

Review

Using heterokaryons to understand pluripotency and reprogramming

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Reprogramming differentiated cells towards pluripotency can be achieved by different experimental strategies including the forced expression of specific ‘inducers’ and nuclear transfer. While these offer unparalleled opportunities to generate stem cells and advance disease modelling, the relatively low levels of successful reprogramming achieved (1–2%) makes a direct analysis of the molecular events associated with productive reprogramming very challenging. The generation of transient heterokaryons between human differentiated cells (such as lymphocytes or fibroblasts) and mouse pluripotent stem cell lines results in a much higher frequency of successful conversion (15% SSEA4 expressing cells) and provides an alternative approach to study early events during reprogramming. Under these conditions, differentiated nuclei undergo a series of remodelling events before initiating human pluripotent gene expression and silencing differentiation-associated genes. When combined with genetic or RNAi-based approaches and high-throughput screens, heterokaryon studies can provide important new insights into the factors and mechanisms required to reprogramme unipotent cells towards pluripotency.

Keywords: reprogramming; embryonic stem cell; pluripotency; cell fusion; heterokaryon; nuclear organization

1. INTRODUCTION

The early 1970s saw the remarkable and scientifically important discovery that cells from different origins can be fused to generate experimental heterokaryons and hybrid cells. Researchers found that they could combine cells representing distinct stages of differentiation, normal and malignant cells and even cells from different species, to trace whether specific cellular traits were dominant or recessive [1,2]. Experimental heterokaryons and hybrid cells have shaped our understanding of malignancy [3] and tumour-suppressor activity [4]. They were forerunners to modern genetics, enabling specific biological properties to be traced to particular chromosomes and domains [5]. The possibility of combining the properties of different parental cells within heterokaryon and stable hybrid cells has also provided the means to produce monoclonal antibodies [6], to interrogate the mechanisms underlying cellular plasticity [7], gene activation and

gene silencing [8,9], and to recapitulate sequential stages of tissue-specific differentiation [10,11].

2. HETEROKARYON-MEDIATED RESETTING OF LINEAGE POTENTIAL

Recently, heterokaryon and hybrid approaches have been used to investigate reprogramming—the restoration of multi-lineage potential to cells with a previously restricted cellular fate—by fusing unipotent somatic cells with pluripotent embryonic stem (ES) cells. Cell fusion results in the formation of heterokaryons in which the two nuclei initially remain discrete. Nuclear fusion occurs later and gives rise to hybrid cells with a single tetraploid nucleus. A proportion of heterokaryons and resulting hybrid cells undergo successful reprogramming, acquiring similar growth and expression characteristics as pluripotent cells [12,13]. By generating interspecies heterokaryons between mouse ES cells and human lymphocytes, where the parental nuclei can easily be distinguished and their activity monitored, it is possible to trace the immediate sequence of events that occur within each cell type as reprogramming is achieved [14,15].

In our studies, mouse ES cells and human B cells were pre-labelled with different cell-surface dyes, mixed together and fused with polyethylene glycol. Heterokaryons containing a human and a mouse nucleus were isolated by fluorescence-activated cell sorting of double-labelled cells. Subsequent

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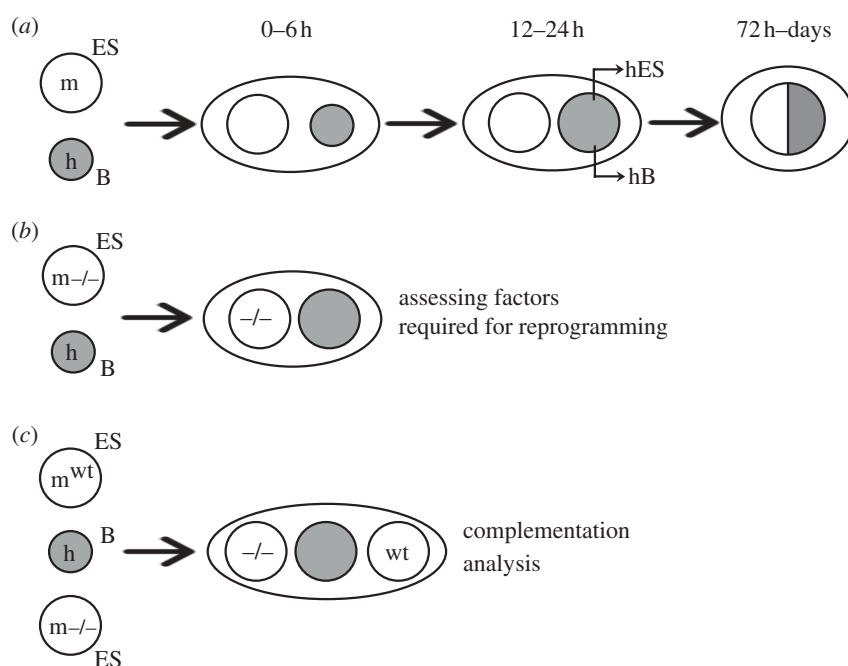


