Video Article

Stable and Efficient Genetic Modification of Cells in the Adult Mouse V-SVZ for the Analysis of Neural Stem Cell Autonomous and Non-autonomous Effects

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URL: http://www.jove.com/video/53282
DOI: doi:10.3791/53282

Keywords: Developmental Biology, Issue 108, Neurogenesis, ventricular-subventricular zone, subependymal zone, lateral ventricle, neural stem cell, ependymal cell, niche, lentivirus.

Date Published: 2/17/2016

Citation: Porlan, E., Martí-Prado, B., Consiglio, A., Fariñas, I. Stable and Efficient Genetic Modification of Cells in the Adult Mouse V-SVZ for the Analysis of Neural Stem Cell Autonomous and Non-autonomous Effects. *J. Vis. Exp.* (108), e53282, doi:10.3791/53282 (2016).

Abstract

Relatively quiescent somatic stem cells support life-long cell renewal in most adult tissues. Neural stem cells in the adult mammalian brain are restricted to two specific neurogenic niches: the subgranular zone of the dentate gyrus in the hippocampus and the ventricular-subventricular zone (V-SVZ; also called subependymal zone or SEZ) in the walls of the lateral ventricles. The development of *in vivo* gene transfer strategies for adult stem cell populations (*i.e.* those of the mammalian brain) resulting in long-term expression of desired transgenes in the stem cells and their derived progeny is a crucial tool in current biomedical and biotechnological research. Here, a direct *in vivo* method is presented for the stable genetic modification of adult mouse V-SVZ cells that takes advantage of the cell cycle-independent infection by LVs and the highly specialized cytoarchitecture of the V-SVZ niche. Specifically, the current protocol involves the injection of empty LVs (control) or LVs encoding specific transgene expression cassettes into either the V-SVZ itself, for the *in vivo* targeting of all types of cells in the niche, or into the lateral ventricle lumen, for the targeting of ependymal cells only. Expression cassettes are then integrated into the genome of the transduced cells and fluorescent proteins, also encoded by the LVs, allow the detection of the transduced cells for the analysis of cell autonomous and non-autonomous, niche-dependent effects in the labeled cells and their progeny.

Video Link

The video component of this article can be found at http://www.jove.com/video/53282/

Introduction

The murine ventricular-subventricular zone (V-SVZ), in the walls of the lateral ventricle facing the striatum, is a very active germinal region in which a continual process of progenitor cell replication and differentiation results in the persistent production of olfactory bulb (OB) interneurons and corpus callosum oligodendrocytes[1]. The lifelong generation of these cells appears to be supported by the presence in this region of neural stem cells (NSCs; also called B1 cells), which express the astrocytic antigen glial fibrillary acidic protein (GFAP) and stem cell markers such as nestin, Id1 and Sox2[2]. GFAP-expressing B1 cells generate transit amplifying progenitor (TAP) cells (C cells), which express transcription factors Dlx2 (distal-less homeobox 2) and Ascl1 (mammalian achaete-schute homolog 1) and divide rapidly a few times before they give rise to migrating neuroblasts (A cells) or oligodendroblasts[3]. Newly-generated proliferative neuroblasts migrate anteriorly, forming the rostral migratory stream (RMS) to the OB, where they integrate into the granular and glomerular layers as differentiated inhibitory interneurons. Migrating young oligodendroblasts move to the CC, where they become immature NG2-positive cells that continue to divide locally or differentiate into mature myelinating oligodendrocytes[1,4].

B1 cells, which derive from fetal radial glial cells, retain the elongated and polarized morphology of their predecessors and exhibit a highly specialized relationship with their niche. They span between the ependyma which lines up the ventricle and the network of blood vessels that irrigate the V-SVZ niche. The small apical process of B1 cells intercalates among multiciliated ependymocytes and ends in a single non-motile primary cilium, whereas their basal process extends long distances to approach the planar vascular plexus that irrigates this niche ending in the basal lamina of the plexus capillaries[2,5-8] .

The most reliable way to distinguish B1-NSCs from non-neurogenic astrocytes, which are also GFAP$^+$, in the intact V-SVZ niche is based on whole-mount preparations of the ventricle lateral wall and their analysis by 3-D confocal microscopy after immunostaining for GFAP to label the thin B1-NSC apical process, β-catenin to delineate cell membranes, and either γ-tubulin as a marker of cilial basal bodies or acetylated α-tubulin to label the extent of each cilium[5,8]. Observations of these whole-mounts from the ventricular surface have indicated that B1 and ependymal cells

are arranged in "pinwheels"[5], in which the uniciliated apical processes of one or several GFAP$^+$ B1 cells are encircled by a rosette of multiciliated ependymal cells.

The characteristic morphology of B1 cells correlates with experimental evidence indicating that blood vessels/endothelial cells and ventricular cerebrospinal fluid (CSF) constitute regulated sources of soluble signals acting on NSCs[2,6,9-11]. At the ventricular surface, homotypic and heterotypic apico-lateral interactions involving ependymal and B1 cells include tight junctions and adherens junctions[5,12]. Moreover, adhesion molecules implicated in the junctional complexes between B1 and ependymal cells, such as N-cadherin and V-CAM, have been shown to regulate not only the highly organized positioning of B1 in the V-SVZ niche, but also their quiescence[12,13]. The ependymal-B1 cell monolayer appears to act as a diffusion barrier allowing the regulated flux of water and small molecules from the CSF, but restricting the intercellular passage of large proteins[10,11]. Experimental evidence indicates that the uniquely positioned B1 cell apical cilium could play a role as a sensor of signaling polypeptides present in the CSF[2,5-7]. Ependymal cells are, per se, also a source of soluble and membrane-bound signals with a role in the regulation of NSC behavior[14,15].

