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Development unchained: how cellular reprogramming is redefining our view of cell fate and identity

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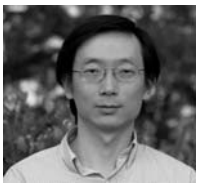
ABSTRACT

Higher eukaryotic development has traditionally been considered a unidirectional and irreversible process. Beginning in 2006, with Yamanaka and colleagues' report on the first successful generation of induced pluripotent stem cells (iPSCs), the field of stem cell biology has experienced perhaps unprecedented rates of growth and discovery. This review is a summary of recent progress in the field of reprogramming. Advances in small molecule-aided reprogramming and transdifferentiation, currently two of the most intensely studied areas of stem cell biology, are emphasized. The field has collectively covered much ground in the past five years, dramatically increasing reprogramming efficiency and successfully eliminating the need for permanent genetic modification, perhaps the biggest obstacle to eventual clinical use of this strategy. Simultaneously, various transdifferentiation strategies are rapidly expanding the scope of cellular plasticity, interconverting unrelated cell types with relative technical ease. While significant challenges remain – such as accomplishing small molecule-only "chemical reprogramming" or ensuring the functional and epigenetic equivalency of reprogrammed or transdifferentiated cells – there is no shortage of enthusiasm in the field.

Keywords: *induced pluripotency, small molecules, cell fate plasticity, transdifferentiation, direct conversion*



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A brief history of reprogramming

The progression of higher eukaryotic development, beginning with the zygote and culminating in a fully formed organism with its myriad specialized cells, tissues, and organs, has long been thought of as a linear and unidirectional process. In this model, cells become progressively restricted in terms of their developmental potential: through a series of divisions, the totipotent zygote capable of

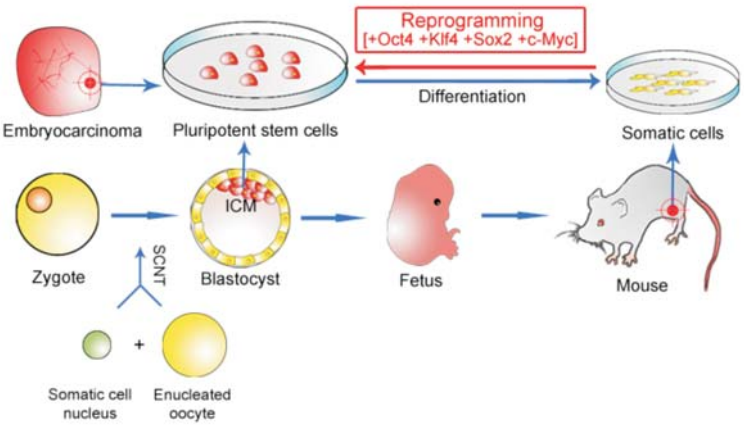

Figure 1 *Deriving pluripotent stem cells from various sources. Development begins with a totipotent zygote, goes through different stages (such as blastocyst and fetus), and ends when a mature adult organism has been formed. Pluripotent stem cells can be derived from the inner cell mass (ICM) of pre-implantation blastocysts, embryocarcinomas (ECs), and by somatic cell nuclear transfer (SCNT). As of late 2006, it is also possible to induce the pluripotent state in somatic cells of adult organisms by ectopically overexpressing just four transcription factors (Oct4, Klf4, Sox2 and c-Myc). The generation of these induced pluripotent stem cells (iPSCs) has had profound implications for cellular plasticity in higher eukaryotic development well as potential therapeutic applications in the longer-term.*

forming all tissues gives rise to a blastocyst with an inner cell mass (ICM) harbouring stem cells[1] that are described as pluripotent, *i.e.* can differentiate into all cell types except extraembryonic tissue. These stem cells further differentiate into multipotent precursors that can only produce a limited number of lineage-specific progeny. Ultimately, such precursors give rise to the terminally differentiated cells that have traditionally been thought of as fulfilling a single, unchanging role (Figure 1).

It has been known for decades, however, that eukaryotic somatic cells are capable of significant de- and re-differentiation under certain circumstances: for example, it was documented in the 1950s that salamanders can regenerate entire limbs following amputation, likely by using this type of strategy[2], the details of which are still being worked out today. It initially appeared that humans and other higher eukaryotes had simply lost this ability to regenerate cells and tissue over evolutionary time. In the coming decades, however, a slow but steady stream of evidence to the contrary began to emerge, eventually sparking great interest in the field of cell fate re-determination. First, somatic cell nuclear transfer

(SCNT) enabled the recreation, *i.e.* cloning, of an entire organism by replacing an oocyte nucleus with that of a somatic cell. Blastocysts resulting from this process could be (a) used to derive pluripotent stem cells that were autologous (*i.e.* of the donor genotype) or (b) allowed to implant in the uterus for full development. Second, the culture of pluripotent cancer cells (embryocarcinoma cells or ECCs, for example) was reported, as well as the observation that such cells can ''reprogram'' a somatic cell nucleus upon forced fusion[3] (Figure 1). Groundbreaking papers on these subjects were published as early as the 1960s, and their most important collective contribution was the demonstration that (a) somatic cells still contained all the genetic information necessary for the *de novo* development of an organism, and (b) unknown trans-acting factors could activate a latent network of genes, resulting in the re-establishment of toti- and pluripotency.

