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Efficiencies and mechanisms of nuclear reprogramming

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Abstract

The differentiated state of somatic cells is highly stable but it can be experimentally reversed and the resulting cells can then be redirected into many different pathways. Nuclear reprogramming has been achieved by nuclear transfer to eggs, cell fusion, and by overexpression of transcription factors. The mechanisms of nuclear reprogramming are not understood, but some insight into them is provided by comparing the efficiencies of different reprogramming strategies. Here we compare these efficiencies by describing the frequency and rapidity with which reprogramming is induced and by the proportion of cells and level of expression in which reprogramming is achieved. We comment on the mechanisms that lead to successful somatic cell reprogramming, and those that resist to help to maintain the differentiated state of somatic cells.

Keywords

nuclear reprogramming; reprogramming efficiency; cell differentiation; egg; oocytes; nuclear transfer; cell fusion; iPS; epigenetic reprogramming; epigenetic memory

Introduction

We define nuclear reprogramming as a switch in gene expression from one cell type to that of an unrelated type. Although the differentiated state of somatic cells is extremely stable, certain experimental procedures can nevertheless reprogram somatic cells or their nuclei to acquire wide developmental plasticity. These procedures include nuclear transfer (to eggs or oocytes), cell fusion, and the overexpression of transcription factors. There are, however, enormous differences between procedures and cell types in the efficiency with which reprogramming takes place. With very low efficiency, there is the concern that an unidentified and minority cell type may be the only cell which is reprogrammed. In some procedures, multiple rounds of cell division take place before reprogramming is evident, and this may allow selection, making it difficult to determine the efficiency with which a cell is reprogrammed. Here, we review the efficiency of nuclear reprogramming in different systems and highlight striking differences in the reprogramming efficiency and speed of reprogramming according to the criteria used to assess reprogramming. There also appears to be great variation in the percentage of the starting population of cells that is reprogrammed, as well as in the level of expression of reprogrammed genes.

We consider that there are three reasons for interest in the efficiency of nuclear reprogramming. One is to help to identify the mechanisms by which reprogramming can be

experimentally induced. Another reason is to understand the basis of the increasing resistance that cells show towards reprogramming as they become progressively more differentiated. The third interest is in reprogramming patient-specific somatic cells to provide genetically matched cells for cell replacement therapies.

The stability of the differentiated state

The fertilized egg is the most completely plastic cell in the sense that its mitotic products can form every cell in the body. This high degree of plasticity is also shared by certain embryonic cells. These include cells of the inner cell mass of the mouse embryo and the derived embryonic stem cells (ES cells); when implanted into a blastocyst, a contribution to all tissues and cell types of the body can occur. Likewise, the animal cap cells of the early amphibian embryo, when supplied with appropriate signals from other underlying cells or when cultured in activin- or retinoic acid-containing media, can form almost all cell types in the body (Asashima and Grunz 1983). However, soon after these early embryo stages, cells start to become restricted in their developmental capacity.

It is useful to distinguish commitment from determination (Slack 1983). Commitment is used to describe cells that will adopt their normal fate when explanted, that is cultured in a neutral medium. This means that these cells are sufficiently committed to their normal fate that they will follow that fate even in the absence of signals with which they would normally be provided when in a whole embryo. A more advanced stage of differentiation is described as determination. The test for this is to transplant cells into different parts of an embryo where they receive signals entirely different from those to which they would be exposed in normal development. A determined cell is unable to diverge from its normal fate, even in the presence of these unusual signals. The term transdetermination applies to those cells which can switch their differentiation pathway under certain conditions, even when they have reached, by normal criteria, a determined state. A number of examples of transdetermination have been reviewed by Okada (1991). The most striking example is that of lens regeneration in newts. In this case, pigmented cells of the iris epithelium can be observed to gradually change, in the course of many cell divisions and over many weeks, to produce lens or other cell types of the eye. Another well-known example is that of *Drosophila* imaginal disc cells. These cells retain their normal fate over many hundreds of cell divisions when serially transplanted to the adult abdomen; their fate is revealed by transplanting the cells to a larval abdomen and passing them through metamorphosis with exposure to ecdysone. Occasionally cells will switch from one fate to another, forming, for example, antenna as opposed to leg. The frequency of this example of transdetermination is very low, and has been estimated at a rate of 10^{-3} to 10^{-4} for most examples of a switch in cell fate (Hadorn 1963; Shearn et al. 1978).

The major conclusion from this section is that cells that have arrived at the determined state only switch to another fate with an extremely low frequency, in the course of numerous cell divisions and under special conditions. The determined state is therefore said to be extraordinarily stable, a very desirable situation so that we do not have inappropriate cells in most of our organs in the body.

Criteria for the efficiency of nuclear reprogramming

Many different measures have been used to estimate the efficiency of nuclear reprogramming. 1. One is to determine the frequency of new gene expression or transcription. The genes scored need to be those of an embryo cell or those of cell fates entirely unrelated to that of the starting cell. 2. More demanding is evidence of a functional cell-type, again unrelated to the cell undergoing reprogramming. 3. The ability to derive ES cells from a somatic cell nucleus opens a route towards several cell-types unrelated to that of

the donor nucleus. 4. In some cases, attention is paid to the extinction of genes characteristically expressed in the cells that are subsequently reprogrammed. 5. Another important measure of reprogramming efficiency, usually not discussed, is the magnitude of the induced new gene expression. Complete reprogramming would ask for expression of the induced genes to be at the same level as that of equivalent cells in normal embryos or adult organs. 6. Finally, it is also important to determine the time and number of cell divisions that are required for the reprogrammed state to become evident. In some cases no cell division or DNA replication is required; in others, extended time and numerous cell divisions, and hence the possibility of cell selection, are required for the reprogrammed condition to become evident. We have assembled the results of different reprogramming procedures into tables, in order to compare the efficiencies of reprogramming by different routes (Table 1 – 2).

