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Biochemical Determinants of Tissue Regeneration

In vivo cell reprogramming to pluripotency: exploring a novel tool for cell replenishment and tissue regeneration

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Abstract

The potential of cell-replacement strategies for the treatment of disorders in which a particular cell type is damaged or degenerated has prompted the search for the perfect cell source. iPSCs (induced pluripotent stem cells) stand out as very advantageous candidates thanks to their self-renewal capacity and differentiation potential, together with the possibility of generating them from autologous somatic cells with minimally invasive techniques. However, their differentiation into the required cell type, precise delivery and successful engraftment and survival in the host are still challenging. We have proposed the transient reprogramming of somatic cells towards a pluripotent state in their *in vivo* microenvironment as a means to facilitate the regeneration of the tissue. The initial reports of *in vivo* reprogramming to pluripotency in the literature are reviewed and the potential clinical applications of this strategy are discussed.

Cell-based therapies for regenerative medicine

Cell-replacement strategies occupy an important place in the growing field of regenerative medicine as they hold encouraging potential for the treatment of numerous disorders including Type 1 diabetes [1], retinal degeneration [2], Parkinson's disease [3], muscular dystrophies [4] and liver diseases [5]. Indeed, the treatment of many injuries or disorders in which a particular cell type is damaged or degenerated could be simplified if a stock of healthy cells was readily available. Unfortunately, this has proved to be a difficult task to achieve, reflected in the many obstacles that need to be overcome for the success of any cell-replacement strategy. These obstacles are shown schematically in Figure 1 using hepatocyte transplantation as an illustrative example [6,7].

The first challenge is the sourcing or generation of the appropriate and functional cells required to ameliorate the particular disorder. Despite the initial success of some studies involving the transplantation of fetal tissue [8], the limited availability of this material together with ethical considerations have narrowed its potential as the ideal source for cell replacement [9]. Primary human cells isolated from unused or rejected organs for transplantation suffer from similar limitations [10]. Autologous grafts from different tissues of the same individual were at first seen as a better alternative, in spite of the invasive techniques that are frequently required for their sourcing [11]. However, the expectations of this approach have not been fulfilled in the long term [12,13]. Further obstacles include the inherent variability in the results of these studies derived from the use of a non-standardized starting material [14]. Pluripotent stem cells represent nowadays one of the most promising sources for replacement cells and hence are discussed in a separate section below [15].

Even when appropriate cells are sourced, their delivery to the precise location in the body is still technically challenging, which has triggered considerable efforts to design appropriate surgical strategies and cell carriers [16]. To date, cell microencapsulation using different formulations [17] and the embedding of cells in matrixes such as fibrin glue [18,19] seem to lead the way of this area. Being able to monitor and track the delivery and correct engraftment

Key words: cell replacement, cell reprogramming, induced pluripotent stem cell (iPSC), pluripotency.

Abbreviation: iPSC, induced pluripotent stem cell.