In the years that followed, many papers were published on the characteristics of toti- and pluripotent cells, using methods ranging from smaller-scale, traditional genetics experiments to large-scale gene expression profiles – all with the eventual goal of identifying how cells establish and sustain the pluripotent state[4,5]. It was assumed that the list of requirements would be fairly complex, and it was far from certain that the requisite set of conditions could be identified in the near-term, let alone artificially recreated. It was against this scientific backdrop that Yamanaka *et al.*[6] published their seminal study on induced pluripotency in late 2006, demonstrating that mouse fibroblasts could be reverted to a stem cell-like state by the viral delivery and overexpression of a mere four transcription factors: *Oct4*, *Sox2*, *Klf4*, and *c-Myc* (Figure 1).

Interestingly, with the exception of *Oct4*, expression of these transcription factors is not restricted to stem and germ cells; they fulfil an array of roles in different cell types, including somatic cells. *Oct4* is a POU-domain containing transcription factor that can heterodimerize with *Sox2* to promote and maintain pluripotency. Aside from stem cell homeostasis, the latter is critical for the development of neural lineages as well. *Klf4*, a member of the Krüppel-like family of transcription factors with higher expression in the skin, intestines, and blood, is a tumour suppressor that regulates cell differentiation, growth, and proliferation. Finally, the potent oncogene *c-Myc*, a known inducer of cellular transformation, also modulates these processes[7]. Interestingly, while *Oct4*, *Sox2*, and *Klf4* all appear to bind multiple pluripotency-related promoter regions and are critical for the eventual re-establishment of the pluripotency program, *c-Myc* appears to be disproport-

ionately involved in the parallel suppression of the somatic program[8].

The reprogrammed cells generated by retroviral overexpression of these transcription factors were dubbed induced pluripotent stem cells (iPSCs). Yamanaka and colleagues' painstakingly methodical and thorough approach demonstrated that transcription factors were most likely the critical components of the complex mix of trans-acting factors required for de-differentiation of somatic cells. The striking extent to which this straightforward process can alter cellular homeostasis further intensified interest in the area of epigenetics, which studies how heritable phenotypic changes come about in the absence of genetic modification. A number of relevant pathways and mechanisms – most notably patterns of histone modifications and DNA methylation – were identified soon thereafter[9], and the scrutiny continues unabated.

Because the process described was as simple as it was groundbreaking, the study is credited with singlehandedly revolutionizing the field and putting the method of cellular reprogramming within most laboratories' reach almost overnight. Using this elegant and powerful paradigm as a springboard, numerous laboratories have been intensely studying mammalian cell fate plasticity, and the field of reprogramming has experienced unparalleled growth in publications and attained mainstream status. However, the drive to uncover the very complex basic biology underlying the reprogramming process can only partially account for this phenomenon; the as-of-yet unrealized potential for therapeutic application of iPSC technology is undoubtedly just as attractive. The ability to quickly and efficiently make autologous pluripotent cells from almost any somatic cell in the body is extremely valuable. Such cells can be re-differentiated along multiple lineages in the dish to model development of a disease state, especially for diseases where no suitable model – *i.e.* animals or cell lines – is available. Moreover, there is potential for cell-based therapies such as transplantation to replace tissues that normally do not regenerate (*e.g.* nervous system and heart muscle). In the context of both disease modelling and transplantation, iPSCs are much more promising than other aforementioned reprogrammed pluripotent cells: the cancer-derived nature of ECCs and their gross abnormalities obviously disqualify them from any therapeutic use, and generating iPSCs is much simpler and more efficient when compared to SCNT.

The remainder of this review aims to serve as a summary of recent progress (*i.e.* post-2006) in the field of reprogramming for a general scientific audience, with an emphasis on chemical repro-

gramming and transdifferentiation – two areas in which our laboratory has had the opportunity to make contributions. The current state of the research and future directions will be discussed, including continually improving prospects for clinical use.

Understanding the reprogramming process

Since Yamanaka and colleagues had defined a surprisingly minimal set of requirements for nuclear reprogramming, everyone in the field was able to rapidly test diverse methods to improve the technique and apply it in various contexts. First, James Thomson's laboratory announced the generation of human iPSCs using the same method with a slightly different set of transcription factors[10]: *OCT4*, *SOX2*, *NANOG*, and *LIN28*. Nanog, one of the master regulators of the pluripotency transcriptional program, has been referred to as the ''gateway to the pluripotent state'' due to its essential role in attaining and stabilizing the iPSC phenotype[11]. Lin28 is an RNA-binding protein that likely contributes to reprogramming success by aiding the (over)expression of pluripotency-related genes[12].

Soon thereafter, the number of required factors for fibroblast-to-iPSC reprogramming was reduced to three in both mice and humans, as it became apparent that *c-Myc* was not absolutely required – although it does increase speed and efficiency considerably[13,14]. Others recognized that certain cell types were either much more amenable to reprogramming (*e.g.* myeloid precursors[15]) or that they could be reprogrammed just as efficiently with even fewer factors (*e.g.* neural precursor cells with *Oct4* alone[16]). Tables 1 and 2 provide a comprehensive list of the various combinations of transcription factors that are able to reprogram different cell types back to pluripotency. Eventually, iPSCs were generated from a diverse array of tissues including the lining of the stomach and adipose cells, as well as from numerous organisms including, for example, monkeys and pigs[3].

