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Mini-Review

Deconstructing age reprogramming

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It has been proposed that age reprogramming enables old cells to be rejuvenated without passage through an embryonic stage (Singh and Zacouto in *J. Biosci.* **35** 315–319, 2010). As such, age reprogramming stands apart from the induced pluripotent stem (iPS) and nuclear transfer-embryonic stem (NT-ES) cell therapies where histo-compatible cells are produced only after passage through an embryonic stage. It avoids many of the disadvantages associated with iPS and NT-ES cell therapies. Experimental evidence in support of age reprogramming is burgeoning. Here, we discuss possible new approaches to enhance age reprogramming, which will have considerable benefits for regenerative therapies.

Keywords. Age reprogramming; Epigenetic rejuvenation; iPS cells; reprogramming factors; Epigenetic clock; eAge

1. Age reprogramming vs developmental reprogramming

Animal cloning using somatic cell nuclear transfer (SCNT) and induced pluripotent stem (iPS) cell technologies have shown that nuclei of mono-potential terminally-differentiated cells undergo 'nuclear reprogramming', whereby they are reprogrammed into pluripotent embryonic stem (ES) cells possessing the well-established characteristics of being able to self-renew and develop into different cell types (Gurdon and Melton 2008; Yamanaka and Blau 2010). However, nuclear reprogramming can also be properly viewed as two processes taking place simultaneously. During nuclear reprogramming, 'developmental' reprogramming to the embryonic state takes place seamlessly along with 'age' reprogramming that resets the ageing clock. In support of this view is the observation that healthy cloned mice have been generated from senescent cells (Mizutani *et al.* 2008). Moreover, iPS cells derived from fibroblasts taken from centenarians can be re-differentiated into fibroblasts that have lost their ageing characteristics (Lapasset *et al.* 2011). Based on these and similar observations a key question was posited: is it possible to age reprogram an old differentiated cell *without* the developmental reprogramming that requires passage through an embryonic stage? Remarkably, the burgeoning experimental evidence falls on the side of the affirmative confirming that age reprogramming can bypass the de-/re-differentiation cycle that characterizes the animal cloning and iPS cell technologies (figure 1). Given this evidence, can we now define experimental approaches that could be used to enable routine age reprogramming of old cells to a youthful state? The benefits to regenerative medicine and the relief of human suffering are self-evident. This mini-review aims to provide a tentative answer to that question.

2. Reprogramming molecular hallmarks of ageing

López-Otín and colleagues (2013) defined nine candidate molecular hallmarks that likely represent evolutionarily-conserved determinants of organismal ageing (box 1). To be raised to a 'hallmark' of ageing the authors showed that each candidate hallmark fulfilled, to a lesser or greater extent, the following criteria: '*(i) it should manifest during normal aging; (ii) its experimental aggravation should accelerate aging; and (iii) its experimental amelioration should retard the normal aging process and, hence, increase healthy lifespan*.' Notably, four of the nine hallmarks, epigenome drift, genome instability, mitochondrial dysfunction and cellular senescence, have been age reprogrammed and this has been achieved *in vivo* also