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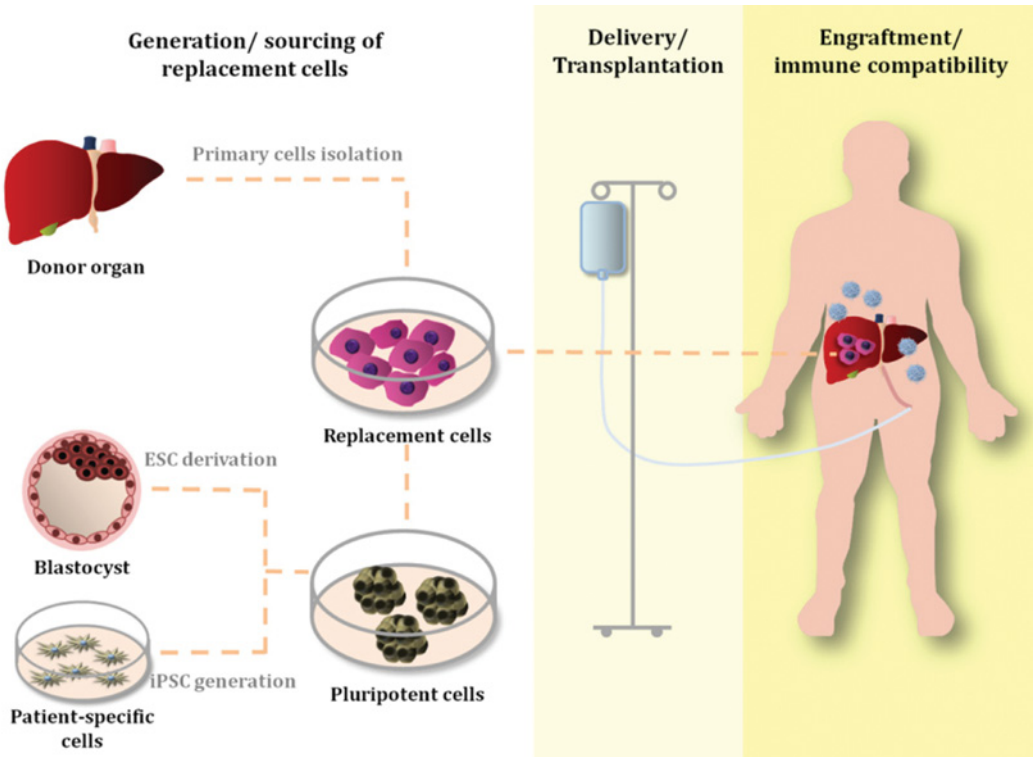
Figure 1 | **Challenges to the success of cell-replacement therapies**
ESC, embryonic stem cell.



of the administered cells, albeit increasing the complexity of the delivery systems, is also desirable [20]. In addition, the viability of the cells in these matrixes, especially after long-term frozen storage, must be carefully characterized [21]. Once the delivery issues are solved, the engraftment in the host tissue needs to be successful and the possible immune reactions against the new cells need to be minimized [22]. Finally, following the fate of the administered cells over time to ensure absence of complications such as cyst formation or graft overgrowth is required [14].

Pluripotent stem cells: the ideal source for cell-based therapies?

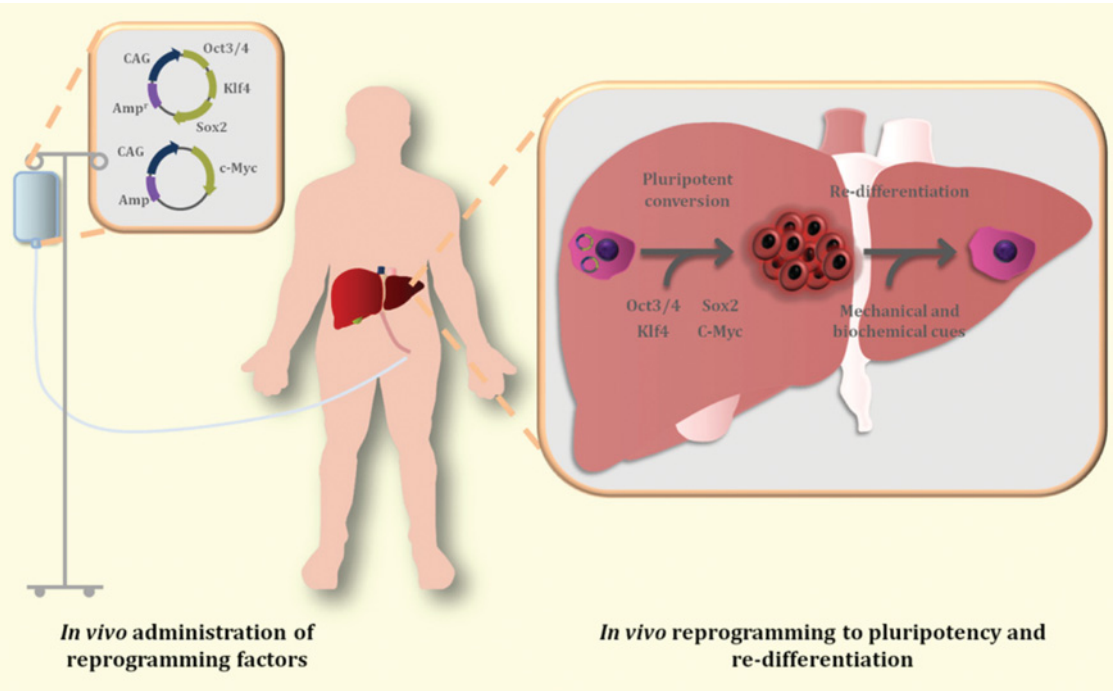
Among the different cell sources that have been explored for cell-based therapeutic strategies, pluripotent stem cells stand out due to their plasticity and ability to differentiate into any developmental lineage. In addition, they are able to self-renew when cultured under the appropriate *in vitro* conditions maintaining their differentiation potential intact [23]. Thanks to these properties, they have been seen as a potentially unlimited source of cells for cell-replacement therapies [15]. The isolation of ESCs (embryonic stem cells) from mice blastocysts [24,25] and human embryos [26] raised the hopes for novel treatments for several incurable diseases, but also prompted a controversial ethical debate [27]. Beyond the ethical or religious considerations, the possible immune complications triggered by an allogeneic cell transplant have also, to some extent, overshadowed the promises offered by these cells [28].

