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Review

Human Pluripotent Stem Cells: Current Status and Future Perspectives

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Abstract

Since human embryonic stem cells (hESCs) were first derived from preimplantation blastocysts, the advent of hESC-based therapy for a wide range of cellular degenerative diseases has been greatly anticipated. However, standard methods for hESC derivation result in embryo destruction and have been under intense ethical debate. Various alternative approaches have been devised since then generate pluripotent stem cells (PSCs) without embryo destruction. These new advances in stem cell derivation include the derivation of human-induced PSCs by introducing genetically-defined factors into adult somatic cells, the generation of hESCs from single blastomeres, and the generation of non-human primate ESCs through somatic cell nuclear transfer (SCNT). They have overcome the major problems inherent in conventional hESCs. With these advances in the derivation of new PSC types, it is essential to carefully examine the nature of the pluripotency expressed by these cells and the mechanism regulating the reprogramming process through large scale genetic and epigenetic screening. We reviewed these new methods of hPSC derivation and addressed some of the problems associated with these advances. A brief summary of new advances in genome-wide chromatin immunoprecipitation–sequencing (ChIP-seq) technology and their implications for stem cell research were provided and discussed.

Key Words: pluripotency, embryonic stem cells, epigenetic, histone modification, reprogramming

Introduction

Human embryonic stem cells (hESCs) are self-renewing, pluripotent, and have the capacity to give rise to cell types representing all three embryonic germ layers *in vitro*. Accordingly, the elucidation of the cellular and molecular mechanisms that control pluripotency and self-renewal in hESCs could lead to treatment of a wide range of human conditions arising from the loss or malfunction of specific cell types. In addition, such knowledge would also contribute to the understanding of mechanisms underlying cellular differentiation and early development. Consequently, researchers have attempted to isolate and propagate ESCs from developing mammalian embryos. To this end, ESCs that maintain pluripotency and self-renewal *in vitro* have been successfully established from embryos of various species, including human (97, 112) and non-human primates (81, 111). Conventional methods of deriving hESCs include the use of whole blastocysts or their associated inner cell mass (ICM) as the starting material (112). However, these methods also result in the destruction of the developing embryos, a step that has triggered ethical concerns with regard to human embryos. In addition to the ethical debate, ESC-based therapies are also subject *in vivo* to immune-incompatibility problems which may significantly hamper their potential clinical application. The long-held dream of stem cell biologists of deriving immune-compatible pluripotent stem cells (PSCs) without

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destroying embryos now seems closer to fruition with recent indications that it is possible to simply induce the transformation of human somatic cell types into ESC-like PSCs with defined transcription factors (110, 124). Furthermore, the recent success in deriving embryonic stem cells through somatic cell nuclear transfer (SCNT) in non-human primate (19) or single human blastomeres (56) also offer advantages in the derivation of patient and disease-specific pluripotent stem cells for both basic research purpose and long-term clinical application. Together, these advances in the generation of embryo-friendly and immune-compatible pluripotent stem cells signal a big step toward the realization of PSC-based therapy.

The potential use of human ESCs in regenerative medicine is predicated on cell availability in large numbers and their ability to be differentiated rapidly and efficiently *in vitro* into the required cell or tissue types. Although it has been demonstrated that various cell types, including hematopoietic (23, 37), cardiomuscular (63, 121), neural (65, 98), pancreatic (28, 52, 92), and germ cells (24) can be differentiated from human ESCs *in vitro*. However, available methods for direct differentiation and isolate signaling molecules responsible for stage-specific ESC development are limited. This is largely due to our ignorance of the genetic and epigenetic mechanisms underlining the induction, maintenance, and maturation of specific cell lineages derived from pluripotent stem cells. Thus, the major challenges associated with human PSC-based therapy are as follows: [1] understanding the factors responsible for the induction and maintenance of specific lineage differentiation from PSC types; [2] developing efficient approaches to produce or purify highly enriched populations of ESC progeny with the desired phenotypes; and [3] ensuring the safety and efficacy of human pluripotent ESCs or their progeny upon transfer to host animals or patients.

In this review, we will discuss recent advances in human pluripotent stem cell derivation, with a focus on reported methods providing alternative means of establishing patient- or disease-specific pluripotent stem cells without embryo destruction and associated problems. Furthermore, we will discuss recent advances in genome-wide epigenetic analysis tools for stem cell research, particularly the application of novel chromatin immunoprecipitation–sequencing (ChIP-seq) technology in the identification of factors regulating maintenance and differentiation of PSCs.