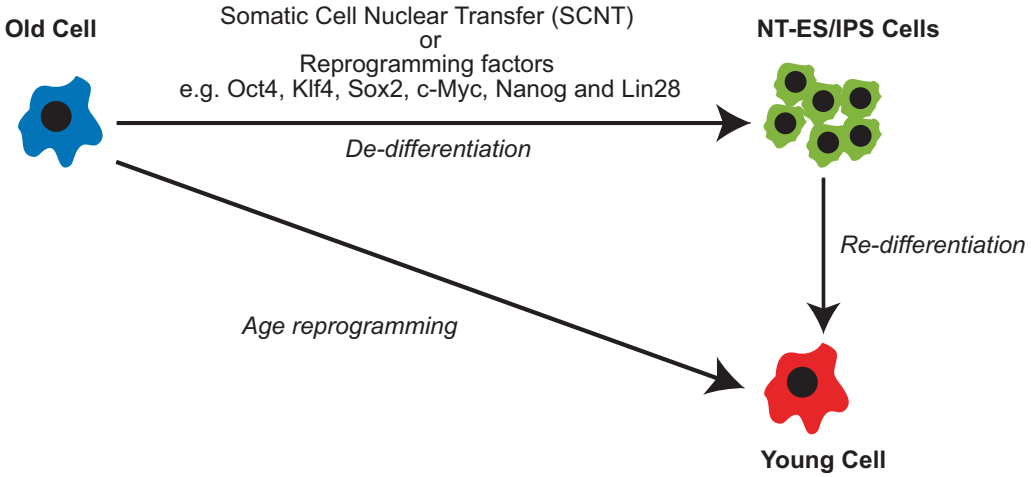
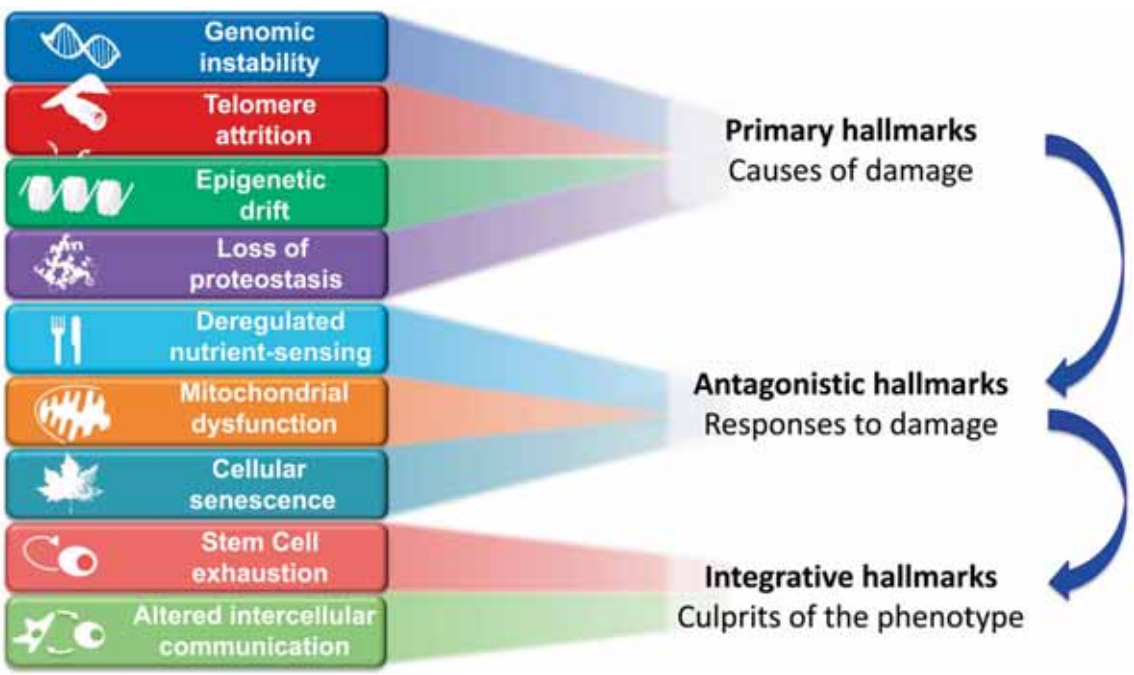


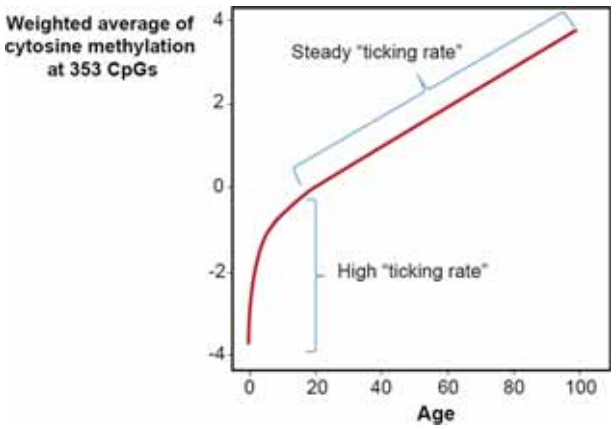
Figure 1. 'Age' reprogramming bypasses the de-/re-differentiation cycle characteristic of the animal cloning (somatic cell nuclear transfer; SCNT) and iPS and technologies. SCNT using nuclei from old cells (in blue) and introduction of 'reprogramming factors' into old cells results in de-differentiation to an ES-cell like state producing NT-ES and iPS cells, respectively. NT-ES cells derived from old nuclei can re-differentiate producing cloned individuals that have a normal lifespan (Mizutani *et al.* 2008): the old nucleus has been rejuvenated to generate a clone consisting of young cells (in red). Likewise, iPS cells generated from aged fibroblasts (in blue) can be re-differentiated into fibroblasts that have lost their ageing characteristics and are again young (Lapasset *et al.* 2011: cell in red). By contrast, age reprogramming bypasses the embryonic (NT-ES/iPS) stage and directly rejuvenates old cells (in blue) into a youthful state (cell in red). Taken and modified from Singh and Zacouto (2010).

