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Totipotency, Pluripotency and Nuclear Reprogramming

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Abstract Mammalian development commences with the totipotent zygote which is capable of developing into all the specialized cells that make up the adult animal. As development unfolds, cells of the early embryo proliferate and differentiate into the first two lineages, the pluripotent inner cell mass and the trophectoderm. Pluripotent cells can be isolated, adapted and propagated indefinitely in vitro in an undifferentiated state as embryonic stem cells (ESCs). ESCs retain their ability to differentiate into cells representing the three major germ layers: endoderm, mesoderm or ectoderm or any of the 200+ cell types present in the adult body. Since many human diseases result from defects in a single cell type, pluripotent human ESCs represent an unlimited source of any cell or tissue type for replacement therapy thus providing a possible cure for many devastating conditions. Pluripotent cells resembling ESCs can also be derived experimentally by the nuclear reprogramming of somatic cells. Reprogrammed somatic cells may have an even more important role in cell replacement therapies since the patient's own somatic cells can be used for reprogramming thereby eliminating immune based rejection of transplanted cells.

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In this review, we summarize two major approaches to reprogramming: (1) somatic cell nuclear transfer and (2) direct reprogramming using genetic manipulations.

Keywords Embryonic stem cells, iPS cells, Pluripotent, Somatic cell nuclear transfer, Totipotent,

Contents

1 Totipotency

Totipotency is defined in Wikipedia as the ability of a single cell to divide and produce all the differentiated cells in an organism, including extraembryonic tissues. Totipotent cells formed during sexual and asexual reproduction include spores and zygotes. In some organisms, cells can dedifferentiate and regain totipotency. For example, a plant cutting or callus can be used to grow an entire plant. Mammalian development commences when an oocyte is fertilized by a sperm forming a single celled embryo, the zygote. Consistent with the definition, the zygote is totipotent, meaning that this single cell has the potential to develop into an embryo with all the specialized cells that make up a living being, as well as into the placental support structure necessary for fetal development. Thus, each totipotent cell is a self-contained entity that can give rise to the whole organism. This is said to be true for the zygote and for early embryonic blastomeres up to at least the 4-cell stage embryo (see Fig. 1). Experimentally, totipotency can be demonstrated by the isolation of a single blastomere from a preimplantation embryo and subsequently monitoring its ability to support a term birth following transfer into a suitable recipient. This approach was pioneered in rats and has been realized in several mammalian species including nonhuman primates [1–4]. In the latter case, we confirmed the ability of isolated blastomeres from 2- and 4-cell stage, IVF produced embryos of the rhesus monkey to support term pregnancies and to produce live animals [5]. As embryo development progresses to the 8-cell stage and beyond depending on the species, the individual blastomeres that comprise the embryo gradually lose their totipotency. It is generally believed that this restriction in developmental potential indicates irreversible differentiation and specialization of early embryonic cells into the first two lineages, the inner cell mass (ICM) that includes cells that will give rise to the fetus and the trophectoderm (TE), and an outer layer of cells that is destined to an extraembryonic fate (Fig. 1).

