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Bacterial Artificial Chromosome Transgenesis Through Pronuclear Injection of Fertilized Mouse Oocytes

Kristina Vintersten, Giuseppe Testa, Ronald Naumann, Konstantinos Anastassiadis, and A. Francis Stewart

Summary

In the mouse, conventional transgenes often produced unpredictable results mainly because they were too small to recapitulate a natural gene context. Bacterial artificial chromosomes (BACs) are large enough to encompass the natural context of most mammalian genes and consequently deliver more reliable recapitulations of their endogenous counterparts. Furthermore, recombineering methods now make it easy to engineer precise changes in a BAC transgene. Consequently, BACs have become the preferred vehicle for mouse transgenesis. Here, we detail methods for BAC transgenesis through pronuclear injection of fertilized oocytes.

Key Words: BACs; pronuclear injection; microinjection; superovulation; pseudopregnant host.

1. Introduction

The mouse has become the premier model for medical studies in part because of the development of advanced ways to engineer its genome *(1)*. Here, we discuss the use of large transgenes, as provided by bacterial artificial chromosomal (BAC) resources. In the past, transgenes were limited in size by the constraints of DNA-engineering methods. Mostly, artificial minitransgenes were made because larger constructs were impractical. However, the average size of a mammalian gene, including its cis regulatory elements, is usually greater than that of a minitransgene. Consequently, most early transgenes suffered from exquisite sensitivity to position effects exerted by chromatin

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surrounding their genomic integration site. Transgenes based on large sections of genomic DNA generally do not suffer from position effects because they encompass entire genes with the cis regulatory elements in the natural position and configuration.

An increasing number of completely sequenced genomes are accompanied online by annotated libraries of BAC genomic clones. Because these BACs are precisely defined and can be readily obtained, a large number of starting options are available. Furthermore, the development of recombineering methodologies permits the precise mutagenesis or alteration of BACs in almost any way *(2)*. Hence, BAC transgenics are now amongst the most useful reagents for applications in mouse experimental biology. Transgenesis through microinjection of DNA into the pronuclei of fertilized oocytes is one of two commonly used methods for gene transfer into the mouse genome *(3,4)*. The first successful attempt to perform this technique was carried out by Lin in 1966 *(5)*, who could show that the early fertilized embryo could survive the mechanical damage of inserting a glass needle into the pronucleus. It was not until 1981, however, that small DNA fragments were integrated into the genome *(6)*. This technique is well described and has now become a standard procedure *(7,8)*, which has permitted its application to larger DNA fragments. Yeast artificial chromosome (YAC) *(9,10)*, P1 artificial chromosome (PAC) *(11,12)*, and BAC DNA *(13,14)* can all be used for the generation of transgenic mice. See Giraldo and Montoliu *(15)* and Ristevski *(16)* for reviews. Although the basic technique for microinjection of large constructs are similar to those used for shorter DNA segments, there are some special requirements *(17)*. In this chapter, we describe relevant steps for microinjection using large DNA constructs.

It is important to keep in mind that, as with any kind of pronuclear DNA injection, the integration takes place in a random manner; hence, the integration site and even the copy number of the integrated transgene are not possible to predict. All the resulting founder animals will carry a unique integration pattern. In most cases, the integration will take place at only one site; occasionally, however, separate integration sites on two different chromosomes can occur *(18)*. When these founder animals are mated for transmission of the construct through the germline, segregating integration patterns may be observed among the offspring. Transgene integration will generally take place shortly after microinjection, before DNA replication in the one-cell stage embryo, also termed the zygote. In some cases though, the integration is delayed, resulting in a mosaic transgenic embryo/animal *(19)*. Occasionally, mosaicism can interfere with the germline transmission of the transgene.

The BAC construct can be injected in one of three forms: supercoiled, linearized, or as a purified insert released from the BAC vector *(15)*. Injection of the supercoiled form is the fastest method and presents the advantage that the DNA, being more compacted, may be less prone to shearing and fragmentation during the injection procedure. Furthermore, suitable restriction sites for cleavage within the BAC vector are not required. Also, solutions of linearized BAC DNA tend to be more viscous, and hence more difficult to inject, than when the BAC is in its supercoiled form.

