Cellular reprogramming – lowering gravity on Waddington's epigenetic landscape

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Summary

During development, cell fate is specified precisely through programming by multiple complex elements and processes, including chromatin modifications that result in epigenetic marks. Once determined, cell fate is specified further only through maturation processes, which include differentiation and senescence. However, recent studies have shown that it is possible to influence cell fate through artificial manipulation. For example, the exogenous expression of a set of transcription factors can result in the reprogramming of differentiated skin fibroblasts to a pluripotent state. In addition, recent reports have demonstrated the directed reprogramming of one type of differentiated somatic cell to another type of differentiated somatic cell, without rejuvenation to a pluripotent state. Reprogramming factors blur the boundaries between different cell fates, which can never meet, as if the hierarchy were flattened by 'lowering gravity'. Although attempts to use direct reprogramming to generate certain cell types, such as those found in the kidneys and the lungs, have remained unsuccessful, recent advances suggest that we are nearing the identification of determinants that allow cells to be directly reprogrammed into cell types from all organs in the not too distant future. This Commentary summarises our current knowledge on cellular reprogramming, and more specifically, recent advances in direct reprogramming to generate a variety of cell types.

Key words: Cell fate, Reprogramming, Transdifferentiation

Introduction

Differentiation has long been thought of as a one-way street that is representative of a downhill progression from an undifferentiated stem or progenitor cell state to a physiologically mature cell, as imagined by Waddington in his famous epigenetic landscape (Waddington, 1957) (Fig. 1). Indeed, one can think of cells rolling down this landscape into deeper, inescapable valleys that represent fate determination during development until they reach stable states (representing their final cell fate) at the bottom (Fig. 1A). In this analogy, changes to cell fate would be prevented by ridges that do not allow the movement from one valley (or specific cell fate) to another. In ancient times, it was believed that the release of genetic information in committed somatic cells could not impact the germ line, a principle referred to as the Weismann barrier (Weismann, 1893). In addition, using nuclear transfer experiments, Briggs and King suggested that irreversible changes take place in somatic nuclei (Briggs and King, 1952). However, following recent discoveries, cell fate now appears to be far more flexible than previously thought. Many terms, such as 'transdifferentiation', 'dedifferentiation', 'transdetermination' and 'reprogramming' are used to express the conversion of cell fate (Box 1). With regards to Waddington's landscape, dedifferentiation refers to the process whereby cells travel back up their differentiation path, through the epigenetic landscape, to become more immature and finally convert into the pluripotent state (Fig. 1B). By contrast, transdifferentiation, transdetermination and reprogramming refer to a process that allows cells to directly progress from one cell fate to another without reaching pluripotency first, which would be equivalent to crossing a ridge in the landscape. However, the precise meaning of these terms has recently become less clear, as our ability to manipulate cell fate has advanced rapidly.

The concept of reprogramming cell fate was first established by John Gurdon's landmark experiments in *Xenopus laevis* in the middle of the last century, at approximately the same time as Waddington's doctrine emerged (Fig. 2) (Gurdon et al., 1958). Later, the birth of a cloned sheep, famously named Dolly, in the late 20th century showed that erasing somatic cell fate was possible, even in mammals (Wilmut et al., 1997). Drawing encouragement from these studies, it has recently been demonstrated that cellular pluripotency can be imposed on a differentiated somatic cell by using a small set of transcription factors, without the requirement for the oocyte cytoplasm (Takahashi and Yamanaka, 2006). Since then, the induction of latent pluripotency in differentiated cells has been termed 'reprogramming'. In the current era, however, the meaning of the word should be used in a broad sense because of rapid advancement of the research area (Box 1).

In addition to reprogramming to a pluripotent state, reports in recent years have shown that it is possible to directly convert somatic cells into other types of differentiated cells, and even to transcend germ layer origin, as illustrated by the conversion of fibroblasts into neurons, cardiomyocytes, cartilage and hepatocytes (Fig. 1C). These feats followed in the wake of the landmark myogenic differentiation 1 (MYOD1, also known as MYOD) experiment, which first showed the direct conversion of fibroblasts to myocytes by a defined factor. At the time, this was referred to as a transdifferentiation event (Davis et al., 1987) (Fig. 2 and see below for more detail). Hence, the conversion of cell fate that does not involve a pluripotent state

intermediate is an example of transdifferentiation. More recently, the term 'direct reprogramming' has gradually become synonymous with *de novo* transdifferentiation.

However, there are important distinctions to be made between transdifferentiation and direct reprogramming. Transdifferentiation and dedifferentiation events might include spontaneous or agnogenic conversion of cell fates. For example, in vivo transdifferentiation of hepatic cells to pancreatic cells can be induced merely by stress, such as copper deficiency or by treatment with dexamethasone (Rao et al., 1989; Shen et al., 2000; Shen et al., 2003). In contrast to spontaneous or idiopathic transdifferentiation, recent examples of direct reprogramming events were induced by defined factors, including transcription factors, chemical compounds or other artificial engineering approaches (Graf, 2011). Hence, direct reprogramming could be defined as transdifferentiation that only occurs under very restricted, artificial control. Certainly, the tremendous success with artificial conversion of cell fate by defined factors is exciting, yet also intriguing, because the mechanisms by which this direct reprogramming occurs remain a virtual black box. However, a pattern is emerging from these studies, whereby it appears that exogenous expression of small sets of lineage-specific transcription factors can often commandeer the fate of another cell.

