Cell Stem Cell
Perspective

Historical Origins of Transdifferentiation and Reprogramming

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Transcription factor-induced reprogramming of specialized cells into other cell types and to pluripotency has revolutionized our thinking about cell plasticity, differentiation, and stem cells. The recent advances in this area were enabled by the confluence of a number of experimental breakthroughs that took place over the past 60 years. In this article, I give a historical and personal perspective of the events that set the stage for our current understanding of cellular reprogramming.

I still remember feeling electrified when at the end of 1987, browsing through the most recent issue of *Cell*, I found an article in which Davis, Weintraub, and Lassar reported the cloning of *Myod*, a gene capable of converting fibroblasts into muscle cells (Davis et al., 1987). Although this paper received much less attention than Yamanaka's description 19 years later of reprogramming somatic cells to pluripotency (also called ''reprogramming'' or ''induced pluripotent stem cell [iPSC] reprogramming'') (Takahashi and Yamanaka, 2006), it foreshadowed the revolution that was to take place in the stem cell and differentiation fields in the years to come. For the purpose of this perspective, I will designate transcription factor-induced cell fate conversion of a somatic cell, whether it is by differentiation, retrodifferentiation, or transdifferentiation, as being under the umbrella of ''transdifferentiation.'' It seems likely that these processes occur by similar mechanisms with the possible exception of retrodifferentiation, which might be mechanistically more related to reprogramming to pluripotency. Our current view of the degree to which transcription factors can dictate and alter the differentiation phenotype of mammalian cells rests on the groundbreaking work of many investigators over the past five decades. Several conceptual and technological breakthroughs were instrumental for the discovery of both transdifferentiation and iPSC reprogramming (Figure 1). In addition to outlining these advances, I will also discuss how they contributed to the intellectual progress that led us to where we are today.

Seminal Experiments and Concepts in Reprogramming

A number of technical and conceptual advances have been crucial to the birth of the reprogramming field; some were more general, whereas others contributed more specifically to our understanding of either transdifferentiation or reprogramming to pluripotency. The concept of differentiation plasticity, for instance, first derived from somatic cell nuclear transfer experiments, was particularly influential for the discovery of iPSC reprogramming, as was the development of embryonic stem cell lines. Other experiments, showing transdetermination of *Drosophila* imaginal discs, the properties of retroviral oncogenes, reactivation of differentiation genes in heterokaryons, and transcription factors establishing regulatory circuits also had a significant impact. Here, I provide a historical perspective of some of these key experimental and conceptual breakthroughs and discuss their influence on the field.

Somatic Cell Cloning

The discovery that animals can be cloned from somatic cell nuclei sparked the idea that it might eventually be possible to identify the cytoplasmic determinants that cause reprogramming. The story began in 1918 when the developmental biologist Hans Spemann working at the University of Freiburg wondered whether the nuclei of a dividing zygote remained totipotent. In what now seems to be an almost impossibly simple experiment, Spemann caused a constriction in a 16-cell salamander embryo by tightening a baby's hair around the midline and pinched off a single nucleus into one side of the embryo. Subsequently, each of these halves generated a viable animal hatching from the same egg, showing that the nucleus remains totipotent for at least four divisions. Although in 1938 Spemann recognized that it would be a ''fantastic experiment'' to use nuclear transplantation to ask whether differentiated cells are inherently plastic, it took another 14 years for Briggs and King to successfully develop the nuclear transplantation technique, using *Rana pipiens* (Briggs and King, 1952). They found that nuclei from blastocyts could generate swimming tadpoles but that more specialized cells from the gastrula stage onward progressively lost their potential for pluripotency, forming aberrant tadpoles at best (King and Briggs, 1955). They therefore concluded that it is impossible to produce a clone from the nucleus of an adult cell and that the cell's genetic potential diminishes during development. John Gurdon later challenged those findings, using *Xenopus laevis* as a model animal, and found that after serial nuclear transfers, nuclei derived from cultured intestinal cells of tadpoles resulted in the formation of mature fertile animals (Gurdon et al., 1958; Gurdon and Uehlinger, 1966). However, subsequent experiments with nuclei from different types of adult *Xenopus* cells only yielded swimming tadpoles (Gurdon and Byrne, 2003) and it took another 30 years before animal cloning could be obtained in another vertebrate species. Ian Wilmut and colleagues at the University of Edinburgh famously created Dolly the sheep, after transplanting the nuclei of cultured epithelial cells into enucleated oocytes (Wilmut et al., 1997), and only a year later the first mice were cloned (Wakayama et al., 1998). Why did it take 30 years to make the leap from *Xenopus* to mammals? It now appears that the key to success is related to the type of recipient cell used

