. Li et al., 2018). Similarly, Surani and colleagues found that 38% of their hits were essential for the maintenance of pluripotency in only one of these culture conditions (Hackett et al., 2018). Both studies found that genes involved in metabolic and biosynthetic processes, such as *Ogdh* and *Dlst*, two enzymes that mediate α -ketoglutarate metabolism, were required for

2i/LIF maintenance but had little effect on Serum/LIF mESC proliferation or survival. These findings emphasize that there are notable distinctions even between the closely related 2i/LIF and Serum/LIF pluripotent states; the differences between the naïve and primed states are likely even greater.

Screens to interrogate the acquisition of pluripotency

The acquisition of pluripotency

The discovery by Yamanaka and colleagues of a way to induce pluripotency in differentiated somatic cells by the overexpression of transcription factors KLF4, MYC, OCT4, and SOX2 (Takahashi et al., 2007; Takahashi & Yamanaka, 2006) has opened up numerous opportunities for the study of development and the treatment of disease. However, reprogramming by transcription factor overexpression was initially inefficient and slow in comparison to reprogramming through somatic cell nuclear transfer (Theunissen & Jaenisch, 2014). Therefore, there was interest in genetic screens to increase the efficiency of reprogramming to enhance the utility of reprogramming in regenerative medicine. Furthermore, such screens could provide insights into the mechanics of pluripotency acquisition. Broadly, such studies may also inform us how cancer cells become malignant due to the similarities between the two processes.

RNAi screens for the acquisition of pluripotency

To identify genetic roadblocks to pluripotency acquisition, RNAi screens have been performed using focused RNAi libraries targeting chromatin factors and kinases (Cheloufi et al., 2015; Sakurai et al., 2014). Ramahlo-Santos and colleagues performed a genome-wide shRNA screen on human foreskin fibroblasts (Qin et al., 2014). 600,000 shRNAs were co-transfected with mRNA expressing the reprogramming factors and RNAi targeting *TRP53*, and after 28 days, cells with pluripotency cell-surface marker TRA-1–81 were enriched for analyses. They identified genes involved in ubiquitination, endocytosis, cellular adhesion, and vesicular; and using a combination of small molecules and shRNAs to inhibit these processes, they were able to improve re-programming efficiency by up to 15-fold.

Concurrently, Rana and colleagues carried out a screen using shRNAs to influence reprogramming efficiency from mouse fibroblasts. They utilized flow cytometry sorting to separate cells at various stages of reprogramming (C. S. Yang, Chang, & Rana, 2014). This strategy allowed them to identify separate gene networks important in the: initiation of reprogramming, transition from the somatic state, early acquisition of pluripotency, and completion of reprogramming. The distinct genetic requirements for reaching each stage of reprogramming supports the hypothesis that acquisition of pluripotency requires both the dissolution of the somatic cell transcriptional network and the acquisition of pluripotency, and that these are distinct events (Brambrink et al., 2008; Samavarchi-Tehrani et al., 2010; Stadtfeld, Maherali, Breault, & Hochedlinger, 2008). To investigate the contribution of various genes to human cell reprogramming in a stage specific manner, Loh and colleagues utilized an arrayed siRNA library on human foreskin fibroblasts in a 384-well based system (Toh et al., 2016). They successfully identified five repressors of reprogramming, *SMAD3*, *SMYD2*, *SFRS11*, *SAE1*, and *ESET*. The combinatorial knockdown of these repressors

increased reprogramming efficiency and resulted in ~85% TRA-1-60- positive cells. Notably, more than half of the genes identified in these reprogramming screens did not change expression levels during reprogramming (Toh et al., 2016; C. S. Yang et al., 2014), which emphasizes that genetic studies should not be guided solely by transcriptome profiling.