This Commentary will review further the history and present state of the field of direct reprogramming. In addition, the limitations and potential applications of this form of transdifferentiation will also be discussed.

Reprogramming cells to a pluripotent state

The reversion of a cell to the pluripotent state has been demonstrated by transferring somatic cell nuclei to eggs, and by fusing somatic cells with pluripotent stem cells (Gurdon et al., 1958; Wilmut et al., 1997; Wakayama et al., 1998; Tada et al., 2001; Cowan et al., 2005). These two approaches suggested that fertilized eggs and pluripotent stem cells contain hidden 'reprogramming factors' that can erase the memory of somatic cells. Indeed, the combination of four transcription factors – OCT3/4 (also known as POU5F1), sex determining region Y (SRY)-box 2 (SOX2), Krüppel-like factor 4 (KLF4) and MYC – is sufficient to revert differentiated somatic cells to an embryonic fate that is similar to that of embryonic stem (ES) cells (Takahashi and Yamanaka, 2006). This finding has strongly endorsed the importance of transcription factor networks for the determination of cell fate, and has, thus, crucially influenced our understanding of direct reprogramming. In fact, the discovery triggered many of the more recent findings regarding direct reprogramming by highlighting that there are no unique reprogramming factors (Fig. 2). These reprogrammed cells have been designated as induced pluripotent stem (iPS) cells, and they can theoretically differentiate into all cell types of the body because they are similar to ES cells (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). In fact, iPS cells derived from mouse fibroblasts were found to be germ-line competent (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). In addition, unlike tissue-specific stem cells – such as, for example, hematopoietic stem cells – iPS cells can grow infinitely in vitro without specific genetic aberrations taking place. As a result of these characteristics, iPS cells are expected to contribute to future research both as source for regenerative medicine and as tools for pathological studies. However, the conversion of cell fate by defined factors was already successful 20 years before the

birth of iPS cells (Fig. 2). Furthermore, numerous studies have focused on achieving transdifferentiation or direct programming to obtain cells of various different lineages, and these will be the focus of the following sections.

Influencing cell fate through direct reprogramming

Twenty-five years ago, the dawn of direct reprogramming was imminent. Transdifferentiation had been demonstrated in *Drosophila* by experimental manipulation of imaginal discs (Hadorn, 1968). Some cell-fusion experiments had shown that mouse teratocarcinoma cells recaptured their differentiation potential, and X-chromosome reactivation of mouse thymus cells had been demonstrated (Miller et al., 1976; Takagi et al., 1983). In addition, Blau and colleagues clearly demonstrated that mouse muscle cells portray a 'dominant' behaviour in heterokaryon hybrids, meaning that they can reactivate the expression of human muscle-specific genes in human amniocytes (Blau et al., 1983; Blau et al., 1985). In this manner, cell programming seemed to be plastic, although the identity of the driving force for changing cell fate had not yet been uncovered.

The first demonstration of direct reprogramming with cloned factors was reported in 1987 (Davis et al., 1987). Davis and colleagues performed complementary DNA subtraction and identified three genes that are expressed predominantly in proliferative myoblasts. One was *MYOD1*, which encodes a basic-helix-loop-helix transcription factor that shares homology with a transactivation domain of the MYC proto-oncogene. Exogenous expression of MYOD1 alone is sufficient to convert fibroblasts into bona fide myoblasts that express myosin. Indeed, MYOD1 turned out to be the main regulator of skeletal muscle differentiation, and this seminal discovery not only revolutionized our understanding of the nature of fibroblast fate, but also had far-reaching implications for developmental biology in general. This pioneering work guided those who followed in the field of direct reprogramming. This year is the silver anniversary of the discovery of direct reprogramming by a defined factor, and the quest for factors that can convert cell fates is hotter than ever. In the following sections, I highlight some of the more recent studies that show how direct reprogramming of various cellular lineages can be achieved and the factors that are important for this.

Blood cells

The discovery of MYOD1-mediated reprogramming suggested that cell fate would be changed by regulatory genetic elements, such as transcription factors. The implication of these results was that a master cellular regulator was considered to be the most likely candidate for converting cell fate. Kondo and colleagues showed that lymphoid progenitor cells, which normally differentiate into T, B and natural killer cells can be converted into myeloid lineages, such as granulocytes and monocytes, following the ectopic expression of interleukin-2 (IL2) and granulocyte-macrophage colony-stimulating factor (GM-CSF, also known as CSF2) receptors (Kondo et al., 2000). Similarly, exogenous expression of CCAAT/enhancer-binding protein (C/EBP), a leucine-zipper-type transcription factor that is essential for the differentiation of myelomonocytes, leads to the reprogramming of mature B cells into macrophages (Xie et al., 2004). These reprogrammed cells are enlarged and contain granules, similar to genuine macrophages. In addition, they not

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Box 1. Glossary

Cell fate: the specific signature of a cell, including cell behavior and epigenetic status.