Meanwhile, other laboratories were making strides in understanding the mechanistic underpinnings of the reprogramming process. As is apparent from the fundamental phenotypic changes involved, the extent of epigenetic modification required to re-establish the pluripotent state in the context of a somatic program is vast; specifically, the more ''open'' or ''relaxed'' chromatin structure (*i.e.* possessing a higher percentage of less condensed euchromatin) characteristic of pluripotent stem cells needs to be reinstated. In ESCs, this structure appears to be maintained by an array of chromatin remodelling factors that (a) ensure a globally

Table 1 *Mouse iPSCs derived from different cell types and through various methods*

Cell type	Delivery method	Factors	Small molecule	Efficiency	Ref.
Fibroblast	Retrovirus	OKSM	–	0.02%	6
Fibroblast	Retrovirus	OKS	–	0.005%	13
Fibroblast	Retrovirus	OKSM	VPA	0.09%	18
Fibroblast	Retrovirus	OKS	CHIR99021	0.2–0.4%	57
Fibroblast	Retrovirus	OKM	VPA, E–616451	~0.1%	58
Fibroblast	Retrovirus	OK	E–616452	~0.05%	58
Fibroblast	Retrovirus	OK	BIX01294, BayK8644	~0.02%	28
Fibroblast	Retrovirus	O	VPA, CHIR99021, E616452, Parnate	0.002–0.03%	59
Fibroblast	Retrovirus	O	A–83–01, AMI–5	0.02%	60
Fibroblast (secondary fibroblast)	Inducible lentivirus	OKSM	–	2%; 4%	14
Fibroblast	Adenovirus	OKSM	–	0.0001%	61
Fibroblast	Transposon	OKSM	–	0.026%	62
Fibroblast	Plasmid	OKSM	–	0.003%	63
Fibroblast	Protein	OKSM	VPA	0.006%	64
Neural stem cell	Retrovirus	OK	–	<0.1%	65
Neural stem cell	Retrovirus	OK	BIX01294	~0.03%	29
Neural stem cell	Retrovirus	O	–	~0.014%	16
Mature B cell	Dox/retrovirus	OKSM + C	–	3%	66
Mature B and T cell	Dox induction	OKSM	–	0.02%	15
Myeloid progenitor	Dox induction	OKSM	–	25%	15
Hematopoietic stem cell	Dox induction	OKSM	–	13%	15
Adipose stem cell	Retrovirus	OKSM	–	~0.42%	67
Dermal papilla	Retrovirus	OKSM	–	1.38%	68
Dermal papilla	Retrovirus	OK	–	0.024%	68
Liver cell	Adenovirus	OKSM	–	0.0018%	61
Hepatocyte	Adenovirus	OKSM	–	0.0006%	61
Pancreatic β cell	Inducible lentivirus	OKSM	–	0.1–0.2%	69
Melanocyte	Inducible lentivirus	OKM	–	0.2%	70

O, Oct4; S, Sox2; K, Klf4; M, c-Myc, C, C/EBPa; Dox, doxycycline.

Table 2 *Human iPSCs derived from different cell types and through various methods*

Cell type	Delivery method	Factors	Small molecule	Efficiency	Ref.
Fibroblast	Retrovirus	OKSM	–	0.02%	71
Fibroblast	Lentivirus	OSNL	–	0.02%	10
Fibroblast	Retrovirus	OSKM	SB431542, PD0325901, thiazovivin	~2%	36
Fibroblast	Retrovirus	OSK	–	<0.001%	13
Fibroblast	Retrovirus	OSK	VPA	~1%	18
Fibroblast	Retrovirus	OS	VPA	~0.005%	30
Fibroblast	Transposon	OKSM	–	0.006%	62, 72
Fibroblast	Plasmid	OSKMNL + SV40LT	–	0.0006%	73
Fibroblast	Protein	OKSM	–	0.001%	74
Fibroblast	RNA	OKSM	–	~1.4%	75
Fibroblast (secondary fibroblast)	Inducible lentivirus	OSKM	–	0.26–2.0%; 1–3%	23, 76
Neural stem cell	Retrovirus	OK	–	0.006%	77
Neural stem cell	Retrovirus	O	–	0.004%	77
Keratinocyte	Retrovirus	OKSM	–	0.28%	78, 67
Keratinocyte	Retrovirus	OKS	–	0.04–0.06%	78
Keratinocyte	Lentivirus	OK	CHIR99021, Parnate	~0.002%	57
Keratinocyte	Lentivirus	OK	CHIR99021, Parnate, PD0325901, SB431542	0.005–0.01%	57
Keratinocyte	Lentivirus	O	A–83–01, PS48 NaB, PD0325901	~0.0006%	37
Mobilized peripheral blood	Retrovirus	OKSM	–	0.01–0.02%	32
Cord blood endothelial cell	Lentivirus	OSNL	–	0.0001–0.03%	79
Cord blood stem cell	Inducible lentivirus	OKSM	–	ND	15
Cord blood stem cell	Retrovirus	OS	–	<0.01%	80
Adipose stem cell	Retrovirus	OKSM	–	~0.31%, ~0.74%	67
Adipose stem cell	Lentivirus	OKSM	–	0.2%	81
Adipose stem cell	Retrovirus	OKS	–	~0.1%	82
Hepatocyte	Retrovirus	OKSM	–	0.1–0.2%	83

O, Oct4; S, Sox2; K, Klf4; M, cMyc; N, Nanog; L, Lin28; ND, not determined.

more permissive transcriptional state while (b) specifically suppressing differentiation-inducing genes and transcriptional networks[17].

