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Lentiviral mediated production of transgenic mice: a simple and highly efficient method for direct study of founders

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Corresponding Author:	P. Dr. Ravassard
Corresponding Author's Institution:	
Corresponding Author E-Mail:	philippe.ravassard@upmc.fr
First Author:	Sébastien Dussaud
Other Authors:	Sébastien Dussaud
	Corinne Pardanaud-Glavieux
	Claire Sauty-Colace
Author Comments:	
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Dr. Philippe Ravassard

*Director of Biotechnology & Biotherapy laboratory
Institut du Cerveau et de la Moelle CNRS UMR 7225, INSERM UMRS975,
Université Pierre et Marie Curie
Hôpital de la Pitié Salpêtrière, 47, Bd. De l'Hôpital, 75013 PARIS, France
Tel: +33 1 57274575
mail : philippe.ravassard@upmc.fr*



Paris, February the 10th 2018

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Philippe Ravassard

TITLE:

Lentiviral Mediated Production of Transgenic Mice: A Simple and Highly Efficient Method for Direct Study of Founders

AUTHORS & AFFILIATIONS :

Sébastien Dussaud^{1,2}, Corinne Pardanaud-Glavieux¹, Claire Sauty-Colace¹, and Philippe Ravassard^{1*}

¹Sorbonne Universités, UPMC Univ Paris 06, INSERM U1127, CNRS UMR 7225, Institut du Cerveau et de la Moelle épinière, ICM, F-75013, Paris, France.

²Sorbonne Universités, UPMC Univ Paris 06, INSERM UMRS1166, Institute of Cardiometabolism and Nutrition, Paris, France.

EMAIL ADDRESSES OF CO-AUTHORS:

Sébastien Dussaud (sebastien.dussaud@upmc.fr)

Corinne Pardanaud-Glavieux (corinne.pardanaud-glavieux@upmc.fr)

Claire Sauty-Colace (claire.sauty@upmc.fr)

Philippe Ravassard (philippe.ravassard@upmc.fr)

CORRESPONDING AUTHOR:

Philippe Ravassard (philippe.ravassard@upmc.fr)

Tel +33 1 57274575

KEYWORDS:

Lentiviral vectors, transgenic mice, transduction of fertilized oocytes, integrative gene transfer, injection in perivitelline space, ubiquitous promoters, cell specific enhancer

SUMMARY:

Here, we present a protocol to promote transgene integration and production of founder transgenic mice with high efficacy by a simple injection of a lentiviral vector in the perivitelline space of a fertilized oocyte.

LONG ABSTRACT:

For almost 40 years, pronuclear DNA injection represents the standard method to generate transgenic mice with random integration of transgenes. Such a routine procedure is widely utilized throughout the world and its main limitation resides in the poor efficacy of transgene integration, resulting in a low yield of founder animals. Only few percent of animals born after implantation of injected fertilized oocytes have integrated the transgene. In contrast, lentiviral vectors are powerful tools for integrative gene transfer and their use to transduce fertilized oocytes allows highly efficient production of founder transgenic mice with an average yield above 70%. Furthermore, any mouse strain can be used to produce transgenic animal and the penetrance of transgene expression is extremely high, above 80% with lentiviral mediated transgenesis compared to DNA microinjection. The size of the DNA fragment that can be cargo by the lentiviral vector is restricted to 10 kb and represents the major limitation of this method.

Using a simple and easy to perform injection procedure beneath the *zona pellucida* of fertilized oocytes, more than 50 founder animals can be produced in a single session of microinjection. Such a method is highly adapted to perform, directly in founder animals, rapid gain and loss of function studies or to screen genomic DNA regions for their ability to control and regulate gene expression *in vivo*.

INTRODUCTION:

The pioneering work of Gordon *et al.* in 1980 showed that after implantation in pseudopregnant mice, the plasmid DNA injection into the male *pronuclei* of fertilized oocytes can yield the production of transgenic animals that integrated the plasmid DNA¹. The demonstration that transgenic mammals can be generated had an enormous impact on global life sciences, opening the way to novel fields of research both for basic sciences and translational biomedical sciences. In the past four decades, DNA microinjection has become a routine practice. Although an enormous number of transgenic mice have been produced, the standard method is not fully usable for all mouse strains and requires time consuming backcrosses^{2,3}. Its application to other species remains challenging⁴ and the overall transgene integration yield is limited to a few percentage of born animals⁵. In addition, the efficacy of transgene integration represents the limiting factor that explains the poor overall yield of pronuclear DNA injection. In this respect, integrative viral vectors are the most efficient tools to cargo and integrate transgenes and thus could provide new means to significantly increase integration yield, the only limitation being that the transgene size that cannot exceed 10 kb⁶.

Lentiviral vectors pseudo-typed with the envelop protein of the Vesicular Stomatitis Virus (VSV) are pantropic and highly integrative gene transfer tools and can be used to transduce fertilized oocytes⁷. The *zona pellucida* surrounding the oocytes is a natural virus barrier and needs to be passed to allow transduction with the lentiviral vectors. Transgenic animals have been generated by transducing fertilized oocytes after micro-drilling or removing of the *zona pellucida*^{8,9}. However, injection beneath the *zona pellucida* in the perivitelline space appears to be the simplest method to transduce the fertilized eggs as initially described by Lois and colleagues⁷.

The perivitelline injection of lentiviral vectors allows high yields in the production of transgenic animals that are above 70% of born animals. Such yield is over 10-fold higher than the best yield that can be achieved using standard *pronuclei* DNA injection^{7,10,11}. In this context, a single session of injections will generate at least 50 transgenic founders (F0). The large number of founders is, therefore, compatible with phenotyping of the transgene effect directly performed on F0 mice without the need to generate transgenic mouse lines. This advantage allows for rapid screening of the transgene effect and is specifically adapted to perform *in vivo* gain and loss of function studies within weeks. In addition, regulatory DNA elements can also be rapidly screened to map enhancers and DNA motifs bound by transcription factors^{11,12}. With pronuclear injections, transgenes usually integrate as multiple copies in a unique locus. With lentiviral vectors, integration occurs in multiple loci as a single copy per locus^{10,13}. Therefore, the multiplicity of integrated loci is most likely associated to the very high expression penetrance observed in the transgenic founders, which makes the new generated model more robust.

Importantly, when using pronuclear injection of DNA, visualization of *pronuclei* during the procedure is absolutely required. This technical limitation prevents the usage of fertilized oocytes originating from a large variety of mouse strains. Therefore, production of a transgenic model in a specific strain for which *pronuclei* are invisible requires the production of animals in a permissive strain followed by at least 10 successive backcrosses to transfer the transgene in the desired mouse strain. With the lentiviral vector injections, perivitelline space is always visible and the injection does not require highly specific skills. As an example, NOD/SCID transgenic mice that are not appropriate for *pronuclei* injection have been obtained with the viral vector injections¹⁴.

Here, a comprehensive protocol is presented to allow simple production of transgenic mice using lentiviral vector injections in the perivitelline space of a one cell stage embryo. Transgene expression controlled with either ubiquitous or cell specific promoters is described in detail.

The pTrip ΔU3 lentiviral backbone was used in this study¹⁵. This vector allows for producing replication defective lentiviral vectors in which the U3 sequence has been partially deleted to remove U3 promoter activity and generate a self-inactivating vector (SIN)¹⁶. Lentiviral vector stocks were produced by transient transfection of HEK-293T cells with the p8.91 encapsulation plasmid (ΔVpr ΔVif ΔVpu ΔNef)⁶, the pHCMV-G encoding the vesicular stomatitis virus (VSV) glycoprotein-G¹⁷, and the pTRIP ΔU3 recombinant vector. The detailed production procedure is provided as supplemental methods.

Production of high titer lentiviral vector stocks is performed under Biosafety Level II conditions (BSL-2). This is true for most transgenes except for oncogenes that have to be produced in BSL-3. Therefore, production in BSL-2 conditions for most cases is sufficient. In addition, the use and the production are usually disconnected for most national regulatory agencies dealing with genetically modified organisms (GMO). Limited amounts of replication incompetent SIN lentiviral vectors (below 2 μg of p24 capsid protein) can be used under BSL-1 conditions as described by the French GMO agency in agreement with the European Union recommendations.

PROTOCOL:

All procedures that include animal work have obtained ethical approval and have been authorized by the French Ministry of Research and Education under number APAFIS#5094-2016032916219274 v6 and 05311.02. The ICM animal facility PHENOPARC has been accredited by the French Ministry of Agriculture under the accreditation number B75 13 19. The overall protocol requires performing each procedure within a precise time frame that is summarized in in **Figure 1**.

1. Animal Purchase and Preparation of Basic Compounds

1.1. Animal purchase

1.1.1. Order 25 vasectomized males B6CBAF1/JRj that are 8 weeks of age (F1 generation from original crosses between ♀C57Bl/6JRj and ♂CBA/JRj).

Note: Isolate males upon arrival. Vasectomized males can be re-used for at least one year.
Change the cages every 3 weeks.

