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REVIEW ARTICLE

Partial Reprogramming as an Emerging Strategy for Safe Induced Cell Generation and Rejuvenation

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***Background*:** Conventional cell reprogramming involves converting a somatic cell line into induced pluripotent stem cells (iPSC), which subsequently can be re-differentiated to specific somatic cell types. Alternatively, partial cell reprogramming converts somatic cells into other somatic cell types by transient expression of pluripotency genes thus generating intermediates that retain their original cell identity, but are responsive to appropriate cocktails of specific differentiation factors. Additionally, biological rejuvenation by partial cell reprogramming is an emerging avenue of research.

***Objective*:** Here, we will briefly review the emerging information pointing to partial reprogramming as a suitable strategy to achieve cell reprogramming and rejuvenation, bypassing cell dedifferentiation.

***Methods*:** In this context, regulatable pluripotency gene expression systems are the most widely used at present to implement partial cell reprogramming. For instance, we have constructed a regulatable bidirectional adenovector expressing Green Fluorescent Protein and o*ct4, sox2, klf4* and *c-myc* genes (known as the Yamanaka genes or OSKM).

***Results*:** Partial cell reprogramming has been used to reprogram fibroblasts to cardiomyocytes, neural progenitors and neural stem cells. Rejuvenation by cyclic partial reprogramming has been achieved both *in vivo* and in cell culture using transgenic mice and cells expressing the OSKM genes, respectively, controlled by a regulatable promoter.

***Conclusion*:** Partial reprogramming emerges as a powerful tool for the genesis of iPSC-free induced somatic cells of therapeutic value and for the implementation of *in vitro and in vivo* rejuvenation keeping cell type identity unchanged.

Keywords: Partial reprogramming, pluripotency, cell identity, rejuvenation, iPSC, somatic cells.

1. INTRODUCTION

The discovery of animal cloning and subsequent development of cell reprogramming technology were quantum leaps of such magnitude that they are rewriting the rules of biology. The achievement of induced pluripotency represents the synthesis of scientific methodologies and principles that were developed over the last six decades. Among the pioneering studies that ultimately led to cell reprogramming, the experiments of John Gurdon and collaborators in the sixties are worth mentioning. Gurdon's group demonstrated that the nucleus of a differentiated intestinal tadpole cell could be turned into a totipotent one when transferred into a functionally enucleated frog oocyte. These newly generated cells were able to give rise to a whole new frog [1, 2]. This research led to the achievement of mammalian cloning by somatic cell nuclear transfer (SCNT), attained in 1996, with the birth of Dolly, the sheep [3]. This work was soon followed by studies reporting cloning in other mammalian species [4-6]. These results revealed that the genome of even fully differentiated cells remains functionally intact and that, under proper conditions, can support the development of a whole organism.

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In 2006, Takahashi and Yamanaka [7] demonstrated that the transfer of only four master genes namely, *oct4*, *sox2*, *klf4* and *c-myc* (OSKM genes), to cultures of adult mouse fibroblasts was able to reprogram them, taking the cells to a pluripotency stage in which they behaved as embryonic stem cells; they were termed induced Pluripotent Stem Cells (iPSC). This seminal paper marked the birth of the field of cell reprogramming and paved the way for the subsequent implementation of cell rejuvenation and the generation of induced somatic cells of any type [8].

1.1. The Emergence of Partial Reprogramming

The first cell reprogramming strategy was the one developed by Yamanaka's group which, as mentioned above, involves converting a somatic cell line into iPSCs, which can be subsequently re-differentiated to specific somatic cell types. This is still the most widely used strategy to convert a somatic cell type into another and is virtually the only procedure so far employed to rejuvenate cells [9]. Despite the advantages of iPSC technology, the procedure as a whole is arduous, lengthy and costly. Since the protocols to generate iPSCs include several steps, the efficiency with which the final cell type is generated may be low. Furthermore, there are a number of concerns about the safety of iPSC-derived cells which need to be addressed before they can be used clinically [10]. Thus, the