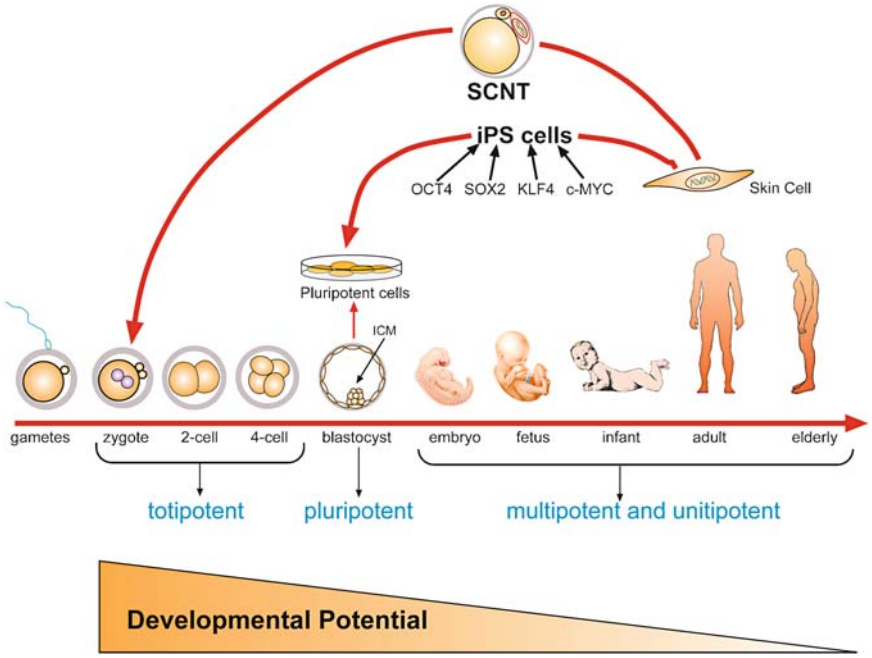

Fig. 1 Development and reprogramming. Ontogeny begins from a single cell, the zygote. The zygote and each blastomere of the early embryo are totipotent with the potential to develop into the whole organism. As development unfolds, the developmental potential of individual blastomeres gradually declines resulting subsequently in pluripotent, multipotent, unipotent and terminally differentiated somatic cells. However, developmental potential of somatic cells can be reinstated to the totipotent stage by SCNT or to the pluripotent state by direct reprogramming

A complication in assessing the state of potency of blastomeres isolated from more advanced stages of development is insufficient cytoplasmic volume. Thus, although the blastomeres may in fact be totipotent, embryonic development of relatively small isolated blastomeres arrests at or near the time of blastulation. Recall that the zygote and early blastomeres undergo several unusual mitotic or cleavage divisions that are not accompanied by a corresponding growth of cytoplasm, that is, there is no change in embryo size despite the presence of more cells or blastomeres and each individual blastomere becomes smaller. The embryonic genome at these early stages is transcriptionally quiescent and development is regulated by maternally inherited factors present at the time of fertilization in the oocyte [6]. The transition in developmental regulation with activation of the embryonic genome and a complete loss of dependence on oocyte factors occurs before the blastocyst stage in a species-specific manner. Additionally, by the late morula or early blastocyst stage the embryo ceases cleavage divisions and resumes normal mitotic divisions with concomitant increases in cell volume during the S-phase. The likelihood that early blastomeres retain totipotency for a major part of preimplantation development but experimentally we cannot prove it is directly supported by the fact that the addition of oocyte

cytoplasm to a blastomere of the 8- to 16-cell stage embryo can restore, or perhaps more appropriately allow expression of, its full developmental potential. This approach, embryonic cell nuclear transfer, has been employed in the monkey to demonstrate the totipotency of 8- to 16-cell stage blastomeres whereby reconstructed embryos when transferred to a recipient resulted in a term birth [7].

It is also known that conglomerates of embryonic cells at a later stage of development can develop into an organism. An experimental manipulation that supports this concept involves blastocyst splitting. Cutting the embryo into halves with an approximately equal distribution of TE and ICM cells can lead to the production of viable infants [5, 8]. Obviously, embryo splitting that creates demi embryos with highly distorted ratios of ICM to TE cells is inconsistent with the production of live births.

2 Pluripotency

The Wikipedia definition in the broad sense means "having more than one potential outcome." In cell biology, the definition of pluripotency has come to refer to a stem cell that has the potential to differentiate into any of the three germ layers: endoderm, mesoderm or ectoderm. Pluripotent stem cells can give rise to any fetal or adult cell type. However, a single cell or a conglomerate of pluripotent cells cannot develop into a fetal or adult animal because they lack the potential to organize into an embryo. In contrast, many progenitor cells that are capable of differentiating into a limited number of cell fates are described as multipotent. Somatic stem cells such as neural, bone marrow-derived, or hematopoietic cells would fit into this latter category.