To identify strong regulators of reprogramming that would not need to be used combinatorically, Hochedlinger and colleagues performed a genome-wide RNAi screen on mouse embryonic fibroblasts undergoing reprogramming (Borkent et al., 2016). Using a serial shRNA enrichment strategy, they found that the ubiquitin-like protein modifier SUMO2 was a strong inhibitor of the acquisition of pluripotency, and that *SUMO2* knock-down reduced reprogramming time to as little as 38 hours. Moving beyond simple readouts based on a small number of pluripotency markers, Gil and colleagues combined shRNA screening with single-cell RNA sequencing (scRNA-seq) to interrogate the reprogramming of human lung fibroblasts (Aarts et al., 2017), shedding light on the dual role of mTOR in the acquisition of pluripotency. Inhibition of mTOR blunted the induction of cyclin-dependent kinase inhibitors, leading to more efficient reprogramming due to decreased senescence, a known barrier to reprogramming (Banito et al., 2009; Krizhanovsky & Lowe, 2009). However, inhibition of mTOR also blunted the senescence-associated secretory phenotype (SASP). As different SASP components may favor or inhibit reprogramming, the exact roles of mTOR in reprogramming await further investigation.

CRISPR-based tools for understanding reprogramming

No CRISPR knockout screens have been performed thus far to identify inhibitors or accelerators of iPSC reprogramming. Based on the successful reprogramming of human fibroblasts through CRISPRa-mediated activation of *OCT4*, *KLF4*, *SOX2*, and *MYC* (Weltner et al., 2018), a genome-scale CRISPRa screen was performed to identify factors capable of reprogramming primed mEpiSCs to a naïve mESC-like state (J. Yang et al., 2019). Induced expression of some hits identified by this screen, most notably *Sal11*, could also increase the efficiency of reprogramming from mouse embryonic fibroblasts to iPSCs. It is foreseeable that CRISPR based loss and gain-of-function screening will continue to inform us about the acquisition of pluripotency in future studies.

Interrogation of the non-coding genome

RNAi screening has been a dominant research tool in functional genomics in the past decade. While RNAi is useful for interrogating protein coding genes, ~98% of the human genome contains important non-coding *cis* and *trans* regulatory sequences (Stower, 2012). Transcription factors and chromatin regulators are targeted to these non-coding regulatory elements to regulate pluripotency and differentiation. CRISPR/Cas9 can be used to directly disrupt the sequences of non-coding regulatory elements, which makes it an excellent tool to investigate the non-coding regions of the functional genome (Shukla & Huangfu, 2018).

CRISPR-based discovery of functional enhancer elements

Large-scale enhancer identification was traditionally driven by comparative genomics (Visel, Bristow, & Pennacchio, 2007), or transgenic reporter assays such as STARR-seq (Arnold et al., 2013). However, functional validation of these predicted enhancers would require analyzing the phenotypes of enhancer knockout at the endogenous locus. CRISPR/Cas tools have made it feasible to interrogate putative enhancers in a high-throughput fashion. One strategy is to use a tiling array of single gRNAs to create indel mutations at the upstream, intronic and downstream regions of a gene. Ren and colleagues performed a CRISPR/Cas9 based screen using a knockin *OCT4-GFP*hESC reporter to interrogate putative *cis*-regulatory elements of the *OCT4* locus (Diao et al., 2016). They designed a CRISPR library with 1,984 gRNAs targeting 174 putative *cis*-regulatory elements within the 1 Mb *OCT4* locus based on previous published ChIP-seq and DNase-seq data (~11 gRNA per element). If an indel mutation generated by the CRISPR disrupts a functional *cis*-regulatory element, *OCT4-GFP* expression level would be reduced, thus the corresponding gRNA should be enriched in the GFP^{low} population. This screen successfully identified gRNAs targeting the well-characterized proximal *OCT4* enhancer and promoter (Hanna et al., 2010; Yeom et al., 1996). In addition, they also identified three DNase I hypersensitive sites (DHSs) in the *OCT4* locus as a new type of enhancers, the disruption of which leads to a temporary and reversible reduction of transcription. Another study, led by Sherwood and colleagues, used a tiling array of gRNAs to target across *cis*-regulatory genomic spaces adjacent to individual genes (including *Nanog*, *Rpp25*, *Tdgfl*, and *Zfp42*) in mESCs (Rajagopal et al., 2016). An important message from this screen is that certain functional enhancers are not associated with any known histone marks or transcription factor binding. This highlights the need of unbiased CRISPR screening, rather than focusing solely on regions associated with known enhancer chromatin features such as DHSs.

