Cell Stem Cell
Review

Defining Human Pluripotency

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Human pluripotent stem cells harbor the capacity to differentiate into cells from the three embryonic germ layers, and this ability grants them a central role in modeling human disorders and in the field of regenerative medicine. Here, we review pluripotency in human cells with respect to four different aspects: (1) embryonic development, (2) transcriptomes of pluripotent cell stages, (3) genes and pathways that reprogram somatic cells into pluripotent stem cells, and finally (4) the recent identification of the human pluripotent stem cell essentialome. These four aspects of pluripotency collectively culminate in a broader understanding of what makes a cell pluripotent.

Cellular plasticity is best exemplified by pluripotency, the unique ability of a single cell type to convert into cells from the three embryonic germ layers (De Los Angeles et al., 2015). Remarkably, pluripotent stem cells (PSCs) can be isolated or generated in the laboratory and grown in culture while maintaining pluripotency and indefinite self-renewal capacities. Both the derivation of embryonic stem cells (ESCs) and the generation of induced pluripotent stem cells (iPSCs) were awarded the Nobel Prizes in 2007 and 2012, respectively (Evans and Kaufman, 1981; Takahashi and Yamanaka, 2006). The derivation of the human counterparts of both of these cell types (Thomson et al., 1998; Takahashi et al., 2007) opened up exciting possibilities for utilizing their differentiated derivatives as a strategy in cell replacement therapies (Trounson and DeWitt, 2016). In addition, human PSCs (hPSCs) also serve as an invaluable resource to model and study human development and disease *in vitro* (Avior et al., 2016). Accordingly, understanding pluripotency remains a pivotal aim in the fields of human development, drug discovery, and regenerative medicine.

Different Views on Pluripotency

Previous work on PSCs focused on different characteristics of the cells and provided at least four different ways of exploring what defines pluripotency (Figure 1). First, there is a developmental view. In the context of development, the inner cell mass within the blastocyst is characterized by pluripotency features, which partially linger until the post-implantation epiblast stage but are eventually lost in somatic cells (De Los Angeles et al., 2015) (Figure 1, top left). The pluripotency of human ESCs (hESCs) is well demonstrated by their ability to differentiate into cells from the three germ layers either *in vitro*, as embryoid bodies or by directed differentiation, or *in vivo*, through teratomas (Thomson et al., 1998; Itskovitz-Eldor et al., 2000; Schuldiner et al., 2000). Second, a transcriptomic view also gives insight on pluripotent cells. Much of the work on hPSCs has focused on identifying their transcriptomic and epigenetic signatures. Gene expression profiling of hPSCs has identified numerous transcription factors and surface molecules as markers of pluripotent cells at different stages of their differentiation (Adewumi et al., 2007; Weinberger et al., 2016) (Figure 1, top right). Third, a reprogramming view assists in characterizing pluripotency. Identification of transcription factors as pluripotency markers in transcriptome analyses suggested that the pluripotent cell identity can be induced in non-pluripotent cells by such master regulators (Takahashi and Yamanaka, 2016). Indeed, about a decade ago, the term "induced pluripotency" was coined when Takahashi and Yamanaka (Takahashi and Yamanaka, 2006) demonstrated that somatic cells can be reprogrammed into PSCs by ectopic expression of a small subset of transcription factors, namely OCT4, SOX2, KLF4, and c-MYC. This study opened a new way to define pluripotency merely by the ability to reprogram non-pluripotent cells into PSCs (Figure 1, bottom left). Fourth, an essentialome view also defines what makes a cell pluripotent. With the advent of genome-wide, scalable gene-editing technologies such as CRISPR-Cas9, the possibility of performing comprehensive essentiality screens in human cells has recently become possible. Initial studies were aimed at identifying essential genes in the human genome by using genetically aberrant cancer cell lines of various tissue origins (Blomen et al., 2015; Hart et al., 2015; Wang et al., 2015). Recently, karyotypically normal haploid hESCs (Sagi et al., 2016) enabled the identification of the essential genes in hPSCs through a genome-wide loss-of-function screen using CRISPR-Cas9 technology (Yilmaz et al., 2016 and 2018). This analysis allows for the utilization of the essentialome of PSCs as another means to define pluripotency (Figure 1, bottom right).

Pluripotency: From Development to Cell Culture

The pluripotent capacity of a cell was initially investigated through mouse embryonal carcinoma cells, malignant pluripotent stem cells derived from teratocarcinomas (Martin and Evans, 1974; Stevens and Varnum, 1974). Normal pluripotent cells in mammals were first isolated from mouse blastocyst embryos (Evans and Kaufman, 1981; Martin, 1981). The ability of the inner cell mass to produce pluripotent ESCs in culture was also recapitulated in humans (Thomson et al., 1998) (Figure 2), opening a new era in human biology (Ben-David et al., 2012). The human zygote-stage embryo has a totipotent capacity, the ability to give rise to embryonic and extraembryonic tissues. During preimplantation development, this highest level of potency found in the zygote becomes more restricted to pluripotency at the blastocyst-stage embryo. Still, hESCs were also isolated from

