19

New Techniques in the Generation of Induced Pluripotent Stem Cells

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1. Introduction

Pluripotent stem cells have the ability to differentiate into cells of the three primary germ layer lineages, ectoderm, mesoderm and endoderm. The most studied type of pluripotent stem cells are embryonic stem cells (ESC), cells derived from the inner cell mass of embryos at the blastocyst stage of development (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). The pluripotent property of human embryonic stem cells (hESC) makes them useful for the development of cellular therapies to replace diseased or degenerated cells in the body. Moreover, hESC also possess the ability to propagate indefinitely *in vitro* while maintaining a normal karyotype, and thus can provide an unlimited source of cells for the development of cell replacement therapies (Pera et al., 2000). However, one of the major hurdles in hESC research has been the ethical implications of using stem cells derived from embryos. Furthermore, generation of patient-specific stem cell lines may overcome some of the issues associated with immuno-compatibility in cell replacement therapy.

The breakthrough studies conducted by Shinya Yamanaka's group demonstrated direct reprogramming of mouse or human fibroblasts back to pluripotent cells, creating so-called induced pluripotent stem cells (iPSC) (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Studies of iPSC revolutionized stem cell research by creating a more reproducible method to generate sufficient amounts of patient-specific pluripotent cells and bypassing the ethical implications surrounding research utilizing human embryos. iPSC also provide an alternative approach to generate disease-specific lines for mechanistic studies in disease modeling, as well as high throughput screening for drug discovery or toxicology studies (Amabile and Meissner, 2009). As the area of iPSC research is rapidly evolving, this review aims to summarize and discuss the current techniques used for the generation of iPSC.

2. Reprogramming factors used in generation of induced pluripotent stem cells (iPSC)

The initial derivation of iPSC by Shinya Yamanaka's group was achieved by overexpressing four transcription factors first in mouse and then human fibroblasts, namely Octamer-binding transcription factor 4 (Oct4), Sex-determining region Y HMG box 2 (Sox2), Krüppel-like factor 4 (Klf4) and v-myc myelocytomatosis viral oncogene homolog (c-Myc), often

referred to as the 'Yamanaka factors' (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Alternatively, a study from James Thomson's lab identified a different combination of factors for the generation of human iPSC, using Oct4, Sox2, Nanog and Lin28 (Yu et al., 2007). Subsequent reports from many labs have contributed to a growing list of reprogramming factors used for iPSC generation, including Estrogen-related receptor beta (Esrrb), Sal-like 4 (Sall4), microRNAs (miRNA), simian virus 40 large-T (SV40LT) antigen and human telomerase reverse transcriptase (hTERT). This section will provide a background of our understanding of these reprogramming factors in regulating the cell fate of pluripotent stem cells and discuss their role during direct somatic cell reprogramming. Other strategies to enhance reprogramming efficiency will also be discussed, such as supplementation with small molecules as well as knockdown of p53, p21 and p16.

2.1 Oct4

Oct4 was one of the first transcription factors identified to be a master regulator of cellular pluripotency (Okamoto et al., 1990; Rosner et al., 1990; Scholer et al., 1989). During mouse development *in vivo*, Oct4 expression is restricted to the inner cell mass, primitive ectoderm and primordial germ cells (Pesce and Scholer, 2001). Similarly *in vitro,* ESC and embryonal carcinoma cells (ECC) have high expression of Oct4, which is reduced upon their differentiation (Assou et al., 2007; Rosner et al., 1990).

Although expression of Oct4 is fundamental for the maintenance of pluripotency and development of the inner cell mass in mice (Nichols et al., 1998), complex regulation of its precise level is required to prevent cells from differentiating into other lineages. A transient increase in endogenous Oct4 levels has been observed upon mesodermal differentiation of mouse embryonic stem cells (mESC) (Zeineddine et al., 2006). Furthermore in various over-expression studies, an increase in Oct4 expression can cause mESC to differentiate into endoderm, mesoderm and neuroectoderm lineages (Niwa et al., 2000; Shimozaki et al., 2003; Zeineddine et al., 2006). On the other hand, repression of Oct4 levels results in a loss of pluripotency and promotes trophectodermal differentiation in mESC and hESC (Matin et al., 2004; Niwa et al., 2000). Consistent with these studies, Oct4 has been shown to directly inhibit the expression of major trophectoderm differentiation regulators such as caudal type homeobox 2 (Cdx2) and Eomesdermin (Eomes) (Liu et al., 1997; Liu and Roberts, 1996; Niwa et al., 2005). Together these studies highlight the significance of the critical range of Oct4 level required to maintain ESC pluripotency (Niwa et al., 2000).

As a master regulator of pluripotency, Oct4 was one of the original four factors utilized by Yamanaka and colleagues in the generation of iPSC in both mouse and human (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). As seen in Oct4 over-expression and down-regulation experiments in ESC, the precise level of Oct4 in combination with other reprogramming factors is also essential for efficient generation of iPSC (Papapetrou et al., 2009). Using bicistronic vectors, Papapetrou *et al*. (2009) showed that a 3-fold higher expression of Oct4 compared to Sox2, Klf4 and c-Myc enhanced iPSC generation (Papapetrou et al., 2009). Interestingly, overexpression of Oct4 alone was sufficient to induce reprogramming in neural stem cells that already express high endogenous level of Sox2, c-Myc and Klf4 (Kim et al., 2009b; Kim et al., 2009c). To date, most protocols for generation of iPSC require ectopic expression of Oct4, underlying the important role of Oct4 during direct somatic cell reprogramming (Feng et al., 2009b). Recently, Heng *et al*. (2010) demonstrated that an orphan nuclear receptor Nr5a2 can functionally replace Oct4 in the generation of

mouse iPSC (Heng et al., 2010). However, the precise role of Nr5a2 in regulating cell fate in pluripotent stem cells remains unclear. Further research is also needed to confirm the ability of Nr5a2 to replace Oct4 in human iPSC generation.

2.2 Sox2

Sox2 is an important transcription factor in pluripotent stem cells as well as precursor cells of the neural compartment. It is expressed in the inner cell mass, epiblast and extraembryonic ectodermal cells during mouse embryo development (Avilion et al., 2003; Miyagi et al., 2004). Unlike Oct4, Sox2 expression is maintained in neural stem cells (Ellis et al., 2004; Graham et al., 2003) and over-expression of Sox2 favors neural differentiation in mESC (Kopp et al., 2008; Zhao et al., 2004). Sox2 is known to interact with several binding partners, including Oct4 in the maintenance of pluripotency (Yuan et al., 1995). In a genome-wide chromatin immunoprecipitation study in hESC, Sox2 and Oct4 were found to share many target genes, many of which are transcription factors important in development (Boyer et al., 2005).

