Nuclear reprogramming and pluripotency

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The cloning of mammals from differentiated donor cells has refuted the old dogma that development is an irreversible process. It has demonstrated that the oocyte can reprogramme an adult nucleus into an embryonic state that can direct development of a new organism. The prospect of deriving patient-specific embryonic stem cells by nuclear transfer underscores the potential use of this technology in regenerative medicine. The future challenge will be to study alternatives to nuclear transfer in order to recapitulate reprogramming in a Petri dish without the use of oocytes.

Cells of a multicellular organism are functionally heterogeneous owing to the differential expression of genes. Historically, differential gene expression had been thought to involve the genetic elimination of those genes that were silenced and the retention of others that were expressed in a particular tissue. Cloning experiments in amphibians and mammals laid this idea to rest[1]. They unequivocally demonstrated that adult cells are genetically equivalent to early embryonic cells, and that differential gene expression is the result of reversible epigenetic changes that are gradually imposed on the genome during development[2,3]. The reversal of the differentiation state of a mature cell to one that is characteristic of the undifferentiated embryonic state is defined here as nuclear 'reprogramming'. Reprogramming by nuclear transfer has been a unique tool for functionally testing nuclear potency, and for distinguishing between genetic and epigenetic alterations of various donor cells[4–8]. The successful treatment of an animal model of disease by nuclear-transfer-derived embryonic stem (ES) cells (NT ES cells)[9] and the prospect of deriving patient-specific human ES cells by nuclear cloning have underscored the potential use of this technology for custom-tailored cell therapy. However, although nuclear transfer remains the tool of choice for studying reprogramming at a functional level, alternative, more amenable approaches are needed for dissecting reprogramming at the cellular, molecular and biochemical levels.

This review discusses the different strategies that have been used to induce the conversion of a differentiated cell into an embryonic pluripotent state, including nuclear transfer, cellular fusion, the use of cell extracts and culture-induced reprogramming (Fig. 1). We critically discuss the criteria for assessing reprogramming at the functional and molecular levels using different approaches, and speculate on the molecular mediators that might facilitate reprogramming and the maintenance of pluripotency. In essence, reprogramming remains largely phenomenological; thus, future efforts should aim to dissect reprogramming at the molecular and biochemical levels.

Reprogramming by nuclear transfer

Most adult tissues contain a heterogeneous population of cells with a hierarchy of multipotent stem cells, progenitor cells and terminally differentiated cells. When 'Dolly' the sheep and other mammals were initially cloned from adult cells, the question remained of whether terminally differentiated cells are genetically totipotent. This was mainly owing to the lack of genetic markers that could unambiguously prove the differentiation state of the donor cell. The cloning of mice from mature lymphocytes that carried differentiation-associated immune-receptor rearrangements[5], and from genetically labelled postmitotic olfactory neurons[6,8], demonstrated that terminal differentiation does not restrict the potential of a nucleus to support development of an animal. In other words, the epigenetic changes that direct terminal differentiation and permanent exit from the cell cycle are reversible.

Cancer cells usually undergo both genetic and epigenetic changes that cause a block in terminal differentiation, and lead to uncontrolled growth. It has been unclear to what extent epigenetic changes contribute to tumorigenesis, and whether these changes are reversible. Nuclear transfer was used to address this question by cloning embryos from brain tumour[7] and melanoma cells[4]. Chimaeric mice generated by injection of embryo-derived ES cells into blastocysts were normal[4], indicating that the phenotype of some, but not all, of the tested cancer cells was largely due to epigenetic alterations that were reversible by nuclear transfer. Together, these observations underscored the importance of epigenetic modifications in regulating normal development, terminal differentiation and disease.

The generation of animals by nuclear transplantation is extremely inefficient, with most clones dying soon after implantation, and the few clones that survive beyond birth often being afflicted with severe abnormalities, such as obesity[10] and premature death[11]. Early experiments with amphibians demonstrated an inverse relationship between the age of the donor cell and clone survival[12]. Therefore, an important question has been whether the state of donor-cell differentiation affects the efficiency of reprogramming in mammals. Reprogramming can be measured functionally by evaluating clone development at several different levels, including the rate of blastocyst formation, the fraction of cloned embryos surviving to birth or adulthood after implantation into the uterus, and the frequency with which pluripotent ES cells can be derived from cloned blastocysts explanted into culture (Fig. 2b). Pre-implantation development of reconstructed oocytes into blastocysts is particularly sensitive to experimental parameters, such as the cell-cycle stage and physical condition of the transferred nucleus, and is therefore not a useful measure of reprogramming efficiency. For example, the cell-cycle stage of the donor nucleus needs to be in synchrony with the metaphase-arrested oocyte. Because different donor-cell populations have different cell-cycle profiles, embryos cloned from asynchronous cell populations will undergo cleavage divisions at different efficiencies. Owing to this experimental variability during cleavage, measuring the fraction of live pups derived from reconstructed oocytes is also not a reliable criterion for quantifying reprogrammability. However, once an embryo has reached the blastocyst stage, it has a relatively consistent probability of developing into a mouse

