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Aggregation Chimeras: Combining ES Cells, Diploid, and Tetraploid Embryos

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Abstract

During the past 40 years, mouse chimeras have served as invaluable tools for studying not only genetics but also embryonic development, and the path from undifferentiated cell populations to fully committed functional cell types. This chapter gives a description of the early events of cell commitment and differentiation in the pre- and postimplantation-stage embryo. Next, a discussion follows highlighting the most commonly used as well as more recently developed applications of various cell types and origins used in the production of chimeras. Finally, detailed protocols and trouble-shooting suggestions will be presented for each of the steps involved.

Keywords

ES cells; mouse; embryo; chimera; tetraploid; aggregation

1. Introduction

The word “chimera” stems from ancient times, describing creatures composed of cells with more than one embryonic origin. In modern science, mythical creatures like the Egyptian sphinx have been replaced by sophisticated combinations of cells and embryos providing us with powerful tools to study mouse development (1). Before entering into a deep discussion on the various chimera combinations, we must take a detailed look at the early steps of differentiation in the developing embryo. As depicted in Fig. 15.1, the blastomeres of morula-stage mouse embryos are all totipotent, that is, each of them is capable of giving rise to all embryonic and extraembryonic tissues. However, at the blastocyst stage, the very first distinct cell types arise: the inner cell mass now consisting of primitive endoderm and primitive ectoderm, and an outer layer of trophectoderm. Each of these three compartments has its unique potential as well as limitation. Trophectoderm cells are committed to the development of the trophoblast cells in the placenta. Primitive endoderm cells are capable of forming the outer layers of the yolk sac, while primitive ectoderm cells will contribute to the embryo proper. It is important to note that this potential is strictly accompanied by a limitation. Each of the three cell types of the blastocyst is restricted to the contribution listed above (2).

Embryonic stem (ES) cells (3–5) are isolated from the primitive ectoderm. Although the concept of referring to “stem cells” in the context of ES cells is very well established, it is somewhat misleading. The basic definition of the stem cell property includes very quiescent and slowly self-renewing cells that reside for a long time in the organism. Primitive ectoderm cells however are a very transient population, existing no longer than a few hours in the blastocyst-stage embryo. It is more useful to think about ES cells as an in vitro artifact; cells derived from the primitive ectoderm and artificially prohibited to follow their natural differentiating program by specific culture conditions. Similar to ES cells, permanent cell lines have recently been established also from the other two compartments of the blastocyst-stage embryo: TS cells from the trophectoderm (6) and XEN cells from the

primitive endoderm (7). Interestingly, all of these faithfully recapitulate both the potential as well as the restriction of the cell types of their origin. TS and XEN cells differentiate exclusively into the trophoblast and extraembryonic endoderm lineages, respectively, when introduced into a blastocyst-stage embryo. ES cells combined with preimplantation-stage embryos, and allowed to develop in vivo, are excluded from the trophoblast of the placenta and primitive endoderm derivatives (8). However, the embryo proper and the resulting chimeric animal will possess a mixture of cells originating from both the original components. A complete separation of ES cells to the embryo proper and host embryo to the placenta can be achieved by the use of tetraploid embryos as hosts. A tetraploid embryo (8,9), which can be made by electrofusing the cells of a two-cell-stage diploid embryo (8,10,11), can efficiently form the trophoblast compartment of the placenta as well as the endoderm layer of the yolk sac. However, it is virtually incapable of contributing to the embryo proper (12). Therefore, in ES cell–tetraploid embryo chimeras, the embryo proper, the amnion, the yolk sac mesoderm, the allantois, and the embryonic mesoderm component of the placenta are completely ES cell-derived, whereas the yolk sac endoderm and the trophoblast cell lineages originate from the tetraploid embryo (8,11,13). ES cell–tetraploid embryo aggregates have an attractive feature in that they are a reliable and simple way of producing completely ES cell-derived embryos and animals from developmentally competent cell lines (4,5,8–11,13–16). This feature is promoting their application in an increasing number of studies. In addition, chimeras between diploid cells (both diploid embryo–diploid embryo, and ES cell–diploid embryo chimeras) are going through a renaissance in addressing specific biological questions (1,17). Figure 15.2 illustrates the various possible combinations of ES cells and diploid versus tetraploid embryos.