Importantly, Sox2 was shown to be indispensable for maintaining pluripotency. Sox2 knockout mouse embryos are unable to form an epiblast and fail to develop past the implantation stage (Avilion et al., 2003). Down-regulation of Sox2 in mESC and hESC results in loss of pluripotency and differentiation towards the trophectoderm cell lineage (Adachi et al., 2010; Fong et al., 2008; Li et al., 2007; Masui et al., 2007). Somewhat surprising was the finding that expression of many Sox2 target genes were not affected by the loss of Sox2 (Masui et al., 2007). The authors in this study suggested potential compensation of Sox2 function by other members of the Sox family. Consistent with this idea, Nakagawa *et al*. (2008) demonstrated that Sox1, Sox3, Sox15 and Sox18 have the ability to replace Sox2 to some extent in iPSC generation (Nakagawa et al., 2008). To date, Sox2 and Oct4 remain to be the two fundamental reprogramming factors and are widely used in various protocols to generate iPSC. Similar to Oct4, it should be noted that ectopic expression of Sox2 can be omitted in the generation of iPSC, if the starting cell type expresses substantial levels of Sox2 (Utikal et al., 2009a).

2.3 Nanog

Nanog is a homeodomain protein that is widely considered as a master regulator for stem cell pluripotency (Chambers et al., 2003; Mitsui et al., 2003). Nanog expression is restricted to the inner cell mass, epiblast and primordial germ cells in the early embryo, as well as a number of pluripotent cells lines such as ESC, ECC and embryonic germ cells (Chambers et al., 2003). During embryo development, Nanog plays a role in suppressing Cdx2, a master regulator of trophectoderm differentiation, and in turn suppression of Cdx2 specifies the inner cell mass fate (Chen et al., 2009). Moreover, Nanog can also physically interact with Oct4 (Wang et al., 2006) and cooperates extensively with Oct4 and Sox2 to form an autoregulated core-transcriptional network that maintain stem cell pluripotency (Boyer et al., 2005; Chen et al., 2008; Kim et al., 2008).

Unlike Oct4 or Sox2, sustained expression of Nanog renders mESC and hESC resistant to differentiation (Chambers et al., 2003; Darr et al., 2006; Ivanova et al., 2006). However in the absence of Oct4, Nanog alone is not sufficient to maintain mESC self-renewal, suggesting that Nanog plays a subservient role in maintaining self-renewal (Chambers et al., 2003). This is also supported by evidence from Nanog knockdown studies. Although early studies

suggested that a reduction of Nanog resulted in differentiation of mESC and hESC (Fong et al., 2008; Hyslop et al., 2005; Ivanova et al., 2006; Mitsui et al., 2003), it was later discovered that transient down-regulation of Nanog can be reversible and does not necessarily mark commitment to differentiation (Chambers et al., 2007). In this respect, mESC can remain undifferentiated in the absence of Nanog, but are more prone to differentiation (Chambers et al., 2007).

Given the important role of Nanog in establishing cell pluripotency, it was somewhat surprising that the initial derivation of iPSC could be achieved without the ectopic expression of Nanog (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). However, Nanog has proved to be a valuable marker for identification of fully reprogrammed iPSC that are germline competent (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). Furthermore, ectopic expression of Nanog in combination with Oct4, Sox2, Klf4 and c-Myc seems to accelerate the reprogramming kinetics of somatic cells to iPSC, but has no effect on the overall reprogramming efficiency (Hanna et al., 2009).

2.4 Klf4

Klf4 and its family members have emerged as important regulators for maintaining pluripotency. Klf4 belongs to the Krüppel-like factor (Klf) family of zinc finger transcription factors. Klf4 can act as an oncogene or a tumor suppressor gene depending on the physiological context (McConnell et al., 2007; Rowland and Peeper, 2006). Klf4 is usually expressed in adult tissues that possess some degree of regenerative capability, including intestine, gut, skin and testis (Nandan and Yang, 2009). Li *et al*. (2005) provided the first evidence that Klf4 plays a role in regulating stem cell pluripotency, by showing that overexpression of Klf4 prevents differentiation of mESC into erythroid progenitors (Li et al., 2005). In conjunction with Oct4, Sox2 and c-Myc, Klf4 was among the first factors to be used to generate iPSC (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). However, the current view is that Klf4 acts only as a secondary factor to enhance somatic cell reprogramming, as iPSC can be generated without Klf4 using a different combination of reprogramming factors (Yu et al., 2007). Klf4 was later discovered to be an important component of the core transcriptional network that regulates expression of Oct4, Sox2, Nanog, Myc and also Klf4 itself (Chen et al., 2008; Kim et al., 2008). Furthermore, Klf4 can directly interact and cooperate with Oct4 and Sox2 to activate a subset of ESC specific genes, including Nanog and Lefty1 (Nakatake et al., 2006; Wei et al., 2009). Klf4 also acts to inhibit apoptosis by suppressing p53 (Rowland et al., 2005), which helps to reprogram somatic cells to a pluripotent state (See discussion below).

Importantly, other Klf family members including Klf1, Klf2 or Klf5, can substitute for Klf4 in iPSC generation (Nakagawa et al., 2008), which suggests that functional redundancies exist among the Klf family members in establishing cell pluripotency. This also explains the observation that Klf4 knockdown in mESC exhibited no obvious phenotype (Jiang et al., 2008; Nakatake et al., 2006), whereas triple knockdown of Klf2, Klf4 and Klf5 resulted in rapid differentiation of mESC (Jiang et al., 2008). However, isoform-specific functions of Klf4 and Klf5 are also observed. Previous studies showed that Klf5 knockout mice result in embryo lethality and defects in implantation (Ema et al., 2008), whereas Klf4 knockout mice are normal during early embryo development but die soon after birth due to loss of skin barrier function (Segre et al., 1999). Furthermore, Ema *et al*. (2008) demonstrated that knocking out Klf5 in mESC results in spontaneous differentiation. Although introduction of

Klf4 can rescue the spontaneous differentiation phenotype, proliferation is significantly decreased (Ema et al., 2008). Further studies are needed to dissect the precise roles of different Klf members in regulating cell pluripotency.