Figure 1. Heterokaryon-based approaches for somatic cell reprogramming. (a) A human B (hB) cell can be reprogrammed following fusion with a murine ES cell. A heterokaryon containing two spatially discrete nuclei, but shared cytoplasm, is formed. After remodelling of the hB cell-derived nucleus, human pluripotency-associated genes are transcribed, whereas human lymphocyte-associated genes are progressively silenced. Tetraploid hybrid cells arise subsequently following nuclear fusion. (b) Factors required for the successful reprogramming of hB cells can be assayed by depleting candidate factors from mES cells ($m^{-/-}$) prior to (during or after) cell fusion. (c) Complementation studies test whether successful reprogramming can be restored (or mutant ES cells are functionally dominant) using tripartite heterokaryons formed between different mES cells (wt and $-/-$ shown here).

changes in gene expression and nuclear morphology were assessed by RT-PCR (using primers that distinguish human and mouse genes) and microscopy (using DAPI (4',6-diamidino-2-phenylindole) staining patterns or DNA probes to identify human and mouse nuclei), respectively. Using this approach, we showed that human B cells initiate the expression of many pluripotency genes within 24–48 h, including human *OCT4*, *NANOG*, *CRIP1*, *TLE1* and *REX1* as well as genes encoding ES-associated enzymes such as *DNMT3b*. Coordinated expression of these human ES-associated genes defines successful reprogramming [15]. Expression of differentiation-associated genes such as human *CD45*, *CD19* and *PAX5* declines within the first 48 h and is progressively extinguished. Hybrid cells, in which parental nuclei are fused, first become evident at 72 h and cellular division can be detected shortly thereafter (figure 1a shows a schematic representation of these events).

In fusions performed between mouse ES cells and human B cells, only a proportion of cells (approx. 15%) express SSEA4, a surface glycoprotein that together with TRA-1-60 and TRA-181 characterizes human ES cells [16]. As the enrichment of these SSEA4-positive heterokaryons co-purifies all cells induced to express human *OCT4*, *CRIP1* and *NANOG* [14,15], SSEA4 provides a useful surrogate marker to estimate the frequency of productive reprogramming. This is particularly relevant since long-term interspecies hybrids are notoriously unstable (they frequently succumb to non-random chromosomal loss), and this can impair their functional characterization.

3. SUCCESSFUL REPROGRAMMING REQUIRES OCT4 AND POLYCOMB REPRESSION

Using the approach described here, we have begun to dissect the factors and processes required for the pluripotent reprogramming of human lymphocytes. These studies employ mouse ES cells in which specific genes are absent or mutated, conditionally deleted (figure 1b) or where expression is knocked down using interfering RNA strategies. For example, we demonstrated that ES cells deprived of *Oct4* can no longer successfully reprogramme human B cells, whereas *Sox2*, a factor believed to be critical for generating induced pluripotent stem (iPS) cells from fibroblasts [17], was dispensable for reprogramming human B cells in heterokaryons [15]. Similar studies in which short interfering RNAs were used to knock down activation-induced cytidine deaminase (AID) in heterokaryons formed between mouse ES cells and human fibroblasts [18] have argued that AID may be essential for active DNA demethylation required for efficient reprogramming. Although the case for replication-independent DNA demethylation remains controversial [19,20], the future application of sophisticated molecular and genetic approaches in heterokaryon studies will allow the stepwise processes contributing to reprogramming to be systematically investigated.

Recent comparisons of the reprogramming activity of mouse ES cells lacking individual chromatin remodelling factors showed that ES cells require Polycomb repressive complex (PRC) activity to successfully reprogramme human B cells. In complementation

assays where trikaryons were generated that contained a wild-type and a PRC-deficient mouse ES nucleus (in addition to the human B cell target), mouse ES cells lacking PRC1/2 dominantly suppressed the reprogramming ability of wild-type ES cells [21] (figure 1c illustrates the approach). This surprising result implied that PRCs were important for silencing factors that if inappropriately expressed by ES cells actively interfered with reprogramming. Although the identity of such factors is currently unknown, the observation that lymphocyte reprogramming by iPS cells is enhanced when *Pax5* (a PRC1/2 target that is important for maintaining B cell identity) is depleted [22], or when DNA methyltransferase activity is inhibited [23], suggests that reprogramming of lineage potential and plasticity can be enhanced when factors maintaining cell identity are ablated.