The main drawback of using intact (supercoiled) BACs is that integration of the BAC transgene will occur upon random linearization, thus possibly interrupting the transgene in a deleterious manner. On the contrary, injection of a linearized transgene is more predictable for reliable integration of the intact transgene. A variation of this approach has been the use of linearized BAC inserts released from the vector and purified by pulsed field gel electrophoresis (PFGE). This is admittedly the cleanest experimental avenue; it is, however, fairly laborious and apparently unnecessary, because, as it has been shown with YACs, the presence of vector sequences integrated to one side of large genomic transgenes does not seem to affect expression *(20)*.

If one wishes to linearize the BAC in the absence of suitable restriction sites, recombineering *(2)* represents a fast and convenient way to precisely introduce a unique site at any desired place in the BAC.

The handling and general conditions surrounding both embryo donor and recipient mice are of major importance for the successful outcome of microinjection experiments. For further reading about optimal husbandry conditions, the reader is referred to Foster et al.*(21)*.

Handling and culture of embryos, the microinjection process, and the final transfer of the embryos back in vivo, all require experience and optimal culture conditions *(8, 22)*. The microinjection process requires expensive, specialized equipment. Therefore, these experiments should not be undertaken without careful consideration (*see* **Note 1**).

1.1. Production of Zygotes for Microinjection

The number of animals needed per experiment will depend on several factors: age and quality of the embryo donors and stud males, physical parameters in the animal facility and the microinjection lab, embryo culture conditions, DNA purity and concentration, and the manual skills of the operator.

Generally, 25–30% of the injected zygotes will immediately lyse because of the mechanical damage. On average, about 30% of the surviving embryos will develop to term, and only 15–20% of those will be transgenics. Therefore, if

all conditions are met, it is usually sufficient to inject 150 fertilized zygotes to produce 3–6 founder animals.

The choice of genetic background of the host embryo should be considered carefully. In most cases, a cross between two F1 animals, such as (C57BL/6J × CBA) F1, (C57BL/6J × SJL) F1, or (C57BL/6J × DBA/2) F1, is used, because these combinations have proven to provide large numbers of good quality embryos. In these cases, however, the genetic background of the founder will be mixed (*see* **Note 2**). It has been shown that transgene expression can be modulated or suppressed by the genetic background of the donor embryo *(23,24)*.

Embryo donor females should be superovulated to increase the number of embryos obtained per mouse (*see* **Subheadings 3.1. and 3.2.**). The exact timing of the developmental stage of the zygotes is of major importance and can only be achieved by careful adjustment of the hormone treatment timing, light cycle in the animal facility, and time of zygote collection (*see* **Note 3**).

1.2. Recovery, Handling, and In Vitro Culture of Pre-implantation Stage Embryos

Great care should be taken to assure the best possible in vitro culture conditions for the embryos. A humidified 5% CO_2 incubator, 37°C is used for the embryo culture. This incubator should be reserved for embryos only and not shared with other tissue culture. The most commonly used culture medium in 5% CO_2 is M16, and the HEPES-buffered equivalent for use at the bench is M2 *(25)*. These media can be purchased from commercial suppliers (Sigma, Specialty Media) or prepared according to published protocols *(5)* (*see* **Note 4**).

The embryos are easiest handled (collected, moved, washed, sorted, and manipulated) by careful mouthpipeting, using a special device *(26)* (*see* **Subheading 3.3.**) (*see* **Note 5**).

Mice tend to ovulate and mate at the midpoint of the dark period (night). The zygotes will soon thereafter reach the swollen ampulla region of the oviduct, where fertilization takes place. The following morning (12 h after fertilization, at embryonic stage E0.5), the ampulla is greatly enlarged, and can easily be located amongst the oviduct coils, which makes the recovery of the zygotes fairly easy (*see* **Subheading 3.4.** and **Note 6**).

The zygotes are tightly packed together and surrounded by a large amount of cumulus cells at the time of recovery. Cumulus cell masses must be removed from the zygotes before injection can take place (**Subheading 3.5.** and **3.6.**) (*see* **Note 7**).