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tumorigenic potential of contaminating iPSCs that fail to differentiate into the desired induced somatic cell type increases the risk for clinical application of induced somatic cells generated by this procedure [11]. These concerns have led to the emergence of other ways of reprogramming cells which involve direct conversion between cell types. One of these strategies was initially called Pluripotency factor-mediated Direct Reprogramming (PDR) [12] which is currently better known as partial reprogramming. This procedure uses fully differentiated somatic cells and converts them into other somatic cell types by transient expression of pluripotency genes, typically for 3 to 5 days, thus generating epigenetically unstable (EU) intermediates which are responsive to appropriate cocktails of specific differentiation factors [13-15].

The PDR strategy originated from early observations indicates that iPSCs are generated in either a sequential or stochastic way [16-20]. This led Kim *et al.* [12] to hypothesize that it could be possible to manipulate cells at an early and EU state induced by the Yamanaka pluripotency factors. In the presence of appropriate differentiation factors, these EU intermediates can be subsequently induced to differentiate into various somatic cell lineages with more stable epigenetic profiles. This new method has several advantages such as the use of a universal pluripotency gene set and the ability to generate rejuvenated multipotent progenitor cell population capable of differentiating into various tissue-specific target cells under proper conditions. Thus, PDR has been used to reprogram mouse fibroblasts into neural stem cells (iNSC) [13], neural progenitors [14] and cardiomyocytes [15]. Partial reprogramming has also been used to generate induced bronchiolar progenitor-like cells using a precisely timed expression of OSKM in non-proliferative bronchiolar Club cells (Clara cells) from R26-rtTA/Col1a1:tetO-4F2A double transgenic mice [21].

Although it was initially thought that in PDR the EU intermediates bypassed the pluripotency state [12], later studies demonstrated that when PDR is used to generate reprogrammed mouse cardiomyocytes or neural progenitor cells from mouse fibroblasts, most of the induced cells derived by OSKM-induced conversion pass through a transient pluripotency state as shown by the reactivation of X chromosome and expression of endogenous OCT4 and NANOG [22]. The evidence that EU intermediates pass through a transient pluripotency state, has been strengthened by a report showing that when PDR is used to generate iNSCs from fibroblasts, iNSC colonies repress retroviral transgenes and reactivate silenced X chromosomes, both of which are pluripotency hallmarks [23]. We now consider the terms EU intermediates and pluripotent intermediates as interchangeable. The fact that PDR involves a transient pluripotency stage implies that partial reprogramming (PDR) applied to somatic cells from old donors may erase at least part of the epigenetic marks of aging.

There is another direct cell reprogramming approach, known as lineage reprogramming (LR) or transdifferentiation, which emerged in 1987 [24] for the generation of specific cell types. It consists of the direct conversion of one somatic cell type into another one by ectopic expression of multiple lineage-specific TF or microRNAs (miRNA) without the cell passing through the pluripotent stem cell stage [25, 26]. This strategy uses factors that show specific expression in target cells. It has been shown that lineage reprogramming does not rejuvenate cells, most likely because it does not involve a transient pluripotency stage [27].

