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ARTICLE

Regulatable adenovector harboring the GFP and Yamanaka genes for implementing regenerative medicine in the brain

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Abstract

Biological rejuvenation by partial cell reprogramming is an emerging avenue of research. In this context, regulatable pluripotency gene expression systems are the most widely used at present. We have constructed a regulatable bidirectional adenovector expressing the humanized green fluorescent protein (GFP) and o*ct4, sox2, klf4*, and *c-myc* genes (known as the Yamanaka genes or OSKM). 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. Vector DNA was transfected in HEK293 Cre cells, which were additionally infected with the helper adenovector H14, unable to package its DNA due to the Cre recombinase produced by the HEK293 Cre cells. The newly generated vector was expanded by six iterated coinfections of the above cells which were lysed at the end of the process and the adenovector purified by ultracentrifugation in a CsCl gradient. The titer of the initial preparation was 1.2×10^{12} physical viral particles/ml. As expected, GFP fluorescence in vector-transduced rat fibroblast cultures declined with the dose of doxycycline (DOX) present in the medium. Immunocytochemical analysis of transduced cells confirmed the expression of the four Yamanaka genes. Additionally, 3 days after vector injection in the hypothalamus of rats, a significant level of fluorescence was observed in the region. Addition of 2 mg/ml DOX to the drinking water reduced the GFP expression. This adenovector constitutes a promising tool for implementing nonintegrative partial cell reprogramming.

Introduction

The first step for cell reprogramming involves the transfer of an appropriate set of pluripotency genes to the somatic cells to be dedifferentiated. Early approaches used monocistronic retroviral vectors in order to integrate the pluripotency genes into the target cell's genome [1]. While transgene integration allows high transduction efficiencies, experience has shown that such integration conveys the risk of insertional mutagenesis and the consequent emergence of tumors in vivo. In the years that followed Yamanaka's seminal paper demonstrating the feasibility of cell reprogramming [1], several viral and nonviral gene transfer methods were employed. 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 [2]. 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 [3]. Consequently, there is a renewed interest in the use of adenoviral vectors for nonintegrative pluripotency gene transfer to somatic cells.

More recently, integrative polycistronic systems harboring the o*ct4, sox2, klf4*, and *c-myc* genes (known as the Yamanaka genes or OSKM) under the control of regulatable Tet-On promoters were constructed in order to generate transgenic cells [4, 5] or transgenic mice [6]. These systems allowed researchers to implement partial reprogramming, a

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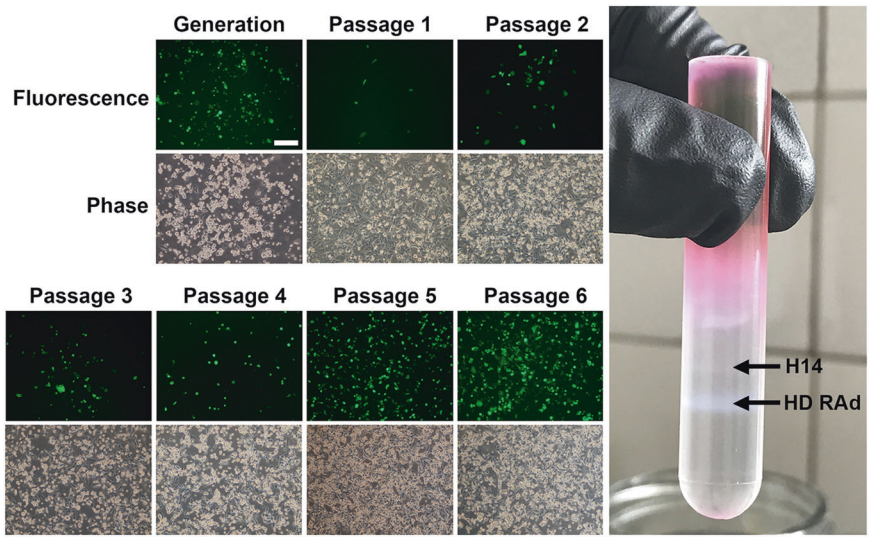

Fig. 1 Vector amplification and purification. Left panel—The STEMCCA adenovector was amplified by iterative coinfections at the end of which 48 preparative 175 cm^2 culture flasks were generated (Fig. 1, left panels). Cells were collected, lysed and the pellet was submitted to two ultracentrifugation steps. Right panel—Image showing the banding of the HD-RAd-STEMCCA (lower arrow) in a CsCl gradient after 18 h ultracentrifugation at 180,000 × *g*, in a swinging bucket SW60Ti rotor. The faint upper band represents contaminant H14 helper virus (upper arrow). Scale bar represents 200 µm

process by which the OSKM genes are transiently expressed by the addition of the antibiotic doxycycline (DOX) to the culture medium or drinking water, as appropriate. Removal of the antibiotic silences the OSKM genes. Transient expression of the OSKM does not lead to the generation of induced pluripotent stem cells (iPSCs) but to cells of the original type that are multi or pluripotent. Furthermore, these cells are epigenetically rejuvenated [6].