Embryo–embryo chimeras are made by aggregating two blastomere-stage embryos. Chimeras with an ES cell-derived component have traditionally been produced by injecting the cells into blastocyst-stage embryos using micromanipulators. However, we have learned that a more simple way of producing such chimeras can be performed by aggregating ES cells with blastomere-stage embryos (18). This alternative method also gives a high efficiency in chimera production as long as optimal culture conditions can be provided. Some combinations, such as embryo–embryo chimeras are only possible to make through the aggregation technique. Hence, in this chapter, we discuss the production and use of aggregation chimeras.

1.1. Uses of Chimeras

As outlined in Fig. 15.2, there are several possible combinations of embryos and cells to produce chimeras. A suitable aggregation combination should be chosen depending on the aim of the experiment.

1.1.1. Generation of Mutants; Germline Transmission and ES Cell-Derived Embryos/Animals—To obtain germline transmission of an ES cell genome, the cells can be aggregated with diploid embryos, resulting in viable and fertile chimeras (18). Due to frequent X chromosomal instability in female ES cells, male ES cells are used for this purpose almost exclusively. In most cases, male chimeras that have a high contribution of ES cell derivatives, scored by using coat color markers, are ideal for obtaining germline transmission of an ES cell genome (19). It should be noted that there are cases in which male chimeras with strong ES cell contribution may not be ideal. With some ES cell lines, high ES cell contribution positively correlates with sterility and decreased viability. The success of germline transmission mostly depends on the quality of ES cells. It is essential to perform genome alteration on ES cells that have a high developmental potential and germline compatibility. However, even in this case, a minor ratio of subclones will lose their original capabilities, and give rise to sterile or non-transmitting chimeras. The most

permissive genetic background for germline-competent ES cell lines is inbred 129. Therefore the 129-derived ES cell lines were dominating the ES cell-based mouse mutagenesis approaches for almost 20 years. As the culture conditions have been more defined, ES cells derived from C57BL/6 embryos can now be used with high efficiency for germline transmission after genetic modifications. Therefore, the international high-throughput gene knockout projects (20) (EUCOMM, NorCOMM, and KOMP) decided to derive mutant ES cells for all the known genes in the C57BL/6 background, which is considered the gold standard among the inbred strains of mice. The developmental potential of inbred lines are generally not high enough to efficiently yield viable completely ES-derived animals following aggregation with tetraploid embryos. However, some F1 hybrid ES cell lines have proven to possess superior developmental potential when applied to this technique (21). Hence, this technology has become highly feasible to analyze *in vivo* phenotypes directly from mutant ES cells (16).

Certain types of mutations, such as dominant mutations or mutations in haploinsufficient or X-chromosome-linked genes, may directly affect the developmental potential of ES cells and are only able to contribute to viable chimeras at a low level. However, even in this situation, it is not impossible to obtain germline transmission through chimeras exhibiting weak contribution of the ES cells (22). Haploinsufficiency could also create an apparently surprising phenomenon, when in the case of germline transmission of the ES cell genome no heterozygous F1 animals are observed. In this case, the heterozygotes might start developing but then die *in utero*. As a consequence, no mutant mouse line can be established. The phenotype of the haploinsufficiency can be analyzed through the chimera-fathered embryos. The only possible way to access the homozygous null phenotype in a severe haploinsufficiency case is the production of homozygous null ES cells followed by the production of completely ES cell-derived embryos (22). Genomic imprinting could create a similar situation. Imprinting is the phenomenon in which the activity of a gene shows a difference depending on the parental origin. Maternally imprinted genes require transmission through the paternal germline for activation. Therefore, one may never find viable progeny carrying the knockout allele of such a gene from a male chimera. In this case, again, female ES cells and chimeras would be a choice to obtain germline transmission. The opposite, the knockout allele of a paternally imprinted gene, does not have germline transmission problems through male chimeras (14,23,24).