Box 1. Hallmarks of ageing.



López-Otín and colleagues (2013) have described nine candidate hallmarks of ageing that can themselves be grouped into either primary, antagonistic or integrative hallmarks. At the top of the diagram are the four primary hallmarks that cause cellular damage and thought to be the proximate cause of ageing. In response to cellular damage the organism mounts three compensatory or antagonistic responses depicted as antagonistic hallmarks in the middle. At first, antagonistic hallmarks ameliorate the damage caused by the primary hallmarks but they too can ultimately contribute to and exacerbate ageing. The integrative hallmarks at the bottom integrate all that has come before and are ultimately responsible for functional decline associated with aging. Taken and modified from Figure 6 in López-Otín *et al.* (2013).

Box 2. The 'Epigenetic clock' and eAge.



Horvath (2013) has developed an 'epigenetic clock' based on age-dependent changes in DNA methylation at 353 'clock' CpGs in the human genome. Methylation at these CpGs is not all in one direction; methylation at 193 of the 353 CpGs increases with age while the remaining 160 CpGs decrease with age. When a weighted average of cytosine methylation over all 353 CpGs is plotted on the ordinate against chronological age on the abscissa a curve is generated that represents an 'epigenetic clock'. A striking feature of the rate of change of the curve is that the epigenetic clock has a high ticking rate until adulthood (~20 years), after which it slows to a constant, steady, ticking rate. Using this epigenetic clock it is possible to predict the age (or epigenetic age, eAge) of multiple tissues with a median error of 3.6 years. iPS and ES cells have an eAge of zero. Importantly, it has been shown eAge can be 'age' reprogrammed *i.e.,* rejuvenated without de-differentiation (Olova *et al.* 2019; figure 2A). Modified from Figure 6B in Horvath (2013).

(Manukyan and Singh 2014; Ocampo *et al.* 2016; Olova *et al.* 2019; reviewed in Singh and Newman 2018). Age reprogramming of the four hallmarks was observed using what has now become the paradigm for studying age reprogramming, namely the expression of iPS cell reprogramming factors (*Oct4*, *Sox2*, *Klf4*, *c-Myc* and, in some cases, along with *Nanog* and *Lin28*) in aged cells. Using this paradigm a recent study has provided a detailed description of the kinetics of reprogramming epigenetic age (eAge) (Olova *et al.* 2019). The measure of eAge is predicted from an 'epigenetic clock' based on cytosine methylation levels at 353 specific CpG dinucleotides in the human genome (Horvath 2013; box 2). eAge of a cell correlates accurately with its chronological age and it has been argued that eAge may measure some aspect of true biological age (Horvath 2013; Hannum *et al.* 2013). Importantly, eAge can be reprogrammed - iPS cells have an eAge of zero, which is much lower than that measured for the differentiated cells from which they were derived (Horvath 2013). As mentioned, the kinetics of eAge reprogramming has been described in a recent *in silico* study of an already published data set where human dermal fibroblasts (HDFs) had been transfected with iPS reprogramming factors (Ohnuki *et al.* 2014). This revealed that reprogramming of eAge can be separated from developmental reprogramming (Olova *et al.* 2019). Specifically, it was shown that eAge declined steadily at 3.8years/day after introduction of reprogramming factors, reaching zero by day 20. Loss of fibroblast molecular identity as measured by extinguishing of fibroblast-specific gene expression was, by contrast, completed only 15 days later (day 35; figure 2A). This exacting study begins the dissection of age reprogramming *in vitro* and raises three different but related questions whose answers, we suggest, are likely to advance practical application of age reprogramming. The first arises directly out of the above study (figure 2A). Is there a '*zone of optimal age reprogramming*' where age reprogramming of the hallmarks takes place with limited or no developmental reprogramming? Identification of such a zone would allow development of regimes that safely balance age reprogramming against unwanted de-differentiation that could increase the risk of teratomas, which form from unrestricted expression of reprogramming factors *in vivo* (Abad *et al.* 2013; Ohnishi *et al.* 2014). Second, are all or only some of the reprogramming factors required for reprogramming the hallmarks of ageing? Identification of critical reprogramming factors that cause reprogramming of hallmarks of ageing will lead to a deeper understanding of the molecular pathways involved, with the possibility of developing interventions that could enhance age reprogramming. Finally, do some cell types require transient de-differentiation for efficient reprogramming of the hallmarks of ageing? If so, then the timing, duration and levels of reprogramming factors will have to be tailored for different cell types, especially *in vivo*, to bring about age reprogramming.