Nuclear transfer to enucleated eggs (metaphase II oocytes)

The original design of nuclear transfer experiments, first established in amphibia, involves the injection of a nucleus (ruptured cell) into an enucleated, unfertilized egg (Fig. 1A) (Briggs and King 1952). Most of the resulting injected eggs begin development as shown by activation (cortical rotation and early cleavage) of the egg. Some of these then cleave normally and develop through normal embryogenesis, eventually reaching adulthood (Gurdon et al. 1958). When a nucleus from an embryonic cell, such as a blastula cell, is used, a high proportion of the embryos reach normal blastula stages (32%) and of these a variable number, but up to 30% in good experiments, will reach adulthood (Gurdon 1960). However, when the nucleus of a specialized cell is used, the success rate is markedly less. For example, the nucleus of an endoderm or skin cell promotes the formation of complete blastulae in 12% and 5% of the total nuclear transfers, respectively (Fig. 2A) (Gurdon et al. 1975). Most of these first nuclear transfer embryos do not develop into tadpoles and therefore do not reach adulthood. Therefore, the efficiency of nuclear reprogramming by nuclear transfer decreases when the nuclei of cells of increasingly differentiated state are used as donor. These and other results are summarized in Table 1.

A stringent test of efficiency asks what proportion of nuclear transplant embryos reach a stage of development in which functional cell-types entirely unrelated to that of the donor are expressed (criterion 2). For example, tadpoles which respond to a stimulus by movement must have functional muscle and nerve cells. If nuclei are transplanted from skin cells, about 30% of such nuclear transplant embryos will develop into partial or complete blastulae, but only a very small proportion will develop into swimming tadpoles (1.3%, Fig 2A). However, when the partial blastulae obtained from such skin cell first nuclear transfers are used for a second round of nuclear transfers, up to 43% of these now reach swimming tadpole stages (Gurdon et al. 1975). This means that serial nuclear transfer can induce a switch in cell lineage with a high frequency. This frequency is up to 30%, when considering a switch between a functional endoderm cell nuclei into functional muscle and nerve cells, by a combination of serial nuclear transfer and grafts (Byrne et al. 2002). This process of reprogramming by single nuclear transfer to the egg involves several cell divisions. For example the muscular response stage of *Xenopus* embryos consists of about 150,000 cells, resulting from an average of 17 cell divisions.

Transplantation of the nucleus of a mammalian somatic cell into an enucleated egg (MII oocyte) also leads to the development of nuclear transfer embryos (Wilmut et al. 1997; Wakayama et al. 1998). The efficiency in mammalian nuclear transfer can be measured as the proportion of nuclear transfer embryos that reaches a particular stage. For example, 50% of mouse tail tip fibroblasts nuclear transfer embryos develop to the blastocyst stage, but only a very few survive to birth (Fig. 2B) (Wakayama and Yanagimachi 1999; Wakayama et

al. 2005). For comparison, the efficiency of morula/blastocyst formation by ES cell nuclear transfer is 44%, with up to 3.3% giving rise to live newborn (Wakayama et al. 1999; Blelloch et al. 2004). Another way of assessing nuclear reprogramming efficiency is to measure the number of ES cell lines that can be derived from nuclear transfer blastocysts (criterion 3). When considering derivation efficiencies of NT ES cells from ES and fibroblast nuclear transfer blastocysts, as much as half or a third of these can give rise to ES cell lines, respectively (Blelloch et al. 2004; Wakayama et al. 2005). While adult nuclear transfer mice can be derived with high efficiencies by using the nuclei of pluripotent ES cells (8%) (Wakayama et al. 1999), those from highly specialized and differentiated cells lead to much lower rates. The efficiencies of mammalian nuclear transfer have been reviewed in detail (Meissner and Jaenisch 2006).

Nuclear reprogramming by nuclear transfer to MII oocytes may involve a very high number of cell divisions. For example, obtaining germ cells by nuclear transfer of mouse somatic cell nuclei involves an average of 25 cell divisions. Yet Oct4 gene reactivation occurs within 1 or 2 cell divisions after nuclear transfer (Boiani et al. 2005; Egli et al. 2008). Similarly, developmentally regulated induction of pluripotency genes in the ICM and reactivation of the silent X chromosome occur within very few cell divisions (Eggan 2000; Bao et al. 2005; Silva et al. 2009).

There are two important conclusions from nuclear transfer experiments. The first is that the nuclei of even the most differentiated cells can be fully reprogrammed efficiently to functional new cell types by nuclear transfer, by the natural components of the egg (MII oocyte) and developmental programmes of the embryo. The second is that the process of cellular differentiation entails a major resistance of somatic cell nuclei to nuclear reprogramming by eggs and oocytes, such that reprogramming is always less efficient for nuclei of cells of increased differentiation states.

Nuclear transfer to oocytes in first meiotic prophase

An altogether different design of nuclear transplant experiment involves transplanting multiple cell nuclei into the germinal vesicle of an amphibian first meiotic prophase oocyte (Fig. 1B) (Byrne et al. 2003). The oocyte germinal vesicle (GV) contains a high concentration of components that are eventually distributed to the egg, after meiotic maturation, and that are necessary for embryonic development (Gao et al. 2002). Importantly, this reprogramming system does not lead new cell types. Somatic nuclei transplanted to an oocyte germinal vesicle do not undergo DNA synthesis or cell division, but are intensely active in RNA synthesis as is the host oocyte itself. This system makes it possible to transplant multiple nuclei from mammalian cells into the amphibian oocyte and to see the activation of many genes expressed in normal early development, including pluripotency marker genes (criterion 1). An advantage of this system is that it is possible to see the direct activation of previously silenced genes in adult somatic nuclei in the total absence of DNA replication. There is therefore a direct switch in gene transcription from a somatic to an oocyte-like type without the intervention of or need for DNA replication. The exchange of molecules involved therefore reflects directly the process of transcriptional reprogramming. In contrast, nuclear transfer experiments to unfertilized eggs (above) are complicated by a period of intense DNA replication and cell division and absence of transcription immediately after nuclear transfer (criterion 6). The time when transcriptional reprogramming takes place is very hard to determine or analyze in egg nuclear transfer experiments.

