Creation of Transgenic Mutants to Study *T-gene* effects in The Post-Implantation Mouse Embryo.

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Abstract

In order to study the effects of Brachyury (T) in the EET (Extra Embryonic Tissues) of the post-implantation mouse embryo, a transgenic mouse was created in which the ET (Embryonic Tissue) originated from foreign ESCs and the EET from the original wild type. The concept of induced tetraploidy (Kubiak 85) and ESC aggregation successfully created a transgenic model whose ET and EET are of separate origins. The proof of concept and validation of protocol on the CD1 wild type mouse model, allows for the procedure to be continued in order to study the effect of the T-mutation on the Extra Embryonic Tissues of the mouse embryo.

Background

In eutharian mammals, the first cell types that are specified during embryogenesis form extraembryonic (placenta and fetal membranes) rather than embryonic structures. Cells at the periphery of the morula (E2.5) become trophoblast, whereas cells on the inside remain undifferentiated embryonic ectoderm, which later gives rise to the fetus as well as the endodermal and mesodermal parts of the placenta and extraembryonic membranes. Genetic studies in mice are beginning to identify growth factors and cell adhesion molecules that mediate interactions between cell types that are essential for morphogenesis of the placenta and fetal membranes, as well as transcription factors that control the differentiation of extraembryonic cell types (Cross 23).

Development of the extraembryonic structures influences the morphogenesis of the embryo because of different cell-fate results and tissue interactions. Trophoblast cells are a cell form unique to eutherian mammals that contributes only to the placenta. They are essential for contacting the uterine wall at implantation, invading into it and producing hormones necessary for maternal recognition of pregnancy (Cross 24). The blastocyst implants into the uterus at E4.5 after conception. Thereafter, trophoblast cells that spread over the surface of the Inner Cell Mass (ICM) continue to propagate, whereas trophoblast cells not contiguous to the ICM no longer divide and differentiate into other cell forms. After implantation, the polar trophectoderm gives rise to extraembryonic ectoderm of the chorion (Cross 26).

Descendents of the ICM produce the entire embryo as well as the mesodermal and endodermal constituents of the placenta and fetal membranes. The primitive endoderm emerges from the ICM in late blastocysts, and later transforms into the extraembryonic parietal and visceral endoderm (Palmieri 62). At about day 6.5 of development, gastrulation begins in the embryonic ectoderm layer and gives rise to the three germ layers of the embryo, as well as to extraembryonic mesoderm, which will form the amnion, visceral yolk sac and allantois. The latter forms the umbilical cord as well as part of the mature chorio-allantoic placenta (Spindle 65)

Recent chimera analysis with FGFR1-mutant ESCs suggests that while these mutant cells are rarely migrate out of the primitive streak and contribute to anterior embryonic structures, they can still contribute to the extraembryonic mesoderm (Ciruna 32). These interpretations suggest that the genes are critical for embryonic but not extraembryonic mesoderm development and suggest that differentiation of the two cell types depends on different signaling pathways (Zhang 84). Until recently, no specific factors have been implicated in extraembryonic mesoderm specification (Cross 27), especially for Brachyury.

Even after the embryonic and extraembryonic cell lineages have been established and segregated by about day 7.5 of mouse gestation, the respective cell types continue to interact (Yost 89-92). Although the factors that account for the processes are unknown, there are also several examples that suggest that extra-embryonic structures could also contribute to patterning in the early embryo. (Nagy24-28) In addition, cell–cell interactions between embryonic and

extraembryonic cell types can be readily studied in chimeric embryos consisting of cells with different genotypes. (Cross 29)

Brachyury is a protein that in humans is encoded by the T gene (Howards 26). The T-mutation (a member of the T-box family of genes) was first described in mice in 1927. It affects the tail length and sacral vertebrae in heterozygous animals (Dobrovolskaïa-Zavadskaïa, 1927).

In humans, homozygous T is lethal and heterozygous T shows immense defects in axial mesoderm and endoderm formation in embryonic stages – giving rise to conditions such as fused lower limbs (e.g. sirenomalia) (Ghebranious, 2008). The T gene encodes a 436 amino acid nuclear transcription factor and defines the mesoderm during gastrulation (Marcellini 52).

