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# Reprogramming somatic cells towards pluripotency by defined factors

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The recent years have seen great advances in reversal of programming of differentiated somatic cells towards pluripotency by methods not involving nuclear transfer. Some of these may present a first step on the way to individual-based cell therapy without the problems connected to collection of mammalian unfertilised oocytes. Although differentiation of cells involves complex genetic and epigenetic changes, it is now possible to generate cells with many properties of pluripotent embryonic stem cells by retroviral transduction of differentiated cells with only four transcription factors: Oct3/4, Sox2, Klf4 and c-Myc. The re-programmed cells contribute to live chimeric mice and are transmitted via the germline.

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## Introduction

Most cells of a multicellular organism will, with increasing levels of differentiation, inevitably become more and more restricted to specific cell lineages. This commitment of cells may already begin between the two-cell and the four-cell stage when some blastomeres already indicate a preference for contributing to either the pluripotent cells of the intracellular mass or the trophoblast [1]. Pluripotent cells can by definition still give rise to all cells of the embryo or the adult organism but they do not have the self-organising capability to form the whole organism [2]. *In vivo* this unique property can only be found in the transient cells of early embryos, including the inner cell mass (ICM) of blastocysts. Pluripotency is *ex vivo* inherited by embryonic stem (ES) cells that are derived from the cells of the ICM. The pluripotency of ES cells in culture has to be maintained by addition of factors that promote proliferation in absence of differentiation, also known as self-renewal [2,3].

Differentiation of pluripotent cells along increasingly defined lineages involves a multitude of genetic and epigenetic changes [4,5] that will influence the long-term fate of each cell and its progenitors. It had been uncertain for a long time if it ever would be possible to completely reverse these changes in adult somatic cells to regain fully pluripotent cells. However, with the first successful cloning experiments in mammals by introduction of nuclei of adult somatic cells into oocytes (SCNT; somatic cell nuclear transplantation) that gave rise to viable offspring, it was clearly demonstrated that the cytoplasm of oocytes must contain sufficient information to reprogram nuclei of at least some types of cells [6,7]. However, the usefulness of SCNT for research or as a step on the road to potential stem cell-based therapies tailored for patients is, especially in research on human cells, limited by low cloning efficiency, and observed abnormalities at different stages of development in test animals [8]. Ethical problems connected to deriving and working with large numbers of human oocytes have so far further restricted the practicality of SCNT.

In future research a new technique of introducing chromosomes into mitosis-arrested zygotes instead of unfertilised oocytes may circumvent some of these difficulties [9<sup>\*</sup>].

This review will briefly discuss other approaches to reprogramming of somatic cells than by nuclear transfer and then focus on the pluripotent cells derived by introduction of the Oct3/4, Sox2, Klf4 and c-Myc transcription factors [10<sup>\*\*</sup>] and the known functions of these factors in ES cells.

## Reprogramming of somatic cells

Differentiation of cells encompasses a vast number of epigenetic changes restricting the unique accessible structure of the genome at the pluripotent stage [11]. It is further accompanied by changes in the pattern of transcription factor expression with rapid downregulation of many of the factors very restrictively expressed in the pluripotent stage, which in turn results in sweeping changes in overall gene expression. So far, there had been no evidence available to assume that this can be achieved by only a few key factors and consequently most efforts to reprogram somatic cells, like SCNT, rely on pluripotent cells to provide the required complex environment. Several alternative approaches to SCNT have been employed (Table 1) in the attempt to revert somatic cells to a pluripotent state [8,11].

## Cell fusion

The creation of cell hybrids by fusion of somatic cells with pluripotent cells of various origins has been shown to