Derivation and Characterization of Human PSCs

In addition to conventional ESCs derived *via* standard procedures, several alternative strategies for deriving human PSCs have recently been proposed and realized. These alternative methods of PSC generation (Fig. 1), including hiPSCs established by introducing defined transcription factors into somatic cell types and single blastomere-derived (SBES) cells, and SCNT ESCs generated *via* somatic cell nuclear transfer, offer various advantages that overcome fundamental problems related to hPSCs derived through the standard procedures.

Human ESCs

Human ESCs were first derived from *in vitro* fertilization (IVF) produced preimplantation embryos by Thomson and colleagues in 1998 (112), using methods similar to those of Evans and Kaufman more than two decades ago in the establishment of mouse ESCs (33) (Fig. 1A). The derivation procedures normally involve culture of ICM isolated from preimplantation blastocysts by immunosurgical procedures or whole blastocysts on mouse embryonic fibroblast (MEF) feeder cells in culture media with high concentrations of fetal bovine serum (FBS) or serum replacement (24, 32). Normally, the embryos used for such research are excess embryos generated by couples undergoing assisted reproductive techniques and are donated with informed consent.

After the initial establishment, the identity of *bona fide* pluripotent stem cells is confirmed by various *in vitro* and *in vivo* assays of their capacity for pluripotency and self-renewal. Human ESCs express surface antigenic markers recognized by antibodies originally raised against pluripotent embryonic carcinoma (EC) cells (3, 59). These surface antigens include the glycolipid stage-specific embryonic antigens 3 and 4 (SSEA-3, SSEA-4), and tumor rejection antigens 1-60 and 1-81 (TRA-1-60, TRA-1-81), glycoproteins associated with a pericellular matrix keratan sulfate/chondroitin sulfate proteoglycan. Analysis of the expression of transcription factors such as Oct4 (a POU domain transcription factor) (88), Nanog (a homeobox transcription factor) (21, 82), Sox2 (a HMG-box transcription factor) (6), and their target genes, which are important in maintaining hESC self-renewal, is also critical for verification of the status of hESCs. These genes are rapidly downregulated upon differentiation but are highly expressed in undifferentiated hESCs. Like their mouse counterparts, hESCs are able to give rise to cell types representing all three embryonic germ layers both *in vitro* and *in vivo via* teratoma formation (43, 105). However, their ability to contribute to somatic and germ cell types has not yet been proven in chimeras due to ethical concerns.

The maintenance of pluripotency and self-renewal in hESCs is achieved through collaboration between various signal transduction pathways and their target gene networks. The signaling pathways required for

