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Abstract

The molecular and phenotypic irreversibility of mammalian cell differentiation was a fundamental principle of developmental biology at least until the 1980s, despite numerous reports dating back to the 1950s of the induction of pluripotency in amphibian cells by nuclear transfer (NT). Landmark reports in the 1980s and 1990s in sheep progressively challenged this dogmatic assumption; firstly, embryonic development of reconstructed embryos comprising whole (donor) blastomeres fused to enucleated oocytes, and famously, the cloning of *Dolly* from a terminally differentiated cell. Thus, the intrinsic ability of oocyte-derived factors to reverse the differentiated phenotype was confirmed. The concomitant elucidation of methods for human embryonic stem cell isolation and cultivation presented opportunities for therapeutic cell replacement strategies, particularly through NT of patient nuclei to enucleated oocytes for subsequent isolation of patient-specific (autologous), pluripotent cells from the resulting blastocysts. Associated logistical limitations of working with human oocytes, in addition to ethical and moral objections prompted exploration of alternative approaches to generate autologous stem cells for therapy, utilizing the full repertoire of factors characteristic of pluripotency, primarily through *cell fusion* and use of pluripotent *cell extracts*. Stunningly, in 2006, Japanese scientists described somatic cell reprogramming through delivery of four key factors (identified through a deductive approach from 24 candidate genes). Although less efficient than previous

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approaches, much of current stem cell research adopts this focused approach to cell reprogramming and (autologous) cell therapy. This chapter is a quasi-historical commentary of the various aforementioned approaches for the induction of pluripotency in lineage-committed cells, and introduces transcriptional and epigenetic changes occurring during reprogramming.

Keywords

Pluripotency • SCNT • Cell-fusion • Cell extract • iPSC • Epigenetics

2.1 Introduction

...reprogramming after transfer into the zygote is impossible in the mammalian embryo, either inherently or because of lack of time, whereas the amphibian nucleus probably has sufficient time to reprogram...

(McGrath and Solter 1984. Science) [8]

Despite the initial seminal work of Briggs and King [1, 2] in the 1950s, and subsequently by John Gurdon [3–7] and colleagues through the 1960s and 1970s, demonstrating the plasticity of a range of primitive and somatic cells in *Xenopus* and amphibia, the fundamental principle of mammalian developmental biology of molecular and phenotypic irreversibility of cellular differentiation persisted until the 1990s [8]. Three landmark studies in sheep challenged this dogmatic assumption, whereby ovine recipient (enucleated) oocytes supported embryonic development of donor nuclei from blastomeres, cultured cells and primary, adult mammary cells, respectively; the latter two studies resulting in both embryonic and fetal development, and live-born progeny [9–11]. With the concurrent development of methods to culture pluripotent stem cells isolated from donated, surplus IVF human blastocysts [12], new strategies for generation of patient-specific (autologous) stem cells for the treatment of human degenerative disorders were proposed and explored, namely (i) nuclear transfer of somatic cell nuclei to recipient oocytes for stem cell isolation from embryos, or (ii) direct reprogramming of somatic cell nuclei via *cell fusion* [13], application of *cell extracts* [14] or ‘*induced pluripotency*’ via

direct delivery of key reprogramming factors (via numerous methods; Fig. 2.1) [15].

In this chapter, we discuss the development of approaches to induce pluripotency in nuclei of lineage-committed somatic cells, and introduce the molecular changes that accompany this process, referred to as ‘[*somatic*] *cell reprogramming*’; namely, transcriptional changes (*JAK-STAT* & *Wnt/Notch* pathways), and epigenetic modifications (chromatin/histone modifications; expanded commentaries of each of these discussions can be found in Chaps. 10, 14 and 17 in this volume).

2.2 Early Nuclear Transfer Experiments in Amphibia (1950–1980): Questioning the Plasticity of the Committed

Much of the early nuclear transfer experiments were undertaken in amphibia. As early as 1952 [1], embryos comprising transferred donor nuclei from undifferentiated frog blastula to enucleated (frog; *Rana pipiens*) oocytes underwent normal embryonic development, prompting consideration of the potential of more committed donor nuclei to contribute to early embryonic development. Briggs and King (1953) [2] noted cleavage rates of constructed embryos, incorporating cells of the frog gastrula (cells undertaking commitment to one of three germ layers), were reduced in comparison to those constructed with frog blastula. Applying these results to more committed donor nuclei, blastulae and later stage (gut) donor cells supported tadpole development and even

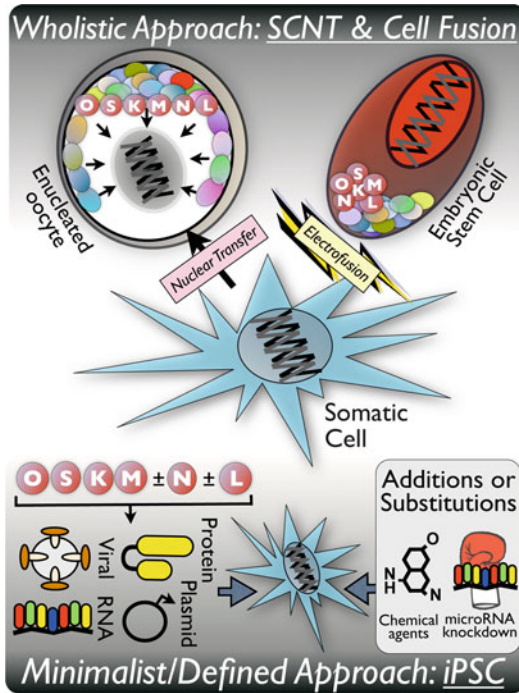


Fig. 2.1 Diagrammatic representation of various methods to reprogram a somatic cell nucleus to pluripotency. ‘Wholistic Approach’ refers to exposure of somatic cells to a *full repertoire* of reprogramming factors (e.g. ooplasmic/cytoplasmic). Somatic cell nuclear transfer of somatic nuclei to oocytes generates cloned stem cells. Fusion of a somatic cell to a pluripotent stem cell via e.g. electrofusion reprograms the somatic cell to pluripotency. The “Minimalist/Defined Approach” refers to reprogramming of somatic cells with *known or defined factors*, in the absence of other factors. Genes such as Oct4 (O), Sox2 (S), Klf4 (K), cMyc (M), Nanog (N), Lin28 (L) have been shown to be key (although not exclusive) to this process [15]. Various chemical agents and microRNA constructs have been used as additions or substitutions to key reprogramming factors

the generation of sexually mature frogs after 9–12 months transplantation [4], proving the ability to undertake complete reprogramming is not limited to primitive nuclei with lineage plasticity. Furthermore, the capacity of lineage-committed, *Xenopus* nuclei to regain pluripotency, and contribute to cleavage-stage embryonic development, is regained in the presence of ooplasmic factors of *alternative Xenopus* species (albeit, of the same *Genus*) [3]. Despite the *Xenopus* species *X. tropicalis* and *X. laevis* being incapable of