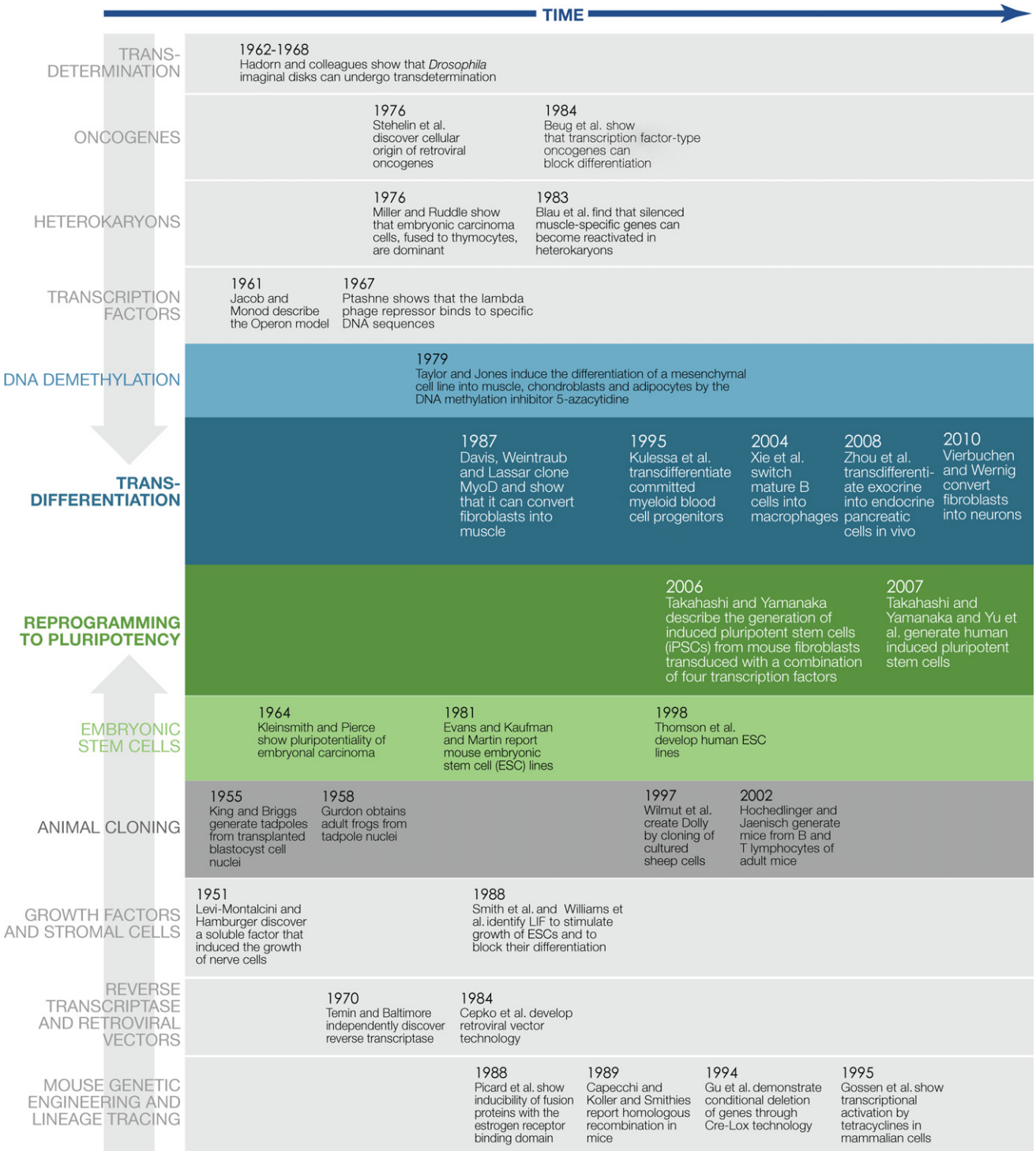

Figure 1. Research Enabling the Discoveries of Transdifferentiation and Reprogramming to Pluripotency

Key advances in the discovery of transdifferentiation and reprogramming to pluripotency are highlighted in the dark blue and green areas, respectively. The light blue and green areas summarize experiments especially relevant to either transdifferentiation or to reprogramming, respectively, and the dark gray area covers nuclear transfer. The findings listed in the light gray areas contributed concepts and technologies important for these processes, as indicated by the arrows on the left. Several of these contributions (especially the more technological ones, at the bottom) were important for both discoveries, as discussed throughout the text.

for enucleation. Earlier attempts to clone mice using fertilized zygotes failed (McGrath and Solter, 1984), probably because these cells were in interphase and the nuclear factors were retained in the nucleus. In contrast, successful experiments with sheep, mice, and other mammalian species performed more recently used unfertilized meiotic stage II nuclei, in which the

nuclear factors are in the cytoplasm because the nuclear membrane had broken down (reviewed in Egli et al., 2007).

In Gurdon and Wilmut's experiments, the frequency of success was 1% or less (Wilmut et al., 1997; reviewed in Gurdon and Byrne, 2003). Wakayama and colleagues succeeded in cloning mice only when using cumulus cell nuclei but none of the other cell types tested (Wakayama et al., 1998). A gnawing doubt therefore persisted that the differentiated cells used to generate adult animals contained a small proportion of stem cells and that cumulus cells are perhaps stem cell-like, whereas truly differentiated cells cannot be reprogrammed. These doubts were put to rest when Konrad Hochedlinger and Rudolf Jaenisch at MIT in Cambridge (MA) succeeded in generating mouse blastocysts and ESCs derived from the nuclei of adult B and T cells. Most strikingly, they obtained viable mice in which all tissues contained either immunoglobulin or T cell receptor rearrangements (Hochedlinger and Jaenisch, 2002). Another issue that had long remained unresolved is whether human somatic cells can also be reprogrammed via nuclear transfer. Until this year, somatic cell nuclear transfer into enucleated human eggs had never resulted in successful generation of blastocysts; cells typically arrested at the 4 to 8 cell stage. However, very recently Dieter Egli and collaborators at the New York Stem Cell Foundation in New York reported that when the oocyte nucleus is left in place it is possible to generate human blastocysts after transplantation of skin cell-derived nuclei. These blastocysts in turn generated stable triploid human ESC lines whose somatic cell nuclei were reprogrammed to resemble ESCs (Noggle et al., 2011). These experiments showed that it is possible, in principle, to reprogram human somatic cells up to at least the blastocyst stage.

Transdetermination

As a student in Tuebingen in the late 1960s, I was fascinated by a *Scientific American* article from Ernst Hadorn of the University of Zürich on the transdetermination of imaginal discs in *Drosophila* (Hadorn, 1968). Imaginal discs are primitive larval structures destined to become appendages in the adult, such as wings, legs, genitals, or antennae. These disks, if dissociated into single cells and serially transplanted to ectopic sites in the larvae, can change their destiny so that, for example, cells from a leg disk can now form a wing. There is also directionality; to "transdetermine" genital disks into wings they must first go through a stage fated either to a leg or an antenna (Ursprung and Hadorn, 1962). In today's interpretation, dividing imaginal disc cells are susceptible to environmental cues that can alter their fate, ultimately through the activation of cell-instructive transcription factors ("selector genes") whose ectopic expression can short-circuit the process. For example, ectopic expression of the *eyeless* gene (*Pax6*) can induce the formation of eye structures in various appendages of the fly (Halder et al., 1995), and similar effects have been observed with other selector genes, including various Hox family members, *distalless* and *vestigial* (Maves and Schubiger, 2003). Although these spectacular in vivo experiments could not address the question whether master regulators act only in progenitors or are also capable of reprogramming differentiated cells, they put an early spotlight on the question of the stability of the differentiated state.

Oncogenic Transcription Factors

Although viral oncogenes are not commonly associated with cell reprogramming, key concepts in this field foreshadowed several observations made subsequently with lineage-instructive transcription factors. The first oncogene, v-*Src*, was discovered in 1976 by Dominique Stehelin, Michael Bishop, Harold Varmus, and Peter Vogt at the University of San Francisco as a cellular gene carried by Rous sarcoma virus (Stehelin et al., 1976), work for which Bishop and Varmus were awarded the Nobel Prize 12 years later. Collaborating with Dominique Stehelin and Martine Roussel at the University of Lille (France), my postdoc Hartmut Beug and I found that the avian leukemia virus E26 and the avian myeloblastosis virus AMV contain the v-*Myb* oncogene (Roussel et al., 1979), corresponding to a transcription factor required for the formation of fetal hematopoietic stem cells (Mucenski et al., 1991). The two v-*Myb*-containing viruses selectively transform myeloblasts, precursors of granulocytes and macrophages (Beug et al., 1979). Several findings suggest that v-*Myb* reprograms the cell state of myeloid cells: specific point mutations in the v-*Myb* DNA binding domain change the gene's transformation specificity, resulting in the proliferation of granulocytes instead of myeloblasts (Introna et al., 1990). In addition, as for the combinatorial action of transcription factors during reprogramming, v-*Myb* cooperates with another oncogenic transcription factor, v-*Ets,* in enhancing the transformed phenotype (Metz and Graf, 1991). Importantly, v-*Myb* also induces the dedifferentiation of macrophages into myeloblasts (Beug et al., 1987; Ness et al., 1987). However, unlike for transcription factor-induced transdifferentiation, v-*Myb* needs to be continuously expressed to maintain the altered cell phenotype because when inactivated in myeloblasts, these differentiate into functional macrophages (Figure 2; Beug et al., 1984), showing that the oncogene specifically blocks, rather than induces, the differentiation of myeloid cells. This suggests that

