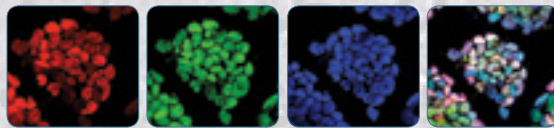


GENERATION OF INDUCED PLURIPOTENT STEM CELLS



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INTRODUCTION

BRIEF HISTORY OF REPROGRAMMING: THE DISCOVERY OF iPSCs

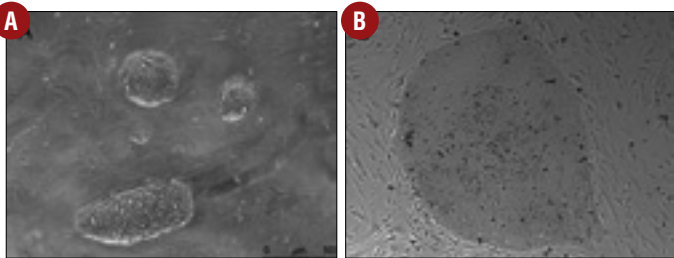


Figure 1: Murine and Human iPSCs. **A.** Murine iPSCs, note that the cells grow in distinct dome-shaped colonies with reflective borders. **B.** Human iPSCs, note the flattened colony morphology.

Pluripotent cells have the ability to generate any cell type present in the adult body, making them a powerful tool for the study of human development and disease. Pluripotency exists only during a brief time window of pre-implantation development in the cells of the inner cell mass (ICM). As development proceeds, ICM cells undergo widespread epigenetic changes, and thus become increasingly lineage-restricted. Embryonic stem cells (ESCs) are the *in vitro* representation of the ICM/epiblast and as such have not yet lost the ability to give rise to all tissues of the embryo proper. While ESCs have been a useful tool in the study of differentiation and development, their potential for generating patient-specific and disease-specific tissues is limited due to their origin from explanted blastocyst-stage embryos. However, different approaches have been developed to reprogram differentiated cells back into pluripotent cells through experimental manipulation. The cloning of animals by somatic cell nuclear transfer (SCNT; Briggs & King 1952; Gurdon et al. 1975; Wilmut et al. 1997; Hochedlinger & Jaenisch 2002; Eggan et al. 2004; Li et al. 2004) is one such approach, which has two key implications. First, it showed that the developmental restrictions incurred during differentiation are not permanent, and second, it implied that factors must be present in the oocyte that have the capacity to restore the developmental potential of a somatic cell. Importantly, the capacity to reprogram somatic cells is not restricted to oocytes, as demonstrated by cell fusion experiments, providing another experimental system to reprogram differentiated cells. Examples include the fusion of somatic cells with ESCs, embryonic germ cells, or embryonic carcinoma cells, which elicits transcriptional and epigenetic reprogramming of the somatic nucleus to a pluripotent state (Tada et al. 1997; Tada et al. 2001; Cowan et al. 2005).

Insights from SCNT and cell fusion experiments led to the breakthrough discovery by Takahashi and Yamanaka, who demonstrated that activation of a defined set of factors in fibroblasts was sufficient to directly reprogram somatic cells to a pluripotent state (Takahashi & Yamanaka 2006). This finding emerged from a screen in which the authors retrovirally overexpressed 24 candidate ESC-associated genes in murine fibroblasts carrying an ESC-specific selection marker for Fbx15. Out of the initial 24 ESC genes, they determined that just four transcription factors – Oct-4, Sox2, Klf4, and c-Myc – were sufficient to convert murine fibroblasts to a pluripotent cell type that was strikingly similar to ESCs. This seminal discovery opened an entire field of research aimed at understanding mechanisms of reprogramming and development, modeling and treating complex genetic diseases in culture, and generating patient-specific stem cells for potential therapies.

In the following sections, we will briefly discuss the current state of the art with respect to (i) gene combinations to reprogram cells to pluripotency, (ii) different methods to introduce those genes, (iii) the influence of somatic cell type on the efficiency and quality of resultant iPSCs, (iv) the equivalency of iPSCs and ESCs, and (v) potential applications of iPSCs in research, disease modeling, and therapy. We will also give our personal recommendations on which approaches to use for specific purposes.

Species	Cell Type	Reprogramming Factors	Reference
Mouse	Fibroblasts	OKSM, OKS, OSE, KSNr	Takahashi & Yamanaka 2006
	Mature B & T cells	OKSMC, OKSM	Hanna et al. 2008; Eminli et al. 2009
	Myeloid progenitors	OKSM	Eminli et al. 2009
	Hematopoietic stem cells	OKSM	Eminli et al. 2009
	Adipose-derived stem cells	OKSM	Sugii et al. 2010
	Dermal Papilla	OKM, OK	Tsai et al. 2010
	Satellite cells	OKSM	Tan et al. 2011
	Pancreatic β -cells	OKSM	Stadtfeld et al. 2008
	Hepatic endoderm	OKS	Aoi et al. 2008
	Neural stem cells	OK	Kim et al. 2008; Kim et al. 2009
Melanocytes	OKM	Utikal et al. 2009	
Human	Fibroblasts	OKSM, OSLN, OKS	Takahashi et al. 2007; Yu et al. 2007; Nakagawa et al. 2008
	Mobilized peripheral blood	OKSM	Loh et al. 2009
	Cord blood endothelia	OSLN	Haase et al. 2009
	Cord blood stem cells	OKSM, OS	Eminli et al. 2009; Giorgetti et al. 2009
	Adipose-derived stem cells	OKSM, OKS	Sugii et al. 2010; Aoki et al. 2010
	Hepatocytes	OKSM	Liu et al. 2010
	Keratinocytes	OKSM, OKS	Aasen et al. 2008
	Neural stem cells	O	Kim et al. 2009
	Pancreatic β -cells	OKSM	Bar-Nur et al. 2011
	Amniotic cells	OKSM, OSN	Li et al. 2009; Zhao et al. 2010

Table 1: Reprogramming factors for mouse and human cells. Factors: O-Oct-4, K-Klf4, S-Sox2, M-c-Myc, E-Esrrb, Nr-Nr5a2, C-C/EBP α , L-Lin28, N-Nanog. Adapted from Stadtfeld and Hochedlinger 2011.

REPROGRAMMING COCKTAILS

Since the derivation of the first iPSCs with transgenes overexpressing Oct-4, Sox2, Klf4, and c-Myc, a number of variations to the initial reprogramming cocktail have been introduced, which are reviewed in Stadtfeld and Hochedlinger (2010). For example, the oncogene c-Myc is dispensable for reprogramming to pluripotency, albeit cocktails including only Oct-4, Sox2, and Klf4 (OSK) give rise to iPSCs about 10-fold less efficiently (Nakagawa et al. 2008; Wernig et al. 2008). In human cells, Klf4 and c-Myc can be replaced by the transcription factor Nanog and the RNA-binding protein Lin28 (Yu et al. 2007). Moreover, cell types that exhibit endogenous expression levels of any of the reprogramming factors can be reprogrammed in the absence of that factor. Murine melanocyte cultures, for instance, express endogenous Sox2 and can be reprogrammed with Oct-4, Klf4, and c-Myc (Utikal et al. 2009), while neural stem cells, which

express endogenous Sox2, Klf4, and c-Myc, can be reprogrammed with Oct-4 alone in both mice and humans (Kim et al. 2009a; Kim et al. 2009b). Notably, even Oct-4, which had seemed to be the only essential reprogramming factor, can be replaced with either the nuclear receptor Nr5a2 or E-cadherin in murine fibroblast reprogramming experiments (Heng et al. 2010; Redmer et al. 2011).

Interestingly, microRNAs have been shown to enhance the efficiency of the reprogramming process in mice and humans. Introduction of mature microRNA from clusters miR-130/301/721 or infection of lentiviral miR-302b and 372 provides a several-fold increase in reprogramming efficiency when combined with OKS or OKSM, respectively (Pfaff et al. 2011; Subramanyam et al. 2011). Remarkably, microRNAs alone have also been suggested to be capable of generating iPSCs. Transfection of mature microRNA from the miR-200c, miR-302s, and miR-369s families or infection with a lentiviral construct overexpressing the miR-302/367 cluster were reported to reprogram mouse and human adipose stromal cells or fibroblasts, respectively, into iPSCs (Anokye-Danso et al. 2011; Miyoshi et al. 2011). While the relevant targets for these microRNAs remain elusive, they may contribute to reprogramming by repressing TGF- β signaling and promoting

a mesenchymal-to-epithelial transition (Liao et al. 2011), which are critical steps during iPSC formation. For most practical purposes, however, a reprogramming cocktail consisting of the classic four factors (OKSM) is sufficient to reprogram the majority of cell types tested at reasonable efficiencies in mouse and human. We therefore recommend using OKS or OKSM for most applications utilizing integrating vector systems.

DIFFERENT APPROACHES TO GENERATE iPSCs

The first sets of iPSCs were generated through infection of fibroblasts with four separate retroviral vectors. While retroviral vectors are most commonly used because of their ease of handling, retroviral reprogramming has some shortcomings. For instance, it is relatively inefficient (0.02-0.1% transfection efficiency) and generates iPSCs with multiple viral transgenes that randomly integrate in the genome, thus increasing the risk of insertional mutagenesis. In addition, faithful reprogramming depends on the epigenetic silencing of the retroviral transgenes, which is sometimes incomplete, giving rise to so-called partially reprogrammed cells. This requires careful screening of established iPSCs for viral silencing and endogenous pluripotency gene reactivation, which is cumbersome.