Broadly speaking, chromatin remodelling factors fall into two categories: DNA modifiers and various histone modifiers that can add an array of post-translational modifications to the N-terminal tail regions of histones. Cytosine DNA methylation, carried out by Dnmt1 and the Dnmt3 family of enzymes, mostly represses transcription. Blocking or reducing this process – for example, using small molecules – can therefore greatly aid reprogramming[18]. In the same fashion, the establishment and maintenance of activating histone modifications is critical: hallmarks of the pluripotent state include trimethylation of histone H3 lysine 4 (H3K4me3) in chromatin regions harbouring pluripotency genes and other relevant factors. Conversely, prevalent inhibitory marks are methylation of lysine 9 on the same histone tail (H3K9me3) and methylation of lysine 27 (H3K27me3). Interestingly, the activating H3K4me3 modification is sometimes observed alongside inhibitory H3K27 methylation in what has been called a ''bivalent'' state[19]. This latter combination is generally observed in regions encoding lineage-specific genes, most likely priming them for expression when differentiation is initiated. As far as key players are concerned, several factors and complexes with specific roles in reprogramming have been identified. The polycomb (PcG) and trithorax (trxG) group of proteins are among the best characterized to date: the former can repress certain genes that the latter may activate[20]. Perhaps not surprisingly, it has been shown that DNA and histone modification mechanisms can interact and reinforce each other; for example, the histone methyltransferase G9a (which mediates H3K9 methylation) has been shown to recruit DNA methyltransferases to its site of action for an added layer of negative regulation[21]. Finally, a recent study showing that pervasive epigenetic changes are among the earliest events observed upon ectopic pluripotency factor expression underscores just how central chromatin modification is to the reprogramming process[22].

Other critical aspects of the reprogramming process are simultaneously being elucidated. Thanks to the generation of transgenic cells capable of doxycycline-regulated Yamanaka factor expression, we now know that there is a minimum requirement for 7 – 8 days of transgene expression (in both mouse and human fibroblasts) before the pluripotent state of iPSCs becomes self-sustaining[14,23]. This type of inducible system can be set up by either infecting somatic cells with viruses carrying the inducible constructs, or by using what have been termed secondary fibroblasts. The latter are

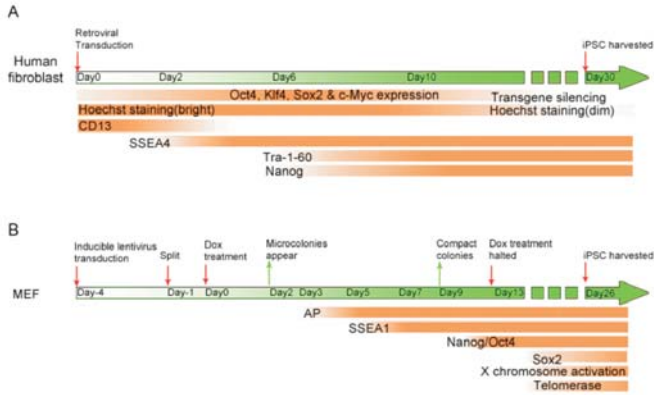

Figure 2 *Timelines for human/mouse induced pluripotent stem cell generation as delineated by marker expression. A, During human fibroblast reprogramming, the somatic marker CD13 is downregulated with ectopic factor expression and the pluripotency-associated marker SSEA-4 is detected as early as day 2. Other markers like TRA-1-60 and Nanog are detected in the later stages. B, In the inducible lentiviral system, mouse embryonic fibroblasts (MEFs) start showing morphological changes on day 2. AP and SSEA-1 are early reprogramming markers. Endogenous Oct4 expression is detectible around day 9. Endogenous Sox2, telomerase, and the silent X chromosome are reactivated during the late stage of reprogramming.*

generated by injecting iPSCs obtained from fibroblasts into blastocysts to generate chimeric mice; secondary fibroblasts derived from the injected iPSCs can then be isolated using drug marker selection. The homogenous population of cells thus generated harbours stably integrated inducible Yamanaka factors, which can then be rapidly and effectively overexpressed just by adding doxycycline to the culture media. The secondary reprogramming process is both considerably faster and more efficient compared to the conventional method (Figure 2 and Tables 1 and 2).

Importantly, we have a much better understanding of the sequence of gene activation events leading up to the establishment of induced pluripotency, enabling rapid determination of reprogramming phase and success based on marker expression[24] (Figure 2). It has been shown that certain markers like SSEA-4 (human) and AP (alkaline phosphatase; mouse) commence expression as early as 48–72 hours into the reprogramming process. In the same timeframe, somatic markers like CD13 are effectively downregulated. The master regulators of pluripotency (*i.e.* Nanog and endogenous Oct4) are not activated until several days later. For mouse and human cells, the process proceeds through the same steps, with each

phase of the process taking somewhat longer in the latter. This stepwise nature of reprogramming is being described in ever-greater detail, with stochastic as well as defined events – at both the molecular and cellular level – driving and defining the process. A recently described example of such an event is a simultaneous decrease in fibroblast surface area and an increase in the rate of proliferation that appears to be highly predictive of successful reprogramming upon retrospective visual analysis[25,26].