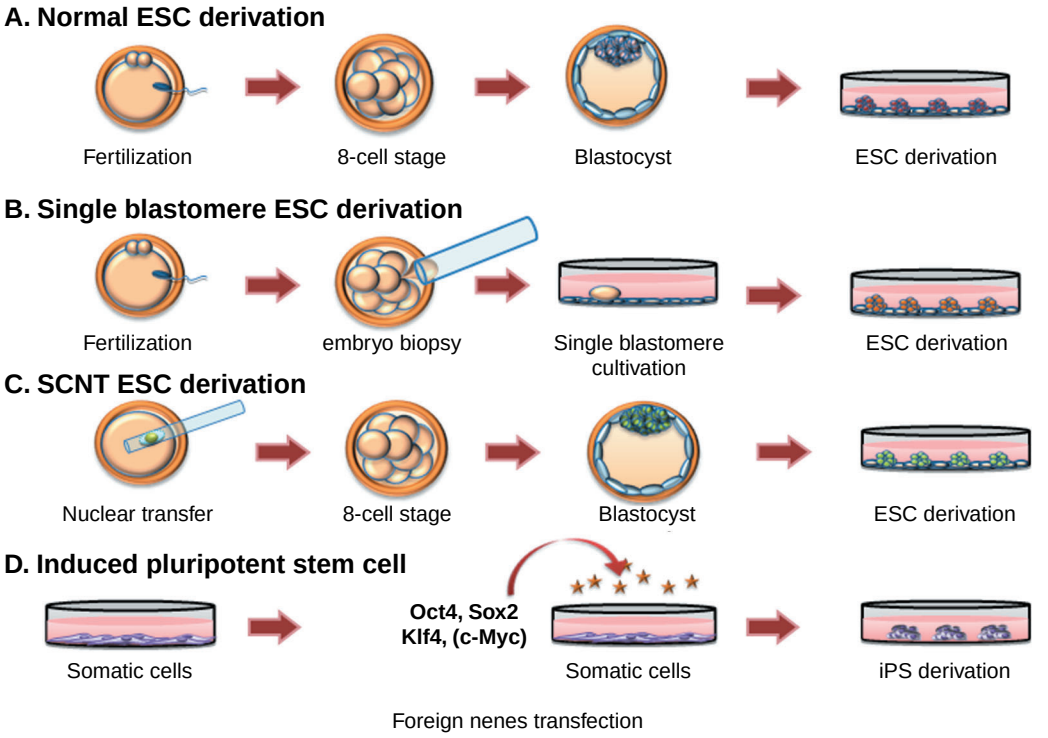

Fig. 1. Methods for derivation of human pluripotent stem cells. (A) Derivation of hESCs by the standard approaches. Conventionally, hESCs were derived from spare IVF-produced human preimplantation blastocysts by outgrowing isolated ICMS or whole embryos. (B) Human PSC lines can also be derived from single blastomeres biopsied from 8-cell stage embryos. (C) Generation of ESCs via somatic cell nuclear transfer (SCNT). Somatic cells were either fused or directly injected into enucleated oocytes to form blastocysts subsequently used for ESC derivation. (D) Generation of human iPSCs by introduction of defined transcription factors into somatic cells to reverse their status into ESC-like cells.

hESCs are known to be considerably different from mouse ESCs though self-renewal of both populations can be maintained on MEF feeder cells in standard hES medium. The Leukemia inhibitory factor (LIF) required for mouse ESC-renewal is obviously not needed for their human counterparts (27, 48), while several other factors such as fibroblast growth factor 2 (FGF2) (120), activin A/Nodal (119) and insulin-like growth factor (IGF) (9) have been identified as critical for hESC self-renewal. It has long been reported that supplementation of FGF2 during hESC culture had beneficial effects on hESC self-renewal (114, 120). However, the pathway by which FGF2 promotes hESC self-renewal remains unidentified until a recent report demonstrates that FGF2 works indirectly by promoting secretion of IGF2 and Activin/Nodal from the surrounding cells, differentiating themselves from ESCs (9). In addition to its indirect role in hESCs, FGF2 may also work directly through activation of the PI3K/AKT signaling pathway and subsequent inhibition of MEK/ERK pathway to promote self-renewal (4, 71). Furthermore, the two major branches of TGFβ signaling have contradictory effects on the maintenance of hESC self-renewal. Members of the TGFβ/Nodal/activin signaling pathway play an important role in maintaining hESC self-renewal, by promoting transcription of genes encoding transcription factors essential for self-renewal, possibly through Smads (50, 114). In contrast, signaling by members of the bone morphogenetic protein class (BMPs) promote differentiation of hESCs into extraembryonic trophoblastic lineages (120). As more information concerning the maintenance of hESCs *in vitro* is revealed, the key extrinsic self-renewal factors and signaling pathways are now beginning to emerge. The crucial role of TGFβ/Nodal/Activin, FGF2, IGF and PI3K/Akt signaling in promoting the proliferation, survival and self-renewal of ESCs has been established.

The POU domain transcription factor *Oct3/4*, the homeodomain transcription factor *Nanog* and the high-mobility group protein transcription factor *Sox2* form a crucial transcriptional network that maintains the pluripotency of hESCs. A genome-scale location analysis revealed that *Oct4*, *Sox2*, and *Nanog* work together in autoregulatory and feedforward regulatory circuitry and bind many of the genes encoding developmentally important homeodomain transcription factors in hESCs (14). This finding implies that the specialized regulatory circuits in ESCs may contribute to pluripotency and

self-renewal by activating genes required for self-renewal and repressing genes required for differentiation, though no clear evidence yet supports the hypothesis.

Single Blastomere-Derived Pluripotent Stem Cells (SBPSCs)

As a possible alternative to the isolation of PSCs from the ICM, a number of researchers have attempted to derive PSCs from single blastomeres of early pre-compacted embryos. This approach has been viewed favorably, in part for the following reasons: firstly, pre-compacted human 8-cell stage embryos are capable of developing normally following the removal of one or two blastomeres (41). The routine removal of single or double blastomeres from 6 to10-cell human embryos in pre-implantation genetic diagnosis procedures has no adverse effect on the biopsied embryo (103) and has resulted in many live births worldwide (39). Secondly, the blastomeres that originate from 2-, 4-, and early 8-cell embryos are traditionally regarded as totipotent because they are able to develop into a normal adult. Previous studies in various species, such as mice (100, 126), pigs (101), and non-human primates (22, 80), have shown that the transfer of single or paired blastomeres isolated by blastomere separation give rise to a normal conceptus and produce genetically identical animals. These findings indicated that it is possible to generate pluripotent ESCs from single isolated preimplantation blastomeres. Thirdly, the use of a single blastomere instead of a whole embryo for stem cell derivation may provide a less controversial scenario for those who disagree with the destruction of embryos. Finally, it allows the generation of immune-compatible tissue or cells for siblings born from biopsied embryos.