Table 1

## Strategies for reprogramming of differentiated cells

Reprogramming method	Description	Restrictions
Somatic cell nuclear transplantation	Introduction of a somatic cell nucleus into an enucleated unfertilised oocyte. For an increasing number of species, a complete organism can thus be formed by the reconstituted oocyte.	Application may be limited by availability of oocytes and the low cloning efficiency. Furthermore, several developmental abnormalities were observed in cloned animals. Ethical and legal obstacles restrict use of this method for human cells.
Cell-cell fusion	Hybrids of differentiated and pluripotent cells exhibit characteristics of pluripotency.	The reprogrammed cell hybrids contain an additional set of chromosomes. The nucleus of the pluripotent cell may be required for reprogramming.
Treatment with extracts of pluripotent cells	Permeabilised cells are exposed to cell-free extracts of pluripotent cells. Treated cells re-express pluripotency markers and re-differentiate into multiple lineages.	Limited experience with primary cells. Reprogrammed cells will regain only some of the properties of pluripotent cells.
Stable expression of defined factors	Exogenous expression of Sox2, Oct3/4, Klf4, and c-Myc and subsequent selection for pluripotency markers gives rise to cells with similarity to pluripotent cells. Reprogrammed cells can contribute to tissues of all three germ layers in live chimeric mice.	Reactivation of transgenes, in particular of the oncogene c-Myc, leads to considerable side effects in offspring of chimeric mice.

successfully re-program somatic cells of mice or humans and to result in the re-expression of pluripotency markers [12,13]. However, the resulting cell hybrids will inevitably contain two sets of chromosomes, which may limit the use of this method for clinical applications. Enucleation of ES cells before fusion may not be feasible in circumventing this problem as it has been shown to abolish the ability of the remaining ES cytoplasm to re-activate expression of pluripotency markers in hybrids with somatic cells [13]. The selective removal of chromosomes is possible but may be unfeasible for the complete set [14].

#### Incubation of somatic cells with extracts of pluripotent cells

Direct exposure of human somatic cell lines to the extracts of *Xenopus* oocytes [15], embryonic germ cell [16], embryonic carcinoma cells, or ES cells [17] seems to have met with partial success in the reversion of some aspects of cell differentiation, mainly in the HEK293T cell line. In most cases re-expression of pluripotency marker, especially Oct3/4, were reported. It has been suggested that some studies cannot properly exclude the possibility that the reported re-expression of pluripotency-associated genes is due to material from the pluripotent cells [8]. Taranger *et al.* [17] found some indicators of multi-lineage differentiation potential in re-programmed HEK293T cells.

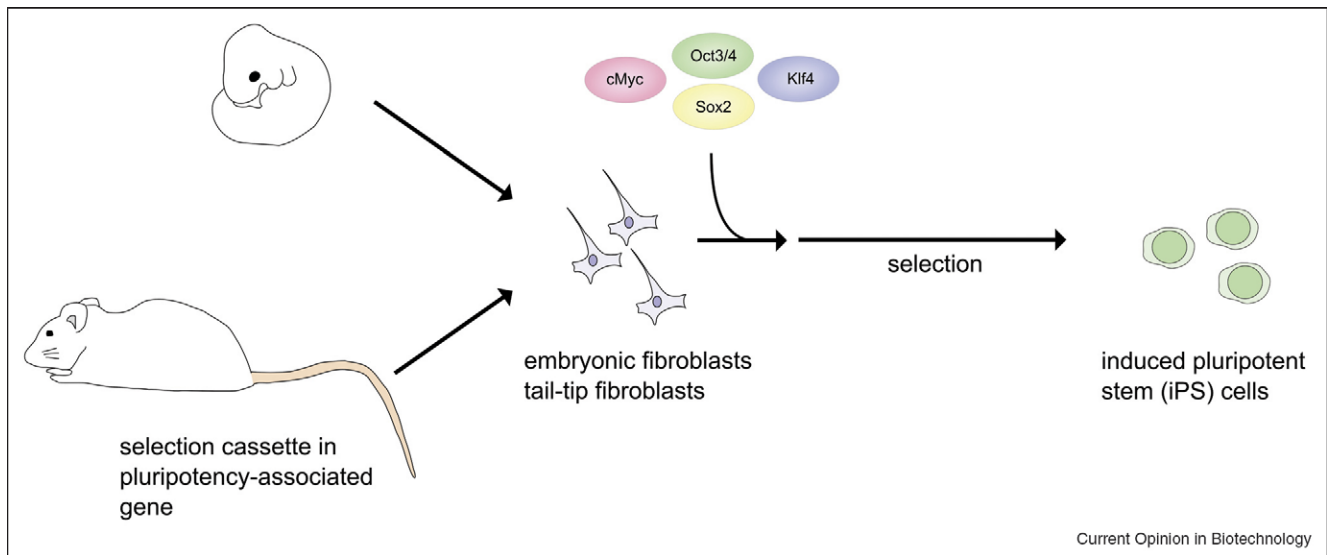
#### Reprogramming somatic cells towards pluripotency by defined factors

A fourth approach (Figure 1) seeks to re-program somatic cells by use of defined factors known to be involved in determining pluripotency in cells [10<sup>••</sup>,18].