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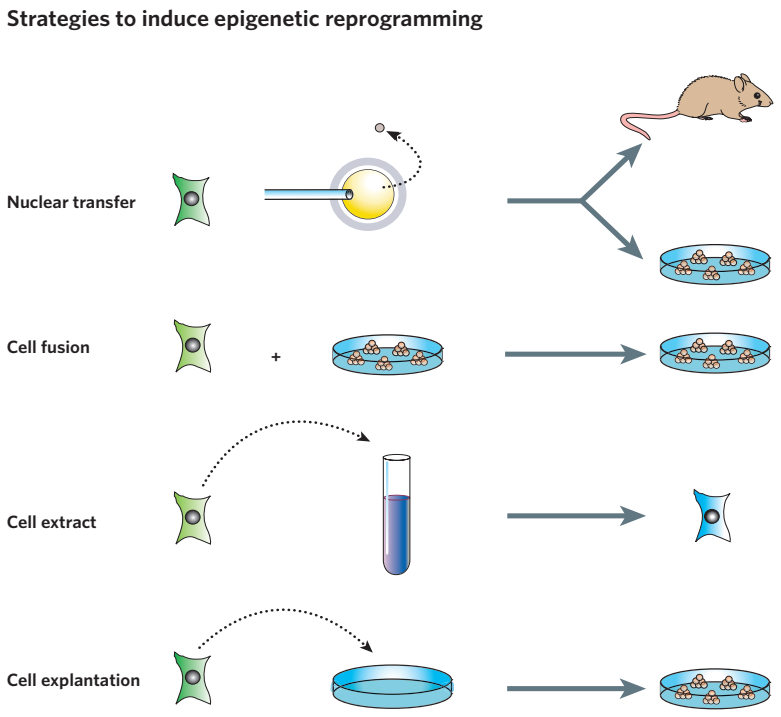

Experimental approach Mechanistic insights	Limitations
Reproductive cloning: functional test for reprogramming to totipotency	Reproductive cloning is very inefficient
Allows epigenetic changes (reversible) to be distinguished from genetic changes (irreversible)	There are abnormalities at all stages of development
Somatic cell nuclear transfer: efficient derivation of genetically matched ES cells with normal potency	
Nuclear reprogramming of somatic genome in hybrids generated with pluripotent cells	Fusion rate is very low
Allows study of genetics of reprogramming	Tetraploid cells are generated
Exposure of somatic nuclei or permeabilized cells to extracts from oocytes or pluripotent cells	No functional reprogramming done
Allows biochemical and kinetic analysis of reprogramming	
Explantation in culture selects for pluripotent, reprogrammed cells	May be limited to germ line cells
Allows study of genetics of reprogramming	

Figure 1 | Different approaches for studying nuclear reprogramming. Illustration of the four major strategies used for studying nuclear reprogramming (left), and summary of the gained mechanistic insights and limitations of each strategy (right). First, nuclear transfer involves the injection of a somatic nucleus into an enucleated oocyte, which, upon transfer into a surrogate mother, can give rise to a clone (reproductive cloning), or, upon explantation in culture, can give rise to genetically matched ES cells (somatic cell nuclear transfer). Second, cell fusion of differentiated cells with pluripotent ES cells results in the generation of hybrids that show all features of pluripotent ES cells. Third, exposure of somatic cells or nuclei to cell extracts from oocytes or ES cells recapitulates early biochemical events of reprogramming without stably changing cell fate. Fourth, explantation of germ cells in culture selects for immortal cell lines that have regained pluripotency.

or giving rise to ES cells. Therefore, measuring the potential of a cloned blastocyst to generate a viable clone or ES cells provides more defined readouts for cloning efficiency than just development through cleavage stages. Figure 2 illustrates the variability in cloning efficiencies when comparing the different experimental readouts.

Previous cloning experiments with blastomeres of the cleavage-stage embryo suggested that pluripotent nuclei support clone development at high efficiency[13,14]. Similarly, the cloning of mice from pluripotent ES cells[15,16] has been shown to be more efficient than cloning from adult cells, such as fibroblast[17], cumulus[2] or Sertoli cells[18] (Fig. 2b; Table 1). Moreover, the derivation of ES cells from cloned blastocysts is significantly more efficient than the generation of mice from cloned blastocysts transferred into the uterus (Fig. 2b; Table 1). Explantation of cloned blastocysts in culture might, unlike fetal development, be under fewer time constraints and could select for the outgrowth of rare reprogrammed cells into stable ES-cell lines, thus resulting in an apparently higher reprogramming efficiency. Alternatively, reprogramming might not be restricted to the oocyte stage, but might continue in the inner cell mass of the blastocyst and, hence, give rise to NT ES cells at high efficiency. This observation might also explain why cloned mice from terminally differentiated neurons could only be generated using a 'two-step' procedure involving the initial derivation of NT ES cells, followed by the subsequent generation of cloned mice from NT ES-cell nuclei by a second round of nuclear transplantation[6]. The finding that mature natural-killer T (NKT) cells can give rise to cloned mice by a single round of nuclear transfer[19] argues, however, that some terminally differentiated cells can generate clones following direct implantation into the uterus. Together, these results suggest the following conclusions: first, that mammalian nuclei, similar to those of amphibians[12], become more refractory to reprogramming with differentiation; second, that blastocyst formation and ES-cell derivation, in contrast to fetal development, are less restrained by genetic and epigenetic abnormalities; and third, that ES-cell derivation from cloned blastocysts is significantly more efficient than the potential of cloned blastocysts to grow into live pups.