Most of the attempts in producing ESC-like PSCs from single embryos have involved embryos at the 2-, 4-, and 8-cell stages of embryonic development. In particular, 8-cell blastomeres from humans (56) and 2-, 4- and 8-cell mouse blastomeres (25, 115) can give rise to PSCs comparable to ESC. By co-culturing the isolated blastomeres with ICM-derived ESCs, Chung *et al*. were the first to derive pluripotent mouse ESCs from single 8-cell blastomeres (25). The ESCs derived by Chung and colleagues are PSCs as they are capable of differentiating into cells representing three embryonic germ layers both *in vitro* and *in vivo via* teratoma formation. Furthermore, they are permissive in germline transmission as evidenced by their ability to produce offspring. The following year, following their success with the mouse model, the same team reported success in deriving ESC-like PSCs from a single human 8-cell stage blastomere by a similar experimental strategy (56). *In vitro* and *in vivo* analysis of the SBESCs showed no discrepancy between human ESCs and SBESCs in surface marker and pluripotent gene expression, *in vitro* differentiation and *in vivo* developmental potency within teratoma (56).

Although the ability to derive PSCs from single human blastomeres represents a crucial advance in avoiding the ethical concerns associated with the destruction of embryos and allows the banking of autologous PSCs for children born from transferred embryos, there are obstacles left to overcome before clinical applications can be considered. Firstly, the current derivation rate of human SBESCs is low (approximately 2%) according to a report by Irina Klimanskaya and colleagues (56). The possible causes of the low derivation rate are complex. It is possible that the developmental potential of some blastomeres of human 8-cell stage embryos is already predetermined toward a non-pluripotent cell fate. Therefore, these cells will not be able to give rise to ICM cells nor subsequently form PSCs. Also, the ESC co-culture system used by Irina Klimanskaya and colleagues may not be optimal for the conversion of totipotent blastomeres into PSCs. Indeed, we have demonstrated that ESC co-culture actually compromises the efficiency of SBESC derivation because the majority of single mouse 4- or 8- cells stage blastomeres were not converted into Oct4+ ICM cells but became either Cdx2-expressing trophectoderm or Gata6-expressing primitive endoderm cells when aggregated and grown with ESCs (60). In contrast, the derivation rate of mouse SBESCs was much higher in an ESC-free culture system created by our group. Our finding has subsequently been echoed by Sayaka Wakayama and colleagues' recent works, which demonstrated an efficient procedure for deriving mouse SBESCs without ESC co-culture (115). Secondly, issues have been raised concerning whether individual human 8-cell stage blastomeres are truly totipotent and capable of generating human life. If this is the case, SBESCs are unlikely to be exempted from the ethical issues. Until now, there is no clear consensus on whether individual 8-cell stage blastomeres posses true totipotency like their earlier counterparts. Some studies have demonstrated that preimplantation cell fate is determined as early as the 2- or 4-cell stages (94, 125). Conversely, other studies show no such tendency at the same stages (45, 62).

Nevertheless, SBESCs and related cell derivation methods provide an excellent research opportunity for both stem cell biologists and developmental biologists to further understand the mechanism underlying totipotency and pluripotency and to explore means to generate autologous PSCs from non-genetic-modified material.

Induced Human PSCs by Defined Factors (hiPSCs)