Using the multiple nuclei to oocyte GV assay system, one important conclusion is that the frequency and speed of gene reactivation is very high. With HeLa cell nuclei, reactivation of

Oct4 can be seen in as little as 8 hours at 18°C (Koziol et al. 2007). When transcriptional reactivation of Sox2 is assayed, reprogramming is seen in all experiments within 48 hours (Halley-Stott et al. 2010). Therefore, *Xenopus* oocytes must contain factors that are able to efficiently induce transcriptional reprogramming. The oocyte type linker histone B4 was recently identified as a factor necessary for efficient induction of gene reactivation (Jullien et al. 2010). After nuclear transfer, B4 is incorporated into transplanted nuclei, an event associated with loss of somatic linker histone H1. This occurs in over 90% of nuclei in most experiments. Genome-wide resetting of histone post-translational modifications of transplanted nuclei has also been shown (Murata et al. 2010). Thus, after nuclear transfer, there is efficient loss of somatic nuclear proteins and incorporation of oocyte factors.

A more stringent measure of reprogramming efficiency includes the magnitude of gene reactivation (criterion 5). With the *Xenopus* oocyte system, the maximum level of gene reactivation is related to the differentiated state of donor nuclei. ES cells that have been induced to differentiate for 3 days by retinoic acid (RA) reactivate transcription of the silenced pluripotency genes Oct4, Sox2 and Nanog to 100% of the level in transplanted ES cells within 4 days (Table 2) (Halley-Stott et al. 2010). However, when the nuclei from specialized cells such as thymus, C2C12 or MEFs are used, the same genes reactivate to a maximum level ranging from 0.3% to 3.3% of the transplanted ES nuclei levels (Fig. 2C). Therefore, the process of differentiation entails a great resistance to transcriptional nuclear reprogramming by oocytes. An important conclusion is that *Xenopus* oocytes have the ability to efficiently induce gene reactivation, in the absence of cell division and within a short time period, using the natural components of the oocyte. Therefore, multiple mammalian nuclear transfer to *Xenopus* oocytes is a good system to investigate the mechanisms of transcriptional reprogramming. Moreover, their large size (1.2 mm) makes them an ideal source of material for the identification of new reprogramming factors.

Cell fusion

Cell fusion can be experimentally induced such that several nuclei are forced to reside within a common cytoplasm (Fig. 1C) (Davidson et al. 1966; Harris et al. 1966). This procedure provides short term multinucleated cells, termed heterokaryons, in which nuclear reprogramming is induced. One cell type usually shows dominance over the other, such that it imposes its state on another nucleus. The early experiments demonstrated that the nuclei of mature hen erythrocytes are induced to resume RNA synthesis when fused to rapidly dividing HeLa cells, an event associated with nuclear swelling (Harris 1967). Reprogramming of previously silenced genes can occur in the absence of cell division in heterokaryons, such that non-muscle amniotic human cells are induced to express muscle genes when they are fused to mouse muscle cells (Blau et al. 1983).

Initiation of reprogramming of gene expression in heterokaryons is direct, rapid (1 to 2 days), and independent of DNA synthesis (Han et al. 2008; Palermo et al. 2009). For example, ES cells that have been treated with the cell division inhibitor Mitomycin C are able to induce reactivation of pluripotency marker Oct4-GFP when fused to neural stem cells (NSCs) (Do and Scholer 2004). In cell fusion experiments, only a minor proportion of cells are induced to fuse (0.6-1%), and a strong selection of fused cells is usually required. However, when ES cells are used, a very high proportion of the fused cells is induced to reactivate previously silent genes (criterion 1). For example, the frequency of pluripotency gene reactivation from human fibroblasts after fusion with mouse ES cells is very high (>70%) (Bhutani et al. 2009). However, the level of gene reactivation (criterion 5) obtained in heterokaryons with lymphocytes is usually very low. One study estimates these levels as <0.01-1% of ES cell levels for Oct4, several days after fusion (Fig. 2D) (Pereira et al. 2008).

Nevertheless, because gene reactivation is initiated frequently in heterokaryons, they constitute a good system to study the mechanisms that initiate gene reactivation.

Heterokaryons eventually give rise to proliferating cell hybrids, following fusion of several nuclei, after nuclear membrane breakdown at cell division. Reprogramming of somatic cells in cell hybrids has been achieved by fusion with ES, embryonic germ (EG) and embryo carcinoma (EC) cells (Miller and Ruddle 1976; Tada et al. 1997; Matveeva et al. 1998; Kimura et al. 2004). Pluripotent cell types are usually dominant over the somatic cell types in cell hybrids, and induce phenotypic and epigenetic changes so that somatic nuclei are induced to resemble pluripotent ones. Full level Oct4-GFP reactivation can be obtained within 2 days of neural stem cells (NSCs) fusion with ES cells (Do et al. 2007). Tissue specific gene extinction can also be seen in pluripotent hybrids (criterion 4). However, gene extinction does not occur in some cases, and is dependant on the cell types and their ratio before fusion (Lee et al. 2009; Kruglova et al. 2010). Somatic gene extinction was shown not to be required for gene reactivation in heterokaryons of human B lymphocytes fused to mouse myotubes (Terranova et al. 2006). Somatic cells, such as thymus cells, are induced to adopt epigenetic modifications resembling those of pluripotent cells when fused to EG cells (Tada et al. 1997). These include reactivation of pluripotency genes and of the inactive X chromosome, self-renewal, and DNA demethylation; reviewed in (Do et al. 2006; Pralong et al. 2006). Cell hybrids obtained by fusion to pluripotent cells therefore possess all the molecular characteristics of pluripotent cells. Tetraploid hybrids contribute to all three germ lineages when introduced into blastocysts, as judged by transgene expression analysis (Matveeva et al. 1998; Tada et al. 2001; Ying et al. 2002). Because evidence for germline contribution has not been reported, whether tetraploid cell hybrids are truly pluripotent remains to be confirmed.

