The novel tool of cell reprogramming for applications in molecular medicine

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Abstract Recent discoveries in the field of stem cell biology have enabled scientists to “reprogram” cells from one type to another. For example, it is now possible to place adult skin or blood cells in a dish and convert them into neurons, liver, or heart cells. It is also possible to literally “rejuvenate” adult cells by reprogramming them into embryonic-like stem cells, which in turn can be differentiated into every tissue and cell type of the human body. Our ability to reprogram cell types has four main implications for medicine: (1) scientists can now take skin or blood cells from patients and convert them to other cells to study disease processes. This disease modeling approach has the advantage over animal models because it is directly based on human patient cells. (2) Reprogramming could also be used as a “clinical trial in a dish” to evaluate the general efficacy and safety of newly developed drugs on human patient cells before they would be tested in animal models or people. (3) In addition, many drugs have deleterious side effects like heart arrhythmias in only a small and unpredictable subpopulation of patients. Reprogramming could facilitate precision medicine by testing the safety of already approved drugs first on reprogrammed patient cells in a personalized manner prior to administration. For example, drugs known to sometimes cause arrhythmias could be first tested on reprogrammed heart cells from individual patients. (4) Finally, reprogramming allows the generation of new tissues that could be grafted therapeutically to regenerate lost or damaged cells.

Keywords Cell fate · Reprogramming · Stem cell biology

Introduction

The fate of a cell is an integral of its morphological and functional makeup that is in turn dictated by its transcriptional, epigenetic, proteomic, and metabolic configuration. Cellular fate is changing during development as the multicellular organism develops from a single totipotent cell to yield billions of specialized cells that make up the human body. Ever since Hans Spemann showed in 1923 that the blastomeres of a 16-cell salamander embryo are all equivalent to the totipotent zygote, it remained an open question whether more differentiated cells irreversibly lose this developmental potential [1]. It was debated whether perhaps even genetic material might be lost during differentiation, which would eliminate the totipotent potential of specialized cells.

One of the first decisive experiments was the nuclear transfer of specialized cell nuclei into oocytes (Fig. 1a). These experiments first done in frogs showed that specialized cells can be reprogrammed to totipotency and can give rise to a new animal [2, 3]. Thus, even specialized cells can activate the entire program of embryonic development. In addition, adult cells can adapt and change quite dramatically upon certain environmental conditions. For example, the respiratory epithelium in the lungs of smokers can convert into squamous cells, and the esophagus epithelium can adopt the morphology of gastric epithelium in a process called metaplasia [4]. But also in hematopoietic tumors, cells have been found to transdifferentiate from one blood lineage to another [5, 6]. There is also evidence that pancreatic α or δ cells can change to β cells upon injury [7, 8]. An additional example for induced lineage plasticity was provided by cell fusion experiments (Fig. 1b) [9, 10].
As briefly stated in the introduction, John Gurdon showed that even somatic cells can be reactivated by the oocyte to form pluripotent cells [13]. These efforts culminated in the discovery that adult mammalian cells can induce insulin-producing cells from exocrine pancreas cells [13]. Overexpression of c-Myc alone in amniocytes is sufficient to induce differentiation and endocrine function in these cells [14, 15]. Overexpression of other transcription factors, such as Pax6, also leads to the induction of insulin-producing cells [14, 15]. These findings suggest that the oocyte has the ability to induce endocrine differentiation in adult mammalian cells.

The power of transcription factors: direct cell fate reprogramming

Developmental biology has focused on the identification of transcription factors that are essential to induce cell type specific genetic programs; those factors are often expressed at distinct stages during differentiation to activate the desired genetic programs and are termed “selector genes.” One such example is the Drosophila eyeless gene (Pax6 in mammals) that is required for eye development [22]. Interestingly, Pax6 overexpression can induce the formation of eye structures in various appendages of the fly [12]. Similar effects have been observed using other selector genes, including the Hox family members distalless and vestigial (reviewed in [23]).