1.1.2. Order 50 B6CBAF1/JRj females that are 10 weeks of age and keep a pool of at least 50 animals.

1.1.3. Order 10-15 C57BL/6JRj fertile males that are 8 weeks of age.

1.1.4. Order 30 C57BL/6JRj females that are 4 weeks of age.

Note: Upon arrival, allow animals a minimum of 1 week to habituate to the facility (no handling or mating). Importantly, any mouse strains including transgenic lines can be utilized as fertile males and fertile females for superovulation. The choice of strain should be made according to the requirements of the scientific question.

1.2. Hormone preparation

1.2.1. Add 910 μ L of PMSG (Pregnant Mare Serum Gonadotropin) buffer into 1 lyophilized PMSG vial, make 100 μ L aliquots, and store at -20 $^{\circ}$ C.

Note: Each aliquot contains 55 UI for 11 mice. Never keep PMSG aliquots for more than 2 weeks after the first use.

1.2.2. Add 2730 μ L of hCG (Human Chorionic Gonadotropin) buffer into 1 lyophilized hCG vial. Make 100 μ L aliquots, and store at -20 $^{\circ}$ C.

Note: Each aliquot contains 55 UI for 11 mice.

1.3. Hyaluronidase preparation

1.3.1. Reconstitute 1 vial of hyaluronidase with 3 mL of M2 Medium to obtain a 10 mg/mL stock solution and make 50 μ L aliquots. Then store at -20 $^{\circ}$ C.

1.4. Surgery tools preparation

1.4.1. Sterilize all surgery tools using the autoclave.

2. Superovulation of Female Donors

2.1. In an animal facility using 12 h day –night cycles, inject PMSG at 2 PM on day -3. Inject hCG at 12 AM on day -1 and mate with fertile males just after hCG injection.

2.2. On day -3, add 1 mL of sterile 0.9% NaCl solution into 1 aliquot of 100 μ L of PMSG (55 UI).

Inject 10 C57BL/6JRj females with 100 μ L intraperitoneally using a syringe without any dead volume.

Note: Each mouse will receive 5 UI of PMSG.

2.3. On day -1, add 1 mL of sterile 0.9% NaCl solution into 1 aliquot of 100 μ L of hCG (55 UI). Inject the mice that received the PMSG injection with 100 μ L of diluted hCG solution (5UI) intraperitoneally. Use a syringe without any dead volume. Perform the injection slowly and wait before removing the needle so that the liquid does not leak.

Note: Each mouse will receive 5 UI of hCG. Injection of hCG should be performed 46 h after PMSG.

2.4. Place each C57BL/6JRj female in the cage of the stud male directly after hCG injection.

2.5. Check vaginal plugs on the morning of day 0 and use the positive females to collect fertilized eggs.

3. Prepare the B6CBAF1/jRj Pseudopregnant Females

3.1. Mate one vasectomized male the day before egg collection (day -1) with 2 B6CBAF1/JRj females at 5 PM.

Note: It is very important to mate with females that are originating from different cages to avoid synchronization of female cycles. This will increase the yield of obtaining vaginal plugs. In addition, do not add a female to a male cage that has been changed within the past 2 days. Efficacy of reproductive behavior in the male is increased when the cage is dirty.

4. Fertilized Eggs Collection

4.1. Preparation

4.1.1. Add 1450 μ L of M2 to hyaluronidase stock solution to prepare the hyaluronidase working solution.

4.1.2. Place one drop of 100 μ L of hyaluronidase working solution per female used to produce fertilized eggs in a 100 mm Petri dish and keep at room temperature.

4.1.3. Add 500 μ L of M16 into 4 wells plates. Use 2 wells per type of lentiviral vectors that will be injected: one well will contain the injected eggs and the other the non-injected ones. Place the 4 well plates in the incubator at 37 °C with a 5% CO₂ atmosphere.

4.2. Prepare pipettes for collection and handling of embryos.

4.2.1. Soften the glass hematocrit capillaries (75 mm/60 μ L) by rotating the center of the hard

glass capillary tubing in the flame.

4.2.2. Remove the capillaries from the heat as quickly as possible and pull to obtain a tube with an internal diameter of about 300 μm . Pull on the cooled tubing to obtain a neat break.

4.3. Collect oviducts.

4.3.1. Euthanize C57BL/6JRj females by cervical dislocation at 9 AM on day 0. For euthanasia, follow IACUC recommendations. CO_2 inhalation can also be used.

4.3.2. Perform a large horizontal incision to open the abdominal cavity with scissors. The oviduct is located between the uterus and the ovary.

4.3.3. Remove the mesometrium and the membrane carrying prominent blood vessels with curved forceps.

4.3.4. Separate the oviduct from the ovary with curved forceps.

4.3.5. Use curved forceps as a guide to cut the oviduct from the ovary using curved scissors.

4.3.6. Pull the oviduct and cut from the uterus with curved scissors.

Caution: Do not touch the swollen ampulla that contains the fertilized eggs. Perform the entire procedure using sterile instruments.

4.3.7. Place all collected oviducts in M2 medium (35 mm culture dish) at room temperature

4.3.8. Place 2 oviducts in the same 100 μL drop of hyaluronidase working solution (0.3 mg/mL).

4.4. Remove cumulus cells from fertilized eggs.

4.4.1. Under a stereomicroscope, use 2 insulin syringes: the first one to hold the oviduct and the second one to tear up the ampulla and disperse fertilized eggs into the hyaluronidase working solution.

4.4.2. Take the glass pipette for collecting eggs and connect it to the tubing with a mouthpiece. Collect all eggs and wash them by successive passage into 6 different drops of 100 μL of M2 medium.

4.4.3. Place washed fertilized eggs into humidified 37 $^{\circ}\text{C}$ incubator with an atmosphere of 5% CO_2 in M16 medium.

5. Making Injection Pipettes

5.1. Use thin-walled glass capillary tubing (10-15 cm long) with an outside diameter of 1 mm and clamp this capillary into horizontal micropipette puller.

Note: In most horizontal pipette pullers, 3 parameters can be adjusted: heat power, pulling strength and time delay between heating and pulling. Adjust these parameters to obtain injecting pipets that resembles the one presented in **Figure 2A**. For users that are routinely performing DNA microinjection, use the standard settings and adjust the delay between heating and pulling to change the global shape of the pipette tip.

6. Making Holding Pipettes

6.1. Use an injecting pipette to prepare the holding pipette.

6.2. Attach an injecting pipette to a microforge. Cut the pipette with the microforge to obtain a sharp symmetrical tip of 80 to 100 μm diameter. Then polish the tip with heat on the microforge to obtain a symmetrical round shape without sharp hedges.

7. Preparation of Injection Pipette Containing the Lentiviral Vector

7.1. Centrifuge the lentiviral vector suspension at 160 x g for 2 min to pellet debris often present in frozen lentiviral stocks.

7.2. Recover the supernatant and transfer it into a new 0.5 mL tube in a class II safety cabinet.

7.3. Transfer 1 μL of supernatant to an injection pipette prepared as described in step 5 using a Micro-loader.

7.4. Set the injection pipette on the instrument holder of the right micromanipulator. Connect the holding pipet to the left micromanipulator.

Note: The transduction titer of the lentiviral vector will be directly correlated with the efficacy of founder production. For high efficacy (>70%), use viral vectors with a titer in the range of 100 ng of p24 capsid protein/ μL . When the titer is expressed as transduction units (TU), the titer should be above 10^9 TU/mL. Lentiviral vector stocks must be produced by transient transfection of 293T cells with the p8.91 encapsidation plasmid, pHCMV-G, encoding the vesicular stomatitis virus (VSV) glycoprotein-G as described in supplemental methods¹⁸.

8. Micro-Injection

8.1. Dispense 8 μL of M2 medium in the center of a depression slide and cover with light paraffin oil (embryo tested) to avoid evaporation.

8.2. Place 20 eggs into the drop as least dispersed as possible.

Caution: Do not make bubbles when depositing the embryos.

8.3. Make sure that the tip of the injection pipette is open. If not, tap the injection pipette with the holding pipette.

8.4. Set the microinjector for an injection time of 20 s.

Note: The viscosity of the viral suspension allows clear visualization of the dispersion of the viral vector in the perivitelline space. The injection pressure should be adjusted in order to fill the entire space within 20 s of injection, which represents a volume of 10 to 100 pL. Injection pressure should not exceed 600 hPa.

8.5. Aspirate one fertilized egg that contains 2 pro nuclei and 2 polar bodies with the holding pipette under the stereomicroscope.

8.6. Inject the egg with the microinjector using settings described in 8.4, in the perivitelline space.

Caution: Do not touch the plasma membrane with injection pipet.

8.7. Inject all fertilized eggs available in batches of 20 eggs and place the injected eggs immediately in pre-heated M16 medium into the humidified 37 °C incubator with an atmosphere of 5% CO₂.

Note: Incubate the injected eggs for a minimum of 30 min after injection before embryo transfers.