Whether the genomes of adult stem cells are similar to ES cells in that they are easier to reprogramme than the genomes of terminally differentiated cells is an open question. To address this, Blelloch *et al.* compared the cloning efficiencies of ES cells with that of cultured neural stem (NS) cells and differentiated fibroblasts[20]. Cloned blastocysts produced from NS cells gave rise to ES cells about as efficiently as do cloned blastocysts derived from ES cells, and about two to three times more efficiently than do cloned blastocysts generated from fibroblast donors. In addition, fibroblasts that had been engineered to contain reduced levels of global DNA methylation were as efficient at being donors as were NS cells and ES cells, thus supporting the notion that the epigenetic state of donor cells affects the reprogrammability of cells, either through experimental manipulation or in the context of differentiation. However, this observation may not be true for all adult stem cells, because haematopoietic stem cells seem to be more efficient nuclear-transfer donors[21] than differentiated B and T cells[5], but less efficient donors than differentiated NKT cells[19] of the same cell lineage (Table 1).

The developmental defects observed in reproductive cloning indicate faulty epigenetic reprogramming that should manifest itself in aberrant gene expression. Indeed, clones at various developmental stages, and even adult clones, show severe dysregulation of gene expression, with some genes being dysregulated in a donor-cell-dependent manner[22–24]. The persistence of donor-cell-specific gene expression in clones indicates the retention of an 'epigenetic memory'[22,25] of the donor nucleus, and might explain the observation that mice cloned from unrelated donor cells may suffer from different abnormalities[10,11]. Faithful reprogramming of the somatic genome and complete elimination of the epigenetic memory of the donor nucleus seem to require passage through the germ line, because abnormalities are not seen in the offspring of clones[10].

The notion that reproductive cloning fails to fully reprogramme a somatic nucleus raises the biologically and therapeutically relevant question of whether reprogramming is ever complete in ES cells derived from cloned blastocysts. Three lines of evidence suggest that this is, indeed, the case. First, NT ES-cell lines, once established, grow as immortal cell lines and produce pluripotent tumours when injected into immunocompromised mice[26]. Second, analyses of the developmental potential of ES cells by tetraploid embryo complementation indicated that NT ES cells can give rise to entirely ES-cell-derived mice that appear normal at an expected frequency[5,6,8,9]. Thirdly, global gene-expression profiling of NT ES-cell lines derived from different donor-cell types reveals transcriptomes that are indistinguishable from those of fertilization-derived ES-cell lines[27]. The process of ES-cell derivation seems to rigorously select for immortal cells that have undergone, or continue to undergo, complete reprogramming to pluripotency, and, therefore, ES-cell lines derived by nuclear transfer are expected to have the same therapeutic potential as those derived from fertilized embryos.

An important question raised by these experiments is whether blastocysts and ES-cell lines can be derived by nuclear transfer from humans. Initial attempts to produce nuclear-transfer blastocysts from somatic donor cells have been unsuccessful[28] (Table 2). However, cloned blastocysts have been generated by injecting human donor nuclei into enucleated rabbit oocytes[29]. These blastocysts failed to grow into stable ES-cell lines, which might have been owing to respiration defects caused by nuclear–mitochondrial incompatibility[30] — a phenomenon that has been observed in interspecies cell hybrids. An initial report on monkey cloning identified spindle abnormalities as a potential obstacle in primate somatic-cell nuclear transfer[31], yet these difficulties were later overcome by modifying the nuclear transfer procedure[32]. Moreover, cloned monkeys have been generated from embryonic donor cells[33], suggesting that the cloning of primates works in principle. Although these and related questions regarding human nuclear transfer, such as the issue of the supply of human oocytes, clearly need to be resolved, alternative approaches to reprogramming deserve attention.

Reprogramming by cell fusion

Fusion between different cell types has been used to study the plasticity of the differentiated state[34] (Fig. 1). In most hybrids, the phenotype of the less-differentiated fusion partner is dominant over the phenotype of the more-differentiated fusion partner. Consistent with this, Miller and Ruddle showed, in 1976, that the fusion of pluripotent teratocarcinoma cells with primary thymocytes resulted in the formation of pluripotent hybrids that shared all their features with the parental embryonal carcinoma (EC) cells, including the potential to induce tumours[35]. The dominance of pluripotent cells over differentiated cells has also been shown in cell hybrids made between somatic cells and murine embryonic germ (EG)[36,37] and ES[36,38] cells, and this reprogramming potential seems to be conserved in human ES cells[39,40].