In an effort to offer PSC lines that could bypass

ethical and political debates, the generation of PSCs through reprogramming or dedifferentiation of somatic cells has been sought by those who feel it is unethical to destroy human preimplantation embryos. Recently, two research teams have reported that human somatic cells can be reprogrammed into a pluripotent state by introducing different combinations of transcription factors (110, 124). By using a combination of transcription factors that they had previously used successfully in reprogramming mouse somatic cells (109) Takahashi, Yamanaka, and their colleagues at Kyoto University, Japan, demonstrate that human somatic cells can easily be directly reprogrammed to a pluripotent state like their mouse counterparts *in vitro* (110). The technique that they use for generation of human iPSCs is adapted from their mouse protocol but without selection procedures. Basically, human fibroblasts were transfected with retroviral vectors carrying human *Oct4*, *Sox2*, *Klf4*, and *c-Myc* genes and colonies resembling human ES morphology emerged from culture at approximately 30 days post retroviral transduction. Subsequently, the ES-like cells could be isolated and propagated in standard human ESC culture conditions. Meanwhile, a team lead by James Thomson of the University of Wisconsin, Madison, has reported success in reprogramming human somatic cells by introducing a different combination of 4 genes: *Oct4*, *Sox2*, *Nanog*, and *LIN28*, by lentiviral vectors (124). The advantage of Thomson's combination is that it avoids the use of the *c-Myc* gene which generated tumors, caused by its reactivation *in vivo* in a significant proportion of mice derived from mouse iPSCs. The hiPSCs created by both groups exhibit the typical characteristics of human ESCs. They not only have similar morphology and growth requirement to human ESCs but also express surface and pluripotent-related markers like their ICM-derived counterparts. Moreover, both *in vitro* differentiation and *in vivo* teratoma formation analysis indicate that hiPSCs are truly PSCs as they give rise to cell types representing all three embryonic germ layers.

The remarkable work on hiPSCs has no doubt created an attractive means for the generation of patient- or disease-specific stem cell lines. Such cell lines will offer scientists an excellent opportunity to understand the mechanism underlining various diseases and also make it possible to create customized patient-specific therapy. The potential of iPSCs for disease treatment has recently been demonstrated in a recent paper published in *Science*. Tim Townes and colleagues reported the successful use of mouse iPSCs to treat mice with sickle cell anemia, by transplantation into the *sickle* animals of hematopoietic stem cells converted from iPSCs in which the *sickle* mutation had been corrected by homologous recombination (42).

Although the creation of hiPSCs by somatic cell reprogramming provides one way to avoid controversial aspects of human ESCs and offers a promising opportunity to generate customized pluripotent stem cell for cellular transplantation, there are many hurdles to clear before they become suitable for therapeutic applications. For example, insertional mutagenesis caused by randomly inserting the required genes for reprogramming in the genome may result in tumorigenesis. Therefore, the development of procedures that can facilitate transient expression of delivered genes is currently critical for future clinical applications of hiPSCs. The genetic and epigenetic stability of hiPSC derivatives is also a concern because their response to differentiation conditions is still unknown, and it may differ considerably from conventional ESCs. The current breakthroughs with hiPSCs also raise many exciting questions regarding the mechanisms responsible for the reprogramming. Our current understanding of pluripotency is insufficient to answer all the questions raised. However, most of the concerns and questions related to hiPSCs should be resolved once we have more insight into the mechanism regulating self-renewal and pluripotency in human ESCs.

Stem Cell Chromatin – An Open State

Chromatin is a form in which DNA is packaged within the cell. The default state of chromatin structure is repressive for biological activities, such as gene transcription, DNA replication, and DNA repair, as most of the DNA is buried inside nucleosomes and packaged into higher-order structures (10, 31, 40, 44, 49, 51). Regulatory factors therefore must alter the conformation of the nucleosomes to gain access to the DNA, an activity called chromatin remodeling. A number of mechanisms are able to loosen or tighten interactions between histone octamers and DNA, including histone modifications, DNA methylation, and ATP-dependent chromatin remodeling (93, 95, 99, 102, 118). An open chromatin state is indicated by an altered higher-order structure, including accumulation of activating histone modifications such as H3 and H4 acetylation, H3K4 methylation, an abundance of chromatin-remodeling factors, and a reduction in DNA methylation (16, 35, 46). Conversely, the closed chromatin state is marked by an abundance of DNA methylation, as well as H3K9, and H3K27 methylation.

The current view of chromatin in ESCs is that they are in an open state (77, 78). As indicated in studies using several systems (5, 36, 85, 86), ESCs are richer in less-compact euchromatin, indicating global de-condensation, and are rich in plasticity. As differentiation progresses, it accumulates inactive, highly condensed heterochromatin regions. The notion that ESCs are able to generate an open chromatin state

at the genome-wide level is supported by several lines of evidence.

First, the pattern of histone modifications in ESCs is more abundant in H3 and H4, as acetylation compared to its differentiated progeny, which is consistent with changes in global genomic activity (77). For example, differentiation of human and mouse ESCs results in decreased global levels of acetylated H3 and H4 histones (54,66), which are usually associated with active chromatin regions and the progressive accumulation of compact heterochromatin. As histone acetylation is important in transcriptional activation by promoting the open state of chromatin, it can also compete with silent histone markers such as histone H3 lysine 9 di- and trimethylation (H3K9me2 and H3K9me3). These observations indicate that ESC chromatin is overall more active, or at least marked with activity-associated histone modifications, and that differentiation is accompanied by a transition to transcriptionally less-permissive chromatin.