4. EARLY MOLECULAR EVENTS THAT DEFINE PLURIPOTENT CONVERSION

Since mouse ES cells depleted of *Oct4* do not initiate pluripotent conversion and mouse ES cells depleted of PRC1/PRC2 fail to successfully complete B cell reprogramming in heterokaryons, both models are useful for discriminating events associated with genuine conversion towards pluripotency from effects associated with heterokaryon formation *per se*. For example, ourselves and others have shown that somatic cells (such as human B lymphocytes) undergo a dramatic increase in the nuclear volume when incorporated within stable muscle heterokaryons [10] or in transient heterokaryons formed with mouse ES cells (mES × hB, illustrated in figure 2a, upper panels) [21]. We estimated that the nucleus of human B cells undergoes a 2.5-fold increase in volume within 6–24 h of mES × hB heterokaryon formation and this is preceded (3 h) by an influx and steady accumulation of mouse (ES-derived) Oct4 protein within the human nucleus (arrowed, figure 2a, lower panel, Oct4 protein shown in green). Accumulation of Oct4 and changes in the nuclear volume are not predictive of productive conversion to pluripotency because they are still induced by non-reprogramming PRC1/2-deficient ES cells [21]. Likewise, the reorganization of human nucleoli and the increase in acetylated histone H4 at lysine 16 (H4K16ac) that characterizes mES × hB heterokaryon formation (figure 2b) are also induced during non-productive reprogramming with PRC1/2-deficient ES cells [21]. Some changes were, however, selectively induced in heterokaryons containing wild-type but not PRC1/2-deficient ES cells. These include global increase in the level of phosphorylated histone H3 at serine 10 (H3S10ph) and the redistribution of heterochromatin protein 1α (HP1α) in human B nuclei (figure 2b) [21]. Phosphorylation of H3S10 is catalysed by the activity of Aurora B kinase [24] and was shown by Gurdon and co-workers [25] to be induced by reprogramming during nuclear transfer. To understand whether the phosphorylation of H3S10 is indeed required for pluripotent induction, we performed cell fusion experiments in the presence of kinase inhibitors. Although inhibition of Aurora B kinase dramatically

reduced H3S10ph levels in human B nuclei, and prevented HP1α redistribution, it did not abolish successful reprogramming (data not shown). This result suggested that although changes in H3S10ph and HP1α mark reprogrammed cells, neither appears to be essential for pluripotent conversion.

It is also worth noting that in heterokaryons formed between human B cells and mouse ES cells lacking PRC1/2 (or conditionally depleted of *Oct4*), expression of human lymphocyte-associated genes was efficiently extinguished—even though pluripotent genes were not properly induced. This indicates that reprogramming is a multi-step and multi-factorial process rather than an ‘all-or-nothing’ phenomenon as suggested by Tada and co-workers [26].

5. OPTIMIZING REPROGRAMMING: FUTURE DIRECTIONS AND TECHNOLOGIES

The ability of ES cells to induce the expression of pluripotency-associated genes by differentiated somatic nuclei in transient heterokaryons is a property shared by other embryo-derived cell types [27]. These include embryonic germ (EG) cells cultivated from primordial germ cells that colonize the genital ridge [28] and embryonic carcinoma (EC) cells derived from spontaneous or induced teratocarcinomas [29]. EG cells, unlike ES and EC cells, are also capable of inducing the erasure of DNA methylation at imprinted genes in somatic cell hybrids [12,30], although exactly how this is achieved is not fully understood. Trophoblast stem and extra-ES cell lines (XEN), derived from the developing trophoblast and primitive endoderm, respectively, have also been shown to dominantly direct the conversion of human B cells towards appropriate extra-embryonic fates within transient heterokaryons [31]. Collectively, these data suggest that stem cell lines derived from primitive cells that emerge at early stages of development possess powerful and often dominant reprogramming capabilities. Whether reprogramming is necessarily uni- or bi-directional has been the subject of much recent [32,33] and past debate [8,29,34], although it seems reasonable to assume that the eventual outcome depends on the relative abundance of certain factors, for example, *Nanog* [35], as well as the ‘compatibility’ of the parental cell types and culture conditions used to select the hybrids. Future studies will need to address these uncertainties, for example, by examining the impact of nuclear ‘dosage’ on lineage conversion (figure 3) and by assessing the reprogramming outcomes using cell-cycle-stage-enriched ES and target cells.