1.2. Rejuvenation by Partial Cell Reprogramming

For many years it was considered that although cells taken from old individuals could be fully rejuvenated *ex vivo*, rejuvenation *in vivo* was not possible due to the fact that continuous expression of the Yamanaka genes in animals had been shown to induce multiple teratomas due to the dedifferentiation of cells in many organs [28, 29]. However, in 2016, it was for the first time reported that cells and organs can be rejuvenated *in vivo* by partial reprogramming [30]. In this seminal study, it was shown that in progeric transgenic mice harboring the OSKM genes under the control of a Tet-On regulatable promoter, *in vivo* cyclic partial reprogramming (achieved by alternatively adding and removing antibiotic doxycycline (DOX) from the drinking water) can erase the epigenetic marks of aging, sparing the epigenetic marks of cell identity. Thus, cyclic partial reprogramming *in vivo* can not only rejuvenate somatic cells from progeric mice but is even able to rejuvenate partially the progeric mice themselves, increasing their survival time [30]. In the same study, Ocampo *et al.* used 12-month-old, transgenic non-progeroid mice. In these animals, cyclic partial reprogramming enhanced the poor regenerative capacity of their pancreas and skeletal muscle and made these tissues more resilient to a subsequent insult. Specifically, after the administration of streptozotocin or mechanical injury, cyclic transient induction of OSKM triggered the proliferation of beta cells in the pancreas and satellite cells in the skeletal muscle. Satellite cells are critical for the maintenance of tissue homeostasis but their number in muscles typically decreases with age. Thus, the benefits of cyclic partial reprogramming may go beyond the rejuvenation of old animals; it could also constitute an effective regenerative treatment [31].

The above results prompted studies aimed at characterizing the cellular changes that take place during partial reprogramming. An analysis of cell age during fibroblast reprogramming to iPSC confirmed that reprogramming leads to a reduction in the biological age of cells and that, on the average, the loss of somatic gene expression (three sets of fibroblast markers were monitored at different time points of the reprogramming process) lags behind the epigenetic age, suggesting that rejuvenation can be achieved *in vivo* by interrupted reprogramming [32]. Partial reprogramming has been proposed as a suitable strategy for treating neurodegenerative diseases [8, 33]. A special case of partial reprogramming was reported in a study in which human mesenchymal stem cells (MSC) were transfected with six nonregulatable mono-transgenic episomal plasmids harboring the OSKM, Lin 28 and siRNA for P53 factors. Some of the MSCs could be taken to the iPSC state provided that they were cultured on mouse embryonic feeder cells (MEFs) with standard iPSC culture conditions. When the achievement of pluripotency by the transfected MSC was prevented by growing the cells in the MSC-culture medium comprising human platelet lysate (hPL), no signs of rejuvenation were detected (for instance, they entered replicative senescence at 42nd day of culture as non-transfected MSC did). The same lack of rejuvenation was observed when transfected MSCs were grown in the iPSC medium for 14 days and then transferred to a standard MSC growth medium [34]. Whether the reported lack of rejuvenation by interrupted reprogramming of MSC was due to the intrinsic features of MSC or due to the unsuitability of the partial reprogramming protocol employed, remains to be clarified.