Traceable nucleosides, such as bromo-deoxyuridine (BrdU), or retroviruses have been widely used to label progenitor cells, including NSCs, *in vivo*. However, these methods are not optimal for long-term fate tracing because BrdU signals dilute through repeated cell divisions and retroviruses appear to preferentially target transiently amplifying cells due to their requirement of cell proliferation for transduction[16,17]. To examine NSC physiology *in vivo*, including interactions with niche components, it is crucial to establish a method to label and trace rarely dividing cells, as B1-NSCs are largely quiescent and their neighboring ependymal cells never divide under physiological conditions[3]. Here, we show that lentiviral vectors (LVs) allow for high-efficiency gene marking and long-term modification of adult NSCs and non-dividing ependymal cells, due most reasonably to their ability to transduce and to integrate into the genome of target cells in a cell cycle-independent way. Moreover, we show how the route of delivery and viral titer help to specifically transduce ependymal cells, but not B1 cells thereby allowing the analysis of niche-dependent, ependymal effects on NSCs.

Protocol

ETHICS STATEMENT: This protocol follows the animal care guidelines of the University of Valencia in compliance with European directive 2010/63/EU.

1. Generation of LV for *In Vivo* Marking Studies (see Figure 1a)

CAUTION: The procedure described herein is biosafety level 2, therefore perform all the following procedures in a biohazard hood. Ensure that research personnel are appropriately qualified and trained in all procedures. Wear personal protective equipment, including gown, double gloves and suitable eye protection. Finally, thoroughly decontaminate all tools and surfaces that could have been in contact with viruses according to approved facility disinfection practices (by wiping with 70% ethanol, 10% bleach and/or autoclaving).

1. Production of LV in Human Embryonic Kidney 293T Cells
    1. Start this protocol by preparing pure DNA for transfection. Prepare and purify each plasmid by double CsCl gradient centrifugation or other commercially available column methods yielding endotoxin-free DNA. In this protocol we have used the transfer vector plasmid pRRL-SIN-PPT.PGK.EGFP.Wpre. Recommended core packaging plasmids are pMDLg/pRRE and pRSV.REV and envelope plasmid pMD2G[13,18,19].
    2. Twenty-four hr before transfection, plate 5 x 10^6 293T cells in Iscove's Modified Dulbecco's Medium (IMDM) (see **Table of Materials**) in a 10 cm plastic dish in order to obtain an approximately 1/4 to 1/3 confluent culture for transfection. Incubate at 37 °C in a humidified incubator in an atmosphere of 5-7% CO_2.
    3. Replace the medium with fresh medium 2 hr before transfection.
    4. In a sterile 1.5 ml microcentrifuge tube mix 10 µg of transfer vector plasmid (containing the cDNA of the transgene or the shRNA to be delivered) with 2.5 µg of the pRSV.REV and 5 µg of the pMDLg/pRRE packaging plasmids, and 3.5 µg of the envelope plasmid pMD2G. Make up the plasmid solution to a final volume of 450 µl with 0.1x TE buffer (see **Table of Materials**) /dH_20 (2:1). Then add 50 µl of 2.5 M $CaCl_2$.
    5. Form the precipitate by dropwise addition of 500 µl of the 2x Hepes Buffered Saline(HBS, see Table of Materials) solution to the 500 µl DNA-TE-$CaCl_2$ mixture while vortexing at full speed.
    6. Add the precipitate to the 293T cells immediately. Gently swirl the plate to mix. Return the cells to the incubator and change the medium 14-16 hr after transfection.
    7. Collect the cell supernatants 30 hr after changing the media. Filter supernatant through a 0.22 µm pore nitrocellulose filter and proceed to concentration.

2. Concentration of LVs
    1. Concentrate the conditioned medium by ultracentrifugation at 50,000 x g (19,000 rpm with SW-28 ultracentrifuge rotor) for 2 hr at room temperature (RT) in a 30 ml polypropylene transparent conical rotor tube.
    Note: Use ultracentrifuge adapters for conical rotor tubes (see table of Materials).
    2. Discard the supernatants by decanting and resuspend the pellets in a small volume (200 µl or less if only one centrifugation is performed) of phosphate buffer saline (PBS; see **Table of Materials**). Then pipette up and down about 20 times.
    3. Pool the suspensions and concentrate again by ultracentrifugation, also at 50,000 x *g* (23,000 rpm with SW-55 ultracentrifuge rotor) for 2 hr at room temperature. Use polypropylene transparent rotor tubes with a nominal volume of 5 ml (see **Table of Materials**).
    4. Resuspend the final pellet in a very small volume (1/500 or 1/1,000 of the starting volume of medium) of sterile PBS and shake on a rotating wheel for 1 hr at RT. Split into small aliquots (5-20 µl) and freeze them at -80 °C.
    5. Treat all empty tubes with 10% bleach before discarding.

3. Lentiviral Titration Using Flow Cytometry

1. The day before, plate 5 x 10^4 HeLa cells per well in 6-well tissue culture plates in 2 ml Dulbecco's Modified Eagle's Medium (DMEM) (see **Table of Materials**). Incubate at 37 °C in a humidified incubator in an atmosphere of 5-7% CO_2 for 24 hr.
2. On the day of titration, thaw an aliquot of the viral stock and prepare serial dilutions, from 10^{-3} to 10^{-8}, in DMEM.
    1. To do so, take a 24-well plate and add 2 ml of DMEM to the first well and 1.8 ml to the following wells. Then add to the first well 2 µl of the concentrated viral stock (to a final dilution of 1:1,000 or 10^{-3}).
    2. After pipetting several times to thoroughly mix the solution, change tip and transfer 200 µl of the 10^{-3} dilution to the second well. Repeat the procedure serially in the following wells until the 10^{-8} dilution is made.
3. Take HeLa cells plated the previous day from the incubator. Carefully remove medium from wells. Add 1 ml of each viral dilution together with 1 µl of 8 mg/ml hexadimethrine bromide to the HeLa cell-containing wells. Gently swirl the plate to mix.
   Note: Hexadimethrine bromide is added to increase the virus adsorption to the cells in culture.
4. Return the cells to the incubator and allow the infection to proceed for 72 hr. After that, remove the medium, wash the cells once with PBS and add 200 µl of trypsin-EDTA (see **Table of Materials**) to each well.
5. After 5 min at 37 °C, add 2 ml of PBS to each well and harvest cells in flow cytometry tubes.
6. Centrifuge at 300 x *g* for 5 min at RT and aspirate the supernatant.
7. Resuspend the pellet with 1 ml of fixing solution (1% formaldehyde electron microscopy grade and 2% fetal bovine serum in PBS), then vortex the tubes.
8. Analyze the cells in a flow cytometer using a 488 nm argon-ionlaser at 15 mW power.
9. Set up the instrument with the standard configuration: forward-scatter (FS), side-scatter (SS), and fluorescence for GFP (525/40 nm). Select cell population gating in a FS *vs.* SS dot plot to exclude cell aggregates and debris. Collect fluorescence in logarithmic scale. Calculate the number of GFP^+ cells in each sample.
10. Calculate vector titer using the following formula: % GFP^+/100 x number of cells infected x dilution factor (DF) = transducing units (TU)/ml.