Claims that the differentiation state of a somatic cell acts as a barrier to efficient reprogramming into iPS [36] can be addressed by measuring the susceptibility of progressive differentiation stages to pluripotent conversion in ES-somatic heterokaryons, for example by comparing the reprogramming success of purified human pro-B, pre-B, immature, mature B and plasma cell targets (figure 3), as well as the effects of chromatin-modifying drugs that may weaken epigenetic memory. Perhaps the most significant future advances are likely to come from

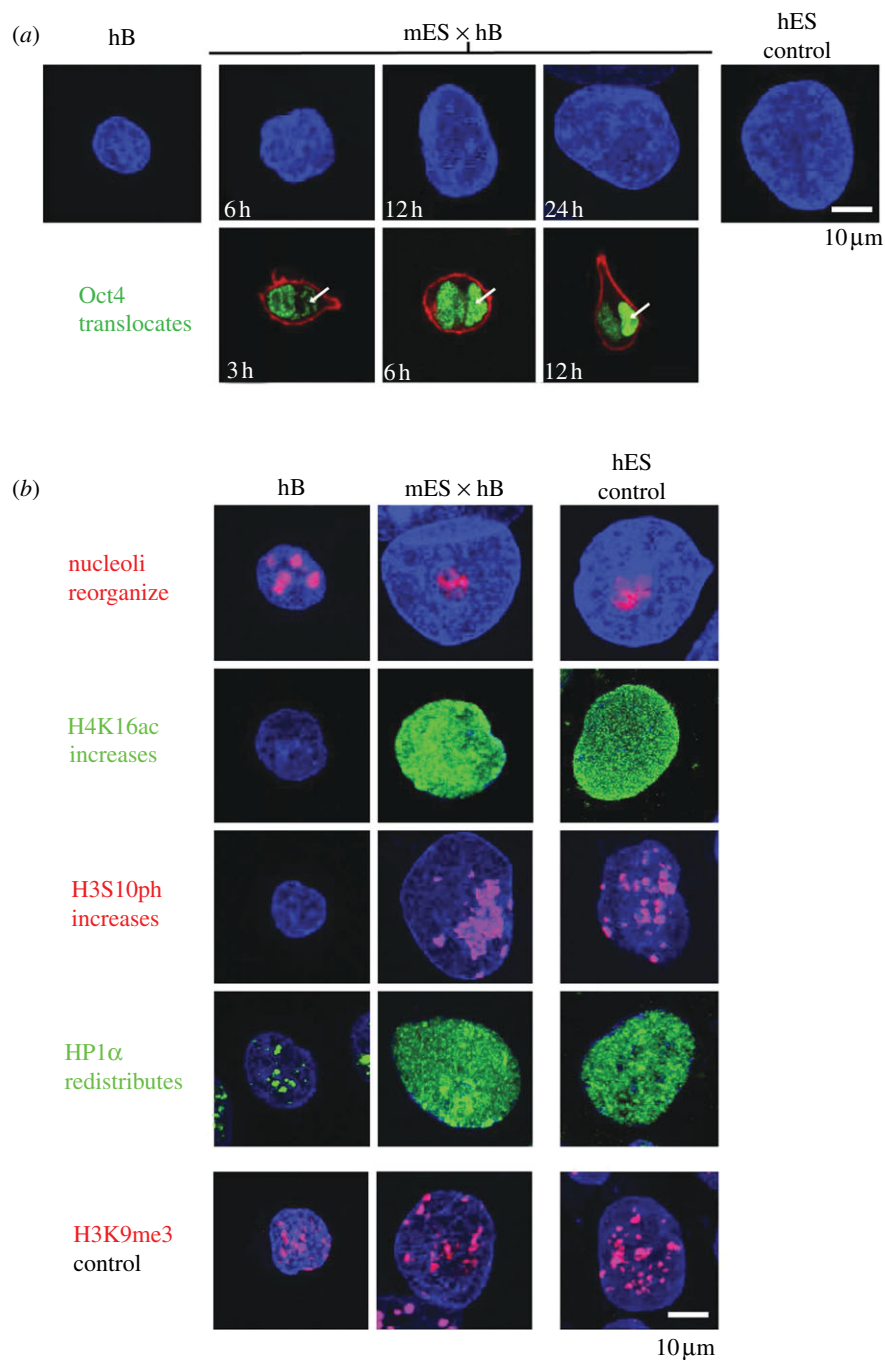


Figure 2. Remodelling of somatic nuclei during pluripotent reprogramming. (a) DAPI-labelled hB cells before and at sequential times (in hours) after fusion and heterokaryon formation with mouse ES cells (mES × hB) reveal an increased nuclear size (blue) and influx of Oct4 protein (green). Oct4 is detected in the hB (arrowed) nucleus within 3 h (and peaks at 12 h), ahead of human Oct4 transcription [15]. Representative confocal images are shown. (b) Human nuclei before (hB) and 24 h after fusion (mES × hB), labelled to reveal specific nuclear changes that occur early during reprogramming. Human ES cells are shown as the control. Antibodies used detect nucleolar marker B23 (nucleophosmin, red), acetylated H4K16 (H4K16ac, green), phosphorylated H3S10 (H3S10ph, red), HP1α (green) and tri-methylated H3K9 (H3K9me3, red). H3K9me3, which does not appreciably change, is provided as a control. Representative confocal images are shown.