Pluripotent cells have also been artificially generated *in vitro* from terminally differentiated cells. A variety of techniques have been used for this purpose, including SCNT (somatic cell nuclear transfer) [29] and cell fusion strategies [30]. More recently, the lessons learnt from those techniques were combined with knowledge of transcription factor expression [31] to identify a combination of four transcription factors (*Oct3/4*, *Klf4*, *Sox2* and c-*Myc*) that were able to revert the differentiated identity of somatic cells to a pluripotent state [32]. This relatively simple, but groundbreaking, approach has revolutionized the fields of stem cell biology and regenerative medicine, because it allows the generation of a pool of pluripotent cells from individual patients, with the capacity for disease modelling, drug discovery and cell transplantation [33]. For the latter, producing replacement cells from the same patient receiving the transplant (autologous) could avoid the immunogenicity complications involved in allogeneic transplantations [34].

In vivo reprogramming to pluripotency: can we put the culture dish aside?

The generation of iPSCs (induced pluripotent stem cells) is now considered to be the preferred source of patient-derived

Figure 2 | Hypothesis that *in vivo* reprogramming to pluripotency can lead to tissue regeneration



cells to be used in cell-based therapies. Proof of this is their rapid progress towards clinical trials facilitated by serious investment [35,36]. The first clinical study to use iPSC derivatives has just been set up in Japan for the treatment of macular degeneration with iPSC-generated retina sheets [37]. iPSC technologies may resolve the issue of cell sourcing; however, they do not overcome some of the classical hurdles of cell-replacement therapies, such as the need for optimized delivery and engraftment [38]. Moreover, long-term *in vitro* culture protocols are required to first derive the iPSCs from the donor's somatic cells, and then to differentiate them into the desired cell types to be implanted. This has raised various alarms regarding the genetic stability and overall safety of the resulting cells, given the genomic abnormalities that may appear [39]. The establishment of feeder- and xeno-free standardized cell culture protocols is essential for the realization of clinically relevant iPSCs [40]. In addition, the long timeframe required to generate pluripotent cells and redifferentiate them into the appropriate phenotypes could also act in detriment of their clinical suitability. This has special relevance in the treatment of certain impairments, as in the case of spinal cord injury, where rapid intervention following trauma is vital to the success of the therapy [41].

In the concept proposed in the present article, we hypothesize that the delivery, engraftment and *in vitro* culture complications surrounding iPSC technology could be circumvented if the damaged or degenerated cells could be reprogrammed back to pluripotency and allowed to redifferentiate into the host cell phenotype within the tissues (i.e. *in vivo*), as represented in Figure 2. The overall comparison between *in vitro* and *in vivo* reprogramming technologies for regenerative purposes is summarized in Table 1.