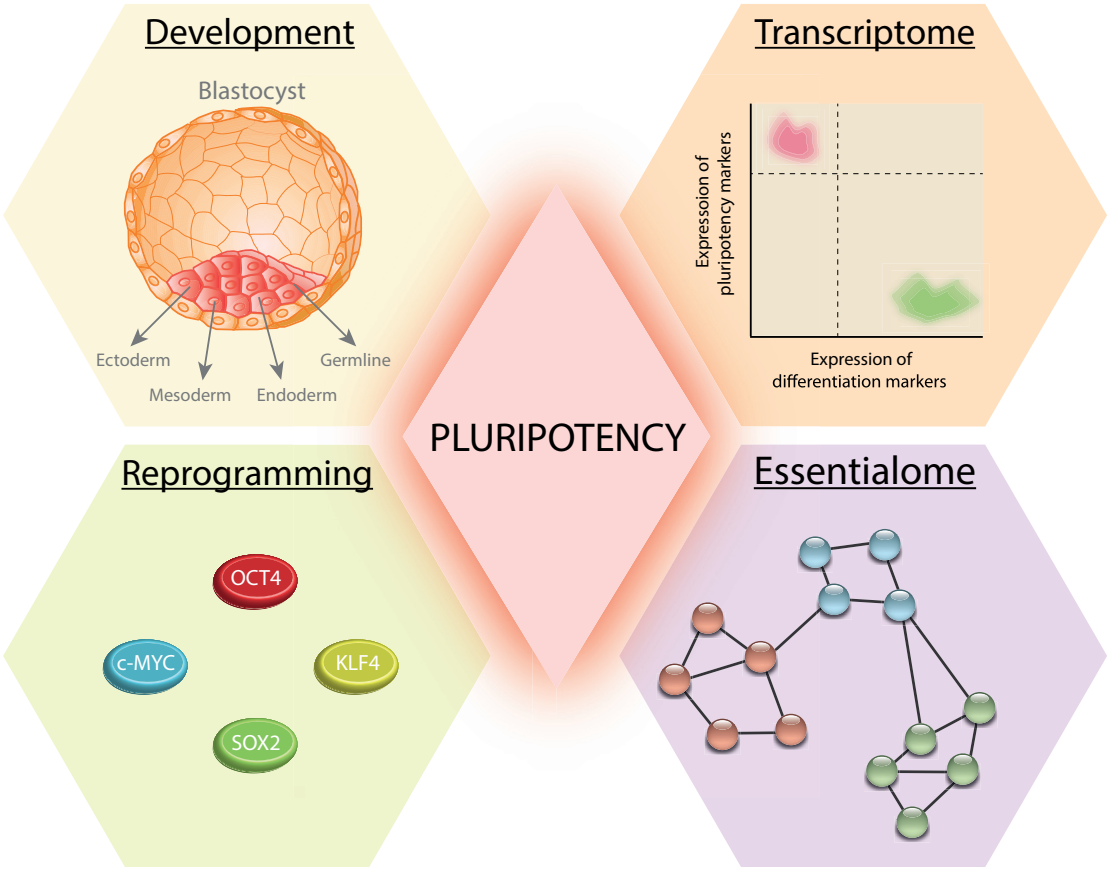


Figure 1. Different Views on Pluripotency
(A) Development: the classical pluripotent stem cells (PSCs) are embryonic stem cells (ESCs) isolated from the inner cell mass of the blastocyst stage embryo. These cells can differentiate into cells from the three germ layers (top left).
(B) Transcriptome: PSCs have a unique expressed-gene profile that distinguishes them from their differentiated derivatives (top right).
(C) Reprogramming: pluripotency can be induced in somatic cells by the expression of a group of factors generating induced pluripotent stem cells (iPSCs) (bottom left).
(D) Essentialome: normal growth and survival of PSCs depend on the expression of a unique set of essential genes specific to these cells (bottom right).

human blastomeres at the morula-stage embryo (Klimanskaya et al., 2006) (Figure 2). Mouse pluripotent cells were derived not only from the inner cell mass but also from post-implantation epiblast-stage cells (Brons et al., 2007; Tesar et al., 2007), and these were named epiblast stem cells. These cells can also generate the cells of the three embryonic germ layers and are thus pluripotent, but they appear to be limited in their ability to contribute to germ cells. When the transcriptome and the methylome of hESCs were analyzed in detail, it was suggested that they were more similar to epiblast cells than the inner cell mass cells. Thus, they were considered primed-ESCs (Nichols and Smith, 2009; Nakamura et al., 2016), and many attempts were made, and are still underway, to convert them into authentic naive-ESCs (Weinberger et al., 2016) (discussed below in Pluripotency States and Transitions) (Figure 2). Although PSCs are lost during gastrulation, pluripotency can be artificially induced by genetic manipulation of somatic cells (see section below, Reprogramming to Pluripotency). In all somatic tissues, pluripotent cells are absent, but the ability to generate pluripotent cells is maintained in primordial germ cells; these cells in the human embryo can generate embryonic germ cells in culture (Shamblott et al., 1998) (Figure 2). Embryonic germ cells were shown to be pluripotent and differentiate into the three embryonic germ layers in both the human and mouse (Shamblott et al., 2001; Leitch et al., 2013). However, the maintenance of human embryonic germ cells in culture is more challenging than that of hESCs, and there have been limited number of reports demonstrating this ''dormant'' pluripotency. Finally, human female primordial germ cells produce oocytes, and even without fertilization by the sperm, these eggs can be induced to proliferate in culture, generating both diploid parthenogenetic ESCs (Kim et al., 2007) and haploid parthenogenetic ESCs (Sagi et al., 2016) (Figure 2). These cultured cells were shown to be pluripotent via *in vitro* or *in vivo* assays, generating either haploid or diploid somatic cells from the three embryonic germ layers (Sagi et al., 2016; Zhong et al., 2016). Thus, pluripotency is maintained during the embryonic, fetal, and adult life, albeit in different cell types, and hPSCs can be derived from different stages during development. Pluripotency is usually viewed to occur at a relatively short period in development, i.e., during the generation of the inner cell mass at blastocyst-stage embryos. Before this stage, the cells are considered totipotent,

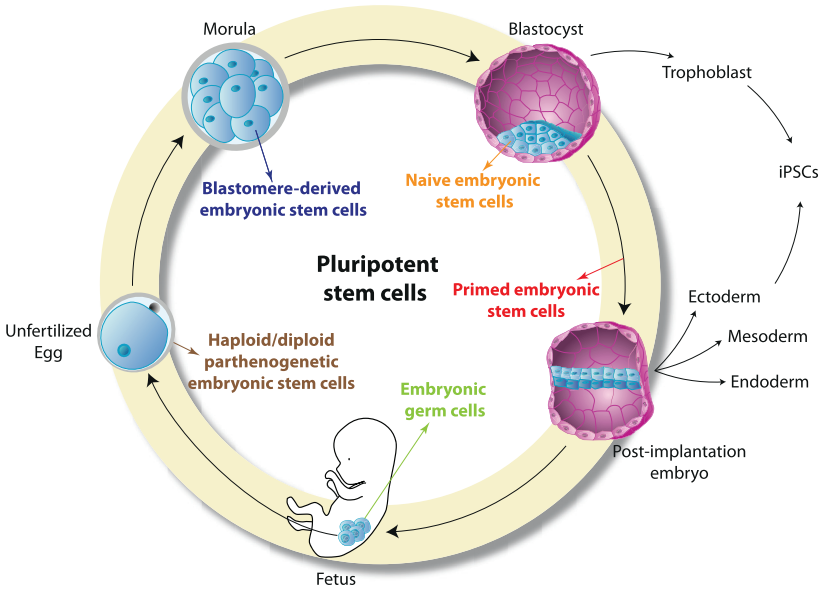


Figure 2. The Developmental Cycle of Pluripotency

Cells with pluripotent character are present at different stages of human development. Oocytes retain the ability to give rise to PSCs via induced parthenogenesis. Pluripotent features can also be captured in the cells derived from 8-cell-stage embryos, early or late blastocysts, and epiblasts, as well as primordial germ cells.

and afterward the stem cells are considered only multipotent. Still, cells that are able to generate PSCs exist at all stages of human development, and although the cultured cell lines that they produce may differ in their molecular characteristics, all of them may differentiate into cells from the three embryonic germ layers.

The Transcriptome of Pluripotency

Each cell type in our body expresses a unique set of genes. Analysis of the transcriptome of cells can enable a clear assumption of the tissue they were isolated from, which cell types in the tissue they represent, and even at which developmental stage they were isolated (GTEx Consortium, 2015). Thus, one of the first aims of international efforts to characterize hESCs was to analyze their transcriptome; this ultimately showed that cells generated in different labs have the same signature of transcribed genes (Adewumi et al., 2007). This was also performed to characterize hiPSCs and to show their high similarity to hESCs (Takahashi et al., 2007). A simple and rather common way to characterize hPSCs is to analyze their global expression, for example through PluriTest analysis (Müller et al., 2011), instead of demonstrating their ability to differentiate into the three embryonic germ layers (International Stem Cell Initiative, 2018).

The transcriptome of a cell is governed by its epigenetic marks, mainly DNA methylation, histone modifications, and chromatin conformation. Hence, analysis of DNA and histone modifications can also define PSCs, their origin, and their developmental stage. This characterization, which became complementary to transcriptome analysis, added another layer to show variability between cells (Bock et al., 2011) and even suggested the consequences of such variability on the differentiation of cells.

One interesting feature concerning the analysis of histone modifications in hPSCs was the ability to show a unique collection of genes that have both activator and repressor bivalent marks (Bernstein et al., 2006). Many of these genes are not expressed in undifferentiated pluripotent cells, and their transcription is initiated upon differentiation. The data suggests that pluripotent cells exhibit a unique feature characterized by these poised genes that are destined to be expressed in the progenies of PSCs, and they are already epigenetically marked in the more primitive cells, which have the distinctive ability to differentiate.