Pluripotency: the potential to differentiate into all cell types in the body, including germ cells.

Reprogramming: the artificial changing of cell fate. This does not include changes to the characteristics of a cell induced by natural phenomena, such as differentiation and senescence. Until recently, this word meant the induction of latent pluripotency in differentiated cells. However, this word has gradually taken on a broader meaning.

Direct reprogramming: artificially changing cell fate without going through the stem or progenitor state.

Transdifferentiation: changing cell fate in a number of different ways.

Dedifferentiation: rejuvenation of cell fate (i.e. conversion to a less differentiated state).

Conversion: changing cell fate in the broad sense of the term, including artificial and natural phenomena.

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only express macrophage-1 antigen (MAC1, also known as ITGAM), granulocyte receptor 1 [(Gr-1, also known as CSF3R), a marker for granulocytes and a subset of macrophages] and F4/80 [(also known as EMR1), a marker for mature macrophages and myeloid dendritic precursors], but they also show phagocytic capacity, which is a typical feature of functional macrophages. The initiation of mature B cell reprogramming into macrophages requires the inhibition of the B-cell-commitment factor paired box 5 (PAX5) and the subsequent downregulation of its target, CD19, by C/EBP. In fact, the conditional depletion of the *PAX5* gene alone is sufficient to induce the dedifferentiation of mature B-lymphocytes into an uncommitted state (Cobaleda et al., 2007), suggesting that the active regulation of cell fate also leads to resistance to reprogramming or transdifferentiation.

C/EBP has also been shown to induce the activation of macrophage-specific genes, such as the gene encoding MAC1, in synergy with the ETS-domain transcription factor, SFFV proviral integration 1 (SPI1, also known as PU.1) (Feng et al., 2008). Indeed, co-transduction of cells with C/EBP and PU.1 induces the reprogramming of committed T cells and fibroblasts to macrophages and dendritic cells, respectively (Laiosa et al., 2006). These data suggest that C/EBP contributes not only to erasing or suppressing the lineage-determining memory of B-lymphocytes, but that it also has an important role in reconstituting the transcription network of macrophages.

In contrast to the examples discussed above, a distinct route for the direct reprogramming of cells to hematopoietic lineages has recently been established. This is based on the direct conversion of fibroblasts into hematopoietic cells (Huang et al., 2011). Through this route, cells of blood cell lineages can be derived from human dermal fibroblasts. Exogenous expression of OCT3/4 in human dermal fibroblasts results in the expression of the pan-leukocyte marker, CD45, in 30% of all transfected cells (Szabo et al., 2010). The reprogramming efficiency is enhanced further by the addition of two factors that promote hematopoiesis in early embryogenesis, namely, the FMS-like tyrosine kinase 3 ligand (FLT3LG) and stem cell factor (SCF, also known as KITLG). The addition of these ligands results in the direct conversion of fibroblasts to blood progenitor cells without the need for reprogramming to early mesodermal progenitors, which

are the common ancestral cells of fibroblasts and blood cells. The reprogrammed cells are able to differentiate into granulocytic, monocytic, megakaryocytic and erythroid lineages following cytokine stimulation, and show engraftment capacity in vivo. Furthermore, treatment of fibroblast-derived CD45-positive cells with erythropoietin (EPO), an inducer of early erythroid differentiation, induces the expression of adult β-globin (Szabo et al., 2010). This indicates that these reprogrammed cells have an adult hematopoietic phenotype, and are, therefore, distinct from hematopoietic progenitors that are derived from pluripotent stem cells.

In many ways, expansion of the direct reprogramming field has been led by the study of hematopoietic cell fates. The generation of adult-type hematopoietic cells from pluripotent stem cells is arduous, because differentiation begins in the fetal state, and is, thus, difficult to recreate in vitro. By contrast, direct reprogramming is expected to allow advances in clinical applications to be made more rapidly, because it can bypass the maturation steps of embryogenesis and produce only adult-type cells. Indeed, the relative ease of conversion of closely related lineages, such as B-lymphocytes and macrophages, which can have a low reprogramming barrier, can be accomplished by a single factor, OCT3/4. Moreover, although it is surprising that exogenous OCT3/4 alone is sufficient to convert fibroblasts into hematopoietic cells, this might be a rare case where a sole reprogramming factor can result in such remarkable changes. Currently, the mechanism whereby OCT3/4 reprograms human fibroblasts into blood cells remains unclear, particularly because expression of OCT3/4 is restricted to pluripotent cells and germ cells in early embryos. It is also perplexing that ectopic expression of OCT3/4 in adult mice does not result in this reprogramming (Hochedlinger et al., 2005). One can speculate that OCT3/4, which can act as one of the reprogramming factors that induces pluripotency, might reverse components that are crucial for the commitment of fibroblasts to a certain extent, subsequently allowing cytokines to drive cells towards hematopoietic lineages.

