Video Article

Trans-inner Cell Mass Injection of Embryonic Stem Cells Leads to Higher Chimerism Rates

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Abstract

In an effort to increase efficiency in the creation of genetically modified mice via ES Cell methodologies, we present an adaptation to the current blastocyst injection protocol. Here we report that a simple rotation of the embryo, and injection through Trans-Inner cell mass (TICM) increased the percentage of chimeric mice from 31% to 50%, with no additional equipment or further specialized training. 26 different inbred clones, and 35 total clones were injected over a period of 9 months. There was no significant difference in either pregnancy rate or recovery rate of embryos between traditional injection techniques and TICM. Therefore, without any major alteration in the injection process and a simple positioning of the blastocyst and injecting through the ICM, releasing the ES cells into the blastocoel cavity can potentially improve the quantity of chimeric production and subsequent germline transmission.

Video Link

The video component of this article can be found at https://www.jove.com/video/56955/

Introduction

For nearly 25 years, the creation of genetically targeted mice has been a slow process, often hampered by a bottleneck at the blastocysts ES cell microinjection stage for the generation of germline transmitting chimeras. Recent advancements, including CRISPR/Cas9[1,2] targeting in ES cells and high-throughput ES cell generation consortiums like KOMP, have improved the generation/availability of genetically modified ES cells. However, the generation of germline chimeras remains a bottleneck in creating genetically modified mice from these ES cells[3,4]. High-throughput ES cell generation projects have been plagued by high variability in ES cell quality, which is critical for successful creation of germline chimeras. Additionally, some of the commonly utilized ES cell lines are known for high aneuploidy, and difficult production of germline competent chimeras[5].

Many methods have been developed for creating mice with either a higher chimera, or completely ES cell derived mice[6,7,8,9,10]. While each of these systems has its own merits, they also have their shortcomings. The generation of tetraploid chimeras, while generating 100% ES cell derived mice, is inefficient, and generally limited to outbred lines [5,6]. Newer methods of injecting morula can yield high percentage chimeras, approaching 100%, but generally require significant additional equipment (lasers or piezos) and training[10,11]. In laboratories where these techniques are already in place, this new methodology may not be necessary.

This study's objective was to find a method that uses common techniques and readily available equipment to increase the rate of chimera generation, increasing the chance of germline transmission of the mutant allele to establish the subsequent mouse colony.

Protocol

All operations were done as part of the normal operation of the NIEHS Knock Out Mouse Core facility. All mice were kept on a 12:12 h light:dark cycle, and were feed a diet of NIH-31 and water *ad libitum.* All experiments were performed in accordance with the Animal Care and Use Committee of the National Institute of Environmental Health Sciences.

1. Animal Preparation

1. Breed donor mice, B6(Cg)-Tyr<c-2J>/J female mice at 8 - 10 weeks of age naturally to B6(Cg)-Tyr<c-2J>/J male mice between 8 and 26 weeks of age. Check plugs visually the following morning (E0.5).
2. Breed recipient mice, Swiss Webster female mice between 6 and 9 weeks of age are naturally bred to vasectomized Swiss Webster male mice. Check plugs visually the following morning (E0.5).

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2. ES Cell Preparation

1. For 129-derieved AB2.2 ES cells, culture cells in DMEM supplemented with 15% FBS, 1% Glutamine, 1% Penn/strep, and 0.1% β-mercaptoethanol. Culture all other ES cells per ES cell supplier's instructions, using their recommended protocols.