3. Defining a 'zone of optimal age reprogramming'

In order to identify a '*zone of optimal age reprogramming*' using the iPS cell method we have taken the approach to first describe the potential outlines of a '*critical window*' in which age reprogramming takes place. The period from day 7 to 15 post-introduction of iPS reprogramming factors into HDFs might represent such a 'critical window'. There are three lines of evidence that support this contention. For one, days 7 to 15 coincide with the plateau of fibroblast-specific expression while over the same period there is a steady, ongoing, decline of eAge (figure 2A; Olova *et al.* 2019). Further, epigenetic rejuvenation of HP1β mobility (box 3) takes place within this window on day 9 (Manukyan and Singh 2014). Lastly, study of iPS cell induction has identified phases of 'epigenetic instability' or so-called 'undefined states' that lie between the differentiated and ES cell phenotypes, which together extend from roughly day 7 to 15 post-introduction of iPS cell reprogramming factors (Nagy and Nagy 2010; Efe *et al.* 2011; Manukyan and Singh 2012). As shown by the yellow band in figure 2A the edges of the 'critical window' would translate to eAges of 50 (day 7) and

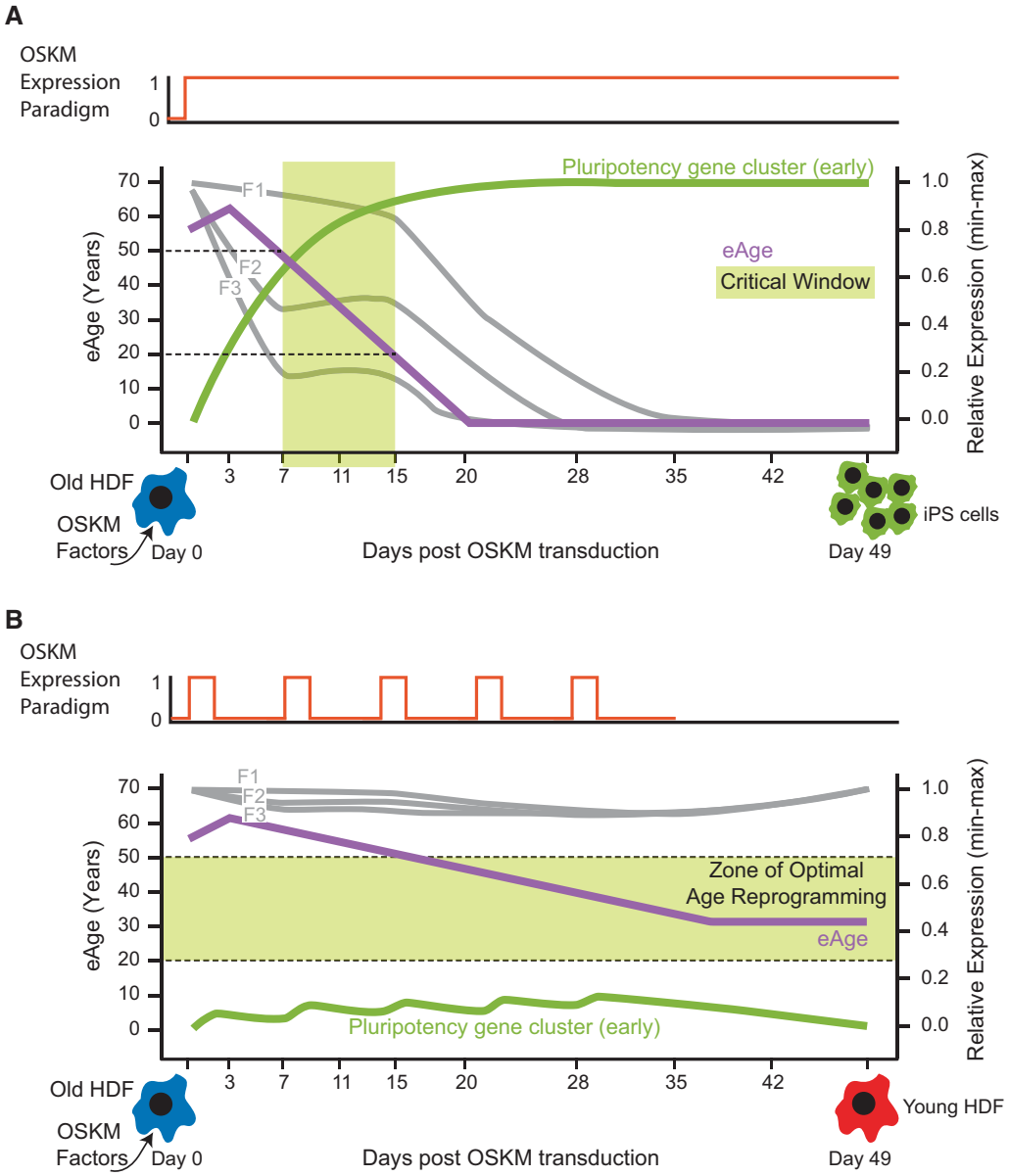
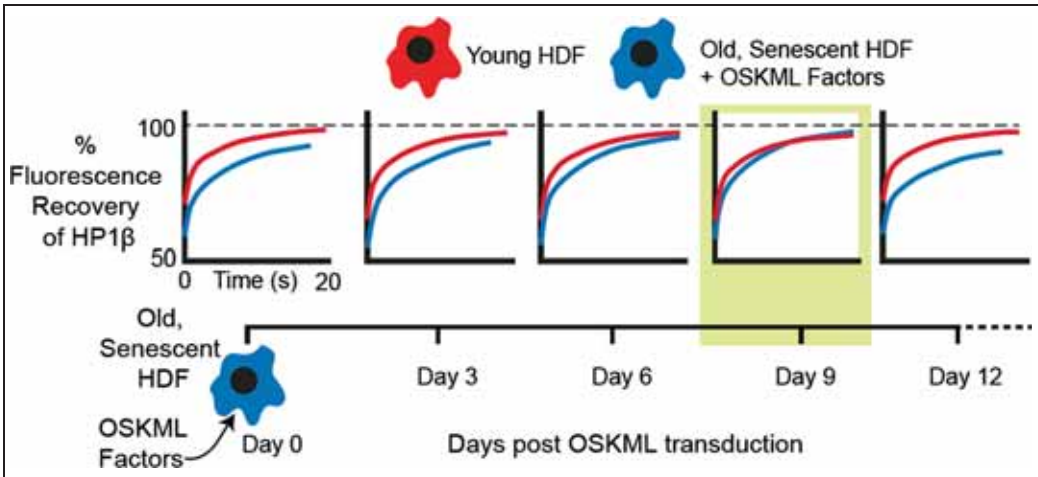