generating a hybrid through natural reproductive means (or artificial fertilization), *X. tropicalis* ooplasm (enucleated) was able to successfully reprogram vegetal hemisphere/endoderm donor nuclei from *X. laevis*. During reprogramming, recipient ooplasm elicits structural and functional changes to transferred somatic cell nuclei. Following transfer to *Xenopus* oocytes, mid-blastula nuclei (mitotically active cells, active in DNA synthesis but with little RNA synthesis) resume RNA synthesis, and from 30 min post-injection, cease DNA synthesis and cell division [5]. Ooplasmic factors of recipient (maturing) eggs also rapidly reverse the hypo-proliferative nature of brain nuclei (0.5–3 h post transfer; [5]). Interestingly, cleavage rates of transfers using cultured cells from numerous organs of tadpole (stage 40) and adult *Xenopus* (kidney, heart, lung, testis, and skin) were similar, even adult donors compared to tadpole stage cells, highlighting the plasticity of nuclei from numerous anatomical sources [6]. Histology proved differentiation of donor nuclei to all cells of the developing tadpoles. Extending these results, transfer of *in vitro* cultured somatic *Xenopus* nuclei to *Pleurodeles waltlii* newt oocytes demonstrated reactivation of previously inactive genes and repression of somatic gene profile [7].

2.3 Generation of (Mouse and Human) Embryonic Stem Cell lines: Realizing the Therapeutic Potential

Blastocyst-derived, embryonic stem cells (ESC) are the ‘natural’ counterpart of induced pluripotent cell populations, and since they originally derive from totipotent blastomeres and not lineage-committed cell sources, they do not strictly satisfying the criteria of ‘induced pluripotency’. However, it is fitting a brief historical perspective of ESC isolation and maintenance be presented here, as it was this seminal work by Evans, Kaufman, Martin and Thomson [16–20] in the 1980s and 1990s that highlighted *in vitro* conditions required to maintain ESC, knowledge that was imperative for subsequent development of

induced pluripotency technologies. Furthermore, the molecular, epigenetic and functional characteristics of ESC remain the ‘gold standard’ to which induced pluripotent cells are compared.

Careful consideration of ideal stage of isolation (epiblast of early post-implantation) and *in vitro* culture conditions led to establishment of the first mouse ESC lines [16, 17]. Evans and Kaufman (1981) [16] plated hatched murine blastocysts (129 mouse strain) in culture dishes, from which inner cell mass (ICM) was isolated and re-plated on inactivated feeders. Expanded lines maintained normal karyotype and displayed differentiation potential *in vitro* and *in vivo*. Soon after, additional mouse ESC lines were isolated and cultured in media pre-conditioned on embryonal carcinoma cell culture [17]. Since then, numerous mouse ESC lines have been established around the world, a feat that has fostered development of technologies for generation of transgenic experimental animals.

The obvious progression from derivation of mouse ESC lines was translation to other species. Bongso et al. (1994) [18] were the first to describe isolation of ICM from donated, human (IVF) blastocysts. Although grown on epithelial feeder layers and displaying stem cell morphology and expressing alkaline phosphatase (AP), this Singaporean group failed to maintain a human ES cell (hESC) beyond 2 passages. Interestingly, the establishment of stable hESC lines expressing stem cell markers, possessing differentiation potential and capable of long term growth was achieved some 4 years later [12], and only after pluripotent lines were established in two species of non-human primate [19, 20]. Subsequently, methods for human ICM isolation have been optimized, including a multi-step procedure using day 8 blastocysts (rather than the usual day 5–7) [21]. Statistically significant improvements in yield of human pluripotent cells were reported using this multi-step approach. hESC lines have also been derived from morula (pre-blastocyst) stage embryos, with approximately 17 % of original explants forming stable pluripotent lines; an efficiency similar to blastocyst derived lines [22]. Furthermore, establishment of stable hESC lines from microsurgical removal of single blastomeres

circumvents the inherent embryo destruction when isolated from ICM cultures [23].

The ethical implications of hESC isolation (i.e. with implied embryo destruction), acquired knowledge of favorable *in vitro* ESC culture conditions, and continuing need for *autologous* stem cells for therapy, collectively drove exploration of alternative sources of stem cells for therapy. Despite SCNT-derived, pluripotent cells being reported in non-human primate (*rhesus macaque*) [24], derivation of stable hESC lines by SCNT still elude us. Alternative, more directed approaches to human somatic cell reprogramming, that circumvent embryo destruction associated with SCNT and ESC isolation, have evolved with varying success and efficiency. We outline mammalian SCNT, and alternative approaches, in the coming sections.

2.4 (Mammalian) Somatic Cell Reprogramming, and the Autologous Therapeutic Cell: Reacquiring Plasticity

2.4.1 Somatic Cell Nuclear Transfer (SCNT)

As outlined in Sect. 2.2, the process of somatic cell nuclear transfer (SCNT) typically involves the removal of maternal chromosomes from an oocyte (‘enucleation’) followed by insertion of the donor cell nucleus, or the fusion of intact somatic cell or nucleus, to the enucleated oocyte. Embryonic development is artificially triggered, by inducing an increase in intracellular calcium, and can continue to pre-implantation stages *in vitro*. If transferred to a recipient animal, embryonic implantation and continued fetal development can give rise to a live-born, cloned animal. This process is referred to as *reproductive cloning*. Alternatively, cells of the inner cell mass of transferred blastocysts (*in vitro*) can be isolated and cultured, giving rise to nuclear transfer embryonic stem cells (ntESC); this process is referred to as *therapeutic cloning*. The resulting ntESC can be used as a tool for biomedical research or as a source of cells for transplantation

back to the somatic cell donor. Since the ntESC are genetically identical to the donor at the genomic DNA level, transplanted cells are unlikely to be rejected by the host immune system. It is important to note that the mitochondrial DNA in clones and ntESC is predominantly inherited from the oocyte.

Despite documented success in deriving nuclear transfer offspring in amphibia, it was widely accepted that the molecular events characterizing mammalian cell differentiation prevented it from re-attaining totipotency and contributing to cloned offspring [8]. This dogmatic assumption was challenged by Willadsen (1996) [9] and Wilmut et al. (1997) [11], who generated viable embryos through nuclear transfer of 8- or 16-cell blastomeres, or live-born sheep from mammary epithelial cells, to enucleated ovine oocytes, respectively. Later, numerous cell types were shown to contribute to embryonic and fetal development, and live offspring [25]. SCNT/cloning has now been performed in a number of mammals, including non-human primates [24]. However, the biggest impact being in its translation to agricultural species. Conceptually, reproductive cloning could create multiple clones of animals with highly valued or desirable traits, including cows with high milk production or bulls that breed offspring with high quality meat [26]. Importantly, the biological properties and nutritional value of milk and meat obtained from cloned individuals does not differ from non-cloned animals [27, 28], and thus is considered safe for human consumption [29, 30].