In many cell types, some of the most highly and specifically expressed genes define the function of the cell or the tissue. Thus, it is valid to expect that the transcriptome of hPSCs will allow us to define their pluripotent characteristics at the molecular level. Much of the previous effort to define the role of genes specifically expressed in PSCs focused on uniquely expressed transcription factors such as OCT4 and NANOG (Nichols et al., 1998; Chambers et al., 2003; Mitsui et al., 2003). It was gratifying to observe that these nuclear factors bind the regulatory regions of many of the genes expressed in PSCs (Boyer et al., 2005). Moreover, they also play a major role in inducing a pluripotent phenotype in somatic cells, as will be discussed in the next sections (Takahashi et al., 2007; Yu et al., 2007). Although the expression of many genes is enriched in PSCs as compared to other cell types, their role in pluripotency is still obscure. One way of defining the role of such genes in PSCs is to check whether they are essential for the growth and survival of pluripotent cells, as discussed below (The Essentialome Perspective on Pluripotency).

Pluripotency States and Transitions

Modeling pluripotency in culture has advanced our understanding of early human development. Classical hESC cultures derived from blastocyst-stage human embryos retain their pluripotent characteristics. Importantly, the molecular characterization of transcriptional signatures and epigenetic marks of human early embryos demonstrated that the classical blastocyst-derived hESC cultures are probably more closely associated with cells of the post-implantation epiblast stage than with blastocyst cells in mouse embryos (Nakamura et al., 2016). Indeed, previous studies performed in mouse ESCs (mESCs) and mouse epiblast stem cells (EpiSCs) suggested two major stages of pluripotency: mESCs resemble the cells of pre-implantation stage embryos and were termed naive PSCs, whereas EpiSCs were termed primed PSCs because they exhibited the features of post-implantation epiblast-stage embryos (Nichols and Smith, 2009). In recent years, several studies have demonstrated that human pre-implantation embryos exhibit stage-specific characteristics that can be used as hallmarks during the derivation of naive hPSCs (Yan et al., 2013; Blakeley et al.,

2015; Petropoulos et al., 2016; Theunissen et al., 2016; Boroviak et al., 2018; Stirparo et al., 2018). Some of these features attracted more consensus and include genome-wide hypomethylation together with the maintenance of the methylated signatures of imprinted loci and a distinct transcriptional profile for coding genes and transposable elements (Smith et al., 2014; Pastor et al., 2016; Theunissen et al., 2016). Other features such as the mode of X chromosome inactivation, however, are still debated. Different studies have suggested either the presence of two active X-chromosomes with bi-allelic dampening, or alternatively, the appearance of X-linked mono-allelic expression in late human blastocysts (Okamoto et al., 2011; Petropoulos et al., 2016; Vallot et al., 2017; Moreira de Mello et al., 2017; Bar et al., 2019).

Derivation and thorough characterization of naive hPSCs might lead to an important *in vitro* model for studying the human development stages that are not easily available due to limited access to early human embryos, which differ significantly from their mouse counterparts at the molecular level. Thus, there has been an extensive effort by several groups to establish culture conditions that would support the maintenance of this pluripotency state (De Los Angeles et al., 2015; Sagi and Benvenisty, 2016).

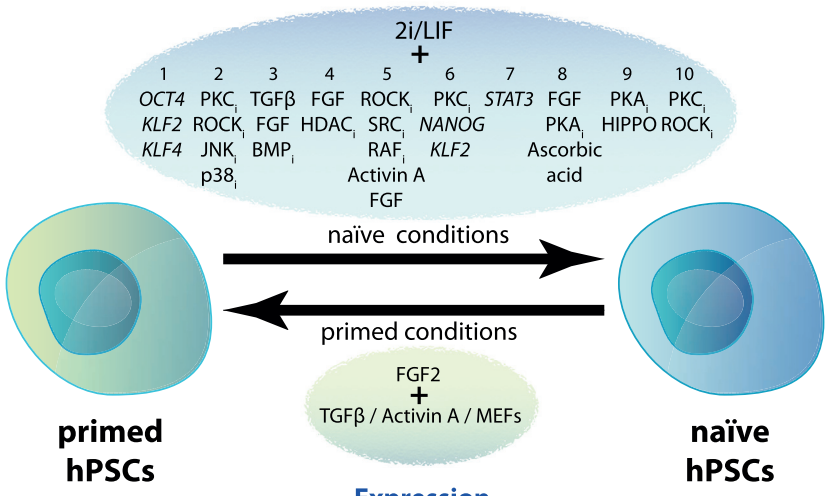
Naive mouse PSCs were obtained by the simultaneous inhibition of two kinases: GSK3β, a member of the Wnt pathway, and MAP2K, a member of the ERK signaling cascade, in the presence of the soluble signaling molecule LIF that activates the JAK/STAT pathway (Silva and Smith, 2008; Ying et al., 2008). This culture formulation was termed 2i/LIF. On the basis of some of the regulators and inducers of this pluripotency state in the mouse, several groups established different protocols that can induce a naive state in hPSCs. The 2i/LIF condition has been a shared requirement across a dozen protocols published during the past few years regarding the induction and maintenance of naive hPSCs. However, each protocol uses a unique set of inhibitors and factors in addition to the 2i/LIF condition (Figure 3).

Initial efforts took a transgene approach, in which the naive state was induced in the presence of 2i/LIF by the overexpression of naive-associated transcription factors, much like in the strategy used during somatic cell reprogramming toward pluripotency (Hanna et al., 2010; Takashima et al., 2014; Chen et al., 2015). Among these transcription factors were the combinations of OCT4, KLF2, and KLF4 or NANOG and KLF2, or more recently, the LIF downstream effector STAT3 that was expressed transiently. Later, transgene-free chemical transition protocols were established to induce and maintain naive hPSCs (Chan et al., 2013; Gafni et al., 2013; Theunissen et al., 2014, 2016; Ware et al., 2014; Duggal et al., 2015; Qin et al., 2016; Guo et al., 2017). Candidate-based and unbiased chemical-screen-based approaches identified kinases, signaling molecules, and pathways and other epigenetic regulator enzymes that can be targeted to stabilize naive pluripotency in the presence of 2i/LIF. These interventions include chemical inhibitions of PKC, PKA, SRC, ROCK, RAF, JNK, p38/MAPK, or the BMP pathways, chemical inhibition of HDACs and facilitation of DNA demethylation, and soluble ligand- or chemical-mediated activators of the TGFβ, Activin A, FGF, and Hippo pathways (Chan et al., 2013; Gafni et al., 2013; Takashima et al., 2014; Theunissen et al., 2014, 2016; Ware et al., 2014; Duggal et al., 2015; Qin et al., 2016; Guo et al., 2017) (Figure 3). These various naive induction conditions led to the derivation and characterization of naive hPSCs from established primed hESC and hiPSC cultures, from *de novo* reprogrammed hiPSCs, and directly from blastocysts (Hanna et al., 2010; Gafni et al., 2013; Chan et al., 2013; Theunissen et al., 2014; Takashima et al., 2014; Ware et al., 2014; Chen et al., 2015; Duggal et al., 2015; Theunissen et al., 2016; Guo et al., 2016; Qin et al., 2016; Guo et al., 2017; Liu et al., 2017).