Figure 2. Maintaining eAge between ages 50 and 20 defines a *'zone of optimal age reprogramming'* that delays de-differentiation thereby inhibiting developmental reprogramming and extending the period in which age reprogramming can take place. (**A**) The classical iPS expression paradigm (Ohnuki *et al.* 2014; Olova *et al.* 2019) is characterized by exogenous OSKM expression after viral transduction. Exogenous expression is eventually replaced by endogenous OSKM expression after the cells are moved onto embryonic stem cell medium. Shown are expression profiles of three clusters of fibroblast-specific genes (F1, F2 and F3) and the early wave of pluripotency gene expression. F1-F3 expression profiles plateau from day 7 to 15 and this period we have termed the '*critical window*' (see text for details), which is highlighted by the yellow band. The critical window that extends for eAge 50 through to 20 years (dotted lines). Taken and modified from Singh and Newman (2018). (**B**) The cyclical iPS expression paradigm (Ocampo *et al.* 2016) is characterized by bursts of iPS reprogramming factor gene expression separated by longer periods where iPS expression is turned off. In the model, cyclical iPS factor gene expression results in an eAge decline with a shallow gradient that remains above or within a '*zone of optimal age reprogramming*' bounded by the eAges of 50 and 20 years. Compared to figure 1A there is low level expression of the early pluripotency gene cluster that follows the cyclic expression of iPS reprogramming factors, while the levels of the fibroblast gene clusters F1-F3 remain elevated. In this way, de-differentiation is delayed allowing a longer period within which age reprogramming may proceed. Using this paradigm in a mouse premature ageing model four hallmarks of ageing have been age reprogrammed, namely, epigenetic modifications, cellular senescence, DNA damage and mitochondrial dysfunction (Ocampo *et al.* 2016).

20 (day 15). Notably, the latter is close to the eAge of ~20, below which the epigenetic clock 'ticks' rapidly compared to the slower, steady, 'ticking' above this age (Horvath 2013; box 2). Accordingly, when eAge falls below ~20 there may be an increased probability of permanent collapse of eAge to zero because the rate of change of eAge increases below

Box 3. Demonstration of age reprogramming - epigenetic rejuvenation of HP1β mobility.



Manukyan and Singh (2014) used fluorescence recovery after photo-bleaching (FRAP) to measure the dynamics of an essential epigenetic modifier, HP1β, in senescent human diploid fibroblasts (HDFs) before and after they had started along the path to becoming iPS cells. Briefly, after OSKML reprogramming factors had been introduced into senescent HDFs (blue) HP1β mobility reached levels found in young HDFs (red) on day 9 (highlighted with the yellow surround), albeit rejuvenation was transient. HP1β mobility returned to levels found in senescent cells on day 12. These observation on 'epigenetic rejuvenation' of HP1β mobility confirmed that an aspect of age reprogramming could be rejuvenated without concomitant developmental reprogramming. Taken from Singh and Newman (2018).

~20. Possessing an eAge of zero is a characteristic of fully de-differentiated iPS cells (Horvath 2013). And reaching zero may be an important step towards crossing the 'commitment point' beyond which cells inexorably de-differentiate towards the pluripotent stem cell state (Nagy and Nagy 2010). Bearing this in mind, we suggest that the paradigm of cyclic expression of iPS factors (Ocampo *et al.* 2016) could be used to delay de-differentiation thereby extending the period in which the process of age reprogramming can take place. Experimentally, this could be achieved by maintaining eAge above or within a *'zone of optimal age reprogramming'* that lies between the eAges of 50 and 20 (yellow range in figure 2B).

4. Direct reprogramming using *SOX2* and *c-MYC* can reprogram hallmarks of ageing

Forced expression of neurogenic transcription factors in mouse and human fibroblasts reprograms them directly into post-mitotic induced neurons (iNs) (Vierbuchen *et al.* 2010; Pang *et al.* 2011; Ladewig *et al.* 2012). Direct reprogramming does not involve passage through an embryonic stage but unlike age reprogramming (Singh and Newman 2018) hallmarks of ageing, as measured by epigenome drift, genome instability and mitochondrial dysfunction were not reprogrammed in iNs (Huh *et al.* 2016; Tang *et al.* 2017; Kim *et al.* 2018). This indicates there is something peculiar to the classic reprogramming factors that enable reprogramming of the hallmarks of ageing. A clue to which of the reprogramming factors are likely to be necessary for age reprogramming has come from the study of *SOX2* and *c-MYC* expression in adult peripheral blood cells, which resulted in a self-renewing oligo-potential population of induced neural precursor cells (iNPCs; Sheng *et al.* 2018). Notably the hallmark of epigenetic drift, as measured by eAge, was reprogrammed in iNPCs (Sheng *et al.* 2018), in contrast to the post-mitotic mono-potential iNs where, as explained, no reprogramming of the hallmarks was observed. It would seem that expression of *SOX2* and *c-MYC* can cause reprogramming of ageing hallmarks in iNPCs and this is associated with the acquisition of some degree of 'stemness' because iNPCs exhibit the capacity for self-renewal and an expanded developmental potential.