1.3. Microinjection Equipment

An inverted microscope with either Differential Interference Contrast (DIC) or Hoffman optics is necessary to locate the pronuclei. A low-magnification lens (×2.5 or ×5) is used to get an overview of the injection, and a high-magnification lens (×32 or ×40) is used for the actual injection process. Two micromanipulators are needed for the movement of the holding and injection capillaries. Commercially available micromanipulators such as Leitz, Eppendorf, or Narishige are all suitable, and the choice of brand is a matter of personal preference. We prefer the Eppendorf TransferMan NK as it is an electronic system with which set positions can be pre-programmed. This feature greatly enhances the efficiency and speed of injection. The control of the holding capillary (capture and release of the embryos) can be achieved by using a micrometer screw-controlled, oil-filled glass syringe, or a commercially available control unit (Eppendorf CellTram air), or by simple mouthpipeting. The flow of DNA in the injection needle is best controlled by an injector (Eppendorf FemtoJet) but can also be done (by experienced experimentators) by using a glass syringe. In all the above cases, an absolutely airtight connection has to be established between the control-device and capillary. Air-filled thin polythene tubing should be used between electronic injectors and the injection needle. If injection is performed by hand, a thick hard silicon tubing (Tygon R3603) is the best alternative. For the connection of the holding capillary, hard silicon (Tygon R3603) or thin polythene tubing can be used, either oil- or air-filled. Oil-filled tubings provide a finer (slower) control but have to be free of any air bubbles to work well. Air-filled tubings provide faster movements, which can be compensated by filling oil only in the holding capillary itself.

The injection takes place in an injection chamber: a small drop of M2 medium covered by embryo-tested light paraffin oil. We recommend to use a simple aluminum frame attached to a clean glass slide with high vacuum grease (Dow Corning) to hold the media drop and oil in place. It is also possible to use a glass slide with compression well or even plastic tissue culture dishes. When using supercoiled BAC DNA preparations, microinjection needles can be obtained from commercial suppliers (Eppendorf FemtoTipII) or prepared on a needle puller such as the model P97 from Sutter Instruments (*see* **Note 8**). For linearized BAC DNAs, we only use pulled needles pulled by our glass equipment because the Eppendorf needles appear to shear linearized BAC DNA. To do this, the needle tip is pulled out further to make it longer than the Eppendorf needle at the smallest bore size. This appears to help laminar flow of the DNA solution through the smallest bore and thereby reduce shearing.

A comprehensive reference about glass capillaries and needle pullers has been published by Flaming and Brown *(27)*.

1.4. Dilution of BAC DNA Solution

After BAC DNA preparation and optional restriction digestion, the DNA should be diluted in BAC injection buffer (*see* **Subheading 3.8.**), which aids to stabilize BAC DNA *(28)*. It is very important to prepare an ultra-pure buffer, completely free of any particles, solvents, detergents, and so on. Even the smallest debris will inevitably clog the microinjection needle, and any other substances then DNA or buffer may be toxic to the embryos. The final concentration should be kept as close as possible to 1.5 ng/μl. It has been shown that integration rate increases, but embryo viability decreases when the DNA concentration is raised *(8)*. It is to some extent possible to increase the rate of single-copy integrations by decreasing the DNA concentration *(7)*. Overall efficiency will, however, be reduced.

1.5. Microinjection Process

Groups of zygotes are moved to the microinjection chamber, not more than can easily be injected within 20 min. The zygotes are picked up one by one with the holding needle and orientated, so that the two pronuclei are readily visible.

The injection needle is inserted through the cell membrane and cytoplasm into the target pronucleus. DNA is injected until a clear swelling of the pronucleus can be observed (*see* **Notes 9–15**).

1.6. Transfer of Injected Embryos to Pseudopregnant Female Mice

Most female mice have an ovulation cycle of 3–5 days, but to get into the hormonal status of pregnancy, there is a need for physical mating to take place. Female mice, which are to be used as recipients for transferred embryos, are therefore mated to sterile (vasectomized) male mice. By detection of a copulation plug in the following morning, pseudopregnant females can be selected for transfer surgery. The vasectomy of male mice *(7)* is a simple surgical procedure, where an incision is made in the scrotal sac or abdominal wall, and an approximately 10-mm long piece of the vas deferens is removed by cauterization. Males can be used for mating 2 weeks after surgery and then for as long as xx months.

Injected embryos are placed in the reproductive tract of pseudopregnant recipient females *(29)*. Anesthesia is induced by an intraperitoneal (i.p.) injection of a suitable anesthetic solution (*see* **Subheading 3.10.**). The oviduct is exposed by a surgical procedure (*see* **Subheading 3.11.**) and the embryos placed in the ampulla region, through the infundibulum (*see* **Notes 15** and **16**).