3. Embryo Collection

1. **Animal Dissection**
    1. Prepare several 60 mm dishes, approximately 1 per 5 mice plus 3 extra, with 10 mL blast collection media (BCM) (10% FBS IN DMEM), and one 4 well dish with 0.5 mL BCM per well. Equilibrate at 37 °C, 5% CO_2.
    2. Euthanize B6(Cg)-Tyr<c-2J>/J female mice at E3.5 using CO_2 asphyxiation, with a flow rate of 10%.
    3. After euthanizing, place the mice in the supine position, and wet the abdomen thoroughly with 70% ethanol.
    4. Make the first incision through the skin only, at the midpoint of the abdomen, making the opening as wide as possible. Next open through the abdominal wall, again making the incision as wide as possible.
    NOTE: Doing this in two steps helps reduce or eliminate fur contamination.
    5. Starting at an ovary, and holding the fat pad of the ovary with blunt forceps, carefully remove the ovary, oviduct, and uterus, taking extra care to keep the adipose tissue to a minimum. Remove the uterus as one single unit, or as individual horns, with no change in the following procedure.
    6. After removal, place the uterus in a 60 mm dish containing blast collection media.

2. **Embryo Removal**
    1. Prepare a 10 mL syringe filled with BCM, and fit with a 25 G needle.
    2. One uterus at a time, remove them from the holding dish of BCM, blot them on a tissue to remove excess blood, and place in a 60 mm dish under a low magnification dissecting microscope.
    3. While carefully and gently holding the uterus just below the distal end (near the oviduct) with dissecting forceps, insert the needle in to the center of the uterus in as parallel a plane as possible. Insert the needle just beyond the tips of the forceps.
    4. Apply some pressure to the forceps to hold the uterus to the needle, and expel approximately 1 mL of BCM through the horn of the uterus.
    NOTE: Care must be taken at this step, as too much pressure on the syringe will cause an uncontrollable stream of media, often leaving the dish.
    5. After flushing the uterus, up to 5 whole uteri per 60 mm dish, place the dish back in the incubator until collection.

3. **Embryo Collection**
    1. Using a hand drawn glass pipette and a mouth pipette apparatus, collect blastocysts under a dissecting scope, set between 25X and 35X. (**Figure 1**).
    NOTE: Mouth pipette apparatus consists of 30 - 40 cm of flexible tubing, under 5 mm I.D. Insert a 1, 000 µL pipette tip, point first in to one end, and affix the glass pipette in it. Add a syringe filter to the other end, with an attached 1 cc syringe barrel, no plunger, to act as a mouth piece.
    2. Removing dishes from the incubator, and using a grid pattern, examine every part of the dish for embryos. Embryos are picked up individually and place on the drop of BCM in a secondary dish.
    3. After collecting from all dishes, wash the embryos once more in a drop of fresh BCM, and then place in the 4 well dish to await injection.

4. Embryo Injection

1. Pick up ES cells individually with the injection needle based on confirmation.
    1. Prepare an injection dish by adding 5 - 7 mL of injection media (DMEM, 10% FBS and 20 mM HEPES) to a glass bottom injection dish. Injections are done at room temperature.
    2. Using either an air or oil microinjector, apply vacuum to the injection needle to draw ES cells in. Take care to keep as little space as possible between cells. Count the ES cells here, or at the injection step.
    3. Select ES cells that are medium to small in size compared to the general population of cells, with a smooth surface, and without obvious defects such as vacuoles.
    NOTE: ES Cell populations vary greatly in morphological quality, and the standards used will need to adjust with them.

2. Inject embryos with approximately 15 ES cells (**Figure 1**).
    1. Attach a holding pipette tip to a second air microinjector, and position an embryo near the opening of the holder. Apply vacuum to secure the embryo to the holder.
    2. Adjust for the z axis by moving the holding pipette up or down so that the embryo is just touching the slide, followed by fine focusing on the zona. Next, adjust the Z axis of the injection needle so that ES cells near the tip are in focus.
    3. Adjust the embryo now by gently pushing from the injection needle at the top or bottom, rotating it to put the ICM in the desired position.
    4. Insert the injection needle through the zona, and either the ICM or the trophoblast, using a firm but controlled push. Apply a slight pressure to the microinjector of the injection needle to help keep the blastocoel from collapsing.
    5. Once the needle has penetrated the blastocoel, apply pressure from the microinjector to transfer ES cells to the blastocoel of the embryo. Pause briefly as the bevel of the needle starts to exit the embryo to allow the pressure in the embryo to return to normal, while still retaining the cells.