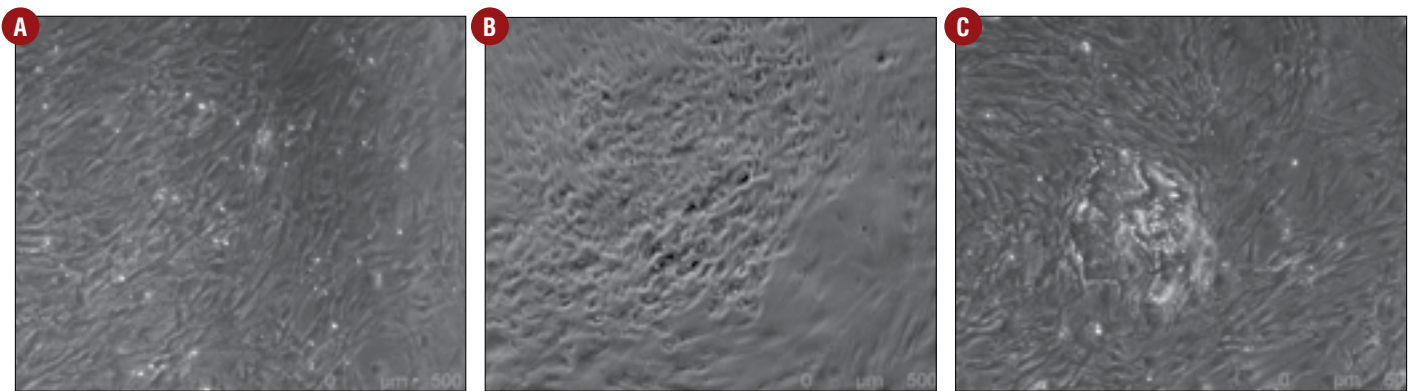


Figure 2: Reprogramming MEFs to iPSCs. A. Early passage MEFs isolated from reprogrammable mice prior to induction of reprogramming factors. B. Day 5-6 of reprogramming, many cells have lost fibroblast morphology and a sheen is noticeable as they begin to grow into ESC-like colonies. C. Day 7-9 of reprogramming, cells are now growing in distinct colonies with a reflective border.

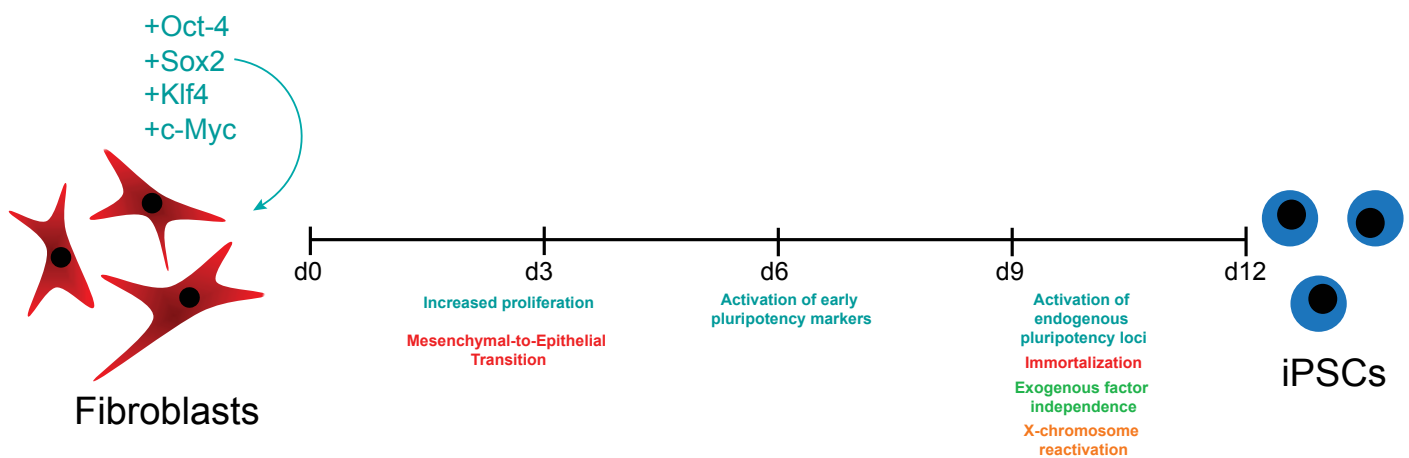


Figure 3: Key events during reprogramming. A timeline of cellular changes that occur during reprogramming. Soon after the introduction of reprogramming factors, cells begin to divide more rapidly and undergo a mesenchymal-to-epithelial transition. This is followed by the expression of early pluripotency markers (e.g. SSEA1 and alkaline phosphatase) and subsequent activation of endogenous Oct-4/Sox2/Nanog. Following the activation of the endogenous core pluripotency loci, the reprogramming cells become factor-independent and undergo immortalization and reactivation of the inactive X-chromosome in female cells. Timeline shown reflects murine reprogramming.

In contrast, the use of doxycycline (dox)-inducible lentiviruses or transposons allows for temporal control of reprogramming factor expression and therefore reduces, if not eliminates, the occurrence of partially reprogrammed iPSCs. Dox-inducible systems also afford researchers the opportunity to determine the minimal duration of factor expression required to produce iPSCs upon experimental perturbations. When combined with polycistronic vectors expressing all four reprogramming factors from one transcript (Sommer et al. 2009; Carey et al. 2009), dox-inducible systems have yielded higher reprogramming efficiencies with fewer integrations. For mechanistic studies involving iPSCs, the latter approach is recommended by the authors; multiple dox-inducible polycistronic vectors are now commercially available, these include: STEMCCA (EMD Millipore®), 4F2A (Stemgent®), and 4F2A-loxP (Stemgent®/Sigma®). Lastly, dox-inducible lentiviruses or transposon vectors can be reactivated in somatic cells derived from primary iPSCs, thus allowing for the generation of so-called “secondary systems,” which are useful to study reprogramming in homogeneous populations of cells (Hockemeyer et al. 2008; Maherali et al. 2008; Wernig et al. 2008). Secondary systems facilitate a significant increase in reprogramming efficiencies (from 0.01%-0.1% up to a few percent), likely due to the fact that the secondary cells already express the transgenes at ratios that are favorable for reprogramming. This system was further improved by the generation of “reprogrammable cells” and “reprogrammable mice” that carry a single dox-inducible polycistronic cassette harboring all four reprogramming factors targeted to the inert ColA1 locus (Carey et al. 2010; Stadtfeld et al. 2010). The use of such transgenic cells eliminates the need for viral infection and ensures that each cell in a given experiment carries only a single copy of each reprogramming factor. Given the relative ease with which reprogrammable cells from different tissues can be collected and reprogrammed with these mice, this system may also provide an effective means for performing large-scale screens for additional factors or molecules that enhance the reprogramming process.

Although retroviruses, lentiviruses, and the reprogrammable system are relatively easy to use, they all require the integration of foreign DNA elements into the future iPSC's genome. This poses a problem for potential therapeutic applications, as random integrations could cause cancer. To circumvent this issue, a number of techniques for generating integration-free iPSCs have been developed, which are also summarized in Stadtfeld and Hochedlinger (2010). By using either floxed viral vectors or transposons, iPSCs can be derived and their integrations subsequently removed through transient expression of Cre or transposase, respectively (Kaji et al. 2009; Soldner et al. 2009; Woltjen et al. 2009; Yusa et al. 2009). This method produces iPSCs at relatively high efficiencies (0.1-1%). However, in the case of floxed viral vectors, a loxP site is left behind. Non-integrating adenoviral vectors can also produce iPSCs in mouse hepatocytes and human fibroblasts, albeit at extremely low efficiencies (0.001%). Interestingly, direct transfections of expression plasmids, protein, and even RNA have the capacity to generate iPSCs (Okita et al. 2008; Zhou et al. 2009; Warren et al. 2010). Protein and plasmid transfections, however, are hampered by low efficiencies (0.001%). The expression of combinations of minimally required sets of reprogramming factors from polycistronic plasmids alleviates some of these issues (Jia et al. 2010). Recent developments of mRNA-based or RNA virus-based reprogramming methods provide other alternatives that yield higher efficiencies (Warren et al. 2010). Notably, mRNA

transfections require multiple rounds of transfections and prior modifications of mRNAs to prevent activation of an interferon response. In contrast, Sendai (RNA) virus-mediated reprogramming, which is fast and efficient, may become a powerful alternative to the more conventional approaches (Fusaki et al. 2009), especially when combined with temperature-sensitive mutations (Ban et al. 2011). Both viral and mRNA systems are commercially available.

MULTIPLE CELL TYPES ARE AMENABLE TO REPROGRAMMING

A number of different somatic cell types, including terminally differentiated lymphocytes and pancreatic β -cells, have been reprogrammed into iPSCs in mice and humans (reviewed in Stadtfeld & Hochedlinger 2010), indicating that the same transcription factors can reinstate pluripotency in cells isolated from different tissues and species. However, reprogramming efficiencies have been shown to vary substantially depending on the starting cell type. For example, reprogramming experiments performed on the entire hematopoietic lineage revealed that, in general, less differentiated cells give rise to iPSCs at greater efficiencies than their more differentiated counterparts (Eminli et al. 2009). This trend was also observed in muscle and adipocyte progenitor populations (Sugii et al. 2010; Tan et al. 2011). Moreover, keratinocytes appear to be a more efficient donor cell type compared with fibroblasts in humans (Aasen et al. 2008; Maherali et al. 2008).

Given that seminal cloning experiments in frogs suggested that cloned embryos exhibit abnormalities that reflect their cell type of origin, it was important to determine if iPSCs derived from distinct cell types were, in fact, equivalent. Interestingly, early passage mouse and human iPSCs display subtle transcriptional and epigenetic differences compared with ESCs, which seems to be due to incomplete silencing of somatic genes and inefficient activation of pluripotency genes. Perhaps more importantly, low-passage iPSCs derived from fibroblasts exhibit impaired differentiation potentials into hematopoietic cells, while iPSC lines produced from blood cells have an increased propensity to differentiate back into their original cell lineage (Polo et al. 2011; Kim et al. 2010). These differences, however, are attenuated in murine iPSCs and in some human iPSCs through extended passaging (Polo et al. 2011; Kim et al. 2011), suggesting that faithful epigenetic reprogramming is actually complete long after the acquisition of pluripotency.

It is likely that proper screening for faithfully reprogrammed iPSCs will have a greater impact on differentiation potential than choice of starting cell population. Therefore, when selecting starting cell populations, one should consider those that are relatively easy to maintain and culture. In the case of murine iPSCs, this is MEFs or tail tip fibroblasts (TTFs), while in humans dermal fibroblasts from skin punch biopsies are most commonly used.

EQUIVALENCY OF iPSCs AND ESCs

iPSCs initially derived by Takahashi and Yamanaka, though clearly pluripotent, lacked several features of ESCs. For example, the endogenous pluripotency loci, as analyzed by promoter DNA methylation and expression, were not completely reactivated, suggesting a dependence on exogenous factors. Moreover, expression profiling revealed that their expression program was intermediate between that of ESCs and the starting fibroblasts. Importantly, chimeras generated with these iPSCs were only low-grade and did not survive to term. These initial limitations, however, were overcome with improved derivation techniques. By selecting for the essential pluripotency genes Oct-4 or Nanog, several groups were able to derive iPSCs that not only gave rise to high-grade chimeras, but could even contribute to the germline of chimeric mice. Moreover, these iPSCs displayed global epigenetic reprogramming to an ESC-like state, an ESC-like transcriptional profile, retroviral silencing, reactivation of the inactive X chromosome, and independence from exogenous factor expression (Maherali et al. 2007; Wernig et al. 2007; Okita et al. 2007).