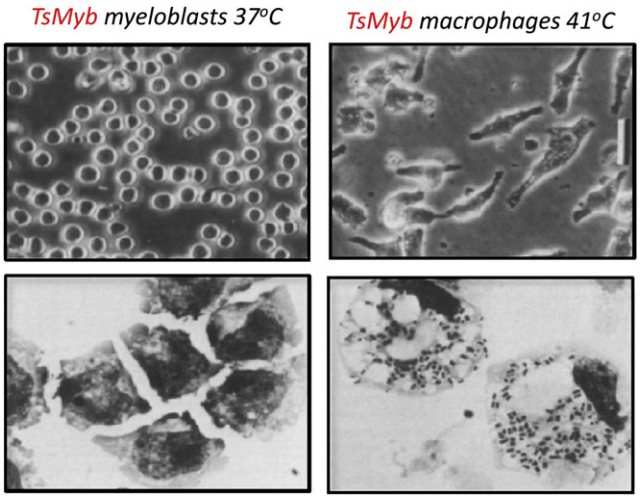

Figure 2. Effect of the v-*Myb* Oncogene on the Differentiation of Myeloid Target Cells

The figure illustrates the ability of v-*Myb* to block the differentiation of myeloid cells, using the *ts*21 temperature-sensitive mutant of E26 leukemia virus, carrying a point mutation in the DNA binding domain of v-*Myb* (Frykberg et al., 1988). Micrographs depict myeloblasts transformed at 37°C with *ts*21 virus and macrophages obtained after shifting the culture for 5 days to 42°C (the physiologic temperature of chickens). The top two panels represent phase micrographs of live cells, the lower of fixed and HE-stained cells preincubated with *E. coli* to illustrate phagocytic capacity of cells shifted to 42°C (Beug et al., 1984). The small dark particles inside the cells represent bacteria.

v-*Myb* perturbs lineage-specific regulatory networks, linking cell proliferation and differentiation. The observed v-*Myb* transgene dependence of cell transformation seems to be a common feature of oncogenes, because in many cancers, inactivation of the causative oncogene, such as cyclin D1 or *Myc*, induces tumor inhibition (Arber et al., 1997; Jain et al., 2002). However, relief from such ''oncogene addiction'' may not necessarily be due to the terminal differentiation of the transformed cells, because shrinking of tumors is typically associated with cell death.

Heterokaryons

The analysis of heterokaryons between embryonic stem cells and various types of somatic cells had a strong impact on the discovery of iPSC reprogramming. Early experiments in which R.A. Miller and Frank Ruddle from Yale University (New Haven, CO) fused embryonal carcinoma cells with thymus cells and injected them into mice revealed the formation of teratocarcinomas containing a range of differentiated tissues (Miller and Ruddle, 1976) and suggested that pluripotency is dominant. Tada and colleagues reached similar conclusions after fusing T cells with ESCs (Tada et al., 2001). The observation of ESC dominance in heterokaryons raised the possibility that ESCs, like oocytes, contain *trans*-acting factors capable of reprogramming somatic cell nuclei (for reviews see Egli et al., 2007; Piccolo et al., 2011; Yamanaka and Blau, 2010).

A separate line of research showed that genes repressed in differentiated cells can be reactivated by experimental manipulations. Henry Harris at Oxford University showed that in hybrids of HeLa cells and chicken red blood cells (which are nucleated), the red cell nuclei swelled and started synthesizing RNA (Harris and Watkins, 1965). However, these early experiments failed to report reactivation of differentiation genes, concentrating instead on cell proliferation, dominance of tumorigenesis, and exchange of nuclear membrane components. Then, almost two decades later, Helen Blau, a young faculty member at Stanford University with a background in genetic counseling, fused human amniocytes and mouse muscle cells in an attempt to develop a prenatal diagnostic test for tissue-specific diseases. To her astonishment, she found that in heterokaryons (which maintained separate nuclei), several human muscle-specific genes became reactivated within 24 hr after fusion, under conditions where there was no DNA replication (Blau et al., 1983). Subsequently she extended these findings to human keratinocytes and hepatocytes, which likewise reactivated muscle genes after fusion with mouse muscle and also found that the relative dosage of ''factors,'' resulting from the skewed nuclear ratio of the fused cells, determined the direction of differentiation, i.e., whether nuclear genes were silenced or activated (Blau et al., 1985). ''I was very excited, as these discoveries brought mammalian differentiation and gene regulation under the broad umbrella of the principles adduced in prokaryotes by the great French scientists Jacob and Monod, and by Ptashne in the U.S.'' (H. Blau, personal communication). In an influential essay, Blau went on to propose that differentiation requires continued regulation, by both positive and negative regulators (Blau and Baltimore, 1991). At the time this suggestion seemed bold, but transdifferentiation and iPSC reprogramming experiments have since fully supported the idea.

Transcription Factors and Regulatory Switches

Studies of regulatory circuits in prokaryotes, yeast, *Drosophila*, and sea urchins also left a permanent imprint on thinking within the reprogramming field. The influential work of Francois Jacob and Jacques Monod at the Institute Pasteur in Paris examining how *Escherichia coli* digests lactose eventually culminated in the Operon model. This model gave a plausible explanation for gene regulation through a circuit containing a *cis* element (the operator) and a *trans*-acting factor acting as a repressor when lactose is absent (Jacob and Monod, 1961), a concept for which Jacob and Monod won the Nobel Prize only 4 years later. Another regulatory circuit, which resembles those later identified for eukaryotes, was discovered in bacteriophage lambda. This phage can exist in either a lytic state or a lysogenic (dormant) state that is controlled by a repressor that binds with high affinity to specific DNA sequences and is responsive to signaling by external factors, thus permitting adaptation to changing environmental conditions. This simple system thus represents a bistable switch that evokes the picture of a transcription factor-driven binary decision during lineage commitment of mammalian cells (Ptashne, 1967, 2011). The similarities to the phage run even deeper: much as GATA-1 antagonizes PU.1 during myeloid to erythroid lineage reprogramming (Graf and Enver, 2009), the same molecule that activates the genetic program for lysogeny represses the genetic program of the lytic cycle and stabilizes the switch by maintaining its own expression, thus perpetuating a defined gene expression state. It is very gratifying and inspiring to understand a switching mechanism in great molecular detail, and so I always keep a copy of Mark Ptashne's book *A Genetic Switch* within reach of my desk. Although vertebrates are vastly more complex, as work by Eric Davidson on sea urchins has impressively documented (Davidson, 2010), the underlying principles remain basically the same.

Embryonic Stem Cell Lines

An early advance was the demonstration that single cells from a teratoma cell line are pluripotent, capable of generating teratomas containing cells from all three major germ layers (Kleinsmith and Pierce, 1964). The development of ESCs provided both a conceptual breakthrough and an indispensable tool for the discovery of iPSC reprogramming. The development of ESCs as the main pillar on which reprogramming to pluripotency is based has been reviewed extensively and the reader is referred to a recent historical review about the subject (Evans, 2011). Nevertheless, the two most important breakthroughs should be mentioned here. The first was the establishment of mouse ESC lines by Martin Evans and Matthew Kaufman at the University of Cambridge (UK) and by Gail Martin at the University of San Francisco (Evans and Kaufman, 1981; Martin, 1981). Martin Evans shared the Nobel Prize with Capecchi and Smithies in 2007 for this discovery. The second breakthrough was the establishment, 17 years later, of human ESCs by James Thomson, at the University of Wisconsin (Madison, WI) (Thomson et al., 1998). Shinya Yamanaka mentioned that Thomson's discovery was one of his main motivations to attempt reprogramming fibroblasts into pluripotent cells (http://www.youtube.com/watch?v=AD1sZU1yk-Y).