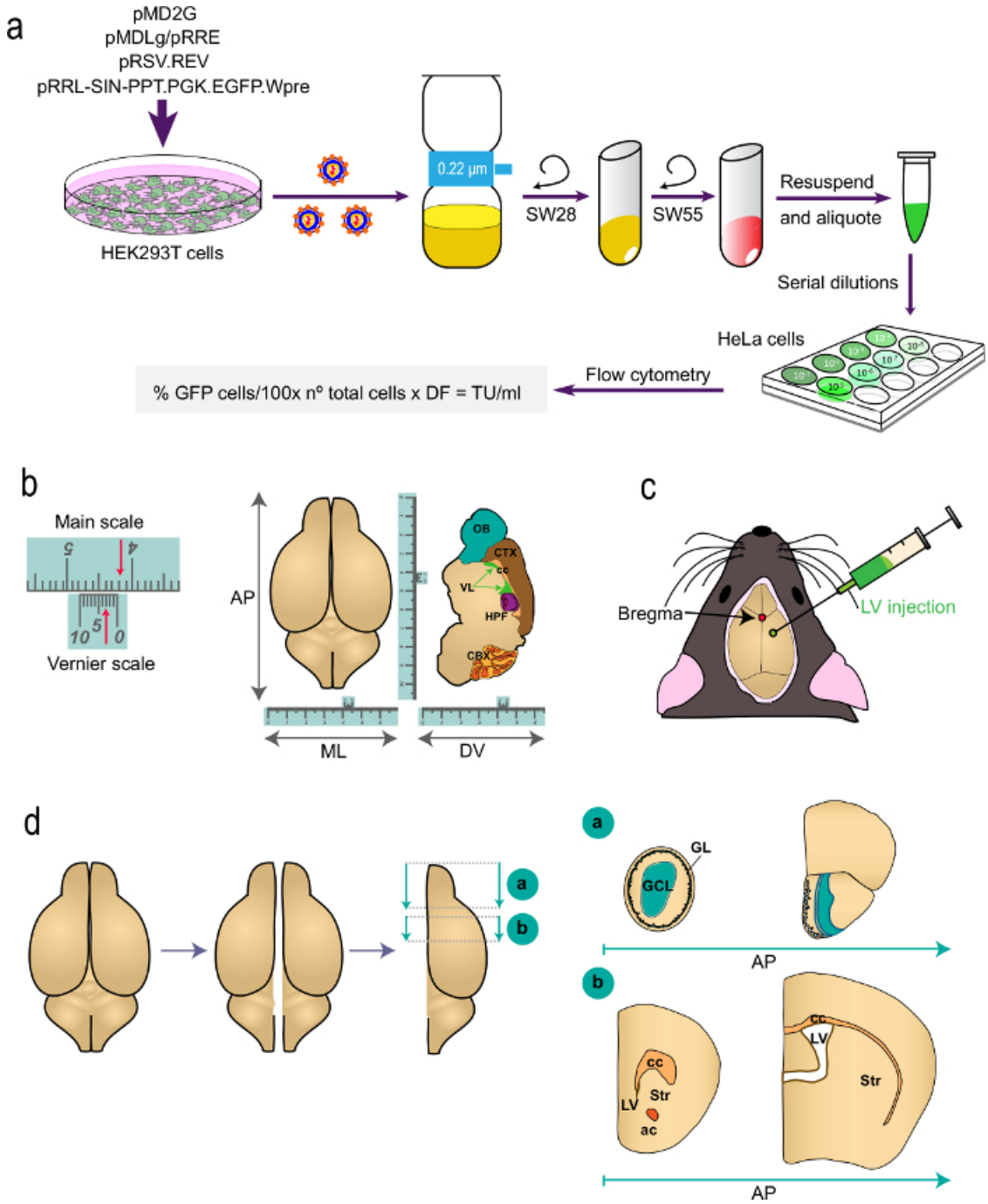

Figure 1: Schematic representation of the different parts of the procedure. (**a**) Part 1 of the protocol: generation of LVs for *in vivo* labeling studies, from the transfection of HEK293T cells with appropriate plasmids to generate the LVs to the determination of the virus titer by flow cytometry using the indicated formula. The names of the plasmids and the centrifuge rotors are indicated. (**b** and **c**) Part 2 of the protocol: stereotaxic injection of LVs. "b" depicts an example of a Vernier scale, a device that is part of stereotaxic instruments and serves for fine measurements. As an example, the arrows indicate 4.23 cm. A Vernier scale is used to determine the coordinates in the antero-posterior (AP), medio-lateral (ML), and dorso-ventral (DV) axis as shown for a top-view (left) and for a sagittal section (right) of the brain. "c" indicates the position of bregma as the intersection between the sagittal and coronal sutures. LVs are injected using a syringe. (**d**) Schematic drawings showing how the brain is processed for analysis. The two hemispheres are split and each one is divided into two blocks. Block "a", containing the OBs, is produced by a coronal cut at the AP level immediately posterior to the OB junction with the telencephalon (bregma 2.46 mm; see Paxinos´ Atlas for a reference). Block "b" is produced by two coronal cuts, one at the level just anterior to the most rostral aspect of the *corpus callosum* (bregma 1.7 mm) and a second one at the level of the junction of the two lateral ventricles (bregma -0.22 mm). GL, glomerular layer; GCL, granule cell layer; st, striatum; cc, *corpus callosum*; ac, anterior commissure; lv, lateral ventricle.