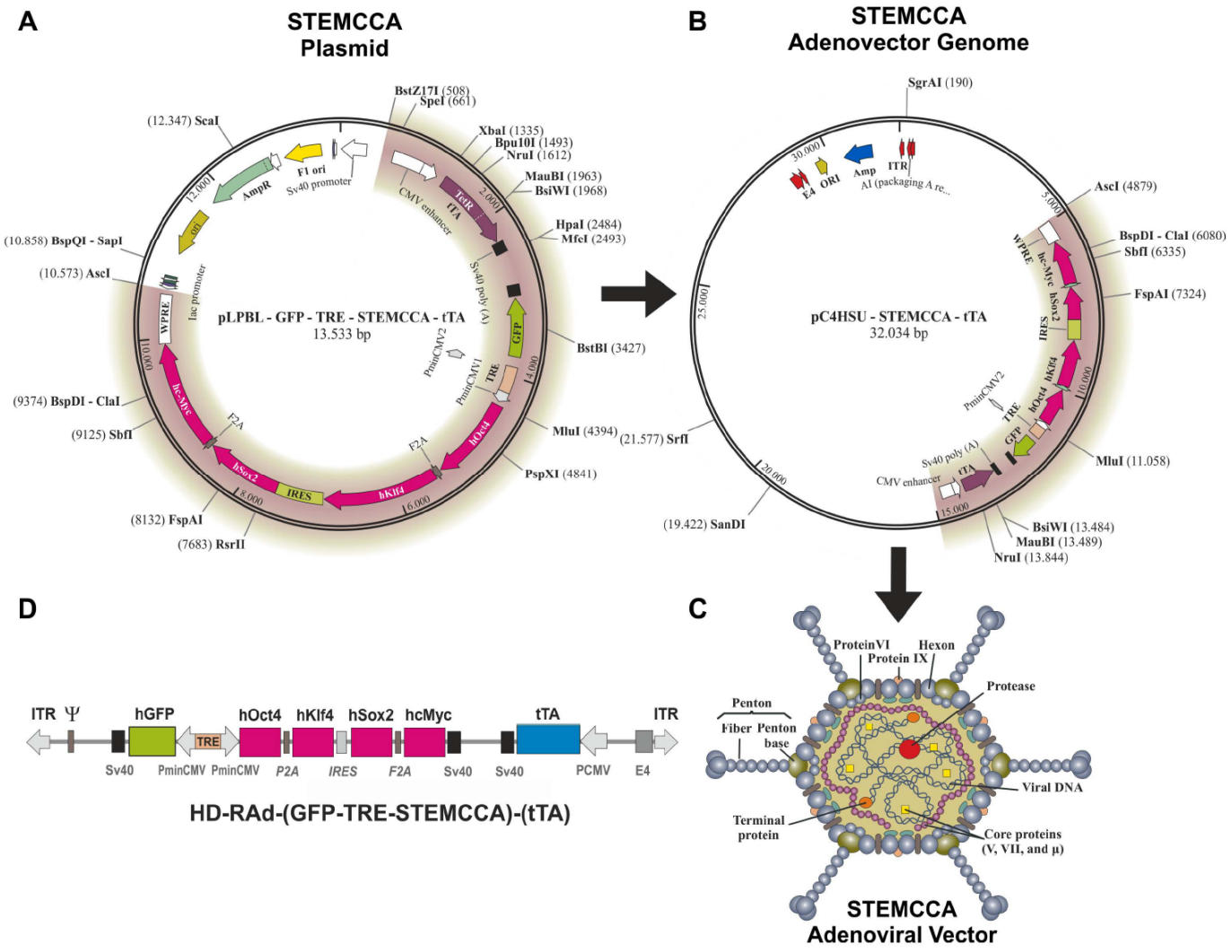

Fig. (1). Set of plasmids used to construct the STEMCCA helper dependent (HD) Adenovector. A) Subcloning plasmid pLPBL harboring the the humanized Green Fluorescent Protein (GFP) and o*ct4, sox2, klf4* and *c-myc* genes (OSKM genes). The OSKM genes are arranged as a bicistronic tandem (hSTEMCCA tandem) which is under the control of a Tet-Off bidirectional promoter that also controls the expression of the GFP gene. Separately, a constitutive cassette expresses the regulatory protein tTA. The whole STEMCCA cassette is shown as a shaded area. **B**) Backbone of a HD adenovector with the STEMCCA system (shaded area) cloned in it. **C**) Diagrammatic representation of the full HD RAd STEMCCA generated. **D**) Backbone of the HD STEMCCA Adenovector constructed. Abbreviations- TRE: Tetracycline responsive element; tTA: chimeric regulatory protein; PminCMV: cytomegalovirus minimal promoter; SV40pA: polyadenylation signal; ITR: inverted terminal repeats; ψ: packaging signal. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