Improving reprogramming: small molecules and alternative methods

Two considerations are very important when evaluating the safety and effectiveness of the Yamanaka method of reprogramming: first, the need for integration of viral constructs into the genome, *i.e.* permanent genetic modification, severely limits downstream clinical potential, especially with the use of oncogenes such as *c-Myc*. Second, the inefficiency and slowness of the reprogramming process (in its first iteration, a time frame of about three weeks and an overall efficiency of 0.02% were reported) raise concerns regarding the acquisition and selection of undesirable genetic and/or epigenetic abnormalities. Two fundamental approaches to alleviating these concerns are: (a) functional replacement of the Yamanaka factors to eliminate the need for genetic modification, and (b) improvements in speed and efficiency to minimize or perhaps eliminate any deleterious side effects of reprogramming.

Small molecules may hold the key to achieving both of the above goals. Over the decades, such compounds have contributed extensively to basic biology by helping to chart cellular pathways and mechanisms. They have also been at the forefront of medicine for at least a century, helping alleviate or cure many human diseases. Stem cell biology, development, and reprogramming are no exception, being significantly aided by small molecule-driven research in multiple ways: (a) hypothesis-driven design of experiments exploiting already known bioactive compounds and (b) the screening of libraries comprising very large numbers of molecules generated through combinatorial chemistry – often referred to as the discovery-driven approach – have led to the identification of compounds that modulate or maintain pluripotency, profoundly influence development and differentiation, and improve reprogramming outcomes.

Regarding small molecules' mechanism of action in the context of reprogramming, one can generally categorize them as specific or

non-specific enhancers. A typical example of the latter are compounds that affect epigenetic processes on a whole-cell level, *e.g*. the DNA methyltransferase inhibitor RG108 or the histone deacetylase inhibitors (HDACs) valproic acid (VPA) and butyrate, which under certain circumstances is able to improve reprogramming up to 50-fold[27]. We have, for example, identified multiple compounds that can functionally replace one or more of the Yamanaka reprogramming factors: BIX01294, a G9a histone methyltransferase inhibitor, can act in concert with the calcium channel agonist BayK8644 to enable two-factor iPSC generation using only *Oct4* and *Klf4* [28]. Further, BIX01294 can even replace the master regulator *Oct4*–arguably the most critical of the four factors–in the reprogramming of neural progenitor cells (NPCs), provided all three of the remaining factors are simultaneously overexpressed[29]. Along the same lines, it has been shown that the non-specific compound VPA can replace both *Klf4* and *c-Myc* in neonatal human fibroblasts[30].

Conversely, some molecules can bring about similar outcomes by very specifically affecting only a small number–or even a single–pathway. In this particular context, we have found that application of the glycogen synthase kinase 3 (GSK3) inhibitor CHIR99021 allows mouse fibroblasts to be reprogrammed with only *Oct4* and *Klf4*. Furthermore, it permits the generation of iPSCs from human fibroblasts overexpressing the same two factors when combined with the compound parnate[31]. Others have been able to replace *Sox2* overexpression with the compound E-616452, which was found to enhance *Nanog* expression by inhibiting the transforming growth factor-beta (TGF-β) receptor[32]. The process termed mesenchymal-to-epithelial transition (MET) is integral to reprogramming, and is also affected by TGF-β signalling[33]. This relationship likely explains how *Sox2* and *c-Myc* can be replaced by using an inhibitor of TGF-β receptor I kinase/activin-like kinase 5 (Alk5)[34]. Lyssiotis *et al*.[35] have recently shown that a GSK3 inhibitor named kenpaullone can substitute for *Klf4* when reprogramming mouse embryonic fibroblasts (MEFs); this substitution does not, however, rely on the inhibition of GSK3 and proceeds through an as-of-yet unknown mechanism. Finally, we have been able to achieve an efficiency boost of over two orders of magnitude (~200-fold) in the derivation of four-factor iPSCs from human fibroblasts using a cocktail consisting of the Alk5 inhibitor SB431542, the MEK inhibitor PD0325901, and thiazovivin[36].

Unlike differentiated somatic cells, which heavily rely on mitochondrial oxidation for their metabolism, pluripotent cells have

mainly adopted glycolysis to meet the demands of a high proliferation rate with less oxidative stress. Thus, reprogramming from somatic cells to iPSCs necessitates a metabolic switch from mitochondrial oxidation to glycolysis. A recent paper published by our lab identified a small molecule activator of PDK1 called PS48, which facilitates reprogramming of human keratinocytes and endothelial cells to iPSCs with *Oct4* only[37]. The activation of AKT/PKB by PDK1 up-regulates the expression of several key glycolytic genes. This phenomenon demonstrates that the reprogramming process can be enhanced by facilitating metabolic conversion. Representative structures of small-molecule enhancers of reprogramming in the major categories outlined above, including many of the individual compounds mentioned, have been provided in Figure 3.