2. Stereotaxic Injection of LV into the V-SVZ/Striatum Border or into the Lateral Ventricle (see Figure 1b)

1. Preparation
    1. Sterilize a 5 µl capacity syringe with a 33 gauge needle by spraying down the body and needle with 70% ethanol with the plunger pulled out all the way. Repeatedly aspirate ethanol from a 1.5 ml microcentrifuge tube and eject it all the way out several times, and rinse the syringe thoroughly with sterile water afterwards. Place the syringe safely aside in the culture hood and allow it to dry.
    2. Prepare a biohazard waste container with 10% bleach to a suitable volume for immersion of all waste from this procedure (generally 200 ml in a 500 ml container).
    3. Prepare and preheat a 37 °C waterbed by filling a sealable plastic storage bag with water and warming it to 37 °C. This will allow mice to recover following injection.
    4. Remove viral stocks from -80 °C freezer storage 1 hr before starting the injections and place the vial on a rotating wheel at RT. After thawing, maintain the viral stock on ice during the time of the injections. Prior to the stereotaxic injection of LV, dilute the concentrated viral stocks to 10^6 TU/µl using PBS in the culture hood.
    5. Sanitize the area selected for performing the surgery with 70% ethanol.

2. Microinjection of LV
    1. Select and sterilize tools needed for surgery (scalpel, drill, and small tweezers).
    2. Anesthetize a 6-8 week-old mouse by intraperitoneally (ip) injecting a veterinary-supervised mixture of ketamine and medetomidine. Weigh each animal and dose each with 50-75 mg ketamine and 0.5-1 mg medetomidine *per* kg of mouse body weight (around 100-125 µl of the ketamine/medetomidine working solution *per* mouse).
    3. Assess the anesthetic plane by pinching the toes, tail or ear and ensuring that the animal shows no reaction.
    4. Once the mouse is anesthetized, inject butorphanol subcutaneously at a final dose of 0.4-0.5 mg per kg mouse weight to minimize post-surgical pain.
    5. Shave the area between the ears and disinfect the skin using an iodophor such as iodopovidone or 70% ethanol. Cleanse using sterile cotton-tipped applicators. Be careful not to excessively wet the animal as this can exacerbate hypothermia.
    6. Place the animal in prone position on a stereotaxic frame and carefully fix the head using the ear bars and the palate support of the apparatus. Keep the mouse with a heating pad set at 37 °C and apply ophthalmic lubricant to the eyes.
    7. Make a 1 cm long incision on the head skin longitudinally using a scalpel, and gently retract the skin to expose the skull using fine tweezers.
    8. Carefully clean the bone surface with a sterile cotton-tipped applicator. Cleanse the exposed skull bone of any remaining tissue.
    9. Mount the sterilized syringe on the stereotactic device using the syringe holder.
    10. Move the syringe holder x, y and z axis until the tip of the syringe needle is positioned on the bregma, the conjunction point where the sagittal (longitudinal and medial) suture is perpendicularly intersected by the coronal suture (**Figure 1b**). Ensure that the "zero" position of the dorso-ventral (DV) axis is at the skull surface at bregma.
    11. Move the syringe to the x and y destination coordinates (see **Table 1** and **Figure 1b**).

Region of injection	**Coordinates**		
	Antero-posterior (AP)	**Medio-lateral (ML)**	**Dorso-ventral (DV)**
SEZ/striatum border	+0.6 mm	+1.2 mm	-3.0 mm
Lateral ventricle	-0.3 mm	+1.0 mm	-2.6 mm

Table 1: Stereotaxic coordinates for the injections. For the AP and ML axis, x and y coordinates are given as a distance (in mm) from bregma. "-" indicates "towards posterior". For the DV coordinates "zero" is the surface of the skull at the bregma point and DV coordinates indicate the distance (in mm) down from this point.

12. Annotate the x, y and z destination coordinates in the Vernier scale in order to be able to come back to the injection site later on. Mark the bone at the x and y coordinates using a surgical marker pen.
13. Move the syringe away from the working area.
14. Using an electric drill make a hole on the skull carefully not to damage the brain. Do not drill the pial surface as this may damage the brain surface.
15. Load the syringe with 1 µl of the 10^6 TU/µl viral solution. Use a 33 gauge sharp beveled needle whose tip has an angle of 10-12°. Position the syringe needle at a 90° angle with respect to the brain surface.
16. Move the syringe back to the site of injection and move it down until the tip touches the pial surface.
17. Penetrate the brain with the syringe to the z coordinate in the DV axis.
18. Slowly release the viral suspension, at a rate of 0.2 µl/min, in order to minimize damage to the brain tissue due to excessive fluid pressure.
19. Wait for 5-10 min to minimize the backflow of viral suspension and then retract the syringe very slowly. Blot any excess of liquid that may appear at the surface as a result of the retraction of the syringe using a laboratory wipe and place it immediately in the bleach-containing biosafety waste container.
20. Take the animal out of the stereotaxic set, place it on a warm pad, and close the wound using skin adhesive. Reverse the sedation using 0.1-1.0 mg/kg body weight atipamezole.
21. Inject Bupenorphrine subcutaneously at a final dose of 0.1 mg per kg mouse weight every 12 hr, starting 4 hr after the administration of the short lasting Butorphanol analgesic.
22. Place the animal in an individualized cage with a warm pad and monitor closely until the mouse recovers from anesthesia. Place one bag of hydrogel in the cage to help the animal hydrate after recovery.

23. Dispose of all bio-contaminated waste in the liquid bleach biohazard disposal. Clean the syringe by aspiration and ejection of ethanol and rinse with water. Disinfect the area, the stereotaxic set and the surgical material that has been used with bleach and 70% ethanol.
24. Keep injected mice isolated in the biosafety level 2 room for 24-48 hr after which they can be transferred to a conventional housing facility

3. Histological Analysis

1. Perfusion, tissue collection, and sectioning
    1. Deeply anaesthetize the mice using a veterinary-supervised mixture of medetomidine and ketamine (assess the anesthetic plane by pinching the toes, tail or ear), as described before.
    2. Transcardially perfuse the mice with 25 ml of saline solution followed by 75 ml of 4% PFA in PB at the same rate[17].
    3. Extract the brain and post-fix it by immersing it in at least 10 times its volume of cold 4% PFA in PB for 1-16 hr (increased post-fixation times may decrease the immunoreactivity of some antigens). Wash thoroughly the remaining PFA with PB.
    4. Cut the brain following indications of **Figure 1d** and glue the resulting block to the holder of a vibratome using cyanoacrylate.
    5. Collect 30 µm-thick serial coronal sections using a vibratome. Store the brain slices in 24-multiwell plates with PB at 4 °C. To prevent contamination, 0.05% sodium azide can be added to the PB solution.