Additional improvements of reprogramming methods have even enabled the derivation of mice derived entirely from iPSCs by using tetraploid embryo complementation (Boland et al. 2009; Kang et al. 2009; Zhao et al. 2009; Stadtfeld et al. 2010), which is the most stringent assay for pluripotency. Interestingly, a study that compared genetically matched murine ESCs and iPSCs revealed that these cell types are nearly identical except for the aberrant silencing of a single imprinted gene cluster termed Dlk1-Dio3 locus (Stadtfeld et al. 2010). While the majority of iPSC clones derived under conventional culture conditions (serum and LIF) seem to undergo aberrant Dlk1-Dio3 silencing, which inversely correlates with their ability to produce adult mice through tetraploid complementation, changes in factor stoichiometry or culture media can profoundly shift the ratio of silenced and non-silenced iPSC clones (Carey, 2011, CSC; Stadtfeld, in press). Thus, it is possible for iPSCs to be derived that are indistinguishable from ESCs by a number of stringent molecular and functional criteria. These results do not exclude, however, that other epigenetic and/or genetic changes are present in iPSCs that escaped detection with the utilized assays.

Soon after the successful derivation of mouse iPSCs, human iPSCs were generated by independent laboratories. Because of the difficulty to genetically manipulate human cells, human iPSCs were derived without drug selection, using colony morphology and/or live staining for surface markers such as TRA-1-60 and TRA-1-81. Though the more stringent assays of pluripotency in mouse (e.g. contribution to chimeras and germline transmission) are not available for human systems, human iPSCs are molecularly and functionally highly similar to human ESCs with respect to the expression of human ESC markers and their potential to give rise to derivatives of all three embryonic germ layers in vitro and in vivo in the context of teratomas (Takahashi et al. 2007; Yu et al. Park et al. 2008). Despite these overt similarities, there has been some controversy regarding the equivalency of human iPSCs and ESCs. An emerging conclusion from these studies is that variables such as line-to-line variation (Bock et al. 2011), vector integration (Soldner et al. 2009), passage number (Chin et al. 2009; Polo et al. 2010), culture conditions (Stadtfeld et al. in press), and genetic background (Stadtfeld et al. 2010) may account for some of the observed differences. In addition, some

iPSCs carry copy number variations and point mutations that are not normally seen in ESCs and appear to be the result of pre-existing mutations in the somatic cells, such as culture-induced and reprogramming-induced genomic alterations (Hussein et al. 2011; Gore et al. 2011). Whether any of these reprogramming-specific alterations have functional consequences remains to be determined. Though additional work is certainly warranted to resolve these issues, especially when considering potential therapeutic applications, human iPSCs have already been proven valuable for modeling certain diseases by providing a renewable istic studies and drug screening efforts.

POTENTIAL APPLICATIONS OF iPSCs

UNDERSTANDING DEVELOPMENT AND EPIGENETIC REPROGRAMMING

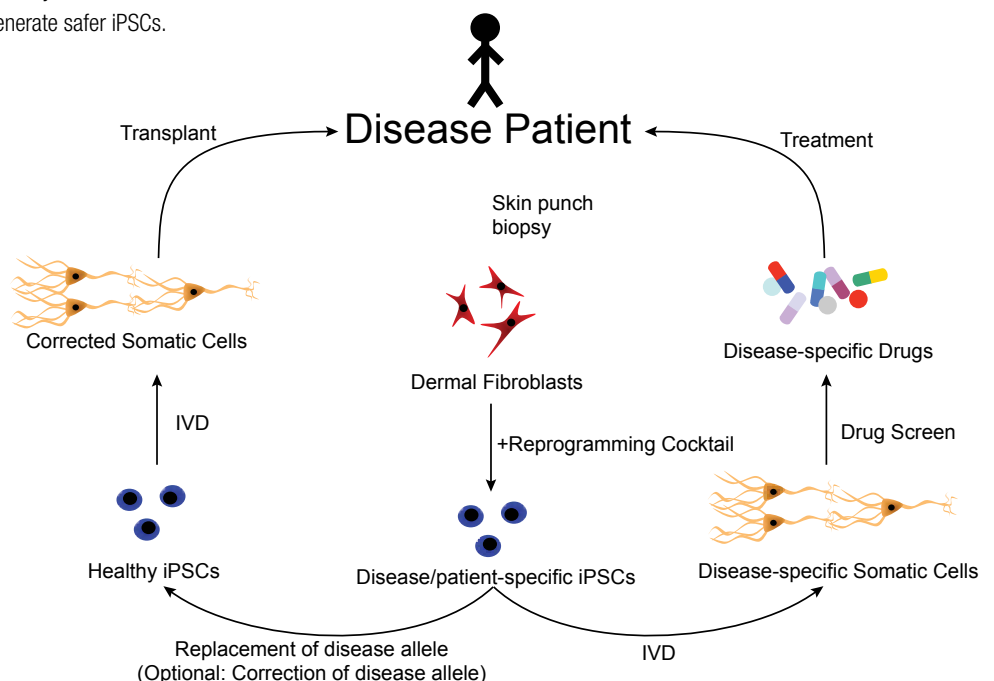
Due to their ability to maintain a pluripotent state in culture, iPSCs can help elucidate the various molecular processes involved in pluripotency, development, and differentiation without destroying human embryos. The study of iPSCs also increases our general understanding of the process of epigenetic reprogramming and the stability of the cellular state by demonstrating the relative ease of converting one given cell type to another. For example, genomic binding studies of the reprogramming factors and of epigenetic modifications during cellular reprogramming towards iPSCs have expanded our knowledge of how pluripotency is attained in a somatic cell (Sridharan et al. 2009; Mikkelsen et al. 2008). Moreover, the observation that more differentiated cells are less amenable to reprogramming than their undifferentiated precursors (Eminli et al. 2009) suggests that certain chromatin or transcriptional states are much more difficult to rewire than others. The use of improved reprogramming systems, such as the secondary system, has allowed researchers to determine that every somatic cell has, in principle, the potential to generate iPSCs following an apparently stochastic mechanism (Hanna et al. 2009). Moreover, the observation that the p53 (Hong et al. 2009; Noginov et al. 2009; Utikal et al. 2009; Marion et al. 2009; Li et al. 2009) and TGF- β (Maherali et al. 2009; Ichida et al. 2009) pathways act as roadblocks for iPSC formation from fibroblasts unveiled interesting parallels between cellular reprogramming and malignant transformation and offered small molecule approaches to enhance iPSC formation. Lastly, identification of intermediate cell populations, based on cell surface marker combinations, has enabled researchers to further study the transcriptional and epigenetic changes that occur during the conversion of a somatic cell into a pluripotent cell (Stadtfeld et al. 2008, Brambrink et al. 2008). In addition to a number of molecules that have already been demonstrated to affect iPSC formation (Esteban et al. 2010; Huangfu et al. 2008; Theunissen et al. 2011; Han et al. 2010; Maekawa et al. 2011), we expect several new molecules to be identified in the next few years whose manipulation will further enhance reprogramming and generate safer iPSCs.

DISEASE MODELING

iPSCs have the ability to self-renew and differentiate into essentially all cell types of the body, which provides the possibility to generate a near limitless supply of cells from patients suffering from diseases for which no cellular models and therefore no effective treatments are currently available. This applies especially to disorders affecting cell types that are inaccessible or difficult to maintain in tissue culture, such as neurons and cardiac cells. The *in vitro* differentiated somatic cells may ultimately be used to identify disease-associated cellular phenotypes and potentially drugs to attenuate them.

iPSC cell lines have been derived from a large number of disease states. Several of these cell lines display specific disease features in culture and show responsiveness to approved drugs, thus validating the disease modeling approach for drug discovery. For example, iPSCs derived from the autism spectrum disorder, Timothy syndrome, were shown to display aberrant Ca^{2+} signaling and abnormally high expression of tyrosine hydroxylase. Interestingly, the tyrosine hydroxylase phenotype could be reversed by treating the cultures with the drug roscovitine (Pasca et al. 2011). iPSCs have also been derived from long QT patients (Moretti et al. 2010; Itzhaki et al. 2011), a potentially fatal congenital disease characterized by delayed ventricular repolarization. *In vitro* differentiated long QT cardiomyocytes displayed a prolonged action potential duration when compared to cardiomyocytes derived from unaffected individuals. Identification of this phenotype allowed researchers to successfully screen for drugs that ameliorate this delay in culture (Moretti et al. 2010; Itzhaki et al. 2011). These proof-of-principle studies demonstrate the feasibility of disease modeling with iPSC technology and can be followed up with large scale screening assays to identify novel therapeutic drugs (discussed in Wu and Hochedlinger 2011).

Figure 4: Therapeutic potential of iPSCs. Human dermal fibroblasts from patients with a given disease can be used to derive iPSCs. Disease-specific iPSCs that are differentiated into a relevant cell type may be used as a tool for disease modeling and therapy. Additionally, patient-specific iPSCs carrying known disease-causing mutations may be corrected through gene targeting prior to *in vitro* differentiation and transplantation back into the patient.



CELL & GENE THERAPY

One exciting possible use for iPSCs is to serve as custom-tailored replacement cells in a therapeutic setting. ESC-derived cells have been successfully used in cell replacement therapies with animal models (Yang et al. 2008; Rideout et al. 2002). However, human studies have been limited due to the various limitations associated with the use of human embryos and donor compatibility issues. These obstacles can be circumvented, however, through the use of iPSCs that are specific to a particular patient. Patient-specific iPSCs are expected to obviate the need for immunosuppressive treatments following transplantation, as these cells are genetically identical to their host. Nevertheless, it should be noted that a recent study in mice suggested that teratomas derived from some syngeneic iPSCs still elicit an immune response from the host animal (Zhao et al. 2011). However, because these experiments were performed with undifferentiated iPSCs rather than with mature cells, which would be transplanted in a clinical setting, and because the authors observed a correlation between the iPSC derivation method and immunogenicity, there is hope that transplantation of mature cells derived from iPSCs using the safest available method will overcome these potential hurdles (Apostolou & Hochedlinger 2011).