A distinct but related body of work is based on the concept of reprogramming as a transition between different pluripotent states, specifically those of embryonic stem cells (ESCs) and epiblast stem cells (EpiSCs). EpiSCs are obtained from post-implantation epiblasts and appear to be pluripotent: they express the requisite core pluripotency network genes including the master regulators Nanog and Oct4, and can give rise to cell lineages of all three germ layers *in vitro*. However, they rely on a different set of molecular signalling cues for survival, divide more slowly, are much more sensitive to single-cell dissociation, and can only poorly contribute to chimerism, indicating decreased robustness and inferior, less ''naïve'' pluripotency[38,39]. Intriguingly, overexpression of *Klf4*, *Nr5a*, and *Nanog* can reprogram mouse EpiSCs to an ESC-like state[40,41]. Accordingly, we have identified a combination of four small molecules that can engender the same conversion; combined application of a MEK inhibitor, a histone demethylase inhibitor targeting LSD1, a ALK5 inhibitor, and a GSK3 inhibitor efficiently converts EpiSCs to the chimerism-competent state that morphologically and functionally mimics ''naïve'' state of mESCs[42].

Given that hESCs very closely resemble mouse EpiSCs in many aspects, it was postulated that the above conversion process might also allow for the isolation of the developmentally earlier/more naïve hESCs. We found that four-factor transduced human fibroblasts produced naïve-state mESC-like human iPSC colonies that can be stably maintained when cultured in mESC medium containing human LIF and a small-molecule cocktail containing inhibitors of MEK, ALK5, and GSK3 (PD0325901, A-83-01, and CHIR99021, respectively). Moreover, this same cocktail of small molecules successfully promoted the establishment and expansion

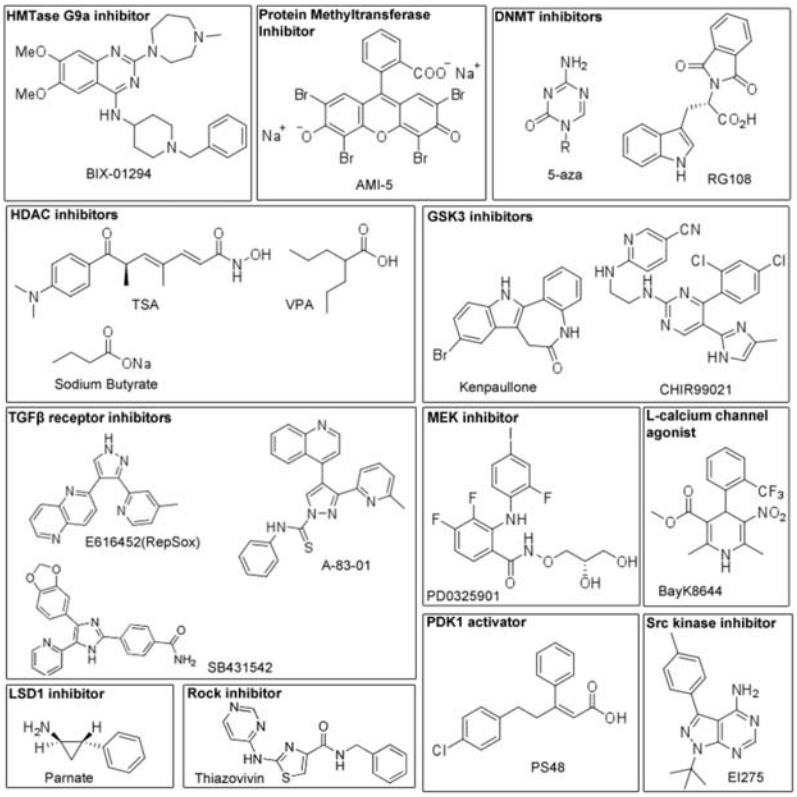

Figure 3 *Chemical structures of small molecules which facilitate direct reprogramming of somatic cells to pluripotent stem cells. Compounds have been grouped according to their functions. Besides the small molecules discussed in the text, also depicted here are 5-aza(cytidine) and TSA (trichostatin A), both of which increase the efficiency of four factor-induced reprogramming of mouse fibroblasts*[18]*. We have found that AMI-5 enables reprogramming of the same fibroblasts with* Oct4 *alone when combined with A-83-01*[60]*. EI-275 is a kinase inhibitor identified in a screen by Ichida* et al.*, and was reported to synergize with VPA and improve three-factor (*Oct4/Klf4/c-Myc*) reprogramming outcomes*[32]*.*

of naïve-state mESC-like rat iPSCs that were capable of contributing to chimerism[43].

Based on their differing single-cell and colony characteristics, conversion of hESCs to a mESC-like state most likely involves important changes in the way the cells interact with extracellular matrix (ECM) and each other. In a large-scale phenotypic screen for cell survival enhancers, we identified two compounds, Thiazovivin and Pyrintegrin, which enhance single hESC survival after extensive trypsinization by more than 30-fold[44]. We showed that this

effect is due to a significant increase in cell-cell and cell-ECM interactions, which promotes cell survival by activating the integrin, PI3K (phosphatidylinositol-3-kinase), and MAPK (mitogen-activated protein kinase) signalling pathways[45,46]. Further, strong interactions with the ECM were found to simultaneously keep inhibitory Rho-ROCK signalling at bay by fostering strong E-cadherin mediated cell-cell interaction. Accordingly, once hESCs underwent a conversion process allowing them to grow under mESC-like conditions[47], E-cadherin on the cell surface was appreciably stabilized and the ill effects of trypsinization dramatically reduced.