A crucial question raised by these experiments was whether the chromosomes of the somatic cell had been reprogrammed to pluripotency, or whether they were simply retained as silent cargo. At the molecular level, the reactivation of the silent X chromosome in female lymphocyte–ES-cell hybrids[38], the demethylation and reactivation of genes essential for pluripotency[38,39], and the expression of genes representative of all three germ layers in teratomas produced from hybrids[36] supported the interpretation that the somatic chromosomes had undergone epigenetic reprogramming. To test reprogramming at a functional level, F9 EC cells that can normally only produce undifferentiated tumours were fused with thymocytes to score for an increase in the differentiation potency of tumours. The majority of hybrid cells gave rise to well-differentiated tumours, consistent with the notion that the thymocyte genome had been functionally reprogrammed to pluripotency by the EC cell[41]. However, independent fusion experiments between EC cells and differentiated cells came to the opposite conclusion[42], and suggested that the differentiated phenotype of tumours might have been due to the loss or dilution of an amplified gene that blocked differentiation in EC cells, rather than the reprogramming of the somatic genome. So far, there has been no convincing functional

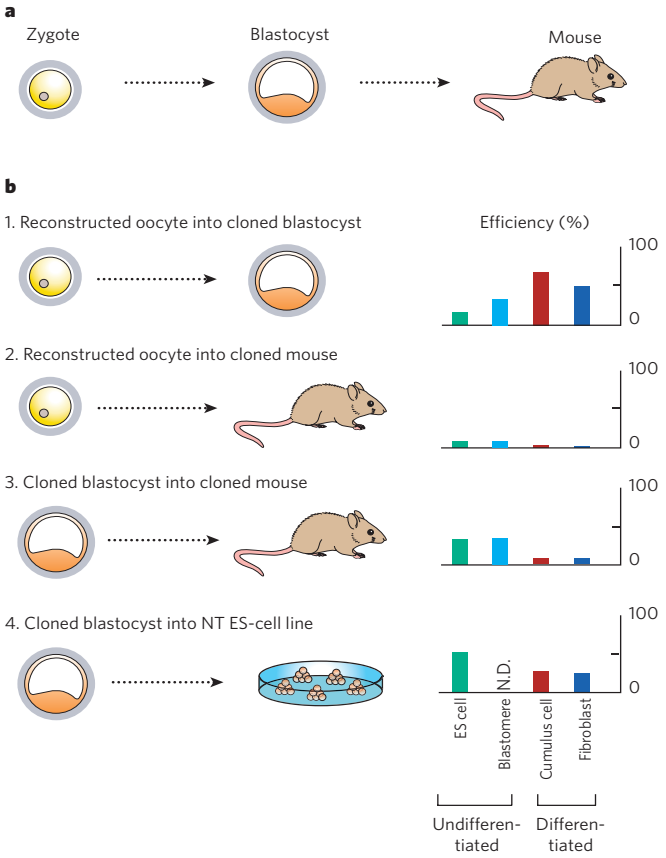

Figure 2 | Differentiation and cloning efficiency. **a,** Illustration of normal development of a mouse from pre-implantation development (zygote into blastocyst) to post-implantation development (blastocyst into mouse). **b,** The left side of the figure depicts four ways of assessing clone development by measuring pre-implantation development (1), a combination of pre-implantation and post-implantation development (2), post-implantation development (3) and the potency of a cloned blastocyst to give rise to ES cells (4). The right side of the figure shows the cloning efficiencies of four different donor-cell types for each category. ES cells and blastomeres are examples of undifferentiated cells, whereas cumulus cells and fibroblasts are examples of differentiated cells. Note the lack of correlation between donor-cell-differentiation state and cloning efficiency when considering the first two categories compared with the second two.

Table 1 | Cloning efficiencies of different donor cell types from different developmental stages

Donor cell	Blast into mouse (%)*	Blast into ES cell (%)†	References
Fertilized egg	60–80	25–68‡	97
Blastomere	13–26‡	ND	14
ES cell	11–23	50	15,16,61
EC cell	ND	50	61
NS cell	ND	64	20
Haematopoietic stem cell	0.7	ND	21
Sertoli cell	6‡	27‡	18,97
Cumulus	1–3‡	9–19‡	2,97
Fibroblast	1‡	13–33‡	17,97
Melanoma	ND	25	4
NKT cell	1–2‡	6‡	19
B/T cell	ND	7	5, unpublished data
Neuron	ND	6–28	6,8

*The efficiency in terms of the percentage of mice obtained from the transfer of cloned blastocysts into surrogate mothers. †The percentage efficiency of deriving NT ES cells from cloned blastocysts explanted in culture. Note the high frequency of NT ES-cell derivation from cloned blastocysts compared with the frequency of mice produced from cloned blastocysts. ‡The combined number of morulae and blastocysts that gave rise to ES cells or mice. ND, not determined.

evidence showing that the somatic donor nucleus has been fully reprogrammed and has re-gained the potential to sustain pluripotency or to direct differentiation in the absence of the ES-cell genome.