Neural cells

Another focus of direct reprogramming studies is the generation of the neural lineage from other cell types. Neural stem or progenitor cells can differentiate into three types of neural cells, including neurons, astrocytes and oligodendrocytes. After commitment to a specific fate, they lose plasticity and are unable to switch between the different lineages. At this point, the cells are considered to have reached a 'point of no return'. However, exogenous expression of paired box 6 (PAX6) in cells that have been committed to the glial lineage has been shown to convert them into neurons (Heins et al., 2002). Recently, Wernig and colleagues screened 19 genes, including genes associated with neural cells and epigenetic modifiers, to identify factors that could reprogram fibroblasts into a neural fate (Vierbuchen et al., 2010), and succeeded in converting mouse embryonic fibroblasts into neural cells expressing Tau by transfecting them with this pool of 19 genes. A secondary screen revealed that three factors, achaete-scute complex homologue 1 (ASCL1), BRN2 (also known as POU3F2) and myelin transcription factor 1-like (MYT1L), were sufficient to induce direct reprogramming into neurons. These factors induced neuronal cells to express multiple neuronal markers, such as tubulin β3, NeuN (also known as RBFOX3) and microtubule-associated protein 2 (MAP2).
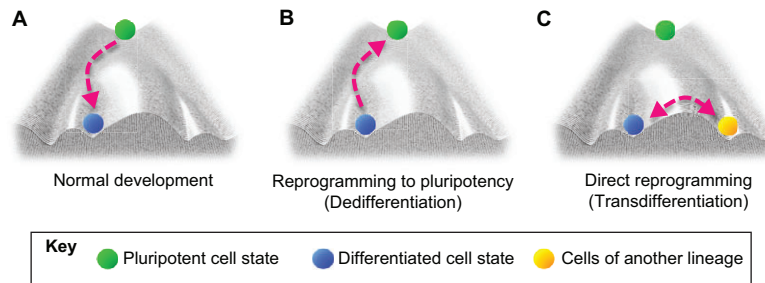
Fig. 1. Changing cell fates on Waddington's epigenetic landscape. (A) The normal development of a cell. 'Rolling down the hill', the cell takes on a specific fate by progressing from the pluripotent state (green) to a terminal differentiated state (blue). **(B)** Reprogramming to a pluripotent state or dedifferentiation. Differentiated cells (blue) return to a less differentiated state and regain pluripotency (green). From here, cells can redifferentiate into another cell type. **(C)** Transdifferentiation and/or direct reprogramming can result in cells of somatic lineage (blue) changing into cells of another lineage (yellow) without 'climbing' the developmental incline (i.e. progressing through a pluripotent state). In this type of cell fate conversion, cells cannot return to an immature state, such as a progenitor or stem cell state. Modified with permission from Waddington (Waddington, 1957).

Moreover, they are also able to generate action potentials and features that are characteristic of a functional synapse.

A subsequent study showed that this combination of reprogramming factors is insufficient for the generation of human induced neuronal cells (iNCs). However, the exogenous expression of neurogenic differentiation 1 (NEUROD1) in addition to the original three factors was found to be sufficient for reprogramming (Pang et al., 2011; Marro et al., 2011). Furthermore, human fibroblasts can be reprogrammed into functional neurons by introducing two microRNA sequences, miR-9/9* and miR-124, as well as ectopically expressing NEUROD2 (Yoo et al., 2011). The combination of ASCL1 and MYT1L with these factors further enhances the conversion of fibroblasts into a neural fate (Yoo et al., 2011). Qiang and colleagues have generated human iNCs from skin fibroblasts of patients with familial Alzheimer's disease by exogenous expression of ASCL1, Brn2 and MYT1L in combination with oligodendrocyte lineage transcription factor 2 (OLIG2) and Zic family member 1 (ZIC1), instead of NEUROD1 (Qiang et al., 2011). Therefore, generating patient-specific iNCs for use as in vitro disease models to study, for example, the processing and localization of amyloid precursor proteins and increased production of amyloid-β, is now possible. Furthermore, Caiazzo and colleagues generated functional dopaminergic neurons from mouse and human fibroblasts by using defined factors (Caiazzo et al., 2011). They identified ASCL1, the orphan receptor nuclear receptor subfamily 4, group A, member 2 (NR4A2), and LIM homeobox transcription factor 1 alpha (LMX1A) as the minimal set of reprogramming factors. Another group succeeded in generating induced dopaminergic neurons (iDAs) from mouse-tail-tip fibroblasts by transduction of genes encoding ASCL1, paired-like homeodomain 3 (PITX3), LMX1A, NR4A2, forkhead box A2 (FOXA2) and engrailed homeobox 1 (EN1, also known as HME1) (Kim, J. et al., 2011). The reprogramming process from fibroblasts into induced dopaminergic cells occurs without the cells reverting to a progenitor cell stage. In addition to generating iDAs from healthy donors, the researchers also reproduced the reprogramming with cells from patients with Parkinson's disease, thus, providing another means to study neurodegenerative diseases in vitro. Induced spinal motor neurons (iMNs) have also been efficiently generated from mouse and human fibroblasts by using a combination of seven factors including ASCL1, BRN2, MYT1L, LIM homeobox 3 (LHX3), HB9 (also known as MNX1), ISL LIM homeobox 1 (ISL1) and neurogenin 2 (NEUROG2, also known as NGN2) (Son et al., 2011). In this way, direct reprogramming into neural lineages is a front-runner for disease modeling, and studies of patient- or disease-specific cell pathology can now be developed using these systems.