2. Materials

2.1. Preparation of Hormones for Superovulation

1. Pregnant Mares Serum Gonadotropin (PMSG) (Intervet, Intergonan, Toenisvorst, Germany).
2. Human Corionic Gonadotropin (HCG) (Sigma CG-10).
3. Sterile distilled H_2O, chilled to 4°C.

2.2. Superovulation of Young Female Mice

1. PMSG, readymade solution 0.05 IU/ml.
2. HCG, readymade solution 0.05 IU/ml.
3. Disposable 1-ml syringe and 27 or 30 G needle.

2.3. Preparation of Transfer Capillaries

1. 50-ml glass capillaries (Brand).
2. Bunsen burner.
3. Capilette (Selzer, Germany, Capilette pipetierhilfe).
4. Flat mouthpieces (HPI Hospital products, Altamonte Springs, Orlando, FL, USA 1501).
5. Flexible silicon tubing with an inner diameter of 3 mm.

2.4. Dissection of Oviducts

1. Anatomical forceps.
2. Fine straight scissors.
3. Fine straight forceps.
4. 3-cm tissue-culture dish.
5. M2 culture medium.

2.5. Preparation of Hyaluronidase Solution

1. Hyaluronidase type IV from bovine testis (Sigma H-4272)
2. M2 embryo culture medium

2.6. Recovery and In Vitro Culture of Fertilized Oocytes

1. 3.5-cm Falcon tissue culture dishes (35-3001).
2. M2 culture medium.
3. M16 culture medium.
4. Embryo-tested light paraffin oil (Sigma)
5. Hyaluronidase solution (*see* **Subheading 2.5.**)
6. Watchmakers forceps.
7. Disposable 1-ml syringe with 30-G needle.

2.7. Preparation of Holding Capillary and Injection Needle

1. Borocilicate glass capillary (Leica 520119) for holding capillary.
2. Bunsen burner.
3. Diamond point pen.
4. Microforge (Bachofer, Alcatel, Narishige), alternatively Bunsen burner.
5. Borocilicate glass capillary with inner filament (Clark Electronic Instruments, GC100TF) for injection needle.
6. Horizontal glass capillary puller (Sutter Instruments, P97 Novato, CA, USA).
7. Alternatively, purchased needles/capillaries can be used (Eppendorf VacuTips and FemtotipsII)

2.8. Preparation of Injection Buffer and Dilution of BAC DNA

1. Spermine (Sigma, tetrahydrochloride S-1141).
2. Spermidine (Sigma, trihydrochloride S-2501).
3. 1 M Tris–HCl pH 7.5, autoclaved.
4. 0.5 M ethylenediaminetetraacetic acid (EDTA) pH 8.0, autoclaved.
5. 5 M NaCl, autoclaved.
6. Sterile ddH_2O.

2.9. Microinjection

1. Inverted microscope as described.
2. Two micromanipulators.
3. Holding capillary (*see* **Subheading 2.7.**).
4. Injection needle (*see* **Subheading 2.7.**).
5. Microloader (Eppendorf Microloader).
6. M2 culture medium.
7. Fertilized oocytes.
8. DNA solution, 1–2 ng per μl.
9. Injector (Eppendorf FemtoJet), alternatively, 10-ml glass syringe (Becton Dickinson, 10CC, 2590,6458)

2.10. Preparation of Ketamine/Xylazine Anesthetics

1. Ketamine hydrochloride (Ketanest, Park Davis).
2. Xylazine 2% (Rompune, Roche).
3. Sterile ddH_2O.

2.11. Embryo Transfer to the Oviduct of Pseudopregnant Female Mice

1. Ketamine/Xylazine (*see* **Subheading 2.10.**).
2. Sterile phosphate-buffered saline (PBS).
3. Transfer capillary.
4. M2 medium.

5. Embryos.
6. 70% EtOH.
7. Paper tissues.
8. Sterile blunt forceps.
9. Two sterile watchmakers forceps.
10. Sterile fine scissors.
11. Sterile seraffin clip.
12. Wound clip with applicator (Clay Adams) or suture needles with ligature.
13. Pseudopregnant female mouse 8–12 weeks old, E0.5.
14. Stereo microscope with cold light lamp.

3. Methods

3.1. Preparation of Hormones for Superovulation

1. Thaw the hormones by adding pre-chilled sterile H_2O directly to the frozen vial (*see* **Note 17**).
2. Mix carefully and dilute the hormones to a final concentration of 50 IU/ml.
3. Aliquot and freeze immediately at –20°C until use.