Knockout Wnt3 mouse models show independence of T in Extra Embryonic tissue (EET). One can thus conclude that T is a transcription factor expressed in Extra Embryonic tissue. A hypothesis was formed based on T being a transcription factor that is expressed in this Extra Embryonic Tissue of the mouse embryo. Since this tissue functions as a scaffold for the formation of the future of the umbilical chord and placenta it was hypothesized that Brachyury (T) expression is crucial for the proper development of the extra embryonic tissues in the mouse embryo.

In order to study T expression in the EET of homozygous T mutants, the EET must contain the mutant gene and the ET must not. Thus, a transgenic mutant, in which the embryonic and extra embryonic tissues are of different origin is necessary. A protocol was generated that would induce such a mutant. In order to guarantee that an embryo's original genetic makeup would not contribute to the

embryonic tissues of the post-implantation embryo, the protocol included tetraploid fusion. By doubling the chromosome number of the diploid zygote, the new tetraploid embryo would not be viable.

The generation of tetraploid embryos by electrofusion was first described by Kubiak and Tarkowski (Kubiak 61). By applying a direct electric pulse, the authors succeeded in generating a single cell through the fusion of two blastomeres from two-cell stage embryos. The replication of the genetic material followed by mitotic division results in a two-cell embryo containing double the diploid content of DNA. This tetraploid embryo can develop further to the blastocyst stage (Naumann 1). The fused embryos can be rescued by embryonic stem cells, which if aggregated with the tetraploid blastomeres can give rise to the embryonic tissues. Due to their pluripotency (instead of totipotency) the cells can give rise to the major germ layers but not to any extra embryonic tissues (which will form vital structures such as the placenta and umbilical chord). Thus, a non-viable zygote (tetraploid) aggregated with embryonic stem cells (ESCs) would yield an embryo whose embryonic tissues would be of ESC descent and extra embryonic tissues of the original T-mutant.

Consequently a secondary hypothesis as it pertains to the success of the protocol is that ESCs can supplant the original embryo if aggregated with early stage, tetraploid blastomeres and form a transgenic mutant whose embryonic and extra embryonic tissues will be of different genetic origins. The extra embryonic tissues containing the mutated gene can then be studied morphologically.

Methodology (materials, procedures)

Figure 1. Timeline for ♀CD1 mating, embryo retrieval and embryo implantation, Ψ♀CD1 mating and uterine transfer, and KT4 ESC culture and aggregation.
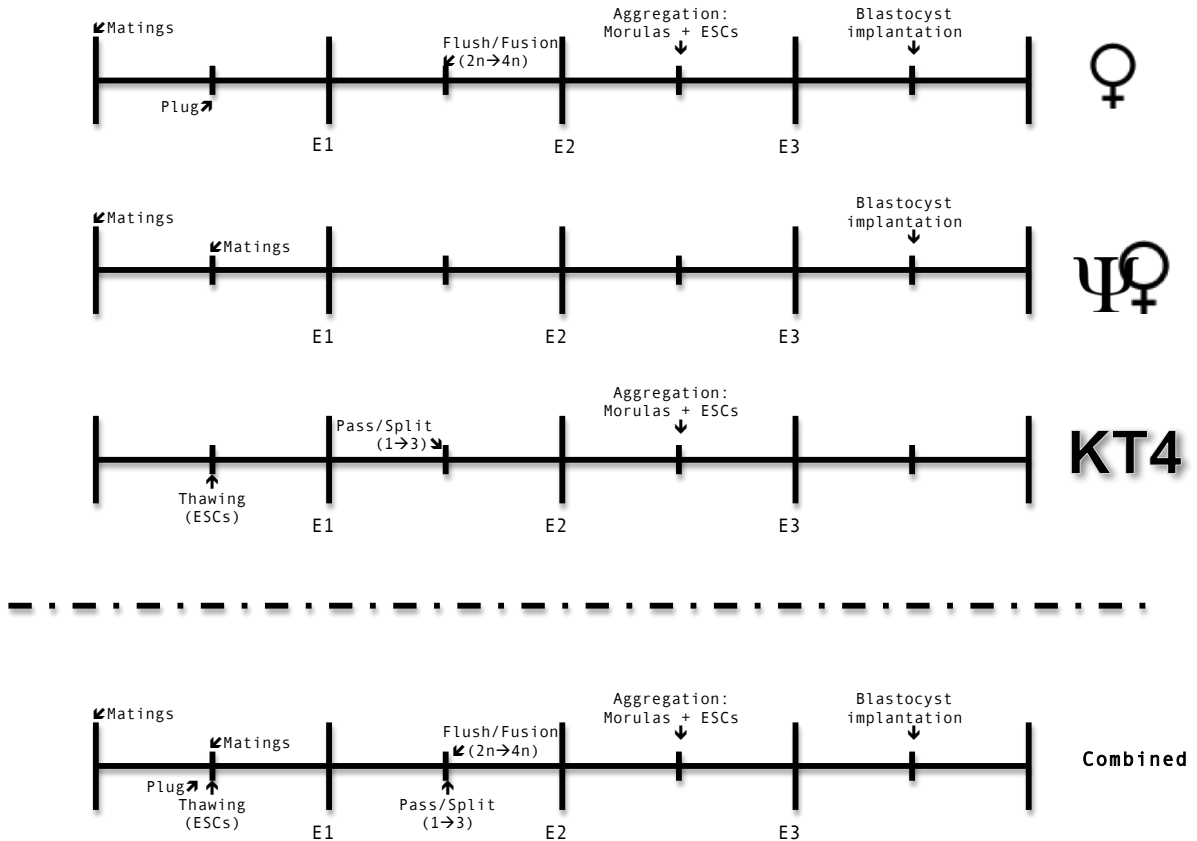