At least some of the embryo's ICM cells are pluripotent, meaning that they can form virtually every somatic and germ cell type in the body. These ICM cells are self sustained and their pluripotency is maintained by endogenously expressed factors. In vivo, pluripotent cells within the ICM exist transiently; as the developmental program unfolds they differentiate into cells of the next embryonic or fetal stage. However, they can be isolated, adapted and propagated in vitro in an undifferentiated state as embryonic stem cells (ESCs) [9, 10]. ESCs were first derived in 1981 from the ICM of the inbred mouse by Martin [10] and Evans and Kaufman [9]. In 1998, ESCs were successfully isolated from surplus, IVF-produced human embryos [11].

ESCs express specific markers or characteristics similar but not identical to the transient pluripotent cells of an embryo. This includes stage specific embryonic antigens, enzymatic activities such as alkaline phosphatase and telomerase, and "stemness" genes that are rapidly down-regulated upon differentiation, including *OCT4* and *NANOG*. Under specific conditions, ESCs can proliferate indefinitely in an undifferentiated state, suggesting that the transcriptional activity and epigenetic regulators capable of supporting pluripotency can be maintained in vitro in ESCs. However, when released from the influence of these culture conditions or following their introduction back into a host embryo, ESCs retain their ability to differentiate into any cell-type, just like ICM cells. Alternatively, they can differentiate in vivo in teratomas into cells representing the three major germ layers: endoderm, mesoderm

and ectoderm or they can be directed to differentiate in vitro into any of the 200+ cell types present in the adult body. Since many human diseases result from defects in a single cell type, pluripotent human ESCs may become an unlimited source of any cell or tissue type for replacement therapy thus providing a possible cure for many devastating diseases.

Parenthetically, one of the challenges before clinical transplantation studies involving hESCs can begin concerns the immune response anticipated after transplantation [12, 13]. Human ESCs are routinely derived from IVF embryos and transplantation of such cells into genetically unrelated patients will incite an immune response and result in rejection. Histocompatibility is one of major unsolved problems in transplant medicine. Rejection of unmatched transplanted tissues is provoked by alloantigens present on graft tissues by the recipient's immune system. The alloantigens or antigenic proteins on the surface of transplant tissues that mostly cause immune rejection are the blood group antigens (ABO) and the major histocompatibility complex (MHC) proteins, also designated in humans as human leukocyte antigens (HLA). Matching donor and recipient HLA types is important to reduce a cytotoxic T-cell response in the recipient, and subsequently improve the chances of survival of the transplant. However, tissue or organ transplantation from one individual to another is a daunting task due to the existence of two classes of HLA molecules (Class I, and II), each encoded by multiple genes and most importantly, each of these genes represented by multiple alleles. For example, there are 22 different alleles identified so far for the class I HLA-A gene and 42 alleles for HLA-B. Thus, due to HLA polymorphism, the chances of finding a donor–recipient match based on just a few HLA genes (HLA-A, -B, and -DR) could be one in several million [14]. Therefore, the need for developing approaches for deriving histocompatible pluripotent cells is commonly recognized.

3 Nuclear Reprogramming

Hochedlinger and Jaenisch define nuclear reprogramming as the reversal of the differentiation state of a mature cell to one that is characteristic of the undifferentiated embryonic state [15]. Let us first look at the forward process of development and differentiation. It is now generally recognized that genetic material is usually not lost during development and differentiation. Consequently, the process of differentiation must reflect the expression at each stage of a unique cohort of specific genes, its transcriptome, and it now appears that such differential expression is determined or regulated by reversible epigenetic changes gradually imposed on the genome during development.