Two proof-of-principle experiments in rodents have already demonstrated the feasibility of using iPSCs for cellular replacement therapy. In the first study, gene targeting was used to correct the mutation in iPSCs derived from a humanized murine sickle cell anemia model. These cells were subsequently transplanted back into an irradiated sickle cell mouse and successfully caused a reversal of the sickling phenotype (Hanna et al. 2007). In another study, transplantation of iPSC-derived dopaminergic neurons into a rat model of Parkinson's disease was sufficient to partially restore neuronal function (Wernig et al. 2008).

In humans, the potential for iPSC-based cell replacement therapy was demonstrated in a study involving the reprogramming of somatic cells from patients afflicted with Fanconi anemia, a rare recessive chromosomal instability disorder caused by mutations in any of the 13 genes associated with this disease (Raya et al. 2009; Wang 2007). Correction of the genetic mutation found in Fanconi anemia-derived iPSCs, followed by their successful differentiation into hematopoietic progenitors with a disease-free phenotype, highlights the promise of this technology in treating diseases caused by known genetic defects (Raya et al. 2009). Additional recent examples of gene correction in human iPSCs include β -thalassemia and Hutchinson-Gilford progeria syndrome (HGPS). In the case of β -thalassemia, where erythrocytes produce insufficient amounts of β -globin, iPSCs were derived from fibroblasts of β -thalassemia patients and infected with an unaffected copy of the β -globin gene. Consequently, these iPSCs were differentiated into erythrocytes that produced increased levels of β -globin (Papapetrou et al. 2011). HGPS is caused by a point mutation in the Lamin A gene, which results in premature aging and a progressive loss of vascular smooth muscle. Liu et al. (2011a) demonstrated that smooth muscle cells derived from HGPS-iPSCs displayed an early onset of cellular senescence when compared to smooth muscle cells derived from control iPSCs. Moreover, the authors were able to ablate this phenotype in a subsequent study by correcting the mutated Lamin A locus in HGPS-iPSCs by targeting with a helper-dependant adenoviral vector (Liu et al 2011b).

TOXICOLOGY

In addition to their potential in cell therapy and disease modeling, iPSCs might be a valuable tool in predictive toxicology. In vitro models using animal-derived cells are not sufficiently representative of humans because of species-specific pharmacotoxicological effects (Laustriat et al. 2010). Since the 1990s, embryonic carcinoma and embryonic stem cells have been recognized as tools for analyzing mutagenic and cytotoxic effects in vitro (Reviewed in Rohwedel et al. 2001). iPSCs offer similar advantages and, therefore provide a new model for drug safety testing and for studying the effects of chemical mutagens on embryonic cells in vitro (Wobus & Loser 2011).

A common limitation for the pharmaceutical industry in the development of novel drugs is the lack of a test system to predict toxicity in a human-specific manner (Kola & Landis 2004). Human pluripotent stem cells, including iPSCs, are ideal for such test systems, because they possess several characteristics that make them amenable to cell-based screening assays and toxicology studies used in drug discovery. This includes their ability to self-renew indefinitely, their capacity to differentiate into any cell in the body, and their ability to be grown in a three-dimensional culture that mimics the microenvironment found in some tissues (Wobus & Loser 2011). Thus, the use of iPSC technology in high-throughput toxicity screening assays has the potential to offer important drug response and toxicity information to aid in drug development and advancing personalized medicine.

PROTOCOLS

CULTURE MEDIA

Fibroblast Complete Media

- 450 ml DMEM
- 50 ml Fetal bovine serum
- 5 ml Pen/Strep
- 5 ml Glutamine 100x
- 5 ml Non-essential amino acids 100x
- 0.5 ml β -mercaptoethanol

mESC Complete Media

- 425 ml Knockout DMEM
- 75 ml Fetal bovine serum
- 5 ml Pen/Strep
- 5 ml Glutamine 100x
- 5 ml Non-essential amino acids 100x
- 0.5 ml β -mercaptoethanol
- 0.5 ml LIF 1000X (commercial or may also use recombinant)

huES Complete Media

- 400 ml DMEM/F12 1:1
- 100 ml Knockout serum replacement
- 5 ml Non-essential amino acids
- 5 ml Glutamine
- 5 ml Pen/Strep
- 0.5 ml β -mercaptoethanol
- 10 ng/ml bFGF

mEB Differentiation Media

- 425 ml IMDM
- 75 ml Fetal bovine serum
- 5 ml Glutamax
- 5 ml Pen/Strep
- 18.9 μ l Monothioglycerol
- 2 ml FE-saturated transferrin
- 250 μ l Ascorbic acid (50 mg/ml)
- 5 ml Non-essential amino acids
- 5 ml Sodium pyruvate (100 mM)

PREPARATION OF MEFS FOR REPROGRAMMING

1. Between days E12.5 and E15.5, euthanize a pregnant female.
2. Lay the mouse on her back and cut through the abdomen.
3. Locate the two uterine horns, the embryos should be clearly visible within them at this stage, and dissect out the uterus by cutting once just below the cervix and through either oviduct.
4. To avoid mycoplasma contamination, submerge the uterus completely in Wescodyne diluted 1:200 in PBS for 30 sec -1 min. Followed by three successive washes in PBS.
5. In a 10 cm tissue culture plate, use a pair of forceps and scissors to cut the embryos out of the uterus.
6. Remove the head and internal organs from the embryos and transfer each embryo to a drop of trypsin in a new 10 cm plate.
Note: If the head and fetal liver are not completely removed there is a risk of contaminating the culture with neural progenitor and hematopoietic cells.
7. Use a pair of scalpels to completely chop up the embryo. It should be minced into fine pieces.
8. Add MEF complete media to the plate and titrate the chopped up embryo with repetitive pipetting.
9. Culture the MEFs at 37°C with 4% O₂. Do not disturb the plate for the first 48 hr to give the fibroblasts a chance to grow out.

Note: While MEFs can be maintained at normoxia (21% O₂) for several passages, reprogramming efficiency declines significantly over time due to cellular senescence caused by oxidative stress. If maintaining MEFs at normoxia, try to use them prior to passage 4 or even earlier for best results.

DERIVATION OF iPSCs FROM MURINE OR HUMAN FIBROBLASTS

Though we most commonly use cells from a reprogrammable mouse or inducible lentiviruses, there are multiple methods that can be used to successfully derive iPSCs (see **Table 2** for suppliers), and there are advantages and drawbacks for each (see above). Below is a generalized protocol for the derivation of iPSC from fibroblasts written for a 6-well format, but can be scaled up or down accordingly.

1. Plate 100,000-200,000 early passage fibroblasts in one well of a 6-well plate in fibroblast complete media and allow them to attach overnight (if viral infection is not needed, skip to step 3).

2. Infect 12-24 hr with high titer virus to deliver reprogramming factors.

Note: Infection time/protocol may need to be adjusted for viral titer/ infection efficiency.

3. During the infection, coat the desired number of 6-well plates in 0.2% gelatin for 30 min at 37°C. Aspirate gelatin and plate irradiated feeders (CF-1 or DR4 MEFs) at 250,000-500,000 cells/well.

Reprogramming System	Factor Delivery Method	Distributor
STEMCCA	Lentivirus	EMD Millipore
Stemgent 4F2A & 4F2A-loxP	Lentivirus	Stemgent; Sigma
Stemgent mRNA Reprogramming Factors	mRNA	Stemgent
CytoTune-iPS	Sendai virus	Invitrogen™
Col1a1tm1(tet0-Pou5f1,-Klf4,-Sox2,-Myc)Hoch/J	Reprogrammable Mouse	Jackson Laboratory
Col1a1tm4(tet0-Pou5f1,-Sox2,-Klf4,-Myc)Jae/J	Reprogrammable Mouse	Jackson Laboratory

Table 2 . Commercially available reprogramming systems.

4. Prior to replating the infected fibroblasts, change the media on the feeders to 3ml mES or huES complete media. If using an inducible system, add doxycycline to the media at a concentration of 1 µg/ml.

5. Trypsinize the infected fibroblasts. Plate between 1,000-10,000 per well of a 6-well plate with gelatin and feeders in mES or huES complete media. If using an inducible system, add doxycycline here at a concentration of 1 µg/ml.

Note: The number of cells per well plated here should be determined based upon the application and expected reprogramming efficiency.

6. Change the media daily and monitor the plates for emerging colonies. See **Figure 2A, B, C** for representative images of reprogramming colonies. Typically, reprogramming cells will display an early burst of proliferation, followed by a mesenchymal-to-epithelial transition (MET), finally forming clusters resembling ESC colonies. Activation of endogenous pluripotency loci, which can be monitored with Oct-4-GFP or Nanog-GFP lines (available from The Jackson Laboratory) in mice, can be seen in a few colonies starting around day 9 of reprogramming.

7. If using an inducible system, doxycycline should typically be removed between days 12 and 15 of reprogramming of murine fibroblasts and between days 14 and 21 of reprogramming of human fibroblasts. Following doxycycline wash off, allow 5 days for regression of exogenous factor-dependent colonies prior to analyzing or picking iPSCs.

GROWTH AND EXPANSION OF MURINE iPSCs

1. iPSC clones may be picked either 5 days following removal of doxycycline for reprogramming with inducible systems or on about day 20 for reprogramming with retroviral and excisable constitutive lentiviral systems. When picking, colonies should closely resemble ESCs, however, they may have additional differentiated cells around and on the colony before the first expansion (see **Figure 1A** for representative images of murine iPSC colonies).

Note: While it is possible to reprogram with non-excisable constitutive lentiviruses, the inability to remove exogenous factor expression makes the analysis of viral silencing essential.

2. To pick clones, prepare a 96-well V-bottom plate with 60µl of PBS without MgCl₂ or CaCl₂ per well.

3. Using an inverted light microscope, manually cut out individual colonies by tracing them with a P10 or P20 pipette tip.

4. Transfer the colony to a 96-well plate with PBS and dissociate it with repetitive pipetting.

5. When the desired number of colonies has been transferred to the 96-well plate, add 30 µl 0.25% trypsin per well and incubate at 37°C for 10 min.

6. Transfer the picked clones to a gelatinized 24-well plate with irradiated feeders for expansion.

7. Change media on iPSCs every other day and split regularly to avoid differentiation. Always keep cells on gelatin with irradiated feeders. iPSCs will grow very fast. To avoid overcrowding, use regular splits of 1:50.

GROWTH AND EXPANSION OF HUMAN iPSCs

1. Pick colonies that resemble human ESC colonies by using a P10 or P20 pipette to cut a grid in the colony (see **Figure 1B** for representative image of human iPSC colony).