In order to perform nonintegrative partial reprogramming, we undertook to construct a regulatable HD-RAd for the simultaneous expression of the OSKM genes and the reporter gene for humanized green fluorescent protein (GFP). The present report describes the STEMCCA Tet-Off adenovector construction process, the scale-up procedure as well as the DOX dose−response curves, OSKM gene expression in fibroblasts and GFP expression in the rat hypothalamus.

Results

Vector expansion and purification

Once an initial inoculum of the HD STEMCCA adenovector was generated, the virus was amplified by six iterative coinfections with the H14 helper virus, at the end of which 48 preparative 175 cm^2 culture flasks were generated (Fig. 1, left panels). Cells were collected, lysed and the pellet was submitted to two ultracentrifugation steps in a swinging bucket SW60Ti rotor, first for 1 h at 180,000 × *g* in a discontinuous CsCl gradient at 18 ºC followed by an 18-h ultracentrifugation at the same gravity in a 1.35 g/ml CsCl isopycnic gradient. A main HD vector band was observed and collected trailed by a faint band corresponding to the H14 helper vector. (Fig. 1, right image). Incubation of 5 µl purified HD vector with HEK 293 cells did not induce lysis plaques, suggesting that the levels of H14 contamination in the HD preparation are negligible.

Transgene expression

In primary rat tail fibroblast cultures, GFP shows a moderately high expression (fluorescence) that remains at steady levels for at least 5 days (Fig. 2). Fluorescence was weaker but still clearly detectable in fibroblasts from 28-month-old rats (data not shown). Immunocytochemical assessment of OSKM gene expression in HD-RAd-STEMCCA-GFP-Tet-Off-transduced fibroblasts (for 3 days) revealed significant expression of OSKM genes, whereas control nontransduced fibroblasts showed no significant immunoreactivity for *Oct4*, *Sox2* and *Klf4*. A weak immunoreactivity was observed for *c-myc*. ICC of transduced cells cultured in the presence of 1000 ng/ml DOX showed a background comparable with control fibroblasts (Fig. 3).

Vector regulatability

The fluorescence levels of HD-RAd-STEMCCA-GFP-Tet-Off-transduced rat fibroblasts incubated with DOX at concentrations, in triplicates, ranging from 0 to 500 ng/ml,

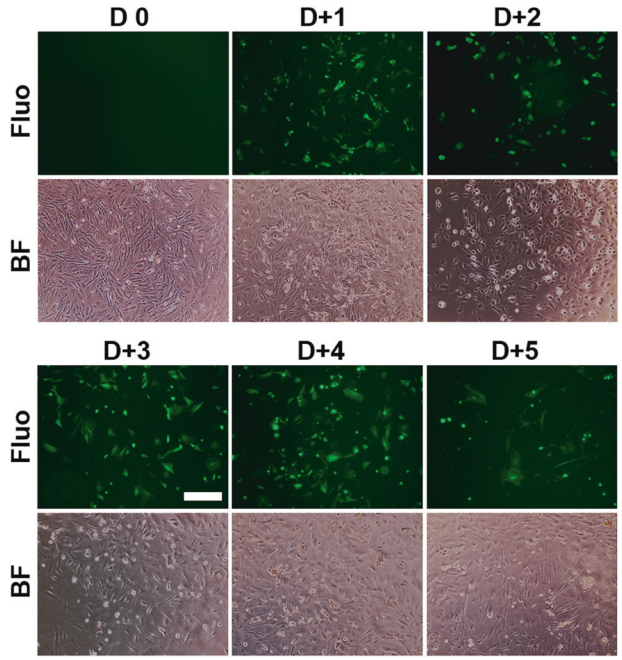

Fig. 2 Time-course expression of GFP in transduced fibroblasts. Fibroblasts grown in 24-well plates at a density of 20×10^3 cells per well were transduced with purified HD-RAd-STEMCCA-GFP-Tet-Off and were photographed daily for 5 days. Upper row shows fluorescence microscopy images whereas the lower row displays phase contrast micrographs. Scale bar represents 200 µm. GFP green fluorescent protein

displayed a sharp DOX-dependent reduction. At about 10 ng/ml, the curve reaches a plateau, showing a 3.9% residual fluorescence (leakage) (Fig. 4).

Transgene expression in the medial basal hypothalamus (MBH)

Stereotaxic injection of HD-RAd-STEMCCA-GFP-Tet-Off in the MBH of young rats induced, after 3 days, a moderate but significant expression of fluorescence in a limited proportion (as revealed by 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining) of cells in the periventricular nucleus, the magnocellular nucleus and to a lesser extent in other nuclei of the MBH (Fig. 5, upper panels). In the MBH of similarly treated rats, but which were given 2 mg/ml DOX in the drinking water, there was a clear fall in the proportion of fluorescing cells (Fig. 5, lower panels).