One study established that the efficiency of cell hybrid formation from ES cell fusion to NSCs can be increased by 200-fold (from <0.014% to ~3%) following overexpression of the pluripotency conferring factor Nanog (Silva et al. 2006). When fibroblasts and thymocytes were used, the effect was also seen, but to a lower extent. Cell fusion experiments have the remarkable property of inducing active DNA demethylation in the absence of cell division. For example, the promoters of the pluripotency genes Oct4 and Nanog become demethylated within a day in mouse ES cells fused to human B cells (Pereira et al. 2008). Cell fusion provides a powerful system for the analysis of nuclear reprogramming. The presence of multiple genomes in the resulting hybrids precludes their use for therapy, but efforts to remove the genome of the dominant cells have been reported (Matsumura et al. 2007).

Several important conclusions can be drawn from cell fusion studies. First, transcriptional reactivation of previously silent genes can be efficiently initiated in the absence of cell division. Second, molecules of differentiated cells can reprogram the nuclei of other somatic cells.

Transcription factor induced lineage switch

It has been known for a long time from developmental biology studies that overexpression of one or a few transcription factors can have profound effects on cell phenotypes and identity. Pioneering studies in this field include the discovery of MyoD, a transcription factor sufficient to convert fibroblasts into myoblasts when overexpressed by transfection (Fig. 1D) (Davis et al. 1987). In these experiments, the proportion of responding cells is very high. For example, about 25 to 50% of fibroblasts are reprogrammed to myoblasts and up to 60% of pre-T cells are reprogrammed to become macrophages by overexpression of MyoD and C/EBP β , respectively (Laiosa et al. 2006). This kind of reprogramming usually involves

several cell divisions, and hence the possibility for selection. However, cultured pancreatic exocrine cells can be transdifferentiated into hepatocytes, in the absence of cell division, following the overexpression of C/EBP β (Shen et al. 2000). The magnitude of gene reactivation can be very high in the reprogrammed cells. For instance, the macrophage marker Mac1 is reactivated to 100% of its macrophage level within 4 days when C/EBP β is overexpressed in pre-B cells (Xie et al. 2004). Moreover, genes that are expressed in the starting cells are also turned off, indicating that reprogramming is complete.

Based on a very elegant strategy designed by Shinya Yamanaka (see induced pluripotency section below), the direct conversion of fibroblasts into functional neurons by defined factors was recently reported (Vierbuchen et al. 2010). A set of 19 genes that are specifically expressed in neural tissues were found to induce neuronal cells when overexpressed in mouse embryonic fibroblasts (MEFs). These experiments suggest that transcription factor overexpression may allow the reprogramming of somatic cells into any desired cell type. However, the conversion of somatic cells into other cell types by transcription factor overexpression is, at present, limited to certain cell types, usually, but not always, of closely related lineages. For instance, MyoD does not lead to myoblasts when expressed in certain non-muscle cell types, such as liver (Davis et al. 1987; Schafer et al. 1990).

A recent study reported the *in vivo* conversion of mouse pancreatic acinar cells into insulin producing β cells, following adenoviral mediated delivery of Pdx1, Ngn3 and Mafk, 3 key genes with established functions in pancreatic embryonic development (Zhou et al. 2008). Insulin was induced in a high proportion of the infected cells (25%), from a low level after 3 days, to a high level after one month. The newly producing insulin cells possessed all the phenotypic characteristics of pancreatic β cells. The conversion seemed to occur with very limited, if any, cell divisions. Altogether, transcription factors involved in the early stages of embryonic development of specific cell types and organs appear as an appealing route for the induction of nuclear reprogramming of one kind of cell into another.

Induced pluripotency

A remarkable advance in the reprogramming field came from the elegant work of Takahashi and Yamanaka (2006), which demonstrated that overexpression of a set of four factors, namely *Oct3/4*, *Sox2*, *KLF4* and *c-Myc* into mouse adult fibroblasts can induce nuclear reprogramming to produce pluripotent cells that closely resemble ES cells (Fig. 1E). The new cells pass the most stringent pluripotency tests, and share many characteristics with mouse ES cells, including morphology, growth, transcriptome and epigenome. Hence they were termed induced pluripotent stem cells (iPS cells). iPS cells show reactivation of the inactive X chromosome (Stadtfeld 2008), restore telomerase activity, and show genome-wide histone methylation patterns characteristic of ES cells (Takahashi and Yamanaka 2006; Maherali et al. 2007). Thus, it is possible to directly reprogram somatic cells from many different origins to acquire pluripotency directly by overexpression of a few factors. Nuclear reprogramming by defined factors has been extensively reviewed by others (Yamanaka 2007; Jaenisch and Young 2008; Amabile and Meissner 2009; Hochedlinger and Plath 2009; Kiskinis and Eggan 2010)