As discussed for cells of hematopoietic lineages above, it has also been shown that relatively similar cell types within the neural lineage, such as glia and neurons, can be converted between each other by a single transcription factor. On the other hand, drastic changes during conversion, such as changing cell fate between different germ layers, have, thus far, required multiple factors. The conversion of fibroblasts to cells of neural fates, including iNCs, iDAs and iMNs, strongly suggests that core cell-type-specific transcription factors initiate direct reprogramming and reconstitute the target transcriptional network activity. Interestingly, the conversion to iNCs, iDAs and iMNs shares common inducers, such as ASCL1, suggesting that direct reprogramming to a neural cell fate generally occurs through common mechanisms.

Cardiomyocytes
Following the discovery of iNCs, various other direct reprogramming strategies for converting cells into other cell types have been achieved. To identify potential reprogramming factors, Ieda and colleagues searched for genes that were expressed at substantially higher levels in mouse cardiomyocytes than in cardiac fibroblasts derived from E12.5 embryos. The subsequent screen for a sufficient combination of factors demonstrated that only three factors, GATA binding protein 4 (GATA4), myocyte enhancer factor 2C (MEF2C) and T-box 5 (TBX5), are required to reprogram fibroblasts into a cardiomyocyte fate. Following ectopic expression of these reprogramming factors, the induced cardiomyocytes (iCMs) express cardiomyocyte-specific genes, such as genes encoding the myosin heavy chain 6 (MYH6), cardiac alpha actin (ACTC1), actinin alpha 2 (ACTN2) and natriuretic peptide precursor type A (NPPA) proteins, and also display sarcomere structures. In addition, iCMs can also be generated from fibroblasts that are derived from the tail-tips of adult mice by using the same factors (Ieda et al., 2010).

Analyses of indicator mice expressing ISL LIM homeobox 1 (ISL1), a marker of early cardiac progenitors, and mesoderm posterior 1 homologue (MESP1), a nascent mesoderm marker,
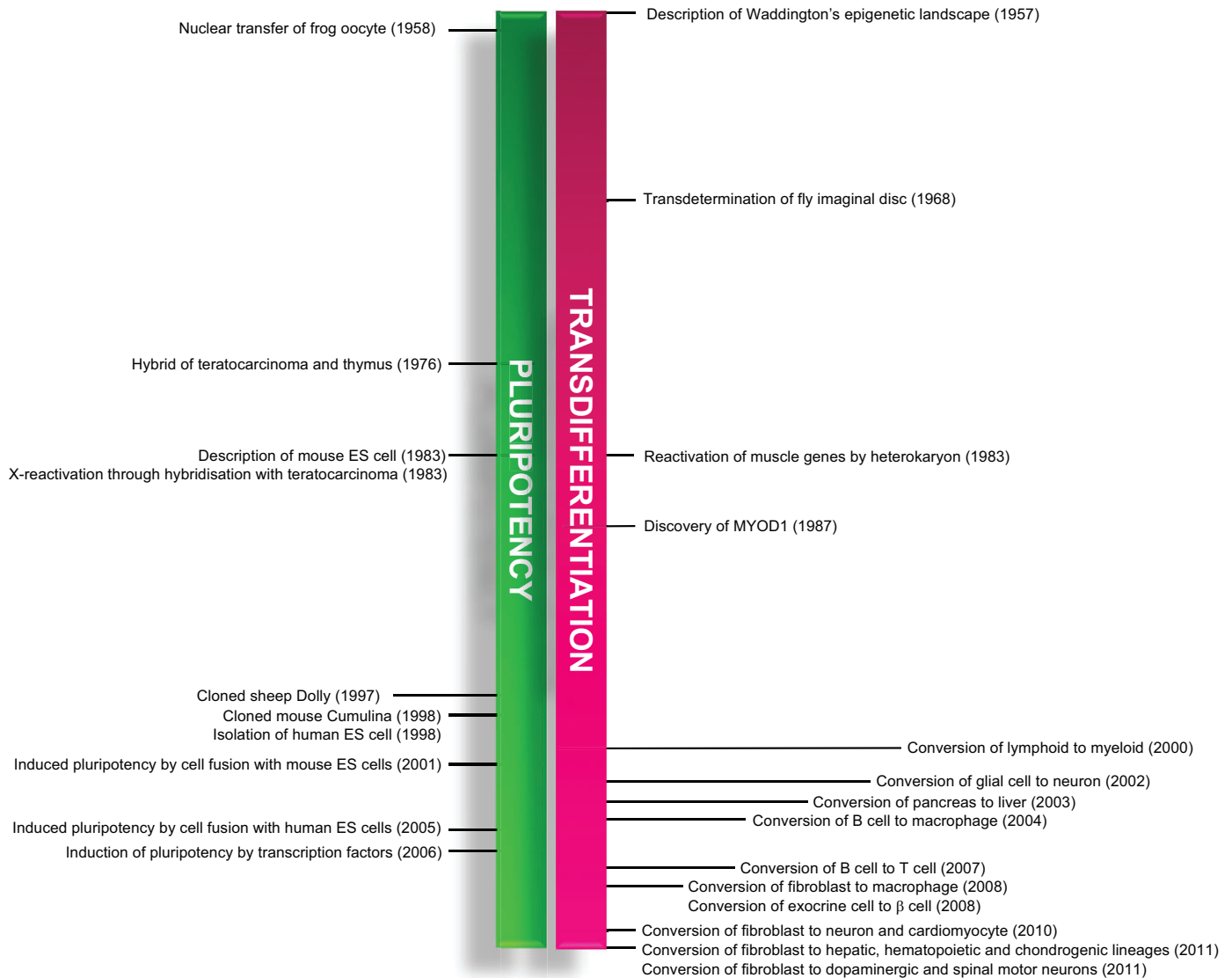
PLURIPOTENCY