technologies that enable high-throughput heterokaryon and hybrid analysis to be established. One approach, reported recently by Voldman and Jaenisch, uses a microfluidic device to trap and efficiently pair cells prior to fusion with electrical or chemical agents [37]. Platforms of this kind, which can be coupled to high-throughput screens using RNAi, or drug libraries, and automated single cell time lapse imaging [38], are set to massively expand our knowledge of the early events and mechanisms that direct lineage-restricted

cells towards pluripotency. With this in mind, it is sobering to remember that while many current discoveries have their origins in work performed nearly 40 years ago, including evidence that reprogramming (by iPS or in hybrids) does not entirely erase epigenetic marking [39] or morphological characteristics [40], we are now entering a decade of research that will bring us far closer to understanding the molecular basis of cellular memory and reprogramming than previously imagined.

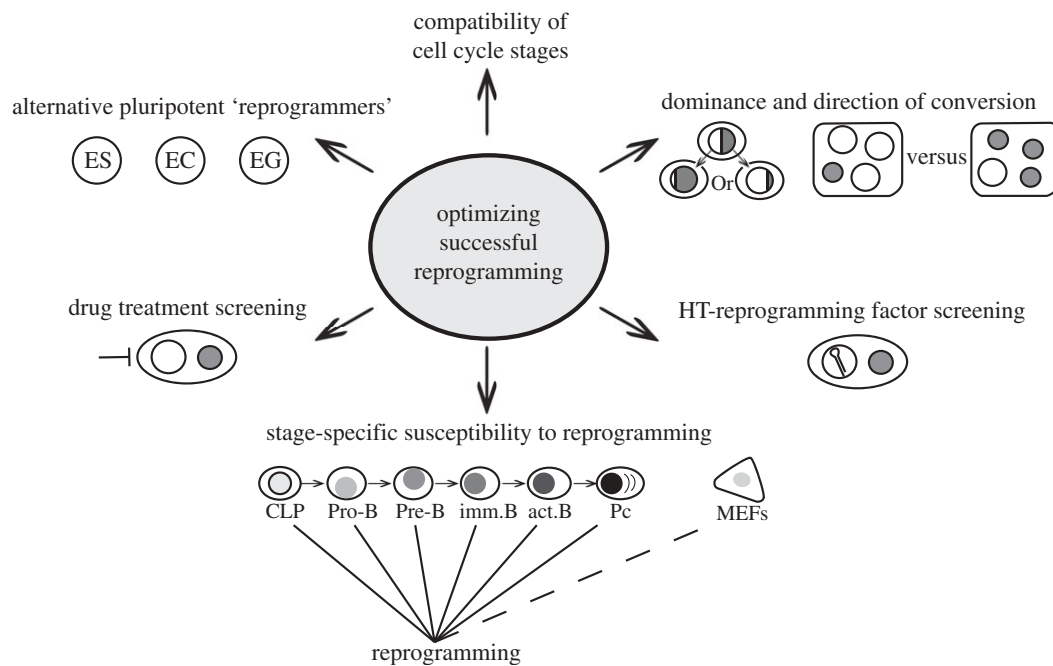


Figure 3. Approaches to advance the understanding of reprogramming. Future strategies aimed at improving our knowledge of the molecular events that define the successful conversion of unipotent differentiated cells to pluripotency. Diploid somatic nuclei, or the contribution of the somatic chromosomes within tetraploid cells, are indicated in grey. HT, high throughput; CLP, common lymphocyte precursor. MEFs, mouse embryonic fibroblasts; Pc, plasma cell.

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