It has been recently reported that an adeno-associated viral (AAV) vector expressing the OSK genes under a regulatable Tet system showed regenerative and rejuvenating effects *in vivo* in mouse retinal ganglion cells (RGCs) and in the human neuronal cell line SH-SY5Y [35]. The human cells were injured by exposing them to vincristine (VCS), a chemotherapeutic agent, and then transduced with th OSK AAV. Expression of the OSK transgenes induced a marked axonal regeneration in the injured neurons which also showed a significant reduction in their epigenetic age. *In vivo*, two models were used, a model based on the crush injury of axons and a glaucoma model. In both models, post-injury injection of OSK AAV had a regenerative effect that did not involve RGC proliferation but rather a functional recovery which was associated with a reduction of epigenetic age. The study also included an assessment of the rejuvenating effect of OSK gene therapy treatment on RGCs from middle-aged mice (12 months). At this age, visual acuity and other vision functions declined significantly when compared with that of young (4 months) counterparts. Interestingly, 4 weeks after OSK AAV intravitreal injection, the visual acuity of the older mice was restored to youthful levels as were other visual functions. Improvement of visual function was also confirmed by transcriptome assessment in RGCs. From the 464 genes that were differentially expressed in middle-aged *versus* young RGCs, 94 % were restored to youthful levels. The rejuvenating action of the treatment was revealed by the observation that after 4 weeks of treatment, epigenetic age was substantially reduced. Interestingly, the knockdown of TET1 or TET2 dioxygenases (enzymes that induce DNA demethylation) blocked these rejuvenating effects of OSK.

Taken together, the results reported in this study are both intriguing and revealing. *In vivo* exposure of the RGCs to OSK expression for as long as 4 weeks induced neither teratomas nor dedifferentiation. It may be possible that the dedifferentiating and teratogenic effect of the OSKM cocktail *in vivo* lies on cMyc, which is not a pluripotent but a proliferative and oncogenic gene. In their seminal 2006 paper, Takahashi and Yamanaka reported that they were forced to include cMyc in their OSKM cocktail due to the fact that the efficiency of OSK to generate iPSC was exceedingly low [7]. This disadvantage of OSK for cell reprogramming turns into a major advantage for *in vivo* rejuvenation. As reviewed above, a transient pluripotency stage seems mandatory to achieve rejuvenation by cell reprogramming. Since this should hold true for RGCs, it remains to be determined whether OSK-treated RGCs undergo a transient pluripotency stage.

1.3. An Adenoviral Vector for Implementing Nonintegrative Partial Reprogramming

There have been numerous efforts to construct viral and non-viral OSKM gene transfer vectors for implementing nonintegrative cell reprogramming. Among the viral vectors assessed were the adenovectors, which do not integrate their genes into the target cell's genome, thus avoiding the risk of insertional mutagenesis [36]. In addition to the safety offered by adenovectors, the high cloning capacity of the so-called helper-dependent adenovectors (HD-RAd) allows them to harbor polycistronic expression cassettes along with their associated regulatory elements [37]. Therefore, there is a renewed interest in the use of adenoviral vectors for non-integrative pluripotency gene transfer to somatic cells. In order to perform non-integrative partial reprogramming, we have recently reported the construction of a regulatable adenovector expressing the humanized Green Fluorescent Protein (GFP) and OSKM genes (known as the Yamanaka genes). The Yamanaka genes are arranged as a bicistronic tandem (STEMCCA tandem) placed under the control of a Tet-Off bidirectional promoter that also controls the expression of the GFP gene. Separately, a constitutive cassette expresses the regulatory protein tTA (Fig. **1**) [38]. The availability of this new adenovector opens a number of possibilities like 1) the implementation of nonintegrative partial cell reprogramming for the genesis of induced somatic cells of therapeutic interest. 2) the implementation of cell rejuvenation by partial reprogramming, which keeps cell type identity unchanged throughout the process and, 3) the implementation of *in vivo* regenerative gene therapy in some specific regions of the brain, like the neuroendocrine system. The rationale behind the latter statement is based on the fact that the transduction efficiency of viral vectors *in vivo* is quite low compared with transgenic animals, therefore only a