2. Transfer the separate chunks of the colony to a new 6-well plate with gelatin and irradiated feeders.

Note: Human ESCs and iPSCs do not tolerate being single cells in a well; do not use enzymatic digestions to passage them.

3. Change media on human iPSCs daily to avoid differentiation. Passage manually with a P10 or P20 pipette as described above.

Note: If experiencing excessive differentiation after passaging, adding ROCK inhibitor (available from EMD Millipore) to the media immediately following passaging may reduce this.

ANALYSIS OF iPSCs:

IMMUNOFLUORESCENCE

Throughout reprogramming and following generation of mouse or human iPSCs, it can be useful to routinely check for the expression of several key transcription factors. Immunofluorescent analysis that includes a panel of antibodies for the detection of Oct-4, Nanog, and Sox2 can be used to track the pluripotent potential of iPS cells (**Figure 5, 6**). The loss of these markers indicates a loss of pluripotency or differentiation of the culture.

When working with human iPSCs, it is also useful to perform immunofluorescent analysis of the culture using a panel of antibodies for the detection of antigens that are specifically expressed on the surface of human pluripotent cells; TRA-1-60, TRA-1-81, and SSEA4 (**Figure 6**). The expression of these three markers on the cell surface of human iPS cells can be used to track the pluripotent potential of the culture, as loss of these surface markers indicates differentiation of the culture. For mouse iPS cells, the expression of SSEA1 on the cell surface can serve as an indicator of pluripotency (**Figure 7**).

IN VIVO DIFFERENTIATION OF iPSCs THROUGH TERATOMA FORMATION

Teratomas are a relatively simple way to assay for iPSC pluripotency in an in vivo context. The assay involves allowing iPSCs to differentiate and form teratomas following subcutaneous injection into immunodeficient mice. A cell's pluripotency in this assay is determined by its ability to give rise to derivatives of all three embryonic germ layers: endoderm, mesoderm, and ectoderm.

1. Grow iPSCs to >60% confluency for murine iPSCs on feeders and gelatin in one well of a 6-well plate for murine iPSCs. For human iPSCs, transfer colonies to feeder-free matrigel-coated (BD Biosciences®) 10 cm plates prior to use to avoid fibroblast contamination; allow the plate to become ~80% confluent before use. Treat human iPSCs with 1 mg/ml Collagenase IV (in DMEM/F12) for 5 min at 37°C. For murine iPSCs, trypsinize and transfer to a T25 flask without gelatin in mES complete media. Incubate the flask at 37°C for 30 min - 1 hr, then collect and pellet cells.

Note: Fibroblasts will attach much more rapidly than iPSCs or ESCs and therefore this method (preplating) can be used to greatly enrich for iPSCs or ESCs.

2. Resuspend cells in PBS with MgCl₂ and CaCl₂ and dilute to 0.5-1 x 10⁶ cells per 300 µl.
3. Subcutaneously inject 300µl of the resuspended cells into the rear flank of a SCID or NOD/SCID mouse with a 23 gauge IM needle. To perform a subcutaneous injection, first anesthetize the mouse with isoflurane. Then tent the skin on the mouse's rear flank, pulling it up away from the peritoneum, with your thumb and forefinger and gently insert the needle into this space, taking care not to enter the peritoneum. Slowly press the plunger on the needle down, you should feel a bubble form just underneath the mouse's skin. Remove the needle and check to ensure that none of the injected PBS seeps out.
4. Allow teratomas to grow for 3-6 weeks, then dissect them out for histology. An H&E stain of the teratomas should allow you to identify derivatives from endoderm, mesoderm, and ectoderm if they are present.

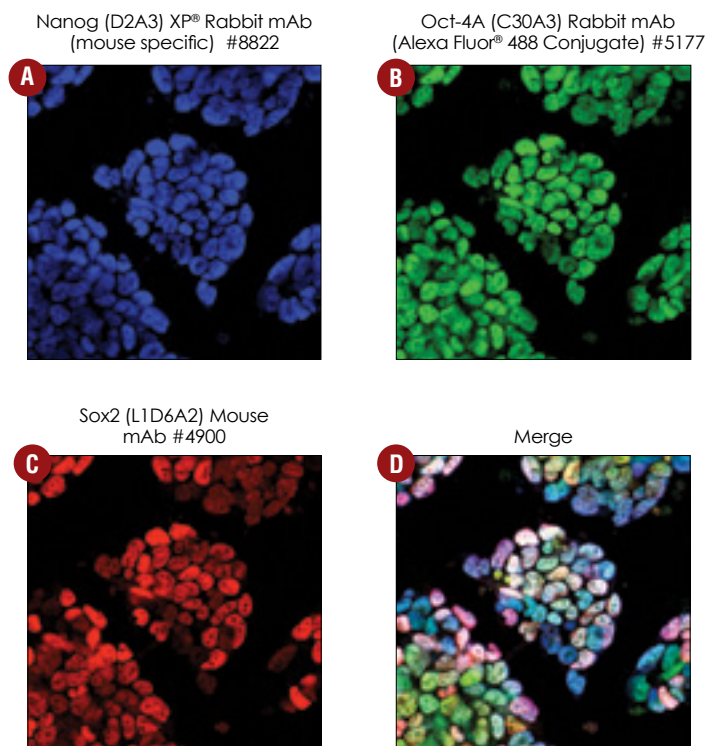


Figure 5: Immunofluorescent analysis of mouse iPSCs using Nanog (D2A3) XP® Rabbit mAb (Mouse Specific) #8822 (**A**) Oct-4A (C30A3) Rabbit mAb (Alexa Fluor® 488 Conjugate) #5177 (**B**), or Sox2 (L1D6A2) Mouse mAb #4900 (**C**). **Panel D** is the merge.*

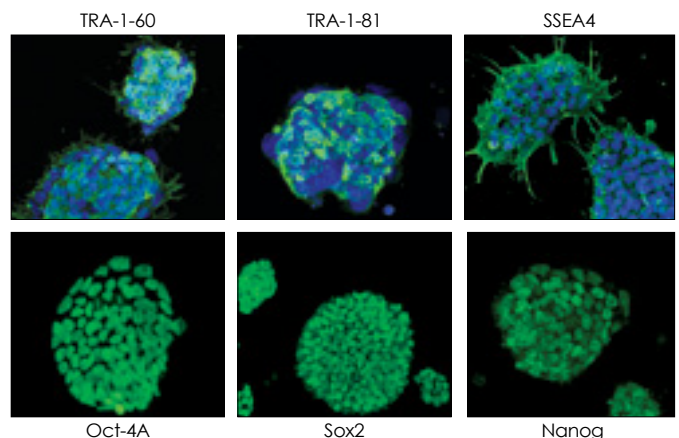


Figure 6: Immunofluorescent analysis human iPS cells using TRA-1-60(S) (TRA-1-60(S)) Mouse mAb #4746 (green, upper right), TRA-1-81 (TRA-1-81) Mouse mAb #4745 (green, upper middle), SSEA4 (MC813) Mouse mAb #4755 (green, upper left), Oct-4A (C30A3) Rabbit mAb #2840 (green, lower right), Sox2 (D6D9) XP® Rabbit mAb #3579 (green, lower middle) and Nanog Antibody #3580 (green, lower left). Blue pseudocolor = DRAQ5® #4084 (fluorescent DNA dye).*

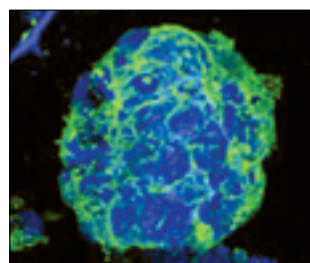


Figure 7: Immunofluorescent analysis of mouse iPSCs using SSEA1 (MC480) Mouse mAb #4744. Blue pseudocolor = DRAQ5® #4084 (fluorescent DNA dye).*

*All antibodies are from Cell Signaling Technology, Inc.

DIFFERENTIATION OF MURINE iPSCs IN CULTURE THROUGH EMBRYOID BODY (EB) FORMATION

1. Grow iPSCs to >60% confluency for murine iPSCs on feeders and gelatin in a one well of a 6-well plate. Ensure that colonies are shiny and do not display signs of differentiation.
2. For murine iPSCs, trypsinize and transfer to a T25 flask without gelatin in mES complete media. Incubate the flask at 37°C for 30 min - 1 hr, then collect and pellet cells.
3. Resuspend iPSCs in EB differentiation medium at 400 cells per 30 μ l.
4. In the lid of a 15cm Petri dish, make 30 μ l drops with a multichannel pipette. Replace the base of the Petri dish and carefully turn the dish over so that the drops are hanging from the lid.
5. Incubate at 37°C for 3-4 days; EBs should become visible at the bottom of each drop by this time.
6. To collect EBs, invert the lid, wash with PBS, and transfer to a conical tube. Allow the EBs to settle, then aspirate the PBS.
7. Transfer the EBs to 10cm Petri dishes in EB differentiation media. Culture the EBs on a shaker, shaking slowly, at 37°C. To prevent too much evaporation, place a water-filled Petri dish on the top and bottom of each stack of EB plates. Change media every other day.

Note: EBs can be collected in the center of the plate by swirling the plate in a circular motion; this allows for easier media changes.

From here the embryoid bodies can be driven to differentiate into a number of diverse cell types derived from all three germ layers. Though this is still an area of active research, multiple protocols have been developed capable of deriving neurons, skin, hematopoietic, vascular, cardiac muscle, skeletal muscle, hepatic, and pancreatic lineages. For reviews of these protocols see Keller 2005, Murry & Keller 2008, and Cohen & Melton 2011.

DIFFERENTIATION OF HUMAN iPSCs THROUGH EB FORMATION

1. To avoid fibroblast contamination, grow human iPSCs on matrigel (BD Biosciences) prior to use. Treat cells from a 10cm dish with 1mg/ml Collagenase IV 5 min at 37°C.
2. Collect cells, spin, and aspirate. Resuspend pellet in 4 ml hES media lacking bFGF and plate onto a 6-well, low attachment plate.
3. Incubate at 37°C on a shaker to avoid attachment and clumping. Change media every other day by spinning the plate in a circular motion, collecting EBs in the center, and aspirating media from the edge.
4. Allow EBs to differentiate for about 16 days, at which point they may be dissociated for analysis or further differentiation.