2. Immunohistochemistry
    1. Incubate the free-floating sections in blocking buffer (PB with 0.05% sodium azide, 1% glycine, 5% normal goat serum, and 0.1% Triton X-100) for 1h at RT with gentle shaking in a rocking platform.
    2. Carefully remove the blocking buffer with a pipette, add an appropriate dilution of anti-GFP rabbit primary antibody (see Table of Materials) in blocking buffer and incubate tissue with this dilution for 48 hr at 4 °C with gentle shaking.
    3. Wash off the primary antibody solution a minimum of 3 times with PB, one wash every 10 min.
    4. Incubate the free-floating sections with a suitable dilution of fluorophore-conjugated secondary antibodies) in blocking solution (see **Table of Materials**) for 1 hr at RT and gentle shaking. Protect the sections from direct light during the incubation.
    5. Wash off the secondary antibody solution with PB, 3 times once every 10 min, and counterstain the tissue by incubating the sections with DAPI (4',6-diamidino-2-phenylindole) at 1 mg/ml in water for 5 min. Wash off the DAPI solution by rinsing twice and quickly with water.
    6. Gently place the sections on a microscope slide using a fine paint brush. Pour a few drops of mounting medium for fluorescent preparations (see **Table of Materials**) over the tissue and carefully place a coverslip on top, checking that the mounting solution is correctly distributed over the entire surface and there are no bubbles. Gently squeeze down the coverslip to drain the excess of mounting medium.
    7. When the mounting solution dries out (2-16 hr), analyze the sample by confocal laser scanning microscopy with the 488 nm laser.

Representative Results

LV-mediated gene delivery system can be used for the long-term *in vivo* transduction of cells in the adult mouse V-SVZ, allowing their tracking and genetic modification during proliferation, migration and differentiation. The infection and the expression are highly effective and yield numerous cells that can be easily distinguished among other non-infected cells by the expression of the reporter included. We have thus far visualized transduced cells with GFP fluorescent reporters, driven by the ubiquitously expressed phosphoglycerate kinase promoter, but other reporters or protein tags can also be used. We routinely use antibodies to GFP instead of relying on the direct detection of the reporter emission as it yields a stronger fluorescent signal. Moreover, the brains can be dissected fresh and immersed in fixative but much better results are obtained by transcardial perfusion.

Purified, concentrated vector stocks are slowly injected under stereotaxic guidance into the V-SVZ at its limit with the striatum (**Figures 2a, b**). Two months after injection, numerous GFP^+ cells were found in the V-SVZ and in an approximate 1 mm radius from the injection site (**Figures 2a, b**). Infection with LVs results in the transduction of all types of cells in the V-SVZ and expression of the reporter by ependymal cells indicates that LVs can also efficiently target non-dividing cells (**Figure 2c**; see also [15]). GFP-labeling pattern in the V-SVZ was similar to that found after 2 weeks[18]. No sign of tissue reaction, however, was detectable at 2 months post injection since the long period allows the recovery of the tissue from the injury inflicted by the surgery. During the two-month period, infected TAP cells progress into A-neuroblasts that migrate out the zone towards the OB. Because this process takes only a few days[3], the presence of GFP^+ neuroblasts in the RMS 60 days after the infection indicates that LVs also target neurogenic B1-NSCs (**Figure 2d**). The transduction of B1, C, and A cells at the time of injection yields GFP^+ interneurons in the OB (**Figure 2e**).

Injection of a small volume of high-titer concentrated LV stocks into the lateral ventricle results in the infection of ependymocytes only (**Figures 2f-h**). Most ependymal cells, which contain the calcium binding protein S100β, express the reporter after only 15 days from infection (**Figure 2i**). In contrast to the injection into the V-SVZ/striatal limit, no GFP^+ cells were observed subependymally located or in the RMS or OB using this procedure (**Figures 2h, j**). Both types of infections can be analyzed in sections through the V-SVZ as explained. They can also be analyzed in whole-mount preparations of the lateral ventricle wall (see protocol in [8]).

Recently, we have described how N-cadherin mediated cell-cell adhesion of B1 cells to ependymocytes regulates their quiescence. We have used the procedure described here to deliver LVs carrying cadherin dominant-negative constructs that resulted in inactivation of N-cadherin. We have demonstrated that loss of N-cadherin in the NSCs and ependymal cells or in ependymal cells only promoted an increase in the number and cell cycle entry of NSCs as a result of the disruption of homotypic cell-cell interactions (**Figures 3a, b**; see [15]).