3.2. Superovulation of Young Female Mice

Day 1, 11 a.m. (*see* **Note 17**):

1. Thaw an appropriate amount of PMSG immediately before use—do not keep the solution at room temperature for longer than 30 min.
2. Inject each female mouse intraperitonealy with 100 µl (5 IU) PMSG solution.

Day 3, 10 a.m. (*see* **Note 17**):

1. Thaw an appropriate amount of HCG as described above.
2. Inject each female mouse intraperitonealy with 100 µl (5 IU) HCG solution.
3. Mate the superovulated females with stud males and check for copulation plugs the following morning.

3.3. Preparation of Transfer Capillaries

1. Hold a glass capillary in the flame of a Bunsen burner until the glass starts to melt.
2. Withdraw the capillary from the flame and immediately apply a sharp pull to the ends. The faster the pull, the thinner the diameter of the capillary will be.
3. Break the thin part of the capillary approximately 5 cm from the thicker shaft.
4. Using a diamond pen, make a mark on the capillary straight across the thin end, 2–3 cm from the thickening shaft.
5. Break the thin end at the mark.
6. Hold the end of the capillary in the flame of the Bunsen burner until it is no longer sharp (*see* **Note 5**).

7. Capillaries that will be used for embryo transfer should be sterilized by baking in an oven at 180°C for 3 h.

3.4. Dissection of Oviducts

1. Kill the plug positive female mice by cervical dislocation or CO_2 gas (*see* **Note 6**).
2. Wet the abdominal wall with 70% EtOH.
3. Wipe off excess EtOH with a paper tissue.
4. Make a cut through the skin across the midline at the lower part of the abdomen with scissors.
5. Tear the skin off the abdomen by grasping the two skin flaps above and below the cut, and simply pulling them apart.
6. Using the fine scissors and forceps cut open the abdominal wall until all the organs are visible.
7. Move the intestines by scooping them aside with the forceps.
8. Grasp the top part of the uterus with the forceps.
9. Carefully tear the connective tissue on the ovary and oviduct with the scissors.
10. Make a cut between the ovary and the oviduct coils, and a second cut across the top part of the uterus horn (*see* **Note 6**).
11. Place the oviducts in a tissue culture dish with M2 medium.

3.5. Preparation of Hyaluronidase Solution

1. Dissolve the hyaluronidase in M2 culture medium obtaining a stock solution of 10 mg/ml.
2. Filter sterilize, aliquot, and store at –20°C (the stock solution is stable for several months).

3.6. Recovery of Fertilized Oocytes from the Oviducts

1. Place the oviducts in a tissue culture dish with 2 ml of fresh M2 medium.
2. Grasp one oviduct at a time with watchmakers forceps and locate the swollen ampulla (*see* **Note 7**).
3. Rip up the ampulla with the tip of the needle and make sure that the zygotes surrounded by the cumulus masses float out in the medium.
4. Thaw the hyaluronidase solution, and add to the embryo containing M2 medium to a final concentration of 300 μg/ml.
5. Incubate at room temperature for 2–3 min.
6. Collect the zygotes using a transferpipet (*see* **Note 7**).
7. Move the zygotes through three drops of M2 medium.
8. Repeat **step 7**, but using M16 medium, and place the zygotes in the CO_2 incubator.

3.7. Preparation of Holding Capillary

1. Follow **steps 1–4 in Subheading 3.3. but** use borosilicate glass capillaries. Pull the glass relatively thin and absolutely straight.

2. Measure the dimensions of the capillary at the cut end; the outer diameter should be 100 μm.
3. Melt the tip of the capillary on the microforge (or alternatively in the flame of the Bunsen burner) until only a small 15-μm-wide opening remains.
4. Pull injection needles on a microcapillary puller. The shaft should be long and very thin, with an inner diameter (ID) of 0.5 μm at the tip (*see* **Note 8**).
5. Alternatively, use purchased needles and capillaries (Eppendorf VacuTips and FemtotipsII)

3.8. Preparation of Injection Buffer and Dilution of BAC DNA

1. 100-mm polyamine mix (1000× stock solution) (*see* **Note 18**): Dissolve the Spermine and Spermidine together in sterile ddH_2O, so that the end concentration is 30 mm Spermine and 70 mm Spermidine. Filter sterilize (0.22 μm) aliquot and store at –20°C.
2. Basic injection buffer: Mix the following in a plastic disposable 50-ml Falcon tube: 0.5 ml of 1m Tris–HCl, pH 7.5, 10 μl of 0.5 m EDTA, pH 8.0, 1 ml of 5 m NaCl. Add sterile H_2O up to 50 ml. Aliquot, filter sterilize (0.22 μm), and store at 4°C.
3. Ready to use injection buffer: 50 ml basic injection buffer, add 50 μl polyamine mix. Use directly, do not store.