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NOTE: Reference group ES cells were injected normally, following a traditional ES cell injection protocol[9], taking care not to touch the ICM. ES cells were also injected through the ICM, pushing the needle directly through the ICM (TICM) (**Figure 2, Supplemental movie**).

5. Transfer of eEmbryos

1. Transfer embryos to Swiss Webster mice at E2.5, using a non-surgical embryo transfer device, per the manufacture's procedures. This non-surgical procedure requires no anesthesia or special post-op care. House mice individually after the transfer of embryos, and through delivery and subsequent weaning.

Representative Results

Treating cell-line as the random effect, a mixed effects linear model was used to analyze the data in the software (e.g. SAS (version 9.2)). Each analysis controlled for the number of embryos transferred and the cell line. The second analysis also controlled for the number of pups that survived.

For this study, each ES Cell line and clone was injected both with traditional and TICM techniques, alternating between the two methods after each embryo. After injection, embryos were segregated on the dish and grouped by transfer, generally between 12 and 15 embryos per recipient female. Embryos that did not have a clearly visible blastocoel, lacked a Zona pellucida, or where the microinjected ES cells failed to enter the blastocoel, were eliminated from this experiment.

The first data examined was pregnancy rate. As shown in **Figure 3**, pregnancy rates were unaltered in the TICM group from the control group. Additionally, an equal proportion of pups survived to weaning (P = 0.50, **Figure 4**). Most importantly, while the number of mice at weaning remains unchanged, the number of pups displaying coat color chimerism increased from 31% to 50% for the TICM group (p = 0.005, **Figure 5**). The degree of coat color chimerism was considered too subjective to be evaluated in this study, and not truly relevant to germline chimerism.

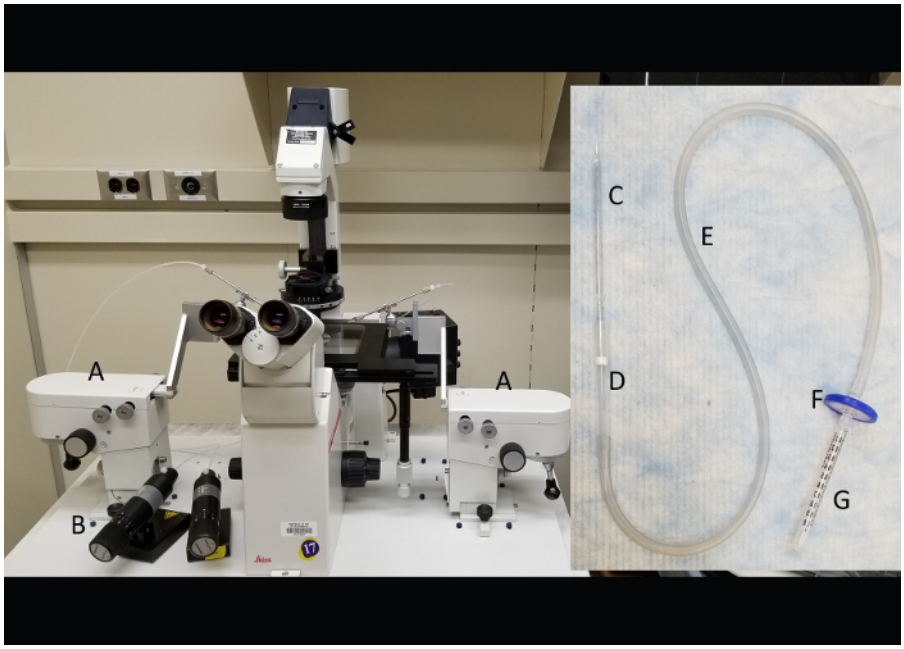

Figure 1: Specialized Equipment. (Left) Injection microscope setup, including **(A)** Micro Manipulators and **(B)** Cell Tram Air. **(Right)** Mouth pipette apparatus, with **(C)** drawn glass pipette, **(D)** 1000µL pipette tip, **(E)** tubing, **(F)** Filter, and **(G)** Syringe barrel. Please click here to view a larger version of this figure.