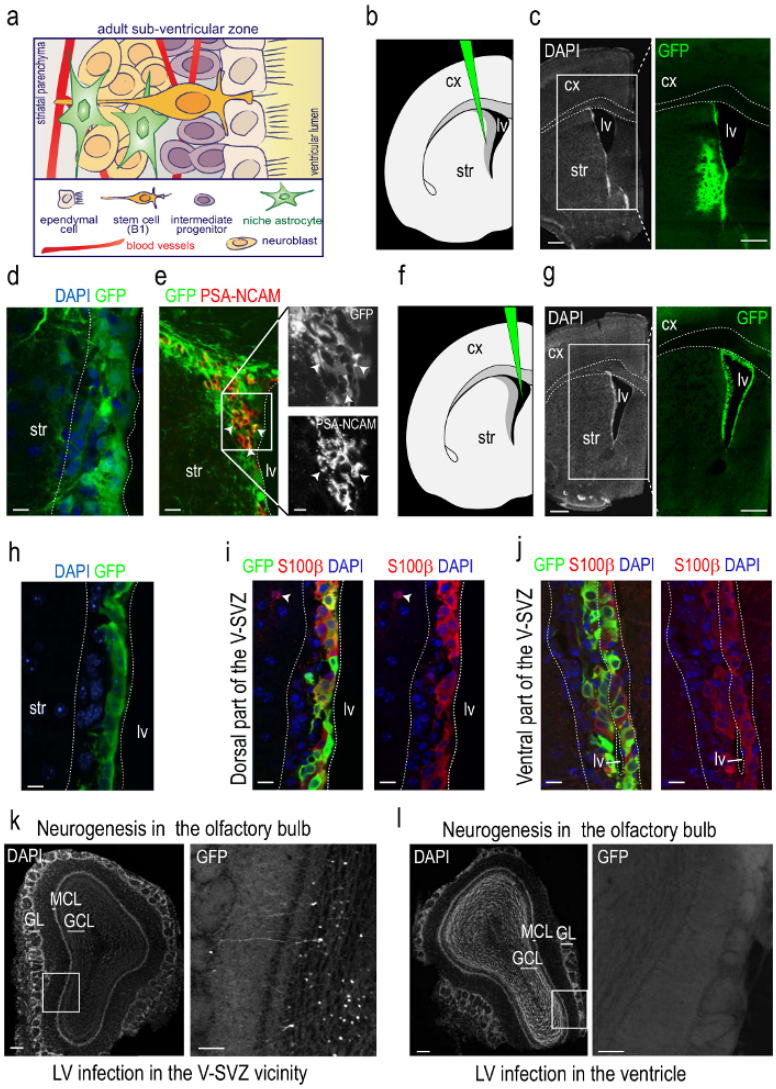

Figure 2: Reporter detection by immunofluorescence in sections of injected mice. (**a**) Schematic representation of the adult sub-ventricular zone niche. A simplification of the cellular components of the niche is depicted. B1 cells are polarized and exhibit a specialized relationship with the niche. They span between the ependyma which lines up the ventricle and the network of blood vessels that irrigate the V-SVZ niche. The small apical process of B1 cells intercalates among multiciliated ependymocytes and ends in a single non-motile primary cilium, whereas their basal process extends to approach the planar vascular plexus that irrigates this niche ending in the basal lamina of the plexus capillaries. B1 cell derivatives are intermediate progenitors that actively proliferate in the surroundings of blood vessels and migrating chains of neuroblasts towards the olfactory bulbs in contact with niche astrocytes. (**b**) Schematic of a coronal section through one hemisphere showing the level of injection and the position of the syringe needle for injections at the V-SVZ/striatum border. (**c**) Micrograph taken at the same level shown in the schematic stained with DAPI (gray; left) and for the immunodetection of GFP (green; right) 60 days after the injection. The dotted lines indicate the limits of the corpus callosum. (**d**) Higher magnification of an immunofluorescent detection of the GFP reporter injected in the V-SVZ vicinity or striatum border in a coronal section through the V-SVZ. The dotted lines indicate the limits of the V-SVZ. Note that many cells are infected in the V-SVZ niche. Ependymal cells can be recognized as cuboidal cells limiting the ventricle lumen. (**e**) Immunofluorescence for GFP (green) and the neuroblast marker PSZ-NCAM (red). Note the presence of doubly positive cells, indicating the generation of neuroblasts from NSCs that were infected 60 days before. White arrowheads point at neuroblasts. Insets correspond to the region enclosed within the white square. (**f**) Schematic of a coronal section through one hemisphere showing the level of injection and the position of the syringe needle for injections at the lateral ventricle. (**g**) Micrograph taken at the same level shown in the schematic stained with DAPI (in grey; left) and for the immunodetection of GFP (in green; right) 15 days after the injection. The dotted lines indicate the limits of the corpus callosum. (**h**) Higher magnification of an immunofluorescent detection of the GFP reporter injected in the ventricle in a coronal section through the V-SVZ. The dotted lines indicate the limits of the V-SVZ. Note that only ependymal cells are infected. (**i-j**) Immunofluorescence for GFP and the ependymal cell marker S100β. Note the presence of numerous doubly positive cells along the dorsal (**i**) and ventral (**j**) V-SVZ. Arrowhead points at an astrocyte in the striatal parenchyma, also labeled with S100β. k. Coronal section through the OB stained with DAPI (gray; left) and immunodetection of GFP (green; right) in the region enclosed by the white square 60 days after a V-SVZ/striatum injection. Notice numerous labeled neurons. (**l**) Coronal section through the OB stained with DAPI (gray; left) and immunodetection of GFP (in green; right) region enclosed by the white square 45 days after a lateral ventricle injection. Notice that there are no OB neurons labeled. cx, cerebral cortex; lv, lateral ventricle; str, striatum; GL, glomerular layer; GCL, granule cell layer; MCL, mitral cell layer. Scale bars: c, g = 500 µm; d, e, h-j = 10 µm; k-l = 250 µm; 100 µm.