A different class are the so-called “terminal selector genes” that regulate the identity of specific neuronal subtypes in C. elegans [24]. Terminal selector genes are transcription factors that are either alone or in combination specifically induced as the corresponding neuronal subtype is generated. Unlike classical selector genes, they stay expressed in these cells throughout the life of the animal and not only induce but also maintain subtype identity by activating key transcriptional modules necessary for the cell’s function and by repressing other terminal selector genes.

The basic helix-loop-helix (bHLH) transcription factor MyoD was the first factor identified that has the power to induce a cell lineage program in an unrelated cell type. Following a subtractive cDNA library screen, Harold Weintraub and colleagues cloned the cDNA coding for MyoD, which was sufficient to convert cultured mouse fibroblasts into beating muscle cells [11]. This work sparked the

![Diagram](image-url)

**Fig. 1** Common technologies to reprogram cell fate. a Somatic cell nuclear transfer (SCNT), in which an oocyte is enucleated to receive a nucleus from a donor cell such as a fibroblast; the cytoplasmic machinery to reprogram the donor cell to pluripotency is then transferred together with the nucleus into enucleated oocytes; similar methods were used to clone entire animals such as Dolly the sheep and generate human stem cell lines. b Analogous to SCNT, diffusible factors can reprogram the expression program of a donor cell such as a human amniocyte upon induced cell fusion with heterologous cells such as mouse myocytes to induce the expression of human muscle genes. c Alternatively, strong cell fate determination transcription factors can be overexpressed using different methods to change a cell fate. For example, the transcription factors Oct4, Sox2, Klf4, and c-Myc (OSKM) can convert a fibroblast into an induced pluripotent stem cell.
search for similar “master” lineage regulators for other cell types. By and large, however, this search was initially unsuccessful, and for many years, it was assumed that MyoD is unique.

Nevertheless, work in hematopoietic lineages continued to provide evidence for the existence of individual powerful lineage determination factors. Thomas Graf showed that the myeloid transcription factor C/EBPα is capable to directly convert B lymphocytes to macrophages in a stunningly efficient and rapid reprogramming process [25]. Another hematopoietic factor Pax5 was shown to maintain the B lymphocyte identity, and loss of function mutations led to transdifferentiation into other hematopoietic lineages [26]. Along similar lines, the eye-inducer Pax6 was also shown in a different cell context to convert neonatal astrocytes into neuronal cells [27].

All this work demonstrates that transcription factors are powerful, but their potency appeared somewhat limited, certainly as single factors. On the other hand, the successful nuclear transfer reprogramming experiments demonstrated that there must be specific reprogramming factors present in the oocyte that allow the installment of a pluripotent program. In 2006, Shinya Yamanaka and Kazutoshi Takahashi set out to screen for factors that could reprogram mouse fibroblasts into pluripotent cells. Establishing a reporter construct within the stem cell-specific Fbx15 locus allowed them to generate and isolate induced pluripotent stem (iPS) cells. Screening 24 candidate factors based on specific expression in pluripotent cells identified the now famous four reprogramming factors that can successfully convert fibroblasts to iPS cells: Oct4, Sox2, Klf4, and c-Myc [14]. A year later, the same group showed that the identical four factors also reprogram human cells (Fig. 1c) [28]. This finding was a game changer for the field and brought up the intriguing question what the limits of cell plasticity are.

Except for iPS cell reprogramming, all other successful transcription factor-based reprogramming examples reported up until that time were limited to conversions of closely related cell types. The question arose if also very distantly related cell types could be directly reprogrammed into each other. Tackling this problem, we attempted to convert mesoderm-derived fibroblasts into ectoderm-derived neural lineages. Assuming that neural reprogramming factors ought to be important lineage determination transcription factors that are also specifically expressed in neural cells, we chose over 20 candidate factors based on these criteria. From those candidates, we identified three factors, Brn2, Ascl1, and Myt1l, that in combination efficiently converted mouse and upon addition of NeuroD1 also human fibroblasts into induced neuronal (iN) cells [15, 29]. The conversion efficiency of about 20% was surprisingly high, and the resulting iN cells had all principal biochemical, morphological, and functional properties of neurons. We subsequently showed that iN cells can also be derived from cells of definitive endodermal origin by converting terminally differentiated hepatocytes [30].