The application of *therapeutic cloning* to the treatment of human, degenerative disease is even more promising. Generation of (cloned) pluripotent stem cells from somatic cells of a diseased individual, in combination with corrective gene therapy *in vitro*, has the potential to treat diseases through autologous cellular transplantation, not withstanding logistical and fiscal considerations. The first proof of principle study on the therapeutic application of SCNT derived cell lines was reported in 2002, whereby ntESC derived from *Rag2^{-/-}* mice were differentiated into hemato-poietic stem cells (HSC) *in vitro* for transplantation after correction of the characteristic *Rag2*

recombinase gene mutation by targeted homologous recombination [31].

However, there are a number of technical hurdles that need to be addressed before SCNT can be a viable source of pluripotent cells for human cell therapy. SCNT is a very resource intensive procedure requiring a large number of oocytes [32], and there are only a handful of reports of human SCNT embryos reaching the blastocyst stage [33–35]. To date, diploid ntESC lines have not been established. Recently, ntESC were isolated following SCNT to an *intact* oocyte (i.e. non-enucleated), resulting in a *triploid* embryo [36]. Interestingly, the triploid ESC isolated from the blastocysts maintained various characteristics of pluripotent cells and could differentiate into cells of all three germ layers.

Most importantly, the process of SCNT demonstrated that adult cells which are programmed to express a subset of specific genes as a differentiated cell, can be reprogrammed or de-differentiated to give rise to an organism karyotypically identical to the donor cell. Due to the ethical issues surrounding SCNT and technical difficulties in translating this to humans, alternate methods of somatic cell reprogramming have been explored including cell fusion, treating cells with cell extracts and viral induction of pluripotency by defined factors.

2.4.2 Cell Fusion

Fusion of interfacing plasma membranes of two cells can be achieved through chemically through treatment with polyethylene glycol (PEG), electrofused or through viral induction [37]. As enucleation does not precede fusion (as for SCNT), the resulting cell hybrids are tetraploid (since nuclear membranes commonly undergo intracytoplasmic fusion) and in most cases the phenotype of the less-differentiated fusion partner dominates the phenotype of the more-differentiated partner. *Reprogramming* of somatic cells by cell fusion involves the hybridization of a pluripotent cell to a somatic cell, resulting in a tetraploid cell hybrid.

In 1976, Miller and Ruddle first demonstrated that the phenotype of the pluripotent cell dominates following somatic cell fusion [13]. Mouse embryonic carcinoma (EC):thymocytes cell hybrids exhibited characteristics of the pluripotent EC cell and could form teratomas containing derivatives of the three germ layers. Other hybrids have adopted a number of pluripotency properties of embryonic germ cells and embryonic stem cells, and shown to modify gene expression profiles of the somatic cell hybrid partner [38–42]. ES-somatic cell hybrids display most properties of pluripotent stem cells; however, since they are tetraploid and have genomic DNA from both parental cells, their use for autologous cell replacement therapy is limited.

Conversion of a tetraploid (pluripotent) hybrid to a reprogrammed diploid cell, carrying the donor (autologous) somatic cell genome, conceptually generates a therapeutically relevant cell. One approach of excluding the ESC-derived DNA genome following reprogramming is maintaining a ‘heterokaryon’ (preventing fusion of the nuclear membranes) for the duration of reprogramming, followed by subsequent enucleation of the ES nucleus [43, 44]. Although a viable cell remains after extrusion of the ESC-derived nucleus, this approach resulted in only partial reprogramming of the somatic nucleus [44]. An alternative approach involves the removal of the specific ESC derived chromosomes responsible for self recognition in the ES-somatic cell hybrid resulting in a hybrid aneuploid cell that is immune matched to the somatic cell [45]. Despite these novel approaches, more advances in the cell fusion field are required before it can be considered a viable alternate method for generating autologous pluripotent stem cells for clinical applications.

2.4.3 Cell Extracts

In contrast to fusing intact pluripotent cells to somatic cells, methods of exposing differentiated cells or nuclei to cell extracts from totipotent and pluripotent cells have been devised as a means of somatic cell reprogramming. This technique

involves the reversible membrane permeabilisation of differentiated cells, using the chemical *streptolysin-O* (SLO; a member of the family of cholesterol-dependent cytolysins), followed by exposure to ‘reprogramming’ cell extracts. The lesions induced in the plasma membrane by SLO are resealed upon application of Ca^{2+} [46]. Earlier reprogramming studies using cell extracts showed that the incubation of somatic cells in *Xenopus* egg extracts resulted in remodeling of chromatin and changes to gene expression [14, 47, 48]. The first demonstration of reprogramming of differentiated cells by exposure to mammalian cell extracts was performed with HEK293T (human embryonic kidney) cells exposed to stimulated T-cell extracts resulting in direct reprogramming toward a lymphoid-specific phenotype [49]. Furthermore, it was demonstrated that cell extract based reprogramming involves transcriptional changes in addition to ATP-dependent chromatin remodeling [49, 50].

The first reprogramming studies using pluripotent stem cell extracts showed that both HEK293T cells and immortalized NIH/3T3 mouse fibroblast cells acquired characteristics of pluripotent stem cells when exposed to extracts made from a pluripotent human carcinoma cell line and cultured [51]. The treated cells were partially reprogrammed and formed colonies, which were alkaline phosphatase (AP) positive and expressed pluripotency markers. They had also deactivated differentiation markers and had undergone epigenetic changes at the promoters of a number of pluripotent gene loci [51, 52]. More recently, human fetal fibroblasts have been shown to form hESC-like colonies when treated with a combination of chromatin inhibitors and hESC extracts [53]. Partial reprogramming was reported when the somatic cells were pre-treated with the epigenetic modifiers 5-aza-2'-deoxycytosine and Trichostatin A prior to exposure to hESC extracts. Treated cells were shown to upregulate a number of pluripotency genes and change morphology and growth characteristics. Although reprogramming to a complete pluripotent state was not achieved, the cells could be trans-differentiated into neurons under differentiation conditions [53].

To date cell extract exposure has been shown to partially reprogram the treated cells toward an embryonic state, predominantly in transformed and immortalized cell lines. However, it is noteworthy that survival and persistence of pluripotent stem cells in fusion preparations, having donated cell extract, constitute a potential source of contamination in subsequent analysis [54].