To establish factors necessary for reprogramming differentiated cells, initially mouse embryonic fibroblasts (MEFs) and tail-tip fibroblasts (TTFs) of mice homozygous for a knockin of a neomycin-resistance cassette into the Fbx15 (also known as Fbxo15) gene locus were employed. Fbx15 expression is restricted to early embryonic development and ES cells but it is dispensable for mouse development and maintenance of pluripotency [19]. Initially, 24 factors with implication in pluripotency were retrovirally transduced into Fbx15-reporter-fibroblasts. It was reasoned that upon becoming pluripotent, cells would express the neomycin resistance gene under control of the Fbx15 promoter and thus become resistant to G418 selection. Selection of cells indeed allowed growth of ES-cell-like colonies and cells derived by sub-culturing these ES-like colonies were designated as 'induced pluripotent stem (iPS)' cells. The initial number of 24 candidate factors was by exclusion of single factors stepwise reduced to only four genes which, on their own, were sufficient to induce iPS cells: Sox2, Oct3/4, Klf4 and c-Myc. Colonies derived with only three factors, either lacking c-Myc or Sox2, were morphologically different from iPS/ES cells.

iPS cells (Figure 2) exhibited morphology and growth properties similar to ES cells and did not undergo senescence as their parental primary fibroblasts. The cells expressed a large number of pluripotency markers, although some only to a lower level compared to ES cells. Notable exceptions were endogenous levels of Oct3/4 and Sox2 that remained low in many clones. Epigenetic changes included a decrease in dimethylation of histone 3 lysine 9 (diMeH3K9) in the Oct3/4 and Nanog promoter

Figure 1



Generation of iPS cells from differentiated fibroblasts. iPS cells are generated from Fbx15-, Oct3/4- or Nanog-reporter mouse embryos or adult animals. After retroviral transduction with the four transcription factors Oct3/4, Sox2, c-Myc and Klf4, cells are selected for upregulation of marker gene activity. After two or more weeks colonies of cells with properties similar to pluripotent embryonic stem cells, induced pluripotent stem (iPS) cells, appear.

regions combined with an increase in histone 3 acetylation. Nevertheless, CpG islands in the Oct3/4 and Nanog regions remained methylated in comparison to ES cells. Microarray analysis of the global gene expression indicated upregulation of many genes in iPS cells that are expressed specifically in ES cells but not in mouse embryonic fibroblasts. However, there were still considerable differences in expression patterns of iPS and ES cells.

iPS cells exhibited, in spite of the various differences to pluripotent ES cells, functional properties that are considered indicators of pluripotency in cells. They contributed in embryoid bodies, teratomas, as well as in chimeric mouse embryos, to tissues originating from all three germ layers. Chimeric mice derived from iPS cells selected for Fbx15 expression were observed up to day E13.5 but no live chimeras were born.

After the initial findings with Fbx15-selected iPS cells, three laboratories demonstrated a marked improvement in quality of iPS cells. All chose either a reporter associated with Nanog [20<sup>•</sup>,21<sup>•</sup>,22<sup>•</sup>] or with Oct3/4 [22] expression in place of Fbx15 expression as indicators of cell reprogramming since both factors are thought to be more stringently associated with pluripotency than Fbx15.

It was commonly observed that the quality of generated iPS cells increased with an increased interval between initial retroviral transduction and onset of selection. Unlike in Fbx15-iPS cells, the expression of retroviral transgenes was subsequently silenced in Nanog- and

Oct3/4-iPS cells. The silencing of retroviral transgenes has been suggested to be mediated by *de novo* methylation as found in silencing of viral genes in embryonic cells [22].