The establishment of reliable culture conditions for naive hPSCs opens up new avenues to study early human development. Despite the high degree of variability in the culture conditions and molecular characteristics of the cells obtained by different published protocols, naive hPSCs can recapitulate several aspects of human blastocysts. Comparisons of existing naive and primed hPSC lines have revealed novel distinct features of both cell states. First, cultured naive and primed cells differ by morphology. Whereas classical primed hPSC cultures form flat colonies in culture, naive hPSC colonies appear dome-shaped. Second, naive cells were also reported to have a higher degree of single cell survival, which can provide an important benefit for certain experimental settings (Gafni et al., 2013).

The two cell states have distinct molecular signatures in terms of their gene expression and epigenome (Figure 3). Comparative transcriptional analysis performed at the single-cell level between naive and primed hPSCs showed SOX2, OCT4, and NANOG as shared regulators of both cell states and also identified unique landscapes of transcription factors for each state. It is noteworthy that although OCT4 is a shared regulator between the two states, its expression was previously suggested to be regulated by differential enhancer activities for each cell type: by a distal enhancer for the naive state and by a proximal one for the primed state (Theunissen et al., 2014). Importantly, the shared regulators, OCT4, SOX2, and NANOG, are expressed at higher levels in naive cells and blastocysts as compared to primed cells (Guo et al., 2016). Cultured naive hPSCs can obtain a highly similar transcriptional signature of coding genes to that of late human blastocysts, marked by the expression of stage-specific regulatory factors (Guo et al., 2016; Theunissen et al., 2016). DNMT3L, DPPA3/5, GATA6, IL6ST, KLF4/5/17, and TBX3 have been found to be among the most upregulated naive hPSC factors, whereas CD24, SFRP2 and ZIC2 were the primed hPSC-enriched factors (Pastor et al., 2016; Chen et al., 2018; Messmer et al., 2019). A recent study also identified a subset of cells under naive conditions that represent an intermediate state between the major group of naive cells and the primed cells (Messmer et al., 2019). These cells are suggested to resemble the recently proposed intermediate pluripotency state in mouse, termed "formative pluripotency" (Smith, 2017; Messmer et al., 2019). Lipogenesis has also been recently suggested to be a hallmark of the transcriptome of naive hPSCs and human pre-implantation epiblasts, enabling the capture of yet another intermediate state of pluripotency (Cornacchia et al., 2019).

Naive and primed hPSCs were also shown to have different expression profiles for transposable elements. Although the SVA family of retrotransposons was overexpressed in naive hPSCs, the overexpression of the HERVH-int family of endogenous retroviruses distinguished the primed hPSC identity from



primed hPSCs		naïve hPSCs
Expression		
CD24, SFRP2, ZIC2	**Expression profile**	DPPA3/5, KLF4/5/17, TBX3
Proximal	***OCT4* enhancer dominance**	Distal
CD24, CD57, CD90	**Surface molecules**	CD7, CD75, CD77, CD130
Low	**E-Cadherin expression**	High
HERVH-int family	**Retrotransposons**	SVA family
Epigenetics		
High	**DNA methylation**	Low
X_aX_i	**XCI**	X_aX_a
High	**H3K27me3 on polycomb-associated genes**	Low
Pathways and differentiation		
Yes	**ERK pathway dependence**	No
Glycolysis	**Energy metabolism**	Oxidative phosphorylation
Poor	**PGCLC differentiation**	Potent
Limitations		
Maintained	**Genomic imprinting**	Erased/aberrant
Low frequency	**Chromosomal aberrations**	High frequency
Non-random	**XCI upon differentiation**	Non-random

Figure 3. Comparisons Between Naive and Primed hPSCs

Primed hPSCs can be reset into a naive state by the inhibition of two kinases, GSK3β and MAPK, and the induction of the JAK/STAT pathway through the ligand LIF (2i/LIF), along with ectopic expression of transcription factors, chemical inhibition of a group of kinases, or activation of specific signaling pathways. Examples of established protocols for primed-to-naive hPSC conversion are summarized in the light blue ellipse (1: Hanna et al., 2010; 2: Gafni et al., 2013; 3: Chan et al., 2013; 4: Ware et al., 2014; 5: Theunissen et al., 2014 and 2016; 6: Takashima et al., 2014; 7: Chen et al., 2015; 8: Duggal et al., 2015; 9: Qin et al., 2016; and 10: Guo et al., 2017). These protocols highly vary in their potency of inducing the distinct features of naive hPSCs. For comprehensiveness, the list of naive hPSC phenotypes in the table includes those documented by at least one protocol. Lowercase i stands for inhibitor. Primed hPSCs can be maintained in the presence of FGF2 along with TGFβ, Activin A, or a feeder layer of mouse embryonic fibroblasts (MEFs) (light green ellipses). Naive and primed hPSCs have distinct defining features with respect to their expression profile, epigenetic status, their active pathways, and their differentiation potentials, although current naive PSC cultures also contain limitations (Abbreviations are as follows: XCI = X chromosome inactivation, X_a = active X chromosome, X_i = inactive X chromosome, and PGCLC = primordial-germ-cell-like cells).

the naive one (Theunissen et al., 2016) (Figure 3). Curiously, expression profiles for transposable elements in naive hPSCs were shown to be highly similar to those of late-morula- and early-blastocyst-stage human embryos (Theunissen et al., 2016).

Expression of cell surface molecules has also been compared between naive and primed hPSCs. An antibody screen identified a small cohort of molecules as naive and hPSC-specific and another subset of molecules as primed and hPSC-specific (Collier et al., 2017). A panel of antibodies against a group of such molecules was reported to distinguish the naive hPSCs (CD7, CD75, CD77, and CD130) from the primed hPSCs (CD24, CD57, and CD90) (Collier et al., 2017) (Figure 3). Further characterization of such markers will help the identification of intermediate stages of pluripotency during the transition between naive and primed hPSCs.

Regarding the epigenome, DNA methylation and H3K27me3 levels were shown to be among the unique identifiers of the two states. Global DNA hypomethylation seen in naive hPSCs is contrasted by a global DNA hypermethylation signature in primed hPSCs. Genome-wide hypomethylation was observed in naive hPSC cultures in levels comparable to those seen in human blastocysts (~30% methylation as opposed to ~80% seen in primed hPSCs) (Guo et al., 2014; Smith et al., 2014; Pastor et al., 2016; Theunissen et al., 2016). However, the pattern of DNA methylation of naive PSCs was suggested to differ from that of the pre-implantation embryo, as will be discussed below. In addition, the repressive chromatin mark H3K27me3 seen at the polycomb-associated genes in primed hPSCs is lost as these cells transition to the naive state (Gafni et al., 2013; Theunissen et al., 2014). Another major difference between the two states is their X chromosome status. Reactivation of the inactive X chromosome in primed hPSCs, hence the presence of two active X chromosomes, and bi-allelic expression of *XIST* have been observed in naive hPSCs, recapitulating other hallmarks of human blastocysts (Petropoulos et al., 2016; Sahakyan et al., 2017) (Figure 3).

The two pluripotency states can also be distinguished by the activity of signaling pathways that are regulating the induction or maintenance of each state. For example, naive pluripotency was induced by the activation of the Hippo pathway through transgenic overexpression or pharmacological upregulation of the Hippo pathway effector YAP (Qin et al., 2016). Conversely, primed hESCs also depend on unique signaling pathways such as the FGF and ERK pathways (Thomson et al., 1998; Brons et al., 2007; Tesar et al., 2007). Finally, there are also differences in the preference over metabolic pathways between naive and primed hPSCs. Although the naive hPSCs rely mostly on oxidative phosphorylation, primed hPSCs use glycolysis for energy metabolism (Takashima et al., 2014).