Discussion

Conventional cell reprogramming involves converting a somatic cell line into iPSC, which subsequently can be re-differentiated to specific somatic cell types of interest [1]. Although it still remains the most widely used cell reprogramming strategy, the procedure as a whole is arduous, lengthy and costly. Since the protocols to generate iPSCs include a number of steps, the efficiency with which the final cell type is generated may be low. Furthermore, significant concerns about safety of iPSC-derived cells need to be addressed before these cells can be used clinically [7]. These hurdles have led to the emergence of other ways of reprogramming cells that involve direct conversion between cell types (for a review on the different direct cell reprogramming strategies to generate neural cells, see ref. [8]). One of the new strategies, initially termed Pluripotency factor-mediated Direct Reprogramming (PDR) but currently better known as partial reprogramming, uses somatic cells and converts them into other somatic cell types by transient expression of pluripotency genes, usually for 3–5 days, thus generating epigenetically unstable (EU) intermediates which are responsive to appropriate cocktails of specific differentiation factors [4, 5, 9]. These studies used mouse fibroblasts in whose genome the OSKM genes, under the control of a Tet-On regulatable promoter, had been integrated. Although adenoviral vectors have been used for cell reprogramming [2], we are unaware of reports documenting the use of viral vectors for partial cell reprogramming. To our knowledge HD-RAd-STEMCCA-GFP-Tet-Off is the first documented adenovector harboring the OSKM and *gFP* genes under the control of a regulatable promoter. It offers the potential to perform nonintegrative partial cell reprogramming and subsequently differentiate the EU intermediates into different types of somatic cells. This potential implies that although partially reprogrammed somatic cells retain their identity, they are transiently pluripotent. In effect, although it was initially thought that in PDR the EU intermediates bypass the pluripotency state [10], subsequent studies showed that when PDR is used to generate induced cardiomyocytes [5] or neural precursor cells [4], most of the induced cells, derived from mouse fibroblasts, pass through a transient pluripotency state (as evidenced by the reactivation of the X chromosome and expression of endogenous *nanog* and *oct4* [11]). The evidence that EU intermediates pass through a transient pluripotency state has been further strengthened by a study showing that when PDR is used to generate induced neural stem cells (iNSCs) from mouse fibroblasts, iNSC colonies repress retroviral transgenes and reactivate silenced X chromosomes, both of which are hallmarks of pluripotency [12]. The process seems safe as in vivo studies have demonstrated that multiple cycles of partial reprogramming in transgenic mice harboring a Tet-On system expressing the OSKM genes do not generate tumors [6]. Although even in the presence of high DOX concentrations our vector shows some leakage (3.9%), it seems unlikely that in a partial reprogramming strategy such a low level of leakage could be enough to keep the reprogramming process in progress.

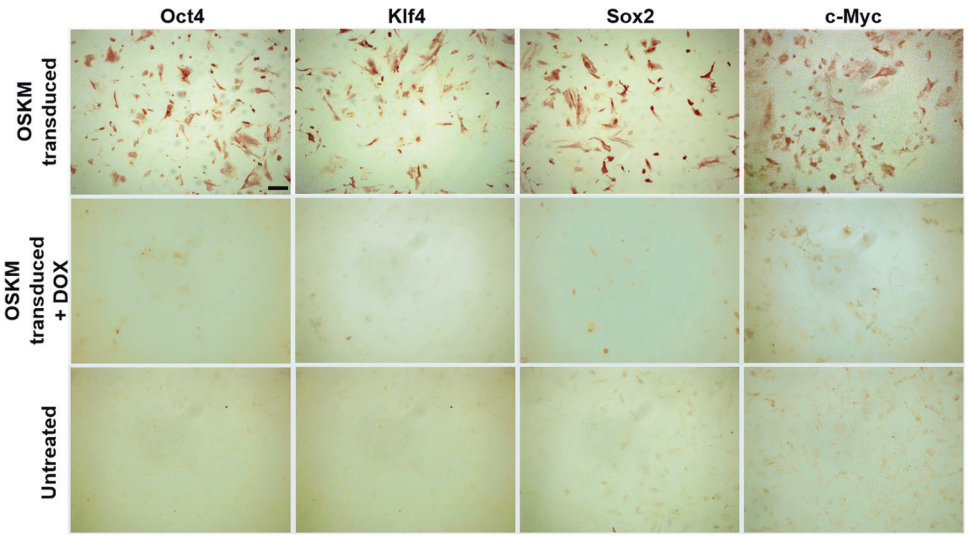

Fig. 3 Immunocytochemical assessment of adenovector-transduced cells. Fibroblasts were grown on glass coverslips in a 24-well plate at a density of 20×10^3 cells per well and were transduced with 2.4×10^9 pvp HD-RAd-STEMCCA-GFP-Tet-Off per well. Immunocytochemistry for OSKM was performed on post-transduction day 3. Upper row shows expression of each of the OSKM genes in vector-transduced cells, middle row shows similarly transduced cells grown in the presence of 1000 ng/ml DOX, whereas the lower row shows expression of the same genes in nontransduced cells. Scale bar represents 200 µm

In parallel with the Tet-Off adenovector described, here we constructed an otherwise identical Tet-On STEMCCA adenovector but preliminary studies in cell culture showed that the Tet-Off system is better suited for partial reprogramming, mainly due to the fact that OSKM silencing is significantly faster in the Tet-Off than in the Tet-On system (unpublished results).