5. Transient de-differentiation may enhance age reprogramming in some cell types

Related to the observations with iNPCs, recent work on 'interrupted reprogramming' in Club cells of the lung (Guo *et al.* 2017) indicates that de-differentiation and the acquisition of 'stemness', even transiently, may aid age reprogramming. Interrupted reprogramming was first used as an approach to generate sufficient numbers of Club cells for transplantation because native Club cells are found in small numbers and have limited proliferative capacity and thus of limited utility in such experiments. It was found that expression of reprogramming factors (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*) for three weeks using a dox-inducible system resulted in the induction of progenitor-like (iPL) cells from Club cells (Club-iPL cells)

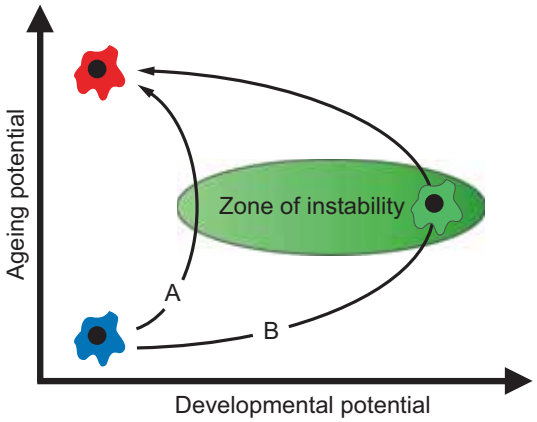


Figure 3. Transient de-differentiation may be required for age reprogramming of some cell types. (**A**) iPS reprogramming factors (OSKM) are introduced into an old cell (in blue), takes a short path through the zone of instability that overlaps with the so-called area of 'undefined states' (Nagy and Nagy 2010). Passage through this zone age reprograms the cell and it re-acquires ageing potential (cell in red). By taking the short path the cell is not subject to de-differentiation. (**B**) An old cell (in blue) from another cell type that is refractory to age reprogramming takes a longer path through the zone of instability. The longer path leads to de-differentiation (cell given in green). De-differentiation is transient because once reprogramming factors have been switched off it will return to its specialized phenotype with the difference that the cell is age reprogrammed and re-acquired ageing potential (in red). Age reprogramming of Club cells may be an example, taking the longer path and passing through a de-differentiated state represented by Club-iPL cells (Guo *et al.* 2017). Taken and modified from Manukyan and Singh (2012).

that proliferated and generated large numbers (Guo *et al.* 2017). Club-iPL cells do not resemble any known progenitors although they possess an expanded developmental potential, but – and this is the nub of the matter - once expression of reprogramming factors was extinguished ('interrupted') Club-iPL cells returned back to being phenotypically identical to native Club cells (Guo *et al.* 2017). The reconstituted Club cells were now in numbers sufficient for engraftment experiments in a mouse cystic fibrosis model. Reprogramming of the hallmarks of ageing was not investigated in these experiments so any interpretation regarding age reprogramming must be made with caution; it will be of interest to determine the status of the hallmarks in native Club cells, Club-iPL cells and reconstituted Club cells. Nevertheless, these data indicate that Club-iPL cells might represent a unique cell type generated by transient de-differentiation of native Club cells. This would be consistent with a prior model where it was posited that limited de-differentiation could be required for age reprogramming (Singh and Zacouto 2010; Manukyan and Singh 2012; figure 3). A degree of de-differentiation might be necessary for specialized cells in which age reprogramming is refractory, as long as re-differentiation once reprogramming has ceased can reconstitute the original specialized cell function(s). It may also be that the degree to which a cell can be de-differentiated whilst still being able to return to its original phenotype is cell type dependent (figure 3).

6. Perspective

A great deal of work needs to be done before cell-type-specific protocols can be developed for the production of large quantities of age reprogrammed cells. However, recent studies using iPS cell reprogramming factors have begun the deconstruction of age reprogramming. In the near future we should expect a more nuanced understanding of the molecular mechanisms and how they might be manipulated *in vitro* and *in vivo* to rejuvenate cells and tissues. This would have significant benefits for Medicine in general and regenerative therapies in particular.

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