Naive and primed hPSCs may exhibit different levels of differentiation potential. Although naive hPSCs were shown to have a restricted, lineage-specific differentiation capacity as compared to primed hPSCs (Lee et al., 2017), they were suggested to be the more potent state in germ cell differentiation owing to their extensive erasure of epigenetic marks normally found in primed hPSCs (Irie et al., 2015; von Meyenn et al., 2016) (Figure 3). Furthermore, naive hPSCs were also shown to express some trophoblast markers and were proposed as a potential model for studying trophoblast differentiation (Theunissen et al., 2016). Interestingly, there has been some evidence for a small contribution of naive hPSCs in the formation of inter-species chimeras in mouse blastocysts, whereas this ability is lost in primed hPSCs (Gafni et al., 2013). Identification of the differences between naive and primed hPSCs may shed light on the mechanisms of *in vitro* and *in vivo* transitions between these pluripotency states and will allow better control of these cell states in culture.

Apart from the similarities mentioned above, it has also been observed that some features of naive hPSCs are not completely aligned with the molecular hallmarks of human blastocysts. Genomic imprinting is vital for maintaining the mono-allelic expression pattern of a few dozens of parent-of-origin-specific genes. Although global DNA hypomethylation is a defining feature of human blastocysts, DNA methylation in differentially methylated regions within imprinted loci is maintained during pre-implantation development and also after lineage commitment and differentiation. Examination of naive hPSCs revealed that global loss of methylation led to the irreversible loss of hypermethylation marks in the imprinted loci (Pastor et al., 2016; Theunissen et al., 2016; Bar et al., 2017) (Figure 3). Loss of genomic imprinting is associated with human disorders and can also potentially misregulate the differentiation of hPSCs into specific cell fates (Tucci et al., 2019). Therefore, optimization of culture conditions will need to address the misregulation of this important feature in naive hPSCs. This can be achieved by altering the exposure and dosage of the factors that are used to erase the methylation marks during the conversion of primed hPSCs into naive hPSCs.

Another aberration in naive hPSCs was observed with respect to their X chromosome inactivation patterns. When naive hPSCs were differentiated, the same reactivated chromosome was inactivated again, suggesting that some remaining molecular memory prevented normal random X-chromosome inactivation (Theunissen et al., 2016) (Figure 3). Further analysis and identification of such molecular memory might give rise to the use of novel inhibitors and factors that can lead to the isolation of naive hPSCs without such bias, enabling studies regarding the mechanisms of random X-chromosome inactivation in human.

Finally, naive hPSCs were found to have a higher degree of genomic instability as compared to primed hPSCs, raising a cautionary warning about their usefulness in disease modeling and clinical applications (Theunissen et al., 2014; Pastor et al., 2016; Liu et al., 2017) (Figure 3). These abnormalities may arise in part due to the fact that most of the naive hPSC lines were derived from existing primed hPSC lines under heavy selection and prolonged culture durations during the transition protocols, facts which might allow the selection and accumulation of mutations. This might be overcome by deriving more naive hPSCs directly from blastocysts. Although genomic instability was detected in most of the blastocyst-derived naive hPSCs, karyotypic stability could be maintained in a small fraction of these cell lines (Guo et al., 2016).

In a recent attempt to identify conditions that would support naive pluripotency, primed hPSCs were reprogrammed into a state with extended developmental potency by the use of small molecules. These cells, termed extended pluripotent stem cells, exhibit a differentiation capacity not only in embryonic but also in extra-embryonic lineages, a feature that distinguishes them from the previously established naive PSCs (Yang et al., 2017a). Cells that have a similar developmental capacity and are named expanded potential stem cells were also established initially in the mouse (Yang et al., 2017b), and recently in the human (Gao et al., 2019), demonstrating a histone expression profile, which is similar to that of human 8-cell- and morula-stage embryos.

Reprogramming to Pluripotency

The ability to convert somatic cells into PSCs changed the way we view pluripotency. A series of studies demonstrated that the ectopic expression of specific transcription factors was sufficient to induce conversions between cell identities, hence attributing master-regulatory roles to these factors for cell fate determination (Davis et al., 1987; Schneuwly et al., 1987; Halder et al., 1995). These studies led to the groundbreaking discovery that pluripotency can also be established through the combination of master regulatory transcription factors, namely, OCT4, SOX2, KLF4, and MYC (OSKM) (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). In parallel to the use of Yamanaka factors in human cells, it was shown that the ectopic expression of a somewhat different combination of factors that included OCT4, SOX2, NANOG, and LIN28 was also able to reprogram the neonate fibroblasts into pluripotency (Yu et al., 2007).

The oncogene *MYC* increases the number of iPSC colonies during reprogramming but also enhances the tumorigenicity of these cells. Therefore, modified protocols in the absence of *MYC* have been tried, and high-quality hiPSCs with fewer tumorigenic properties were successfully generated (Nakagawa et al., 2008). This was followed by other studies demonstrating that somatic cells could be reprogrammed with only OCT4 and SOX2 in the presence of histone deacetylase inhibitors, and also with OCT4 alone by using cell types that express SOX2, KLF4, and MYC (Huangfu et al., 2008; Kim et al., 2009). Recently, activation of endogenous *Oct4* or *Sox2* (by the CRISPR activation system) was sufficient to induce pluripotency (Liu et al., 2018) in mouse somatic cells. These observations demonstrate

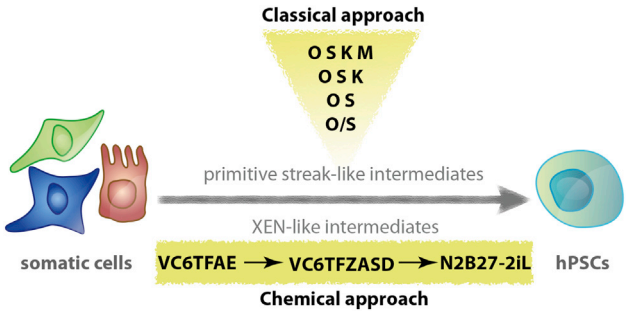


Figure 4. Reprogramming into Pluripotency by Genes and Small Molecules

The classical transgene approach can induce pluripotency through the collective ectopic expression of transcription factors *OCT4*, *SOX2*, *KLF4*, and *MYC* (OSKM) in somatic cells (Takahashi and Yamanaka, 2016). Induction of pluripotency was also achieved by the expression of subsets of these factors and recently by OCT4 or SOX2 alone via the CRISPR activation system (Liu et al., 2018). The chemical approach is based on the induction of pluripotency by the use of small molecules to inhibit or activate signaling pathways or a wide range of enzymes. During their reprogramming route into pluripotency, somatic cells can pass through different intermediate stages such as primitive streak-like intermediates in the classical approach (Takahashi et al., 2014) and extraembryonic endoderm-like (XEN-like) intermediates in the chemical approach (Zhao et al., 2015). Multiple combinations of molecules were tested for their ability to induce pluripotency: V = VPA, a histone deacetylase inhibitor; C = CHIR99021, a GSK3β inhibitor; 6 = 616452, a TGFβ inhibitor; T = Tranylcypromine, a histone demethylase inhibitor; F = Forskolin, a cAMP agonist; A = AM580, a retinoic acid agonist; E = EPZ004777, a DOT1L inhibitor; Z = DZNEP, a SAH hydrolase inhibitor; S = SGC0946, a DOT1L inhibitor; D = 5-aza-dC, a DNA methylation inhibitor; 2i = a combination of a GSK3β inhibitor (CHIR99021) and ERK inhibitors (PD0325901); and L = LIF.

that the individual factors from the original reprogramming cocktail could be omitted and suggested a hierarchy between them, placing the OCT4/SOX2 complex as the master regulators in inducing and maintaining the pluripotent cell identity (Liu et al., 2018).