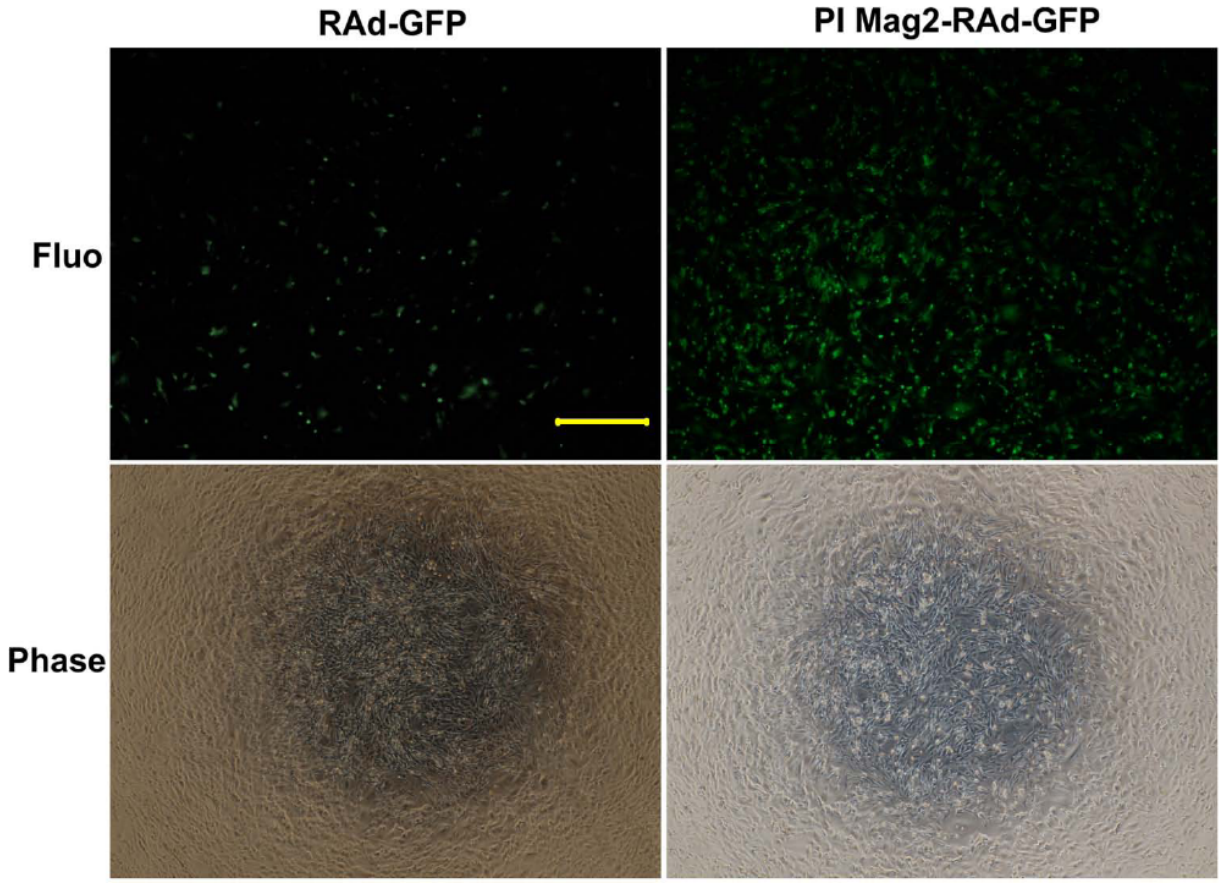

Fig. (2). Magnetofection in rat fibroblasts using RAd-GFP- Cells were plated on 12-well plates and incubated with either a low dose of RAd-GFP alone (left column) or a low dose of RAd-GFP vector complexed with PI Mag2 magnetic nanoparticles (0.5 µl/well – 200 µl medium /well) in the prescence of an external magnetic field (right column). Upper images show fluorescence microscopy of the lower images which show phase contrast BF microscopy. Scale bar, 200 µm. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 1. Summary of key points of the review.