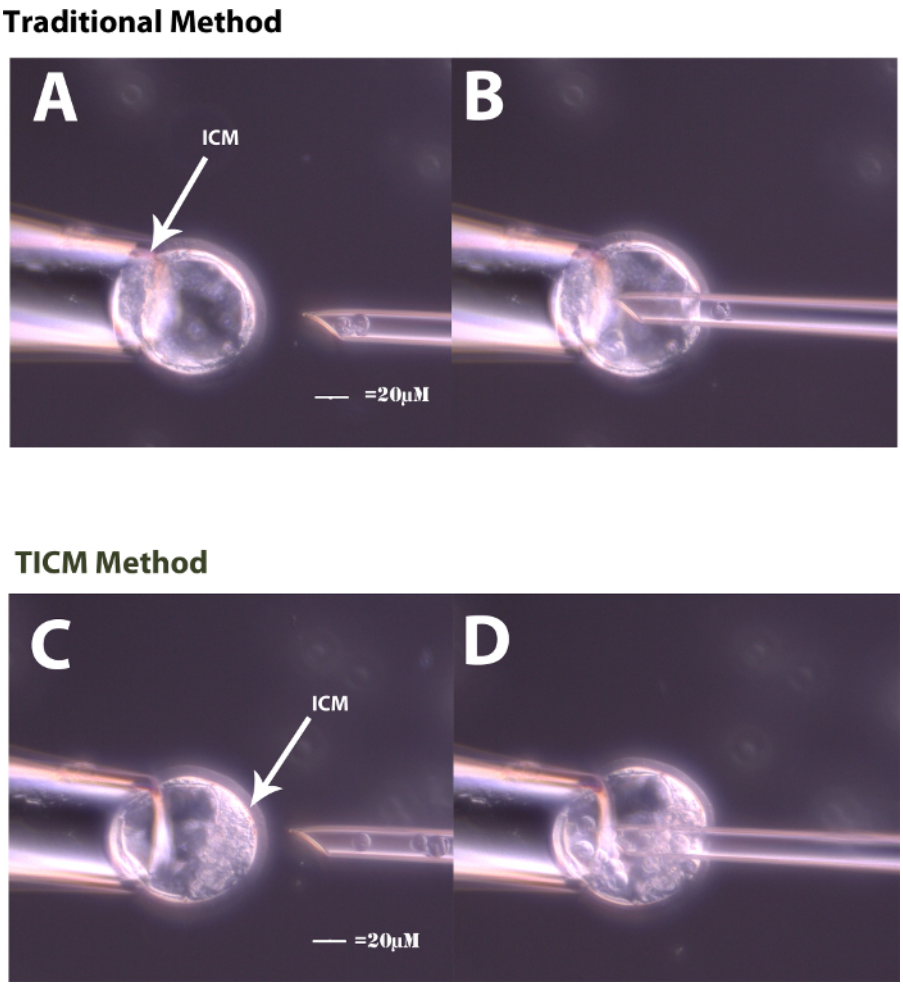

Figure 2: Example of blastocyst embryo injection. (**A, B**) Traditional injection method, where the injection needle enters opposite of the ICM, avoiding any interaction with the ICM. (**C, D**) TICM method, where the injection needle enters directly through the ICM in to the blastocoel. Please click here to view a larger version of this figure.