Epigenetic mechanisms that have been implicated in the regulation of differential gene activity include modifications to the histones (such as acetylation, methylation, phosphorylation, ubiquitination, and ADP-ribosylation) and methylation of DNA at CpG dinucleotides (see reviews by [16, 17]). These specific epigenetic modifications regulate expression or silencing of genes at the level of transcription, mediated by

the level of packaging DNA into chromatin. For example, acetylation of histones H3 and H4 and methylation of H3 at the lysine 4 position (H3 Lys-4) unfolds and loosens up the DNA template and makes it accessible to transcription factors. Thus, these epigenetic mechanisms are generally associated with active gene transcription. Conversely, methylation of H3 Lys-9 and H3 Lys-27 induce DNA compaction and subsequently gene silencing.

In this review, we will summarize two major approaches to nuclear reprogramming or reversing the developmental process: (1) somatic cell nuclear transfer (SCNT) and (2) direct reprogramming using genetic manipulations. It should be noted that interest in both of these strategies derives, in large measure, from the potential production and use of histocompatible human ESCs in regenerative medicine.

4 Epigenetic Reprogramming by SCNT

The concept of reprogramming of a patient's somatic cells into pluripotent ESCs was conceived based on two independent breakthroughs in the field of developmental biology in the late 1990s: success with cloning of animals by SCNT [18, 19] and derivation of human ESCs [11]. SCNT, or cloning, dates back to 1962 when John Gurdon first demonstrated that somatic cells from *Xenopus laevis* could be reprogrammed back into an early embryonic state by factors present in an egg cytoplasm and support development of an adult frog [20]. Thus, it became clear that the cytoplasm of the oocyte has the ability to reprogram gene expression and that a single somatic cell nucleus has the capacity to yield a whole new organism [21].

Research in SCNT involving other vertebrates including mammals continued for several decades and culminated in groundbreaking announcements, first in 1996 [18] and then in 1997 [19] that sheep could be produced by SCNT using fetal and adult somatic cells. This accomplishment was quickly reproduced in other mammals including mice [22], cattle [23, 24], pigs [25], goats [26], rabbits [27], cats [28], mules [29], horses [30], rats [31], and dogs [32].

As mentioned above, ESCs were first derived in 1981 in the mouse [9, 10]. Exploiting the ability of mouse ESCs to contribute to germ-line chimeras and homologous recombination technology for the creation of knock-out mice and mammalian gene function analysis revolutionized the field of experimental biology [33]. To date, an estimated 10,000 mutated mice have been generated worldwide using the gene targeting technique. In recognizing their enormous contribution to the advances in every field of biology and medicine, the 2007 Nobel Prize in Physiology and Medicine was awarded to three scientists who pioneered the derivation of mouse ESCs and gene targeting [34].

The establishment of mouse ESCs has instigated similar studies in other mammals. Working with nonhuman primates, James Thomson of the Wisconsin National Primate Research Center reported in 1995 the successful isolation of ESCs from rhesus macaque, in vivo flushed blastocysts [35]. Unlike mouse ESCs, monkey ESCs grew as flat colonies and expressed slightly different surface markers than did mouse

cells. Primate ESCs are relatively cumbersome to maintain and manipulate, requiring considerable technical expertise and attention confounded by a requirement for manual passaging and their slow growth rate. Nevertheless, these cells were indeed pluripotent and capable of differentiating into cell types of all three germ layers. ESCs were also successfully isolated in other nonhuman primate species including marmosets and cynomolgus macaques [36, 37]. In the rhesus macaque, an additional 25 cell lines were produced from in vitro produced embryos at the Oregon National Primate Research Center [38]. In 1998, following protocols and markers developed in the monkey, the isolation of ESC lines from surplus IVF-produced human embryos was reported [11]. Subsequently, approximately 65 human ESC lines were approved in the United States for Federal research support in August of 2001; however only a few of those lines are currently available and under study (http://stemcells.nih.gov/research/registry/).

Despite the remarkable strides that have been made to date with mouse and primate ESCs, success in other species has been limited. ESC-like cells have been described in several species including sheep [39], cattle [40], pigs [41], rabbits [42] and rats [43]. However, the pluripotency of these cells and their ability to maintain an undifferentiated phenotype over long term culture remains questionable.