-	Key Points	Relevant References
The Emergence of Partial Reprogramming	- Description of Pluripotency factor-mediated Direct Reprogramming (PDR), now known as partial reprogramming - Introduction of Epigenetically Unstable (EU) intermediates, and its transient pluripotency state	[12, 13]
Rejuvenation by Partial Cell Reprogramming	- Partial reprogramming as an approach to erase part of epigenetic marks of aging - Report of *in vivo* cyclic partial reprogramming - Comment on partial reprogramming as a strategy for treating neurodegenerative diseases	[26, 28]
An adenoviral vector for implementing nonintegrative partial reprogramming	- Advantages of adenoviral vectors among other tools to perform partial reprogramming - Outline of a regulable adenoviral vector harboring OSKM - Account of proposed experiments to restore TIDA neurons function	[33, 35, 39]
Magnetofection	- Brief description of magnetofection technique to improve viral transduction into cells	[41]

small % of cells will be transduced even in the injection area. Such a low percentage of rejuvenated (or restored) cells will not have a significant impact on tissue function. However, the situation is different in the hypothalamus. It is well-established that old female rats typically develop chronic hyperprolactinemia [39, 40] which is consequential to progressive dysfunction and loss of the hypothalamic tuberoinfundibular dopaminergic (TIDA) neurons, the physiologic role of which is to exert a tonic inhibitory control on prolactin (PRL) secretion and lactotroph proliferation [41, 42]. If a single intrahypothalamic injection of the STEMCCA adenovector rejuvenates only a small percentage of TIDA neurons, the increased release of dopamine into the portal system of the hypothalamic median eminence will reach the lactotrophic cell population of the anterior pituitary gland, an increase that is likely to reduce PRL secretion and lactotroph proliferation. In other words, the beneficial effect of rejuvenating a small % of TIDA neurons would be amplified by an increased dopamine output reaching the anterior pituitary gland. This amplifying effect of gene therapy in the hypothalamus of old female rats has been demonstrated for insulin-like growth factor –I (IGF-I) gene therapy [43, 44] and other neurotrophic factors [45].

1.4. Magnetofection

Initially, the safety offered by adenovectors was in part offset by their relatively low transduction efficiency. This is no longer the case thanks to the methodologies like magnetofection, which significantly increases the transduction efficiency of adenovector systems [46]. The concept of magnetofection emerged in the early 2000s with the discovery that magnetic nanoparticles (MNPs) complexed to nonviral or viral vectors can, in the presence of an external magnetic field, markedly improve gene transfer into cells, an observation that has raised substantial interest. This technique, called magnetofection, is particularly suitable for improving viral vector-mediated gene transfer in cell cultures but it has also been successfully used *in vivo* [47-50].

This capability is illustrated in Fig. (**2**) which shows the substantial increase in transduction efficiency that can be achieved by magentofecting an adenovector expressing GFP in primary cultures of rat fibroblasts. With the aid of this technique, adenovector-based partial cell reprogramming can be more effectively implemented.

CONCLUDING REMARKS

While the discovery of animal cloning and cell reprogramming technology was a watershed in biology as it led to the achievement of biological rejuvenation which in turn constitutes a paradigm shift in gerontology, the observation that partial reprogramming erases epigenetic marks of aging sparing marks of cell identity has opened the possibility of implementing iPSC-free reprogramming and rejuvenation of cells and organs in animals and eventually, in humans. There is a keen interest in understanding the mechanisms underlying the properties of partial cell reprogramming which, in turn, may lead to refinements that substantially increase the effectiveness and safety of the procedure. Table **1** summarizes the key points of the review.

LIST OF ABBREVIATIONS

Yamanaka genes	=	o*ct4, sox2, klf4* and *c-myc* genes
OSKM	=	oct4, *s*ox2, klf4 and c-myc genes
iPSC	=	induced Pluripotent Stem Cells
PDR	=	Pluripotency factor-mediated Direct Reprogramming
EU	=	Epigenetically Unstable
DOX	=	Doxycycline

MSC	=	Mesenchymal Stem Cells
MEFs	=	Mouse embryonic Feeder Cells
HD-RAd	=	Helper-Dependent Adenovectors
GFP	=	Green Fluorescent Protein
TIDA	=	Tuberoinfundibular Dopaminergic
MNPs	=	Magnetic Nanoparticles
BF	=	Bright Field
RGC	=	Retinal Ganlion Cells
AAV	=	Adenoassociated Vector
VCS	=	Vincristine

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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