TROUBLE SHOOTING GUIDE

COMMON REASONS REPROGRAMMING FAILS

1. Low viral infection efficiency or viral titer.

As reprogramming is relatively inefficient, it is key that the proportion of the starting population expressing all reprogramming factors be maximized. It is also important to determine the viral infection efficiency in the cell type being reprogrammed. Keep in mind that retroviruses can only infect dividing cells; if the starting population is slow-dividing, a lentivirus may improve your results. Infection efficiency can typically be done by using immunofluorescence (see "Protocols" section) to quantify the percentage of cells overexpressing the exogenous reprogramming factor. It may be necessary to use either an IRES-GFP or a tagged version of the reprogramming factor here if the reprogramming factor is already expressed in the starting population (e.g. Klf4 or c-Myc in fibroblasts). Expression of the exogenous reprogramming factors can also be checked on a population level by RT-qPCR.

2. Elevated cellular senescence or death in the starting population or during reprogramming.

Activation of the cellular senescence pathways greatly impedes the reprogramming process. If possible, the starting population should be maintained at 4% O₂ from the time of derivation to the start of reprogramming. Reprogramming can also be performed at 4% O₂ for an increased efficiency, which is about 2.5- to 3-fold in the case of secondary MEFs. Cell death could also be a reason for reduced reprogramming efficiency. In this case it is possible that the viral infection is too toxic; try reducing titer or infection time.

3. Inhibitory media conditions.

Media conditions, particularly serum quality, can have a considerable effect on reprogramming cultures. Reprogramming efficiency varies significantly between different lots of serum. It is worthwhile to run a pilot test of reprogramming efficiency in multiple serum lots. Alternatively, knockout serum replacement media can be used for reprogramming (see "Protocols" section). Though it is less permissive for fibroblast growth, ESCs and iPSCs thrive in knockout serum replacement and can yield higher reprogramming efficiencies than some serum lots. Additionally, there are several additives that have been shown to boost reprogramming efficiency, such as ascorbic acid, and TGF- β and HDAC inhibitors.

4. Media changes are too infrequent or cells are too dense.

Media should be changed on reprogramming cells every other day at a minimum; if reprogramming is being performed at 4% O₂, media should be changed daily. Do not allow the media to turn yellow. Failure to maintain reprogramming cultures in fresh media will often result in very inconsistent reprogramming efficiencies and may cause reprogramming to fail altogether. This can also occur if the reprogramming culture is seeded too densely, as the reprogramming cells will overcrowd, become contact inhibited, and use up their resources too quickly.

REFERENCES

1. Aasen, T., Raya, A., Barrero, M.J., Garreta, E., Consiglio, A., Gonzalez F., Vassena, R., Bilic, J., Pekarik, V., Tiscornia, G., Edel, M., Boue, S., and Izpisua Belmonte, J.C. (2008). Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. *Nat. Biotech.* 26(11), 1276–1284.
2. Anokye-Danso, F., Trivedi, C.M., Jühr, D., Gupta, M., Cui, Z., Tian, Y., Zhang, Y., Yang, W., Gruber, P.J., Epstein, J.A., and Morrissey, E.E. (2011). Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. *Cell Stem Cell* 8(4), 376–388.
3. Apostolou, E. and Hochedlinger, K. (2011) Stem cells: iPSCs under attack. *Nature* 474, 165–171.
4. Baker, M. (2010). Testing time for stem cells. *Nature*. 463(7282), 719.
5. Ban, H., Nishishita, N., Fusaki, N., Tabata, T., Saeki, K., Shikamura, M., Takada, N., Inoue, M., Hasegawa, M., Kawamata, S., and Nishikawa, S. (2011). Efficient generation of transgene-free human induced pluripotent stem cells (iPSCs) by temperature-sensitive Sendai virus vectors. *Proc. Nat. Acad. Sci. U.S.A.* 108(34), 14234–14239.
6. Bar-Nur, O., Russ, H.A., Efrat, S., and Benvenisty, N. (2011). Epigenetic memory and preferential lineage-specific differentiation in induced pluripotent stem cells derived from human pancreatic islet beta cells. *Cell Stem Cell* 9(1), 17–23.
7. Bock, C., Kiskinis, E., Verstappen, G., Gu, H., Boulting, G., Smith, Z.D., Ziller, M., Croft, G.F., Amoroso, M.W., Oakley, D.H., Gnirke, A., Eggan, K., and Meissner, A. (2011). Reference maps of human ES and iPSC cell variation enable high-throughput characterization of pluripotent cell lines. *Cell*. 144(3), 439–452.
8. Boland, M., Hazen, J., Nazor, K., Rodriguez, A., Gifford, W., Martin, G., Kupriyanov, S., and Baldwin, K. (2009). Adult mice generated from induced pluripotent stem cells. *Nature* 461, 91–95.
9. Brambrink, T., Foreman, R., Welstead, G.G., Lengner, C.J., Wernig, M., Suh, H., and Jaenisch, R. (2008). Sequential expression of pluripotency markers during direct reprogramming of mouse and somatic cells. *Cell Stem Cell* 2(2), 151–159.
10. Briggs, R. and King, T. (1952). Transplantation of Living Nuclei From Blastula Cells into Enucleated Frogs' Eggs. *Proc. Nat. Acad. Sci. U.S.A.* 38, 455–518.
11. Carey, B., Markoulaki, S., Beard, C., Hanna, J., and Jaenisch, R. (2009). Single-gene transgenic mouse strains for reprogramming adult somatic cells. *Nat. Met.* 7, 56–65.
12. Carey, B., Markoulaki, S., Hanna, J., Saha, K., Gao, Q., Mitalipova, M., and Jaenisch, R. (2009). Reprogramming of murine and human somatic cells using a single polycistronic vector. *Proc. Nat. Acad. Sci. U.S.A.* 106, 157–219.
13. Chin, M.H., Mason, M.J., Xie, W., Volinia, S., Singer, M., Peterson, C., Ambartsumyan, G., Aimiwu, O., Richter, L., Zhang, J., Khovorostov, I., Ott, V., Grunstein, M., Lavon, N., Benvenisty, N., Croce, C.M., Clark, A.T., Baxter, T., Pyle, A.D., Teitell, M.A., Pelegri, M., Plath, K., and Lowry, W.E. (2009). Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures. *Cell Stem Cell*. 5(1), 111–123.
14. Cohen, D.E. and Melton, D. (2011). Turning straw into gold: directing cell fate for regenerative medicine. *Nat Rev. Gen.* 12, 243–252.
15. Cowan, C., Atienza, J., Melton, D., and Eggan, K. (2005). Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. *Science* 309, 1369–1442.
16. Doi, A., Park, I.H., Wen, B., Murakami, P., Arjee, M.J., Irizarry, R., Herb, B., Ladd-Acosta, C., Rho, J., Loewer, S., Miller, J., Schlaeger, T., Daley, G.Q., and Feinberg, A.P. (2009). Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. *Nat. Gen.* 41(12), 1350–1353.
17. Ebert, A.D., Yu, J., Rose, F.F., Mattis, V.B., Lorson, C.L., Thomson, J.A., and Svendsen, C.N. (2009). Induced pluripotent stem cells from a spinal muscular atrophy patient. *Nature* 457(7227), 227–280.
18. Eggan, K., Baldwin, K., Tackett, M., Osborne, J., Gogos, J., Chess, A., Axel, R., and Jaenisch, R. (2004). Mice cloned from olfactory sensory neurons. *Nature* 428, 44–53.
19. Eminli, S., Foudi, A., Stadtfeld, M., Maherali, N., Ahfeldt, T., Mostoslavsky, G., Hock, H., and Hochedlinger, K. (2009). Differentiation stage determines potential of hematopoietic cells for reprogramming into induced pluripotent stem cells. *Nat. Gen.* 41, 968–1044.
20. Esteban, M.A., Wang, T., Qin, B., Yang, J., Qin, D., Cai, J., Li, W., Weng, Z., Chen, J., Ni, S., Chen, K., Li, Y., Liu, X., Xu, J., Zhang, S., Li, F., He, W., Labuda, K., Song, Y., Peterbauer, A., Wolbank, S., Redl, H., Zhong, M., Cai, D., Zeng, L., and Pei, D. (2010). Vitamin C enhances the generation of mouse and human induced pluripotent stem cells. *Cell Stem Cell*. 6(1), 71–79.
21. Fusaki, N., Ban, H., Nishiyama, A., Saeki, K., and Hasegawa, M. (2008). Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proc. Jap. Acad. Ser. B, Phys. Bio. Sci.* 85, 348–410.
22. Gore, A., Li, Z., Fung, H.L., Youg, J.E., Agarwal, S., Antosiewicz-Bourget, J., Canto, I., Giorgetti, A., Israel, M.A., Kiskinis, E., Lee, J.H., Loh, Y.H., Manos, P.D., Montserrat, N., Panopoulos, A.D., Ruiz, S., Wilbert, M.L., Yu, J., Kirkness, E.F., Izpisua, Belmonte, J.C., Rossi, D.J., Thomson, J.A., Eggan, K., Daley, G.Q., Goldstein, L.S., and Zhang, K. (2011). Somatic coding mutations in human induced pluripotent stem cells. *Nature*. 471(7336), 63–67.
23. Gurdon, J.B., Laskey, R.A., and Reeves, O.R. (1975). The developmental capacity of nuclei transplanted from keratinized skin cells of adult frogs. *J. Embr. Exp. Morph.* 34(1), 93–112.
24. Han, J., Yuan, P., Yang, H., Zhang, J., Soh, B.S., Li, P., Lim, S.L., Cao, S., Tay, J., Orlov, Y., Lufkin, T., Ng, H.H., Tam, W.L., and Lim, B. (2010). Tbx3 improves the germ-line competency of induced pluripotent stem cells. *Nature* 463(7284), 1096–1100.
25. Hanna, J., Saha, K., Pando, B., van Zon, J., Lengner, C.J., Creighton, M.P., van Oudenaarden, A., and Jaenisch, R. (2009). Direct cell reprogramming is a stochastic process amenable to acceleration. *Nature* 462(7273), 595–601.
26. Hanna, J., Wernig, M., Markoulaki, S., Sun, C.-W., Meissner, A., Cassady, J., Beard, C., Brambrink, T., Wu, L.-C., Townes, T., and Jaenisch, R. (2007). Treatment of sickle cell anemia mouse model with iPSCs generated from autologous skin. *Science* 318, 1920–1923.
27. Heng, J.-C.D., Feng, B., Han, J., Jiang, J., Kraus, P., Ng, J.-H., Orlov, Y., Huss, M., Yang, L., Lufkin, T., et al. (2010) The nuclear receptor Nr5a2 can replace Oct-4 in the reprogramming of murine somatic cells to pluripotent cells. *Cell Stem Cell* 6, 167–241.
28. Hochedlinger, K. and Jaenisch, R. (2002). Monoclonal mice generated by nuclear transfer from mature B and T donor cells. *Nature* 415, 1035–1043.
29. Hockemeyer, D., Soldner, F., Cook, E., Gao, Q., Mitalipova, M., and Jaenisch, R. (2008). A drug-inducible system for direct reprogramming of human somatic cells to pluripotency. *Cell Stem Cell* 3, 346–399.
30. Hong, H., Takahashi, K., Ichisaka, T., Aoi, T., Kanagawa, O., Nakagawa, M., Okita, K., and Yamanaka, S. (2009). Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. *Nature*. 460 (27259), 1132–1135.
31. Huangfu, D., Osafune, K., Maehr, R., Guo, W., Eijkelenboom, A., Chen, S., Muhlestein, W., and Melton, D.A. (2008). Induction of pluripotent stem cells from primary human fibroblasts with only Oct-4 and Sox2. *Nat. Biotech.* 26(11), 1269–1275.
32. Hussein, S.M., Batada, N.N., Vuoristo, S., Ching, R.W., Autio, R., Narva, E., Ng, S., Sourour, M., Hamalainen, R., Olsson, C., Lundin, K., Mikkola, M., Trokovic, R., Peitz, M., Brustle, O., Bazett-Jones, D.P., Alitalo, K., Lahesmaa, R., Nagy, A., and Otonkoski, T. (2011). Copy number variation and selection during reprogramming to pluripotency. *Nature*. 471(7336), 58–62.
33. Ichida, J.K., Blanchard, J., Lam, K., Son, E.Y., Chung, J.E., Egli, D., Loh, K.M., Carter, A.C., Di Giorgio, F.P., Koszaka, K., Huangfu, D., Akutsu, H., Liu, D.R., Rubin, L.L., and Eggan, K. (2009). A small-molecule inhibitor of tgfbeta signaling replaces Sox2 in reprogramming by inducing Nanog. *Cell Stem Cell* 5(5), 491–503.