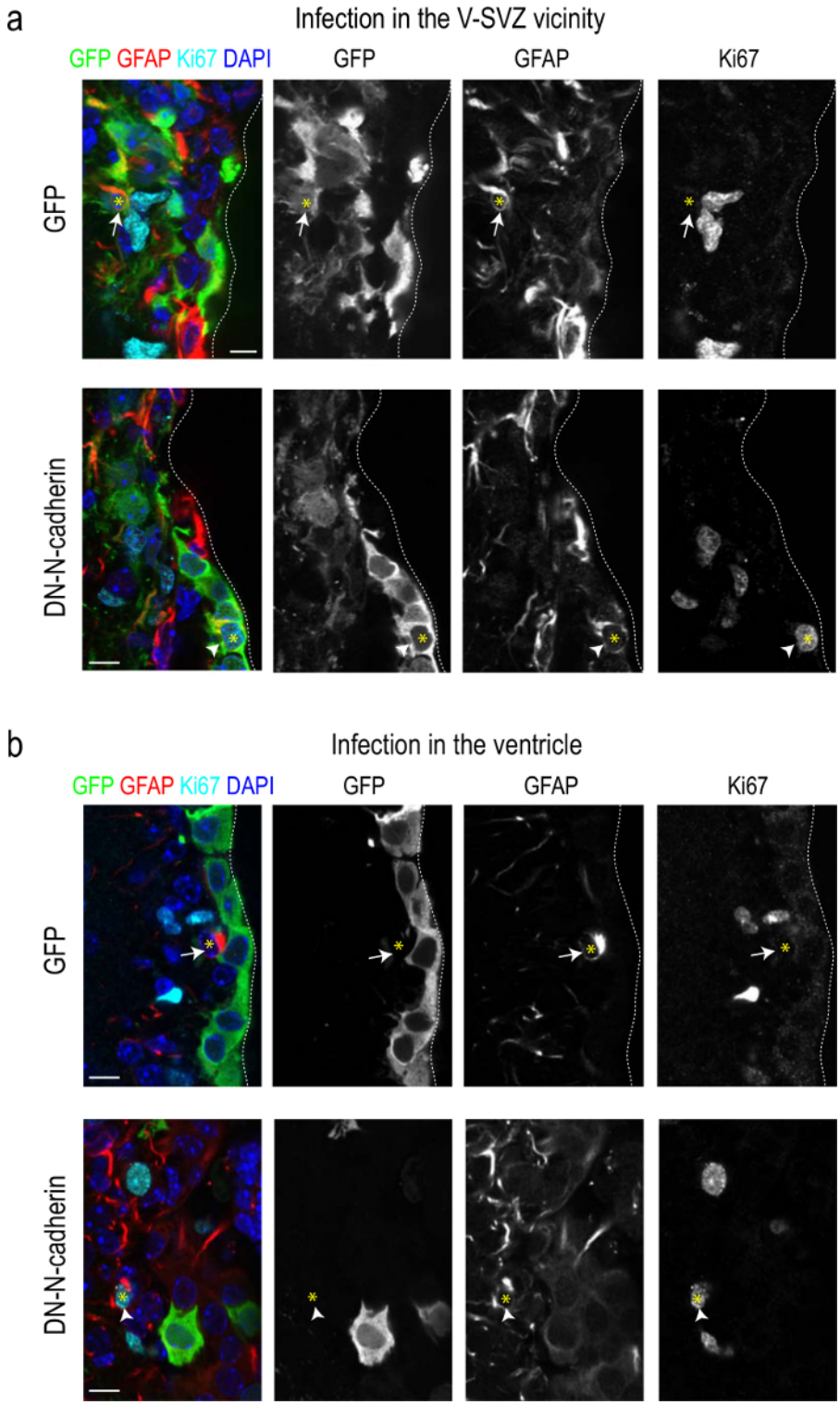

Figure 3: Adult NSCs proliferate more actively when they become detached from their niche. (**a**) Confocal micrographs of sections through the V-SVZ of mice that received striatal injections of empty LVs (upper panel) or LVs carrying a dominant-negative cadherin construct (bottom panel), immunostained for the detection of the LV reporter GFP (green), the NSC marker GFAP (red), the cell cycle marker Ki67 (cyan), and DAPI (blue) 60 days after the infections. Independent panels for each marker are shown (grey; right). (**b**) Confocal micrographs of sections through the V-SVZ of mice that received injections of empty LVs (upper panel) or LVs carrying a dominant-negative cadherin construct (bottom panel) into the lateral ventricle immunostained for the detection of the LV reporter GFP (green), the NSC marker GFAP (red), the cell cycle marker Ki67 (cyan), and DAPI (blue), 15 days after the infections. Independent panels for each marker are shown (gray; right). White arrows point at GFAP/GFP double positive cells and white arrowheads point at GFAP/Ki67/GFP triple positive cells whose nuclei in both cases are indicated by a yellow asterisk. Quantitative analysis indicated more cycling NSCs when the N-cadherin levels were decreased in the NSCs themselves or only in ependymal cells[15]. Scale: 10 μm.

Discussion

LVs offer important advantages over other viral systems for the genetic modification of adult NSCs[16,18]. Stereotaxic delivery of lentiviruses to the V-SVZ niche represents an efficient method to label and trace infrequently dividing B1-NSCs overcoming the limitations of other commonly used methods such as BrdU, which is diluted after multiple cell divisions, or retrovirus, which only target cells that are proliferating at the moment of application. LVs, together with adenoviruses, can infect cells independently of their cycling status and this is especially relevant for cells that do not cycle, such as ependymal cells, or are relatively quiescent, and thus divide rarely, like adult NSCs[16,18]. The transgenes become stably integrated and expressed with high efficiency allowing morphological characterization of transduced cells and their progeny.

NSCs are harbored in a highly specialized niche with neighboring cells such as ependymal or vascular cells as well as their own cell progeny thus creating a tightly regulated environment[2]. Like many fetal NSCs, B1 cells lie next to the ventricle and the ventricle contact its fluid through small, apical processes that end up in a single non-motile primary cilium immersed in the ventricular CSF[1] (**Figure 2a**). This detailed organization can be exploited to specifically target ependymal cells or all cells in the niche through the control of the viral titer and delivery area. Adherens and tight junctions in polarized epithelia have been shown to hinder entry of foreign viruses into cells and tissues, sometimes due to the restricted location of viral entry receptors (*i.e.* CAR receptors) in the junctional complexes. Disruption of intercellular adhesion therefore favors infection by different types of viruses, including the lentivirus (LV). Interestingly, LVs have been shown to disrupt junctional complexes when used at high concentrations in lung epithelium[20-23]. This could explain previous reports of infections of B1-NSCs by LVs injected into the lateral ventricle[24].

Since B1-NSCs produce progeny that migrate anteriorly and dorsally, GFP-positive cells can be found at all levels of the RMS as well as in the OB and corpus callosum where they terminally differentiate into neurons and oligodendrocytes, respectively. However, we do not use the reporter expression to determine effects of the genetic modification on the rates of neurogenesis or oligodendrogenesis. On one hand, the corpus callosum is in the path of the needle track and, therefore, some cells that appear labeled may be the result of a direct infection during the injection process. On the other hand, because all types of cells in the V-SVZ can be infected it is not possible to distinguish whether the labeled OB neurons are the result of a genetic modification in the B1 cells or their progenitor derivatives. In the case of experiments in which only the ependyma is infected, B1 cells and their progeny are not labeled with the reporter and therefore neurogenesis or oligodendrogenesis cannot be traced. However, one can take advantage of BrdU retention to analyze B1-NSC dependent neurogenesis to the OB or oligodendrogenesis to the corpus callosum as well as the proliferative activity of slowly-dividing NSCs in the V-SVZ of the infected mice. For example, the mice can be injected with BrdU following previously published protocols[17] 10 days after the infection with the LVs and allowed to survive for 21 days. BrdU detection in the OB/corpus callosum can then be used to determine the rate of neurogenesis/oligodendrogenesis, whereas BrdU retention in the V-SVZ can be taken as an estimate of the numbers of activated B1-cells[13].