Of special interest here is the efficiency with which somatic cells can be reprogrammed to produce iPS cells. This was originally calculated as the number of iPS cell colonies obtained 2 weeks after addition of the four factors to MEFs (Takahashi and Yamanaka 2006). By this route, 0.01% to 0.05% of the starting cells are induced to form iPS cell colonies over 2 weeks (Takahashi and Yamanaka 2006; Maherali et al. 2007; Meissner et al. 2007; Okita et al. 2007; Wernig et al. 2007). This raises the possibility that reprogrammed cells arise from a subpopulation of cells, more prone to reprogramming. Variable reprogramming

efficiencies of iPS formation have been reported (Kiskinis and Eggan 2010). During the reprogramming process, the reactivation of pluripotency markers AP1, SSEA1 and Oct4 was shown to be gradual (Brambrink 2008), culminating in the reactivation of Nanog and the acquisition of ground state pluripotency (Silva et al. 2009). It is thought that induction of pluripotency during iPS cell formation occurs stochastically (Hanna et al. 2009). When successful, the full pluripotency transcriptional network is induced, such that pluripotency genes are reactivated to the same levels as in ES cells (criterion 5), and differentiation genes initially expressed in the starting cells are silenced (criterion 4). As for nuclear transfer, the efficiency of nuclear reprogramming by defined factors decreases with the increased differentiation state of cells. For example, the reprogramming efficiency is much higher from less differentiated cells compared with terminally differentiated cells (Eminli et al. 2009). When the four factors are expressed in donor cells by means of stably integrated inducible expressing cassettes, the overall efficiency is increased (from 0.05% to 0.5%) but still remains low (Stadtfield et al. 2008). Striking improvements in efficiencies have been reported following the use of small molecules (Feng et al. 2009). The histone deacetylase inhibitor valproic acid (VPA) (Huangfu et al. 2008), DNA methyltransferase inhibitors (Huangfu et al. 2008; Mikkelsen et al. 2008), histone methyltransferase inhibitor BIX-01294 (Shi et al. 2008) and Vitamin C (Esteban et al. 2009) have been shown to substantially increase the efficiency of nuclear reprogramming, and even to substitute for some of the factors, such VPA for Klf4 and c-Myc. VPA was found to increase the frequency of Oct4-GFP reactivation in MEFs from 0.03% to 11% (Fig. 2E) (Huangfu et al. 2008).

Because nuclear reprogramming of somatic cells by defined factors is a long process, the origins of iPS cell colonies have been hard to investigate. A recent study addressed this issue by using time-lapse imaging to track the reprogramming process over several weeks from MEFs to pluripotent iPS cells (Smith et al. 2010). This allowed a calculation of the normalized efficiency of nuclear reprogramming, taking into account only those iPS cell colonies that can be tracked back to single MEFs. It is found that apart from those colonies that originate from reprogramming events of single MEFs, secondary satellite iPS colonies also appear during the culture process, and cannot be tracked back to single cells; these therefore do not represent new reprogramming events. Moreover, iPS colonies were stringently assessed for full acquisition of pluripotency as judged by expression of AP, Nanog and Chd1. The use of less rigorous criteria can lead to an overestimate of reprogramming efficiencies (Smith et al. 2010). For example, the normalized efficiency of MEFs nuclear reprogramming to Cdh1 + Nanog + iPS cell colonies is 1.15%, to be compared with 3.7% obtained without normalization. A series of earlier reports which established that the deletion of p53 in MEFs is sufficient to increase the efficiency of Nanog-GFP positive colony formation from 0.2% to 20% (Hong et al. 2009; Kawamura et al. 2009; Li et al. 2009). However, it is noteworthy that the efficiency of iPS formation from p53 depleted MEFs, as judged by the number of Cdh1 and Nanog positive colonies is markedly less, approaching 0.2% (Fig. 2E) (Smith et al. 2010). The main conclusions from iPS cell work is that overexpression of a set of few factors can induce, with low frequency, the full reactivation of genes leading to the complete erasure of somatic cell identity, providing pluripotent cells capable of differentiating into all other cell types. Because iPS cells can be generated from human patient cells, without the need for oocytes, this is the most hopeful route to regenerative medicine.

Mechanisms

Efficient reprogramming requires opening up of the genome

One of the mechanisms of nuclear reprogramming is an efficient opening up of the genome. This is required in order to give reprogramming factors access to regulatory regions, facilitate chromatin remodelling, and mediate changes in gene expression. Eggs and oocytes

very efficiently induce nuclear swelling and decondensation after somatic cell nuclear transfer (Gurdon 1968; Gurdon 1976). Moreover, nuclear decondensation correlates with nuclear reprogramming in many reprogramming systems. These include nuclear transfer to eggs and oocytes, cell fusion (Harris 1967), and reprogramming in the mouse germline (Hajkova et al. 2008). Nuclear swelling may also help removal of chromosomal proteins present in the nuclei undergoing reprogramming, which could contribute to chromatin decondensation, through loss of proteins such as Ring1b, shown to mediate chromatin condensation independently of its catalytic domain (Eskeland et al. 2010). Reprogramming by eggs uses the natural components of the egg, which have been synthesised and stored in the oocyte during oogenesis. These factors are used at fertilization to reprogram sperm and induce its reprogramming with a 100% efficiency. By use of egg extracts, Nucleoplasmin, an abundant egg protein, was identified as a factor greatly contributing to decondensation of sperm and somatic nuclei (Philpott et al. 1991; Tamada et al. 2006). It is noteworthy that factors required for opening up of the genome, such as Chd1, are reactivated and required for the induction of pluripotency in iPS cells (Gaspar-Maia et al. 2009). The main concept here is that efficient reprogramming requires opening up the genome, in order to give transcriptional regulators access to regulatory regions. This is done very efficiently by eggs, which possess the natural ability to decondense and remodel sperm with high efficiencies.