In addition to histone hyperacetylation, manipulating the methylation of histone provides more options for keeping chromatin open in ESCs. The methylation at the H3 lysine 4 residues has been linked to transcriptional activation in a variety of eukaryotic species (29, 75). H3 lysine 4 trimethylation (H3K4me3) can be manipulated by the activities of histone methyltransferases (HMTs) and histone demethylases (HDMases). HMT-dependent H3K4 trimethylation presumably results in keeping chromatin open. In contrast, activation of HDMases leads to unmethylated H3. Interestingly, it was recently reported that unmethylated H3 is specifically recognized by a member of the DNA methyltransferases, Dnmt3L (90). Dnmt3L lacks DNA methylation activity but is able to recruit Dnmt3a to generate de novo DNA methylation, and potentially triggers heterochromatin formation (90). Conversely, methylation at lysines 9 and 27 of H3 represents repressive markers (58,107). These methylations recruit heterochromatic proteins to form heterochromatin. Therefore, removing these repressive histone markers (10, 58) signifies a possible mechanism for generating open chromatin in ESCs. A recent finding that histone demethylases of H3K9me2, H3K9me3, Jmjd1a, and Jmjd2c are positively regulated by Oct3/4 supports the notion that the euchromatic state of ESCs can be generated by excluding repressive histone markers (73, 74).

In agreement with the observation of histone modifications, the expression level of several chromatin-remodeling factors is elevated in ESCs (61). This indicates that high levels of chromatin-remodeling activity might contribute to the open state of chromatin in ESCs. It is noteworthy that several ATP-dependent chromatin-remodeling factors are essential for early embryonic development, including BRG1 (18), SNF5 (57), SSRP1 (20), and SNF2H (106). These remodeling factors belong to different families of remodeling complexes: BRG1 and SNF5 are subunits of SWI/SNF; SNF2H is the human homolog of yeast ISW1; and SSRP1 is a member of FACT complex. Significantly, although different molecular mechanisms can be used to facilitate relaxation of chromatin structure, the disruptions caused by these remodeling factors all lead to embryonic lethality at the blastocyst stage, when the inner cell mass, the source of all ESCs, is formed.

Chromatin Dynamics during Differentiation

Pluripotent ESCs have the capacity of entering any one of several distinct differentiation pathways as their genome is in a highly plastic state. It is clear that many properties of chromatin in ESCs are distinct from those in differentiated cells. As ESCs are not committed to any particular function, a possible model is that these cells express, in addition to their housekeeping genes, a minimal set of genes that are responsible for self-renewal and maintenance of pluripotency. Following differentiation, those genes that are specific for stem cells are silenced, and the expression of lineage-specific transcription factors triggers sets of lineage-specific genes (77, 96).

Emerging data point to a key role of epigenetic mechanisms in these fundamental processes of self-renewal, maintenance of pluripotency, and lineage specification (2, 13, 15, 68). Advances in creating induced pluripotent stem cells (iPSCs) using a combination of transcription factors have provided insights into the concept of epigenetic control development. Before the dramatic demonstration of creating iPSCs, somatic cell nuclear transfer (NT) was the only way to reprogram the adult nucleus and lead to successful development of cloned animals. However, both the reprogramming of NT cloning (83) and iPSCs are tremendously inefficient (89, 109, 110). Most cloned animals have epigenetic defects, particularly in DNA methylation (83). Similarly, iPSCs have been noted to bear epigenetic defects such as a lack of demethylation of the Oct4 promoter which affects the expression of the encoded transcription factors (109). The molecular mechanisms for self-renewal, maintenance of pluripotency, and lineage specification are still poorly understood (70, 113). However, recent advances in genetic and epigenetic regulation of pluripotency have begun to uncover the mysterious process of determining cell and lineage commitments during development.

Pluripotency-associated genes are stably silenced during differentiation through epigenetic mechanisms. For example, genes such as Oct4 and Nanog are silenced during ESC differentiation (34), and this process can involve both histone methylation, such as methylation

of H3K9 mediated by G9a, and DNA methylation. However, there is recent evidence for the temporary inactivation of differentiation-specific genes in ESCs. For example, lineage-specific transcription factors of Hox, Dlx, and Pax genes (7, 15, 108) are silenced in ESCs by the Polycomb group (PcG) in mice and humans. The promoters of these genes are enriched in repressive histone H3K27 trimethylation. Surprisingly, many of those non-transcribed genes in ESCs also carry chromatin markers (11) that are normally associated with active transcription, including high levels of trimethylated H3K4 (H3K4me3). Thus, both active and inactive modifications are physically present at the same or adjoining nucleosomes, termed a "bivalent chromatin structure" (7, 11). This indicates that those genes that are required for later stages are transiently held in a repressed state by histone modifications during the early stages of development, which is highly flexible and easily reversed when expression of these genes is required.