Subsequently, Hanna *et al*. have also succeeded in reprogramming hESCs to a mESC-like state by overexpressing Oct4 and simultaneously treating the cells with LIF and small molecules. The compounds used included inhibitors of GSK3 and ERK1/2, and forskolin, a protein kinase A (PKA) pathway agonist that can induce *Klf2* and *Klf4* expression[48].

Aside from small-molecule efforts, the reprogramming field is benefiting greatly from the development of alternative methods of transcription factor overexpression to generate iPSCs without permanent genetic modification. However, even in these cases, small molecules can play critical supporting roles in the process. Successful examples of such efforts to date include transfection of RNA, recombinant protein, various non-integrating plasmids, and transposon-mediated reprogramming. In terms of efficiency, non-integrating methods have often lagged significantly (*e.g.* $>$20-fold less efficient) compared to viral transduction, but mRNA-based reprogramming of human cells is a very encouraging outlier in this regard (1.4% *versus* 0.02% for viral methods, provided no small molecules are used). A summary of these approaches, their corresponding efficiencies, and references can be found in Tables 1 and 2.

Transdifferentiation: the new frontier

Initially, most in the stem cell field were truly surprised to find out that iPSCs could be produced by overexpressing only four genes, and that the entire process took a mere two-to-three weeks. As new ways to more efficiently and rapidly generate iPSCs were quickly developed and reported, many began to wonder if this powerful new paradigm could be applied in a related but distinct context: direct reprogramming of one somatic cell into a different developmentally mature cell, *i.e*. transdifferentiation. Attempts at trans-

differentiation were modelled after Yamanaka and others' iPSC work, in that overexpression of a group of transcription factors known to establish and sustain the transcriptional program of another differentiated cell type were overexpressed in various combinations. Ectopic expression of such lineage-specific transcription factors was not a completely novel concept; in fact, some success – *e.g.* in the form of limited intra-lineage fate switching in some cases or incomplete phenotypic conversion in others – had been achieved years earlier[49,50]. Armed with new reprogramming knowledge and much improved global profiling data to pinpoint exactly what factors may be required for various transcriptional programs to be jump-started, multiple groups have been able to make significant progress in the past few years. In two separate, carefully crafted studies, it was shown that mouse fibroblasts can be directly reprogrammed to neurons or cardiomyocytes that are for the most part properly developed and functional[51,52]. As was the case with iPSC work, it did not take much to bring about such striking change; interconversion only required transient overexpression of three master regulator transcription factors in each case. For neuronal conversion, *Ascl1*, *Brn2*, and *Myt1l* were able to generate functional neurons from mouse fibroblasts within 12 days at the efficiency of 1.8% for MEFs and 7.7% for tail-tip fibroblasts (TTFs)[51]. The critical genes for direct cardiac reprogramming include *Gata4*, *Mef2c*, and *Tbx5*. This transcription factor combination transforms mouse postnatal cardiac and/or dermal fibroblasts to αMHC (α myosin heavy chain) and cTnT (cardiac troponin T)-expressing cardiac cells in a few days (although development of contraction takes several more weeks)[52].

During our own work on reprogramming and transdifferentiation, one hypothesis that we considered was that Yamanaka factor-based reprogramming might be capable of generating cells other than iPSCs. We formulated this hypothesis over the course of many reprogramming experiments, during which we always observed a low but always noticeable and persistent level of ''background'' reprogramming, *i.e.* the formation of differentiated cells of other lineages as a by-product of the protocol. Given our current knowledge that reprogramming proceeds in a step-wise and likely stochastic manner[26], this observation is perhaps not entirely surprising. Nonetheless, the idea had apparently not been seriously entertained before, as there were no studies proposing a more general role for the Yamanaka factors in any type of reprogramming process other than the generation of iPSCs. If these factors could be used as generic mediators of transdifferentiation, it might have

critical long-term implications for cell-based clinical treatments: such a technology could potentially be a much safer, faster, and more efficient way to make autologous cells for eventual transplantation.

We focused our efforts on transdifferentiation to a cardiac fate, not least because spontaneous contraction of cardiomyocytes represents an easily detectable and quantifiable phenotype as an assay endpoint. Starting with MEFs, we overexpressed numerous combinations of the Yamanaka factors under different conditions, and in a couple cases ($\pm$ *c-Myc*) observed a weak, but clear and reproducible, activation of the early cardiac transcriptional program. After testing myriad culture conditions – including the combinatorial addition of cytokines and small molecules at varying concentrations and for varying durations – we found that the addition of the cytokine BMP4 dramatically improved activation of the late-stage cardiac program, ultimately leading to the formation of spontaneously contracting patches that express markers of mature cardiomyocytes[53]. We also found that use of a small-molecule JAK (Janus kinase) inhibitor (JI1) could further increase the generation of cardiomyocytes. Since the JAK/STAT (signal transduction and activator of transcription) pathway is critical for the establishment and maintenance of pluripotency, this finding implies that the generation of iPSCs and cardiomyocytes represent mutually exclusive outcomes of our reprogramming protocol (Figure 4). Accordingly, we conducted a series of experiments to show that a pluripotent intermediate is not generated during the process of cardiac reprogramming.