TRANSDIFFERENTIATION

Nuclear transfer of frog oocyte (1958)

Description of Waddington's epigenetic landscape (1957)

Transdetermination of fly imaginal disc (1968)

Hybrid of teratocarcinoma and thymus (1976)

Description of mouse ES cell (1983)
X-reactivation through hybridisation with teratocarcinoma (1983)

Reactivation of muscle genes by heterokaryon (1983)

Discovery of MYOD1 (1987)

Cloned sheep Dolly (1997)
Cloned mouse Cumulina (1998)
Isolation of human ES cell (1998)

Conversion of lymphoid to myeloid (2000)

Induced pluripotency by cell fusion with mouse ES cells (2001)

Conversion of glial cell to neuron (2002)
Conversion of pancreas to liver (2003)
Conversion of B cell to macrophage (2004)

Induced pluripotency by cell fusion with human ES cells (2005)
Induction of pluripotency by transcription factors (2006)

Conversion of B cell to T cell (2007)
Conversion of fibroblast to macrophage (2008)
Conversion of exocrine cell to β cell (2008)
Conversion of fibroblast to neuron and cardiomyocyte (2010)
Conversion of fibroblast to hepatic, hematopoietic and chondrogenic lineages (2011)
Conversion of fibroblast to dopaminergic and spinal motor neurons (2011)

Fig. 2. Key discoveries in reprogramming history. Some of the key discoveries marking the progress in cellular reprogramming are shown in the form of a simplistic timeline. The pink ribbon shows the history of cell fate conversion, including transdifferentiation. The green ribbon shows the history of cell fate rejuvenation, such as recapturing pluripotency.

to allow lineage tracing have revealed that the route of reprogramming to iCMs is direct, and does not occur through the formation of mesoderm or the differentiation of existing cardiac progenitors. The authors also tested whether such a conversion to iCMs could be achieved in vivo. Transplantation of fibroblasts that express the three exogenous factors (GATA4, MEF2C and TBX5) into the hearts of immunodeficient mice demonstrated the successful reprogramming of fibroblasts into iCMs, and their subsequent engraftment in vivo. Therefore, the iCM technology is not only useful for disease modeling with patient fibroblasts, but it is also expected to contribute to the establishment of regenerative treatments for cardiac diseases.

Cartilage cells

Even though hyaline cartilage is a suitable source for curing cartilage injury, it has been difficult to collect and maintain in vitro. Hiramatsu and colleagues demonstrated that ectopic expression of KLF4 and MYC, together with a chondrogenic factor SOX9, effectively converts skin fibroblasts derived from adult mice into polygonal chondrocytes, which can produce hyaline cartilage (Hiramatsu et al., 2011). These colony-forming cells expressed chondrocyte-specific genes, but not fibroblast markers. In addition, the cytosine of the cytosine–guanine dinucleotide at the promoter region of the gene encoding type-I collagen, which is a fibroblast marker, is highly methylated in these cells. These chondrocyte-like cells can proliferate for more than 45 days, and have the potential to form cartilage tissue in three-dimensional cultures. In addition, when subcutaneously administering these cells to immunodeficient mice, it was shown that many clones of these cells are able to differentiate into hyaline cartilage in vivo, although some non-teratoma hyperplasia has also been observed. This conversion of

fibroblasts to chondrogenic cells does not involve a pluripotent state (Outani et al., 2011), although two out of the three factors used to achieve direct reprogramming, KLF4 and MYC, are also reprogramming factors that push cell development towards the pluripotent state (Takahashi and Yamanaka, 2006). Interestingly, a previous study showed that co-transduction of KLF4 and MYC immortalizes rat kidney cells (Foster et al., 1999), which suggests that in the reprogramming process from fibroblasts to chondrogenic cells, KLF4 and MYC support the action of SOX9 by inhibiting cellular senescence or cell death.