Figure 1 presents a timeline in embryonic days (e.g. E1) for three aspects of the experiment – Female CD1s on the 1st line, pseudo-pregnant CD1s on the 2nd line and KT4 ESC culture on the 3rd. The 4th line is a combination of the previous three.

- ## CD1 Mouse Manipulation (♀/Ψ), Embryo manipulation

  o CD1 strain female mice are crossbred with CD1 male studs. Plugs are checked the following morning (E0.5) and the plugged females are transferred into a separate cage until E1.5 for embryo retrieval.

  o At E0.5, CD1 females are crossbred with CD1 vasectomized males. The plugs are checked the following morning on E1.5. When the female is successfully crossed with an infertile male, the corpus luteum persists without an embryo, leading to pseudo-pregnancy. The female will develop mammary glands, lactate, and build nests in the pseudo-pregnant state. Thus, the stimulus of pseudo-pregnant mating elicits the hormonal changes needed to make her uterus receptive.

  o The embryos are retrieved on E1.5 from the ♀CD1 x ♂CD1 cross.

    ▪ The female mouse is killed by cervical dislocation.

    ▪ It is then dorsally placed on a surgical pad in a prone position and doused with 70% ethanol (to facilitate the imminent incision).

    ▪ A transverse superficial incision is made above the abdominopelvic cavity revealing the diaphragm. A second transverse incision of the lining will reveal the abdominal viscera (Figure X).

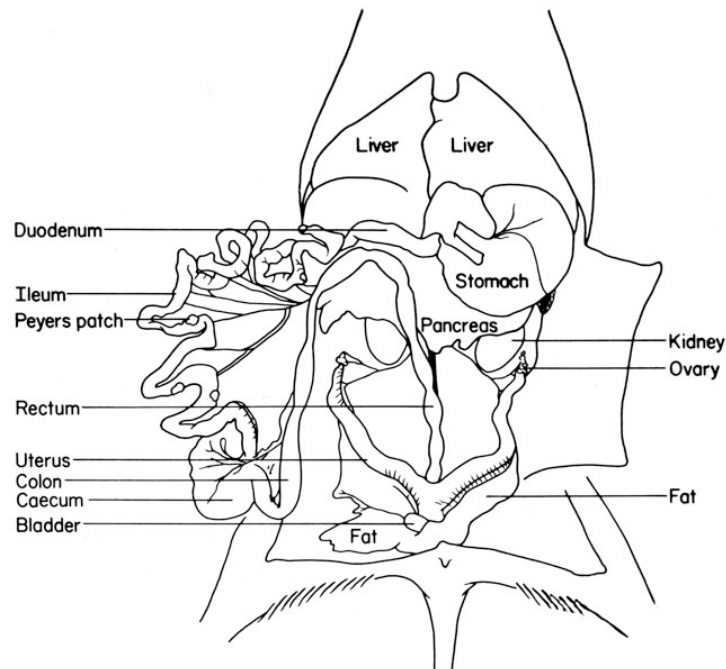**Figure 2**: Abdominal viscera displayed.