Although our adenovector is effective to transduce fibroblasts from adult rats, the fact that it does not integrate its five transgenes in the host cell genome will cause a progressive dilution in the number of transduced cells as they proliferate. Although this well-known limitation of adenovectors significantly reduces their efficiency for conventional cell reprogramming (generation of iPSCs), where 2–3 weeks of continued OSKM expression is necessary, this is not the case in partial reprogramming. In effect, transgene expression in partial reprogramming strategies is usually required for 5 or less days. As shown in Results, with our vector the number of fibroblasts expressing GFP remains rather steady for at least 5 days post-transduction. Another application of partial reprogramming is epigenetic rejuvenation [13], a reprogramming process in which cells are epigenetically rejuvenated without changing their cell type. Rejuvenation by cyclic partial reprogramming has been achieved both in vivo and in cell culture using transgenic mice and cells expressing the OSKM genes controlled by a regulatable promoter [6]. We believe that our STEMCCA adenovector is well suited for in vitro rejuvenation despite the fact that only some of the cells in culture will be transduced. Profiting from their fluorescence, these transgenic cells can be concentrated by

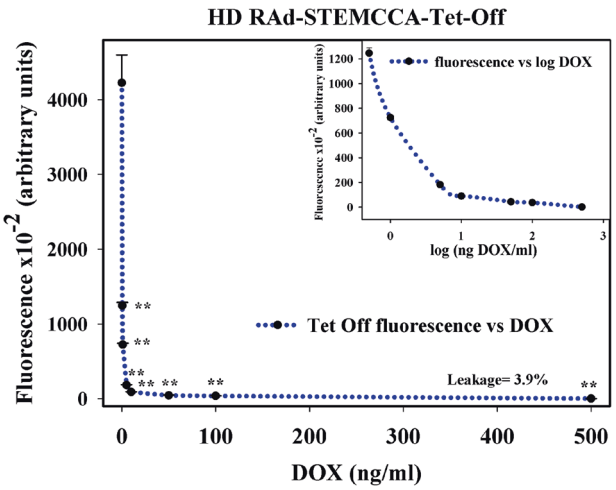

Fig. 4 DOX-dependent expression of GFP of HD-RAd-STEMCCA-GFP-Tet-Off in rat fibroblasts. Fluorescence expression in HD-RAd-STEMCCA-GFP-Tet-Off-transduced rat fibroblasts decreased sharply as DOX concentration increased in the culture medium. Cells were lysed 3 days post-transduction. The fluorescence plateau was reached at about 10 ng/ml DOX. At this point the fluorescence intensity was 3.9% of 0 ng/ml DOX levels (leakage). DOX doxycycline. Inset–Semi-logarithmic dose−response curve of the same data. $^{**}P < 0.01$ versus fluorescence at DOX dose 0

cell sorting and furthermore, each transgenic cell can be identified by its fluorescence, followed and studied during the rejuvenation process. In the above OSKM transgenic mice, cyclic partial reprogramming in middle-aged animals submitted to pancreas injury by streptozotocin or muscle damage by mechanical injury proved to possess regenerative properties [6]. Since our HD-RAd-