The conceptual unification of SCNT and embryonic stem cell derivation technology suggested that it might be possible to produce preimplantation human embryos by SCNT and then derive isogenic embryonic stem cells from the resulting SCNT embryos [44, 45]. Human ESCs produced by this approach called "therapeutic cloning" would subsequently be differentiated into therapeutically useful cells and transplanted back into a patient suffering from a degenerative disease. The proof of the concept was first demonstrated in the mouse in 2000 with the isolation of pluripotent ESCs from adult somatic cell nuclei [46]. These SCNT-derived ESCs expressed canonic pluripotent markers and were able to differentiate readily into various somatic cell types in vitro or in vivo in teratomas and chimeras. Despite multiple abnormalities observed in cloned offspring, mouse ESCs derived by SCNT were transcriptionally indistinguishable from their counterparts derived from fertilized embryos [47, 48], consistent with the notion that ESCs derived from reprogrammed somatic cells have an identical therapeutic potential with "wild type" IVF-derived ESCs. This exciting scientific advance indicated that it may soon be possible to provide patients with pluripotent cells tailored for a given therapeutic purpose.

However, despite this remarkable progress, the feasibility of therapeutic cloning in primates remained questionable. Early attempts demonstrated that human and nonhuman primate SCNT embryos were unable to develop efficiently into blastocysts and typically arrested at early cleavage stages [49, 50]. This indicated an inability of primate SCNT embryos to activate embryonic genes and sustain the developmental program, possibly due to lacking or incomplete nuclear reprogramming. These challenges along with retraction of two high profile papers that contained fabricated data on human SCNT [51] significantly dampened scientific enthusiasm. The ability to derive primate ESCs by SCNT until recently was uncertain.

We initially reported incomplete nuclear remodeling following standard SCNT in the monkey, including nuclear envelope breakdown (NEBD) and premature

chromosome condensation (PCC), and correlated this observation with a decline in maturation promoting factor (MPF) activity [52]. Although, a direct link between NEBD, PCC and successful reprogramming was not clear, we presumed that remodeling could be particularly beneficial for efficient nuclear reprogramming by allowing access of reprogramming factors to the somatic cell's chromatin. We introduced several modifications to SCNT protocols that prevented MPF decline and induced robust NEBD and PCC. Importantly, these modifications resulted in improved SCNT embryo development and significantly increased blastocyst rates, suggesting that MPF activity is essential for efficient nuclear reprogramming. The modified protocols allowed routine production of SCNT blastocysts from various donor somatic cells providing the foundation for rapid advances in the derivation of ESCs. More recently, we succeeded in the derivation of two ESC lines from rhesus macaque SCNT blastocysts using adult male skin fibroblasts as nuclear donors (Fig. 2) [53]. DNA analysis confirmed that nuclear DNA was identical to donor somatic cells and that mitochondrial DNA originated from oocytes. Both cell lines exhibited normal ESC morphology, expressed key stemness markers, were transcriptionally similar to control ESCs and differentiated into multiple cell types in vitro and in vivo. These results represent a significant advancement in understanding the role of nuclear remodeling events in reprogramming following SCNT and demonstrate the first successful reprogramming of adult primate somatic cells into pluripotent ESCs. Currently, we are focused on further improvements in reprogramming by SCNT and efficient derivation of ESCs in the nonhuman primate model. In our initial report, the efficiency of this approach was quite low, requiring approximately 150 oocytes to produce a single ESC line [53]. However, based on our current SCNT outcomes yielding nearly threefold higher blastocyst development and ESC derivation rates over our previously reported efficiency, as few as ten or less monkey oocytes are required to produce one ESC line (Mitalipov, unpublished results). These results suggest that systematic optimization of SCNT approaches to define critical reprogramming factors will likely succeed in the efficient generation of patient-specific ESCs for therapeutic applications.