2.5 Lin28

Lin28 encodes for a cytoplasmic RNA binding protein that acts as a translational enhancer (Polesskaya et al., 2007). It was first identified as a heterochronic gene that regulates the developmental timing pathway in *Caenorhabditis elegans* (Moss et al., 1997). A previous transcriptome study has shown that Lin28 is a hESC-specific gene, suggesting that it may play a role in regulating stem cell pluripotency (Richards et al., 2004). However, functional studies of Lin28 in hESC and mESC yielded opposing results. In mESC, Lin28 knockdown resulted in decreased cell proliferation while overexpression of Lin28 enhanced cell proliferation (Xu et al., 2009a). In sharp contrast, Lin28 knockdown in hESC had no obvious phenotype, whereas Lin28 overexpression reduced proliferation and promoted extraembryonic endoderm differentiation (Darr and Benvenisty, 2009). Further studies are clearly needed to elucidate whether Lin28 has a different role in maintenance of pluripotent stem cells in mice and humans.

Lin28 was first used in combination with Nanog, Oct4 and Sox2 to generate human iPSC, acting as an enhancer for somatic cell reprogramming much like Klf4 (Yu et al., 2007). However, it remains unclear how Lin28 contributes to induction of pluripotency. It was demonstrated that Lin28 can block the processing of let7 microRNA family members, a group of pro-differentiation microRNA that also act as tumor suppressors (Melton et al., 2010; Viswanathan et al., 2008). Members of the let7 microRNA family have been shown to repress expression of oncogenes such as c-Myc and Ras. Hence, down-regulation of let7 by Lin28 could increase cell proliferation and drive cellular transformation (Viswanathan et al., 2009). Consistent with this idea, a recent study in mice demonstrated that Lin28 accelerates reprogramming kinetics by enhancing cell proliferation (Hanna et al., 2009). Another proposed mechanism of Lin28 action is that it can selectively regulate gene expression at a post-transcriptional level, enhancing translation of anti-differentiation mRNAs while degrading pro-differentiation mRNAs to maintain pluripotency. A previous study demonstrated that Lin28 can reside in polysomal ribosome fractions, in which mRNAs are translated (Balzer and Moss, 2007). Indeed, Lin28 has been shown to bind directly to Oct4 mRNA in hESC to facilitate translation via interaction with RNA helicase A (Qiu et al., 2010). Lin28 can also reside in P-bodies, in which mRNAs are degraded (Balzer and Moss, 2007). Therefore, it remains speculative that Lin28 may be able to selectively degrade certain pro-differentiation mRNA to support stem cell pluripotency. Further studies are needed to confirm this hypothesis.

2.6 c-Myc

The basic helix–loop–helix/leucine zipper transcription factor c-Myc has a well documented role in cellular transformation and tumor progression, by controlling cell cycle, apoptosis, protein biosynthesis and metabolism (Kendall et al., 2006; Patel et al., 2004). c-Myc has been shown to regulate its target genes through interactions with the transcription machinery, as well as exerting epigenetic regulation via interactions with chromatin remodeling complexes, DNA methyltransferases and histone modifying enzymes (Eilers and Eisenman, 2008). Subsequent studies also identified a critical role of c-Myc in mESC maintenance.

Overexpression of c-Myc enables mESC to be resistant to differentiation, whereas expression of a dominant negative form of c-Myc promotes differentiation (Cartwright et al., 2005). However, a functional study of c-Myc in hESC yielded rather different results. Overexpression of c-Myc drives hESC to apoptosis and differentiation into extraembryonic endoderm and trophectoderm (Sumi et al., 2007).

c-Myc was identified as one of the four 'Yamanaka factors' initially used to generate both mouse and human iPSC (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Other reports have also shown that c-Myc can be substituted by two other related family members, N-Myc and L-Myc, during somatic cell reprogramming (Blelloch et al., 2007; Nakagawa et al., 2008). Subsequent studies demonstrated that somatic cell reprogramming can be achieved without c-Myc, albeit with significantly reduced efficiency and slower kinetics (Nakagawa et al., 2008; Wernig et al., 2008). Furthermore, reactivation of c-Myc has been observed in iPSC following blastocyst incorporation, resulting in tumor formation in the chimeric mice (Okita et al., 2007). This finding raises concerns about the safety of using iPSC generated with c-Myc for clinical applications. Understanding the molecular mechanism of c-Myc contributions during somatic cell reprogramming may help identify alternative enhancers for iPSC generation that are less tumorigenic.

Recent studies have shed light on the function of c-Myc during somatic cell reprogramming. Genome-wide analysis of promoter binding demonstrated that c-Myc regulates a different set of target genes compared to other pluripotency factors Oct4, Sox2 and Klf4 in mESC and iPSC (Chen et al., 2008; Kim et al., 2008; Sridharan et al., 2009). This suggests that c-Myc may have a very different function than the other transcription factors associated with induction of pluripotency. One proposed function is that c-Myc acts to repress expression of somatic genes during the early reprogramming stage, a process that is necessary before the activation of pluripotency gene networks (Sridharan et al., 2009). Another proposed mechanism of action of c-Myc is that it may induce a cell cycle program that is necessary for self-renewal of stem cells, activating genes which promote proliferation (i.e. cyclin A, cyclin E or E2F) and repressing genes associated with growth arrest (i.e. p21, p27) (Vermeulen et al., 2003). Finally, c-Myc may exert epigenetic control by modifying the chromatin structure to become suitable for activation of the self-renewal gene program, thus allowing somatic cells to revert back to a pluripotent state (Knoepfler et al., 2006).

2.7 Esrrb

Esrrb belongs to a subfamily of orphan nuclear receptors that are closely related to estrogen receptors (Giguere, 2002). The natural ligands for Esrrb are currently unknown. Nevertheless, Esrrb and its family members can bind to DNA and function as transcriptional activators without exogenous ligands (Giguere, 2002). Most of our knowledge of the role of Esrrb in regulating self-renewal comes from studies in mice. Overexpression of Esrrb is sufficient to maintain self-renewal of mESC in conditions that favour differentiation, possibly by maintaining the level of Oct4 expression (Zhang et al., 2008). Also, knockdown of Esrrb level in mESC induces differentiation (Ivanova et al., 2006; Loh et al., 2006). These results identify Esrrb as a positive regulator of ESC pluripotency.