2.5 Induction of Pluripotency (Post-2006) Identification of the Critical Reprogramming Factors for Direct Induction of Pluripotency

A major turning point in international stem cell research came in 2006 with the generation of ‘induced pluripotent stem cells’ (iPSC), the significance of this discovery was recognized by the joint-award of the Nobel prize for Physiology or Medicine in 2012 to Shinya Yamanaka to its discoverer. Ectopic expression of just four transcription factors resets the transcriptional profile and epigenetic state of the host cell to one resembling an ESC [15]. The most widely used set of reprogramming factors, *Oct4*, *Sox2*, *Klf4* and *c-Myc*, was identified initially by screening 24 pre-selected factors by Takahashi and Yamanaka [15]. Since this discovery, research papers characterizing properties of iPSC have flood scientific literature: murine (and subsequently human) iPSC can be generated without the oncogenic factor cMyc [55, 56], are germ line competent [57], and contribute all cell types to fertile offspring via the tetraploid complementation assay [58–60]. Human iPSC can be generated using the same set of factors [61, 62] or an alternative set of 4 factors, namely *Oct4*, *Sox2*, *Nanog* and *Lin28* [63], suggesting that *Oct4* and *Sox2* are essential whereas *Nanog*, *Klf4*, *Lin28* and *c-Myc* are alternative reprogramming supporting factors. iPSC can be generated without permanent integration of transgenes [64–67] and be generated from diseased patient cells [68–71]. Like ESC, they are capable of differentiating into multiple cell types of the germ layers, including heart, blood, islet, nerve, liver, and muscle [72–79]. Therefore, the potential

benefits of iPSC for regenerative medicine are immense, for example, skin biopsies (notably, terminally differentiated readily accessible cells) could be taken from patients of degenerative disease or injury for conversion to pluripotent iPSC before their directed differentiation to the cell type of interest. Transplantation of the differentiated progeny to the afflicted organ or tissue could manage or cure disease/condition, and since the original donor cell came from the patient, the risk of immune rejection of the grafted cells is minimal.

Despite the therapeutic potential, technical and logistic issues surround iPSC technology. Efficiency of cell reprogramming remains low, attributable in part by incomplete transcriptional and epigenetic reprogramming, and actual/risk of mutagenesis during conversion makes the differentiated cells possibly oncogenic. If iPSC are to achieve therapeutic relevance, methods that ensure complete differentiation of all cells within a pool of iPSC are required to negate the possible tumourgenicity of rare pluripotent cells once transplanted. Also the time taken to convert somatic cells to clinically relevant and regulatory body approved therapeutic cells may prevent autologous use of the cells *per se*, rather cells matched to potential populations may require to be banked. Therefore, research is currently underway that address these, and other iPSC-related problems; some of which are discussed here.

2.5.1 Improvement of Efficiency, Quality and Purity of iPSCs Production

In Yamanaka’s landmark study, the efficiency of reprogramming mouse embryonic fibroblasts (MEF) into iPSC was reportedly 0.01–0.1 % [15]. Despite prolonged expression of the Yamanaka’s four factors, only a small percentage of cells achieve full reprogramming. In contrast, reprogramming by somatic cell nuclear transfer and cell fusion is quicker and more efficient [44, 80], indicating that additional key reprogramming factors may yet to be identified. Previous studies showed that in differentiated mouse ESC, COUP-TFs silences the *Oct4* locus by binding to

RAREoct, a composite RA responsive element in the *Oct4* promoter [81, 82]. RA receptors (RARs) and members of Nr5a steroid hormone receptor family (Lrh-1) form heterodimers, which compete with COUP-TFs for RAREoct and maintain *Oct4* expression [83]. By adding *Rarg* (RAR- γ) and *Lrh-1* to the Yamanaka reprogramming cocktail, Liu and co-workers report 100-fold improvements in reprogramming efficiency [84]. Key indicators of iPSC reprogramming, namely, activation of *Oct4* and *Rex1* genes, were observed from as few as 3–4 days of (six) factor induction, a temporal indicator comparable to somatic cell nuclear transfer [85] or cell fusion [86].

One central question related to the molecular mechanisms of iPSC formation is how transcriptionally restrictive chromatin at loci of inactive pluripotent genes (e.g. *Oct4*, *Sox2*, and *Nanog*) in somatic cell is relaxed upon exogenous factor induction to permit resumption of expression. Chemical agents that relieve the restrictive conformation of heterochromatin, such as inhibitors of histone deacetylation and DNA methylation, increase the efficiency of iPSC generation [87, 88], indicating that modifications to chromatin structure in somatic cells is key to full reprogramming. Knockdown of *p53* in B cells shortens cell cycle length results in relief of repressive heterochromatin conformation during DNA synthesis, greater access of reprogramming factors to previously inaccessible genomic loci and therefore reduces the time required to form iPSC twofold [89]. MyoD, a transcription factor for skeletal myogenesis, can recruit various transcription factors and chromatin remodeling proteins to its target genes more efficiently than *Oct4*, leading to activation of suppressed genes embedded in repressive chromatin [90]. Hirai and co-workers discovered that fusing a fragment transactivation domain (TAD) of MyoD to *Oct4* (M₃O) improves the iPSC reprogramming process [91]. Transduction of TAD-*Oct4* with *Sox2*, *Klf4*, and *cMyc* to fibroblasts effectively remodeled patterns of DNA methylation, chromatin accessibility, histone modifications, and protein binding at pluripotency genes, raising the efficiency of mouse/human iPSC generation more than 50-fold in comparison to the Yamanaka four factors (OSKM). The resultant human iPSC colonies appeared in around 5 days, in contrast to

two weeks with OSKM, and the purity of the iPSC was much higher with the M₃O-SKM gene introduction (98 % of the colonies) compared with OSKM (5 %).

2.5.2 Epigenetic Characteristics of iPSC

Although iPSC exhibit many of the morphological and molecular characteristics of ESC, a number of recent reports have questioned the extent to which reprogrammed iPSC adopt an epigenetic signature characteristic of their ESC counterparts. iPSC appear to retain an ‘epigenetic memory’ of the donor tissue from which they were derived and exhibit somatic genome-wide messenger RNA and microRNA expression patterns, thus questioning their differentiation potential. Although sharing pluripotency status assessed by various criteria, iPSC derived from fetal fibroblasts, neonatal fibroblasts, adipose stem cells, and keratinocytes differ in expression profiles of core sets of donor genes [92]. Expression profiles in fetal fibroblasts-derived iPSC bore closer resemblance to human ESC followed by adipose, neonatal fibroblasts, and keratinocyte-derived iPSC.