The procedure described here can be used for the expression of genes of interest for gain-of-function experiments but can also be used for the delivery of silencing constructs based on RNA interference or for the delivery of cre recombinase constructs to inactivate germ-line transmitted floxed alleles in the V-SVZ cells. In addition, this technique could also be employed for CRISPR-mediated genome editing if injection of lentivirus-single guide RNAs is used in combination with a Cre-dependent Rosa26 Cas9 knockin mouse[25].

Critical step 1: lentivirus production and manipulation. *In vivo* gene transfer applications with LVs into specific brain areas require high titer vector preparations, since only very small volumes can be injected stereotaxically without damaging the brain tissue. Lentiviral preparations need to be purified and efficiently concentrated before injection into the brain due to the fact that LVs are generated in transient co-transfection systems. The procedure described herein is biosafety level 2, therefore all procedures that require the manipulation of viruses are performed in BSL2 facilities. Research personnel need to be appropriately qualified and trained in all procedures. In addition, surgery must be performed by fully trained investigators following routines approved by the local authorities for animal welfare, and should be done under aseptic conditions with properly sterilized instruments in biosafety level 2 areas. Adequate pre-operative and post-operative care of animals must be carried out and should be in accordance with established veterinary medical and nursing practices. More specifically, all precautions must be taken to minimize the pain and stress inflicted to the animal including administration of anesthesia and analgesia. Every step concerning the use of viruses in animals must be authorized by the institutional authorities and performed in accordance with local regulations. Personal protective equipment must also be worn, including a gown, double gloves and suitable eye protection. Finally, all tools and surfaces that could have been in contact with viruses must be thoroughly decontaminated according to the facility´s approved disinfection practices (by wiping with 70% ethanol, 10% bleach and/or autoclaving).

In our experience, the calcium phosphate transfection method is a very efficient and cheap technique of introducing DNA into 293T cells. However commercial DNA transfection reagents can also be used as per manufacturer's protocol in this step. This protocol is optimized for the collection of the viral supernatant at 30 hours after media replacement of the 293T cells. Lentiviral supernatant can also be collected 24 and 48 hr after media replacement. However, in our experience a single collection at 30 hr produces a high-titer lentivirus vector.

Critical step 2: practical example of the calculations of the vector titer using the formula: % GFP^+/100 x number of cells infected x dilution factor (DF) = transducing units (TU)/ml. In this example, 5 x 10^4 cells (293T) are seeded the previous day allowing for the transduction of 10^5 cells. After obtaining the % of transduced cells using flow cytometry as described in the protocol, two values of transduced cells are chosen for the calculations, those of which correspond to the dilutions at which transduction is neither saturated nor too low. For example: Dilution Factor 10^{-6} yield 8.08% cells infected, and dilution factor 10^{-5} yield a 61.78% cells infected. The titer is the average of the values rendered from applying the formula using the values of the cells transduced and its corresponding dilution factor. In this example:

Titer for the dilution factor 10^{-6} = (8.08/100) x 100,000 x 10^6 = $8.08 \cdot 10^9$ TU/ml

Titer for the dilution factor 10^{-5} = (61.78/100) x 100,000 x 10^5 = $6.178 \cdot 10^9$ TU/ml

Average = $[(8.08 + 6.178)/2] \cdot 10^9 = 7.1 \cdot 10^9$ TU/ml

Critical step 3: stereotaxic injection. The method described here relies on the use of low volumes of high titers of LVs stereotaxically guided delivery into specific locations. The stereotaxic coordinates given in this protocol have been established for C57/BL6 mice and may not be suitable for other mouse strains. Stereotaxic coordinates should be determined for each strain and age using a dye like fast green prior to the experiment. To do this, the dye is injected and after the mouse is sacrificed, the brain is extracted and frozen for 1 hrat -20 °C. Once the brain is hardened, it can be sliced with a blade at the site of the injection and observed under a dissecting microscope to determine whether the injection site is optimized for specific experimental purposes.

Critical step 4: post-survival times. Post-survival times will be different depending on the site of injection. A long-term survival time allows brain tissue to recover from the injection lesion and, more importantly, cells observed in the RMS and OB after 60 days are derivatives of the relative quiescent B1-NSCs. Injections into the lateral ventricle do not physically harm the V-SVZ. Therefore, a 15 day period is enough to ensure expression of the transgenes by ependymal cells.

Critical step 5: post-fixation times. Excessive post-fixation of the brain tissue may generate masking of some antigens, due to the crosslinking activity of the reagents, such as paraformaldehyde. These are good at preserving cell structure, but may reduce the antigenicity of some proteins as the crosslinking can obstruct antibody binding to the epitopes. Antigen retrieval techniques may be required, particularly if there is a long fixation incubation time or if a high percentage of crosslinking fixative is used. Alternatively, shorter periods of post-fixation of 1 hr have proved sufficient in our experience to maintain the structure of the tissue as well as ensuring a good antigenicity within the cells without the use of antigen retrieving procedures.

Disclosures

All the manipulations were made in a biosafety level 2 room. Animal protocols were approved by the ethics committee of the University of Valencia and were all in compliance with European directive 2010/63/EU.

Acknowledgements

We acknowledge the help of M.J. Palop and the technical support of the SCSIE of the Universidad de Valencia. We also thank Antonia Follenzi for helpful comments and discussion of the manuscript. I.F is supported by Fundaciòn Botìn, by Banco Santander through its Santander Universities Global Division, and by grants from Generalitat Valenciana (Programa Prometeo, ACOMP, and ISIC) and Ministerio de Economìa y Competitividad (MINECO: SAF2011-23331, CIBERNED and RETIC TerCel). This work was also supported by BFU2010-21823 and RETIC TerCel grants from MINECO and the European Research Council (ERC) 2012-StG (260511- PD-HUMMODEL) to A.C. B.M-P. is the recipient of a Spanish FPI fellowship of the MINECO.

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