Indeed, Esrrb has been used as a factor to reprogram somatic cells back to a pluripotent state. In the presence of Oct4 and Sox2, Feng *et al*. (2009) demonstrated that Esrrb could replace Klf4 as an enhancer to reprogram mouse fibroblasts into iPSC, albeit with lower reprogramming efficiency (Feng et al., 2009a). Furthermore, another family member Esrrg

also possesses a similar reprogramming ability when used in conjunction with Oct4 and Sox2 (Feng et al., 2009a). Genome-wide analysis of promoter binding suggested that Essrb shares many target genes with Oct4 and Sox2. Further studies demonstrated that Esrrb is a binding partner for Oct4 and Nanog in mESC (van den Berg et al., 2010; Wang et al., 2006), but whether it physically interacts with Sox2 remains to be determined. In summary, Esrrb was found to have a partially overlapping role with Klf4 in enhancing somatic cell reprogramming in mice, by cooperating with other pluripotency factors Oct4 and Nanog. To date, it has not yet been determined whether Esrrb plays a similar role in human cells.

2.8 Sall4

Sall4 belongs to the family of *Spalt* transcription factors that are characterized by highly conservative C2H2 zinc-finger domains (Sweetman and Munsterberg, 2006). Mutations of Sall4 in humans results in Okihiro syndrome, a disease characterized by limb deformities and eye movement deficits (Kohlhase et al., 2002). Sall4 is highly enriched in ESC, and is one of the 'embryonic cell associated transcripts' identified by Shinya Yamanaka's group. A previous study indicated that knockdown of Sall4 promoted mESC differentiation, most notably into the trophectoderm lineage (Zhang et al., 2006). However, follow-up reports showed that Sall4-null mESC are able to remain pluripotent, albeit with impaired proliferation (Tsubooka et al., 2009; Yuri et al., 2009). A study by Yuri *et al*. (2009) also demonstrated high expression of trophectoderm markers in Sall4-null mESC (Yuri et al., 2009). These results suggest that Sall4 is not essential to maintain pluripotency in mESC, but rather functions to stabilize the stem cell phenotype by promoting proliferation and possibly repressing trophectoderm differentiation. Furthermore, it is becoming clear that Sall4 is an integral part of the autoregulatory transcriptional network of Oct4, Sox2 and Nanog in mESC (Lim et al., 2008; Yang et al., 2008). These results suggest Sall4 is a possible candidate reprogramming factor for induced pluripotency.

Recently, Wong *et al*. (2008) discovered that Sall4 can enhance the efficiency of reprogramming mouse fibroblasts through fusion with mESC (Wong et al., 2008). Sall4 also increases the reprogramming efficiency of mouse fibroblasts when used in combination with Oct4, Sox2 and Klf4 (Tsubooka et al., 2009). However, this enhancing effect of Sall4 in reprogramming is inconsistent in different human fibroblast cell types, possibly due to variations in endogenous levels of Sall4 in different samples (Tsubooka et al., 2009). Further studies of Sall4 in human pluripotent stem cells will clarify whether the role of Sall4 in regulating cell pluripotency is conserved between mouse and human.

2.9 miRNA

miRNA are small RNAs that provide post-transcriptional control of gene regulation. Once transcribed, primary miRNA undergo multiple processing steps to become mature miRNA that promote degradation or repress translation of target mRNA (Siomi and Siomi, 2010). A previous report provided evidence that miRNA play an important role in the inter-connected transcriptional network regulated by pluripotency factors Oct4, Sox2 and Nanog in mESC (Marson et al., 2008). The pluripotency factors Oct4, Sox2 and Nanog are able to bind and regulate expression of specific miRNA, activating ESC specific miRNA while repressing those associated with differentiation (Barroso-delJesus et al., 2008; Marson et al., 2008). It is believed that miRNA serve as a mechanism for Oct4, Sox2 and Nanog to fine-tune the expression level of their target genes.

Recently, Robert Blelloch's group described a subset of miRNA that play an important role in regulating the cell cycle of mESC, termed ESC-specific cell cycle regulatory miRNA (ESCC miRNA) (Wang et al., 2008b). These ESCC miRNA promote G1 to S transition in mESC by repressing expression of various cyclin E-Cdk2 inhibitors (Wang et al., 2008b). In a follow-up study, the same group demonstrated that ESCC miRNA, in particular mir-291-3p, mir-294 and mir-295, are able to enhance reprogramming efficiency when used in combination with Oct4, Sox2 and Klf4. These ESCC miRNA are found to be downstream effectors of c-Myc, and thus are able to act as substitutes for c-Myc albeit to a lesser extent (Judson et al., 2009). Another set of ESCC miRNA, the mir-302 cluster, is able to reprogram human melanoma and prostate cancer cells to ESC-like cells in the absence of any other reprogramming factors (Lin et al., 2008). However, it remains unclear whether miRNA on their own can reprogram normal human primary cells to obtain genuine iPSC, as the effectiveness of miRNA-based reprogramming strategies may be cell-type dependent. Alternatively, others have shown that suppression of pro-differentiation let-7 miRNA can also enhance reprogramming efficiency in mouse fibroblasts when used in combination with the Yamanaka factors (Melton et al., 2010). This result is consistent with the previous identification of Lin28 as a reprogramming factor for iPSC generation, which presumably acts by blocking the processing of the let-7 family of miRNA (see discussion above). Together, these results demonstrate opposing roles played by different miRNA in somatic cell reprogramming and identify miRNA as important regulators of cell pluripotency. Future research to screen for reprogramming effects of other miRNA members will prove helpful in deriving a more efficient somatic cell reprogramming method. For instance, a recent report showed that miRNA-145 can regulate expression of Oct4, Sox2 and Klf4 and represses pluripotency in hESC (Xu et al., 2009b). Therefore, it will be interesting to see whether miRNA-145 can contribute to somatic cell reprogramming.

2.10 SV40 LT antigen and hTERT

The SV40LT antigen and the catalytic subunit of hTERT are well documented for their roles in establishing immortalized cells. Overexpression of SV40LT and hTERT along with another oncogene Ras are sufficient to confer tumorigenic transformation of normal human cells (Hahn et al., 1999). One proposed mechanism by which SV40 functions in tumorigenesis is by perturbing cellular senescence pathways through suppression of p53 activity. As discussed below, reduced p53 activity during reprogramming has been shown to improve the efficiency. On the other hand, reduction in telomere length during the normal aging process results in replicative senescence and limits the cellular lifespan of human cells. Studies have shown that this can be prevented by ectopic expression of hTERT to drive cellular immortalization (Bodnar et al., 1998). High telomerase activity is observed in the vast majority of tumors and is vital for the progression of malignant tumor cells (Kim et al., 1994). Interestingly, a previous study has also shown that c-Myc overexpression can activate hTERT activity (Wu et al., 1999). Therefore, SV40LT and hTERT may be able to contribute to somatic cell reprogramming by activating a cell cycle program that is required for pluripotent cells, much like the role of c-Myc in induced pluripotency.