Overall, iPSC and ESC share a well-defined core pluripotency network, although select core genes are often hypo-expressed in iPSC. George Daley’s lab found iPSC harbor residual DNA methylation signatures characteristic of their somatic tissue of origin, which could be reset by differentiation and serial reprogramming, or by treatment of iPSC with chromatin-modifying drugs (TSA and AZA) [93]. The DNA methylation of nuclear transfer-derived pluripotent stem cells resembles classical ESC than iPSC. However, other studies report resetting of epigenetic memory and cell function upon continuous passaging, suggesting that complete reprogramming is a gradual process that continues beyond the acquisition of a *bona fide* iPSC state assessed by activation of endogenous pluripotency genes, viral transgenes-independent growth and the ability to differentiate into cell types of all three germ layers [94]. Guenther et al. compared both global chromatin structure and gene expression

profiles of a panel of human iPSC and ESC [95]. Genome-wide maps of nucleosomes with histone H3Kme3 and H3K27me3 modifications indicate that there is little difference between ESC and iPSC with respect to these marks. Gene expression profiles confirm that the transcriptional programs of ESC and iPSC show very few consistent differences. Importantly, the observed differences in these cell lines did not discriminate iPSC from ESC [95].

2.5.3 Generation of iPSC for Clinical Applications

Numerous modifications to the original, retroviral-based Oct4, Sox2, Klf4 and cMyc method have been reported since its original description with the aim to improve reprogramming efficiency or create iPSC for clinical application [15, 87, 88, 96, 97]. Retroviral or lentiviral vector-mediated transduction of reprogramming genes involves random integration of exogenous DNA into the genome of the recipient cells, representing a preventative obstacle to therapeutic use of the cells and their derivatives. iPSC can be obtained with removable *PiggyBac* transposons or episomal systems [65, 98–100], but these approaches are either (i) at least temporarily mutagenic, or (ii) still require delivery of exogenous DNA construct to the nuclear compartment of target cells, thus increasing risk of genomic recombination or insertional mutagenesis. Sendai virus, an RNA genome, has been used to deliver transgenes, but undesirable in the therapeutic arena given the required purging of reprogrammed cells of replicating virus [101, 102]. iPSC have been generated with recombinant proteins of Oct4, Sox2, Klf4 and cMyc incorporating cell-penetrating peptide moieties [103, 104] and synthetic modified mRNA of the four factors [105]. A recent study showed reprogramming of mouse and human cells to pluripotency by direct transfection of a combination of mir-200c plus mir-302 and mir-369 family microRNAs (miRNAs), albeit at considerably lower efficiency [106].

iPSC appear to share much of the differentiation potential characteristic of ESC. Recently, iPSC (from murine and human cell sources) have

been differentiated into functional SM/C-2.6⁺ skeletal cells [107], IFN- γ expressing T-cells [108], human hematopoietic precursors [109], coagulation factor-expressing, liver-like cells [110], and hepatocyte- and cardiomyocyte-like cells [111], to name a few. The considerable health and economic burden of neurological disease has prompted development of protocols for directed differentiation to neural cell (progenitor and mature) and related lineages (e.g. glial cells; [112–119]). These cells enable immediate study of neurological disease development in vitro, and represent a potential transplantable pool of cells for the treatment of human neurological disease in vivo. Interestingly, Kim et al. (2011) [115] report transient induction of Oct4/Sox2/Klf4/cMyc, in combination with FGF2, FGF4 and EGFP-containing media, (trans) differentiate mouse fibroblasts to expandable neural progenitor cells and glia. Mature cells expressed numerous neuronal markers, could be induced to generate action potentials and formed functional synapses. Secreted protein from chick dorsal root ganglia in culture also directs differentiation of murine iPSC to motor and sensory neurones [116]. iPSC technology enabled the identification/study of novel/known molecular pathways misregulated in diseased neural cells, through neural differentiation from human fibroblast donor cells, as well as in vitro screening of proposed corrective measures for efficacy (genetic Parkinson's disease and schizophrenia fibroblast donors; [113, 117]).

Whilst the propensity to differentiate to oligodendrocyte precursor cells (marker A2B5⁺) from a pluripotent phenotype is comparable between mouse ESC and iPSC, rates of differentiation directly to mature oligodendrocytes (marker O4⁺) differ markedly (24 % ESC v 2.4 % iPSC; [112]). It is noteworthy that the differentiation protocols employed in this study were optimized to differentiation of ESC; subtle differences between iPSC/ESC phenotypes may account for this discrepancy and specific, iPSC-related protocols may need to be devised for improvements in differentiated cell yield to be realized. In a three-step protocol, (i) induction of mouse iPSC for (ii) differentiation to an intermediate oligodendrocyte precursor cell yielded (iii) functional (i.e. myelinating) oligodendrocytes in vitro

when co-cultured with dorsal root ganglial neurones [114]. It will be interesting if these results can be recapitulated in a human system.

Despite these impressive results, concerns surround pluripotent iPSC retaining a ‘memory’ of their original, differentiated donor cell state, and thus a propensity to spontaneously differentiate to their original phenotype [120, 121]. Partial DNA methylation in low passage iPSC, permits re-activation of the original somatic cell-related transcriptional profile [121]. This has obvious implications for cell transplantation *in vivo*.

Also, the non-immunogenicity of pluripotent ESC transplanted to allogenic recipients (leading to teratoma formation) raised hope that autologous iPSC transplantation would have applications in clinical medicine. However, Zhao et al. (2011) [122] recently questioned the clinical applicability of iPSC. Although ESC derived from B6 embryos circumvent the immune response in B6 recipients, leading to teratoma formation, transplantation of reprogrammed iPSC to syngeneic hosts initiates T-cell-mediated infiltration and necrosis (Zhao et al. 2011) [122]. The behavioral differences between transplanted ESC and iPSC to syngeneic hosts are perhaps attributable to gene expression profile differences. These concerning and unresolved issues highlight the need for caution when considering transferring current cell induction and differentiation protocols from bench to bedside.

In addition to being a cellular source for transplantation therapy, human iPSC also have great potential for disease modeling and drug development. Human iPSC can be generated from a variety of diseased individuals, display similar differentiation capacity to control iPSC derived from normal individuals [68, 70], and (diseased) iPSC-derived cells (e.g., motor neurons, cardiomyocytes) recapitulate disease-specific effects *in vitro* [69, 123–125]. iPSC technology enabled modeling of diseases such as dyskeratosis congenital and Friedreich’s ataxia, leading to identification and exploration of novel therapeutic strategies [126, 127]. The ability to derive iPSC and create disease models lacking high quality or appropriate animal models will facilitate disease biology studies and drug

discovery in the future, notably, for diseases that are complex or polygenic and are not easily recapitulated by gene modifications in mice, and personalized medicine.