A novel and potentially useful aspect of our reprogramming protocol is that one can transiently generate cardiac precursor cells expressing characteristic markers such as *Isl1*, *Nkx2.5*, and *GATA4*. Since this population of cells is known to be capable of proliferation and expansion in culture[54], this feature could ultimately allow a much greater number of reprogrammed cells to be derived from a limited number of starting cells like fibroblasts. In our current model, we have hypothesized that the multipotent precursor cells derive from an early, epigenetically highly unstable, intermediate population of cells that can rapidly and effectively transition to multiple lineage-primed states (Figure 4). In fact, we have already established that our cardiogenic protocol can be modified by using different culture conditions and cytokines such that these unstable intermediates give rise to cells of other germ layers: a neurogenic protocol using the Yamanaka factors has recently been developed in our laboratory (Kim *et al.*, in press)

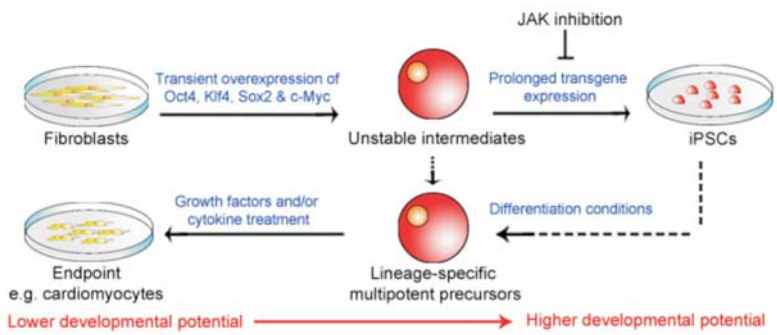

Figure 4 *Model of direct reprogramming from fibroblasts to alternative fates using the Yamanaka factors. We hypothesize that overexpression of three or four factors (* $\pm$ c-Myc*) leads to the formation of a heterogeneous intermediate cell population that is highly transient and epigenetically unstable. These intermediates can either be (a) fully reprogrammed to induced pluripotent stem cells (iPSCs) with prolonged transgene expression under mESC culture conditions or (b) directly switched to multipotent lineage progenitors (and perhaps even terminally differentiated cells like cardiomyocytes) by turning off transgene expression and providing empirically identified conditions and/or terminal differentiation cues.*

and exhibits the same characteristic of being able to generate multipotent precursors, which in this case can even be isolated and stably expanded (a feat that has not yet been accomplished in the cardiac system).

Based on these results, we are inclined to speculate that Yamanaka factor-based reprogramming may have much broader applicability than first realized. One can also imagine that further enhancements (*e.g.* by using small-molecule screens to identify potentiators) might render the method significantly more powerful. It is our hope that this process may someday lead to the development of a general platform for transdifferentiation.

Perspectives: lingering questions and the potential for clinical application

Research on iPSCs and transdifferentiation is still to a large extent a basic science endeavour; of course, clinical application in humans remains the key long-term goal. Before this goal can be realized, however, the molecular details of reprogramming will need to be better characterized. Included in this effort are, for example, attempts to assess how equivalent iPSCs are to ESCs, both genetically and epigenetically. Reports of successful mRNA and

protein-based reprogramming bode well for the generation of iPSC free of genetic modifications, but recent studies have indicated that iPSCs may retain significant epigenetic characteristics of their cells of origin and/or acquire other aberrant epigenomic traits, and that this can lead to markedly different differentiation propensities[55,56]. Another key issue impeding clinical applications is a paucity of well-defined and highly efficient directed differentiation protocols, which are critical for cell/tissue generation from pluripotent cells. Small molecules have had a major impact on research concerning all of these potential problems, and will no doubt play key role(s) in their resolution.

With regard to transdifferentiation, the concerns are virtually the same; just like iPSC technology, before it can be successfully applied in the clinic, it needs to be first accomplished without permanently modifying the genome of the starting population of cells. Given the aforementioned successes in iPSC reprogramming (using small molecules, mRNA, and proteins), it would not be surprising for this issue to be resolved in the not-so-distant future. This is especially true of our direct cardiac and neural reprogramming strategy, where a much shorter period of transgene overexpression (relative to iPSC generation) is required.

As methods for the generation of transdifferentiated cells become much safer and more efficient, the resolution of another set of issues will become all the more critical: for iPSCs and transdifferentiated cells alike, we will have to ensure equivalency and longevity. The recent studies cited above indicating that iPSCs may have critical shortcomings in terms of epigenetic status and differentiation potential demonstrate that this will not be a trivial problem to resolve. Achieving functional longevity and stability – especially for cell-based transplantation – could be equally challenging, and will most likely require significant advances in bioengineering (in the form of inductive or protective biomolecular scaffolding, for example) for eventual clinical application. As usual, small molecules will likely have critical roles to play as well.

In terms of small-molecule breakthroughs, the most significant would likely be full ''chemical reprogramming'', *i.e.* the replacement of all four Yamanaka factors simultaneously. This concept is perhaps not as far-fetched as it may initially seem, as all of these factors have already been individually replaced by small molecules. From a mechanistic standpoint, chemical reprogramming would undoubtedly represent a completely novel, and by necessity indirect, path to reprogramming. Achieving the potency and specificity of transcription factors – with their powerful and versatile

DNA-binding domains honed by millions of years of evolution – is a daunting yet equally alluring challenge.

Ultimately, the current era could end up being the most exciting for the reprogramming field or even stem cell biology in general. By revealing the surprising plasticity inherent in our cells, recent breakthroughs have once and for all changed our view of cell fate and development. And while the challenges are formidable, opportunities, potential, and promise abound – and the enthusiasm is palpable.

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