This work sparked great interest in the field and triggered several labs to further develop iN cell reprogramming techniques [31]. The successful generation of iN cells also inspired scientists to apply similar strategies to other cell lineages. To date, many important cell types can be generated through direct conversion from fibroblasts including cardiomyocytes, hepatocytes, intestinal cells, and blood progenitor cells (reviewed in [32]).

**Direct induction of progenitor cells**

Often, strong lineage determination factors induce terminal differentiation. For example, MyoD induces mature skeletal muscle fibers skipping the proliferative myoblast stage, and also, the three reprogramming factors we found induce postmitotic neurons without a transient induction of neural precursor intermediate. For several applications, in particular for cell transplantation, the more plastic precursor cells would be desired because they are likely to better integrate into pre-existing host tissues than fully matured cells. Therefore, ensu- ing work has focused on the generation of precursor cell states from various different lineages.

We and others demonstrated that this can also be accomplished using transcription factor combinations unique for the desired progenitor cell population including oligodendrocyte precursor cells and tripotent neural progenitor cells [33–36]. However, an alternative approach was to transiently induce a pluripotent state using only brief exposure to iPS cell reprogramming factors such as Oct4, Sox2, Klf4, and c-Myc, without proper establishment of iPS cell lines, and rapidly followed by environmental differentiation cues. This approach has been successful to generate progenitor types of neural, hematopoietic, osteoblast, cardiac, and endothelial cells [37–42].

Two recent studies confirmed that this “indirect” reprogramming approach involves the transient induction of an authentic pluripotent state using elegant genetic lineage tracing techniques [43, 44].

**In vivo reprogramming**

Another application of lineage reprogramming, potentially of clinical interest, is the in vivo reprogramming of endogenous cells to regenerate or replace damaged tissues (reviewed in [45]). Gliial cells for example are the most abundant cells in adult brain and have progenitor-like features; therefore, they are perfectly suited to repair diseased or injured brains characterized by loss of neurons. Indeed, it has already been shown that glial cells such as astrocytes and oligodendrocyte
previously generated induced neurons could also be converted from reactive glial cells in Alzheimer’s disease mouse models, this technology might be applicable for in vivo brain repair in the future [52].

Recent data suggest that it may even be possible to switch the identity of postmitotic neurons within the brain, because ectopic expression of the cortical transcription factor Fezf2 has been shown to reprogram upper layer neocortical neurons to lower layer 5 neurons [53, 54]. This conversion, however, was only successful in early postmitotic stages suggesting that neurons become less plastic as they mature. Another cell type successfully targeted for in vivo reprogramming is insulin-producing pancreatic β cells that can be converted from pancreatic exocrine cells in the adult mouse to decrease hyperglycemia caused by insulin deficiency observed in diabetes [13]. Even mouse intestinal and liver cells could recently be reprogrammed to insulin-secreting cells, suggesting potential therapeutic value for diabetic patients by in vivo reprogramming of non-pancreatic cell types [55, 56]. In addition, it is possible to generate hepatocyte-like cells from myofibroblasts that could reduce early signs of chemical and cholestasis-induced liver fibrosis in the mouse [57]. Besides those cell types, cardiomyocytes are an attractive target for regenerative reprogramming. Indeed, induced cardiomyocyte-like cells could be efficiently generated through in vivo reprogramming from endogenous cardiac fibroblasts and enhanced cardiac function after heart injury in mice [58, 59]. However, cardiomyocytes generated by reprogramming exhibit phenotypic and electrophysiological heterogeneity causing a potential risk of arrhythmias [60]. Like for β cells, induction of cardiomyocytes is much more efficient in vivo then in vitro, highlighting the importance of the in vivo niche for the reprogramming process [58, 59, 61].