Figure 2: Female abdominal viscera displayed. Ovaries labeled on distal ends of uterus.

- Both ovaries are severed from the distal ends of the uterus and placed in separate 40μL drops of M2 media on separate 3cm tissue culture plates.

- The ovary is transferred to a dissection area under a dissecting light microscope.

- Number 1 micro-dissection forceps are used to manipulate the oviduct and locate the infundibulum (the end of the mammal oviduct nearest to the ovary).

- A hamilton needle attached to a 1mL syringe filled with M2 media is inserted into the infundibulum and clamped with the forcep.

- The injection of the M2 media then flushes the oviduct of its contents.

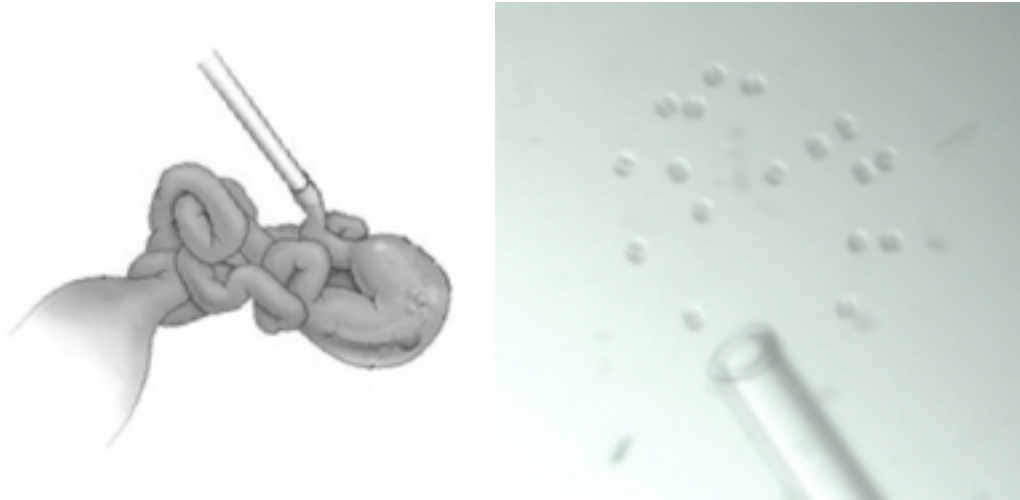**Figure 3**: Embryo Retrieval E1.5

Figure 3: Diagram of Hamilton needle inserted into infundibulum (left). Collection of 2-cell stage (E1.5) embryos after flushing (right).

- The E1.5 2-cell embryos are then transferred via glass blastocyst pipette and mouth-pipetor to a drop of KSOM submerged in Mineral Oil on a 3cm plate.
- The plate is incubated at 37°C/5% CO_2

- Still at a 2-cell stage, the embryos are then fused to induce tetraploidy.
  - The embryos are transferred into a series of media during the process – 1.) M2, 2.) Mannitol (0.3M), 3.) M2, 4.) KSOM.
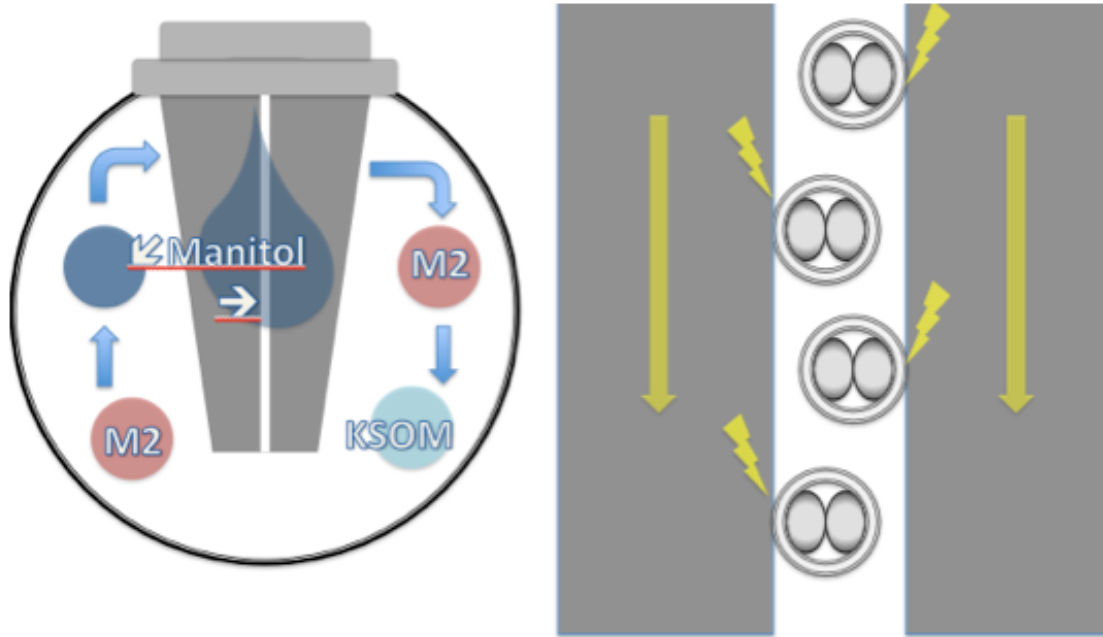
Figure 4: Plate with 100μL drops of M2, 0.3M Mannitol and KSOM. Electrofusion slide in the center (left). Enlarged view of slide corridor with embryos.