9. Transferring Embryos into B6CBAF1/JRj Pseudopregnant Females

9.1. Check copulation plug 16 h after mating B6CBAF1/JRj females with B6CBAF1/JRj vasectomy males. Do this just before starting the egg collection.

9.2. Prepare implantation pipettes for the injected embryo.

9.2.1. Make implantation pipettes for embryos as described for collecting and handling embryos (step 4.2). Select pipettes with an internal diameter of about 150 µm with a narrow part around 4-5 cm in length.

Note: The tip should be flame polished, in order to reduce possible damage to the eggs or the oviduct.

9.2.1.1. Fill with light paraffin oil (embryo tested) just above the pipette shoulder.

9.2.1.2. Aspirate a small air bubble, then M2 medium, and finally a second air bubble.

9.2.1.3. Draw up embryos one behind each other to minimize the total volume of medium that will be injected in the oviduct along with the embryos.

9.2.1.4. Finish by loading a very little drop of light paraffin oil (embryo tested) of about one embryo width.

Caution: Be gentle while handling the pipette.

9.3. Embryo transfer.

9.3.1. Sterilize all instruments.

9.3.2. Anesthetize females with isoflurane (2.5% in the induction chamber, 1.5% during the surgery). Alternatively, anesthetize female using an intraperitoneal injection of 300 μ L of sterile 0.9% NaCl containing 150 μ g per g of body weight of Ketamine and 0.15 μ g per g of body weight of Xylazine.

9.3.3. Estimate the depth of anesthesia by pinching the tail of the animal with forceps.

9.3.4. Shave 2 cm on both sides of the back along the spinal cord at the level of the last rib.

9.4. Place the female mouse on a heating pad and in a sterile field. Cut a 2 cm x 2 cm window in the middle of the mouse back.

9.4.1. Apply an antiseptic solution (10% Povidone iodide) on the skin and make a 1 cm transverse incision with scissors, then slide the skin laterally until the ovary (orange color) is visible through the body wall.

9.4.2. Make a 5 mm incision with the fine scissors under a binocular surgical microscope.

9.4.3. Pick up the fat pad with an atraumatic bulldog clamp and pull out the ovary, the oviduct and the top of the uterus.

9.4.4. Visualize the ampulla and make a hemisection with vannas scissors on the oviduct segment that links the ovary to the ampulla.

9.4.5. Introduce the transfer embryo pipette and deliver eggs into the ampulla, stopping at the first air bubble in the implantation pipette.

9.4.6. Repeat the procedure on the second oviduct.

9.4.7. Close the skin up with wound clips.

9.4.8. Inject intraperitoneally 0.1 mg/kg analgesic (Buprenorphine) and place the animal in the

recovery chamber (39 °C, 30-60 min) until fully awake.

9.4.9. Repeat analgesic injection after 12 h and 48 h in case of signs of pain or distress.

9.4.10. Remove wound clips 7-10 days after surgery.

9.4.11. Check implanted females for pregnancy by following their weight curve every 3 days after implantation. A significant weight gain can be observed 10 to 12 days after implantation and will be indicative for pregnancy.

Note: All embryos that will develop here will represent putative transgenic founders. The phenotype of these founders can be analyzed at any developmental stages or after birth according to the scientific question linked to the generation of these transgenic animals.

10. Genotyping Transgenic Founders

10.1. Prepare genotyping buffer containing 10 mM Tris-HCl, pH 8; 5 mM EDTA, pH 8.0 with 0.2% SDS (w/v), 50 mM NaCl. Sterilize the genotyping buffer through a 0.22 µm filter and store at room temperature for several months.

10.2. Put extraembryonic membranes (for embryos) or little piece of tail (for born animals) into 500 µL of filtered genotyping buffer and add 15 µL of proteinase K (20 mg/mL). Incubate overnight at 55 °C.

10.3. Centrifuge the lysate at 15,000 x g for 5 min and then use 1 µL of supernatant for the PCR reaction. Lysate can be stored at 4 °C for several months.

10.4. Perform the PCR amplification of a fragment of the transgene in a 20 µL reaction volume containing 1x PCR buffer, 1.5 mM MgCl₂, 200 µM of dNTPs, 0.2 µM of each PCR primers, 1 UI of Taq DNA polymerase and 1 µL of each digested sample. As a negative control, use 1 µL of H₂O. As a positive control, use DNA from the lentiviral vector plasmid containing the transgene.

10.5. For detection of eGFP described use:

eGFP Forward Primer: 5' GACCACATGAAGCAGCAGCACTTCT 3'

eGFP Reverse Primer: 5' TTCTGCTGGTAGTGGTCGGCGAGCT 3'

10.6. Perform PCR amplification in a thermocycler: 4 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 60 °C, and 2 min at 72 °C.

10.7. Load the PCR product on a 2% agarose gel in order to visualize a 300 bp eGFP PCR product as illustrated in **Figure 2B**.

Note: All individuals showing a 300 bp PCR band have integrated the eGFP transgene and can be considered as transgenics.

10.8. Analyze transgene expression in transgenic animals. For example, perform histological and immunostaining as illustrated in **Figures 3 and 4** and described in supplemental methods.

Note: Both transgene expression analysis and phenotype analysis should be performed using pertinent methods according to the global scientific question.

11. Quantification of Transgene Copy Number

11.1. Prepare DNA samples for quantitative PCR.

11.1.1. Extract genomic DNA (gDNA) from the Proteinase K lysate obtained in step 10.3 using a commercial kit according to manufacturer instructions.

11.1.2. Quantify gDNA by spectrophotometry at 260 nm.

11.1.3. Dilute each gDNA sample to a 10 ng/μL final concentration.

11.1.4. For each sample, prepare 5 serial dilutions (1:5) in H₂O to obtain 6 tubes at the following concentrations: 10 ng/μL, 2 ng/μL, 0.4 ng/μL, 0.08 ng/μL, 0.016 ng/μL and 0.0032 ng/μL

11.2. Prepare the quantitative PCR (qPCR) reaction mix.

11.2.1. Prepare the primer mix for qPCR. For each primer couple to use for qPCR, add 10 μL of forward primer (100 μM primer stock solution), 10 μL of reverse primer (100 μM) and 80 μL of H₂O.

Note: To amplify eGFP, use Forward TCCAGGAGCGCACCATCTTCTTCA and Reverse TTGATGCCGTTCTTCTGCTTGTCG primers. *Cdx2* gene is used as normalizer for the qPCR (2 copies per genome). For *Cdx2* normalizer use Forward GCCAGGGACTATTCAAACACTACAGG and Reverse GACTTCGGTCAGTCCAGCTATCTT primers

11.2.3 Prepare 2 qPCR mixes, one with the eGFP primer mix and one with *Cdx2* primer mix. Prepare sufficient qPCR mix to amplify the 6 dilutions in duplicates of each gDNA. One qPCR reaction contains 3.8 μL of H₂O, 5 μL of fluorescent green 2x reaction mix and 0.2 μL of primer mix.

Note: For each transgenic animal to test, 24 qPCR reactions will be performed. The reaction mix is provided for a 384 well qPCR machine.

11.2.3 For each animal to test, distribute: 12 wells with 9 μL of eGFP qPCR mix and 12 wells with 9 μL of *Cdx2* qPCR mix. Add 1 μL of each gDNA dilution to 2 wells containing the eGFP qPCR mix and 2 wells containing the *Cdx2* qPCR mix.

11.2.4. Leave 2 wells for each qPCR mix in which gDNA was replaced by H₂O as negative control.

11.3. Place the 384 well plate in the qPCR machine and apply the following running protocol: 10 min at 95 °C then 50 cycles of 10 s at 95 °C and 1 min at 60 °C.

11.4. Analyze data. For each gDNA to test, plot the Ct values as a function of the Log of total gDNA amount (6 points in duplicates). Fit the curve using linear regression with the least square method and extrapolate the Ct value corresponding to the intercept with the y axis. Use the extrapolated Ct values for eGFP and the normalized Cdx2 to calculate the eGFP copy number relative to Cdx2 (2 copies) using the standard $2^{\Delta\Delta Ct}$ method¹¹.

REPRESENTATIVE RESULTS:

Transgenic animals were generated using the protocol presented here. Representative results both ubiquitous and cell type specific transgene expression are illustrated.

Constitutive expression of transgenes

Ubiquitous promoters are basic research tools to express transgenes in a sustained and efficient manner. Such promoters are used for a very large variety of application from *in vitro* cell transfection to *in vivo* transgenesis in small and large animals.

Lentiviral vectors were constructed to express the green fluorescent reporter gene (eGFP) under the control of either the cytomegalovirus (CMV) promoter or the composite promoter CAG based on the fusion of the chicken actin promoter and the CMV enhancer. Both lentiviral vectors were produced (supplemental methods) and the titer was determined in 293T cells as transduction units (TU) based on eGFP expression. Both lentiviral vector constructs were injected in the perivitelline space of fertilized oocytes at a concentration of 10⁹ TU/mL and implanted in pseudo-pregnant female mice. Implanted embryos were next collected just before birth and genotyped by PCR to follow eGFP integration. 73% (n=22) and 83% (n=32) of collected embryos had integrated the transgene for the CMV and the CAG lentiviral construct, respectively (**Table 1**). Transgenic embryos were then sectioned and immuno-stained for eGFP. As illustrated in **Figure 3**, only scattered eGFP positive cells are observed with the CMV promoter (**Figure 3**, top panel) whereas all cells expressed GFP when the CAG promoter was used (**Figure 3**, middle and bottom panels).