By transferring mouse ESCs into a blastocyst that is then implanted in vivo, they can contribute to the germline and therefore after breeding can generate animals entirely derived from the ESCs. This technique has become the gold standard for the

pluripotency, or even totipotency, of iPSCs, especially when using the technology of tetraploid morula aggregation developed by Andras Nagy and Janet Rossant from the Samuel Lunenfeld Research Institute, Mount Sinai Hospital (Toronto, ON, Canada) (Nagy et al., 1990). The fact that ESCs can also be induced to differentiate in vitro into a large number of different cell types enabled the identification of transcription factors that are essential for the maintenance of their phenotype (Nichols et al., 1998), such as Oct4, which is expressed in preimplantation mouse embryos (Scholer et al., 1990). ESCs also represent a key technological advance that provided important cellular parameters, such as growth conditions and markers, which could be used as a blueprint for the cells to be generated by reprogramming.

Reprogramming Technologies

Aside from the conceptual advances discussed so far and the development over the past five decades of basic tools for cellular and molecular biology, such as tissue-culture methods with defined media, restriction enzymes, molecular cloning, and DNA sequencing, a number of technologies, discussed below, were more specifically required for the discoveries of transdifferentiation and reprogramming to pluripotency.

Growth Factors and Stromal Cells

The first fibroblast cultures grown in defined medium needed to be supplemented with bovine serum (which we now know contain a number of growth factors such as EGF, PDGF, and FGF), and it soon became evident that many specialized cell types require additional tissue-specific growth factors. The first growth factor identified, nerve growth factor, was described by Rita Levi-Montalcini and Viktor Hamburger at Washington University (St. Louis, MO), who discovered that conditioned medium from a tumor cell line triggered the outgrowth of chick neurons in culture (Levi-Montalcini and Hamburger, 1951). Levi-Montalcini shared the Nobel Prize in 1986 with Stanley Cohen, who purified the factor. We have since learned that specialized cells require specific environments (niches) to develop in the body. These niches consist of both soluble factors and direct cell-cell interactions that can activate specific receptors, thus triggering signaling pathways. The signaling pathways in turn ultimately activate transcription factors that regulate genes involved in differentiation and growth control, among other cellular parameters (Jones and Wagers, 2008). The first example of the lineage-instructive effect of cytokine receptors was discovered when the IL-2 and GM-CSF receptors were ectopically expressed in lymphoid-committed progenitors. After exposure to GM-CSF, the cells acquired a myeloid fate, producing granulocyte and macrophage colonies, and lost their lymphoid potential (Kondo et al., 2000). It is likely that this cell fate change was produced by activation of C/EBPα in these cells, known to act as a powerful myeloid lineage-instructive transcription factor (Xie et al., 2004). Even more directly, time-lapse recordings showed that bipotent myeloid precursors treated with M-CSF differentiated preferentially into macrophages while G-CSF induced them to become granulocytes (Rieger et al., 2009), putting a long-lasting controversy to rest (Enver et al., 1998; Metcalf, 1998). Without detailed knowledge about the growth requirements of specialized cells, it would not be possible to perform transcription factor-induced transdifferentiation experiments. For example, the conversion of pre-B cells into macrophages by C/EBPα required cytokines and stromal cells appropriate for both B cell and macrophage development (see below) (Xie et al., 2004). Cytokines also had an important role in reprogramming to pluripotency, because murine iPSCs, like ESCs, require LIF. The discovery of LIF as a factor essential for ESC growth and maintenance of pluripotency was made in 1988 at the University of Oxford and at the European Molecular Laboratory in Heidelberg (Smith et al., 1988; Williams et al., 1988). Later, Austin Smith and collaborators at the University of Cambridge (UK) found that inhibitors of GSK3 and of phosphorylated ERK can replace LIF in serum-free medium, leading to the proposal that these conditions maintain ESCs in a self-renewing ground state (Ying et al., 2008).

Retroviruses, Reverse Transcriptase, and the Transduction of Cellular Genes

RNA tumor viruses, later called retroviruses, provided essential toolkits for reprogrammers. The way these viruses replicate remained a mystery for a long time. Then, in 1970, Howard Temin at the University of Wisconsin and David Baltimore at MIT (Cambridge, MA) described an enzyme contained in RNA tumor viruses that transcribes RNA into cDNA (Baltimore, 1970; Temin and Mizutani, 1970). The discovery of reverse transcriptase, for which Temin and Baltimore received the Nobel Prize in 1975 (shared with Renato Dulbecco), showed that retroviruses are unique among animal viruses in that they can integrate into the host's genome and can behave as endogenous DNA. Based on this knowledge, Rudolf Jaenisch generated the first transgenic mice by obtaining germline integration of a mouse retrovirus (Jaenisch, 1976). Of course, modern research on gene regulation, differentiation, and cell reprogramming would be unthinkable without the existence of reverse transcriptase with which to synthesize cDNA. The second important technological contribution of retroviruses is their natural ability to transduce cellular genes, thus making them ideally suited to introduce genes of interest into almost any dividing cell type and at high efficiencies. The first retroviral vectors were developed by Constance Cepko and Richard Mulligan at the Whitehead Institute of MIT (Cambridge, MA) (Cepko et al., 1984). Lentiviral vectors, which were developed more recently (Naldini et al., 1996), have the advantage that they can infect even nondividing cells. The use of retro- and lentiviral vectors for the combinatorial expression of transcription factors has greatly accelerated if not critically enabled the discovery of reprogramming to pluripotency and of transdifferentiation.

Mouse Genetic Engineering

One of the artifacts that can obscure claims of transdifferentiation is the presence in the starting population of a rare cell selected for under the experimental conditions. The advent of mouse genetic engineering made it possible to test such claims rigorously by the use of lineage-tracing experiments. The most fundamental discovery was made by the laboratories of Mario Capecchi at the University of Utah and Oliver Smithies at the University of North Carolina (Chapel Hill) in 1989, who succeeded in deleting specific genes in ESCs by homologous recombination and then used these cells to generate the first knockout mice (Capecchi, 1989; Koller and Smithies, 1989). For their work, Capecchi and Smithies shared the Nobel Prize with Evans in 2007. Then, Klaus Rajewsky's group at the University of Cologne in Germany developed the Cre-Lox technology for mice by