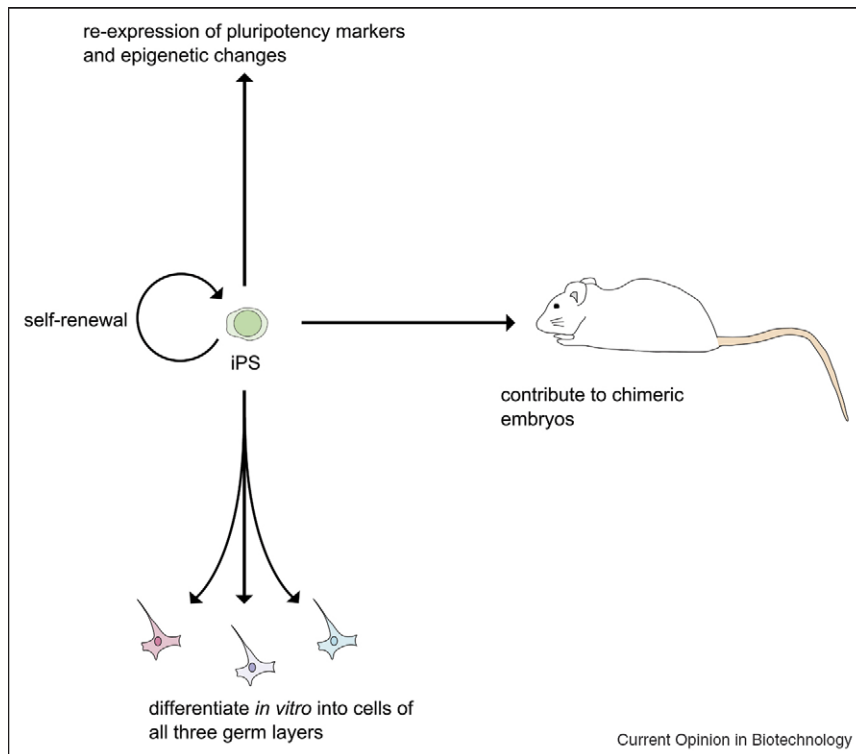
Sox2 and Oct3/4, as well as other markers of pluripotency, were continuously and stably expressed from endogenous loci and the endogenous expression of Oct3/4 was demonstrated to be sufficient for the maintenance of pluripotency [20<sup>•</sup>].

The Nanog- and Oct3/4-promoter regions were unlike in Fbx15-iPS cells efficiently de-methylated and global gene-expression patterns were remarkably similar in iPS and ES cells [21<sup>•</sup>,22<sup>•</sup>]. Although Nanog-iPS gene expression patterns were more similar to ES cells than were Fbx15-iPS expression patterns, there were still differences, notably in the case of the pluripotency marker Rex1 with a stronger presence in ES cells [21<sup>•</sup>].

Nanog- and Oct3/4-iPS share with ES cells a bivalent pattern of histone trimethylation on histone 3 lysine 4 (triMeH3K4), associated with transcribed genes, and on histone 3 lysine 27 (triMeH3K27) that is associated with silenced genes [20<sup>•</sup>,22<sup>•</sup>,23].

Functionally, Nanog- and Oct3/4-iPS cells contributed in teratomas to tissues derived from all three germ layers. Both types of iPS cells also contributed, in contrast to Fbx15-iPS cells, to live chimeric mice and demonstrated potential for germline transmission [20<sup>•</sup>,21<sup>•</sup>,22<sup>•</sup>].

Figure 2



iPS cells mimic properties of pluripotent cells.

It was possible to raise offspring of chimeric mice derived from one Nanog-iPS clone. However, the disadvantages of the retroviral transduction system were emphasised by frequent occurrence of mainly neck tumours associated with reactivation of the c-Myc-transgene expression in the tumour tissue [21].

ES-like Nanog- and Oct3/4-iPS cells are clearly an important step towards the generation of fully pluripotent cells by only four transcription factors. A closer look at the four

factors reveals their deep involvement in the maintenance of pluripotency and may explain at least part of their individual contribution to iPS cell generation (Table 2).