34. Itzhaki, I., Maizels, L., Huber, I., Zwi-Dantsis, L., Caspi, O., Winterstern, A., Feldman, O., Gepstein, A., Arbel, G., Hammerman, H., et al. (2011). Modelling the long QT syndrome with induced pluripotent stem cells. *Nature* 471, 225–234.
35. Jia, F., Wilson, K.D., Sun, N., Gupta, D.M., Huang, M., Li, Z., Panetta, N.J., Chen, Z.Y., Robbins, R.C., Kay, M.A., Longaker, M.T., and Wu, J.C. (2010). A nonviral minicircle vector for deriving human iPSCs. *Nat. Meth.* 7(3), 197–199.
36. Kaji, K., Norrby, K., Paca, A., Mileikovsky, M., Mohseni, P., and Woltjen, K. (2009). Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature* 458, 771–776.
37. Kang, L., Wang, J., Zhang, Y., Kou, Z., and Gao, S. (2009). iPSCs can support full-term development of tetraploid blastocyst-complemented embryos. *Cell Stem Cell* 5, 135–143.
38. Kim, J., Greber, B., Arauzo-Bravo, M., Meyer, J., Park, K., Zaehres, H., and Schagler, H. (2009a). Direct reprogramming of human neural stem cells by Oct-4. *Nature* 461, 649–652.
39. Kim, J., Sebastiano, V., Wu, G., Arauzo-Bravo, M., Sasse, P., Gentile, L., Ko, K., Ruau, D., Ehrlich, M., van den Boom, D., et al. (2009b). Oct-4-induced pluripotency in adult neural stem cells. *Cell* 136, 411–420.
40. Kim, K., Doi, A., Wen, B., Ng, K., Zhao, R., Cahan, P., Kim, J., Aryee, M., Ji, H., Ehrlich, L., et al. (2010). Epigenetic memory in induced pluripotent stem cells. *Nature* 467, 285–375.
41. Kim, K., Zhao, R., Doi, A., Ng, K., Unternaehrer, J., Cahan, P., Hongguang, H., Loh, Y.-H., Aryee, M., Lensch, M., et al. (2011). Donor cell type can influence the epigenome and differentiation potential of human induced pluripotent stem cells. *Nat. Biotech.* 29(12), 1117–1119.
42. Kiskinis, E. and Eggan, K. (2010). Progress toward the clinical application of patient-specific pluripotent stem cells. *J. Clin. Invest.* 120(1), 51–59.
43. Kola, I. and Landis, J. (2004). Can the pharmaceutical industry reduce attrition rates? *Nat. Rev. Drug Discovery* 3(8), 711–715.
44. Laustriat, D., Gide, J., and Peschanski, M. (2010). Human pluripotent stem cells in drug discovery and predictive toxicology. *Biochem. Soc. Trans.* 38(4), 1051–1057.
45. Lee, G., Papapetrou, E.P., Kim, H., Chambers, S.M., Tomishima, M.J., Fasano, C.A., Ganat, Y.M., Menon, J., Shimizu, F., Viale, A., Tabar, V., Sadelain, M., and Studer, L. (2009). Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. *Nature* 461(7262), 402–406.
46. Li, H., Collado, M., Villasante, A., Strati, K., Ortega, S., Cañamero, M., Blasco, M.A., and Serrano, M. (2009). The Ink4/Arf locus is a barrier for iPSC cell reprogramming. *Nature* 460(7259), 1136–1139.
47. Li, J., Ishii, T., Feinstein, P., and Mombaerts, P. (2004). Odorant receptor gene choice is reset by nuclear transfer from mouse olfactory sensory neurons. *Nature* 428, 393–402.
48. Li, R., Liang, J., Ni, S., Zhou, T., Qing, X., Li, H., He, W., Chen, J., Li, F., Zhuang, Q., Qin, B., Xu, J., Li, W., Yang, J., Gan, Y., Qin, D., Feng, S., Song, H., Yang, D., Zhang, B., Zeng, L., Lai, L., Esteban, M.A., and Pei, D. (2010). A mesenchymal-to-epithelial transition initiates and is required for the nuclear reprogramming of mouse fibroblasts. *Cell Stem Cell* 7, 51–63.
49. Liao, B., Bao, X., Liu, L., Feng, S., Zovoilis, A., Liu, W., Xue, Y., Cai, J., Guo, X., Qin, B., et al. (2011). MicroRNA cluster 302-367 enhances somatic cell reprogramming by accelerating a mesenchymal-to-epithelial transition. *J. Biol. Chem.* 286, 17359–17423.
50. Liu, G.H., Barkho, B.Z., Ruiz, S., Dipe, D., Qu, J., Yang, S.L., Panopoulos, A.D., Suzuki, K., Kurian, L., Walsh, C., Thompson, J., Boue, S., Fung, H.L., Sancho-Martinez, I., Zhang, K., Yates III, J., and Izpisua Belmonte, J.C. (2011a). Recapitulation of premature ageing with iPSCs from Hutchinson-Gilford progeria syndrome. *Nature* 472, 221–225.
51. Liu, G.H., Suzuki, K., Qu, J., Sancho-Martinez, I., Yi, F., Li, M., Kumar, S., Nivet, E., Kim, J., Soligalla, R.D., Dubova, I., Goebel, A., Plongthongkum, N., Fung, H.L., Zhang, K., Loring, J.F., Laurent, L.C., and Izpisua Belmonte, J.C. (2011b). Targeted gene correction of laminopathy-associated LMNA mutations in patient-specific iPSCs. *Cell Stem Cell* 8, 688–694.
52. Maherali, N., Ahfeldt, T., Rigamonti, A., Utikal, J., Cowan, C., and Hochedlinger, K. (2008). A high-efficiency system for the generation and study of human induced pluripotent stem cells. *Cell Stem Cell* 3, 340–345.
53. Maherali, N. and Hochedlinger, K. (2009). Tgfbeta signal inhibition cooperates in the induction of iPSCs and replaces Sox2 and cMyc. *Current Biology* 19(20), 1718–1723.
54. Maherali, N., Sridharan, R., Xie, W., Utikal, J., Eminli, S., Arnold, K., Stadtfeld, M., Yachechko, R., Tchieu, J., Jaenisch, R., et al. (2007). Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. *Cell Stem Cell* 1, 55–125.
55. Maekawa, M., Yamaguchi, K., Nakamura, T., Shibukawa, R., Kodanaka, I., Ichisaka, T., Kawamura, Y., Mochizuki, H., Goshima, N., and Yamanaka, S. (2011). Direct reprogramming of somatic cells is promoted by maternal transcription factor Glis1. *Nature* 474(7350), 225–229.
56. Marión, R.M., Strati, K., Li, H., Murga, M., Blanco, R., Ortega, S., Fernandez-Capetillo, O., Serrano, M., and Blasco, M.A. (2009). A p53-mediated DNA damage response limits reprogramming to ensure iPSC cell genomic integrity. *Nature* 460(7259), 1136–1139.
57. Mikkelsen, T.S., Hanna, J., Zhang, X., Ku, M., Wernig, M., Schorderet, P., Bernstein, B.E., Jaenisch, R., Lander E.S., and Meissner, A. (2008). Dissecting direct reprogramming through integrative genomic analysis. *Nature* 454(7200), 49–55.
58. Miyoshi, N., Ishii, H., Nagano, H., Haraguchi, N., Dewi, D., Kano, Y., Nishikawa, S., Tanemura, M., Mimori, K., Tanaka, F., et al. (2011). Reprogramming of mouse and human cells to pluripotency using mature microRNAs. *Cell Stem Cell* 8, 633–641.
59. Moretti, A., Bellin, M., Welling, A., Jung, C., Lam, J., Bott-Flugel, L., Dorn, T., Goedel, A., Hohnke, C., Hofmann, F., et al. (2010). Patient-specific induced pluripotent stem-cell models for long-QT syndrome. *N.E. J. Med.* 363, 1397–1806.
60. Nakagawa, M., Koyanagi, M., Tanabe, K., Takahashi, K., Ichisaka, T., Aoi, T., Okita, K., Mochizuki, Y., Takizawa, N., and Yamanaka, S. (2007). Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat. Biotech.* 26, 101–107.
61. Noginov, M.A., Zhu, G., Belgrave, A.M., Bakker, R., Shalae, V.M., Narimanov, E.E., Stout, S., Herz, E., Suteewong, T., and Wiesner, U. (2009). Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. *Nature* 460(7259), 1132–1135.
62. Okita, K., Ichisaka, T., and Yamanaka, S. (2007). Generation of germline-competent induced pluripotent stem cells. *Nature* 448, 313–320.
63. Okita, K., Nakagawa, M., Hyenjong, H., Ichisaka, T., and Yamanaka, S. (2008). Generation of mouse induced pluripotent stem cells without viral vectors. *Science* 322, 949–1002.
64. Papapetrou, E., Lee, G., Malani, N., Setty, M., Riviere, I., Tirunagari, L., Kadota, K., Roth, S., Giardina, P., Viale, A., et al. Genomic safe harbors permit high β -globin transgene expression in thalassemia induced pluripotent stem cells. *Nat. Biotech.* 29, 73–81.
65. Park, I.-H., Zhao, R., West, J., Yabuuchi, A., Huo, H., Ince, T., Lerou, P., Lensch, M., and Daley, G. (2008). Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* 451, 141–147.
66. Pasca, S., Portmann, T., Voineagu, I., Yazawa, M., Shcheglovitov, A., Pasca, A., Cord, B., Palmer, T., Chikahisa, S., Nishino, S., et al. (2011). Using iPSC-derived neurons to uncover cellular phenotypes associated with Timothy syndrome. *Nat. Med.* 17(12), 1657–1662.
67. Pfaff, N., Fiedler, J., Holzmann, A., Schambach, A., Moritz, T., Cantz, T., and Thum, T. (2011). miRNA screening reveals a new miRNA family stimulating iPSC cell generation via regulation of Meox2. *EMBO Reports* 12, 1153–1162.
68. Polo, J., Liu, S., Figueroa, M., Kulalert, W., Eminli, S., Tan, K., Apostolou, E., Stadtfeld, M., Li, Y., Shioda, T., et al. (2010). Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. *Nat. Biotech.* 28, 848–903.