Alternative cocktails of transcription factors have been used with different degrees of reprogramming efficiencies and applicability to a narrower range of cell types. *Sall4*, *Essrb*, and *Lin28*, along with *Nanog* or *Dppa2*, were shown to induce pluripotency in mouse somatic cells (Buganim et al., 2012, 2014). *RCOR2*, a member of a nucleosome demethylation complex, was shown to replace *SOX2* in the classical Yamanaka factors both in the mouse and the human (Yang et al., 2011). A combination of three mature miRNAs that have an enriched expression in PSCs was also used to induce pluripotency in the absence of ectopic expression of transcription factors (Miyoshi et al., 2011). Interestingly, lineage specifiers were also shown to induce pluripotency (Montserrat et al., 2013). The mesendodermal lineage specifier *GATA3* was able to replace *OCT4*, whereas the ectodermal lineage specifiers *ZNF521*, *SOX1*, or *SOX3* could replace *SOX2* during reprogramming, suggesting that pluripotency is a balanced cell state between opposing lineage-specification pathways (Montserrat et al., 2013).

Induction of pluripotency has also been linked to the activity of histone-modifying enzymes, which directly regulate gene expression. *Wdr5*, a member of the Trithorax complex that deposits the activator H3K4 methylation mark on histones, and the H3K27 methyltransferase *EZH2*, a member of the polycomb repressive complexes 1 and 2, were shown to be required for the efficient generation of iPSCs (Ang et al., 2011; Onder et al., 2012). In contrast, the inhibition of another subset of epigenetic modifiers such as the context-dependent transcriptional activator or repressor *YY1*, H3K9 methyltransferase *SUV39H1*, and the H3K79 methyltransferase *DOTL1* increases the efficiency of iPSC generation. Interestingly, shRNA-based or pharmacological inhibition of *DOTL1* could replace *KLF4* and *MYC* in the Yamanaka factors during the induction of pluripotency (Onder et al., 2012). A specific subunit of the nucleosome remodeling and deacetylase (NuRD) co-repressor complex, Gatad2a, has been shown to have a repressive activity on the pluripotency circuitry during iPSC reprogramming. Inhibition of this factor was suggested to facilitate deterministic induction of naive pluripotency in the mouse (Mor *et al.*, 2018). These observations indicate that the establishment of the pluripotent state is strongly regulated by epigenetic modifiers that can also take over the role of master regulatory transcription factors of this process.

A series of studies have shown the counteracting roles of pathways such as the P53 and INK4/ARF pathways for the wiring of pluripotency, indicating their significance in restricting pluripotency (Banito et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009a; Marión et al., 2009; Utikal et al., 2009). Indeed, several members of these pathways were recently identified in a genome-wide loss-of-function screen as the major growth-restricting players in hPSCs (Yilmaz et al., 2018).

Although studies on the transcription-factor-mediated induction of pluripotency have demonstrated transcriptional end-point direct effectors and master regulators of this state, recent studies focusing on chemical reprogramming strategies revealed epigenetic modulators and signaling regulators of pluripotency. Chemically induced PSCs (CiPSCs) have been generated using a multitude of small molecules in the absence of any transgene expression (Hou et al., 2013; Zhao et al., 2015). These small molecules included inhibitors of GSK3, TGFβ, DOT1L, histone deacetylase or demethylase, and agonists of cAMP and retinoic acid (Figure 4). The inhibitory or agonistic functions of these small molecules suggest that the establishment of pluripotency requires the regulation of a wide range of epigenetic modifiers, along with modulators of signaling pathways. The finding that DOT1L inhibitors enhanced reprogramming efficiency was also in accordance with a previous screen that identified DOT1L as an inhibitor of reprogramming (Onder et al., 2012). Studies on the temporal progression of reprogramming steps revealed that the reprogramming route of CiPSCs is distinct from that of classical OSKM-derived iPSCs and undergoes an extra-embryonic, endoderm-like intermediate stage with diverse lineage potentials (Zhao et al., 2015; Li et al., 2017) (Figure 4). In contrast, OSKM-driven reprogramming of fibroblasts into human or mouse iPSCs was suggested to go through a transient state resembling the primitive streak in the post-implantation embryo, although this intermediate state could not be detected in reprogrammed keratinocytes and neutrophils in the mouse (Takahashi et al., 2014; Cacchiarelli et al., 2015; Nefzger et al., 2017) (Figure 4). These observations highlight the presence of alternative ways of reconstructing the pluripotency machinery.

Molecular mechanisms of induction of pluripotency have been recently investigated via single-cell RNA sequencing for CiPSCs

and also OSKM-driven reprogramming of mouse embryonic fibroblasts (Zhao et al., 2018; Schiebinger et al., 2019). These studies led to the identification of intermediate cell types and alternative fate transitions during reprogramming. During chemical reprogramming, two intermediate cell types were identified: a previously described extraembryonic endoderm and another intermediate that was similar to two-cell-stage embryos (Zhao et al., 2018). In OSKM-driven reprogramming, multiple cell lineages, including stromal-, trophoblast-, and neural-like cells, were identified parallel to the generation of pluripotent stem cells (Schiebinger et al., 2019). However, it is yet to be determined whether these developmental programs are identical in human cells.

The Essentialome Perspective on Pluripotency

Small-scale and genome-wide genetic screens were previously performed with RNA interference (RNAi) in both mouse and human ESCs, and they identified genes involved in self-renewal and determination of cell identity (Ivanova et al., 2006; Chia et al., 2010). These studies suggested that a group of pluripotency network genes inhibit differentiation of PSCs into their progeny in one or more germ layers. These screens also identified multiple basic cellular processes, including cell-cycle-related processes, essential for self-renewal and maintenance of pluripotency in PSCs. RNAi does not lead to complete loss-of-function alleles, and previous RNAi screens lacked robust targeting complexity. Both of these disadvantages could be overcome with CRISPR screens, which targeted genes with numerous different sgRNAs and allowed robust quantification and increasing confidence in screen results.

Recently, several CRISPR screens that were performed in mouse ESCs identified regulators of maintenance and exit from naive pluripotency and the genes controlling the acquisition of primordial germ cell fate (Li et al., 2018; Hackett et al., 2018; Villegas et al., 2019; MacDougall et al., 2019). The analysis of the essentialome of human PSCs by a genome-wide CRISPR screen was initially performed in haploid cells (Yilmaz *et* al., 2018). A comparison of the essential genes identified in hPSCs with those identified in transformed cells has shown striking differences between the landscapes of the essentialomes of hPSCs and various cancer cell types (Yilmaz et al., 2018). Although nearly 80% of the genes found to be essential in hPSCs were also essential in at least one other cancer cell line, more than 350 genes scored as uniquely essential in hPSCs, adding yet another view on what defines pluripotency (Yilmaz et al., 2018). In order to further analyze these hPSC-unique essential genes to suggest essential cellular pathways for hPSC biology, we first sought to identify specific protein complexes that might indicate functional pathways in which hPSC-unique essential genes have a role. This analysis found a group of interaction clusters that suggested the presence of protein complexes that function in specific pathways, which were divided into four main functional categories: (1) a pluripotency network, (2) the cell cycle and DNA repair, (3) metabolism and signaling, and (4) protein and RNA modules (Figure 5A).