### Functions of the individual transcription factors necessary for somatic cell reprogramming

#### Oct3/4

The transcription factor Oct3/4 is strongly involved in the maintenance of self-renewal of pluripotent cells. Its

**Table 2**

#### Minimum number of factors required for iPS cell generation

Transgene	Known functions in maintenance of pluripotency
Oct3/4	Oct3/4 is a tightly regulated transcription factor that is associated with a large number of target genes implicated in maintenance of pluripotency. Regulatory elements in target genes are often in close vicinity of Sox2-binding sites. Oct3/4 is likely to be a key factor in the transcriptional framework of self-renewing stem cells.
Sox2	The transcription factor Sox2 is necessary for embryonal development and to prevent ES cell differentiation. Although many ES cell pluripotency-associated genes are co-regulated by Sox2 and Oct3/4, Sox2 may also cooperate with other transcription factors, for example Nanog, to activate transcription of pluripotency markers.
c-Myc	c-Myc, a helix-loop-helix/leucine zipper transcription factor, takes part in a broad variety of cellular functions. It has been implicated in LIF receptor signalling as a downstream effector of STAT3. In Wnt signalling c-Myc is a substrate for GSK3 $\beta$ . In iPS cells, c-Myc may compensate anti-proliferative effects of Klf4.
Klf4	Klf4, the fourth member of the quartet, is a Krueppel-type zinc finger transcription factor. It can act as an oncogene but also as a tumor suppressor protein. Klf4 is like c-Myc a STAT3 target in the LIF pathway and its overexpression inhibits differentiation of ES cells. Klf4 upregulates, in concert with Oct3/4, Lefty1 transcription but the role as co-factor for Oct3/4 may be limited to only a few targets. Klf4 can repress p53, a negative regulator of Nanog.

expression level appears to require tight regulation as repression in ES cells leads to differentiation into trophoectoderm-like cells while overexpression induces differentiation into various lineages including primitive-endoderm-like cells [24]. Genome-wide studies in human and mouse revealed a large panel of target genes with Oct-regulatory elements and many targets have frequently been implicated in ES cell signalling. A large number of these genes possesses regulatory elements for the transcription factors Sox2 and Nanog in close proximity that were found to be co-occupied in genes specifically positively or negatively regulated in ES cells [25,26]. Target genes of Oct3/4 in co-regulation with Sox2 include Sox2 and Oct3/4 in a positive regulatory loop [27] and the Nanog protein [28,29]. A number of putative regulatory factors for Oct3/4 have been identified [2]. These include the enhancers Lrh1 [30], retinoic acid receptor:retinoid X receptor heterodimers [31,32] and SF1 [33] and the repressors Cdx2 [34], COUP I+II [31,32] and Gcnf [35]. Gcnf has been shown to recruit the de-novo methyltransferase Dnmt3 to the Oct3/4 promoter and to promote its methylation [36].

The exogenous expression of Oct3/4 is likely to support, in cooperation with the transcription factor Sox2, the maintenance of the basic transcriptional framework required for the ES-like properties of iPS cells.

### Sox2

The transcription factor Sox2 (SRY-type high mobility group box 2) is part of a large family of 20 proteins that share a similar HMG box DNA-binding motif. So far, it is the only Sox-protein found to have a crucial function in sustenance of ES cell pluripotency. The related Sox-family member Sox15 is also present in ES cells, however, unlike its counterpart Sox2, it is not required for embryonal development [37,38]. Downregulation of Sox2 in murine ES cells by RNA interference promotes ES cell differentiation [39]. Sox2 regulatory elements in gene promoter regions are often found in close proximity to Oct3/4 and Nanog binding sites [25]. Several genes specific to ES cells are transcriptionally regulated by the combined action of Sox2 and Oct3/4. These include apart from Sox2, Oct3/4 and Nanog genes, as mentioned in the Oct3/4 section, also Fgf4 [40], Utf-1 [41], Fbx15 [19] and Zfp206 [42]. It is possible that other ES-cell associated genes are regulated by combination of Sox2 with different transcription factors as in the case of Rex1, which is mainly activated by a combination of Sox2 and Nanog [43].