In spite of the appeal of this strategy due to its simplicity, several aspects need to be proven and validated before its potential can be fully assessed. First, the feasibility to reprogramme somatic cells *in vivo* at high enough efficiency that can be expected on the basis of the experience gathered in the *in vivo* transdifferentiation field (i.e. direct conversion from one into another somatic cell type) [42–46] and has indeed already been demonstrated in a number of studies [47–49]. Secondly, the role of the tissue-specific cues and whether the *in vivo* microenvironment will be able to redifferentiate the reprogrammed cells to the correct cell type will have to be studied in detail.

Somatic cells can be reversed to the pluripotent state *in vivo*: what we already know about *in vivo* cell reprogramming to pluripotency

Although we are still learning how the intricate interplay of mechanical and biochemical cues present in a particular tissue are able to control the differentiation of cells into the specific phenotypes needed [50], a number of studies have already shown that somatic cells can also be transcriptionally forced to transdifferentiate (i.e. be directly reprogrammed to a different cell type) *in vivo* [42–46]. However, the first study in which somatic cells were transcriptionally reprogrammed to

Table 1 | Comparison between *in vitro* and *in vivo* reprogramming to pluripotency strategies for tissue regeneration
OKSM cocktail, combination of *Oct4*, *Klf4*, *Sox2* and c-*Myc*.

	Cell-replacement strategies (*in vitro* iPSCs)	*In vivo* reprogramming
Generation of pluripotent cells	Requires extraction of starting cells (e.g. fibroblasts); OKSM cocktail and other formulas	Has only been studied in limited organisms and tissues; OKSM cocktail
Delivery of reprogramming factors	Requires safe and efficient vectors	Requires safe and efficient vectors; specific for target tissue
Generation of differentiated cells	Requires long *in vitro* culture protocols; specific for each type of cell	Driven by signals of tissue microenvironment (to be demonstrated)
Cell delivery	Requires specific delivery depending on target tissue; may require invasive techniques	N/A
Engraftment	Requires engraftment of the transplanted cells in the host tissue	N/A
Immunogenicity	Possible immune reactions if allogeneic transplantation	Less predicted risk
Timeframe	Long protocols needed for the derivation of pluripotent cells from the patient and their differentiation	Rapid

the pluripotent state while in their *in vivo* microenvironment was only published in 2012 [47]. In this work, Vivien et al. [47] administered plasmid DNA encoding *Oct4*, *Sox2* and *Klf4* in the tail muscle of *Xenopus* tadpoles, to later observe the generation of proliferating cell clusters within that tissue. These clusters not only recapitulated the main molecular features of pluripotent cells, but also were able to differentiate into derivatives of the three developmental lineages *in vitro* and into ectoderm and mesoderm representatives *in vivo*, hence confirming their functional pluripotency [47].

The capability of transcriptionally reprogramming somatic cells in their *in vivo* microenvironment was first reported in fully developed mammalian (mouse) tissue in our laboratory. Yilmazer et al. [48,51] benefited from the techniques developed in the gene therapy field and utilized for that purpose the hydrodynamic delivery of plasmid DNA. Hydrodynamic tail vein injection has been used in a number of studies to specifically transfect with high efficiency the hepatocyte population in the intact liver tissue without the need for viral vectors or tissue damage [52]. The hydrodynamic tail vein injection of two plasmid cassettes encoding the Yamanaka factors resulted in a fast and transient up-regulation of pluripotency markers and down-regulation of hepatocyte-specific genes. The gene expression profile of the hepatocytes returned to baseline levels 8 days after the administration of the *Oct4*, *Klf4*, *Sox2* and c-*Myc* factors and more importantly, no teratomas or any other disruption in the liver anatomy and function were evident up to 120 days after the induction of pluripotency [48,51].