- While in the slide corridor, immersed in 0.3M Mannitol, the embryos are fused at two pulses – 30V/25μS – then transferred to the M2 drop for one minute and subsequently to the KSOM drop.

- The fused embryos are transferred in a final KSOM drop immersed in Mineral oil.

- The plate is incubated at 37°C/5% CO_2

- Within one hour, the embryos return to a 1-cell stage. Embryos that do not return to a 1-cell stage are discarded as diploid.

- Embryo/ESC Aggregation

  - 24-30 hours after tetraploid fusion the embryos are at a 4-cell to 8-cell stage (morulae).

  - The zona pellucida is removed as the embryos are quickly micro-pipetted into acidic tyrode solution:
  - One liter preparation of tyrode:
    - NaCl 8 g 137 mM
    - KCl 0.2 g 2.68 mM
    - 26.5% $CaCl_2$
    - $2H_2O$ 1 mL 1.8 mM
    - 4.42% NaH_2PO_4
    - H_2O* 1 mL 0.32 mM
    - Glucose 1 g 5.56 mM
    - $NaHCO_3$ 1 g 1.16 mM
    - Add distilled water up to 1000 mL pH=7.4

  - The naked blastomeres are then briefly transferred into M2 and again incubated in KSOM at 37°C/5% CO_2

  - A dimple is made into a 3cm tissue culture plate with a darning needle and covered with a KSOM drop.

  - The naked tetraploid blastomeres and KT4 ESCs are aggregated into the darning needle dimple and incubated in KSOM at 37°C/5% CO_2
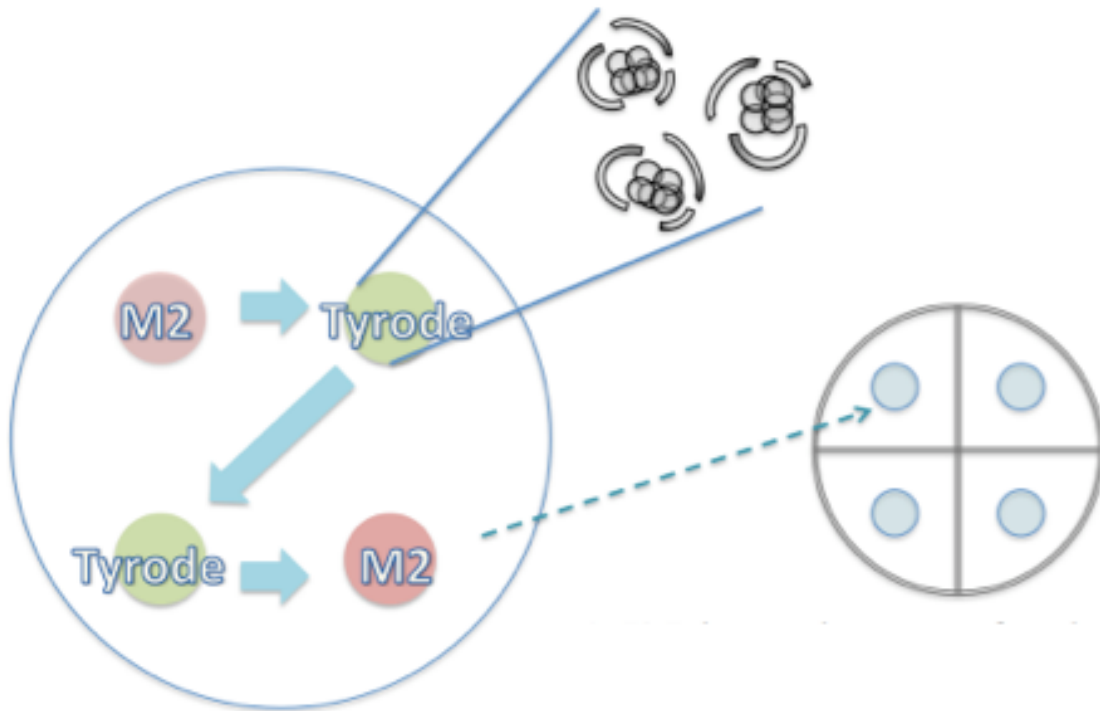
Figure 5: Removal of Zona Pellucida