Sensory receptor cells that reside in the retina, olfactory epithelium, and inner ear are also clinically relevant cell types for potential therapeutic reprogramming. Along these lines, Ascl1 has been shown to convert retinal Müller glia to neuronal fate in injured mice [62]. Importantly, young mice responded more efficiently to Ascl1 overexpression than older mice [62], suggesting that age-associated changes restrict reprogramming as recently observed in vitro [63]. The sequence-related bHLH factor Atoh1 (Math1) was also successfully used for induction of hair cells in mouse and rat inner ear, another important sensory cell type [64, 65].

**Mechanism, mediators, and roadblocks of reprogramming**

Understanding the mechanism and identifying the key roadblocks and mediators that hinder and enable cell fate changes, respectively, will be an essential task for the field in order to ultimately orchestrate the reprogramming process in a tightly controlled manner required for disease modeling and regenerative medicine. Below, we will discuss some of the key aspects of the reprogramming mechanism, and we will consider some principal obstacles cells are facing when induced to change fate (Fig. 2).

**Pioneer transcription factors**

The DNA binding of most transcription factors is highly dependent on the chromatin configuration in a given cell. There is ample evidence for the notion that the chromatin modifies the binding affinity of ordinary transcription factors in addition to the affinity based on DNA sequence. One of the most obvious barriers for transcription factor access are the multiunit nucleosomes that have high affinity for DNA and bind about 146 bp of linear DNA sequence in about 1.7 superhelical turns. As so often in biology, there are exceptions from the rule. A small handful of transcription factors seem to behave differently and have been found to directly access nucleosomal DNA (or “closed chromatin”). This class of transcription factors has been termed “pioneer” factors. For instance, FoxA and GATA factors were shown to initiate liver and heart development in a pioneering mechanism (reviewed in [66]). It turns out that many of the reprogramming factors, identified in independent functional screens, belong in fact into this “pioneer” category [67–69]. Three of the four iPS cell reprogramming factors, namely Oct4, Sox2, and Klf4 (OSK), but not c-Myc have been shown to possess pioneer function.

![Fig. 2](image-url)
factor activity [67, 68]. More recent work has shown that
despite their pioneering potential, the binding of these three
factors is still highly context dependent illustrated by the ob-
servation that their binding varies along different
reprogramming stages and also changes when other
reprogramming factors are co-expressed in fibroblasts [70].
Thus, despite their pioneering capability, these iPS cell
reprogramming factors are still binding in a chromatin-
dependent manner.

Ascl1, one of the key reprogramming factors to generate iN
cells from fibroblasts, seems to behave in a fundamentally dif-
f erent way. Its binding pattern in fibroblasts immediately after
overexpression and its binding pattern in normal neural precu-
sor cells is in fact very similar, even though these two cell types
exhibit a completely distinct chromatin state [69]. Ascl1, there-
fore, appears to have additional qualities compared to other
pioneer factors, in the sense that it actually binds its neuronal
targets seemingly independent of the chromatin state of the
cells. We called this property “on target” pioneer factor activity.

Finally, the main reprogramming factor that converts B lym-
phocytes to macrophages, C/EBPα, has recently been reported
to also act as pioneer factor during reprogramming [71].

In summary, pioneer factor activity seems to be a common
property of reprogramming factors. Since they engage with
silent chromatin and can at least in part override chromatin
barriers, their expression must be tightly regulated during nor-
mal development, to ensure proper lineage specification.

**Donor program repression**

Much attention is being devoted to the induction of target cell
programs as cells differentiate or are reprogrammed. However, equally important is the downregulation of the
donor cell program and the silencing of undesired transcriptional
programs during new lineage acquisition [72]. In some cases,
continued expression of exogenous reprogramming factors is
required to maintain the newly acquired cell state, and down-
regulation of the factors leads to their reversion towards the
donor fibroblast identity [73]. Failure to silence the expression
programs of the initial cell population and induction of un-
wanted programs might explain immature phenotypes ob-
served upon reprogramming [74, 75]. Most reprogramming
regimes seem to repress the donor cell specific program before
induction of the target program such as in the reprogramming
of fibroblasts to iPS cells [76], pre-B cells to macrophages
[77], fibroblasts, and hepatocytes to neurons [30, 69, 72].
The addition of three mature hepatocyte-enriched transcrip-
tion factors, C/EBPα, ATF5, and PROX1, in combination
with hepatic reprogramming factors HNF1A, HNF4A, and
HNF6 resulted in the induction of human-induced hepatocytes,
suggesting that specific factors might contribute to do-
nor program silencing [78].