Two key questions arising from fusion experiments are whether the ES-cell nucleus or cytoplasm is required, and whether DNA replication is needed for reprogramming. The first question was addressed by separating the nuclear compartment (karyoblast) from the cytoplasmic compartment (cytoblast) of an ES cell; these elements were then individually fused with neuronal cells isolated from neurospheres[43]. In hybrids produced with the ES-cell karyoblasts, reactivation of an *Oct4*–green fluorescent protein transgene was detected. By contrast, fusion of neurosphere cells with ES-cell cytoplasts gave no green fluorescent protein signal, suggesting that nuclear factors are essential for molecular reprogramming. This conclusion is consistent with cloning experiments in amphibians[44] and mice[2], which indicate that successful reprogramming depends on direct injection of nuclei into the germinal vesicle or into a metaphase oocyte, where nuclear factors are available in the cytoplasm.

The requirement for DNA replication for reprogramming is less clear. Although one ES cell–somatic cell fusion experiment suggested that replication is essential for reprogramming[43], nuclear transfer experiments indicated the presence of a replication-independent mechanism, possibly involving an active DNA demethylase[45]. The different results might be due to biological differences in the cell types (ES cell versus oocyte) and/or technical differences in the assays (cell fusion versus nuclear transfer) used.

Reprogramming of somatic cells to pluripotency is a potentially attractive approach to generate customized cells for therapy without having to rely on nuclear transfer[39]. However, for this approach to be viable, the ES-cell nucleus needs to be removed from the hybrid in order to generate diploid customized cells for transplantation therapy. If DNA replication and cell division are required for complete reprogramming it will be difficult, if not impossible, to selectively eliminate the entire set of ES-cell chromosomes from the hybrids.

Reprogramming by cell extracts

Using cell-free systems to study reprogramming is an attractive alternative to nuclear transfer or cell fusion (Fig. 1). Importantly, extracts can be used that might allow the purification of protein complexes involved in reprogramming. For example, exposure of permeabilized somatic frog cells to extracts prepared from *Xenopus* eggs showed that the somatic-cell-specific TATA-binding protein TBP is actively dissociated from chromatin through the activity of the ATP-dependent chromatin-remodelling factor ISWI[46], suggesting that major chromatin-remodelling complexes are involved in reprogramming. In a different set of experiments, human cells were exposed to *Xenopus* cell extracts and elevated *OCT4* transcript levels were detected following treatment[47]. However, the latter study could not exclude the possibility that the detected *OCT4* signal came from a transcribed *OCT4* pseudogene or a *Xenopus Oct4* homologue, rather than from the reactivation of the endogeneous OCT4 locus. In addition, no stable reprogramming was seen in reversibly permeabilized somatic cells that were subsequently passaged in culture, suggesting that an intact oocyte might be required for functional de-differentiation to a pluripotent state.

Xenopus egg extracts have proved useful in solving an old puzzle in frog cloning. A recent study showed that efficient chromatin remodelling of differentiated nuclei depends on their exposure to mitotic egg extract, thereby facilitating embryonic DNA replication[48]. Why frog cloning works better when using serial nuclear transfer has remained a mystery for decades[49]. Serial nuclear transfer involves injecting the nucleus of a differentiated cell into an enucleated egg, allowing the cloned embryo to undergo a few cell divisions and then transferring the nucleus from one of the daughter cells into another enucleated egg. The biochemical approach now explains why embryos cloned serially from nuclei that have undergone previous rounds of embryonic DNA replication are more efficient donors than embryos cloned directly from adult cells.

In a conceptually simpler approach to induce reprogramming, permeabilized 293T cells were exposed for 1 h to extracts from EC cells[50] or T-cell lines[51], and OCT4 or T-cell-receptor (TCR) expression, respectively, was detected by RNA and protein analyses. However, concluding that this indicated reprogramming of the somatic donor cells into other cell types is problematic. The presented data cannot exclude the possibility that gene products of the cells that were used to prepare the extracts were detected. A case in point is the reported TCR activation in reprogrammed fibroblasts. TCR expression requires functional genomic rearrangement of the TCR locus, which was not demonstrated in the reprogrammed fibroblasts, suggesting that the detected signal was due to transient uptake of TCR molecules from the extracts rather than activation of the endogenous TCR locus.

Table 2 | Facts and challenges in primate nuclear transfer

Human nuclear transfer	Initial attempts by the company ACT unsuccessful[28]; recent reports of successful derivation of human NT ES cells fraudulent[98]; supply of human oocytes limiting for human nuclear transfer; possibility of generating ES-cell-derived oocytes[99,100]
Human–rabbit xenotransplantation	Successful generation of cloned blastocysts and inner-cell-mass outgrowths from the injection of human donor nuclei into enucleated rabbit oocytes[29]; however, no stable ES-cell lines generated; possible respiration defects owing to mitochondrial incompatibility[30]
Monkey cloning	Successful with embryonic donor cells[33]; spindle abnormalities observed in one report of somatic nuclear transfer clones[31]; abnormalities were alleviated by optimizing the nuclear transfer procedure[32]

ACT, Advanced Cell Technology.