Efficient reprogramming of gene expression requires epigenetic reversal

Epigenetic regulation of gene expression mediated by DNA methylation, histone modification and chromatin remodelling plays an important role in the efficiency of nuclear reprogramming. Epigenetic defects have been widely documented in cloned embryos (Bourc'his et al. 2001; Dean et al. 2001). These result from the inability to revert donor cell epigenetic states, causing some genes to be inconsistently expressed in reprogrammed cells or embryos. The failure to reverse an epigenetic state is seen in the continuing expression of MyoD in muscle nuclei transplanted to amphibian eggs, but in the wrong lineages, such that endoderm cells are found to express muscle specific genes (Ng and Gurdon 2008).

Another example of epigenetic defects in cloned embryos comes from studies on the inactive X chromosome of female mouse somatic cells. After nuclear transfer from female somatic cells, the inactive X chromosome is reactivated upon developmentally programmed induction of pluripotency in the ICM (Eggan et al. 2000; Silva et al. 2009). However, the chromatin state of the inactive X chromosome is not fully reversed in early nuclear transfer embryos (Bao et al. 2005), and aberrant expression of the X inactivation inducing non-coding RNA *Xist* is seen in the differentiating cells of the epiblast, with cells harbouring one, two, or no *Xist* clouds (Nolen et al. 2005). These defects indicate that the *Xist* locus is incompletely reversed in some cells of cloned embryos. This could be due to DNA methylation (see below).

Epigenetic defects can also arise during the reprogramming process itself. One such recent development in this area includes the inactivation of the imprinted *Igf2* locus during iPS cell formation (Stadtfeld et al. 2010). This leads to iPS colonies with limited developmental potential. The resulting iPS colonies share all the epigenetic characteristics of ES cells except for this locus, which can nevertheless be reactivated by exposure to TSA, providing fully pluripotent iPS cells.

The low efficiencies of nuclear reprogramming have been suggested to result from incomplete epigenetic reprogramming. Epigenetic defects are best revealed by the inefficiency of gene reactivation. Inconsistent reactivation of pluripotency genes in nuclear transfer embryos may be correlated with the inability to demethylate gene promoters (Boiani et al. 2002; Yamazaki et al. 2006). The general conclusion here is that during cell

differentiation, cells progressively acquire epigenetic modifications that make them resistant to nuclear reprogramming, and decrease its efficiency.

Efficient reprogramming requires DNA demethylation

Efficient nuclear reprogramming requires epigenetic reversal of donor cell nuclei. It is well known that the inefficiency of nuclear reprogramming by nuclear transfer to enucleated eggs correlates with DNA methylation. Nuclear transfer embryo development is often associated with defects in DNA methylation (Dean et al. 2001; Kang et al. 2001; Ohgane et al. 2001; Yamazaki et al. 2006). Surprisingly, treatment of donor nuclei with unmethylatable cytosine analogue 5-Aza-dC is usually not found to increase the efficiency of nuclear transfer embryo development (Enright et al. 2003; Enright et al. 2005). However, when using Dnmt1 mutant MEFs, the efficiency of NT ES cell derivation from nuclear transfer blastocysts is increased threefold (Blelloch et al. 2006). Efficient active DNA demethylation in heterokaryons was recently suggested to require AID (Activation-induced cytidine deaminase) (Bhutani et al. 2010). Another study demonstrated genetically that efficient DNA demethylation during germline reprogramming involves AID (Popp et al. 2010). There are several other factors that could initiate active DNA demethylation, and may include components of the DNA repair pathway (Niehrs 2009). The major conclusion here is that efficient reprogramming requires DNA demethylation of silenced genes, a process that can be achieved passively, when cell divisions occur, or actively, by mechanisms that remain to be further explored.

Histone modifications

Epigenetic memory of gene silencing could also involve mechanisms that are independent of DNA methylation. During cell differentiation, gene silencing is generally seen to precede DNA methylation. Post-translational modifications of histone tails is well known to play an important role in regulation of gene expression (Kouzarides 2007). In particular, histone acetylation loosens the highly compact nucleosomal structure, making chromatin more accessible to transcriptional regulators such as transcription factors. Part of the epigenetic restriction imposed by modifications such as DNA methylation and histone modifications result in the recruitment of histone deacetylases (HDAC). HDAC inhibition after nuclear transfer was shown to enhance chromatin remodelling, histone modification, DNA replication and transcriptional activity (Kishigami et al. 2006; Bui et al. 2010). The efficiency of gene reactivation is also dramatically increased in iPS by HDAC inhibitors (Huangfu et al. 2008).

Cell divisions are not required for initiation of nuclear reprogramming

Transitions through mitosis are opportunities to reverse somatic cell characteristics, and has been proposed to greatly facilitate nuclear reprogramming (Egli et al. 2008). For example, many factors such as HP1, Bmi1 or Brg1 are excluded from mitotic chromatin. After cell division, the pool of transcriptional regulators present in the cell instructs the nucleus which products to make. However we see that while cell divisions facilitate reprogramming, these are not strictly required for the induction of transcriptional reprogramming. Gene reactivation has been demonstrated to occur in the complete absence of cell division after nuclear transfer to *Xenopus* oocytes and in heterokaryons (Blau et al. 1983; Byrne et al. 2003). Furthermore, previously methylated pluripotency genes can be reactivated in the absence of cell division, as demonstrated by cell fusion experiments (Pereira et al. 2008). In addition, cell type switches can occur without cell divisions (Shen et al. 2000; Zhou et al. 2008). Altogether, we conclude that the initiation of transcriptional reprogramming does not require cell division.