This bivalent state is resolved to a primarily active or repressive chromatin conformation with differentiation depending on the on-or-off state of the involved genes (7, 11). In differentiated cells, including T cells (7) and neural progenitors (15), H3K4me3 is present at the promoters of many transcribed developmental genes, but the opposing "repressive" marker is no longer retained. This may indicate that after the inactivating marker, H3K27me3, has been removed, these genes are automatically poised for activation through an H3K4 methylation marker. This process involves active histone demethylation to achieve this transition.

The anticipated H3K27 demethylase was very recently identified. Several groups discovered that UTX and JMJD3 are H3K27 demethylases, which perform demethylation of differentiation-specific genes, such as Hox genes (1, 30, 64, 67). Interestingly, both of the H3K27 demethylases were found in a complex with MLL, a member of H3K4 methyltransferases. This suggests a model for transcription regulation during cellular differentiation through the coordinated removal of repressive H3K27me3 and deposition of the activating marker, H3K4me3.

In addition to the dynamics of histone modifications, an ATP-dependent remodeling complex appears to be actively engaged during development. Recent studies have uncovered specific roles of the SWI/SNF complex in tissue-specific development and germ layer formation. SWI/SNF complexes have at least two sub-classes in mammals: BAF and PBAF (116, 123). The two complexes share most of their common subunits but are distinguished by four components: BAF250a and BAF250b, which are unique to BAF, and BAF180 and BAF200 both of which exclusively belong to PBAF. By ablating BAF250a in early mouse embryos, Gao and colleagues clearly demonstrated that the BAF complex has specific roles in promoting self-renewal and sustaining pluripotency in ESCs (38). The possible mechanism could be the contribution of BAF250a to proper expression of genes involved in ESC self-renewal, including Sox2, Utf1, and Oct4. They further revealed that BAF250a-mutated ESCs are defective in differentiating into mesoderm but are capable of developing into an ectoderm-specific lineage. Their results suggest that the SWI/SNF complex may work with ESC-specific transcription factors to target regulatory networks in pluripotent cells. While BAF is involved in early embryo development, the function of PBAF is little understood. BAF180 is a polycomb protein uniquely present in the PBAF complex (84, 122). In an effort to identify the function of PABF, BAF180 was shown to be critical for coronary development (47, 117). Therefore, ATP-dependent remodeling complexes may be involved in the different stages of developmental and differential processes by working with stage- or tissue-specific subunits to facilitate global or regional chromatin remodeling and achieve specific transcription networks for lineage-specific requirements.

The New Research Frontier: Searching for the Epigenetic Identity of Stem Cells

A powerful tool for analyzing patterns of chromatin variations is chromatin immunoprecipitation (ChIP), which targets genomic regions that carry a particular type of chromatin modification as well as sites of protein binding to DNA (91). With this approach, formaldehyde is usually used to locally crosslink proteins to proteins, and to DNA. The crosslinked chromatin is then fragmented by sonication or nuclease treatment, and immunoprecipitated with antibodies for a specific protein or histone modification. In this way, DNA sequences that are bound by this protein are also pulled out and, after crosslinking is reversed, can be analyzed by quantitative polymerase chain reaction (qPCR). qPCR detection is effective and convenient, but is limited to loci that are selected for study.

The last few years have seen the rapid development of technologies for deciphering chromatin modifications in the context of the entire genome. Two new approaches have expanded the coverage of the analysis. The first one, designated ChIP-chip, takes advantage of high-resolution genomic tiling microarrays (15, 73). Tiling microarrays contain short DNA oligonucleotides that represent a nearly continuous genome. The immunoprecipitated DNA fragments are hybridized to these microarrays, and the regions bound by a particular antibody target are identified by probes that show significant enrichment compared to control DNA.

In the last few years, the ChIP-chip method has provided high-resolution and genome-wide views of histone modifications (12, 55, 76).

However, ChIP-chip usually requires total amplification and labeling with fluorescent dyes before hybridization, a process which can introduce bias (17, 87). Moreover, the complexity of higher eukaryotic genomes makes it difficult to design unique oligonucleotide probes for a large number of regions. Therefore the resolution and coverage are limited.