Our recent data also strongly support the notion that oocyte-induced reprogramming of primate somatic cells results in complete erasure of somatic memory and the resetting of a new ESC-specific epigenetic state. Imprinted gene expression, methylation, telomere length and X-inactivation analyses of SCNT-derived primate ESCs were consistent with accurate and extensive epigenetic reprogramming of somatic cells by oocyte-specific factors ((Mitalipov, unpublished results).

A variation on the SCNT theme that has received recent attention is called altered nuclear transfer (ANT). Reprogramming by oocyte-specific factors after SCNT employs endogenous epigenetic pathways/programs. Thus SCNT provides a paradigm for identification of natural epigenetic factors in an egg that accompany nuclear reprogramming and promotes utilization of these factors for direct reprogramming. However, utilization of an SCNT approach for reprogramming of human somatic cells into pluripotent ESCs poses ethical concerns since it involves the creation and subsequent destruction of preimplantation stage embryos with potential for full-term development. Thus, ANT proposes the creation of pluripotent stem cells by preemptive

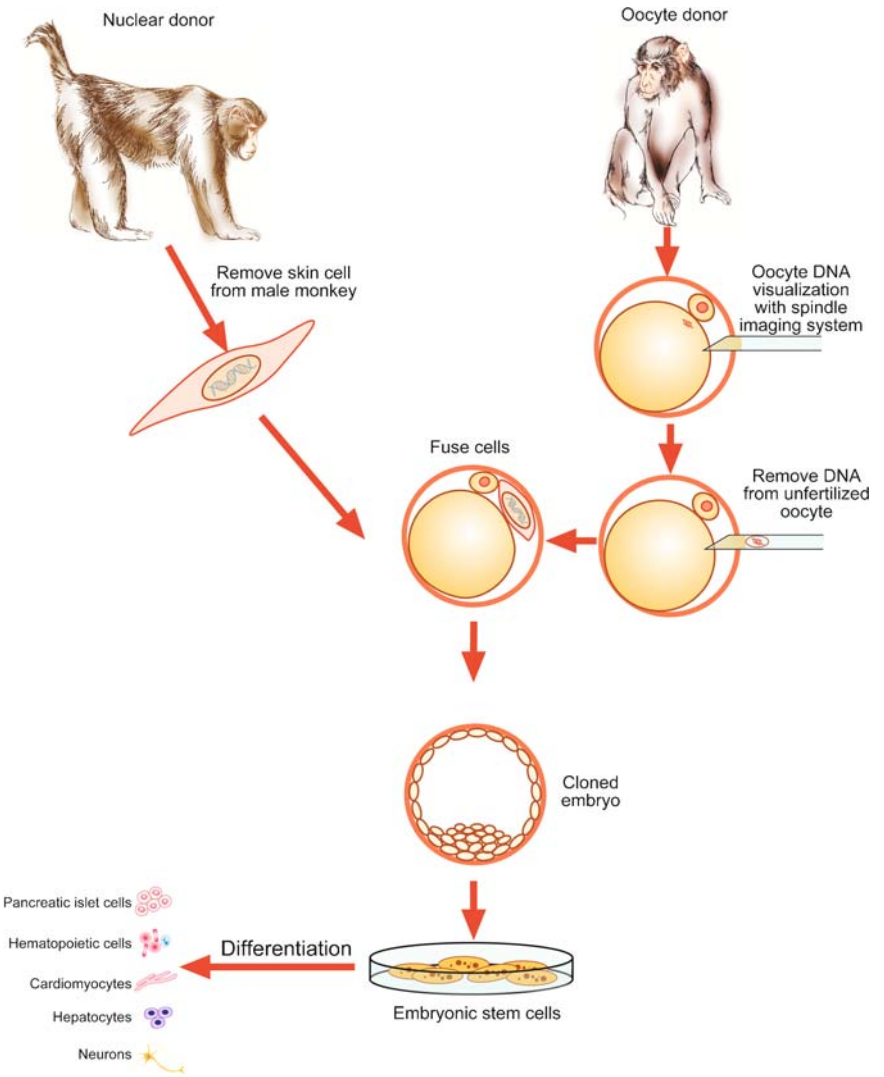

Fig. 2 A schematic diagram showing experimental steps in reprogramming of adult primate somatic cells into pluripotent embryonic stem cells via SCNT. A donor nucleus from a skin cell was introduced into an enucleated oocyte and the resulting embryo gave rise to embryonic stem cells (copied from [53], supplementary information)