Another strategy to functionally discover non-coding elements is to use paired gRNAs to generate large deletions instead of using individual gRNAs to generate relatively small indel mutations. Ren and colleagues used this paired gRNA (11,570 pairs) approach to expand on their previous *OCT4* enhancer screen (Diao et al., 2017). The tiling deletion method scanned the whole 2 Mb region at the *OCT4* locus with deletions of an average size of ~2 kb and an overlap of ~1.9 kb between deletions, thus saturating each nucleotide in the locus by ~20 distinct genomic deletions. A surprisingly large number of the *cis*-regulatory sequences (17 out of 45 *cis*-regulatory elements identified) are also promoters of functionally unrelated neighboring genes, a feature known as enhancer-like promoters.

The current CRISPR enhancer screening strategies have their own unique advantages and limitations. The gRNA tiling array on putative enhancers is a great way to validate enhancers with precision, but it relies on previous epigenomic characterization in the cell type of interest and it could miss functional enhancers that are not associated with known enhancer chromatin features. The gRNA tiling array across the whole *cis*-regulatory region of a gene can offer a high resolution mapping of the functional elements and can identify enhancers that do not have the typical molecular features of an enhancer; however, it is challenging to scale up. The paired-gRNA screening strategy is suitable for mapping regulatory elements in a larger genomic window, however, the result from deletion of DNA segment reduces the

resolution of enhancer mapping and performing large-scale deletion screens remain technically difficult. Overall, the tremendous size of non-coding genome remains perhaps the biggest challenge for unbiased interrogation, which needs to be overcome through future innovations of CRISPR based tools and screening strategies.

Screening of non-coding RNAs in pluripotency regulation

Long noncoding RNAs (lncRNAs) and microRNAs (miRNAs) constitute another major branch of the non-coding genome. For lncRNAs, small indel mutations generated by CRISPR/Cas are often insufficient to fully disrupt their activities. Paired gRNA CRISPR deletion can be used for lncRNAs knockout screens (S. Zhu et al., 2016), but this method is not ideal as lncRNAs vary greatly in length and sometimes reside in coding regions. Lim and colleagues performed a CRISPRi based lncRNA screen in seven diverse cell lines (including one hiPSC line) to identify lncRNA required for cellular growth and survival (S. J. Liu et al., 2017). This screen identified cell type specific lncRNAs for cell fitness, highlighting the need to study lncRNAs in specific cellular contexts. Interestingly, only 9 out of 326 lncRNA hits affecting hiPSC cell growth played a role in maintaining *OCT4* expression, suggesting that the majority of the lncRNAs hits identified from this screen affected general cell fitness instead of regulating pluripotency. lncRNAs play important roles in the regulation of pluripotency and differentiation (Guttman et al., 2011). Therefore, future lncRNA screens could make use of similar screening strategies discussed for protein-coding genes to further the understanding of pluripotency regulation.

Around 2,000 miRNAs have been discovered in the human genome (Hammond, 2015). A few families of miRNAs play important roles in the regulation of pluripotency (Y. J. Lee et al., 2016; Leonardo, Schultheisz, Loring, & Laurent, 2012). One big hurdle towards understanding the exact roles of individual miRNAs is their overlapping functions. No CRISPR screen specifically targeting miRNA has been performed to interrogate pluripotency. Newly developed miRNA focused CRISPR libraries may offer a powerful way to uncover novel miRNAs that influence pluripotency and differentiation in future (Kurata & Lin, 2018). At the same time, one strategy to overcome the overlapping miRNA function is to reintroduce individual miRNAs into miRNA-deficient cells. For instance, miRNAs that influence pluripotency have been identified by reintroducing 266 miRNA mimics into mESCs lacking *Dgcr8*, which is responsible for the generation of precursor miRNAs (Y. Wang et al., 2008). *Dgcr8*^{-/-} mESCs have proliferation defects due to the prolonged G1 phase of the cell cycle. Reintroducing ESC-specific cell cycle regulating miRNAs (ESCC: miR-291a-3p, miR291b-3p, miR-294, miR-295 and miR-302) rescued these defects (Y. Wang et al., 2008). Similarly, our group has made inducible hESC knockout lines for *DICER1*, which is responsible for processing of precursor miRNA hairpins into their mature functional forms. *DICER1* is required for hESC self-renewal, and a targeted miRNA screening strategy has uncovered essential pro-survival roles of mir-302–367 and mir-371–373 family members (Teijeiro et al., 2018).