inserting the bacterial recombinase Cre in ESCs in loci controlled in a tissue-specific manner (Gu et al., 1994). Crossing such mice expressing Cre in distinct tissues or lineages with mice containing a reporter gene repressed by a stop cassette flanked by LoxP sites inserted into the ubiquitously expressed *Rosa26* gene (Soriano, 1999) permits lineage-tracing experiments (Zinyk et al., 1998). For example, employing a B cell-specific lineage tracing mouse made it possible to show that mature B cells in the spleen can be transdifferentiated in vivo into macrophages by C/EBPα (Xie et al., 2004). Another application of gene engineering, which was singularly important for the discovery of iPSC reprogramming, was the generation of a mouse line with a "knockin" of a reporter construct (lacZ/neoR) into the ESC-specific Fbx15 locus. This permitted the selection of reprogrammed cells that express this marker, facilitating the isolation of the first iPSC colonies (Takahashi and Yamanaka, 2006). Finally, methods to express transcription factors in an inducible manner have become versatile tools for reprogramming experiments. The first such tool was developed in 1988 by the laboratory of Keith Yamamoto at the University of San Francisco. This group showed that the estrogen receptor hormone binding domain, when fused to a transcription factor (E1A), can confer inducibility by estrogen or related drugs, through shuttling of an inactive form of the fusion protein from the cytoplasm into the nucleus (Picard et al., 1988). The second tool was developed in 1995 by Herrmann Bujard and collaborators at the University of Heidelberg (Germany) who adapted the tetracycline-dependent repressor from *E. coli* to mammalian cells. Treatment with doxycycline of cells expressing the Tet transactivator as well as genes that contain the Tet operator permits gene activation and repression in a reversible manner (Gossen et al., 1995). Both induction methods work not only in cell culture but also in genetically modified mice. For example, experiments showing that C/EBPα is only needed transiently to induce immune cell transdifferentiation have used an estrogen receptor fusion protein (Bussmann et al., 2009), whereas similar experiments with Yamanaka factors used the doxycyclin system (Stadtfeld et al., 2008). And "reprogrammable mice" have been generated by inserting Tet operator-containing Yamanaka factors constructs as well as a transgene encoding the tetracycline transactivator. With these mice, all tissues can be interrogated in vitro and in vivo by doxycycline treatment for their response to the reprogramming factors (Stadtfeld et al., 2010; Wernig et al., 2008).

Transdifferentiation: From MyoD to Inducing Neurons

The discovery that it is possible to convert one cell type into another was not made in a single experiment, such as the first description of iPSC reprogramming. Instead, it occurred in increments that started from the directed differentiation of fibroblasts into muscle cells by *Myod*, followed by the demonstration that committed and fully differentiated cells can be switched within the hematopoietic system, and then finally finding that cell types from different germ layers can be interconverted. These experiments and developments are discussed below.

The MyoD Story

The main observation that led to the discovery of *Myod* gene is a classical example of serendipidity in science. In 1973 Peter Jones, working at the Children's Hospital in Los Angeles, was performing a screen for the effect of chemotherapeutic drugs on cultured fibroblasts when "…a large mold seemed to be growing in a dish exposed to azacytidine, a new drug from Czechoslovakia. When I examined the presumed mold I was amazed to see a huge syncytium of multinucleated cells visible to the naked eye… a total switch of phenotype into muscle" (Jones, 2011). In addition to muscle, the drug, 5-azacytidine (AzaC), also induced the differentiation of the 10T1/2 fibroblasts into adipocytes and chondrocytes (Taylor and Jones, 1979), probably because this cell line is an immortalized mesenchymal stem cell. It did not take Jones long to discover that AzaC worked through the inhibition of DNA methylation (Jones and Taylor, 1980), although the relevant target gene would not be revealed for several years. Enter Harold Weintraub, a young faculty member at the Fred Hutchinson Research Center in Seattle, interested in epigenetic changes during differentiation, who had found that the chromatin of globin genes became accessible to DNase1 during the transition from avian red blood cell precursors to their differentiated derivatives via an inducible system that we had developed (Graf et al., 1978; Weintraub et al., 1982). Weintraub had trained under Howard Holtzer, a muscle development researcher at the University of Pennsylvania who postulated that differentiation requires "master switch" genes separate from those that regulate housekeeping genes; therefore, the stage was set for when Andrew Lassar joined Weintraub's lab as a postdoc in 1984 and proposed to test whether Jones' AzaC-treated 10T1/2 fibroblasts expressed a gene capable of inducing muscle differentiation. Indeed, by using cDNA transfection (Graham and van der Eb, 1973), they discovered that the drug-treated cells contained an RNA that induced muscle formation (Lassar et al., 1986). A year later they succeeded in cloning a cDNA with muscle-inducing activity and after sequencing found that it encoded the helix-loop-helix transcription factor MyoD (Davis et al., 1987).

Stephen Tapscott relates the moment of the discovery: "I was working in the tissue culture room on a day when Andrew Lassar was scanning plates of 10T1/2 fibroblasts transfected with cDNAs from his subtraction screen. He found a plate full of fused cells and became very excited. He showed them to me, called in Hal [Weintraub] and others. We then gathered by the chalkboard and Andrew and Hal soon turned the discussion to what artifact could have caused the outcome. After Hal was walking away from the group he commented, 'This is a really sick profession. We finally find what we have been looking for for so long and the first thing we need to do is try to disprove our finding, try to show how our logic or experiments are wrong' " (S. Tapscott, personal communication). Subsequent experiments showed that *Myod* can also induce the conversion of pigment, nerve, fat, and liver cell lines into cells that express muscle markers, but the muscle cells looked aberrant and were generated only at very low frequencies (Weintraub et al., 1989). Tapscott and colleagues went on to show that AzaC inhibits methylation of *Myod* in 10T1/2 cells, thereby inducing reactivation of the gene, as suggested by Jones' earlier findings (Tapscott, 2005). Ironically perhaps, the methylation of *Myod* in 10T1/2 cells appears to be an artifact of the cell line because the gene is not methylated in primary fibroblasts, where it is repressed by a different mechanism (reviewed in Tapscott, 2005). This series of coincidences illustrates how chance, luck, and a keen eye often result in important advances in science.

Transcription Factor-Induced Blood Cell Transdifferentiation

My work on mechanisms of leukemogenesis by acute avian leukemia viruses led me to study how cells differentiate. I had found that the v-*Myb*-v-*Ets*-containing E26 leukemia virus induces the proliferation of myeloblasts as well as MEP cells (megakaryocyte-erythrocyte precursors). MEPs turned out to be a simple surrogate "stem cell" system because they could be converted into myeloblasts (and eosinophils) by activating the Ras pathway. Importantly, during this process, MEPs downregulated the erythroid transcription factor GATA-1 (Graf et al., 1992). When Holger Kulessa joined my lab as a graduate student at the European Molecular Laboratory in Heidelberg in 1993, he studied the effect of ectopic expression of GATA-1 in myeloblasts. My hope was that we would find upregulation of MEP markers, reproducing Weintraub's findings in blood cells. That was indeed the case, but to our great surprise the cells also downregulated a myeloid surface antigen and converted into MEP cells, as well as eosinophils in a dose-dependent manner (Kulessa et al., 1995). Our findings immediately suggested that transcription factors not only activate novel gene expression programs but also repress the programs specific to the starting cell, a hallmark of transdifferentiation. The other insight was that inducing the commitment to differentiation does not require a set of separate "master regulators" because GATA-1 was known to activate hemoglobin expression (Orkin, 1990). Later, we found that GATA-1 represses the myeloid regulator PU.1 and that overexpression of PU.1 in MEP cells converts them into myeloblasts (Nerlov and Graf, 1998). These findings led us to propose that transcription factor cross-antagonisms, by repressing alternative cell fate options, are a driving force for binary decisions during cell fate specification (Graf, 2002). However, these experiments did not address the question of whether normal progenitors are equally plastic. As will be discussed later, Tariq Enver showed that they are (Heyworth et al., 2002).