alterations prior to SCNT insuring that no totipotent embryo is involved [54, 55]. These alterations should preclude the integrated organic unity and developmental potential that are the defining characteristics of a living organism, while still allowing the formation of the ICM cell lineage from which pluripotent stem cells can be derived. ANT proposes to alter the nucleus of a somatic cell and/or the cytoplasm of an enucleated oocyte prior to SCNT that would prevent formation of a totipotent zygote. However,

residual oocyte factors should be capable of reprogramming an introduced nucleus with subsequent development to a stage that would support pluripotent stem cell isolation in the absence of a trophectodermal lineage.

Mature metaphase II (MII) oocytes are one of the largest cells produced by the human body. They contain key maternally inherited transcriptional and epigenetic factors essential for "natural" reprogramming of highly specialized gametic genomes into totipotent and pluripotent cells. Therefore, it is not surprising that oocyte-specific factors are able to reprogram transplanted somatic nuclei, although with less efficiency than that which occurs in the embryo after fertilization. As indicated above, maternally inherited factors in the oocyte convert a transcriptionally-quiescent embryonic genome into an active one during early embryonic development and following embryonic genome activation, control of the developmental program is gradually shifted to embryonic factors. Among these maternal transcription factors, whose functions have been well defined, are Oct4 and Sox2, which are both essential for formation of the ICM in mouse preimplantation embryos. Cdx2 plays a similar role in the development of TE. In early cleavage-stage mouse embryos these transcription factors are expressed in all blastomeres. At the blastocyst stage, Oct4 and Sox2 are detected exclusively in the ICM cells, while Cdx2 is confined to the TE [56]. The role and expression pattern of these factors is poorly studied in other species including primates. However, we have shown a similar expression profile for OCT4 in monkey preimplantation embryos [57]. The homeodomain protein, Nanog, is also detected exclusively in the ICM of mouse embryos and cooperates with Oct4 and Sox2 to control a set of target genes that have important functions in maintaining pluripotency and ICM formation. Currently, little is known about maternal epigenetic factors that induce histone modifications and DNA methylation. Recent work suggests that expression of Nanog in embryos may be regulated by the histone arginine methyltransferase Carm1 [58]. Interestingly, overexpression of Carm1 upregulates both Nanog and Sox2 and was able to direct development of individual blastomeres into an ICM fate.

As stated above, Cdx2 is one of the earliest known transcription factors that is essential for formation and function of the TE lineage [56]. Cdx2-deficient mouse embryos fail to maintain a blastocoel and cannot form the TE, but nonetheless, development of the pluripotent lineage of the ICM is relatively unaffected [59, 60]. Additional evidence for a key role of Cdx2 comes from Tead4 knockout embryos which are devoid of both the TE lineage and Cdx2 expression [61, 62]. Interestingly, Cdx2-deficient ICMs can generate functional ESCs. Recent evidence also suggests that somatic cells lacking Cdx2 can be used for SCNT, resulting in formation of the single ICM lineage suitable for isolation of ESC lines [63]. This demonstrates that inhibition of TE specific factors during SCNT can significantly alter the developmental program and prevent formation of a totipotent embryo without compromising reprogramming to the pluripotent state, thus providing a scientific basis for the ANT concept.

On the other hand, Cdx2-deficient nuclei in this study were complemented by maternal factors including Cdx2 before the onset of embryonic genome activation. Therefore, SCNT embryos were not obviously abnormal until the

maternal-to-embryonic transition point. To solve this ethical dilemma, maternal Cdx2 transcripts must be inactivated as well. Moreover, it remains to be determined whether this approach will work in other species including primates.