Emerging technologies and future directions

Expansion of CRISPR perturbation methods with dCas9

We have focused most of this review on how RNAi and CRISPR/Cas tools have enabled genetic screens on the regulation of pluripotency. It is notable that CRISPRa and CRISPRi based approaches could offer more precise regulation of gene expression that could be further explored in future screens. For instance, Qi and colleagues applied the CRISPRa approach in mESCs with 55,562 gRNAs targeting computationally predicted transcription factors and DNA-binding factors to identify genes driving neuronal differentiation (Y. Liu et al., 2018). 19 genes were validated as promoting neuronal differentiation and further studied using a paired gRNA library that co-activate two genes in the same cell. The CRISPRa approach has also been used to investigate the reprogramming of mEpiSCs to an ESC-like state (J. Yang et al., 2019). Metzakopian and colleagues found that a number of factors previously shown to have no effect on reprogramming using cDNA overexpression. They posit that this discrepancy may be due to differences in gene dosage between cDNA and CRISPRa. Importantly, gene dosage with CRISPRa can vary with gRNAs targeting adjacent sequences (Y. Liu et al., 2018). It is likely that improved methods will be developed in future to allow more precise up or downregulation of gene expression, which could be used in large-scale screens for a more comprehensive understanding of pluripotency and development.

CRISPR screening coupled with complex readouts

The CRISPR/Cas technology has numerous potential applications in the stem cell field. It has already proven useful for fast generation of knockin reporters to study pluripotency and differentiation (Roberts et al., 2017; Z. Zhu, Verma, González, Shi, & Huangfu, 2015). Reporters generated using these improved methods will greatly facilitate functional interrogation of the coding and non-coding genome. Overcoming the limitation of relying on single reporters, it is now possible to combine CRISPR screens with single-cell RNA sequencing (scRNA-seq) for comprehensive transcriptional readouts (Adamson et al., 2016; Dixit et al., 2016; Gasperini et al., 2018; Xie, Duan, Li, Zhou, & Hon, 2017). scRNA-seq has already been used to reveal the heterogeneous gene expression profile of mESCs in Serum/LIF (Kumar et al., 2014). CRISPR screening in combination with scRNA-seq will likely reveal novel factors and mechanisms of action underlying heterogeneity in ESCs. A recent screen for factors influencing chromatin accessibility during human definitive endoderm differentiation demonstrates the utility of combining CRISPRi screening with scRNA-seq (Genga et al., 2019). Maehr and colleagues performed this screen using a gRNA library targeting transcription factors implicated by ATAC-seq as potential initiators of endoderm differentiation. They found that *FOXA2* depletion did not impair definitive endoderm formation but affected the competence of endoderm cells to form endoderm-derived hepatic cells. Indeed, in our own independent work, we have found that *FOXA2*^{-/-} hESCs formed definitive endoderm normally, but failed to form endoderm-derived pancreatic progenitor cells efficiently due to the requirement of *FOXA2* in enhancer priming (K. Lee et al., in press). Screening with scRNA-seq allowed for the identification of hits based on a more complex readout rather than a single reporter; and instead of the typical binary “on-or-off” signal indicated by a reporter, it also enables more quantitative measures

of gene expression. However, the current technology does not yet support large-scale perturbation combined with scRNA-seq. Unlike the genome-wide screen performed by our group also interrogating the endoderm fate (Q. V. Li et al., 2019), the screen performed by Maehr and colleagues was only able to perturb 50 transcription factors (Genga et al., 2019), an aspect that will almost certainly improve in future. Other single-cell epigenomic methods are on the horizon, including scATAC-seq and scChIP-seq (Clark, Lee, Smallwood, Kelsey, & Reik, 2016). Incorporating these emerging methods can further expand our knowledge of pluripotency and differentiation, as well as provide tools to improve the use of hPSCs to study human development and treat disease.