With the CAG promoter, 96% of collected transgenic embryos ubiquitously expressed the eGFP transgene (**Table 1**). Although both promoters are ubiquitous, only the CAG promoter is able to drive robust expression of the transgene in all cells. Alternative ubiquitous promoters were used such as phosphoglycerate kinase (PGK) and ubiquitin-C promoters and yielded similar results as the ones obtained with the CAG promoter with lower expression levels of eGFP (data not shown).

***In vivo* mapping of regulatory genomic regions to test tissue specific control elements.**

For a large number of applications, expression of transgenes in a cell specific manner in transgenic animals is required. In addition, generation of transgenic animals can be highly instrumental to screen the ability of putative regulatory genomic DNA fragments to control cell

specific expression of a given gene. As an example, lentiviral mediated production of transgenic animals was used to map cell specific enhancers that control Neurogenin 3 (*Neurog3*) expression¹¹. *Neurog3* is a basic Helix-Loop-Helix (bHLH) transcription factor that controls the commitment of pancreatic progenitors towards the endocrine fate. In *Neurog3* null mutant mice, no endocrine cells in the pancreas can differentiate¹⁹. A 2.2 kb DNA fragment localized between positions -5284 and -3061 relative to *Neurog3* transcription start site was cloned into a lentiviral vector upstream a beta globin minimal promoter to drive expression of an eGFP reporter gene as previously described¹¹. A control construct was similarly generated by cloning a 2.4 kb intergenic fragment localized on mouse chromosome 6 (chr6: 14237279-14239685 relative to mm9 mouse genome assembly) in the same lentiviral backbone. This genomic region is localized within a 1 mega-base long gene desert between *Gpr85* and *Ppp1r3a* genes. High titer lentiviral vectors were next produced using both constructs and named *Neurog3*-enh-eGFP and Chr6-eGFP.

Both lentiviral vectors were constructed and produced (supplemental methods). Since no cells expressing *Neurog3* were currently available, the TU titer could not be determined. Alternatively, the titer was measured as concentration of p24 capsid protein. The 2 vectors were injected in the perivitelline space of fertilized oocytes and implanted in pseudo-pregnant female mice. The implanted embryos were collected at embryonic day 14.5 (E14.5) as this developmental stage corresponds to the maximal expression of *Neurog3* in the pancreas. Embryos were next genotyped to follow eGFP integration. 84% (n=47) and 71% (n=48) of collected embryos had integrated the transgene for *Neurog3*-enh-eGFP and Chr6-eGFP lentiviral constructs respectively (**Table 1**). For each embryo, the pancreatic bud was dissected and then sectioned to perform immunostaining. 92% of *Neurog3*-enh-eGFP transgenic embryos expressed eGFP in the pancreas as illustrated in **Figure 4** top panel (representative immunostaining). Importantly, the vast majority of eGFP positive cells were also *Neurog3* expressing cells (**Figure 4**) indicating that the 2.2 kb *Neurog3* enhancer is able to restrict eGFP expression within the *Neurog3* cell population. By opposition, none of the Chr6-eGFP embryos expressed eGFP (**Figure 4** bottom panel and **Table 1**) in the pancreas or outside of the pancreas. In addition, no ectopic expression of eGFP was observed outside of the pancreas in *Neurog3*-enh-eGFP embryos¹¹.

For the 4 experiments presented above, a precise quantitative description of each step of the procedure is presented in **Table 1**. This illustrates the global efficacy of the procedure. Indeed, when comparing the numbers of collected animals that integrated the transgene with the number injected fertilized eggs, the global yield of the procedure is 44% in average of. The same yield with a pronuclear DNA injection of a construct containing the *Neurog3* enhancer fused to the beta-galactosidase reporter does not exceed 3.1%.

Transduction of fertilized oocytes with a lentiviral vector leads to transgene integration that can occur at multiple sites^{10,13}. The relative number of transgene integration sites were evaluated using quantitative PCR on genomic DNA (**Figure 5**). Quantification of eGFP integration was determined by quantitative PCR (qPCR) and normalized to *Cdx2* gene that is present at 2 copies per genome as described previously¹¹. The average number of integration sites was 19.36 ± 2.468 (S.E.M.) and 9.537 ± 1.186 (S.E.M.) in embryos generated from *Neurog3*-enh-eGFP and Chr6-

eGFP construct, respectively. Interestingly, the two lentiviral vectors used to produce these animals presented different viral titers. The concentration of p24 capsid protein were of 124 ng/μL for *Neurog3*-enh-eGFP vector and of 52 ng/μL for the control Chr6-eGFP vector. It is most likely that such titer difference will account for the significant difference observed in integration site numbers in both population of transgenic embryos (**Figure 5**). This suggests that the average number of integration sites obtained in a batch of founder transgenic mice could be modulated by using viral stocks with different titers.

Importantly, no direct correlation was observed between the expression of eGFP in *Neurog3*-enh-eGFP transgenic embryos and the number of transgene copies that were integrated. In other words, embryos that integrated either single or multiple copies of the *Neurog3*-enh-eGFP transgene were similarly found to express eGFP in *Neurog3* positive cells.

FIGURE AND TABLE LEGENDS:

Figure 1: Flow chart of the overall procedure

Figure 2: Preparing the micro injection pipettes and genotyping.

(A) Schematic drawings of microinjection pipettes to highlight main differences between pipets used for DNA or lentiviral vector injections. **Left panel:** the overall shape of both pipette types is drawn. The dashed circle highlights the enlarged area of the pipet tip. Pictures of the pipet tips are also presented. Note that for lentiviral injection the tip needs to be broken as indicated with the dotted line and the corresponding picture. **Right panel:** example of egg injection setting with the holding pipet on the left, the fertilized egg and injection pipet either in one *pronucleus* or in the perivitelline space. Scale bars = 50 μm. **(B)** Visualization on agarose gel of eGFP PCR product amplified from genomic DNA extracted from 8 different embryos (lane 1 to 8). Only embryos 1, 2, 3, 5, 6 and 8 had integrated the eGFP transgene. The DNA plasmid pTrip PGK-eGFP used for lentiviral vector production was used as PCR positive control. For the negative control, H₂O replaced DNA in the PCR reaction. **MWM:** molecular weight marker. **bp** = base pair.

Figure 3: Ubiquitous promoters drive expression of the eGFP reporter in transgenic embryos.

10 μm cryo-sections of transgenic embryos were stained to visualize eGFP expression (green) and nuclei (blue). Embryos generated with the CMV promoter lentiviral construct (top left label) were collected at E11.5. Embryos generated with the CAG promoter lentiviral construct (bottom left label) were collected at E18.5. **Pb:** pancreatic bud, **VSC:** ventral spinal cord, **Vt:** vertebra, **Li:** liver, **Ms:** muscle of the abdominal belt. Scale bars = 50 μm

Figure 4: Cell specific expression of reporter gene in transgenic embryos is driven by the *Neurog3* enhancer. 10 μm cryo-sections of E14.5 pancreatic buds of transgenic embryos were stained to visualize the expression of *Neurog3* (red), eGFP (green) and nuclei (blue) as described in supplemental methods). *Neurog3* expression is scattered in the pancreas. Transgenic embryos that integrated the *Neurog3*-enh-eGFP construct express eGFP and most of eGFP positive cells are *Neurog3* positive (top panel). Embryos generated with the Chr6-eGFP construct were not expressing eGFP (bottom panel). Scale bars = 50 μm

Figure 5: Relative copy number of integrated transgenes. Quantification of eGFP integration sites relative to CDX2 gene as described in protocol section. Box plot from 25th to 75th percentile. Dots represent the different transgenic embryos that were generated. The comparison of transgenes integrated sites between the two lentiviral constructs is significantly different (Unpaired parametric t-test, $p = 0.001$).

Table 1: Step by step quantitative report during the complete procedure. During the course of the procedure, the total numbers of eggs or embryos were counted. The first column represents the total number of eggs that were retrieved from the oviducts of superovulated females. Only eggs with clear 2 polar bodies and/or visible pronuclei were injected and are reported. After injection and a few hours in culture only the injected eggs that were not lysed and had a normal morphology were implanted. Next the total number of embryos that were collected from the pseudopregnant females are counted. Finally, embryos that had integrated the transgene and expressed the reporter are listed in the last two columns. The same features are also given for comparison with an experiment using standard pronuclei DNA injection. Here the transgene contained the *Neurog3* enhancer driving expression of a beta-galactosidase reporter gene (*Neurog3*-enh-LacZ).