Conclusion

We have discussed six criteria that have been used to assess the efficiency of nuclear reprogramming by nuclear transfer, cell fusion, and transcription factor overexpression. A general concept that emerges from a comparison of these procedures is that the differentiated state of a cell is very dynamic; it seems to be continually maintained by a cyclic reprogramming of gene expression by cytoplasmic factors that are encoded by the nucleus. We suggest that the proteins synthesized in the cytoplasm under the direction of the nucleus include ones, like transcription factor, that move back into the nucleus, and continually reprogram genes characteristic of that cell. The differentiated state will then persist in that cell and in its mitotic progeny. When the cytoplasmic environment around a nucleus is changed by cell fusion, and even more massively by nuclear transfer to eggs, then these reprogramming proteins will be changed. In the case of iPS, the enforced overexpression of transcription factors will have a similar effect. The survey of efficiencies, described here, shows that the transcription of nuclei from even the most differentiated cells can be rapidly re-set. The efficiency of reprogramming is much greater by the natural components of eggs and oocytes than by the overexpression of a few transcription factors. Also the efficiency of reprogramming as judged by new gene expression (criterion 1) is much greater than by fully functional cell types or ES cells (criteria 2 and 3).

The second generalization based on our survey of efficiencies is that, as cells differentiate, they become increasingly resistant to the reprogramming components of eggs and cultured cells, and to transcription factor overexpression. This is seen by the decreasing percent of cells that become reprogrammed (criterion 4) as well as by the reduced magnitude of reprogramming in a cell (criterion 5). This resistance is not explained by the lower division rate of more specialized cells (criterion 6). It is thought to reflect a progressively tighter association of repressors with the regulatory regions of pluripotency genes (Gurdon and Melton 2008). To identify the molecular basis of this resistance is a major challenge for the future.

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A. Single somatic cell nuclear transfer to egg (metaphase II oocyte)

B. Multiple somatic cell nuclear transfer to prophase I oocyte

C. Cell fusion

D. Transcription factor induced lineage switch

E. Induced pluripotency

Figure 1. Nuclear reprogramming strategies

A. Nuclear reprogramming by nuclear transfer involves the transplantation of a somatic cell nucleus into an enucleated egg (metaphase II oocyte), which can lead, in some cases, to the production of fertile adults, genetically identical to the donor nucleus. B. Direct transplantation of multiple mammalian somatic cell nuclei into the nucleus of a *Xenopus* prophase oocyte I does not lead to new cells, but induces fast and direct transcriptional reprogramming of previously silenced genes. C. Reprogramming of somatic cells by cell fusion produces heterokaryons and cell hybrids, possessing many molecular characteristics of pluripotency when pluripotent cells are fused to somatic ones. D. Overexpression of transcription factors can induce a lineage switch. E. Ground state pluripotency can be induced in somatic cells by overexpression of a few factors such as Oct4, Sox2, KLF4 and c-Myc.

Figure 2. Nuclear reprogramming efficiencies judged by different criteria

The left part of the figure shows the reprogramming steps of each system. The graphs on the right indicate the efficiency (%) of reprogramming as judged by different criteria. The origin of donor nuclei is indicated. A. Efficiency judged by the proportion of nuclear transfer *Xenopus* embryos obtained from somatic cell nuclear transfers to egg (metaphase II oocytes) that reach a particular stage of development. * Includes results from serial nuclear transfer experiments. B. Efficiency judged by the proportion of mouse embryos obtained by somatic cell nuclear transfers to mouse eggs (MII oocyte) that reach a particular stage of development. C. Efficiency judged by the level of transcriptional reactivation of pluripotency genes after nuclear transfer to *Xenopus* oocytes. D. Reprogramming efficiency after cell fusion, as judged by the number of colonies obtained or the extent of gene reactivation. E. Induced pluripotency: efficiency judged by the number of colonies obtained from starting cells by using various factor delivery approaches. Numbers in parentheses indicate normalized efficiencies (see text).

Table 1

Efficiencies: Maximum number of reprogrammed cells and number of cell divisions.

System	Starting Cell	Resulting Cells	Starting Nuclei Reprogrammed (%)	Number of Cell Divisions	Reference
SCNT to amphibian egg (MII oocyte)	Blastulae cell nuclei	Blastula stage embryo	32	~7	(Gurdon 1960)
SCNT to amphibian egg (MII oocyte)	Blastulae cell nuclei	Swimming tadpole	22	~17	(Gurdon 1960)
SCNT to amphibian egg (MII oocyte)	Skin outgrowth	Swimming tadpole	1,3	~17	(Gurdon et al. 1975)
SCNT to amphibian egg (MII oocyte)	Skin outgrowth	Swimming tadpole	43 [†]	~17	(Gurdon et al. 1975; Byrne et al. 2002)
SCNT to mouse MII oocyte (egg)	ES	Blastocyst	29	5	(Wakayama et al. 1999)
SCNT to mouse MII oocyte (egg)	Cumulus	Blastocyst	55	5	(Wakayama et al. 1998)
SCNT to mouse MII oocyte (egg)	Tail tip fibroblasts	Blastocyst	50	5	(Wakayama and Yanagimachi 1999)
SCNT to mouse MII oocyte (egg)	ES	Live born mice	2.4	>25	(Wakayama et al. 1999)
SCNT to mouse MII oocyte (egg)	Cumulus	Live born mice	1-3	>25	(Wakayama et al. 1998; Wakayama et al. 2005)
SCNT to mouse MII oocyte (egg)	Tail tip fibroblasts	Live born mice	1	>25	(Wakayama and Yanagimachi 1999; Wakayama et al. 2005)
SCNT to mouse MII oocyte (egg)	ES	NT ES cells	13	>15	(Blelloch et al. 2004)
SCNT to mouse MII oocyte (egg)	Cumulus	NT ES cells	5-10	>15	(Wakayama et al. 2005)
SCNT to mouse MII oocyte (egg)	Tail tip fibroblasts	NT ES cells	0.5-3	>15	(Wakayama et al. 2005)
NT Blastocyst	ES	NT ES cells	10-50	>10	(Blelloch et al. 2004)
NT Blastocyst	Cumulus	NT ES cells	12-19	>10	(Wakayama et al. 2005)
NT Blastocyst	Tail tip fibroblasts	NT ES cells	13-33	>10	(Wakayama et al. 2005)
Multiple mammalian NT to <i>Xenopus</i> prophase I oocyte	3 days RA mouse ES	Nuclear transfer oocyte	>90 [*]	0	(Jullien et al. 2010)
Cell fusion	Human fibroblasts × mouse ES	Heterokaryons	0.6-1 [*]	0	(Bhutani et al. 2010)
Cell fusion	ES × NSC	Tetraploid hybrids	0.00014 [*]	~10	(Silva et al. 2006)
Cell fusion	ES × NSC + Nanog	Tetraploid hybrids	~3 [*]	~10	(Silva et al. 2006)
MyoD overexpression	Fibroblasts	Muscle myoblasts	25-50	0 to 1	(Davis et al. 1987)
C/EBP overexpression	Pre-T cell	Macrophages	60	0 to 2	(Laios et al. 2006)
Pdx1, Ngn3, Maf: adenovirus mediated expression	<i>in vivo</i> adult pancreatic acinar cells	cells	20	0 to 2	(Zhou et al. 2008)
OSKM: retrovirus mediated overexpression	MEFs	iPS colonies	<0.02	>15 ?	(Takahashi and Yamanaka 2006)