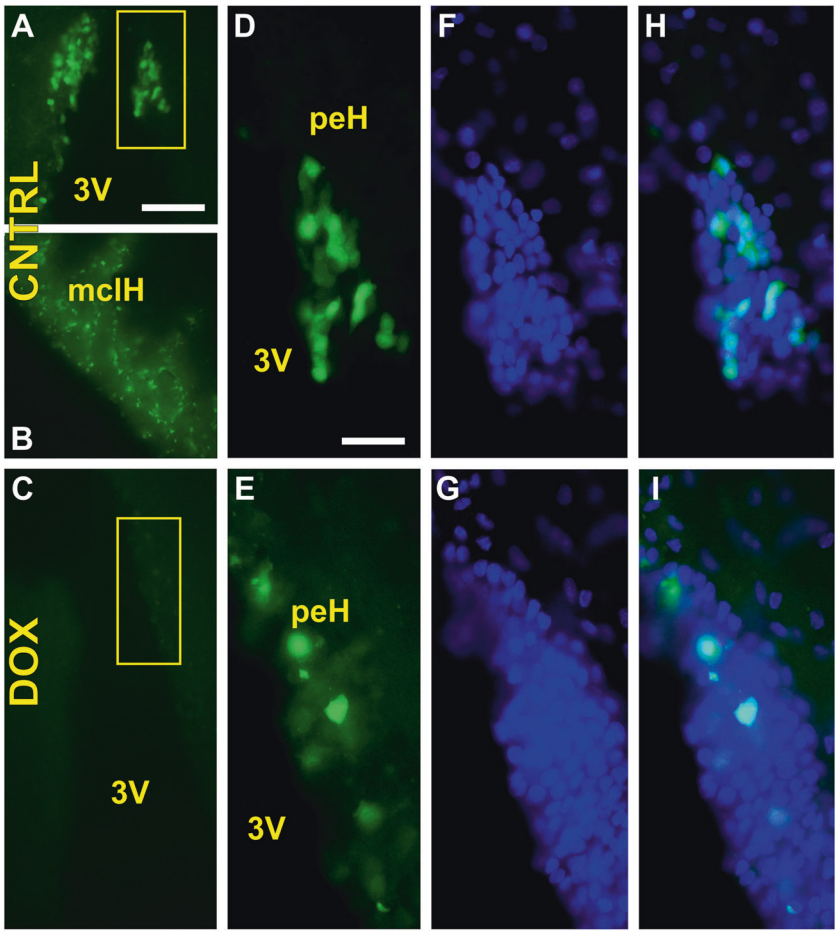

Fig. 5 GFP transgene expression in the MBH of rats stereotaxically injected with HD-RAd-STEMCCA-GFP-Tet-Off. Upper panels (**a**, **b**, **d**, **f**, **h**) correspond to animals that did not receive DOX in the drinking water. Lower panels (**c**, **e**, **g**, **i**) correspond to rats that received 2 mg/ml DOX in the drinking water for 3 days. Insets in panels (**a**) and (**c**) are magnified in right panels **d** GFP, **f** DAPI, **h** merge of (**d**) and (**f**) and in panels **e** GFP, **g** DAPI, **i** merge of (**e**) and (**g**), respectively. Notice in the lower panels, the significant reduction in the number of fluorescent cells effected by DOX. Scale bar in the panoramic images (**a**), (**b**) and (**c**) represents 200 µm. Scale bar in panel (**d**) corresponds to all other panels and represents 50 µm. 3V third ventricle, peH periventricular hypothalamic nucleus, mclH magnocellular nucleus of lateral hypothalamus, DOX doxycycline, MBH Medialbasal hypothalamus, DAPI 4′,6-diamidino-2-phenylindole dihydrochloride, GFP green fluorescent protein

STEMCCA-GFP-Tet-Off vector is functional in the MBH of female rats, we hypothesize that it could be used in the neuroendocrine system of, for instance, old female rats, in order to reverse, completely or in part, their well-documented neuroendocrine deficits [14]. The neuroendocrine system lends itself well to the implementation of restorative gene therapy as even the transduction and consequent rescue of a few target neurosecretory hypothalamic neurons will lead to increased release of specific hypophysiotropic hormones into the portal vascular system and from there to the pituitary gland where the signal will be amplified by the secretion of specific pituitary hormones and finally by the target peripheral glands. The feasibility of implementing restorative gene therapy in the neuroendocrine system of old female rats has been previously documented [15–17].

Concluding remarks

The availability of the STEMCCA adenovector opens new possibilities namely, (1) the implementation of non-integrative partial cell reprogramming for the genesis of induced somatic cells of therapeutic value. (2) The implementation of ex vivo cell rejuvenation keeping cell type identity unchanged. (3) Implementation of in vivo regenerative gene therapy in certain regions of the brain, particularly the neuroendocrine system.

The human OSKM genes have been successfully used in mouse [18] cells and also for cell reprogramming in a number of farm animals [19]. Therefore, our STEMCCA vector offers the possibility of implementing partial reprogramming in a number of species, human fibroblasts being of particular interest to us.

The effective achievement of the above aims is likely to face experimenters with numerous technical hurdles, but virtually every new step in regenerative medicine (and most other biomedical fields) has been riddled with challenges; however, in every instance, ingenious ways were found to overcome the challenges. One such hurdle comes from the fact that while the Tet-Off system shows a rapid suppressive response of transgene expression in response to DOX, after removal of the antibiotic, the DOX bound to the tTA protein remains partially protected from normal catabolism which causes a slow reactivation of the silenced transgenes. A possible way around this problem