Our reports that *in vivo* reprogramming of mammalian tissue to the pluripotent state was feasible were later confirmed by Abad et al. [49] using a transgenic model of 'reprogrammable mice' in which the ubiquitous expression of *Oct4*, *Klf4*, *Sox2* and c-*Myc* was induced by the administration of doxycycline [49]. Upon oral administration of the drug (in drinking water), cells with totipotent capabilities were isolated from the tissues of these transgenic species to confirm that somatic cells can be reprogrammed *in vivo* despite the pro-differentiation signals present in the tissues. As expected, the survival of the transgenic mice employed in this study was rapidly and severely compromised, due to the appearance of teratomas. This was interpreted by some as confirmation of a general rule that reprogramming *in vivo* leads to teratoma formation [49,53]. However, it is very important to underline that the generation of such tumours occurred in the specific transgenic species due to doxycyline-mediated switch-on of the reprogramming transcription factors. The original work carried out by Yilmazer et al. [48] found experimental evidence that the transient induction towards pluripotency triggered by a boost of reprogramming factor overexpression will not lead to teratoma formation.

In vivo reprogramming to pluripotency as a universal tool for tissue regeneration

In vivo reprogramming to pluripotency can be, at first glance, considered a very similar approach to *in vivo* somatic cell transdifferentiation. Indeed, both technologies make use of the roles of defined transcription factors in determining cell fate and identity. Nevertheless, *in vivo* reprogramming to pluripotency can not only benefit from the lessons learnt in the more advanced field of *in vivo* transdifferentiation, but it can also add an advantage: the universality of the approach. In order to directly convert a cell type into a different one, it is imperative to identify the specific transcription factors whose expression needs to be altered. For this purpose, extensive research that requires sufficient knowledge of the cell phenotypes and gene expression

patterns is needed. In contrast, and based on the several *in vitro* studies that have demonstrated the generation of iPSCs from starting somatic cells from diverse tissues [54], we envisage that *in vivo* reprogramming to pluripotency can be theoretically achievable in any tissue using a 'universal' cocktail of reprogramming factors to pluripotency. This, of course, will only be achieved provided that the appropriate delivery vectors are designed to be capable of transfecting the target tissues at sufficiently high efficiency.

The role of the *in vivo* microenvironment and the potential of *in vivo* reprogramming to pluripotency in regenerative medicine

On the basis of the initial studies on *in vivo* reprogramming to pluripotency, it can be concluded that the tissue microenvironment is permissive enough to allow the transient dedifferentiation of the cells towards a pluripotent state rapidly [47,48]. However, it is imperative to elucidate the extent, kinetics and capacity at which the mechanical and biochemical cues in each tissue are able to achieve the redifferentiation of the pluripotent cells to each phenotypic identity. Extensive work has already been carried out in understanding the influence of specific signals in the fate of tissue-specific stem cells to orchestrate their differentiation to particular phenotype characteristics of the host tissue [50,55]. Specific studies investigating these factors and each tissue niche in the context of *in vivo* iPSC commitment and redifferentiation are yet to appear.

Lastly, even if the tissue microenvironment acts in favour of the redifferentiation of the reprogrammed pluripotent cells, it will be necessary to address whether the effect of induced *in vivo* reprogramming will be sufficient to result in clinically relevant acceleration in the regeneration of the damaged tissue. To achieve this goal, *in vivo* reprogramming to pluripotency studies will have to be carried out using appropriate injury models.

Concluding remarks and future perspectives

In view of recently generated data, we propose that *in vivo* reprogramming to pluripotency in adult somatic cells can be feasible, efficient, rapid and safe. We hypothesize further that it will be achievable in any mammalian tissue provided that the appropriate vectors are engineered to efficiently, transiently and safely express the reprogramming factors in the particular tissue. *In vivo* reprogramming to pluripotency could be a desired alternative to the *in vitro* generation of cells for cell-replacement therapies, circumventing barriers related to the delivery, engraftment, *in vitro* culture and overall long timeframe required for the clinical translation of such strategies. However, for the use of *in vivo* reprogramming to pluripotency technologies to move forward, it will be necessary to comprehensively characterize the role of the tissue microenvironment in the redifferentiation of the pluripotent intermediates and guarantee the safety of the approach. Most importantly, use of appropriate disease models should determine whether the transient induction of reprogrammed cell pluripotency can result in clinically relevant regeneration of damaged tissue.

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