Culture-induced reprogramming

The approaches discussed so far require the exposure of somatic nuclei to nuclear/cytoplasmic factors of an oocyte or ES cell to elicit nuclear reprogramming. However, under certain physiological conditions, entire cells can de-differentiate or transdifferentiate into another cell fate (Fig. 1). Examples of cellular reprogramming include blastema formation during newt or zebrafish appendage regeneration, transdetermination of imaginal disc cells in flies, and reprogramming of germline cells in mammals and *Drosophila*. This review focuses only on cellular reprogramming in mammals, and the reader is referred to an excellent review in ref. 52 on the different forms of transdetermination and regeneration.

Teratocarcinoma cells were the first pluripotent cells discovered in adult mammals[53]. Teratocarcinomas are members of a class of germ-cell tumours that are composed of a rare population of undifferentiated embryonic cells known as EC cells, as well as a range of differentiated cell types. These tumours have been experimentally shown to originate from primordial germ cells[53] (PGCs), which normally differentiate into oocytes or spermatozoa. The discovery of EC cells within teratocarcinomas prompted scientists to find the equivalent cells in normal embryos, leading to the isolation of pluripotent ES cells[54,55] from pre-implantation-stage embryos, and pluripotent EG cells[56,57] from isolated PGCs. Despite the phenotypic similarities of ES, EG and EC cells, functional and molecular differences exist that probably reflect their different cellular origins. For example, EG and EC cells show a more restricted developmental potential than ES cells, which presumably reflects their origin from PGCs that have lost genomic imprints[58,59]. In addition, EC cells can form tumours when reintroduced into blastocysts[60] and this behaviour correlates with chromosomal changes that have accrued during tumour growth or *in vitro* culturing[61]. Nonetheless, germline contribution has been demonstrated for at least some EG[58,62] and EC[63] cells, thus demonstrating their pluripotency.

The reprogramming of PGCs into EG and EC cells can be detected when comparing the developmental potencies of the cells of origin with their *in vitro* products (Fig. 3). For example, inner-cell-mass cells of the blastocyst and derivative ES cells are both pluripotent, and can give rise to all mouse cell types, including germ cells. Although the derivation of ES-cell lines[54,55] from inner-cell-mass cells probably induces epigenetic changes that facilitate immortal growth, no differences in the developmental potentials have been observed before and after culturing of the cells. In contrast to inner-cell-mass cells and ES cells, PGCs do not contribute to tissues upon transfer into blastocysts[64]. However, EG cells derived from explanted PGCs, and EC cells isolated from teratocarcinomas form

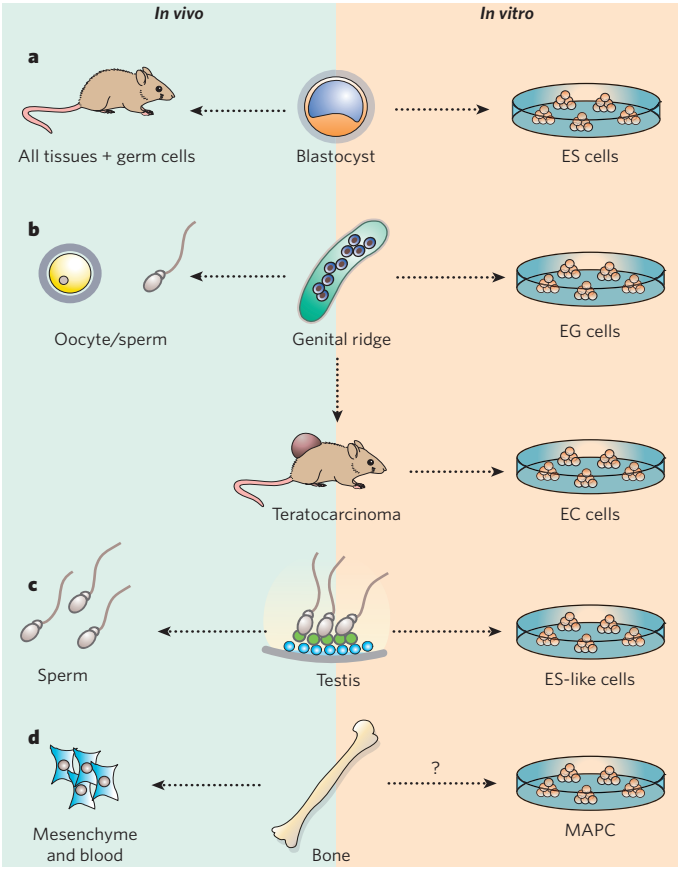

Figure 3 | Culture-induced reprogramming. Inner-cell-mass cells of the blastocyst give rise to all cell types of the body during normal development. **a,** Explantation of blastocysts in culture facilitates the outgrowth of immortal ES-cell lines but no change in developmental potential. **b,** PGCs of the genital ridge give rise to oocytes and spermatozoa *in vivo,* and facilitate the outgrowth of pluripotent EG cells *in vitro*. In teratocarcinomas derived from genital ridges, pluripotent EC cells are found that resemble EG cells. **c,** Spermatogonial stem cells from newborn or adult testes normally differentiate into spermatozoa, but occasionally give rise to pluripotent ES-like cells in culture. **d,** Bone-marrow-derived multipotent adult progenitor cells (MAPCs) might be the *in vitro* counterpart of mesenchymal or blood stem cells that have gained differentiation potential in culture.