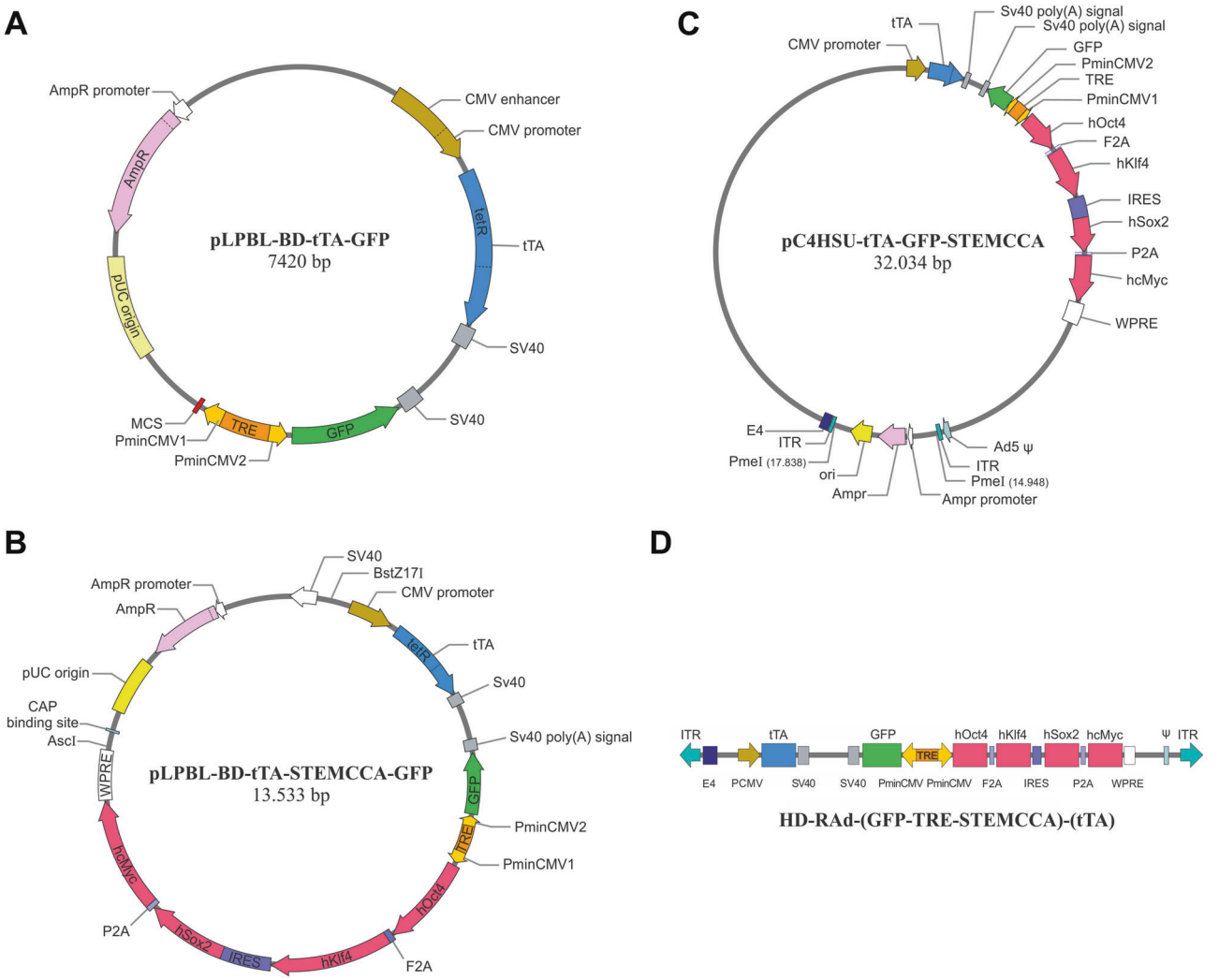

Fig. 6 Diagrammatic representation of main intermediate constructs generated during the construction of HD-RAd-STEMCCA-GFP-Tet-Off. **a** shows the base plasmid, pLPBL-BD-tTA-GFP, from which we departed in the generation of the above adenovector. It harbors the CMV1-TRE-CMV2 regulatable promoter flanked on one side by the gene for GFP and by a multiple cloning site (MCS) on the other side. Separately, the plasmid also harbors the gene for the chimeric tetracycline transactivator (tTA) protein, created by fusing one protein, TetR (tetracycline repressor), with the activation domain VP16, from the Herpes Simplex Virus type 1. This construct is under the control of the human cytomegalovirus (CMV) promoter. **b** displays the above plasmid now harboring the STEMCCA tandem, which was cloned into the MSC site. **c** shows the full STEMCCA construct (including all associated regulatory components) cloned into the HD genomic plasmid which includes a bacterial sequence between its ITRs, flanked by PmeI sites. **d** illustrates the basic components of HD-RAd-STEMCCA-GFP-Tet-Off genome after removing the bacterial sequence from pC4HSU-tTA-STEMCCA-GFP. GFP humanized green fluorescent protein, TRE Tetracycline responsive element, tTA chimeric regulatory protein, PminCMV cytomegalovirus minimal promoter, SV40pA polyadenylation signal, ITR inverted terminal repeats, ψ packaging signal

is to add DOX for only 1 day and then remove it. Transgene expression will remain relatively low for 2–4 days which seems reasonable as a resting period. Although we have already constructed the genome of the Tet-On version of the STEMCCA HD vector, our preliminary tests showed, as expected, that while addition of DOX to the systems rapidly activates transgene expression, removal of the antibiotic does not lead to a rapid silencing of transgenes, for the reasons given above. Since we considered that the latter limitation is more serious than the former for our rejuvenation purposes, we chose to use the Tet-Off system for our in vitro studies. Another potential limitation of viral vectors for implementing regeneration or rejuvenation of most brain regions is that the % of brain cells transduced in vivo is very low, a fact that is likely to compromise the functional recovery of the region in question.

The construction of our STEMCCA adenovectors (Tet-Off and Tet-On) was a 4-year effort in which we sometimes had to face daunting difficulties.