2.6 Transcriptional and Epigenetic Changes During Reprogramming

Wholesale changes to the transcriptional and epigenetic architecture of somatic cells must precede, and are a feature of, full adoption of the pluripotent phenotype. Molecular signatures of ESC provide a yardstick to which induced pluripotency is assessed in somatic cell reprogramming. In this section, we discuss the known transcriptional and epigenetic profile characteristics of ESC, and highlight their counterparts in the process, and full adoption, of (induced) pluripotency in somatic cells.

2.6.1 Changes to Transcriptional Profile During Reprogramming

Here, we provide a brief preview of transcriptional changes relating to two well-characterized gene families during reprogramming; (i) the *Wnt/NOTCH* Pathway and (ii) the *JAK-STAT Pathway*. We hope this provides an introduction to more comprehensive appraisals of these important gene families in Chaps. 10 and 14 of this volume, respectively.

2.6.1.1 *Wnt/NOTCH* Pathway And *Wnt* Signaling Pathway

The membrane-bound Frizzled and LRP5/6 (low-density-lipoprotein (LDL)-receptor like protein 5 or 6) heterodimer forms the dual receptor complex for the canonical Wnt signaling pathway after binding with Wnt proteins [128–131]. Upon binding of Wnt ligand to the trans-membrane receptor, dual pathways are activated; (i) the cytosolic scaffolding phosphoprotein Disheveled is phosphorylated via CK1 activated by Wnt signaling [132] (ii) the function of serine threonine kinases glycogen synthase-3(GSK3-β)

is deactivated through binding of Axin GSK3- β complex to LRP5/6 receptor co-factor. Both mechanisms lead to activation of the β -catenin; (i) phosphorylation of Dishevelled prevents phosphorylation of β -catenin by the recruitment and binding of Axin GSK3- β complex to phosphorylated LRP5/6 via the PPPSP motif [133, 134] and (ii) deactivation of GSK3- β suppresses its ability to phosphorylate β -catenin, phosphorylated β -catenin is targeted for degradation by the 26S proteasome. Accumulation of cytosolic β -catenin results in its translocation to the nuclear compartment and binding of lymphoid enhancer factor (LEF)/T cell factor3 (TCF3) transcription factors to regulate expression of target genes [135]. The subsequent recruitment of specific co-factors to the β -catenin/Tcf3 heterodimer, from a range of possible co-binders, dictates activating or repressive functions on gene expression.

The Wnt signaling pathway contributes to the maintenance of pluripotency in mouse and human ESC [136–141] as well as the self-renewal of undifferentiated adult stem cells in multiple tissues [138]. Application of Wnt1 or Wnt3a to culture media, or over-expression in feeder cells, enhances proliferation of hESC and maintains pluripotency [140]. Inhibition of GSK-3 maintains the undifferentiated phenotype and sustains expression of pluripotent state-specific transcription factors, Oct-3/4, Rex-1 and Nanog [135, 140]. Indeed, chemical inhibition of GSK-3 by CHIR99021 permits generation and maintenance of rat ESC [141]. In ESC, the Tcf3 (\pm β -catenin) and Oct4/Sox2/Nanog, combine to maintain protein levels within desirable thresholds and thus regulate the balance between pluripotency and differentiation [142, 143]. To illustrate, Tcf3 co-precipitates with Oct4 and/or Nanog in genome-wide ChIP-ChIP experiments [142], and occupies the Myc promoter. Tcf3 is a key player in the regulation of Nanog expression, maintaining mRNA and proteins levels, and regulating promoter activity through binding to regulatory elements [144]. Furthermore, Nanog levels are elevated in Tcf3 null ESC. Wnts also act synergistically with LIF through the JAK/STAT pathway, the former increases STAT3 mRNA levels while the latter phosphorylates it [145].

The effect of Wnt signaling in pluripotent ESC can be applied to a cell reprogramming setting. Wnt3a enhances somatic cell reprogramming by cell fusion [145]. Wnt3a-containing conditioned medium can substitute for exogenous cMyc in Oct4-Sox2-Klf4 mediated reprogramming, and improve reprogramming efficiency by as much as 20-fold [146]. Reprogramming of neural progenitor cells (NPCs) through exogenous expression of Oct4 and Klf4 alone is facilitated by the knock down of Tcf3, suggesting that Tcf3 represses β catenin activated genes, which are relevant in the efficient formation of iPSC. Similar successes can be recapitulated in cell fusion of NPCs with Tcf3 null ESC, resulting in large epigenetic changes in the genome permitting endogenous Oct4 to bind previously inaccessible target promoters [147].

Notch Signaling Pathway

The Notch pathway is involved in differentiation processes and lineage fate in fetal and postnatal development, as well as in adult self-renewing organs [148]. Five mouse/human Jagged and Delta proteins (Jagged1, Jagged2, Delta-like (Dll)-1, -3 & -4) represent ligands for Notch receptors (Notch-1 to -4 in mouse/human) [148, 149]. The ligand–receptor interaction at the cell surface (of neighboring cells) leads to the proteolytic cleavage of the Notch receptor, leaving a membrane-bound cleavage product, referred to as Notch Extracellular Truncation (NEXT). The intracellular portion of NEXT is further cleaved by cytosolic γ secretase to the Notch intracellular domain (NICD), which translocates to the nucleus [150, 151]. Notch fragment NICD binds CBF/CSL in the nucleus and along with MAM(mastermind)/Lag3(mammals) forms a transcriptionally active ternary complex which recruits general transcription factors CBP/p300 and PCAF, promoting chromatin acetylation and increased expression of Notch target genes [152, 153], such as Hes/Hey [154], bHLH like Id family of proteins, Sox9, Pax6, Lineage specific transcription factors (LSTFs) [155]. Here we focus on pluripotent stem cells and Notch signaling in that context.

Notch signaling activates Hes/Hey transcription, which leads to repression of Hes/Hey target

genes such as tissue specific transcriptional activators, thereby preventing differentiation [154]. Furthermore, CHiP-Seq mapping of 13 transcription factors has revealed that core pluripotency transcription factors associated with Oct4, Sox2 and Nanog as well as signaling effectors Smad1 and STAT3 co-localize with enhancer-associated transcriptional co-activator p300 at non-promoter region [156].

In the nervous system, Notch signaling influences balance between progenitors and its differentiating progeny corroborated by evidence in which forced expression of Notch-1C promotes neurogenesis [157–159]. Furthermore Jag1 exposure to hematopoietic stem cells increases the proportion of stem cells as opposed to differentiating cells [160]. Disruption of Notch1 signaling in ESC results in commitment to mesodermal lineage through upregulation of mesodermal and cardiac markers [161]. Thus, Notch regulates lineage commitment, particularly relevant to mesodermal and neuro-ectodermal fates [161].