Conclusions

The dynamic pluripotent states in an early developing embryo can be captured as “static snapshots” using an array of defined *in vitro* culture methods. While we have gained understanding of this dynamism through gene expression profiles of the early human embryo (Blakeley et al., 2015; Petropoulos et al., 2015; Yan et al., 2013), the transient nature of pluripotency *in vivo* has made it a particularly compelling subject for genetic screens *in vitro*. The ability to distinguish between, and interrogate direct transitions within, the pluripotent state, the exit from pluripotency, and the disorganized dissolution of the pluripotent state *in vitro* informs our understanding of this state *in vivo*. The recent development of protocols for the 3-D modeling of mouse and human embryogenesis *in vitro* (Beccari et al., 2018; Deglincerti et al., 2016; Harrison, Sozen, Christodoulou, Kyprianou, & Zernicka-goetz, 2017; Morgani, Metzger, Nichols, Siggia, & Hadjantonakis, 2018; Turner et al., 2017; Warmflash, Sorre, Etoc, Siggia, & Brivanlou, 2014) may in future be combined with CRISPR screening technology to interrogate influences on pluripotency, lineage decisions, and the breaking of axial symmetry in the early embryo. Though in this review we have focused on genetic screens in cells corresponding to early developmental stages, these techniques can be applied to the study of later stages of development. While the screens discussed were performed in wild-type cells, with the increasing flexibility and ease of CRISPR directed mutagenesis and large-scale screening, future screens may be performed in disease-sensitized backgrounds, such as hESC lines with engineered patient mutations or patient iPSC lines paired with isogenic controls. Such screens will allow us to understand complex disease phenotypes at varying stages of development.

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Figure 1: Pluripotency and its regulation.

Pluripotent stem cells have both the ability to self-renew to maintain the pluripotent state and the potential to differentiate to all three somatic lineages (endoderm, mesoderm and ectoderm). During lineage differentiation, pluripotent stem cells undergo the process of exiting pluripotency in which they transition identity from pluripotent stem cells to a particular somatic cell lineage. Differentiated somatic cells can acquire pluripotency through the process of reprogramming by overexpression of pluripotency inducing factors. The state of pluripotency itself exists on a spectrum encompassing the naïve and primed states, which utilize different mechanisms to achieve self-renewal and pluripotency maintenance.

Figure 2: Schematic of arrayed and pooled genetic screens.

Most arrayed screens use a reverse transfection/infection approach. Synthetic siRNA, gRNA or lentiviral particles are pre-distributed in 96/384-well plates. After reagent reconstitution according to manufacturer's instruction, cells are distributed to each well by using an automatic liquid dispensing instrument and mixed with RNAi and gRNA reagents. A forward transfection/infection approach can be used as an alternative solution if cells need to be pre-seeded one day earlier before transfection/infection. After the initial transfection/infection step, cells are cultured with media to promote normal cell growth, drug selection or differentiation. The cellular phenotype of interest (overgrowth, death, differentiation) can be evaluated by high-content imaging based on the quantitative signals from of reporter cell line (GFP) or immunostaining. In a typical pooled screen, a lentivirus library is first prepared to express an array of shRNAs or CRISPR gRNAs. With a low multiplicity of infection, most of the infected cells will carry one shRNA or gRNA integration. The infected cells will then be subject to positive selection, negative cell selection, or marker selection. For positive and negative cell selection, the target population is the population that survives the selection, and the control population can be the infected cells either before selection or cultured in parallel without selection. For marker selection, the target and control populations can be the population with or without the marker expression, respectively. Deep sequencing and sequence deconvolution will be performed to quantify the degree of enrichment or depletion of each shRNA or gRNA in the target population compared to the control population.

Table 1.

A summary of screens conducted to interrogate the acquisition, maintenance and exit of pluripotency.