DISCUSSION:

The perivitelline injection of lentiviral vectors in fertilized oocytes described here resulted in the production of transgenic embryos that yielded more than 70% of transgenic embryos relative to total number of collected embryos. This result is consistent with previous reports and exemplifies the specificity of the procedure^{2,7,10-12}. When comparing all the data presented in **Table 1**, important features can be highlighted. First, the number of implanted eggs corresponded to all injected eggs that had a normal morphology or were not lysed after few hours in culture. 93% of injected eggs were implanted suggesting an almost complete absence of rapid toxicity due to the injection of lentiviral vector in the perivitelline space. The situation is dramatically different when considering DNA injection since only 44% of injected eggs had survived and were implanted. Furthermore, the ratio of collected embryos relative to implanted eggs is identical between the two procedures, suggesting no exacerbated long-term toxicity of lentiviral vectors. Second, when expressing the number of embryos that integrated the transgene relative to the number of injected eggs the global yield is more than 10 times higher with lentiviral vector injection compared to DNA injection. An 86-fold difference is even found when comparing the numbers of embryos expressing transgene between the two procedures using the same *Neurog3* enhancer construct.

Importantly, transgenic production yield appears to be dependent of the transduction titer of the used lentiviral vectors. In other words, lentiviral vectors produced with a titer above 10⁹ TU/mL are sufficient to obtain such high yield. As described in the protocol section, the injected volume in the perivitelline space is in the range of 10 to 100 pL. This volume will represent 10 to 100 active lentiviral particles. In comparison to standard *pronuclei* DNA injections, the total number of founder animals generated with the same amount of born animals is at least 10-fold higher when using lentiviral vectors. In addition, the expression penetrance of the transgene is

extremely high with this protocol and was observed both with ubiquitous and cell specific promoters with the exception of the CMV promoter. By opposition to cellular ubiquitous promoters, the CMV promoter is actively shut down by DNA methylation²⁰ and has been shown to be unable to maintain long term expression upon transduction in pluripotent stem cells²¹. This could explain the very limited number of eGFP expressing cells observed in the transgenic embryos. Therefore, lentiviral vectors are well adapted to produce transgenic animals in which expression of a transgene is controlled by a cell specific enhancer. Importantly, the protocol can be used to screen for enhancer activity *in vivo* and to find map transcription factor binding sites within regulatory regions^{11,12}. This screening approach can hardly be performed using standard transgenesis. The total number of founder animals needed to test all the different constructs and reach statistical significance would require dozens of injection sessions whereas it can be obtained rapidly with lentiviral mediated transgenesis.

One of the main differences between the standard procedure and the lentiviral based method resides in the transgene integration. Using pronuclear injection, transgenes integrate randomly as multiple copies in a unique locus. Using lentiviral vectors, integration can occur at multiple loci (one copy per locus) without being strictly random. By cloning integration sites using Linear Amplification Mediated PCR (LAM-PCR), the group of D. Trono has shown that transgenes integrate preferentially into open chromatin regions of the fertilized eggs¹³. The integration bias should not interfere or contribute to the transgene expression in the transgenic mice. Integration during lentiviral transduction in a one cell stage embryo occurs in open chromatin that may not be still in the open configuration later during development or in the adult.

In addition, when analyzing copy numbers of integrated transgenes in first generation animals (F0) or embryos, a large variation in number of integrated transgene is observed. In this study, an average of 19 integrated copies was found with the Neurog3-enh-eGFP construct. This large copy number could reflect high levels of mosaicism. Sauvain *et al.* have performed an extensive study of integrated loci in F0 animals generated with the lentiviral mediated method described here¹³. They followed 70 individual integration sites in 11 F0 animals and examined the rates of transmission for each site from F0 transgenic mice to their F1 progeny. The overall rate of transmission of 44% for individual integrated transgene suggests that they were most often established either after the S phase of one cell embryos or before the S phase at the two-cell stage. Indeed, integration prior to S phase would transmit the integrated transgene to both daughter cells, while integration after the S phase would transmit it to only one daughter cell. Thus, the degree of mosaicism for individual integrated transgenes is minimal in transgenic mice obtained through this technique. This further indicates that most integration will occur within the first 12 h corresponding to the average time of production of the first cleavage in the used culture conditions. This integration kinetic is consistent with the one described for lentiviruses in T-lymphoid cells²².

Importantly, with a high number of loci bearing integrated transgenes, establishing mouse lines would not be reasonable. The number of crossings to segregate all these loci would be considerably high. This represents one important limitation of this method that should be used either for rapid screening of transgene effects or for simultaneous analysis of multiple

transgenes. Nevertheless, mouse lines can still be established by selecting from the F0 animal the ones with the lowest integrated transgene copy number.

Since the first description of the pronuclear DNA injection method¹, improvements have been made that circumvent many of the drawbacks of the initial procedure. The first set of improvements was based on the targeted integration in a precise locus using a cassette exchange strategy. Pronuclear injection is performed using either CRE, Flip or PhiC31 recombinases together with an integrative DNA fragment flanked with loxP, FRT or attB sites, respectively. In this situation, the integrative DNA is exchanged with an integrated fragment flanked with the same recombinase specific site^{23,24}. Although up to 60% of first generation animals can be transgenic²³ using such method, the limitations linked to the technology of pronuclear injection still apply. The second set of improvement is based on cytoplasmic injections of two circular DNA, one carrying the fragment to integrate and one allowing expression of either the Tol2²⁵, Sleeping Beauty²⁶ or piggyBac²⁷ transposases. Using these methods, high yields are obtained (>30%), but more importantly, the cytoplasmic injection is easy to perform and circumvents the restrictions due to pronuclear injection as the lentiviral based protocol. Furthermore, very large DNA fragments, such as bacterial artificial chromosomes, can be integrated.

It is clear that lentiviral mediated transgenesis will not replace the standard nor the improved procedures. Still this method represents a powerful tool for rapid animal model production and characterization as it considerably reduces the required time to generate proper number of animals with the least genetic variability. Furthermore, this technology can be directly applied to all mouse strains including any transgenic lines. In addition, it is crucial to mention that the global landscape of generation of novel animal models is about to change with the recent development of the CRISPR/Cas9 technology. Today, pronuclear injections of Cas9 protein along with guide RNA allows production of genome edited animal models with an efficacy of 40%²⁸. This approach could largely benefit from the use of lentiviral mediated transgenesis. Indeed, the use of non-integrative lentiviral vectors²⁹ to transiently express both Cas9 and guide RNAs could result in even higher production yields. The combination of the newest technologies to produce relevant and robust animal models would benefit most international research groups involved in studying disease pathogenesis and therapeutic approaches.

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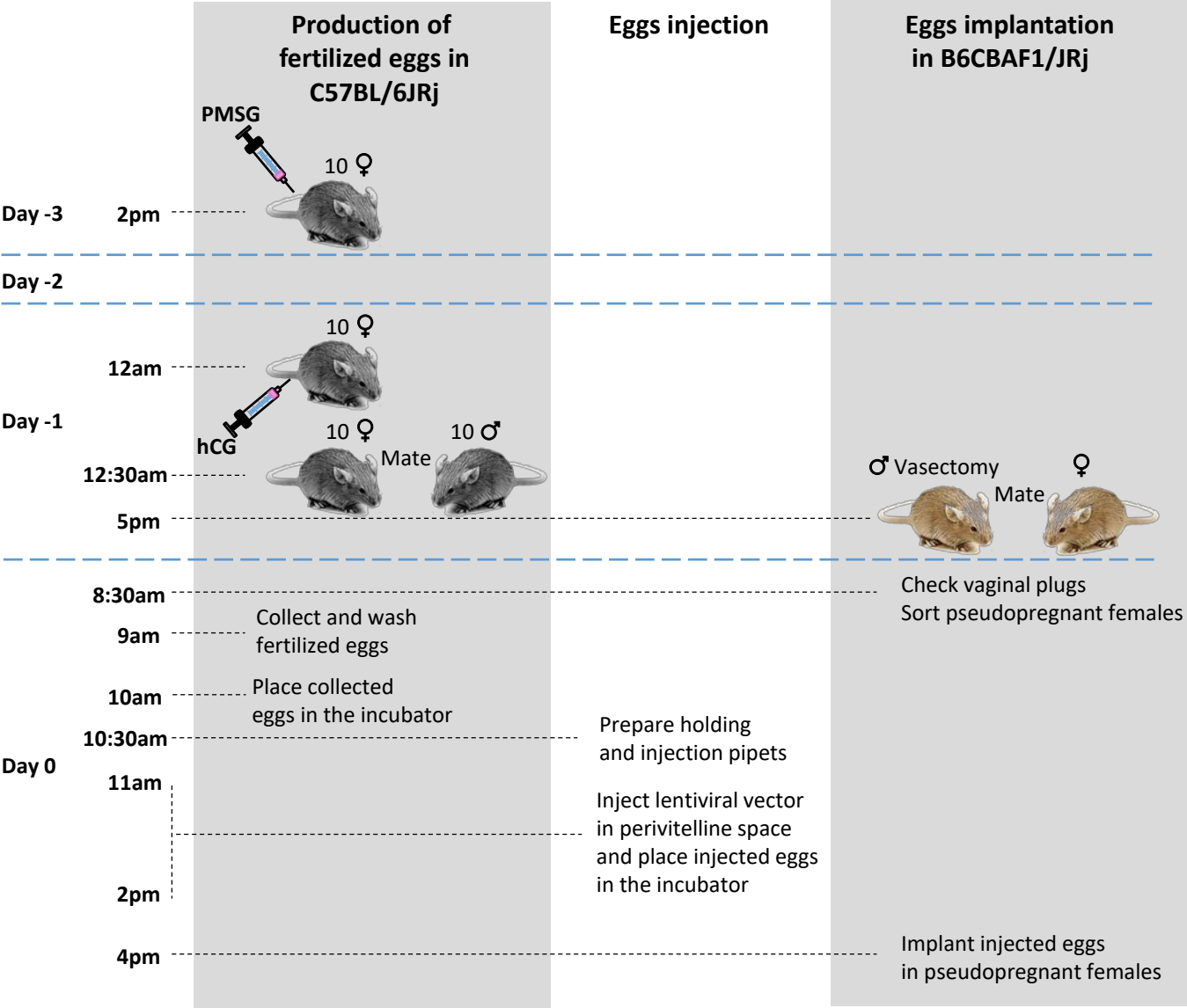
DISCLOSURES:

The authors have no conflict of interest to disclose.