Can mature hematopoietic cells also be induced to switch lineage? We stumbled onto the answer in 2003, when testing the erroneous hypothesis that the acute myeloid leukemia-associated fusion oncoprotein AML-ETO can reprogram lymphoid cells into myeloid cells. Infecting a pre-B cell line with the fusion oncoprotein showed no effect. However, unexpectedly, among the transcription factors that we used as controls, the granulocyte/macrophage-restricted transcription factor C/EBPα induced a dramatic, dosage-dependent switch into myeloid cells, while the erythroid lineage-associated factor FOG-1 had no effect. C/EBPα converted nearly 100% of primary pro- and pre-B cells and around 35% of mature, antibody-producing B cells into macrophages. Importantly, the reprogrammed macrophages, which retain immunoglobulin rearrangements, are functional by a number of criteria, including Fcγ receptor-dependent and -independent phagocytosis (Di Tullio et al., 2011; Xie et al., 2004). As work by Dan Tenen showed, ablation of C/EBPα in hematopoietic cells results in the loss of granulocytes/macrophages and the proliferation of immature cells (Zhang et al., 2004). Furthermore, C/EBPα is mutated in about 10% of acute myeloid leukemia (Wouters et al., 2009), suggesting that it can act as a tumor suppressor.

A Forgotten Tissue-Culture Dish and Retrodifferentiation of B Cells

The experiments described so far suggest that tissue-restricted transcription factors can dictate cell fate, but what happens to committed cells in which a master regulator is *inactivated*? The answer came from yet another chance discovery. Stephen Nutt, an Australian postdoc in Meinrad Busslinger's laboratory at the Institute for Molecular Pathology in Vienna, was asked to analyze a mouse knockout model lacking the B cell-specific regulator Pax5. He found that B lineage cells were blocked at the pro-B cell stage and could be propagated in the presence of the early lymphoid cytokine IL-7. One day, returning from vacation, he cleaned out the incubator and found a dish of forgotten Pax5-defective pro-B cells. Based on an instinct that proved decisive, he put them under the microscope and discovered that they had turned into macrophages! As he and Busslinger later described, *Pax5*$^{-/-}$ pro-B cells selectively express genes of alternative lineages and can differentiate into functional macrophages, osteoclasts, dendritic cells, granulocytes, and natural killer cells when cultured with the appropriate cytokines (Nutt et al., 1999) and into T cells when transplanted into immunodeficient mice (Rolink et al., 1999). The forgotten dish of Pax5-defective pro-B cells had been cocultured with M-CSF-producing stromal cells; once the culture ran out of IL-7, the M-CSF induced macrophage differentiation (M. Busslinger, personal communication). These observations could still have been due to a developmental blockage of *Pax5*$^{-/-}$ B cell progenitors at a very early stage, generating an abnormal type of multipotent progenitor. To address that possibility, Busslinger's group also looked at what happens when Pax5 is deleted in fully differentiated B cells and found that they reacquire T cell potential, as revealed by transplantation into immunodeficient mice (Cobaleda et al., 2007). Here it needs to be stressed that the effect of Pax5 ablation appears to differ from the transdifferentiation induced by ectopic expression of lineage-restricted transcription factor(s) or ablation of *Bcl11b* (see below), which occur at much higher frequencies and do not involve overt retrodifferentiation (see for example Di Tullio et al., 2011).

Cell Conversions with Therapeutic Potential

Although of considerable academic interest, the blood cell switching examples discussed so far have no obvious therapeutic applications. A breakthrough in this area was made in 2008 by Douglas Melton and colleagues at Harvard University (Cambridge, MA) who showed that insulin-producing cells in the pancreas could be obtained by in vivo transdifferentiation (Zhou et al., 2008). These authors screened 1,100 transcription factors by in situ hybridization in the pancreas and identified 20 that are expressed in mature β cells and their precursors, of which 9 led to β cell phenotypes when mutated. By using adenoviral vectors to introduce a mixture of these 9 factors into the pancreas of immunodeficient mice, they noted an increase in the number of β cells, which they could attribute to the effects of *Ngn3*, *Pdx1*, and *Mafa*. Lineage-tracing experiments showed that these three factors converted >20% of endocrine cells into cells closely resembling β cells, but they had no effect when expressed singly or as a combination in fibroblasts. Remarkably, by using a mouse model for diabetes type one, Zhou et al. (2008) showed that the in vivo transdifferentiation alleviated the hyperglycemia caused by insulin deficiency.

Brown fat cells are specialized to dissipate chemical energy in the form of heat, as a physiological defense against cold and obesity. Bruce Spiegelman and colleagues at the Dana Farber Center of Harvard University have shown that these cells derive

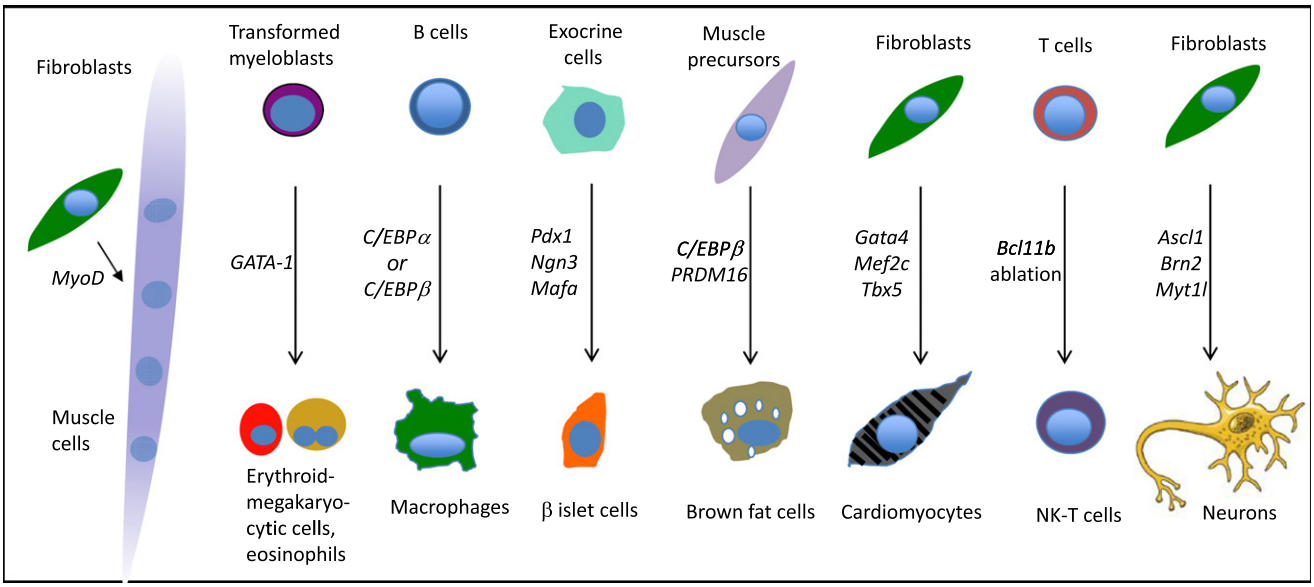

Figure 3. Examples of Transcription Factor-Induced Transdifferentiation
The examples shown are discussed throughout the text. Models (left to right) based on work from Davis et al. (1987), Kulessa et al. (1995), Xie et al. (2004), Zhou et al. (2008), Kajimura et al. (2009), Ieda et al. (2010), Li et al. (2010a) and (2010b), and Vierbuchen et al. (2010).

from a muscle progenitor by the action of PRDM16 (Seale et al., 2008). More recently, Kajimura et al. (2009) identified C/EBPβ as a partner of PRDM16 and showed that these two transcription factors together can induce a highly efficient switch from skin-derived mouse and human fibroblasts into brown fat cells. As a third example of a transdifferentiation with therapeutic potential, Deepak Srivastava and colleagues at the University of California, San Francisco, succeeded in converting mouse fibroblasts into beating cardiomyocytes with a combination of *Gata4*, *Mef2c*, and *Tbx5*. Importantly, transplantation of freshly transduced fibroblasts into the heart of mice showed functional cardiomyocytes derived from the infected cells 2 weeks after transplantation (Ieda et al., 2010).