Materials and methods

Construction of a regulatable HD-recombinant adenoviral vector (RAd) Tet-Off adenovector harboring the GFP and Yamanaka genes

The adenovector was constructed using a commercial kit (Microbix Inc., Ontario, Canada) that provides the shuttle plasmid pC4HSU, the helper adenovirus H14 and the HEK293 Cre4 cell line. As starting point we used a derivative of plasmid pLPBL-1 (a kind gift from Dr. KCL Oka) into which we cloned a regulatable Tet-Off bidirectional construct where we had already inserted the gene for GFP in one of the two multiple cloning sites (MCS) flanking the bidirectional promoter PminCMV-TRE-PminCMV. On a separate site on the plasmid we cloned a constitutive cassette that expresses the regulatory protein tTA. The plasmid so designed was called pLPBL-BD-tTA-GFP (Fig. 6a). In the second MCS we cloned the bicistronic tandem *Oct4*-f2A-*Klf4*-ires-*Sox2*-p2A-*cMyc* (excised from the pHAGE2-hSTEMCCA-loxP plasmid, generously provided by Dr. G. Mostoslavsky), which harbors the four Yamanaka genes grouped in pairs placed downstream and upstream of an internal ribosome entry site. In turn, each pair of genes is separated by a type 2A CHYSEL (*cis*-acting hydrolase element) self-processing short sequence that causes the ribosome to skip the Gly-Pro bond at the C-terminal end of the 2A sequence, thus releasing the peptide upstream the 2A element but continuing with the translation of the downstream mRNA sequence. This allows near stoichiometric coexpression of the two cistrons flanking a 2A-type sequence [18]. The construct so generated was called pLPBL-TRE-STEMCCA-tTA (Fig. 6b).

Next, the whole expression cassette (STEMCCA cassette 10,065 bp) was excised from pLPBL-TRE-STEMCCA-tTA with nucleases BstZ171 and AscI and was subsequently cloned into the pC4HSU HD shuttle at the *Asc*I and *Swa*I sites giving rise to pC4HSU-STEMCCA-tTA (Fig. 6c). The pC4HSU HD shuttle consists of the inverted terminal repeats (ITRs) for Ad 5 virus, the packaging signal and part of the E4 adenoviral region plus a stuffer noncoding DNA of human origin which keeps a suitable size (28–31 Kbp) of the viral DNA. Between the two ITRs there is a bacterial sequence flanked by *Pme*I sites. The pC4HSU-STEMCCA-tTA plasmid was digested with *Pme*I in order to remove the bacterial sequence, thus generating the desired HD-RAd-STEMCCA-GFP-Tet-Off genome (Fig. 6d).

The linearized DNA backbone of the new HD-RAd was transfected in Cre 293 cells. Next day, purified helper H14 virus was added to the cell cultures at a multiplicity of infection (MOI) of 5. In H14, the packaging signal is flanked by lox P sites recognized by the Cre recombinase expressed by the 293 Cre4 cells. Therefore, the helper virus provides in *trans* all of the viral products necessary for generation of the desired HD-RAd. The infected 293 Cre4 cells were left for 2–3 days until cytopathic effect (CPE) was evident. Cells and medium were collected and submitted to three freeze-thaw cycles to lyse them. Clear lysates were obtained, mixed with H14 helper virus and added to a fresh culture of Cre4 293 cells at MOI 1. When CPE appeared, passage 2 (P2) cell lysates were prepared. This iterative coinfection process was carried on five more times in order to generate enough HD-RAd particles as to proceed to the purification step. The newly generated HD-RAd was rescued from P6. The HD-RAd so generated was purified by ultracentrifugation in CsCl gradients. Final virus stock was titrated by lysing the viral particles, extracting their DNA and determining its concentration in a Nanodrop spectrophotometer. For the first preparation, titer was 12×10^{11} pvp/ml.

Cells

HEK293 Cre4 cells

This cell line was provided by Microbix along with the HD-Ad construction kit. These cells have the same features than the standard HEK293 line but additionally harbor the gene for the Cre recombinase. They were grown in Eagle's minimum essential medium, 16.8 mM Hepes buffer (pH 7.0), 2 mM glutamine, 0.1 mM nonessential amino acids, 20 mg/l penicillin/streptomycin, 3.3 mg/l amphotericin B, 2.2 mg/l $NaHCO_3$, geneticin (0.4 mg/ml) and 10% (v/v) fetal bovine serum. They were grown at 37 ºC in a humidified atmosphere of 95% air - 5% CO_2. Cells were fed every 3–4 days and split when confluent.

Fibroblasts

Two-month-old intact rats were anesthetized with isoflurane and fibroblasts obtained from the distal half of the animal tail. After sterilization with 70% ethanol, 2-cm-long tissue specimens were cut, and placed for 5 min in 70% ethanol. They were let dry and transferred to a dish with complete medium (DMEM high glucose + ATB/ATM + 10% SFB). Following hair removal, they were diced in <5 mm pieces and digested with type I collagenase (SIGMA Chem. Co, St Louis, MO) dissolved in complete culture medium (0.01 mg type I collagenase per tail) as previously reported [20]. After digestion at 37 °C, the cell suspension was centrifuged at 200 rpm for 90 min. Then, samples were filtered through a 40 µm filter, resuspended in complete medium and centrifuged for 8 min at $1000 \times g$. Cell pellets were resuspended in 6 ml fresh complete medium. Centrifugation and resuspension were carried out two more times. Each sample was seeded into 25 cm^2 tissue culture flasks (Greiner,

Pleidelsheim Germany). Complete medium was replaced every 3 days.