Two recent studies sought to enhance the efficiency of generating human iPSC by supplementing reprogramming factors with hTERT and/or SV40LT (Mali et al., 2008; Park et al., 2008). In the study by Mali *et al*. (2008), the reprogramming cocktails were supplemented with the SV40LT transgene and resulted in accelerated reprogramming kinetics and up to a 70-fold increase in reprogramming efficiency, depending on the

combination of reprogramming factors used (Mali et al., 2008). Similar results were obtained by Park *et al*. (2008) when hTERT and SV40LT were included in their reprogramming strategy (Park et al., 2008). Interestingly, while the addition of both SV40LT and hTERT was reported to increase cell proliferation, there were no viral integrations of either transgene in the genomes of the iPSC derived by this method (Park et al., 2008). This suggests that SV40LT and hTERT might be acting indirectly on supportive cells to enhance reprogramming. However, concerns remain about the safety of using SV40LT and hTERT in somatic cell reprogramming for clinical purposes. In this regard, future research studying whether iPSC generated using these two factors are more tumorigenic will help address this issue.

2.11 Silencing of the p53/p21/p16 pathway

One of the major roadblocks in iPSC generation is overcoming cellular senescence. p53 is known as the guardian of the genome and deregulation of p53 function promotes cell immortalization and bypasses cell senescence (Bond et al., 1994). Recent discoveries suggested that high-passage somatic cells with short telomeres show a dramatic decrease in the efficiency for iPSC generation (Marion et al., 2009; Utikal et al., 2009b). In this regard, bypassing cellular senescence may help improve the reprogramming efficiency of iPSC generation.

Key studies have shown that knockdown of senescence factors like p53, $p21^{CIP1}$ or $p16^{INK4a}$ enhances the efficiency of generating iPSC (Banito et al., 2009; Hanna et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Marion et al., 2009). Furthermore, p53 knockdown can be used to replace c-Myc and/or Klf4 in the Yamanaka factors (Hong et al., 2009; Kawamura et al., 2009). When used in combination with UTF1, a chromatin bound factor highly expressed in pluripotent stem cells, the addition of p53 further enhanced reprogramming efficiency by 100-fold when used with the Yamanaka factors (Zhao et al., 2008). Together these studies show that the p53 pathway not only acts as a roadblock for cancer but also for iPSC generation. As p53 is a major tumor suppressor, further studies will be required to evaluate the safety of p53 knockdown in iPSC generation before these iPSC can be used in clinical applications.

2.12 Small molecules used to enhance somatic cell reprogramming

It is becoming clear that reversion of somatic cells to a pluripotent state involves epigenetic changes to chromatin that allow different sets of genes to be expressed (Feng et al., 2009b). Several reports have investigated the use of small molecules to enhance current cellular reprogramming methods towards developing a completely transgene-free strategy. Huangfu *et al*. (2008) discovered that the addition of valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, can increase the efficiency and kinetics of reprogramming (Huangfu et al., 2008a). Moreover, two other HDAC inhibitors, suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA), can also improve the reprogramming efficiency, albeit to a lesser extent. This provides supporting evidence for the notion that chromatin modifiers can help to overcome the epigenetic barrier to achieving complete reprogramming. Furthermore, addition of VPA was also able to substitute for c-Myc and Klf4 during reprogramming, thus reducing the number of reprogramming factors required to derive iPSC (Huangfu et al., 2008b). Similar effects on enhancing reprogramming efficiency were also observed with DNA methyltransferase inhibitors, including azacytidine

(AZA) (Huangfu et al., 2008a) and RG108 (Shi et al., 2008a), as well as a histone methyltransferase inhibitor, BIX-01294 (Shi et al., 2008b). In addition, a calcium channel agonist, BayK864, was shown to enhance the effect of BIX-01294 and further improve the efficiency of reprogramming (Shi et al., 2008a). However, it remains unclear whether or not the enhancing effect of these small molecules is dependent on the cell type used for iPSC generation. Altogether, it is thought that histone deacetylase inhibitors (VPA, TSA, SAHA), methyltransferase inhibitors (AZA, RG108, BIX-01294) and possibly other, yet to be identified, chromatin modifiers may function by relaxing chromatin to allow ectopically expressed transcription factors to bind.

Other researchers have screened small molecule libraries to identify chemical compounds that can directly substitute for the known reprogramming factors. This led to the discovery of RepSox (replacement of Sox2), a small molecule used to substitute for Sox2 in the reprogramming strategy (Ichida et al., 2009). RepSox acts by inhibiting transforming growth factor-ß (TGF-ß) signaling, in turn increasing Nanog expression that ultimately promotes partially reprogrammed cells to become fully reprogrammed (Ichida et al., 2009). However, future research will need to address the specificity of RepSox in order to fulfill its potential in generating iPSC for clinical purposes.

Finally, an interesting study by Esteban *et al*. (2010) demonstrated that antioxidants, in particular vitamin C, also help to enhance somatic cell reprogramming when used in combination with Oct4, Sox2 and Klf4 (Esteban et al., 2010). During early stages of reprogramming, vitamin C is able to overcome, at least partially, the cellular senescence roadblock by down-regulating p53 to allow the conversion of partially reprogrammed cells into fully reprogrammed iPSC (Esteban et al., 2010). This study provides a natural alternative to synthesized small molecules and may be easier to obtain approval for clinical usage.

3. Techniques for delivery of reprogramming factors

Since the seminal iPSC work by Shinya Yamanaka and colleagues, the field has moved forward at a rapid pace. Significant progress has been made in identifying new strategies to enhance the reprogramming efficiency and new methods to improve clinical safety. In this section, we will discuss the current techniques employed to introduce the reprogramming factors required for iPSC generation. It is important to note that the reprogramming efficiencies discussed in this section are only subject to the context described in the particular studies. Actual efficiency can be highly affected by many factors including the cell type of origin, the reprogramming factors and enhancer molecules used, as well as the methods to calculate reprogramming efficiencies.