System	Starting Cell	Resulting Cells	Starting Nuclei Reprogrammed (%)	Number of Cell Divisions	Reference
OSKM: dox inducible lentivirus	Tail tip fibroblasts	Oct4-GFP iPS colonies	0.06-0.5	>15 ?	(Stadtfeld et al. 2008; Sommer et al. 2009)
OSKM: dox inducible lentivirus + VPA	MEFs	Oct4-GFP iPS colonies	12	>15 ?	(Huangfu et al. 2008)
OSKM: retrovirus mediated overexpression	p53 ^{-/-} MEFs	Nanog-GFP iPS colonies	16 *	>15 ?	(Hong et al. 2009)
OSKM: dox inducible lentivirus	MEFs	Nanog+ Cdh1+ iPS colonies	3.7	>15 ?	(Smith et al. 2010)
OSKM: dox inductiondox inducible lentivirus	MEFs	Nanog+ Cdh1+ iPS colonies	1.15	>15 ?	(Smith et al. 2010)
OSKM: dox inducible lentivirus + p53 shRNA	MEFs	Nanog+ Cdh1+ iPS colonies	<0.2	>15 ?	(Smith et al. 2010)

[†] Experiment involves serial nuclear transfer.

* Includes incompletely reprogrammed cells.

normalized efficiency. NT, Nuclear transfer. SCNT, Single Cell Nuclear Transfer. MII, Metaphase II. O, Oct4. S, Sox2. K, KLF4. M, c-Myc. MEFs, Mouse embryonic fibroblasts. NSC, Neural stem cell. RA, Retinoic Acid. VPA, Valproic Acid.

Table 2

Efficiencies: Frequency, speed and magnitude of response to reprogramming cue.

System	Starting cell	Frequency of gene reactivation (%)	Magnitude of gene reactivation (%)	Starting cell gene extinction (%)	Time	Reference
SCNT to amphibian egg (MII oocyte)	Stage 21 somite	Xbra, >90	100	50	< 10 hours	(Ng and Gurdon 2005)
SCNT to mouse MII oocyte (egg)	Cumulus	Oct4, 88	0-400?	~Yes	3.5 days	(Boiani et al. 2002; Bortvin et al. 2003)
Multiple mammalian NT to <i>Xenopus</i> prophase I oocyte	3 day RA mouse ES	Sox2, >90	100 of ES level	No	24 h	(Jullien et al. 2010)
Multiple mammalian NT to <i>Xenopus</i> prophase I oocyte	C2C12	Sox2, >90	0.4 of ES level	No	1-3 days	(Halley-Stott et al. 2010)
Cell fusion	Human fibroblasts × mouse ES	Oct4, >70	<0.01	Sometimes	24-48h	(Pereira et al. 2008; Lee et al. 2009; Bhutani et al. 2010)
C/EBP overexpression	pre-B cell	Mac1, 60	100	Yes	4 days	(Xie et al. 2004)
Pdx1, Ngn3, Maf overexpression	<i>in vivo</i> adult pancreatic acinar cells	Insulin, 25	low (3 days) high (1 month)	Yes	3 days to 1 month	(Zhou et al. 2008)
OSKM: retrovirus mediated overexpression	MEFs	Oct4	100	Yes	2 weeks	(Takahashi and Yamanaka 2006)
OSKM: retrovirus mediated overexpression	MEFs	Oct4-GFP, 0.03	100	Yes	1 week	(Huangfu et al. 2008)
OSKM: retrovirus mediated overexpression + VPA	MEFs	Oct4-GFP, 11.8	100	Yes	1 week	(Huangfu et al. 2008)
OSK: retrovirus mediated overexpression	MEFs	Nanog-GFP, 0.22	1 to 100	Yes	1 month	(Hong et al. 2009)
OSK: retrovirus mediated overexpression	p53 ^{-/-} MEFs	Nanog-GFP, 10	1 to 100	Yes, but not always	1 month	(Hong et al. 2009)

NT, Nuclear transfer. SCNT, Single Cell Nuclear Transfer. MII, Metaphase II. O, Oct4. S, Sox2. K, KLF4. M, c-Myc. MEFs, Mouse embryonic fibroblasts. NSC, Neural stem cell. RA, Retinoic Acid. VPA, Valproic Acid.