69. Raya, A., Rodríguez-Piza, I., Guenechea, G., Vassena, R., Navarro, S., Barrero, M.J., Consiglio, A., Castella, M., Rio, P., Sleep, E., Gonzalez, F., Tiscorina, G., Garreta, E., Asen, T., Veiga, A., Verma, I.M., Surrallés, J., Bueren, J., and Izpisua Belmonte, J.C. (2009). Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. *Nature* 460(7251), 53–59.
70. Redmer, T., Diecke, S., Grigoryan, T., Quiroga-Negreira, A., Birchmeier, W., and Besser, D. (2011). E-cadherin is crucial for embryonic stem cell pluripotency and can replace Oct-4 during somatic cell reprogramming. *EMBO Reports* 12(7), 720–726.
71. Rideout, W.M., Hochedlinger, K., Kyba, M., Daley, G.Q., and Jaenisch, R. (2002). Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. *Cell* 109(1), 17–27.
72. Rohwedel, J., Guan, K., Hegert, C., and Wombus, A.M. (2001). Embryonic stem cells as an in vitro model for mutagenicity, cytotoxicity and embryotoxicity studies, present state and future prospects. *Toxicology in vitro, an international journal published in association with BIBRA*. 15(6), 741–753.
73. Samavarchi-Tehrani, P., Golipour, A., David, L., Sung, H.K., Beyer, T.A., Datti, A., Wolftjen, K., Nagy, A., and Wrana, J.L. (2010). Functional genomics reveals a BMP-driven mesenchymal-to-epithelial transition in the initiation of somatic cell reprogramming. *Cell Stem Cell* 7(1), 64–77.
74. Soldner, F., Hockemeyer, D., Beard, C., Gao, Q., Bell, G., Cook, E., Hargus, G., Blak, A., Cooper, O., Mitalipova, M., et al. (2009). Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. *Cell* 136, 964–1041.
75. Sommer, C., Stadtfeld, M., Murphy, G., Hochedlinger, K., Kotton, D., and Mostoslavsky, G. (2009). Induced pluripotent stem cell generation using a single lentiviral stem cell cassette. *Stem Cells* 27, 543–552.
76. Sridharan, R., Tchieu, J., Mason, M.J., Yachechko, R., Kuoy, E., Horvath, S., Zhou, Q., and Plath, K. (2009). Role of murine reprogramming factors in the induction of pluripotency. *Cell* 136(2), 364–377.
77. Stadtfeld, M., Apostolou, E., Akutsu, H., Fukuda, A., Follett, P., Natesan, S., Kono, T., Shioda, T., and Hochedlinger, K. (2010). Aberrant silencing of imprinted genes on chromosome 12qF1 in mouse induced pluripotent stem cells. *Nature* 465, 175–256.
78. Stadtfeld, M. and Hochedlinger, K. (2011). Induced pluripotency: history, mechanisms, and applications. *Genes & Development* 24, 2239–2302.
79. Stadtfeld, M., Maherali, N., Borkent, M., and Hochedlinger, K. (2009). A reprogrammable mouse strain from gene-targeted embryonic stem cells. *Nature Methods* 7, 53–58.
80. Stadtfeld, M., Nagaya, M., Utikal, J., Weir, G., and Hochedlinger, K. (2008). Induced pluripotent stem cells generated without viral integration. *Science* 322, 945–954.
81. Subramanyam, D., Lamouille, S., Judson, R., Liu, J., Bucay, N., Derynck, R., and Blelloch, R. (2011) Multiple targets of miR-302 and miR-372 promote reprogramming of human fibroblasts to induced pluripotent stem cells. *Nat. Biotech.* 29, 443–451.
82. Sugii, S., Kida, Y., Kawamura, T., Suzuki, J., Vassena, R., Yin, Y.Q., Lutz, M.K., Berggren, W.T., Izpisua Belmonte, J.C., and Evans, R.M. (2010). Human and mouse adipose-derived cells support feeder-independent induction of pluripotent stem cells. *Proc. Nat. Acad. Sci. U.S.A.* 107(8), 3558–35563.
83. Tada, M., Tada, T., Lefebvre, L., Barton, S., and Surani, M. (1997). Embryonic germ cells induce epigenetic reprogramming of somatic nucleus in hybrid cells. *The EMBO Journal* 16, 6510–6530.
84. Tada, M., Takahama, Y., Abe, K., Nakatsuji, N., and Tada, T. (2001). Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. *Current Biology* 11, 1553–1561.
85. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861–933.
86. Takahashi, K. and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663–739.
87. Tan, K., Eminli, S., Hettmer, S., Hochedlinger, K., and Wagers, A. (2011). Efficient Generation of iPS Cells from Skeletal Muscle Stem Cells. *PLoS one* 6(10), e26406.
88. Theunissen, T.W., van Oosten, A.L., Castelo-Branco, G., Hall, J., Smith, A., and Silva, J.C. (2011) Nanog overcomes reprogramming barriers and induces pluripotency in minimal conditions. *Current Biology* 21(1), 65–71.
89. Utikal, J., Maherali, N., Kulalert, W., and Hochedlinger, K. (2009). Sox2 is dispensable for the reprogramming of melanocytes and melanoma cells into induced pluripotent stem cells. *Journal of Cell Science* 122, 3502–3512.
90. Wang, W. (2007). Emergence of a DNA-damage response network consisting of Fanconi anaemia and BRCA proteins. *Nature reviews. Genetics*. 8(10), 735–748.
91. Warren, L., Manos, P., Ahfeldt, T., Loh, Y.-H., Li, H., Lau, F., Ebina, W., Mandal, P., Smith, Z., Meissner, A., et al. (2010). Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell* 7, 618–648.
92. Wernig, M., Lengner, C., Hanna, J., Lodato, M., Steine, E., Foreman, R., Staerk, J., Markoulaki, S., and Jaenisch, R. (2008). A drug-inducible transgenic system for direct reprogramming of multiple somatic cell types. *Nat. Biotech.* 26, 916–940.
93. Wernig, M., Meissner, A., Cassady, J., and Jaenisch, R. (2008). c-Myc is dispensable for direct reprogramming of mouse fibroblasts. *Cell Stem Cell* 2, 10–12.
94. Wernig, M., Meissner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B., and Jaenisch, R. (2007). In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* 448, 318–342.
95. Wernig, M., Zhao, J.-P., Pruzsák, J., Hedlund, E., Fu, D., Soldner, F., Broccoli, V., Constantine-Paton, M., Isacson, O., and Jaenisch, R. (2008). Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. *Proc. Nat. Acad. Sci. U.S.A.* 105, 5856–5917.
96. Wilmot, I., Schnieke, A., McWhir, J., Kind, A., and Campbell, K. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature* 385, 810–813.
97. Wobus, A.M. and Loser, P. (2011). Present state and future perspectives of using pluripotent stem cells in toxicology research. *Archives of Toxicology*. 85(2), 79–117.
98. Wu, S.M. and Hochedlinger, K. (2011) Harnessing the potential of induced pluripotent stem cells for regenerative medicine. *Nat. Cell Bio.* 13(5), 497–505.
99. Yang, D., Zhang, Z.J., Oldenburg, M., Ayala, M., and Zhang, S.C. (2008). Human embryonic stem cell-derived dopaminergic neurons reverse functional deficit in parkinsonian rats. *Stem Cells* 26(1), 55–63.
100. Yazawa, M., Hsueh, B., Jia, X., Pasca, A., Bernstein, J., Hallmayer, J., and Dolmetsch, R. (2011). Using induced pluripotent stem cells to investigate cardiac phenotypes in Timothy syndrome. *Nature* 471, 230–234.
101. Yu, J., Vodyanik, M., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J., Tian, S., Nie, J., Jonsdottir, G., Ruotti, V., Stewart, R., et al. (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318, 1917–1937.
102. Yusa, K., Rad, R., Takeda, J., and Bradley, A. (2009). Generation of transgene-free induced pluripotent mouse stem cells by the piggyBac transposon. *Nature Methods* 6, 363–372.
103. Zhao, T., Zhang, Z.-N., Rong, Z., and Xu, Y. (2011). Immunogenicity of induced pluripotent stem cells. *Nature* 474, 212–217.
104. Zhao, X., Li, W., Lv, Z., Liu, L., Tong, M., Hai, T., Hao, J., Guo, C.-I., Ma, Q., Wang, L., et al. (2009). iPS cells produce viable mice through tetraploid complementation. *Nature* 461, 86–176.
105. Zhou, H., Wu, S., Joo, J.Y., Zhu, S., Han D.W., Lin, T., Trauger, S., Bien, G., Yao, S., Zhu, Y., et al. (2009) Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell* 4, 381–384.



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