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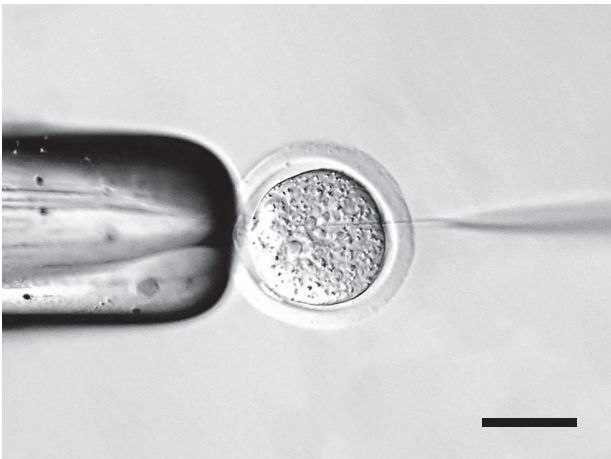
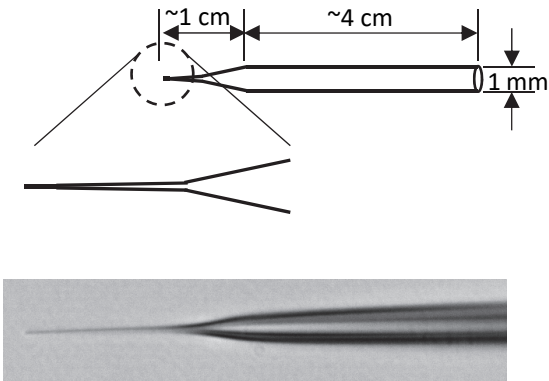
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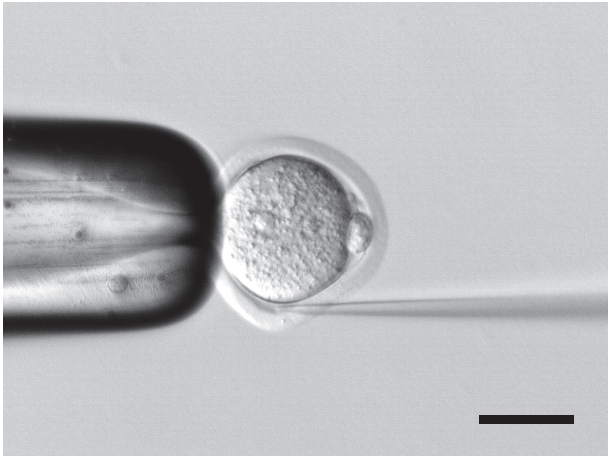
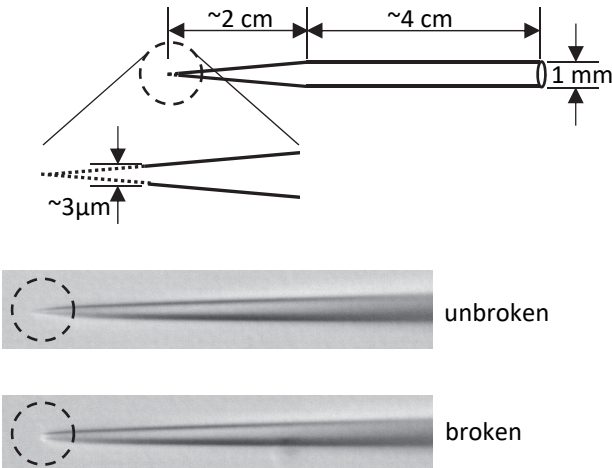


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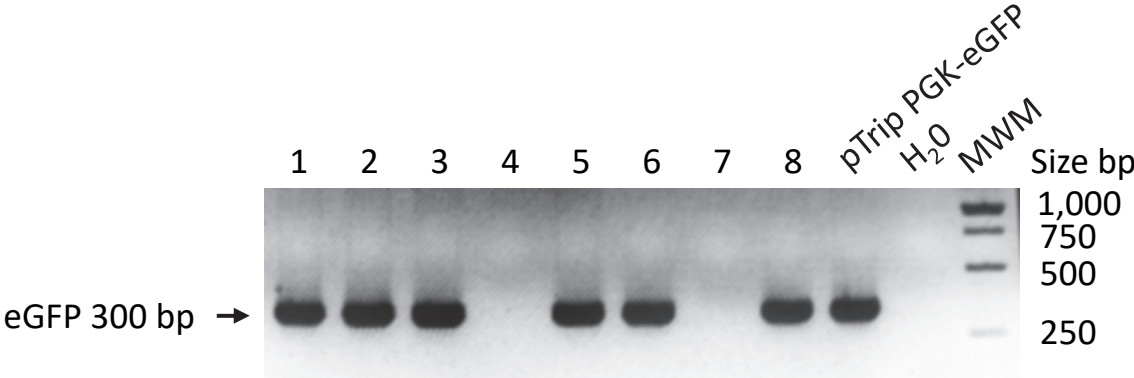
Pipet for pronuclear injection