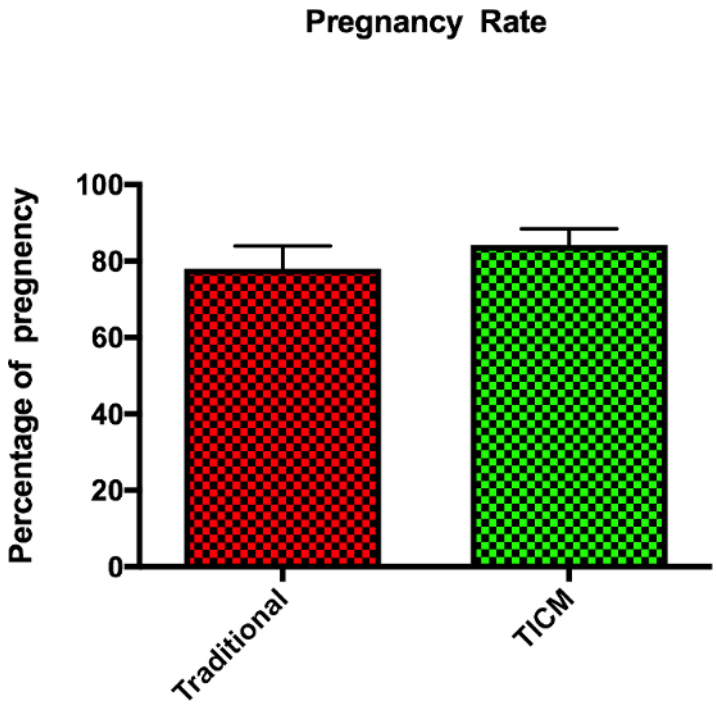

Figure 3: Pregnancy rate. The pregnancy rate showed no significant difference between traditional (39/50) and TICM (64/76). Averages plus standard error of the mean (SEM). Please click here to view a larger version of this figure.

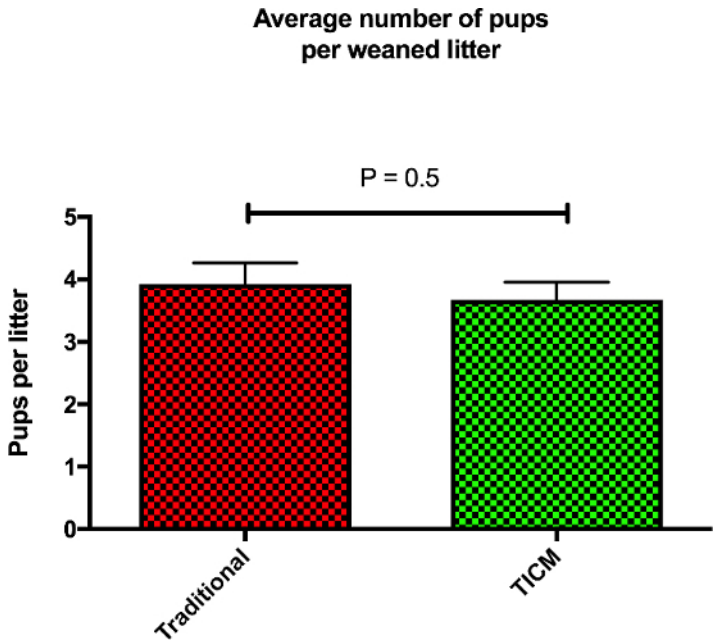

Figure 4: Survival rate. The survival rate is the number of pups that survived to weaning, where there was a litter to wean. Pups that did not survive to weaning, and litters where no pups survived to weaning, were not included in this data. Averages plus standard error of the mean (SEM). Please click here to view a larger version of this figure.