Finally, two studies have shown that ablation of the transcription factor Bcl11b results in mice that have no T cells but instead an increased number of NK-T cells (Li et al., 2010a, 2010b). When *Bcl11b* was deleted in committed T cell progenitors in culture, they switched into cells with natural killer (NK) cell properties at nearly 100% efficiency. Remarkably, the induced NK cells killed tumor cells in vitro and prevented tumor metastasis in vivo (Li et al., 2010b). These and other direct lineage conversions selectively covered in this review are illustrated in Figure 3. Although the phenotype of the transdifferentiated cells might not be identical to that of their normal counterparts, the fact that they function in vivo raises hopes that specific transcription factor perturbations can eventually be harnessed to generate therapeutically useful cells "a la carte."

Crossing Germ Layers: Converting Fibroblasts into Neurons

One of the fundamental questions in the reprogramming field that remained difficult to answer for a long time was whether transcription factors can induce direct conversions between developmentally distant cells, such as between cells belonging to different germ layers. In an early attempt to address this issue, we ectopically expressed C/EBPα and its cofactor PU.1 in fibroblasts and found that the cells acquired a macrophage-like morphology, gene expression pattern, and phagocytic capacity (Feng et al., 2008), although they were not stably reprogrammed because inactivation of the exogenous transcription factors led to their reversion into fibroblasts. However, Marius Wernig and colleagues at Stanford University (Palo Alto, CA) described definitive experiments showing that a cocktail of transcription factors can convert mouse and human fibroblasts into functional neurons (Pang et al., 2011; Vierbuchen et al., 2010) and, more recently, hepatocytes into neurons (Marro et al., 2011). In all of these cases, the cells retained their newly acquired phenotype even when the exogenous factors were inactivated and thus appeared to be stably reprogrammed. These results show that it is possible to drive changes in cell states across germ layer barriers (mesoderm-ectoderm and endoderm-ectoderm). Of note, the approach used for the fibroblast-to-neuron conversion experiments, as well as others that require several transcription factors for transdifferentiation, was inspired by that used for iPSC reprogramming (Takahashi and Yamanaka, 2006; see also review by Yang et al., 2011, this issue).

Reprogramming to Pluripotency

In my opinion, Shinya Yamanaka's discovery that somatic cells can be reprogrammed into pluripotent cells represents the single most important finding in the stem cell/differentiation fields in the 50 years since John Gurdon demonstrated that somatic cell nuclei have the potential to generate all germ layers in an adult animal. Almost overnight, Yamanaka showed that the arguably most important change induced after somatic cell nuclear

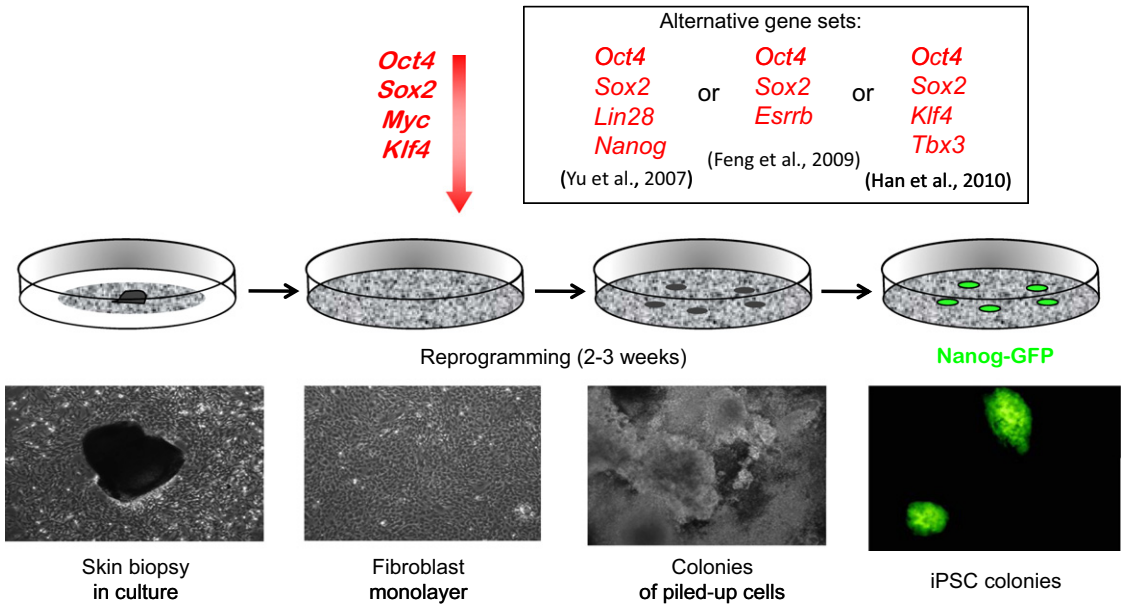

Figure 4. Reprogramming to Pluripotency

The figure outlines the methodology developed by Takahashi and Yamanaka for mice and humans (Takahashi et al., 2007; Takahashi and Yamanaka, 2006) as modified by Maherali et al. (2008). The micrographs (courtesy of Matthias Stadtfeld and Konrad Hochedlinger) illustrate the changes in morphology of skin-derived fibroblasts infected with retroviruses carrying *Oct4*, *Sox2*, *Klf4*, and *Myc*, with a *Nanog*-driven GFP reporter for iPSC formation. The process is very inefficient, typically occurring in less than 1% of the cells. A selection of alternative combinations of transcription factors capable of generating human and mouse iPSCs are indicated in the box (Feng et al., 2009; Han et al., 2010; Yu et al., 2007, 2009).

transfer, the switch to pluripotency, can be reproduced in culture with just a few molecules (Figure 4). This finding opened up a vast number of new research avenues that are being pursued in hundreds of laboratories around the globe and raised great hopes for regenerative medicine, although accumulating evidence suggests that iPSCs are quite heterogeneous and not necessarily identical to embryonic stem cells (ESCs) (see for example Kim et al., 2010). I will not further discuss the discovery of iPSC reprogramming, because this has been covered extensively in several excellent reviews (Hochedlinger and Plath, 2009; Orkin and Hochedlinger, 2011; Silva and Smith, 2008; Stadtfeld and Hochedlinger, 2010; Sterneckert et al., 2011; Yamanaka, 2009a, 2009b; Yamanaka and Blau, 2010). In addition, the reader is referred to an entertaining and informative talk given by Yamanaka at the U.S. National Institutes of Health in 2010, in which he describes his path to iPSC reprogramming (http://www.youtube.com/watch?v=AD1sZU1yk-Y).