3.9. Microinjection

1. Fill approximately 5 μl of the BAC DNA solution into the injection capillary using a microloader.
2. Attach the injection needle to the right-hand side micromanipulator handle, and position it correctly in the media drop in the microinjection chamber.
3. Attach the holding capillary to the other micromanipulator handle and position it in the media drop.
4. Move a group of 10–15 embryos from the incubator into the media drop.
5. Open up the tip of the injection needle by carefully tapping it on the holding needle (*see* **Note 9**). It is important to break up the tip to a size where the DNA readily flows without having to apply too high pressure. The size should not be so large, however, that it damages the embryos.
6. Clear the needle tip by flushing a small amount of DNA at high pressure. Set a constant flow of DNA to a low level (*see* **Note 10**).
7. Pick up one embryo with the holding capillary and position it, so that both pronuclei can be seen at the midplane of the embryo.
8. Set the focus on the nearest pronucleus, making sure that the border can be seen clearly (*see* **Note 11**).

9. Move the injection needle to the same y-axis position as the targeted pronucleus (either 6 O'clock or 12 O'clock of the embryo) and adjust the height of the needle, so that the tip of the needle appears completely sharp (without changing the focus!). This procedure will allow for the needle to exactly target the pronucleus.
10. Move the injection needle to a 3 O'clock position.
11. Insert the injection needle straight into the embryo, pushing through the zona pellucida, cell wall, cytoplasm, and into the targeted pronucleus (*see* **Notes 12** and **13**).
12. Apply a higher pressure to the DNA flow and keep injecting until a clear increase in the size of the pronucleus can be seen.
13. Withdraw the injection needle and release the embryo.
14. Repeat with the remaining embryos and move them back into the incubator, placed in M16 media (*see* **Notes 14** and **15**).

3.10. Preparation of Ketamine/Xylazine Anesthetics

1. Dissolve 100 mg Ketamin hydrochloride and 0.8 ml Xylazine 2% in 11 ml sterile ddH_2O.
2. Mix and store at 4°C for up to 2 weeks.

3.11. Embryo Transfer to the Oviduct of Pseudopregnant Female Mice

1. Anesthetize the mouse by injecting 10 μl Ketamine/Xylazine solution per gram bodyweight (*see* **Note 19**).
2. Load the transfer capillary with M2 medium, adding two large air bubbles for better control of the movement. Add one small air bubble, pick up the embryos, and add one more small air bubble. The embryos should now be enclosed in a small amount of medium, close to the tip of the capillary, and surrounded by two small air bubbles (*see* **Note 16**).
3. Wait until the mouse has reached surgical anesthesia. This can be checked by pinching the tail or a hind leg. The mouse should not react to these stimuli.
4. Moisten the eyes of the mouse with a drop of PBS (*see* **Note 20**).
5. Place the mouse on its right side, with the legs toward the operator.
6. Remove the fur around the area just behind the last rib.
7. Wipe with 70% EtOH.
8. Make a 12-mm incision in the skin right under the muscles surrounding the vertebra and immediately behind the last rib. A blood vessel and a nerve should be seen running in the abdominal wall, vertically across this area.
9. Make a 10-mm incision in the abdominal wall. Locate the fat pad between the ovary and the kidney.
10. Lift out the fat pad and secure it with a seraffin clip outside the body wall. Take care not to touch the ovary or oviduct coils!
11. Place the mouse under a stereomicroscope and focus on the oviduct area.

12. Tear the bursa surrounding the ovary and oviduct coils with the two pairs of watchmaker forceps.
13. Locate the infundibulum. If bleeding is obscuring the view, place a very small piece of paper tissue in the cleft between ovary and oviduct coils.
14. Insert the transfer capillary into the infundibulum and expel the embryos. Check that the two small air bubbles have entered into and behind the first turn of the coils (the air bubbles can easily be seen through the wall of the oviduct).
15. Remove the seraffine clip and place the organs back into the abdominal cavity.
16. Seal the skin incision with a wound clip.
17. Repeat the procedure on the other side.
18. Place the mouse in a quiet, dark and warm area until it recovers (*see* **Notes 21** and **22**).