Hepatocytes

The generation of hepatocytes is not only useful for the purpose of regenerative medicine, but is also useful for in vitro toxicology studies, including drug side-effect studies. Recently, two independent groups have reported the direct conversion of mouse fibroblasts into hepatocyte-like cells, designated induced hepatocytes (iHeps) (Huang et al., 2011; Sekiya and Suzuki, 2011). The transduction of cells with 14 candidate factors was found to generate epithelial-like cells that express hepatocyte markers, such as albumin, tryptophan 2,3-dioxygenase (TDO2) and transthyretin (TTR, also known as TTHY). The combination of GATA4, HNF1 homeobox A (HNF1A) and forkhead box A3 (FOXA3) proteins was subsequently identified as being the most effective inducer of these epithelial-cell-like colonies. Another study investigated 12 candidate genes, which are all relevant to hepatic development, as reprogramming factors (Sekiya and Suzuki, 2011). The authors of that study succeeded in generating iHeps from mouse embryonic fibroblasts by introducing only two factors, HNF4A and FOXA1, FOXA2 or FOXA3. As observed for the combinations of factors that generate iNCs, the combinations of reprogramming factors that are effective for the conversion of fibroblasts into hepatic cell fates varies between models. Indeed, with regards to hepatocytes, the fact that hepatic tissues can be transdifferentiated to other cell types by drug stimulation (Rao et al., 1989; Shen et al., 2003) and hepatocytes are easily reprogrammed to become iPS cells (Aoi et al., 2008) suggests that the reconstitution of epigenetic networks from or to a hepatic cell fate is particularly flexible.

Pancreatic cells

The transplantation of pancreatic islets, which are mainly composed of beta cells, is a known cure for juvenile diabetes. However, effective differentiation of pluripotent stem cells into functional mature beta cells has not yet been fully realized. Zhou and colleagues first analyzed 1100 transcription factors that are predominantly expressed in the embryonic pancreas (Zhou et al., 2008). From this set, they found that the combination of just three factors, neurogenin 3 (NGN3), pancreatic and duodenal homeobox 1 (PDX1) and v-maf musculoaponeurotic fibrosarcoma oncogene homologue A (MAFA) are sufficient to reprogram acinar cells into beta cells. Although the reprogrammed cells in that study did not form islet structures, they were indistinguishable from endogenous beta cells in terms of their morphology. The in vivo transduction of reprogramming factors for the conversion of beta cells has also been shown to improve the level of blood glucose in a mouse model for diabetes. Thus, these results provide a new model for diabetes therapy that does not require any steps or treatment of cells in vitro, including reversion to the pluripotent state. Under carefully determined and monitored conditions, the successful use of

human iCMs and iHeps might also be possible. However, a lot of work still needs to be done before functional human pancreatic beta cells can be generated in vitro. Although these challenges are substantial, the potential benefits mean that such studies are of high importance, and additional progress will hopefully soon be made.

Direct reprogramming for clinical applications – advantages and disadvantages

Many types of committed cell, such as neural cells, hepatocytes and hematopoietic cells can be obtained through the differentiation of pluripotent stem cells. Because most differentiation protocols have attempted to mimic developmental processes, they often depend on extrinsic factors, such as cytokines, extracellular matrix molecules and specific growth niches (i.e. the growth environment during differentiation, such as three-dimensional culture). Although these signals eventually converge in the regulation of core transcriptional networks, such relationships are not always direct. Thus, following the differentiation processes from the pluripotent state makes it is difficult to understand the molecular mechanisms, such as epigenetic alterations, that occur during cell fate changes. This problem is mitigated by direct reprogramming strategies, which makes direct reprogramming a useful strategy for obtaining a variety of differentiated cells. However, recent studies have shown that pluripotent stem cells can differentiate into cerebral cortex, retina and pituitary gland cells, which suggests that the self-organization of tissue development is a great advantage of pluripotent stem cells (Eiraku et al., 2008; Eiraku et al., 2011; Suga et al., 2011).

Because pluripotent stem cells are theoretically able to differentiate into all cell types, they have been expected to provide an important source of tissues for regenerative medicine. Although the clinical use of pluripotent stem-cell-derived tissues is promising, there are some issues that need to be overcome. One problem is that the differentiated cells that are derived from pluripotent stem cells are often embryonic cell types. In humans, maturation to adult cell types might require the length of the typical gestational period, or even longer (Saha and Jaenisch, 2009). By contrast, hematopoietic cells derived from fibroblasts by direct reprogramming with ectopic expression of OCT3/4 immediately show features that are typical of adult cells, such as the expression of adult β-globin (Szabo et al., 2010). Therefore, direct reprogramming without the transition through the pluripotent state is expected to shorten the time required for the generation of differentiated cells for transplantation. Whether hematopoietic cells derived from fetal fibroblasts exhibit embryonic or adult phenotypes will be crucial for their use in research and clinical applications, and needs to be investigated further.