The ''pluripotency network'' category consists of a single strong cluster with well-established pluripotency factors such as OCT4, SALL4, PRDM14, and TDGF1, but it also contains other proteins such as IGF2BP1, VRTN, NANOS1, STRBP, ACVR1B, SMAD2, and EOMES, hinting at novel factors for the maintenance and regulation of pluripotency.

The ''Cell Cycle and DNA repair'' category includes clusters enriched in DNA repair, as well as cell cycle and chromatin organization proteins. The exemplary proteins in these clusters range from epigenetic modifiers such as KDM1A, KDM2A, and KDM2B, to specific histone variants, such as HIST1H2BI, HIST1H2BM, and HIST1H2BN, and also DNA repair proteins such as FANCG, LIG4, and MSH6.

Another functional category, ''Metabolism and Signaling,'' is composed of clusters with enrichment in insulin signaling, phosphate modification, cholesterol biosynthesis ,and the glycosylphosphatidyl-inositol (GPI)-synthesis pathway. The IGF1 signaling pathway that shares many of its members with the insulin signaling pathway was shown to be essential for the maintenance of hESCs. Phosphatases and kinases in these clusters and signaling proteins IGF1R, FGFR2, IRS2, IRS4, and AXIN1 further highlighted the specific signaling events essential for hPSCs. Moreover, the GPI-synthesis pathway, represented by the proteins PIGC, PIGF, PIGL, PIGM, PIGO, PIGU, and PIGW, and the cholesterol biosynthesis pathway, suggest roles for cell membrane-related signaling events.

The last category ''Protein and RNA modules'' has interaction clusters enriched in processes such as RNA splicing, RNA binding, protein ubiquitination, and protein localization. The finding that genes associated with such basic cellular processes can exhibit cell-type selective essentiality suggests that these processes can be regulated differentially in order to maintain pluripotency. For example, the RNA binding protein LYAR, which has a role in ribosome biogenesis and is one of the hPSC-unique essential genes, was also shown to be involved in the maintenance of normal levels of nuclear factors that are essential for the self-renewal of ESCs (Li et al., 2009b). Similarly, the identification of RNA-splicing molecules PQBP1, PTBP1, and CD2BP2 within the hPSC-unique essential genes may suggest an hPSC-selective regulation of this basic cellular process.

A previous analysis of the hPSC-essentialome identified 50 genes that were both essential for the normal growth and survival of hPSCs and that also had a highly enriched expression in hPSCs as compared with their expression in somatic and transformed cells (Yilmaz et al., 2018). We aimed at demonstrating the protein interaction network upon this combinatorial analysis between the essentialome and transcriptome (Figure 5B). This analysis yielded two main protein interaction clusters. One interaction cluster was enriched with genes related to the pluripotency network, suggesting that this cluster contributes to the maintenance of the undifferentiated state and inhibition of differentiation. Indeed, several of these genes, such as OCT4, NANOG, SALL4, PRDM14 or DPPA3, are known to actively maintain pluripotency. The second cluster was enriched in cell cycle-related factors, suggesting that the proteins in this cluster are essential for the self-renewal capacity of hPSCs. These two clusters are interconnected via two bridging interactions that are mainly mediated by LIN28B, arguing a role of this hPSC-essential gene in coordinating two core essential biological pathways in pluripotent cells.

To assess the specificity of this hPSC-essentialome landscape derived with the aid of transcriptome data, we analyzed

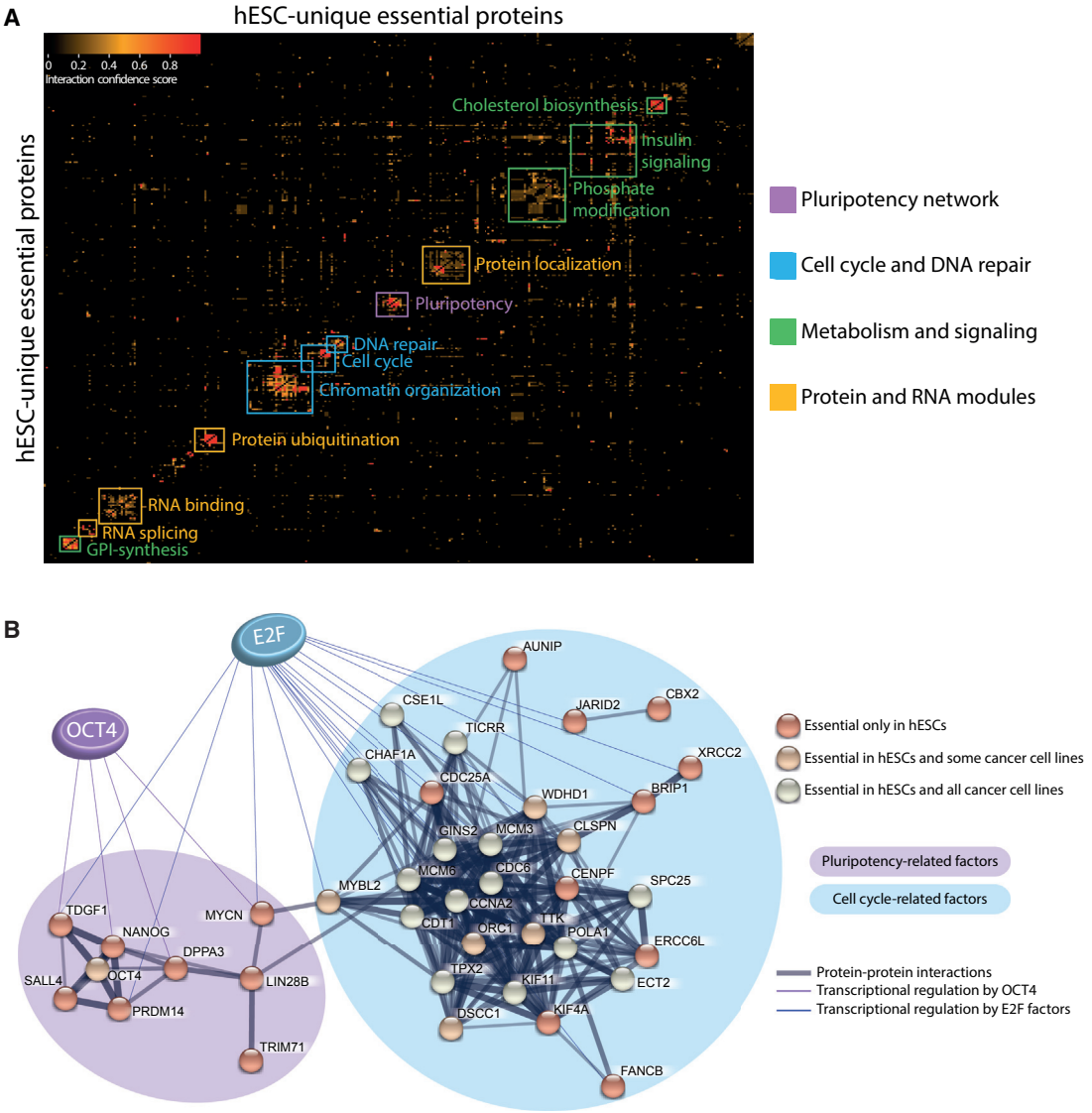


Figure 5. hESC-Essential Protein Networks, Their Functions, and Transcriptional Regulation