Figure 5: Tetraploid embryos are transferred into acidic tyrode solution for removal of zona pellucida and subsequently transferred into M2 and finally incubated in KSOM.

Figure 6: Embryo/ESC Aggregation

Figure 5: Membrane-less blastomeres are aggregated with ESC in darning needle dimple (left). After 16 hours of aggregation, blastocyst stage (E3.5) forms in dimple (center).

- o Embryo transfer

  - At E2.5, the pseudo-pregnant female is prepped for surgery.

  - The mouse is anesthetized by intraperitoneal (IP) injection with freshly prepared Avertin.

  - The anesthetized mouse is placed prostrate on a surgical plate and a transverse superficial incision is made with fine dissection scissors to reveal the body wall and a second one to reveal the abdominal viscera.

  - The testicular fat pad layer is pulled out with blunt forceps until the ovary and uterus is revealed attached to the adipose layer.

  - A micro bulldog clamp is used to weigh the adipose layer and uterus outside the mouse.

  - The uterus is punctured with a 10g hypodermic needle.

  - The blastocysts are then transferred from KSOM to M2 and from M2 through the uterine lining puncture into the uterus via glass mouth-micro-pipettor.

  - The uterus and adipose tissue are reinserted into the abdominal cavity and the incision is stapled closed with a surgical staple gun.

- The anesthetized mouse is then allowed to recover in a cage while on a heated plated (to aid with any drop in body temperature).

  o Retrieval

- At E10.5 the pseudo-pregnant female is dissected via the same procedure and the embryos are dissected in M2 media.

KTA Embryonic Stem Cell Culture

  o ESC Medium

- ES cells are grown at 37°C/5% CO_2/ 95% humidity in dishes coated with a feeder layer of mitotically inactivated primary mouse embryonic fibroblast.

- DMEM (high glucose, Gibco 41966-052, store in fridge) minimal medium supplemented before use with 15% (v/v) FBS (Fetal Bovine Serum). 1X-BME, 1X-PenStrep, 1X-Glutamax.

  o MEF (Mitomycin Treated Embryonic Fibroblasts)

- This composes the feeder (bottom) layer of the plates on which ESCs are grown.

- The ESC and MEF medium differ in FBS content (MEF:10% FBS).

  o MEFs and ESCs are thawed for 30 seconds in a 37°C water bath.

- They are transferred into a tube with 10mL of their respective medium.

- The tube is centrifuged at 1000rpm for 5mins.

- The media is aspirated and the pellet is resuspended in medium and transferred to cell culture plate.

    - The MEFs are transferred on gelatinized cell culture plates.

    - The ESCs are transferred on the plates already containing MEFs.

- Aggregation

    - On aggregation day, the ESC plates are aspirated of medium and washed twice with 1X PBS, which is aspirated.

    - 700μL of 1X Trypsin-EDTA is added to the plate, which is incubated for 4-mins in 37°C/5% CO_2

    - The resulting dissociation of the cell body is inactivated of trypsin with 2-3mL of medium.

    - The cells are removed from dish by gently pipetting up and down.

    - They are then transferred to a 3cm plate and incubated at 37°C/5% CO_2 for 20mins (this allows the heavier MEFs to descend to the bottom of the plate).