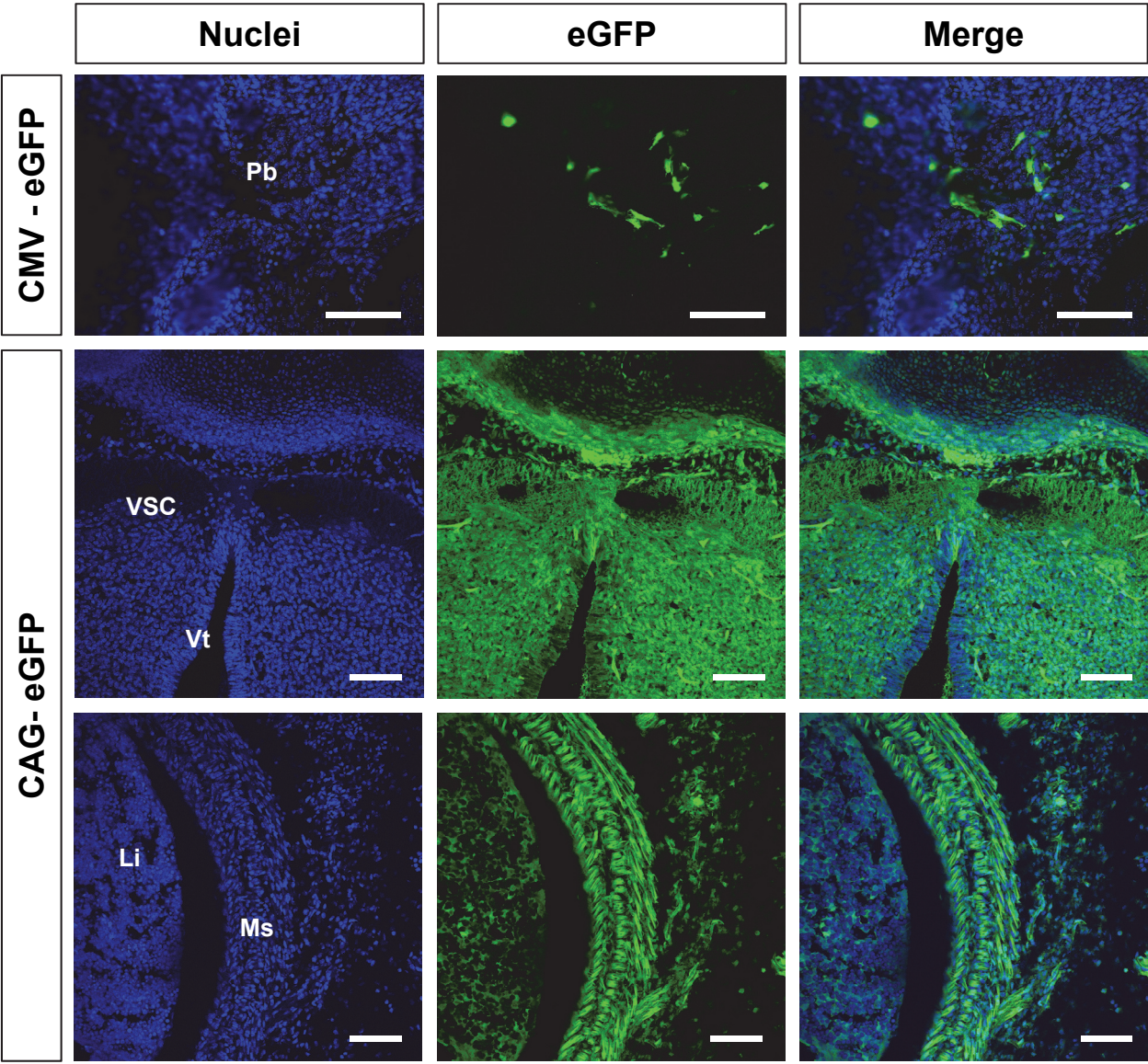


Pipet for lentiviral vector injection



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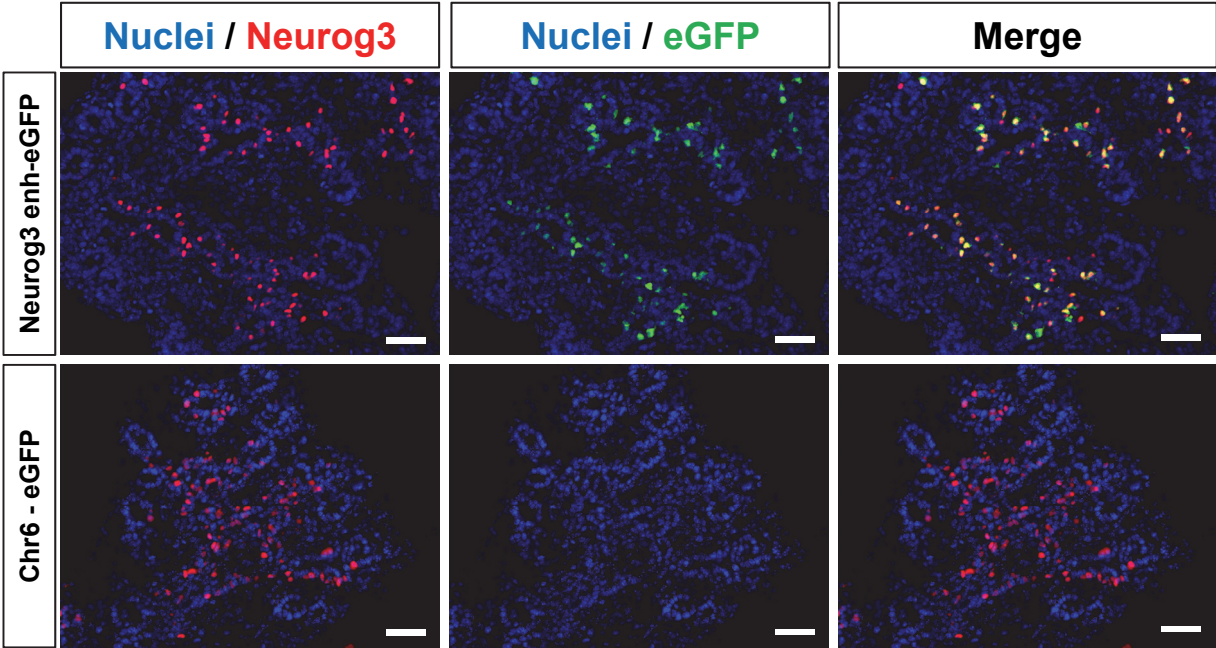
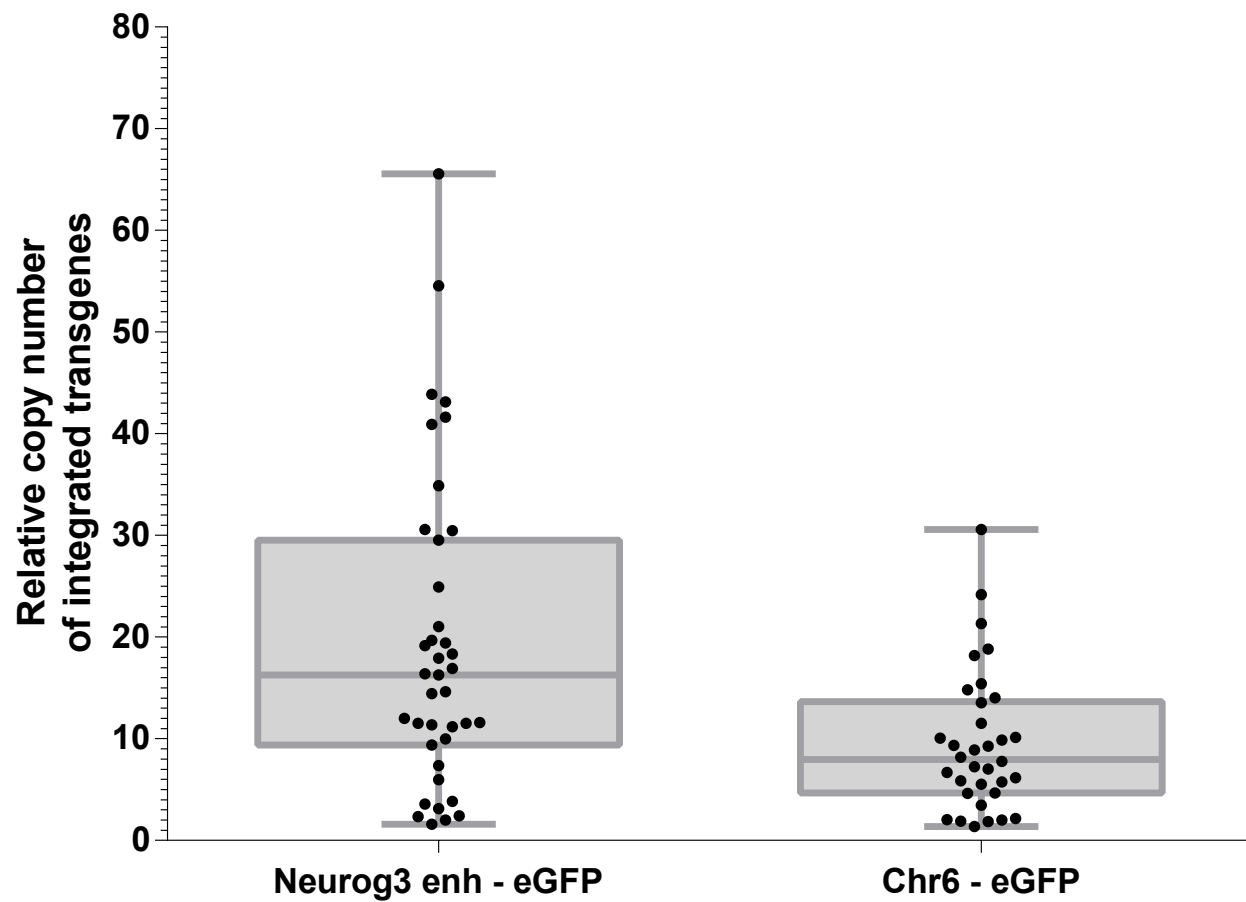


Figure 5



Transgenes	Number of collected eggs	Number of injected eggs	Number of implanted eggs	Number of collected embryos	Number of embryos with transgene integration	Number of embryos expressing transgene
<i>Lentiviral mediated transgenesis</i>						
CMV-eGFP	48	36	34	22	16	16
pCAG-eGFP	63	57	51	32	27	25
<i>Neurog3</i> -enh-eGFP	94	94	83	47	39	36
Chr6-eGFP	122	82	82	48	34	0
<i>Pronuclear injection</i>						
<i>Neurog3</i> -enh-LacZ	1302	1234	542	263	38	3