Dose−response curve

Primary rat tail fibroblasts (passage 5) were seeded at a density of 20×10^3 cells per well in a 24-well plate. Cells were grown in DMEM medium (Invitrogen, Argentina) at 37 °C, 5% CO_2, and treated with concentrations of DOX ranging from 0 to 500 ng/ml. Next day (day 1), they were transduced with 2.4×10^9 pvp HD-RAd-STEMCCA-GFP-Tet-Off per well for 3 h and maintained in culture. Three days postinfection (day 4), cells were washed twice with PBS for 10 min and treated with lysis buffer (TRITON X-100 0,1%, EDTA 1 mM, PBS) for 30 min at 4 °C. The cell lysate was centrifuged at $14{,}000 \times g$ for a minute. The supernatant was transferred to a microplate. Fluorescence was measured in a Beckman Coulter DTX 880 Multimode Detector, using an excitation filter of 485/20 nm and an emission filter of 535/25 nm.

Immunocytochemistry of OSKM gene products

Primary rat tail fibroblasts were seeded on sterile glass coverslips in a 24-well plate at a density of 20×10^3 cells per well. Cells were grown in DMEM plus 10 % fetal bovine serum, at 37 °C and 5% CO_2. Next day (day 1) fibroblasts were transduced with 2.4×10^9pvp HD-RAd-STEMCCA-GFP-Tet-Off per well for 3 h and maintained in culture. Immunocytochemistry was performed on day 4. Cells were washed with PBS, fixed with 4% formaldehyde for 30 min and incubated with primary antibodies for 45 min. The following antibodies were used, as appropriate, mouse anti-hOct4 (1:10, BD Pharmingen, San Jose, CA), mouse anti-hSox2 (1:40, BD Pharmingen, San Jose, CA), mouse anti-hKlf4 (1:100, Ab Cam, Cambridge, MA), and mouse anti-hc-Myc (1:50, BD Pharmingen, San Jose, CA). Then, cells were incubated with biotinylated horse anti-mouse serum (1:300, Vector Labs, Burlingame, CA) for 45 min and with avidin-biotin-peroxidase complex for 30 min. Cells were finally incubated with 3,3′-diamino-benzidine-tetrahydrochloride (DAB). Cells were washed with PBS, mounted with Fluoromount™ (Electron Microscopy Sciences, Hatfield, PA), cover slipped and observed with an Olympus BX51 microscope equipped with a DP70 CCD Video Camera (Tokyo, Japan). The subsequent image processing was performed with the Image-Pro® Plus software (Version 5.1.2, Media Cybernetics, Inc).

Animals

Young (3 mo.) female Sprague−Dawley rats were used. Animals were housed in a temperature-controlled room (22 ± 2 ºC) on a 12:12 h light/dark cycle. Food and water were available ad libitum. All experiments with animals were performed in accordance to the Animal Welfare Guidelines of NIH (INIBIOLP's Animal Welfare Assurance No A5647-01) and approved by our Institutional IACUC (Protocol # T09-01-2013).

Surgical procedures

Rats were allotted to a control (no DOX) and experimental (2 mg/ml DOX in the drinking water) group. On experimental day 0, control and experimental animals received bilateral 15-µl intrahypothalamic injections containing $1.2{\times}10^{12}$ pvp/ml HD-RAd-STEMCCA-GFP-Tet-Off vector. For this purpose, rats were anesthetized by injection of ketamine hydrochloride (40 mg/kg, i.p.) and xylazine (8 mg/kg, i.m.), and placed in a stereotaxic frame. In order to access the arcuate-periventricular (ARC-PeH) region, the tip of a 22 gauge needle fitted to a 100 µl syringe was brought to the following coordinates relative to the bregma: −3.0 mm posterior, −10.0 mm ventral and 0.6 mm right and left [21]. On experimental day 3, they were placed under deep anesthesia and perfused with phosphate-buffered formaldehyde 4%, (pH 7.4) fixative. Each brain was removed and trimmed down to a block containing the whole hypothalamus. The block was then serially cut into coronal sections 40 µm thick on a vibratome (Leica, Nussloch, Germany).

Hypothalamic section assessment by fluorescence microscopy

Hypothalamic and other brain sections were counterstained with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma Chem Co, St Louis, MO), mounted with Fluoromount water-soluble mounting medium. Images of hypothalamic and other brain sections were captured using an Olympus DP70 digital camera attached to an Olympus BX51 fluorescence microscope (Tokyo, Japan). Digital images were analyzed using the ImagePro Plus (IPP™) v5.1 image analysis software (Media Cybernetics, Silver Spring, MA).

Statistical analysis

The analysis of variance was used, as appropriate, to evaluate group differences. Tukey's method was chosen as a post hoc test.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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