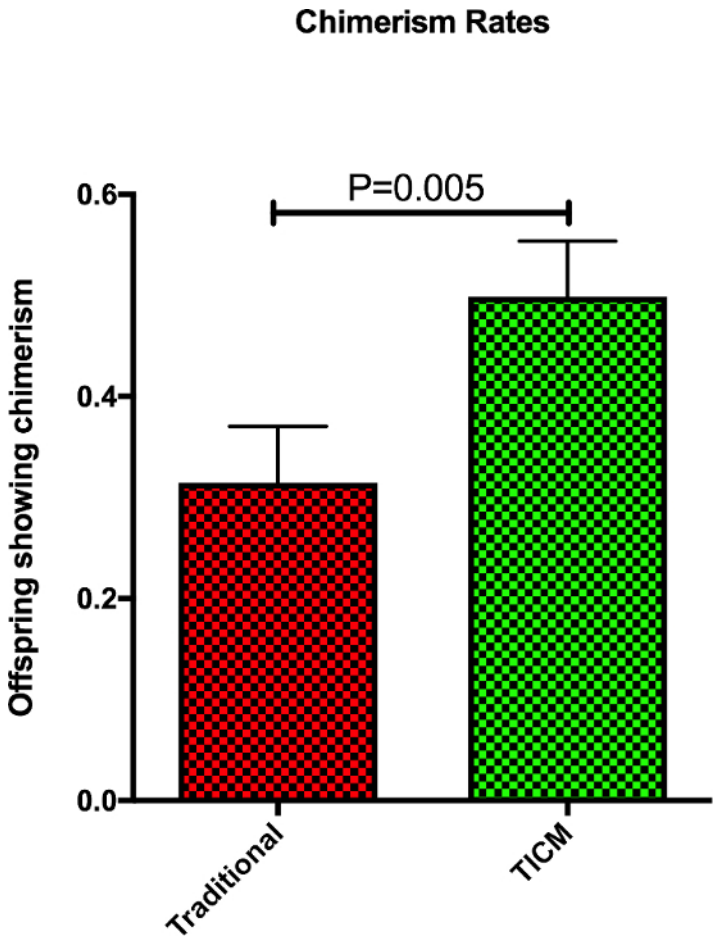

Figure 5: Chimerism rate. This data only represents the number if chimeras produced, and does not address the quality of chimerism. In the traditional group, 31% (34/110) of the offspring showed chimerism, where the TICM group had an average of 50% (79/158). Average plus standard error of the mean (SEM). Please click here to view a larger version of this figure.

Discussion

It has long been thought that any disturbance of the ICM could lead to mortality, in fact, current blastocyst microinjection protocols still warn of this[12,13]. What we have shown here is that microinjection through the ICM is not detrimental to the embryo, and increases the yield of chimeras.

The exact mechanism for this effect has not been identified. However, the mechanical disruption of the ICM, and the accompanying hypoblast, should increase the incidence of ES cell integration into the newly formed ICM following microinjection. Recent work in the field of iPSCs has shown that mechanical stimulation helps in the reprogramming of human iPSC[14]. If mechanical manipulation has a similar effect in the ICM of the mouse embryo, it could have the effect of returning cells of the ICM which had potentially started to differentiate, back to a pluripotent state, removing a potential 'head start' of the blastocyst donor derived cells in forming the embryonic tissue. Lastly, it is possible that the injection through the ICM results in the death of an unknown number of cells in the ICM, lowering the level of competition for the microinjected genetically modified ES cells.

The primary benefits of this method modification are that it requires no new learning, troubleshooting, or equipment. All the techniques carry over from standard blastocyst injection. The most critical steps also remain unchanged; proper and timely collection of embryos, and proper culture conditions for the embryos before, during, and after injection. When using the non-surgical embryo transfer technique, it does become more critical to select proper recipient mice. With surgical transfers, the surgeon had the opportunity to examine the mouse to verify proper reproductive state, where with non-surgical transfers, this is not possible. Mice should be of prime reproductive age (6-9 weeks) and only mice with visible plugs should be used. The most important aspect of this technique, and all ES cell injections, is the quality of the ES cells.

When dealing with high quality, robust cell lines, this technique may yield only minimal differences, but with suboptimal ES cell lines or difficult to work with lines, any, even minor improvements, can be significant and make the difference between project success and failure. It is also of note, that over the course of this study, several commercially available ES cell lines generated germline chimeras, and nearly all of the commercial ES lines that generated germline chimeras were TICM injections. Here we have shown that a simple change in technique can result in a significant improvement in productivity, without incurring any additional costs for new equipment, training, or increasing the use of animals.

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Disclosures

The authors have no competing interests to disclose.

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