    - After 20mins, the supernatant (containing ESCs) on the plate is pipetted into another 3cm and incubated at 37°C/5% CO_2 until aggregation.

Results

• Control/Diploid/Tetraploid retrieval

Three CD1 embryos were transferred into the uterus of a pseudopregnant female CD1 mouse. The first embryo was retrieved on E1.5 and incubated until the blastocyst stage at E3.5, on which it was transferred. No tetraploid fusion or ESC aggregation was performed on the embryo. This embryo served as a control – seeing as how when administered to X-Gal testing, it would not show lacZ+ tissue. The embryo retrieved at E10.5 also indicates no tetraploid fusion since fusion would promote an early resorption site and miscarriage of the litter.

The second embryo also did not undergo tetraploid fusion but was subjected to ESC aggregation with KT4 ESCs. Because KT4 ESCs are lacZ+ and were aggregated with the original embryo blastomeres, it was hypothesized that the transgenic mutant would exhibit two different lineages for its tissues. The tissues descended from KT4 ESCs proved lacZ+ when subjected to X-Gal testing, while the tissues descended from the original embryo did not prove lacZ+.

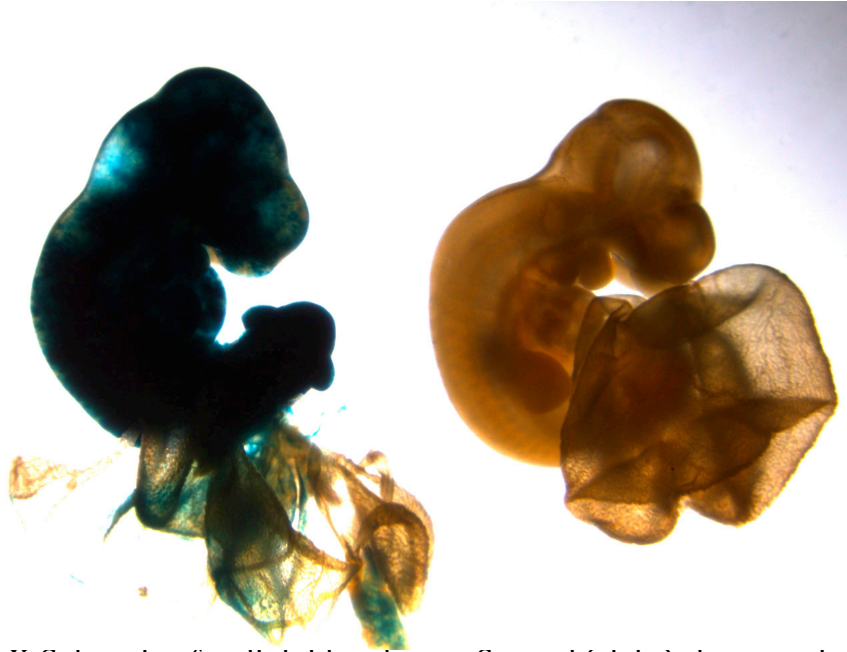**Figure 7**: X-Gal Testing for Diploid Embryos (E10.5)

Figure 7: X-Gal testing for diploid embryos. Control (right) shows no indigo color. Diploid KT4 Aggregate (left) shows both indigo and normal tissue – indicating two distinct lineages for cell makeup.

Figure 8: Close-up of Diploid Aggregate

Figure 8: Close-up of diploid KT4 Aggregate (E10.5) distinctly shows partial indigo dying of cells and indicates two different lineages of tissue – blue tissue indicates KTA lacZ+ descendant and normal tissue indicates embryonic lacZ- descendant.

The third embryo underwent tetraploid fusion and KT4 ESC aggregation. The tetraploidy was hypothesized to guarantee no original embryonic DNA contribution. The KT4 aggregation would then solely contributed KT4 genetic makeup in the tetraploid embryonic tissues. The retrieved embryo exhibited complete indigo staining, indicating that KT4 ESCs had been the sole contributors to the embryonic tissues.