3.1 Integrating Viral Vectors

3.1.1 Retroviral vectors

Retroviruses are efficient gene delivery vectors widely used in a broad range of dividing cell types. They can integrate into the host cell genome to produce continuous transgene expression. However, slow dividing or non-dividing cells are extremely resistant to retroviral transduction and the random sites of transgene integration can lead to unpredicatable genetic mutations within the genome and aberrant transgene expression. The initial derivation of iPSC utilized retroviral-mediated introduction of Oct4, Sox2, Klf4 and c-Myc to convert mouse fibroblasts back to a pluripotent state (Takahashi and Yamanaka, 2006), and subsequently human iPSC derived from adult dermal fibroblasts,

fetal and neonatal cells (Lowry et al., 2008; Park et al., 2008; Takahashi et al., 2007). Also, it was observed that retroviral mediated expression of transgenes were silenced during the iPSC reprogramming process (Hotta and Ellis, 2008). Even with a low efficiency (0.001%-0.5%), these pioneering studies revealed a potential alternative to the controversial use of ESC as a cell source for cellular transplantation therapies.

3.1.2 Lentiviral vectors

At the same time Yamanaka and colleagues reported the generation of human iPSC by retroviral transduction, Yu *et al*. (2007) demonstrated successful derivation of human iPSC using lentiviral methods to deliver a different set of factors Oct4, Sox2, Nanog and Lin28 (Yu et al., 2007). Lentiviruses, a subclass of retroviruses, offer the capability of high-efficiency infection in both dividing and non-dividing cells with stable expression of the transgenes and low immunogenicity. These distinguishing characteristics allow lentiviral vectors to be used for reprogramming a broader range of somatic cell types. However, lentiviruses integrate randomly into the host genome, similar to other retroviruses, which may hinder the use of iPSC generated using these methods for clinical applications. Initial derivation of human iPSC using lentiviral transduction yielded reprogramming efficiencies of 0.01%, significantly lower than previous retroviral methods (Yu et al., 2007). Subsequent improvements have been made by using lentiviral vectors to deliver SV40T, UTF1 or p53 shRNA to supplement the reprogramming cocktails, resulting in a 70-100 fold increase in reprogramming efficiency (Mali et al., 2008; Zhao et al., 2008). Moreover, reporter and antibiotic selection casettes have also been incorporated into lentiviral vectors to aid in the isolation of iPSC (Hotta et al., 2009a; Hotta et al., 2009b).

Previous studies indicated that transient expression of reprogramming factors is sufficient to activate the endogenous pluripotent gene program to allow for direct cell reprogramming (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). This led to the development of inducible lentiviral vectors for direct cell reprogramming. Inducible lentiviral methods provide for improved temporal control over the levels of reprogramming factor expression and have been used to study the timing of reprogramming as well as the molecular changes that occur during the process. This system relies on inclusion of an additional vector that constitutively expresses the reverse tetracycline transactivator (rtTA). In the presence of the drug doxycycline, the rtTA functions to drive expression of the reprogramming factors, while in the absence of doxycycline the reprogramming transgenes are not expressed. Utilizing this type of inducible system, it has been established that exogenous transgene expression is necessary for 8-12 days to induce reprogramming of mouse fibroblasts and dispensable thereafter (Brambrink et al., 2008; Maherali et al., 2008; Stadtfeld et al., 2008a). Moreover, when doxycycline was removed eight days after initial transduction, the partially reprogrammed cells were unable to survive in the ESC growth conditions due to incomplete reactivation of their self-renewal programs. This provides a useful system to select for cells that have completely reverted back to a pluripotent stem cell state.

A major obstacle encountered when attempting to transduce cells with multiple viruses is that only a small proportion of the total cells will become infected with all the viruses. During reprogramming of somatic cells, those cells infected with few viruses may fail to become reprogrammed; leading to a low reprogramming efficiency. In this regard, development of a system to express the transgenes from a single vector may substantially improve the efficiency. One method to express the four transgenes from a single promoter is to insert the self-cleaving 2A and 2A-like sequences between each cDNA sequence

(Donnelly et al., 1997). These 2A sequences act by triggering ribosomal skipping that result in expression of each sequence in a stoichiometric fashion. Importantly, efficient polycistronic expression by 2A-mediated separation of transgenes was achieved in hESC (Hasegawa et al., 2007). This strategy was recently applied to derive mouse and human iPSC using a single lentiviral vector expressing Oct4, Sox2, Klf4, and c-Myc (Carey et al., 2009; Shao et al., 2009; Sommer et al., 2009). One of the advantages of using polycistronic vectors is that it reduces the difficulty of handling multiple lentiviral vectors for different reprogramming factors. Moreover, the mouse and human iPSC generated using this method had less viral integration sites compared to those developed using lentiviral delivery with individual gene. Indeed, the results showed as few as a single viral integration was sufficient for reprogramming (Carey et al., 2009; Shao et al., 2009). Minimizing the number of viral integrations reduces the risk of tumorigenesis and genomic instability. Also, the use of polycistronic vectors ensures expression of all four factors in the transduced cells. However, reprogramming efficiencies using this method were significantly lower (0.05%) than previous methods and reprogramming kinetics were also notably slower. One possible explanation for this low reprogramming efficiency is that the reprogramming factors are required to be expressed at an optimal stoichiometry in order to achieve efficient direct cell reprogramming (Papapetrou et al., 2009). Further studies are needed to clarify if the use of polycistronic vectors is an ideal technique to generate iPSC.

3.2 Non-integrating and excisable approaches

A major concern with employing retroviral or lentiviral-based methods to derive iPSC is random, uncontrollable integrations of the foreign transgenes into the host chromosomes. While many of these integrations prove harmless to the cells, residual viral portions have been shown to contribute to tumor formation. Moreover, it has been suggested that viral integrations may be a possible cause for some of the gene expression and differentiation potential differences observed between blastocyst-derived ESC and iPSC. These differences could affect the interpretation of results during mechanistic studies and, due to the safety concerns, severely limit the clinical applicability of these genetically modified cells. The previous approaches were extremely inefficient processes and most required multiple integration sites to induce reprogramming. Therefore, many groups have focused on developing novel, non-integrating methods for deriving iPSC. Some of these methods include the use of non-integrating vectors, excisable vectors, as well as RNA- or protein-based reprogramming.