This may suggest that Notch does not appear to be a general inhibitor of ESC differentiation but functions as a regulator of cell fate decisions in multipotent ESC that must choose between mesodermal and neuro-ectodermal fates [161]. Although maintenance of pluripotency is not affected by Notch signaling, whether or not it affects induction of pluripotency from somatic cells is yet to be established.

2.6.1.2 JAK-STAT Pathway

Binding of the Leukemia Inhibitory Factor (LIF) to its receptor triggers three intracellular cascades: the *JAK/STAT3* (Janus kinase/signal transducer and activator of transcription 3), the *PI3K* (phosphoinositide 3-kinase)/*AKT* and the *SHP2* [SH2 (Src homology 2) domain-containing tyrosine phosphatase 2]/*MAPK* (mitogen-activated protein kinase) pathways [162, 164]. The *JAK/STAT* pathway is of particular interest due to its pleiotropic nature and its regulation of proliferation, differentiation, cell migration, apoptosis cell renewal and pluripotency [165]. Herein we focus on the main events during the transduction of LIF signal through *JAK/STAT3* cascade on mouse ES

and iPSC *in vitro* (Fig. 2.2); more comprehensive reviews can be found elsewhere [163].

Several activators of the *JAK/STAT* pathway have been identified to date including, the growth hormone, erythropoietin, interferons and interleukin family members. Following development of methods for murine ESC isolation and culture, persistent activation of the *JAK/STAT* pathway was found to be essential for the maintenance of pluripotency, with LIF driven *JAK1/STAT3* activation [167–168]. The considerable expression of cytokines including LIF from initial feeder (e.g. Buffalo rat liver BRL epithelial cell line) shown to be responsible for repressing differentiation in murine ES *in vitro* [169]. Disruption of *JAK1/STAT3* signaling promotes differentiation of ESC [170], additionally, constitutive *STAT3* expression, using a fusion protein composed of *STAT3* and the estrogen receptor is sufficient to maintain ES in an undifferentiated state [167].

LIF is a “helical type 1”, interleukin (IL)-6-type family protein [162]. The LIF receptor comprises a heterodimer of (i) A common IL-6 family subunit (gp130) [171], and (ii) A low-affinity, LIF-specific subunit (gp190 or LIF receptor beta/LIFR β) [170]. Upon formation of the ligand-receptor trimeric complex, numerous phosphorylation events characterize activation of the *JAK/STAT* pathway. The four members of the mammalian *JAK* family (*JAK1*, *JAK2*, *JAK3* and *TYK2*) share seven regions of homology, named JH1 to JH7. They are functionally divided into three domains: amino-terminal region (N), a catalytically inactive kinase like (KL) domain and a tyrosine kinase (TK) domain [172]. Among the four *JAK* proteins, only *JAK1* and *JAK2* are involved to LIF signal as is suggested by knock-out murine model experiments [173]. *JAK1* is bound to gp130. Upon LIF mediated activation a reciprocal phosphorylation occurs between gp130 and *JAK1*. Firstly, phosphorylation of *JAK1* at its TK domain (Tyr1022), followed by phosphorylation of four tyrosine residues on gp130, providing a docking site for the following component: *STAT3* [163, 174, 175].

The seven isoforms of *STAT* share 6 conserved domains: an amino-terminal domain (NH2), a coiled-coil domain, the DNA binding domain

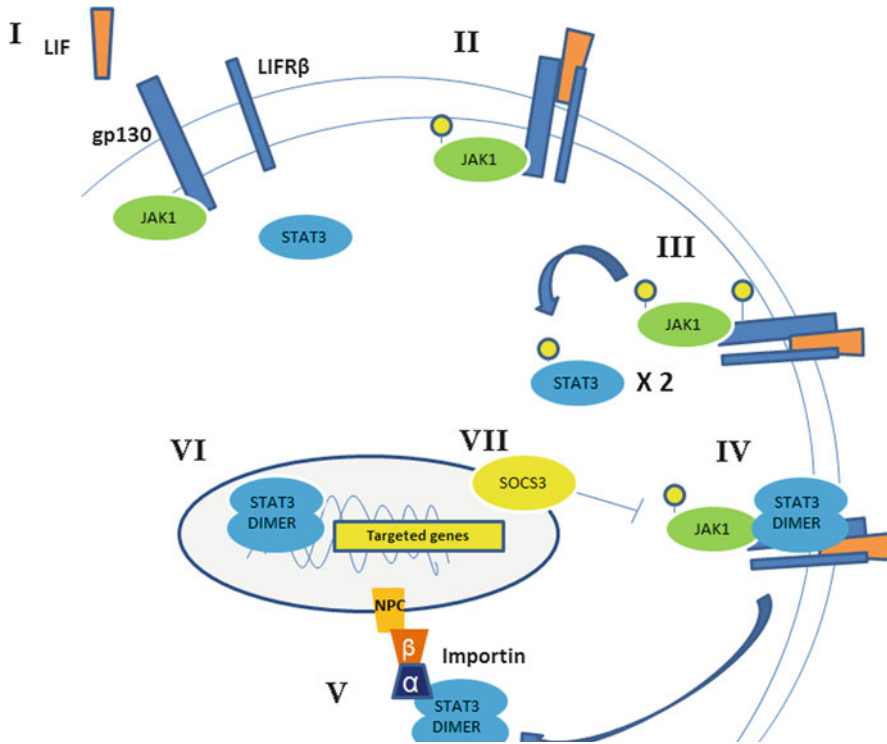


Fig. 2.2 Simplified schematic LIF-dependent JAK1/STAT3 signaling in pluripotent stem cells. I The LIF receptor comprises two glycoproteins (gp130 and gp190/LIFRβ). JAK (intracellular transducer) and cytosolic STAT3 (second messenger). II JAK1 is phosphorylated after LIF ligand binds the receptor. III gp130 and STAT3 are sequentially phosphorylated IV After dimerization of STAT3 the homodimer is released V STAT3 dimer translocates to the nucleus through the nuclear pore complex

NPC; translocation is mediated by subunits importin-α3 or importin-α6 and subunit importin β. VI STAT3 mediates the expression of several target genes related to self renewal and stemness and inhibition of mesoderm and endoderm differentiation. VII SOCS3 is also upregulated under STAT3 signaling. SOCS3 binds the phosphorylated JAK and mediates its degradation. The latter provides a mechanism of negative feedback for attenuation and regulation of STAT3 signaling

(DBD), a linker domain, an SH2 domain, and a tyrosine activation domain and a carboxy-terminal transcriptional activation domain (TAD); the latter being conserved in function but not in sequence [173]. STAT3 is also phosphorylated by JAK at Tyr (705) [166], triggering formation of a STAT3 homodimer (STAT3h), through the N-terminal SH2 domain; STAT3h is subsequently released to the cytoplasm [176]. The translocation of STAT3h to the nuclear compartment is mediated by two importin protein family members, importin-α3 and importin-α6 [177, 178]. Then STAT3h binds to the STAT3-related enhancer region harboring the consensus sequence *TTCC(C/G)GGAA* on target genes [179].