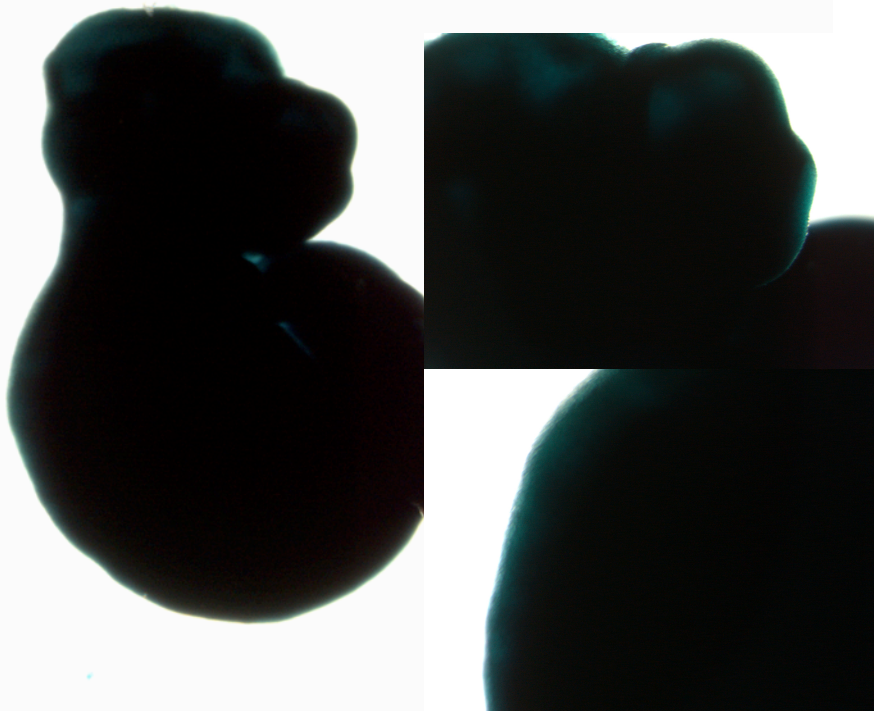**Figure 9:** Tetraploid KTA Aggregate (E10.5)

Figure X: The tetraploid embryo (E10.) shows complete indigo staining and lacZ+ tissue, indicating that the embryonic tissue in its entirety is of KT4 ESC origin and not of the original embryo.

Discussion

The tissue staining results of the embryos retrieved at E10.5 validated the secondary hypothesis. It was hypothesized that if embryonic stem cells were aggregated with tetraploid blastomeres, the resulting transgenic mutant would have embryonic tissues derived completely from ESC genetic origin. By applying a direct electric pulse, a single cell was created through the fusion of two blastomeres inside two-cell stage embryos. The replication of the genetic material followed by mitotic division results in a two-cell embryo containing double the diploid content of DNA. This tetraploid embryo developed further to the blastocyst stage. When aggregated with ESCs, the original tetraploid cells were not able to contribute to the embryo itself, but instead created the primitive endoderm derivatives and the trophectoderm. Because ESCs are pluripotent, they generated the three germ layers but could not contribute to extra-embryonic tissue. Upon aggregation with embryo blastomeres, the ESCs contributed only to the embryonic tissues.

A diploid embryo aggregated with ESCs had both viable original embryonic cells and viable embryonic stem cells to contribute to the transgenic mutant. The diploid mutant displayed this. X-Gal testing showed both lacZ positive tissues and lacZ negative tissues. This exhibits that the KT4 lacZ+ ESCs contributed to the embryonic tissue along with the original genetic makeup of the diploid zygote.

The tetraploid embryo aggregated with ESCs had no original contribution from the zygote. This was evident in the embryonic tissues showing complete lacZ+

staining from the X-Gal assay. This validates the secondary hypothesis that the KT4 ESCs alone provided the genetic contribution for the embryonic tissues.

The control embryo was not subjected to genetic manipulation and was not lacZ+ when treated with X-Gal. The viability of the embryo and its ability to be retrieved at E10.5 proved that it did not undergo tetraploid fusion.

The validation of the secondary hypothesis shows that tetraploid embryos (E1.5) aggregated with lacZ+ ESCs and transferred into pseudo-pregnant females (E3.5) after successful formation of blastocyst, will yield mutants with Embryonic Tissues and Extra Embryonic Tissues of separate genetic origins. This paves the way for the method to be used on homozygous T mutants in order to study the primary hypothesis. The proof of concept and of the protocol shows that the extra embryonic tissues of T mutants can develop to late embryonic stages and can be studied morphologically.

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