tumours in mice with severe combined immunodeficiency disease and contribute to chimaeric animals after injection into host embryos[58,62–64]. Differences between PGCs and derivative pluripotent cells are also seen when comparing the effect of deleting the *Oct4* gene on the phenotype of the cells. Deletion of *Oct4* in PGCs results in apoptosis[65], whereas loss of *Oct4* in ES cells causes differentiation[66]. It might be that selective pressure imposed on the cells by transplantation to ectopic sites or by explantation in culture can relieve PGCs from certain restraints that normally control the terminal differentiation of germ cells. Loss of this control might facilitate proliferation and a gain of developmental potential. Convincing and reproducible evidence for the derivation of pluripotent cells has been confined to cells of the pre-implantation-stage embryo[54,55,67] and the germ line[56,57,68,69]. Germline cells, in contrast to somatic cells, undergo major epigenetic changes during their differentiation, which might render them more suitable for epigenetic reprogramming to pluripotency than somatic cells. It has been suggested, in fact, that all pluripotent cell lines characterized so far, including ES cells, are the product of germ-cell precursors[70]. Therefore, an important issue has been whether pluripotent cells can be derived not only from the embryo but also from adults without previous manipulation of their nuclei.

Several reports have described the derivation of multipotent or pluripotent cell lines from adult tissues, including multipotent adult progenitor cells (MAPCs; Fig. 3) from adult bone marrow[71] and unrestricted somatic stem cells (USSCs) from human newborn umbilical cord blood[72]. These cells were shown to differentiate into cell types indicative of all three germ layers in culture and, when a single MAPC was injected into blastocysts, one extensive chimaera was reported[71]. This is surprising, as MAPCs divide infrequently, and to contribute to somatic tissues of the chimaera must successfully compete with the host epiblast cells that divide with a 6-h cell cycle[73]. Although these results are intriguing, they await confirmation by independent laboratories. Also, it remains to be seen whether MAPCs and USSCs can functionally contribute to somatic tissues in animal models of disease or injury.

Recently, neonatal[68] and adult[69] testis cells were shown to give rise to ES-like cells when exposed to a specific combination of growth factors. ES-like cells expressed all the markers of pluripotent cells, formed teratomas after transplantation and gave rise to chimaeric animals that transmitted to the germ line. Thus, these cells represent the only clear example of the derivation of pluripotent cells from a normal neonatal or adult mammal, and might be useful for studying genetic diseases in different cell lineages. However, a potentially serious concern for any therapeutic application of these cells is the unbalanced genomic imprinting. Parental imprints are erased in PGCs and sequentially re-established in a male-specific or female-specific pattern during subsequent gametogenesis[74–76]. Androgenetic ES cells are derived from two male gametes and have a male-specific pattern of imprinting, whereas parthenogenetic ES cells are derived from two female gametes and have a female-specific pattern of imprinting[77] (Table 3). Androgenetic ES cells are unable to contribute extensively to chimaeras, and fibroblasts derived in culture display an overtly transformed phenotype. By contrast, parthenogenetic ES cells show a broader differentiation potential, and fibroblasts rescued from chimaeric embryos undergo premature senescence[77]. ES cells that have been genetically manipulated to be essentially 'imprint-free' develop into normal-appearing high-grade chimaeras upon injection into blastocysts[78]. However, these chimaeras develop multiple types of cancer by 1 year of age[78]. Spermatogonial stem cells, which originate from PGCs, must have undergone complete elimination of their imprints and probably have re-established some male-specific imprints. Thus, the ES-like cells derived from the spermatogonial stem cells are expected to have an unbalanced imprinting status that falls between that of 'imprint-free' ES cells[78] and androgenetic cells[77]. This observation predicts that chimaeras derived from ES-like cells should be at least as prone to develop cancer as chimaeric mice derived from imprint-free ES cells (Table 3). Consequently, the therapeutic application of testis-derived ES-like cells might be problematic, because the unbalanced male-specific pattern of imprinted gene expression might inevitably result in tumorigenesis.

Molecular mediators of reprogramming and pluripotency

Cell-extract and nuclear transfer experiments have implicated chromatin-remodelling factors, DNA and histone modifications in the reprogramming of somatic nuclei. However, little is known about which genes are targeted by these modifications and are critical for reprogramming. There is a much better understanding of the genes that are important for sustaining pluripotency in ES cells and, as ES cells have reprogramming activity themselves, some of these genes might have a role in somatic-cell reprogramming.

The pluripotency of ES cells is maintained by a combination of extracellular and intracellular signals. Extracellular signals include generic signalling pathways, such as the signal transducer and activator of transcription 3 (STAT3), bone morphogenetic protein (BMP) and WNT cascades, whereas intrinsic signals comprise ES-cell-specific factors that execute the maintenance of pluripotency at the transcriptional level[79]. So far, there has been no functional analysis of the effect of pluripotency-sustaining genes on cellular reprogramming. Indirect evidence, however, suggests that there is a correlation between aberrant reactivation of the pluripotency genes *Oct4*, *Nanog* and *Sox2* in blastocysts cloned from somatic cells and the abnormal development of the clones[80–82]. By contrast, clones derived from ES, EG or EC cells that already express pluripotency genes showed faithful activation of OCT4 and exhibited an increased cloning efficiency[61,81,82].