Several genes have been described as targeted genes by STAT3 with a majority of them involved on the subsequent inhibition of mesoderm and endoderm differentiation [180]. To illustrate, STAT3 expression promotes self-renewal of ESC in part through transcriptional control and regulation of c-Myc and Klf4 [181, 182], as well as binding to the enhancer element of Nanog gene (in mice, [183]). Chromatin Immunoprecipitation (ChIP) experiments show co-localization of STAT3 at loci share with pluripotency regulators Nanog, Sox2, Oct4 and Smad1 [179]. Finally, among those genes which are targets of STAT3, is SOCS3, a regulator that attenuate LIF signaling by binding the phosphorylated JAK1 and mediates

its proteosomal degradation after a previous conformational change by gp130 binding; this provides a negative feedback to the STAT3 signal [184, 185].

The dependence of LIF to maintenance of murine ESC pluripotency directly translates to the induced pluripotent phenotype [186, 187]. The JAK/STAT3 molecular cascade is activated during somatic cell reprogramming to pluripotent iPSC and in EpiSC reprogramming under Nanog or Klf4 over expression and specific conditions [188]. Additionally, the clonal yield is reduced if JAK/STAT3 cascade is inhibited [188].

iPSC generated from Neuronal Stem Cells (NSC) and embryonic fibroblast depends on JAK/STAT3 pathway to maintain a ground state. Furthermore, in the presence of LIF a 3–4-fold increase in the number of colonies was observed; an effect only attributable to JAK/STAT3 cascades [188]. This effect has been proved in experiments using chimeric receptors [188, 189]. Over expressing a JAK/STAT3 activating receptor GY118F, LIF independent and responsive to granulocyte colony stimulating factor (GCSF), was possible to reprogram EpiSc into chimera-competent iPSC [188]. In a similar approach, but using the expression of a tamoxifen inducible form of STAT3 (STAT3MER) was possible to retain a pluripotent phenotype in murine iPSC even in absence of LIF stimulus, but not once tamoxifen was removed from the media [189].

2.6.2 Epigenetic Changes During Reprogramming

Chromatin refers to the collective DNA/histone complexes within the nucleus of a cell, the modification of which regulates access of transcriptional machinery to genes and regulatory elements in the genome [190]. Epigenetic marks at specific loci throughout the genome of somatic and pluripotent stem cells are likely to differ markedly, and require considerable global remodeling when converting from the former to the latter cell type.

Analysis of the epigenetic state provides a meaningful way of determining the degree of

reprogramming in iPSC. Pluripotent stem cells contain a characteristic chromatin signature, termed ‘bivalent domains’ [89]. These are regions enriched for repressive histone H3 lysine 27 trimethylation (H3K27me3) and simultaneously for histone H3 lysine 4 trimethylation (H3K4me3), an activating mark [191]. Bivalent domains are indicative of genes that remain in a poised state and iPSC are found to contain large numbers of bivalent domains. Consequently, pluripotent cells were found to contain a large number of bivalent domains compared with, for example, multipotential neural progenitor cells (NPC) cells. Several of the murine iPSC studies have investigated a small number of representative loci for their chromatin and DNA methylation patterns [192–194].

In a more expanded survey of the iPSC epigenome, Maherali and colleagues (2008) [192] suggested that the epigenetic profile of iPSC closely mirrored their ESC counterparts, with 94.4 % of 957 ‘signature’ genes (defined as genes that have a different epigenetic state between MEF and ESC) being reset to an ESC state in the respective iPSC line. H3K4me3 patterns were also similar across all samples, indicating that reprogramming was largely associated with changes in H3K27me3 rather than H3K4me3 [192]. Applying ChIP-Seq to determine genome-wide chromatin maps in several iPSC lines that were derived in distinct ways; namely, (i) through drug selection using an Oct4–neomycin-resistance gene [194], (ii) through drug selection using a Nanog–neomycin-resistance gene [196], and (iii) by simply isolating reprogrammed cells through their morphological appearance [197], Mikkelsen et al. (2007) [198] found overall global levels of repressive H3K27me3 and the characteristic bivalent chromatin structure were restored across the different iPSC lines.

In addition to histone modifications, DNA can be modified directly through attachment of methyl groups to CpG islands [199]. DNA methylation is stable and heritable (yet reversible) and influences many biological processes, including gene regulation, genomic imprinting and X-chromosome inactivation. DNA methylation patterns are dynamic during early embryonic development

and are essential for normal post-implantation development [199]. Overall DNA methylation levels remain stable during ES-cell differentiation. However, these marks are not static [200] and regulate chromatin structure and function in concert with histone modification. To illustrate, H3K4me3 and DNA methylation are considered mutually exclusive and rarely co-exist at a given loci [201]. The re-establishment of H3K4me3 and the associated loss of DNA methylation in particular at ‘ES-cell-associated transcript’ (ECAT) genes seem to be a crucial and potentially rate-limiting step during reprogramming [202]. At reprogramming, expression from ECAT’s in somatic cells is reinstated from a state of dormancy, with associated demethylation marks of promoter regions [203]. This illustrates the importance of demethylation of key genomic loci for complete reprogramming to be achieved. Consistent with this notion, application of DNA demethylation agent 5-azacytidine accelerated fourfold increase in the reprogramming of lineage-restricted cells to iPSC [209].

Despite similarities in overall epigenetic state of iPSC and ESC, iPSC line-to-line variation complicates the comparison. Application of different/fewer reprogramming factor combinations or methods (e.g. RNA, recombinant protein delivery), use of chemical substitutes as well as choice of target cell, and prolonged periods of culture is likely to alter the epigenetic state of the respective cells [201, 202]. In particular, imprinted genes can only be reset in the germ line and are unstable in murine ESC cultures [203], although apparently not in human ESC [204]. More studies are required to test these hypotheses and confirm whether epigenetic stability is cause for concern when considering iPSC for therapeutic applications.

2.7 Conclusion

Convincing the scientific community that the observed plasticity of amphibian somatic cell nuclei translates to the mammalian system took some 30 years. The cloning of *Dolly* was conclusive proof mammalian somatic cells can acquire

totipotency in certain situations. The concurrent development of cell culture techniques for ESC facilitated application of the techniques to cultivate cloned, human ESC by SCNT; however this eventuality still eludes us. Since 2006, much of the stem cell research has adopted a direct approach for derivation of patient-specific stem cells for therapy, but numerous issues require addressing before their therapeutic potential is realized. However, the current state of iPSC allows a unique means for understanding development and disease and provides an unprecedented resource for drug discovery.

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