When differentiated cells derived from pluripotent stem cells are used for clinical applications, the risks of teratoma formation resulting from residual undifferentiated cells also needs to be carefully considered. By contrast, when using direct reprogramming, the formation of teratomas is unlikely, because cells are never found in the pluripotent state. Therefore, direct reprogramming can expand the possibilities for auto-grafting to patients in urgent need, such as those that have experienced spinal cord injury. However, partially reprogrammed cells, which can be tumorigenic, must be excluded. For example, partially reprogrammed iNCs derived from fibroblasts might be

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fundamentally different from neural stem cells or progenitor cells. We also know too little about the epigenetic memories in directly reprogrammed cells and the possibility of reversion, which is a highly debated issue with regards to iPS cells (Kim et al., 2010; Polo et al., 2010; Bar-Nur et al., 2011; Kim, K. et al., 2011; Ohi et al., 2011). Clearly, further detailed analyses will be required for the realization of clinical applications of these cells.

Concluding remarks

Research into direct reprogramming is currently attracting a lot of interest because of its relevance to clinical applications. The phenomenon has not only given rise to a biomedical revolution, but has also led to increased interest in cell plasticity. The goal of recapturing pluripotency is to take a cell back to the top of the Waddington's landscape, from where it originated. It is still unclear whether several different paths to the top exist for any particular fate. However, it now appears that transdifferentiation, or direct reprogramming, effectively lowers the force of gravity on this landscape, and allows cells to move from one valley to another with relative ease, thereby, bypassing the requirement for reverting to a pluripotent state before redifferentiating into another cell type. Indeed, in the experiments discussed here, the efficiencies of direct reprogramming were relatively higher than those achieved in the experimental generation of iPS cells.

In many cases, the reprogramming factors used to obtain certain lineages are also essential or have important roles in the maintenance of cell fate and development of specific tissues in vivo. In addition, although there are some exceptions, the combination of just two or three factors seems to be all that is required for the conversion of cell fate. These observations suggest that a small set of transcription factors can gradually change cell fate by inducing secondary ectopic expression of endogenous genes. The activated gene products probably act synergistically with exogenous reprogramming factors, and are subsequently, able to stimulate tertiary genes to further reconstitute transcriptional circuits. As a result, reprogramming factors can lead to a gradual change of the intracellular environment. In contrast to previous expectations, the pairing of reprogramming factors seems to be flexible, because different gene sets can all achieve reprogramming, at least to iNC, iHep and iPS lineages (Takahashi et al., 2007; Yu et al., 2007; Huang et al., 2011; Sekiya and Suzuki, 2011; Pang et al., 2011; Yoo et al., 2011). It is likely that the interchangeable factors share common direct or indirect targets and, therefore, lead to the same outcome.

During the developmental process – throughout which cells can be thought of as moving from the epigenetic mountain top to the bottom of a valley – cell lineages generally progress through intermediate states, such as lineage progenitors, and might also transition through fetal states before reaching maturation. Hence, the maturation of cells that are differentiated from pluripotent stem cells is still inefficient because it takes a long time. In fact, there is a sense of reassurance when cells are differentiated from pluripotent stem cells, because, as far as we can tell, they follow normal fate determination paths. By contrast, the 'valley hopping' that occurs during direct conversion remains a mechanistic black box with consequences that are not yet known. As with reprogramming to a pluripotent state, further detailed analyses will be required to fully understand each type of direct reprogramming.

In addition to its potential for therapeutic approaches, direct reprogramming is highly promising for pathological analyses with patient-derived cells. As mentioned above, because the production of adult-type differentiated cells from disease-specific iPS cells is difficult, the pathogenic representation of diseases with a late onset, such as Alzheimer's disease and amyotrophic lateral sclerosis, remain largely out of reach. The direct conversion of patient cells to iNCs enables a model for Alzheimer's disease to be generated in vitro. In addition, hematopoietic cells that were derived directly from skin fibroblasts exhibit adult-type signatures (Szabo et al., 2010). These technologies will provide powerful tools for the discovery of new drugs to treat such diseases, and for obtaining a better understanding of the currently undefined mechanisms underlying many diseases. However, for age-dependent cellular phenotypes, such as Alzheimer's disease, it remains to be seen whether a newly converted cell will be consistent with the age of the patient and express the pathological changes, or whether it will have its own age after becoming a neuron, and as a young neuron, would not express the pathological changes. As described, both approaches to generating cells with specific properties have advantages and disadvantages. However, these technologies represent a new method for generating cells for both basic research and clinical applications by providing new ways to traverse across Waddington's epigenetic landscape.

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