The understanding of how the donor cell program is si-
enced in these induced cell conversions is only in its infancy.
A recent study on iPSC cell reprogramming suggested that
the reprogramming factors themselves initially bind and decom-
mission fibroblast enhancers and gradually activate
pluripotency enhancers [70]. Investigating the iN cell
reprogramming mechanism, we found that Myt1l, one of the
three reprogramming factors, appears to be dedicated to sup-
press the fibroblast and many other non-neuronal programs
whereas activation of the neuronal program is accomplished
by the “on target pioneer” factor Ascl1 [69, 79]. Therefore,
repressing alternative cell fates along with concomitant induc-
tion of cell type specific programs enable faithful and efficient
binary decisions during cell fate reprogramming.

**Epigenetic regulators**

Cell identity is largely driven by the overall gene expression,
which in turn is regulated by the chromatin state. It therefore
seems likely that also epigenetic mechanisms constitute im-
portant barriers for reprogramming (reviewed in [80]). In fi-
broblasts, genes required to establish pluripotency were
shown to be “locked” initially within H3K9me3-enriched het-
 erochromatin domains that restrict the access of the
reprogramming factors [67]. Accordingly, reducing
H3K9me3 levels by knockdown of the histone methyltrans-ersases SUV39H1/H2 lowered this barrier to reprogramming
[67]. Inversely, a “trivalent” chromatin signature consistent of
H3K4me1, H3K27ac, and H3K9me3 was enriched at the
binding sites of the pioneer factor Ascl1 in fibroblasts. Its cell
type specific presence is predictive of the reprogramming out-
come and “erase” of H3K9me3 by overexpression of the
histone demethylase KDM4D impaired neuronal
reprogramming, further suggesting that epigenetic barriers
are essential for cell fate conversion [69].

Direct methylation of DNA is considered another robust
epigenetic mechanism stabilizing cell lineage programs. Its
global depletion by treatment with the drug 5-azacytidine re-
lieves this break, inducing the differentiation of fibroblasts
into several lineages including muscle cells, adipocytes, and
chondrocytes [81]. New approaches to specifically rewrite the
epigenome in a sequence-specific manner might allow direct-
redifferentiation of cells that are blocked by epigenetic bar-
riers [82, 83].

In addition, there are multiple examples that transcriptional
regulators work in conjunction with chromatin-modifying fac-
tors. In Pax6-mediated reprogramming of mouse glia to neu-
rons, it was shown that the chromatin remodeling complex
member Brg1 (also known as Smarca4) is required for this
process [84]. Compatible with this insight, the formation of
iNS cells from human fibroblasts with a combination of trans-
scription factors and the microRNAs miR9/9* and 124
involved the accurate induction and assembly of the neuronal-specific Brg1-associated factor (BAF) complex, also known as SWI/SNF complex [85, 86]. Some of the microRNAs used in this reprogramming protocol block expression of the chromatin complex REST, which is a specific repressor of neuronal genes and therefore needs to be silenced in neurons. Another target of miR124 is a protein called PTB, which in turn was shown to regulate iN cell reprogramming and it was proposed that just reducing PTB levels promotes iN cell formation from fibroblasts [87, 88]. Since PTB blocks miRNA-mediated activity of the REST complex, its depletion enables expression of multiple miRNA-regulated neuronal genes.

An early study showed that the combination of transcription and chromatin factors enables the reprogramming of non-cardiac fetal cells into cardiomyocytes [89]. This study further showed that BAF60c, a cardiac specific subunit of the BAF complex, enabled the binding of Gata4 to cardiac-specific genes. Moreover, depletion of the polycomb complex member Bmi1 appeared to de-repress cardiac genes and enhance reprogramming to cardiomyocytes [90].