Phase of Pluripotency	Starting cell type	Perturbation method	Scale	Screen Format	Phenotypic selection	Reference
acquisition	human foreskin fibroblast	RNAi	Genome-wide, 600,000 shRNAs	Pooled	TRA-1–81	Qin 2014
acquisition	mouse embryonic fibroblast	RNAi	734 kinase genes, 3,686 shRNAs	Arrayed	<i>Oct4-GFP</i>	Sakurai 2014
acquisition	mouse embryonic fibroblast	RNAi	Genome-wide, 57,000 shRNAs	Pooled	Thy1, SSEA-1, <i>pMX-DsRed</i>	Yang 2014
acquisition	mouse embryonic fibroblast	RNAi	615 chromatin regulators, 5,049 shRNAs	Pooled	<i>Oct4-GFP</i>	Cheloufi 2015
acquisition	human foreskin fibroblast	RNAi	Genome-wide, 21,121 siRNAs	Arrayed	TRA-1–60	Toh 2016
acquisition	mouse embryonic fibroblast	RNAi	Genome-wide, 60,642 shRNAs	Pooled	<i>Oct4-GFP</i>	Borkent 2016
acquisition	human lung fibroblast	RNAi	554 candidates from an shRNA screen, 3,154 shRNAs	Pooled	scRNA-Seq	Aarts 2017
acquisition	mEpiSC	CRISPRa	Genome-wide, 87,863 gRNAs	Pooled	<i>Oct4-GFP</i>	Yang 2019
maintenance	mESC	RNAi	70 genes downregulated upon RA differentiation, 110 shRNAs	Arrayed	cell growth	Ivanova 2006
maintenance	mESC	RNAi	1,008 chromatin proteins, esiRNAs	Arrayed	morphology	Fazio 2008
maintenance	mESC	RNAi	Genome-wide, 25,057 esiRNAs	Arrayed	<i>Oct4-GFP</i>	Ding 2009
maintenance	mESC	RNAi	Genome-wide, 16,683 siRNAs	Arrayed	<i>Oct4-GFP</i>	Hu 2009
maintenance	hESC	RNAi	Genome-wide, 84,484 siRNAs	Arrayed	<i>OCT4-GFP</i>	Chia 2010
maintenance	mESC	RNAi	104 protein phosphorylation genes, 181 shRNAs	Arrayed	cell growth	Lee 2012
maintenance	mESC	RNAi	640 ubiquitin proteasome genes, 2,560 siRNAs	Arrayed	<i>Nanog-GFP</i>	Buckley 2012
maintenance	hESC	CRISPR/Cas9	Genome-wide, 64,751 gRNAs	Pooled	cell growth	Shalem 2014
maintenance	hiPSC	CRISPRi	16,401 lncRNA loci, 164,010 gRNAs	Pooled	cell growth	Liu 2017
maintenance	haploid hESC	CRISPR/Cas9	Genome-wide, 18,1660 gRNAs	Pooled	cell growth	Yilmaz 2018
maintenance	hESC	CRISPR/Cas9	Genome-wide, 70,948 gRNAs	Pooled	cell growth	Mair 2019
maintenance	hESC	CRISPR/Cas9	Genome-wide, 91,726 gRNAs	Pooled	cell growth, OCT4	Ihry 2019
maintenance	mESC	CRISPR/Cas9	323 epigenetic regulators and	Pooled	<i>Oct4-GFP</i>	Seruggia 2019

Phase of Pluripotency	Starting cell type	Perturbation method	Scale	Screen Format	Phenotypic selection	Reference
			transcription factors, 2,335 sgRNAs			
maintenance, exit	mESC	CRISPR/Cas9	Genome-wide, 90,230 gRNAs	Pooled	<i>Rex1-GFP</i>	Li 2018
Exit	mESC	RNAi	321 genes involved in chromatin regulation, 642 shRNAs	Pooled	<i>Nanog-GFP</i>	Schanuel 2009
Exit	mESC	RNAi	Genome-wide, siRNAs	Pooled	<i>Rex1-GFP</i>	Yang 2012
Exit	mESC	RNAi	9,900 genes, 39,600 siRNAs	Arrayed	<i>Oct4-GFP</i>	Betschinger 2013
Exit	haploid mESC	Transposon	Piggy-Bac in haploid cells	Pooled	<i>Rex1-GFP</i>	Leeb 2014
Exit	hESC	RNAi	13,764 siRNAs	Arrayed	<i>NANOG-GFP</i>	Gonzales 2015
exit	mESC, mEpiSC	RNAi	31 genes with role in naïve pluripotency, siRNAs	Pooled	<i>Oct4-GFP</i>	Geula 2015
exit, differentiation	mESC	CRISPR/Cas9	Genome-wide, 87,897 gRNAs	Pooled	<i>Stella-GFP, Esg1-tdTomato</i>	Hackett 2018
exit, differentiation	hESC	CRISPR/Cas9	Genome-wide, 123,411 gRNAs	Pooled	<i>SOX17-GFP</i>	Li 2019
exit, differentiation	hESC	CRISPRi	50 candidate transcription factors, 160 gRNAs	Pooled	scRNA-Seq	Genga 2019

The perturbation methods are color coded. Screens conducted on human cells are shown in shaded rows to distinguish from those performed on mouse cells.