(A) A heatmap illustrating protein interactions between the hESC-unique essential genes identified by comparing the essentialome dataset derived from hESCs to those derived from cancer cell lines of various tissue origins. Protein network analysis was performed for genes that scored as essential in hESCs (Yilmaz et al., 2018) but not in any of the cancer cell lines used in three independent studies (Blomen et al., 2015; Hart et al., 2015; and Wang et al., 2015). The STRING database (Szklarczyk et al., 2015) was used to determine the predicted interaction strengths of each possible pair of proteins for all 352 hPSC-unique essential genes. Predicted interaction scores for every protein pair were clustered and plotted on the heatmap. Gene ontology analyses were performed in the DAVID and GSEA databases for the clusters that represent protein complexes, and they are labeled with colored rectangles that denote four functional categories: (1) pluripotency network (purple), (2) cell cycle and DNA repair (blue), (3) metabolism and signaling (green), and (4) protein and RNA modules (orange). Each cluster was also labeled with a gene ontology term significantly enriched within the group of genes in that cluster. Darker red and orange interactions indicate the highest confidence levels for the corresponding interactions.

(B) A protein interaction network of essential genes that also have an enriched expression in hESCs. Two distinct protein interaction clusters are shown for the pluripotency-related factors (purple) and the cell-cycle-related factors (blue). The STRING database was used to map the interactions. Shown are the genes that are essential only in hESCs (red), in hESCs and at least one cancer line (orange), and in both hESCs and all cancer cell lines (white). The thickness of the gray lines between the protein nodes demonstrates the level of confidence for the interaction. Regulatory transcription factors are connected to the target proteins with purple (OCT4) and blue (E2F) lines (for the highest-scoring transcriptional targets). The PASTAA method for predicting transcription factor affinities to DNA was used for predicting the regulatory transcription factors for the interaction clusters (Roider et al., 2007).

the essentiality screen datasets from the cancer cell lines for the genes in two distinct interaction clusters that we identified. This analysis revealed that nearly all of the genes in the pluripotency cluster were uniquely essential in hPSCs. In the case of self-renewal cluster, one-third of the genes were uniquely essential in hPSCs, one-sixth of them were essential in hPSCs and in some but not all cancer cell lines, and half were essential in both hPSCs and all tested cancer cell lines. This observation indicates that the regulation of hPSC self-renewal at least partially relies on a unique set of essential genes and that this gene network distinguishes PSCs from other proliferative cells such as transformed cells.

A small group of cell cycle-related genes were previously identified in RNAi screens in mouse and human and were shown to be essential for the maintenance of self-renewal of PSCs (Ivanova et al., 2006; Chia et al., 2010). The extended list of cell cycle-related genes found in CRISPR screens with highly enriched expression and specific essentiality in hPSCs supports the notion that the differentiation and cell cycle regulation of hPSCs are closely linked to each other. Furthermore, these findings also suggest that hPSCs govern their cell cycle with a unique gene circuitry that can be turned off for differentiation.

Analysis of the predicted regulatory transcription factors for these two interaction clusters in the hPSC-essentialome, on the basis of the highest-ranking transcription factor predictions, showed that the pluripotency genes were expected to be specifically regulated mainly by OCT4. On the other hand, self-renewal genes were predicted to be regulated by the E2F family transcription factors (Figure 5B). These predictions suggest that the regulators of the pluripotency and self-renewal genes, namely OCT4 and E2F family transcription factors, respectively, are the master regulators of the hPSC-essentialome and hence the pluripotency state. Overall, these analyses indicate that primed hPSCs depend on two major protein networks; one that is responsible for the maintenance of the undifferentiated state and is mainly regulated by OCT4 and another that regulates the self-renewal feature of hPSCs and is mainly regulated by the E2F family transcription factors.

The analyses identifying essential genes and pathways in recent studies that used genome-wide CRISPR screens in hPSCs (Yilmaz et al., 2018; Ihry et al., 2019; Mair et al., 2019) have been, by and large, in agreement, although the use of haploid hESCs as compared to diploid ones has been shown to lead to the identification of more essential genes (Mair et al., 2019). Interestingly, differences in the hPSC-essentialome have been identified according to the substrate the cells are grown on (Mair et al., 2019), suggesting that the cells can also re-wire the genetic circuits they depend on in a non-autonomous manner, and the culture adaptations can re-define the landscape of the essentialome to a certain extent. Future genome-wide loss-of-function screens will be of interest in order to define the essential genes for the transition between different stages of pluripotency and will facilitate the understanding of these rapid cell-state changes in early human embryogenesis.

Interplay and Differences between Different Views on Pluripotency

The transcriptome of the inner cell mass during embryo development or of cultured hPSCs has been classically used to identify and study the regulators of pluripotency. The level and specificity of expression are often considered as being correlated with essentiality. In this respect, a specific transcriptome signature can predict gene essentiality for a given cell type. However, gene redundancy by homology or function limits the prediction power of the transcriptome analysis, and it can only be observed by analyzing the essentialome. For example, TET1 is an important demethylase with a highly-enriched expression in hPSCs and contributes to the maintenance of pluripotency. Nevertheless, it was not identified as an essential gene for the normal growth and survival of the hPSCs (Yilmaz et al., 2018), most likely because its absence is compensated for by its close homolog TET2, which is expressed at much lower levels in hPSCs.

A gene that is not specifically enriched in expression but is specifically essential in a certain cell type may suggest a cell-type selective regulatory mechanism of a commonly shared cellular process. Our analysis revealed examples of this case for cell cycle regulation, RNA splicing, and RNA binding processes in hPSCs (Figure 5A).

Studying the hPSC-essentialome can also reveal a hierarchy of essentiality because pluripotency exhibits varying degrees of sensitivity to disturbances in the functions of different essential genes. However, a combinatorial analysis between the essentialome and transcriptome also proved to be informative in identifying the core essential processes for a specific cell type, as was the case for the identification of two main interaction clusters for the self-renewal and pluripotency of hPSCs.

Although several novel reprogramming factors have been suggested in the last decade since the first demonstration of induced pluripotency in mouse and human somatic cells by transcription-factor transduction, the original Yamanaka factors are still being used as the gold-standard cocktail in the generation of iPSCs. Although these factors can drive the induction of pluripotency, only OCT4 was found to be essential in the essentiality screen in hPSCs, arguably because of functional redundancy by homologs or proteins from the same family for the other three Yamanaka factors. Indeed, potential c-MYC-like cell-cycle activators MYBL2 and MYCN were identified within the hPSC-essentialome. Similarly, instead of KLF4, other well-characterized members of the pluripotency network, e.g., PRDM14, SALL4, and DPPA3, were identified as part of the hPSC-essentialome. These observations raise the question of whether the master regulators and drivers of pluripotency can also be redefined in the context of somatic-cell nuclear reprogramming to yield iPSCs with more efficient and rapid reprogramming.

Another interesting suggestion from our protein network analysis would be that the E2F family of transcription factors and OCT4 might be sufficient to induce pluripotency because they are predicted to regulate the self-renewal- and pluripotency-related interaction clusters in the hPSC-essentialome, respectively.

In light of different individual and combinatorial analyses between the different views on pluripotency, the essentialome brings with it a new definition and a novel way of assessing pluripotency. The network of essential genes for the normal growth and survival of the hPSCs also provides a platform to study and redefine master regulators of pluripotency, as well as to study the hPSC-specific regulation of cellular processes commonly shared across many cell types.

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