In an alternative approach, ChIP-seq (Fig. 2), the immunoprecipitated DNA is directly analyzed by high-throughput sequencing (8, 79). Recent studies have used next-generation sequencing (NGS), which allows millions of short DNA sequence tags to be assigned to individual transcription factors or histone markers (53, 79). Those short threads of DNA sequences from antibody precipitation can serve as molecular tags to uniquely locate the genomic positions of the immunoprecipitated DNA fragments. Sophisticated statistical algorithms are required to interpret the locations of the short DNA sequences on the complete genomic sequence. The statistically enriched genomic regions are thus identified as potential sequences associated with antibody targets.

Two very recent reports simply demonstrated that epigenomic sequencing has come of age. Both independent reports presented the DNA methylation pattern of *Arabidopsis* using NGS technology at a single-base resolution (26, 72). Mapping of DNA methylation at single-base resolution reveals that the local sequence context has a strong effect on cytosine methylation, which was not evident from previous studies that mapped methylation in *Arabidopsis* using tiling arrays. To further exhibit the capability of NGC, Jacobsen and coworkers demonstrated that their newly developed library construction and computational methods can be applied to large genomes such as that of the mouse (26).

It would also be interesting to compare global profiles of DNA methylation and histone modifications in human ESCs and investigate their changes in the course of differentiation. This information will be extremely useful as a basis for novel strategies for modifying the differentiation of hESCs to produce functional cells for therapy, and perhaps to minimize their tumorigenic potential.

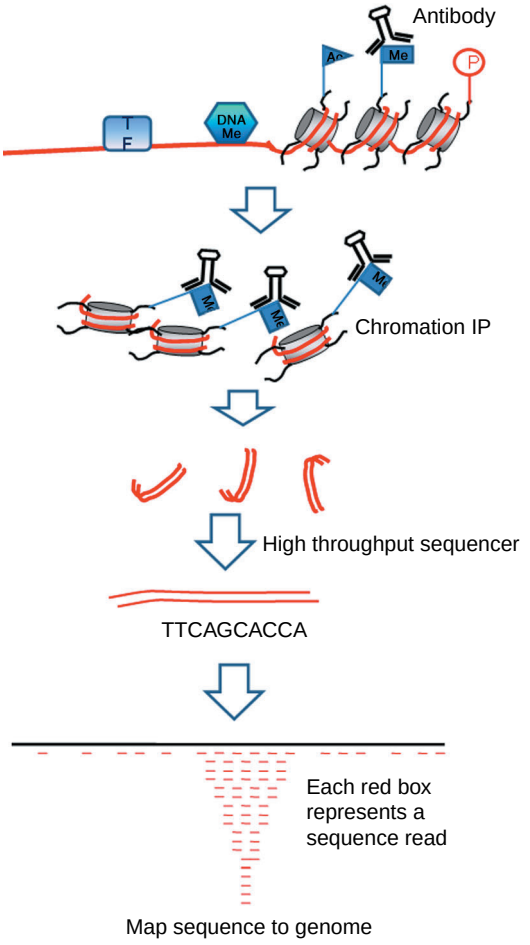

Fig. 2. Generalized scheme of ChIP-Seq. An initial ChIP experiment is followed by standard preparation for high-throughput sequencing. The final panel shows a cluster of individual sequence reads that map back to the same region of the genome and point to a transcription factor or chromatin modification.

Conclusions and Prospects

Mammalian development requires the specification of over 200 cell types from a single totipotent cell. Investigations of the regulatory networks that are responsible for pluripotency in embryo-derived stem cells and of how cells maintain the balance between self-renewal and differentiation are fundamental in understanding mammalian development and realizing the therapeutic potential of PSCs. Our emerging knowledge of the pathways that regulate proliferation, self-renewal, and survival of embryonic stem cells, and lineage-committed stem cells has been obtained from pluripotent ESC lines derived from the ICM. However, our understanding of the genetic and epigenetic bases of pluripotency in iPSCs, SCNT ESCs, and other PSCs derived from embryos and the relationship between these cells is still relatively limited. Considerable further study will be needed to unveil the discrete mechanisms that sustain cell identities among different PSC types, and hopefully this will also shed light on mechanisms of cancer and suggest better modes of treatment.

Given the technical features of ChIP-Seq (high throughput, low cost and input requirement), it is now appropriate to consider the generation of catalogues

of chromatin-state maps representing a wide range of human and mouse cell types. These should include cells of varied developmental stages and lineages, from totipotent to terminally differentiated, with the aim of precisely defining cellular states at the epigenetic level and observing how they change over the course of normal development.

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