Table 3 | Developmental and tumorigenic potential of pluripotent cells derived from donor cells with different imprinting states

Cells of origin	Imprinting status	Derivative pluripotent cell type	Developmental potential (chimaeras)	Tumorigenic potential (chimaeras)	References
Inner cell mass (from biparental embryo)	Biparental imprints	ES cells	Normal chimaeras	None	53–55
Imprint-free ES cells*	Erased imprints	ES cells	Overgrown chimaeras (no methylation imprints)	Multiple tumours	78, unpublished data
PGCs	Erased imprints	EG cells	Overgrown chimaeras	Not assessed	56,57,62,64,74–76
Inner cell mass (from uniparental embryo)	Male-only imprints	Androgenetic ES cells	Chimaeras abnormal, restricted contribution	Chimaera-derived fibroblasts transformed	77
	Female-only imprints	Parthenogenetic ES cells	Broad but uneven contribution to chimaeras	Fibroblasts undergo premature senescence	77
Spermatogonial stem cells (GS cells)	Erased and partly re-established male imprints	ES-like cells (mGS/maGS cells)	Normal chimaeras?	Not assessed	68,69

*Removal of all methylation imprints by sequential inactivation and reactivation of the DNA-maintenance methyltransferase 1 gene[78]. GS cells, germline stem cells; mGS/maGS cells, multipotent/adult germline stem cells.

A key issue for current research is defining the downstream target genes of pluripotency genes. Genome-wide location analysis for OCT4, SOX2 and NANOG in human[83] and mouse[84] ES cells showed that these transcription factors collaborate to form specialized regulatory circuitry in ES cells. The target genes of these three regulators frequently encode other developmentally important transcription factor genes that are silent in the pluripotent undifferentiated cells, supporting the notion that inhibition of differentiation pathways is essential for the maintenance of pluripotency. Polycomb group (PcG) proteins are transcriptional repressors that function in maintaining cellular identity during metazoan development through epigenetic modification of chromatin structure[85]. Recent evidence suggests that PcG proteins function to transcriptionally repress developmental genes in ES cells, the expression of which would otherwise promote differentiation[86,87]. Many of the OCT4, SOX2 and NANOG target genes previously identified were also PcG targets, indicating that chromatin modifiers might act in concert with these three key pluripotency regulators to directly repress developmental pathways in ES cells. Interestingly, the chromatin conformation associated with many of these key developmental genes is composed of 'bivalent domains' consisting of both inhibitory histone H3 lysine 27 methylation marks and activating histone H3 lysine 4 methylation marks[88]. These bivalent domains are lost in differentiated cells, suggesting that they play an important part in maintaining developmental plasticity of ES cells. Thus, the pluripotency factors OCT4, SOX2 and NANOG might act in concert with PcG proteins to silence key developmental regulators in the pluripotent state, while, for the same genes, positive epigenetic regulators are recruited and are poised to activate transcription upon differentiation. Once the transcriptional regulatory circuitry that confers pluripotency and self-renewal on ES cells is fully understood at the molecular level, it might be possible to reprogramme one cell type into another by affecting the activity of the key components of the transcriptional network.

Looking ahead

Will it be possible to fully reprogramme a somatic cell into an ES-like cell without exposure of the nucleus to the reprogramming factors of the oocyte? Will the genes and pathways essential for reprogramming be identified? Evidence from adult cells supports the notion that deletion or activation of single genes can facilitate the de-differentiation of mature cells into an immature state. For example, ectopic expression of CCAAT/enhancer-binding protein-α (C/EBPα) and C/EBPβ in B cells facilitates their reprogramming into macrophages[89]; similarly, loss of PAX5 (ref. 90) or ectopic expression of β-catenin[91] in lymphoid progenitors endows them with multilineage differentiation potential. In the nervous system, oligodendrocyte precursors reprogramme into multipotent central nervous system-like stem cells when exposed to certain combinations of extracellular signalling factors[92]. Likewise, astrocytes lacking the *Ink4a/Arf* tumour-suppressor locus have the potential to reprogramme into NS cells in the presence of endothelial growth factor[93]. Our laboratory has found that the ectopic activation of the pluripotency factor OCT4 in adult mice results in the expansion of adult progenitor cells and tumour formation[94]. Importantly, the tumours completely regress when OCT4 is turned off, owing to the instant differentiation of expanded progenitor cells. This finding suggests that adult progenitor cells remain responsive to this key embryonic transcription factor, and, thus, might identify adult progenitors as ideal targets for future reprogramming efforts. Some of the pluripotency-sustaining genes of ES cells have been identified through genetic screens[95]; hence, it should be possible to devise gain-of-function and loss-of-function strategies to identify genes that facilitate reprogramming of somatic cells into ES-like cells. Lastly, it might be possible to further select for reprogrammed cells by exploiting the resistance of ES cells to loss of DNA methylation[96], which is a manipulation that is not tolerated by any other cell type. ■

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