4. Notes

1. The reader is strongly recommended to gather further information and experience with mouse embryo micromanipulation before attempting the actual microinjection. Useful protocols, advice, and consideration are available at the following Websites: Thom Saunders, http://www.med.umich.edu/tamc/BACDNA.html, and Dr. Lluis Montoliu, http://www.cnb.uam.es/~montoliu/prot.html. in **ref. 7**
2. We have used embryos obtained by crossing CD1 outbred females with C57 inbred males, and this combination has worked very efficiently in our hands. The number of embryos that can be obtained per donor female in these crosses is usually high, the embryo viability is good, the embryos are easy to inject and survive the microinjection process well. If an inbred background is desirable for the transgene, it is possible to use for example C57BL/6J embryos, although the microinjection process will be more difficult, and the viability of the embryos is lower.
3. The highest numbers are usually obtained from very young female mice, just at weaning age. The embryo quality is, however, often variable and low, and very small females may suffer significantly during the stressful experience of being mated by a large and often less careful male. For this reason, we recommend using 5- to 6-week-old females. Both the time-point at which each of the hormone injections are given and the light cycle in the room where the animals are kept will have influence on the ovulation. The optimal time during which microinjection can take place is as short as 2–4 h. If the embryo development is not advanced enough, the pronuclei will be very small and difficult to target. In this case, the hormones should be given earlier in the morning, and/or the light cycle should be set back. On the contrary, if the embryos are at too late a stage of development, the pronuclei will already be fused, and the first division will have taken place. The solution here is to give the hormones later, and/or set the light cycle forward. We use a light cycle of 14 h light/10 h dark, with the light switching on at 5 a.m.

4. Each batch of oil and all ingredients in homemade embryo culture media should be tested for supporting optimal embryo development before use. M16 should be pre-equilibrated in 5% CO_2 incubator for a minimum of 2 h before use.
5. Care should be taken to use glass capillaries with a smooth end, as sharp edges can harm the zona pellucida. It is also important to minimize the time which the embryos spend outside the incubator as much as possible. One-cell stage embryos should not spend more then 30 min in room air and temperature.
6. Care should be taken not to damage the ampulla during dissection, as this would inevitably result in losing the embryos. The choice of method to kill the donor mice (cervical dislocation or CO_2 gas) does not have any influence on embryo viability and is therefore a matter of personal preference and local legislation.
7. The cumulus masses can easily be seen through the wall of the swollen ampulla. By grasping the oviduct with watchmaker's forceps and tearing the wall of the ampulla with a sharp 27-G needle, the embryos are allowed to float out in the M2 media-filled tissue-culture dish. It is essential to remove the embryos from the hyaluronidase solution as soon as the cumulus masses are dissolved.
8. Settings for P97:

   a. Borosilicate glass capillaries with inner filament; outer diameter 1.0 mm, inner diameter 0.78 mm (Clark Electronical Instruments GC100TF-15).
   b. Filament = B032TF.
   c. Pressure = 100.
   d. Heat = 305 (~10 values below ramp test result).
   e. Pull = 100.
   f. Velocity = 150.
   g. Time = 100.

9. Before attempting to inject the embryos, the tip of the injection needle should be broken up to a larger size. Because BAC DNA is more viscous than solutions with smaller DNA fragments, it is more likely to clog the needle during injection. There is also a risk for shredding the DNA in case it is pushed through a small opening with a high pressure. The final size of the needle tip should be around 3–4 μm.
10. If the Eppendorf FemtoJet is used, the Pc should be set to 5–10 and the Pi to 40–50. The injection should take place in "Manual" mode, where the injection time is adjusted to each pronucleus, and kept until a clear swelling is achieved.
11. It is easier to target the larger of the two pronuclei and/or the one that is closest to the injection needle. If the embryos are well timed, the pronuclei should both appear in the center of the embryo, both be large and clearly visible. At the optimal time for injection, it is often difficult to tell which pronucleus is the male and which is the female. There is no advantage in choosing one or the other. The only criteria for the choice should be the ease of injection: the pronucleus that is largest and/or nearest to the injection needle should be targeted. If the pronuclei

are small, the embryo has not yet reached the optimal time-point for injection. It may help to bring the embryos back to the incubator for an hour and start the injection process later. If ever the embryos are left for too long at 37°C before the injection is started, the two pronuclei will fuse, and injection will be impossible. The timing of the developmental stage can be influenced by the superovulation protocol (*see* **Subheading 3.2.**).