3.2.1 Adenoviral vectors

The use of adenoviral vectors is advantageous for somatic cell reprogramming as they lack the machinery to integrate into the host's genome. This allows for high-level expression of exogenous genes with a low risk of integration of viral transgenes into the host genome. The viral titer becomes diluted after every cell division, which allows transient expression of the transgenes. On the other hand, multiple rounds of infections can achieve prolonged expression of transgenes, but it can be difficult to control gene expression levels. Reprogramming somatic cells with adenoviral vectors was first reported by Stadtfeld *et al* (Stadtfeld et al., 2008b). Using adenoviral vectors, mouse and human iPSC were generated using the Yamanaka factors in various donor cell types, albeit with a low reprogramming efficiency as compared to integrating viral vectors (0.0001%-0.001%) (Stadtfeld et al., 2008b;

Zhou and Freed, 2009). Similarly, a low reprogramming efficiency was also observed using polycistronic adenoviral vectors (Okita et al., 2008). One explanation for this is that it is difficult to maintain high enough transgene levels for multiple days to allow for reprogramming in many of the cells. Also, roughly 20% of the transduced cells were tetraploid, a phenomenon which was not seen in retroviral or lentiviral induced iPSC (Stadtfeld et al., 2008b). The reason for this observed tetraploidy is not clear, but it was suggested that cellular fusion or the presence of a rare aneuploid cell population in the starting culture may account for this result. Therefore, further research is needed to refine the use of adenoviral vectors in reprogramming somatic cells back to pluripotency.

3.2.2 Plasmids

Another non-integrating approach to transiently express reprogramming factors is the use of conventional plasmids. Previous reports have successfully generated integration-free mouse iPSC using polycistronic plasmids to express the Yamanaka factors (Gonzalez et al., 2009; Okita et al., 2008). However, a substantial amount of iPSC colonies contained integration of the transgenes. Therefore, screening of transgene integration sites is still necessary for iPSC generated using this technique to ensure their safety for clinical purposes. Furthermore, multiple rounds of transfection are required to sustain transgene expression at the level required to induce reprogramming and the observed reprogramming efficiency remained significantly lower than seen with the retroviral vectors (Gonzalez et al., 2009; Okita et al., 2008). Improvements to this plasmid-based method were made by the use of a polycistronic nonviral minicircle plasmid to reprogram human adult adipose stem cells (Jia et al., 2010). Minicircle DNA offers a higher transfection rate and is diluted at a slower rate than conventional plasmids when the cells divide. As a result, fewer rounds of transfections are required to generate iPSC. Using this method, Jia *et al*. (2010) generated integration-free human iPSC with a reprogramming efficiency of 0.005%, an efficiency still much lower than the integrating viral methods (Jia et al., 2010).

Episomal plasmids are another non-integrating method used to reprogram somatic cells into iPSC. Unlike conventional plasmids where transient transgene expression is gradually depleted after each cell division, episomal plasmids can be stably established in a number of cell types by drug selection and removed when the drug selection is withdrawn. Using this technique, Yu *et al.* (2009) generated a polycistronic episomal vector to co-express seven transgenes to reprogram human foreskin fibroblasts into iPSC (Yu et al., 2009). It was observed that different positioning of the transgene in the polycistronic vector resulted in varying reprogramming efficiencies, with the highest efficiency achieved being 0.006%. In summary, these studies provide proof-of-principle of the derivation of human iPSC free of genomic integration. However, all plasmid-based methods used to date yield a lower reprogramming efficiency compared to integrative viral methods, possibly due to difficulties in sustaining high transgene expression. Further research combining enhancing factors, such as small molecules, may help improve the reprogramming efficiency of these plasmid-based methods.

3.2.3 Cre recombinase /loxP system

Early non-integrating methods, such as those utilizing adenoviral and plasmid introduction of reprogramming factors, were substantially less efficient than the retroviral methods. Excisable integrating vectors offer a plausible reprogramming strategy to overcome the

shortcomings of both the integrating retroviral and the transient expression methods. These excisable systems allow high initial transgene expression followed by subsequent removal of exogenous factors. Soldner *et al*. (2009) used inducible lentiviral vectors to derive human iPSC from fibroblasts collected from Parkinson's disease patients followed by Cre-recombinase mediated excision of the viral transgenes (Soldner et al., 2009). In this study, a constitutively active reverse tetracycline transactivator lentivirus was infected along with doxycycline-inducible lentiviruses for expression of the reprogramming factors, Oct4, Sox2, Klf4 and c-Myc. The inducible lentiviruses were engineered to contain a loxP site in the 3′ LTR that becomes duplicated into the 5′ LTR during viral replication, producing loxP sites flanking the transgenes. Subsequent expression of Cre-recombinase by electroporation allows the transgenes to be excised. Using this technique, the authors reported successful derivation of integration-free human iPSC (Soldner et al., 2009). Interestingly, the authors also demonstrated that the gene expression profile of these iPSC are more similar to hESC following transgene excision, suggesting that residual integrated reprogramming factors perturb the transcriptional profile of human iPSC (Soldner et al., 2009). Although the Cre-loxP system is the most efficient recombination system known, screening of integration-free iPSC clones is still required, as the authors reported that only sixteen out of 180 clones analyzed were integration-free following excision of transgene. Moreover, Cre-mediated excision of the transgenes does not remove the loxP sites, which raises concern of the possibility of disruption of endogenous gene expression. In this regard, a recent report by Chang *et al*. (2009) demonstrated successful generation of integration-free human iPSC where residual loxP sites did not interrupt expression of any genes or other functional sequences (Chang et al., 2009).

3.2.4 piggyBac transposon-based system

Unlike the Cre-loxP system, the advantage of transposon-based system is that transposases can remove all exogenous transposon elements from the host DNA. In particular, the piggyBac transposons have been demonstrated to be an efficient system for excisable gene delivery, delivering up to 10kb DNA fragments (Ding et al., 2005). Following transfection of the piggyBac transposons, transient expression of the transposase enzyme catalyzes the insertion or the excision event (Fraser et al., 1996). The advantage of the piggyBac transposon system is that it can be completely removed from the host genomes without altering the DNA sequences at the integration sites (Wang et al., 2008a). This led to the development of piggyBac-based reprogramming strategy that allowed generation of mouse and human iPSC using tetracycline-inducible or polycistronic expression of reprogramming factors (Kaji et al., 2009; Woltjen et al., 2009; Yusa et al., 2009). A high reprogramming efficiency of 2.5% was reported in the generation of mouse iPSC using this technique (Kaji et al., 2009). Furthermore, the integration sites were sequenced to confirm that excision of transgenes did not alter the host genome. Therefore, the piggyBac transposon system represents an efficient non-integrative approach to generate iPSC. However, excision of the transgenes by the transposase may still lead to micro-deletions of the genomic DNA (Wang et al., 2008a), which could hinder the clinical application of iPSC generated using this method.