5 Direct Reprogramming

Possibly one of the greatest developments in the stem cell research field in the past 2 years is the discovery that introduction and ectopic expression of several genes can induce pluripotency in somatic cells. A research group led by Shinya Yamanaka of Kyoto University found that murine somatic cells transduced with retroviral vectors carrying only four transcription factors, namely Oct4, Sox2, c-Myc and Klf4 can revert their epigenetic state to become ESC-like [64]. These cells termed induced pluripotent stem (iPS) cells were similar in their properties to ESCs in terms of marker expression, transcriptional activity and the ability to differentiate into a variety of cell types in chimeras. The relative simplicity with which iPS cells can be generated compared with SCNT makes this technique an attractive approach for studying the principles of nuclear reprogramming and also to evaluate their potential for clinical applications. Indeed, mouse iPS cells were quickly developed in several laboratories and have recently been used to successfully treat sickle cell anemia in mice [65].

In November of 2007, two independent groups led by Shinya Yamanaka and James Thomson reported that using a similar transduction approach they were able to generate iPS cells from human somatic cells [66, 67]. These human ESC-like cells also expressed markers of ESCs and were capable of differentiating into cell types of all three germ layers. Yamanaka's group used the same quartet of four factors that worked in the mouse, while the Thomson lab demonstrated that a slightly new combination, OCT4, SOX2, NANOG and LIN28 can also generate human iPS cells. It is interesting to note, that the efficiency of reprogramming was lower with adult somatic cells than with cells of fetal or embryonic origin. Moreover, some adult somatic cell-derived iPS cells did not contribute to all cell types following differentiation in teratomas. It is likely that there could be additional factors that may enhance production of iPS cells from adult somatic cells. Indeed, a recent report suggests that hTERT and SV40 large T can enhance the reprogramming efficiency of Yamanaka's factors on human adult somatic cells [68].

Recent reports also suggest that the kinetics of reprogramming significantly differs between iPS and SCNT approaches. Direct reprogramming of somatic cells to iPS cells appears to be a much slower process with activation of the endogenous Oct4 or Nanog in the mouse observed on day 16 post-transduction [69]. In contrast, Oct4 expression in mouse SCNT embryos can be detected after the 4-cell stage or on day 2 after SCNT [70].

The direct genetic manipulation of somatic cells into iPS cells carries an advantage over SCNT since it does not produce totipotent cells and does not require human eggs. From a bioethical viewpoint this approach would resolve concerns about producing

and destroying human embryos. However, this approach currently has serious limitations as a source of cells for regenerative medicine. Reprogramming using c-Myc results in tumor development in approximately 20% of chimeric mice derived by injection of iPS cells [71]. Recent findings suggest that c-Myc is not absolutely necessary for iPS cell induction, although it appears that reprogramming efficiency is much lower when the oncogene is omitted [72]. Another concern is that introduction of multiple copies of transgenes may cause insertional mutations and disrupt the function of many endogenous genes. Continuous overexpression of transgenes is also problematic due to the possibility of incomplete silencing of these transgenes during differentiation. The residual incidence of even a few pluripotent cells in transplanted tissues may cause tumors. Although the retroviral-delivered genes are silenced in most iPS cells, there is the likelihood of reactivation of these transgenes in differentiated cells and the possibility of spontaneous reversion of transplanted cells back to the pluripotent state, leading to the risk for malignant progression.

To avoid these pitfalls each patient-specific iPS cell line must be rigorously tested in animal models before therapeutic applications. These concerns suggest that further advances in the derivation of iPS cells without gene transfer will be required to overcome these problems. In the near future, novel reprogramming approaches that involve transient gene delivery system or small molecules may prove to be a safer way of generating iPS cells suitable for clinical applications. It will be necessary to carry out a detailed analysis of iPS cells to understand fully the mechanisms of reprogramming and their role in regenerative medicine. It is also essential to continue to study SCNT-induced reprogramming and to compare carefully the properties of iPS cell lines to those derived by SCNT.

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