### c-Myc

The helix-loop-helix/leucine zipper transcription factor Myc is associated with a number of cellular functions including cell growth, differentiation and proliferation but also with oncogenic transformation. In myeloid [44] and neuronal [45] cell lineages Myc has been found to

block differentiation and for epidermal [46] and haematopoietic [47] stem cell populations it has also been associated with the maintenance of the stem cell pool. c-Myc has been proposed as a major downstream target for two pathways that support maintenance of pluripotency: the LIF (leukaemia inhibitory factor)/STAT3 and the Wnt signalling cascades. Its potential in stem cell renewal has been demonstrated by ability of ES cells expressing a stable c-Myc mutant, to contribute to chimeric mice even when cultured in absence of LIF [48].

However, its specific role in self-renewal of ES cells has not been thoroughly characterised yet. The first pathway associated with c-Myc, LIF signalling, is routinely used in murine ES cell culture but it does not appear to be necessary for the culture of human ES cells [49]. LIF triggers by binding to a hetero-dimeric LIF-receptor a signalling cascade that results in activation and nuclear translocation of the transcription factor STAT3. STAT3, when overexpressed, is sufficient for the continued self-renewal of mouse ES cells even in absence of LIF [50]. c-Myc transcriptional activation was found to be one of the downstream targets of STAT3 in ES cells [48].

Wnt signalling can, independently of the LIF/STAT3 pathway, maintain murine and human stem cell populations. The Wnt signal is thereby thought to act via inhibition of GSK3 $\beta$ . BIO, a GSK3 $\beta$ -inhibitor, can bypass the need for Wnt-activation and supports self-renewal of ES cells in the absence of Wnt-ligand [51]. c-Myc had been described in a different cellular context as a substrate for GSK3 $\beta$  kinase activity that, when phosphorylated, undergoes subsequently quick ubiquitination and proteasomal degradation [52]. A similar regulation mechanism also appears to apply for c-Myc-levels in ES cells. Here, the activation of Wnt signalling maintains increased total levels of c-Myc [48]. Some evidence points to a crosstalk between the LIF and the Wnt pathways via PI3K activation by LIF [53] or by Wnt-mediated upregulation of STAT3 [54].

Apart from its direct involvement in supporting self-renewal, c-Myc may, in iPS cells, also counter anti-proliferative activation of p21Cip1 by Klf4 [55]. In addition, c-Myc may open up chromatin of somatic cells by binding to numerous sites in the genome and by recruiting histone acetylase complexes [56].

### Klf4

The Kruepel-type zinc-finger transcription factor Klf4 is, like c-Myc, a downstream target of activated STAT3 in LIF-induced ES cells. Its overexpression leads to sustained expression of Oct3/4 and inhibition of differentiation in ES cells [57]. Similar to Sox2, Klf4 can also act as a co-factor for Oct3/4-mediated regulation of gene transcription. However, this seems to apply only to a very limited number of genes, including Klf4 itself and Lefty1

[58]. There is so far no evidence that Klf4 is required to exert a similar function for other target genes of Oct3/4 or Sox2.

Klf4 may be indirectly involved in the upregulation of Nanog protein by repressing p53 [59], a negative regulator of Nanog [60]. In addition, Klf4, by suppressing p53, could in iPS cells act as a counterweight for pro-apoptotic properties of c-Myc.

## Conclusion

iPS cells, generated by introduction of Oct3/4, Sox2, Klf4 and c-Myc transcription factors into fibroblasts, share many properties with ES cells. Additionally, contribution of iPS cells to live adult chimeric animals and germline transmission clearly demonstrate that re-programming, to cells very similar to pluripotent ES cells, was achieved. However, considering the high number of tumours in offspring with c-Myc-transgene reactivation, it will be critical for any application of re-programmed somatic cells in a medical setting to bypass the stable overexpression of retroviral transgenes. Future work will have to address this problem either by using a transient expression system or possibly by substitution of c-Myc with another factor that can replace its role in re-reprogramming without the side effects. Other oncogenes had been included in the initial panel of 24 factors but only a combination of c-Myc and Klf4 was able to fulfil the requirements for iPS induction.

It is also still unknown why the efficiency of iPS cell generation is very low. This may be due to only limited subpopulations of fibroblasts being susceptible to conversion or only a small number of cells that achieve an optimal balance of transgene expression. It also possible that optimising yet still unknown factors as for example time intervals will increase the efficiency.

And finally one of the most important questions remains unanswered: Will it be possible to generate iPS cell from human cells?

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