3.2.5 RNA and protein-based reprogramming

Since all of the reprogramming strategies highlighted above create the risk of unexpected genetic modifications, many groups have begun to devise ways to reprogram somatic cells

in the absence of genetic modification. Yakubov *et al*. (2010) recently developed a reprogramming technique by transfecting RNA synthesized *in vitro* from cDNA of Oct4, Lin28, Sox2, and Nanog to generate iPSC from human fibroblasts (Yakubov et al., 2010). This method harnesses the power of the endogenous translational machinery for proper protein folding and post-translational modifications. Also, this method of generating iPSC eliminates the risks associated with the use of viruses and DNA transfection methods. However, at least five consecutive transfections were necessary to reprogram these human fibroblasts as the transfected RNAs have a limited half-life. Finally, the reprogramming efficiency (0.05%) remained lower than those observed for integrating viral methods. Further characterization is also needed to confirm the pluripotency of the iPSC-like cells generated using this method and to prove the feasibility of RNA-based reprogramming.

An alternate approach to somatic cell reprogramming without genetic modification is using protein transduction. Importantly, a previous study demonstrated that protein tagged with a C-terminus poly-arginine domain allows efficient protein transduction through the cell membrane (Matsushita et al., 2001). Using direct protein delivery of reprogramming factors, two groups have report successful derivation of iPSC with the Yamanaka factors (Kim et al., 2009a; Zhou et al., 2009). Zhou *et al*. (2009) was the first to use recombinant reprogramming factors tagged with the poly-arginine domain to generate mouse iPSC. This virus-free and DNA-free method yielded a reprogramming efficiency of 0.006% with the use of an enhancer molecule VPA (Zhou et al., 2009). In addition, Kim *et al*. (2009) used whole cell extract from human embryonic kidney cells overexpressing the Yamanaka factors for the generation of human iPSC, yielding a reprogramming efficiency of 0.001% (Kim et al., 2009a). Both studies generated iPSC without any genetic modification, making them suitable for clinical applications. This direct protein transduction method also eradicates the need to screen for integration-free iPSC, thus shortening the time required for generating clinical grade iPSC. However, the reprogramming efficiency achieved with this method is still far lower than those obtained with viral mediated reprogramming. Moreover, multiple rounds of treatment are required during the reprogramming process as the recombinant proteins become degraded overtime. Nevertheless, these studies proved the feasibility of protein-based reprogramming methods and improvements to this technique could be important for final clinical application of iPSC.

4. Conclusions

The clinical potential of hESC for cell replacement therapies and for studying human diseases is undeniable. However, the use of these cells has been constantly burdened by both ethical (destruction of human embryos) and practical concerns (lack of available embryos, difficulties with generation of histo-compatible hESC). The conversion of somatic cells into pluripotent cells may overcome many of these roadblocks. Large numbers of embryos are no longer needed to create banks of patient-matched lines, since cells can now be harvested directly from each patient to create iPSC that are genetically identical and immune-compatible. Since the initial derivation of iPSC using the four Yamanaka factors, a growing list of reprogramming factors have been identified that either permit or enhance the reprogramming process. Moreover, researchers have begun to study other ways to improve the reprogramming efficiency, including the use of miRNA, small molecules, and several different methods to overcome barriers that prevent direct somatic cell reprogramming.

The first generation iPSC methods involved integration of viral transgenes, but the clinical necessity for deriving iPSC without these viral transgenes has pushed many researchers to develop alternative methods. The use of inducible polycistronic lentiviral vectors has already evolved to the utilization of excisable Cre-loxP and piggyBac expression systems, non-integrating plasmids, and recombinant proteins and RNA transfection as tools for generating iPSC. Advances in these non-viral, non-integrating methods will presumably continue until a method is discovered that can, with high efficiency, be used to derive patient-specific iPSC lines in a technically simple manner which can be adopted by many researchers and clinicians. In this regard, pursuing the development of protein-based and small molecule-based reprogramming methods may be most beneficial. However, these methods are still at an early stage and further improvements are needed to achieve high reprogramming efficiencies. Interestingly, a recent report utilized lentiviral vectors to deliver three transgenes and demonstrated direct conversion of mouse fibroblasts into neuronal like cells, bypassing the need of reprogramming back to a pluripotent state (Vierbuchen et al., 2010). Further research is needed to develop similar non-viral methods and to translate these techniques to human cells for clinical applications. Many methodologies used in the derivation of iPSC can be applied to study direct conversion of a somatic cell into another cell type of interest. However, the disadventagous of such direct reprogramming of a somatic cell into another lineage is that the reprogrammed cells are terminally differentiated and are not proliferating. Therefore, this reprogramming strategy is not ideal for large scale production to yield cells for the development of cell replacement therapies. In this regard, direct reprogramming of a somatic cell to a progenitor stage, where the progenitor cells remain proliferative, may prove advantageous. Recent studies also showed that iPSC retain an 'epigenetic memory' of their origins, where differentiation of iPSC to their tissue of origin is more efficient than other lineages (Kim et al., 2010; Polo et al., 2010). This could be used as a strategy to derive efficient protocols for differentiating iPSC to a particular cell type of interest. In summary, the field of stem cell biology was radically altered by the derivation of iPSC. Since their generation, the field has moved forward at a staggering speed, in large part due to the potential of iPSC to transform modern medicine as well as our understanding of human development.

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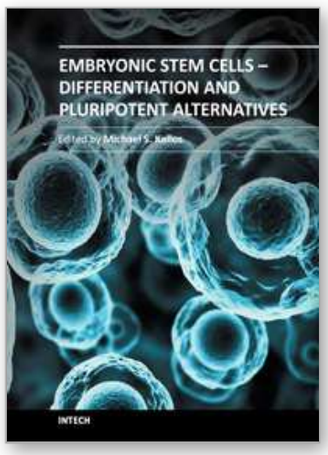

Embryonic Stem Cells - Differentiation and Pluripotent Alternatives
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The ultimate clinical implementation of embryonic stem cells will require methods and protocols to turn these unspecialized cells into the fully functioning cell types found in a wide variety of tissues and organs. In order to achieve this, it is necessary to clearly understand the signals and cues that direct embryonic stem cell differentiation. This book provides a snapshot of current research on the differentiation of embryonic stem cells to a wide variety of cell types, including neural, cardiac, endothelial, osteogenic, and hepatic cells. In addition, induced pluripotent stem cells and other pluripotent stem cell sources are described. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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