Name	Company	Catalog Number	Comments
PMSG 50UI	Sigma	G4527	
hCG 5000UI	Sigma	CG5-1VL	
NaCl	Sigma	7982	
100 mm petri dish	Dutsher	353003	
4 wells Nunc dish	Dutsher	56469	IVF dish
M2 medium	Sigma	M7167	
M16 medium	Sigma	M7292	
0,22 µm Syringe filter	Dutsher	146611	
Hyaluronidase Enzyme 30mg	Sigma	H4272	mouse embryo tested
Insulin serynge	VWR	613-3867	Terumo Myjector
Curved forceps	Moria	2183	
Curved scissors	Moria	MC26	
Aspirator tube assemblies for calibrated microcapillary pipettes	Sigma	A5177-5EA	
Borosilicate glass capillaries	Harvard apparatus	GC 100-10	
Horizontal micropipette puller	Narishige	PN-30	
Microforge	Narishige	MF-900	
Inverted microscope	Nikon	Transferman NK2 5188	Hoffman modulation contrast illumination is
Micromanipulator	Eppendorf	Celltram air	
Controler of holding pipet	Eppendorf	Femtojet	
Mineral oil	Sigma	M8410	mouse embryo tested
Microinjector	Eppendorf	Femtojet	Can be used to inject DNA or viral vectors
Dumont # 5 forceps	Moria	MC 40	
vannas micro scissors	Moria	9600	
Isoflurane	centravet	ISO005	ISO-VET 100% 250ml
ocrygel	centravet	OCR002	
Povidone iodure	centravet	VET001	vetedine 120ml
Buprenorphine	centravet	BUP002	Buprecare 0,3Mg/ml 10ml
Tris-HCl	Sigma	T5941	Trizma hydrochloride
EDTA	Sigma	E9884	
SDS	Sigma	436143	
NaCl	Sigma	S7653	powder
proteinase K	Sigma	P2308	
oneTaq kit	NEB	M0480L	
Primers	Eurogentec		
Strip of 8 PCR tube	4titude	4ti-0781	
96 well thermal cycler	Applied Biosystems	4375786	Veriti
Genomic DNA mini kit	invitrogen	K1820-02	
Nanodrop 2000	Thermo Scientific	ND-2000C	
qPCR Master mix	Roche	4887352001	SYBR Green
Multiwell plate 384	Roche	5217555001	
qPCR instrument 384 well	Roche	5015243001	LightCycler 480



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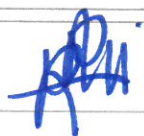
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CORRESPONDING AUTHOR:

Name:	Philippe Ravassard	
Department:	Biotechnology and biotherapy	
Institution:	ICM, CNRS UMR7225	
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1. The editor has formatted the manuscript as per journal's style. Please retain the formatting.

[No modification of the format were made.](#)

2. Please proofread the manuscript well. There are still some grammatical errors. Please use American English throughout.

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3. Please address specific comments marked in the manuscript.

[All specific comments were addressed in the manuscript and all changes can be visualized in the tracked copy of the manuscript. Importantly, figure 2 was modified to include results of PCR genotyping as requested by the editor.](#)

4. Regarding highlighting, I have highlighted all the steps suggested by you in yellow. The highlights seem to be way more than 2.75 pages which is our hard cut limit including headings and spacings. Please adjust the highlights to fit this limit. Highlights can be removed for notes and anesthesia steps. Also, few small steps can be combined into one.

[Highlights have been changed to fit into the 2.75 page limit.](#)

Production of high titer stocks of lentiviral vectors

The efficacy of the lentiviral production is highly dependent on the quality of the 293T cells used. Order fresh ATCC CRL-3216 cells that can be amplified and banked according to ATCC recommendations. For lentiviral vector production never use cells that have been passaged more than 30 times.

According to safety regulations, production of high titer lentiviral stocks has to be performed in BSLII environment.

Day 1: plating 293T cells

Prepare 20 10cm culture plates in the morning of day 1. Such amount of cells is sufficient for a single production. For a double or triple production, multiply every amount by 2 or 3.

Use confluent 293T cells for the plating. The average number of cells per confluent T75 flask is around 20 million. You can change medium one day before plating, to obtain full confluence.

- 1) Remove the medium and wash the flasks with 2 ml of PBS. Remove the PBS
- 2) Add 1.5 ml trypsin per flask and incubate for several minutes at 37°C.
- 3) Detach the cells by knocking on the side of the flask. Cells really have to be in a single cell suspension before counting them.
- 4) Add 8.5 ml of medium and put the cell suspension in 50 ml tubes.
- 5) Count the cells in a Burkett counter
- 6) Prepare cell dilutions in 293T cell culture medium in order to plate 20 10 cm plates. The number of cells to be used depends on the day of the lentiviral vector harvest a should be performed according to the table below.

Friday	Saturday	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday
1 million/plate			transfection		harvest		
			5 million/plate	transfection		harvest	
			2,5 million/plate		transfection		harvest

- 7) Mix well because the cells sediment very fast, and then put 10ml of cell dilution per plates.

- 8) Put the plates in an incubator at 37 °C under 5% CO₂ atmosphere.

Day 3: transfection of the 293T cells (in the morning or early afternoon)

9) Change the medium 4 hrs before the transfection, except when you plate on monday. The cells should be 50 to 70 % confluent.

10) 1 hour before the transfection, place all transfection solutions at room temperature

11) Preparation of the DNA/CaCl₂ mix (the DNA should be sterile)

in a 50 ml tube, add in the following order :

H₂O (up to a final volume of 10 ml)

pMD2G vector DNA : 100 µg

p8.91 vector DNA: 200 µg

pTRIP –vector DNA: 200µg

CaCl₂ (2.5M) : 1 ml

12) Mix well and put (droplet by droplet) on 10 ml of HEPES buffered saline 2X concentrate (Sigma 51558) while making very fine bubbles. To make such bubbles use a 50 ml syringe with 0.2 µm filter linked to a glass capillary tube (1.4 mm diameter) connected to a rubber tube. This will allow you to make fine bubbles just by pressing on the syringe.

13) The mix should be opal. If large precipitates are formed try to brake them using a pipette or by shaking the tube vigorously

14) Add 2 ml of Chloroquine diphosphate salt 2.5mM (use solution 100x) to the tube

15) Put 1.1 ml of the DNA mix per 10 cm plate. Move the plate (left to right, from back to forth) to obtain a homogeneous suspension

16) Change the medium after 4-6 hours (to remove Chloroquine), put 9 ml of fresh medium on the cells

Day 5: Harvest of the lentivirus, 48 hrs after the transfection

17) Cool the ultracentrifuge buckets and the rotor to 4 °C. Set the water bath at 37 °C

Note that ultracentrifugation has to be performed using a swinging rotor such as swing-out SureSpin 630 (Thermo Fisher) with 6 buckets and 36 ml conical ultracentrifugation tubes (Beckman 358126). Do not forget to add the conical adapters in the rotor's buckets.

18) Collect the cell medium of the plates in 50 ml tubes. Spin down the cells debris by centrifugation for 10 min at 1 000 x g.

- 19) Transfer the supernatants into new 50 ml tubes
- 20) Add DNaseI (1mg/ml Roche) 1/1000, and 1/1000 MgCl₂ (1M),
- 21) Mix and incubate at 37 °C for 15 to 20 min
- 22) Filtrate using a 0.45 µm filter.
- 23) Transfer the filtrate into ultracentrifuge tubes and centrifuge at 4°C for 1hr 30 min at 60 000 x g
- 24) Remove as much as possible of the supernatants
- 25) Put the tubes upside down on sterile paper towel and clean the inside of the tubes with the paper towel, without touching the virus pellet.
- 26) Put 50 µl PBS on each pellet and put at 4 °C for at least 2 hrs (best overnight). Then resuspend the pellet using a P200 pipette (10-20 up and down) WITHOUT making bubbles. Place the resuspended viral stock in a 1.5ml tube.
- 27) Remove debris by successive centrifugation steps (30 sec, 2 000 x g) in a microfuge. As long as you see a white pellet, continue the centrifugation. This step will dramatically reduce the toxicity of the viral stock for in vivo and in vitro use.
- 28) Make aliquots of 5 to 10 µl and store à -80 °C. Note that lentiviral vectors should not be thaw several times.
- 29) Determine viral titer using a HIV-1 p24 Antigen ELISA kit.

Tissue preparation for histological analysis

Transgenic embryos were collected at different stages. The date of reimplantation of the one cell stage embryos in pseudo-pregnant females was considered as embryonic day 0.5 (E0.5). Embryos were dissected and a tissue block containing the stomach; pancreas and duodenum was removed and fixed by immersion in 4% PFA for 24 h. Tissues were cryoprotected by incubation for 48 h in 15% sucrose prepared in PBS and next embedded in 15% sucrose, 7% gelatin prepared in PBS then frozen in isopentane at -50°C. 10 µm sections were performed using a cryostat (Leica). Whole embryos were fixed in 4% PFA for 20 min, cryoprotected and embedded in gelatin sucrose as described above. 10 µm sections were performed using a cryostat (Leica)

Immunostaining

10µm cryosections were used for immunofluorescence staining as described previously¹. The following primary antibodies were used: mouse anti-Neurog3 (1/1000 Beta Cell Biology Consortium Ref. AB2013) and chicken anti-GFP (1/1000 Antibodies-Online GMBH Germany, Ref. ABIN 147441). The corresponding secondary antibodies were used: Alexa 488 Goat anti Chicken (1/2000 Life Technologies Ref.

[A11039](#)). Neurog3 detection was amplified as described by Zahn et al. 2004 ². TSA amplification (Perkin Elmer TSA-Direct NEL702) was performed prior to GFP staining.

Références

- 1 Khalfallah, O. *et al.* Zinc finger protein 191 (ZNF191/Zfp191) is necessary to maintain neural cells as cycling progenitors. *Stem Cells*. **27** (7), 1643-1653, doi:10.1002/stem.88, (2009).
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