The Ups and Downs of "Transdifferentiation"

Acceptance of the concept that the lineage of cells can be switched was slow to come. I still remember my deep embarrassment when, after a presentation of our preliminary findings about GATA-1-induced lineage conversion at a 1993 meeting in Austin, Texas, a prominent developmental biologist briskly walked out, murmuring "… all artifacts of cell lines and overexpression!" Then, 8 years later, Tariq Enver visited me at the Albert Einstein College of Medicine in New York and gave a talk about the effects of GATA-1 in programming multipotent cells to the erythroid lineage. He had observed a large increase in erythroid colonies and interpreted these results as evidence for an instructive effect of GATA-1 on cell fate choice. When I saw his data I said, "Tariq, I bet what you see is the reprogramming of committed myeloid precursors." Tariq gave me a long incredulous look and responded, "These GATA-1 effects could simply point to a selective mechanism whereby GATA-1 allowed the survival and proliferation of covertly committed erythroid cells without altering their initial commitment decisions." We agreed that this problem could not be resolved in multipotent cells that retained erythroid potential. So he went home to test the idea directly by expressing GATA-1 in committed myeloid cells. A few months later, I got an e-mail that started: "You were bloody right." By using clone-marking and daughter cell experiments, his lab showed that myeloid precursors infected with an inducible form of GATA-1 generated erythroid colonies when GATA-1 was induced and granulocyte/macrophage colonies in the controls (Heyworth et al., 2002). After his "conversion," Tariq concluded, "Our results demonstrate that the cell type-specific programming of apparently committed primary progenitors is not irrevocably fixed, but may be radically respecified in response to a single transcriptional regulator" (Heyworth et al., 2002). However, such gain-of-function experiments still do not formally define the role of GATA-1 in specifying the commitment of multipotent progenitors in vivo.

In the meantime, at the turn of the century the field experienced a burst of papers claiming that during normal development blood cells can turn into neurons, cardiomyocytes, and liver cells; muscle and neural cells into blood cells, etc. Unfortunately, attempts to reproduce these claims failed. To rigorously examine whether cells can physiologically transdifferentiate, Amy Wagers, Irving Weissman, and colleagues at Stanford University used GFP-labeled hematopoietic stem cells in mice whose hematopoietic system had been destroyed by irradiation.

They concluded that transdifferentiation of circulating hematopoietic stem cells and/or their progeny into distantly related cells is an extremely rare event, and whenever observed it is mostly attributable to cell fusions (reviewed in Wagers and Weissman, 2004). By using lineage-tracing experiments, Matthias Stadtfeld in my group reached similar conclusions (Stadtfeld and Graf, 2005). The claims of physiological conversions gave the term "transdifferentiation" somewhat shady connotations for the years to follow and reinvigorated some of the old skeptics. When in 2004 Rudolf Jaenisch visited me in New York, I asked him, euphoric by our recent finding that B cells can be transdifferentiated into macrophages, what he thought about the idea of testing whether transcription factor combinations could reprogram fibroblasts into ESCs. Rudolf replied emphatically, "you are totally out of your mind, that is never going to work." Rudolf's main argument was that the two cell types are developmentally so far apart that chromatin modifications would present an insurmountable barrier. Reflecting this attitude, after Yamanaka first presented his results at a Keystone meeting in 2005, skepticism prevailed among the colleagues I talked to. And during that same year we submitted an article to a high-profile immunology journal showing that C/EBPα and PU.1 can reprogram committed T cell progenitors into macrophages and dendritic cells. The paper came back unreviewed: "It is the editorial policy of the journal not to publish articles that are based on overexpression experiments." That turned out to be bad timing, because only one year later, after Yamanaka's discovery of iPSCs, the gates were opened and the journal started publishing lineage conversion experiments. So, ironically perhaps, the tremendous impact of the iPSC reprogramming discovery helped restore the reputation of transcription factor-induced transdifferentiation (or "lineage conversion," as it was less controversially called [Zhou and Melton, 2008]), leading to a renewed and sustained explosion of papers reporting transcription factor-induced conversion of one specialized cell type into another. The number of papers listed in PubMed under the terms "transdifferentiation" and "iPS cell reprogramming" reflect these developments. The transient increase of transdifferentiation papers between 1999 and 2004 represent the "physiological transdifferentiation" bubble, while the steep increase after 2006 is at least in part attributable to the discovery of iPSC reprogramming (Figure 5). As a footnote it should be added that there are credible examples of both physiological and pathological transdifferentiation, such as the endothelial-blood cell transition in embryonic development (Eilken et al., 2009; Zovein et al., 2008) or the epithelial-mesenchymal transition during tumor formation (discussed in Slack, 2007; Yang and Weinberg, 2008).

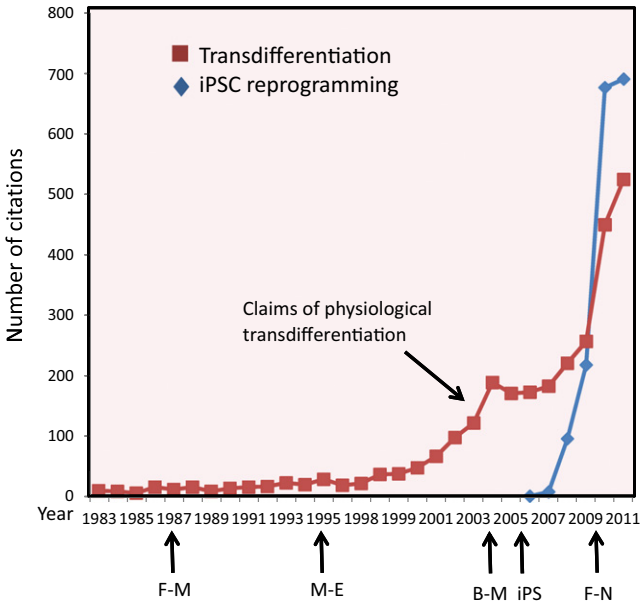

Figure 5. Time Course of PubMed-Listed Papers Containing the Terms "Transdifferentiation" and "iPSC Reprogramming"
The transient transdifferentiation peak may be attributed to papers claiming physiological transdifferentiation. F-M, conversion of fibroblasts into muscle cells; M-E, switch of transformed erythroid into myeloid precursors; B-M, conversion of B cells into macrophages; iPS, reprogramming of fibroblasts into iPSCs; F-N, conversion of fibroblasts into neurons.

The Future of Reprogramming

At this point, new lineage conversions are reported almost every month, for example the switch of fibroblasts into hepatocytes (Huang et al., 2011; Sekiya and Suzuki, 2011). However, the hurdles that remain for use in cell therapy are still formidable, whether the cells are obtained by direct lineage conversion or iPSC reprogramming, making it hard to predict whether and when these approaches will enter the clinic and which will ultimately prevail (Cohen and Melton, 2011). Today's explosion in cell reprogramming research recapitulates that of cancer research between the late 1970s and the 1990s propelled by the discovery of retroviral oncogenes. Although oncogene research did not lead to the rapid development of new cancer therapies, as had been widely expected, the wealth of knowledge for the understanding of basic cellular processes has been enormous and some of it has been translated into the clinic. Similarly, cell reprogramming experiments have already dramatically increased our understanding of cell differentiation and enabled the creation of tissue culture models for human cell degenerative diseases that could not be studied previously.

The discovery of transdifferentiation and iPSC reprogramming is only a decade or so old and many basic questions remain to be resolved. How do the mechanisms of transcription factor-induced transdifferentiation and iPSC reprogramming differ? How does the regulatory network of one cell collapse while a new one is generated? How important is the role of chromatin modifications in these processes? Why does only a minority of the cells respond during iPSC reprogramming and what happens to the others? Future research in cellular reprogramming will undoubtedly generate new discoveries beyond our current imagination, with likely potential for benefiting human health.

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