12. Care should be taken to avoid touching any of the nucleoli within the pronucleus. These structures are extremely sticky and will immediately attach to the needle. If this should happen, the injected embryo must be discarded, and the injection needle changed.
13. The cell membrane is sometimes very difficult to penetrate. Because it appears very elastic, it will simply follow the tip of the injection needle into the embryo and sometimes all the way into the pronucleus. If this happens, a small "bubble" will form at the tip of the needle, and the pronucleus will not expand. The needle can in these cases be pushed further on, through the pronucleus, and then slowly withdrawn into the pronucleus again, which usually solves the problem.
14. Embryos of lower quality should be discarded. Under the high magnification during injection, it is easy to detect those embryos with only one or more than two pronuclei, embryos with sperm under the zona, and so on. It is important to sort away these embryos and those that could not be successfully injected. Later, it will be impossible to distinguish between well-injected healthy embryos and those of poor quality.
15. Embryos can be transferred immediately after injection. It is also possible to culture the embryos overnight in vitro and transfer only the well-developing two-cell stage embryos the next day. Great care should be taken to make sure all embryos are well in the oviduct and to minimize the damage during the surgery. The anesthesia should be carefully optimized to give a short but deep sleep, and the mouse should be kept warm until recovery.
16. Generally not more than 25–30% of the transferred one-cell stage embryos (slightly more if two-cell stage embryos are transferred) will develop to term. To keep the litter size at an optimal level (6–8 pups), the number of embryos to transfer should be kept in the range of 25–30. We highly recommend transferring half of the embryos to each oviduct, although it is possible to put all into one side.
17. It is essential to prepare the hormones as soon as possible after thawing, to work fast, and to freeze the solution as soon as it is prepared. Repeated freeze-thawing should be avoided. Hormones are stable in –20°C for at least 3 months.
18. Polyamine mix is stabile at –20°C for several months. The microinjection buffer without polyamines added is stabile at +4 for several months. The ready-to-use buffer (polyamines added) should be prepared fresh for each experiment and not stored.
19. We recommend using a mixture of Ketamine and Xylazine, as this combination has proven to be nontoxic, has a high safety marginal in terms of overdoses and

gives a deep but short surgical anesthesia. These measures will all contribute to increasing the possibility of maintaining a pregnancy.

20. During anesthesia, the eyes of the mouse will stay wide open. The cornea will quickly dry out, leading to severe pain when the animal recovers. It is therefore highly recommendable to moisten the eyes with a drop of PBS as soon as the mouse has fallen asleep.
21. The normal gestation time in most mouse strains is 19–20 days. Outbred CD1—which is one of the most commonly used mice as recipients for embryo transfer—tends to deliver very reliably on E19. A commonly encountered problem following embryo transfer is low implantation rates and dying/reabsorbing fetuses. If the number of fetuses that develop to term is too low, the size of each conceptus will be larger then normal, which often results in difficulties at birthing. Litter sizes of less then four pups often cause delivery difficulties in recipient females. These pups generally do not survive, and the mother suffers pain unless a caesarean section is performed. The procedure is fairly simple and the survival rate of the sectioned pups very high *(7)*. During the first half of the pregnancy, the weight gain of the mother is moderate, but weight increases dramatically during the last week. By weighing the recipients every other day, it is possible to predict in which cases the litter size could be expected to be small, because the curve of weight gain will reliably indicate litter size and possible problems during pregnancy. A female that has not given birth on E20 should promptly be opened for caesarean section in any case.
22. It is essential to get the pups dry and warm immediately after sectioning. The breathing can be stimulated by wiping them roughly with a soft paper tissue. A common mistake is to be too soft with the pups. Because they have not gone through the physical stress of birth, they usually do not start breathing regularly unless they are turned around, pinched, and gently massaged with cotton tips or paper tissue pieces. Place the pups with a new foster mother when they are completely freed from all blood, regularly breathing, and have gained a normal body temperature.

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