Finally, the histone chaperone complex CAF-1 has recently been shown to limit reprogramming towards several cell types, including iPS cells and neurons [91]. Together, these studies indicate that chromatin factors and transcriptional regulators are highly dependent on each other and work together to accomplish the remodeling of the chromatin that in turn dictates lineage identity.

### Future perspectives and biomedical applications

Our experimental command on lineage reprogramming, discovered by basic researchers driven by their scientific curiosity, has transformed biomedical research over the last few years. Rather than being studied in a handful of laboratories, today every major academic institution and pharmaceutical company entertains stem cell facilities that serve their scientists to provide human cell types for research. Lineage reprogramming has become a new asset in the arsenal of research with the goal to investigate pathomechanisms and develop therapeutic approaches for various human diseases.

There are four main areas where lineage reprogramming and pluripotent stem cells are or could be applied to enhance biomedical research (Fig. 3):

1. **Disease modeling**: It is now possible to obtain skin or blood cells from patients and convert them into essentially any other desired cell type relevant for the particular underlying disease. This new kind of disease modeling has the great advantage that actual human patient cells are used rather than cell line or animal models, which might not always reflect the complexity of human-specific traits including the mechanisms of human diseases. This application is perhaps the one with the highest impact of lineage reprogramming on biomedical research. With all excitement about this new way to study diseases, it is also clear that there are currently obvious limitations. Cultured cells are not comparable to three-dimensional organs of the body and only minimally reflect the complex interaction of multiple different cell types. More sophisticated models will be needed combining tissue engineering with reprogramming and stem cell approaches. Efforts are on the way to manufacture “organs on a chip” to mimic at least some aspects of physiological organ interactions [92]. An intriguing alternative approach is three-dimensional differentiation as so called “organoids.” Pluripotent stem cells have the remarkable property to self-organize, thus imitating early embryonic structures that can be exploited to generate at least embryonic or fetal embryoid tissue structures [93].

2. **Drug discovery**: Reprogramming could be used to generate specific cell types from a large cohort of patients representing various ethnicities and genetic backgrounds. Newly developed drugs could be evaluated in such cells as a “clinical trial in a dish” before the drugs would be tested in people. This approach could be used for both general efficacy and side effect evaluation. Given the
often extensive financial burden of clinical trials, such intermediate in vitro assays would be of high interest to the pharmaceutical industry.

3. Precision medicine: Many already approved drugs have deleterious side effects like heart arrhythmias in a small and unpredictable subpopulation of patients. Reprogramming methods could facilitate precision medicine by testing drugs with known serious side effects first on reprogrammed patient cells before administration. For instance, potential arrhythmic-inducing drugs could be first tested on reprogrammed cardiomyocytes derived from individual patients. Assuming that such reprogrammed cardiomyocytes express the full panel of ion channels and pumps present in the heart, it would seem in principle straightforward to identify drugs that bind and alter the function of these channels in individual patients that may carry unknown genetic variants predisposing them to develop side effects. Given the complexity of the genome, such variants are very difficult to predict and identify. The advantage of the reprogramming approach outlined here is that no prior knowledge on risk factors is needed, since the tested cardiomyocytes will be derived from reprogrammed patient cells that carry the identical genetic background in a personalized manner.

4. Regenerative medicine: Finally, but not least, reprogramming could be used in novel regenerative medicine approaches. Principally, any cell type could be manufactured from easily accessible tissue such as skin or blood. Therefore, current cell replacement therapies such as envisioned for neurodegenerative diseases could be performed using autologous cells, which would eliminate the complication of an immune rejection of the graft. In cases where immune rejection is of major concern, an autologous source could justify the higher development and manufacturing costs that are associated with a more involved manufacturing procedure using cellular reprogramming. Importantly, reprogramming could be combined with gene editing, thereby allowing new therapeutic approaches for rare monogenetic diseases, but also the genetic engineering of autologous cells to deliver therapeutic factors to otherwise inaccessible structures. An intriguing shortcut for cell replacement could be an in vivo reprogramming approach, discussed above. Rather than reprogramming cells ex vivo followed by cell transplantation, therapeutic reprogramming could be accomplished in vivo by direct delivery of reprogramming vectors to the target organs.

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