Other potential difficulties could arise if the mutation introduced into the ES cell line itself is the cause of the sterility, resulting from, for example, a defect in spermatogenesis. A possible way to circumvent this problem can be provided by the occasional germline transmission through female chimeras (25). However, one must keep in mind that the Y chromosome usually is lost or becomes nonfunctional in fertile transmitting female chimeras.

1.1.2. Determination of Cell Autonomy of Particular Mutations—Chimeric analysis has proven to be a powerful method for studying the cell-autonomous requirements of genes of interest (26–29). Mutant cells can be ES cells as well as diploid and/or tetraploid embryos depending on the question that one would like to address. The suitable aggregation combination for chimeric analysis to address cell autonomy will be discussed in further detail in Section 1.2.

1.1.3. Separation of Embryonic and Extraembryonic Phenotypes—Chimeras also provide an excellent way to separate embryonic from any extraembryonic phenotypes of a genetic alteration (30). The complementary restricted developmental potential of ES cells and tetraploid embryos as mentioned in the introduction makes this feasible. An example highlighting this scenario is the relatively common case where mutant embryos die from

placental failure or other extraembryonic defects. By aggregating mutant diploid with wild-type tetraploid embryos, one can rescue the defect in the extraembryonic compartment, and gain access to mutant embryos for further phenotypic analysis. In this method – often referred to as the tetraploid complementation assay – the wild-type tetraploid embryos provide functionally normal placentae, while being excluded from the embryo proper (14,25,31,32). The reverse scenario is also possible: an embryonic phenotype can be rescued in order to allow the study of extraembryonic defects by aggregating mutant diploid embryos with wild-type ES cells (29). In this case, the ES cells will provide primitive ectoderm derivatives, but will never contribute to the primitive endoderm or trophoblast lineages. The aggregation combination for this use will be discussed further in Section 1.2.4.

1.1.4. Accessing Phenotypes Without Germline Transmission—ES cells carrying dominant genome alterations that may cause a phenotype in primitive ectoderm cell lineages can be aggregated with wild-type tetraploid embryos to study the phenotype directly, without going through the potentially problematic and time-consuming germline transmission breeding scheme (22). In the case of recessive mutations, the production of ES cell lines homozygous for the mutation is required (33,34). However, dominant-negative, gain-of-function mutations or stable transgenesis-based RNAi knockdowns can be analyzed directly from ES cell-derived embryos/animals after a single genetic alteration (16,35).

1.2. Aggregation Combinations

As discussed above, different aggregation combinations are required depending on the aim of each experiment. In this section, all possible aggregation combinations using ES cells, diploid and tetraploid embryos are listed and the expected contribution of mutant cells in resulting chimeras from each aggregation combination is discussed with examples of their practical use.

1.2.1. Wild-Type Diploid Embryo–Mutant Diploid Embryo—The contribution of mutant diploid cells is expected to be present in all cell lineages in the resulting chimeras made by this aggregation combination (*see* Fig. 15.3) unless cells from the mutant embryo have developmental restrictions. This makes it possible to assess the question of cell autonomy of mutations (13).

1.2.2. Wild-Type Diploid Embryo–Mutant Tetraploid Embryos—In this scenario, the contribution of mutant tetraploid cells is limited to the trophoblast and primitive endoderm derivatives (*see* Fig. 15.3). The resulting chimeras are expected to have chimeric extraembryonic tissues with no contribution of mutant cells in the primitive ectoderm derivatives, such as the embryo proper. This combination could be used in order to address cell autonomy of the mutation specifically in the extraembryonic lineages, when there are multiple cell-autonomous defects in both the extraembryonic as well as the embryonic lineages.

1.2.3. Mutant Diploid Embryo–Wild-Type Tetraploid Embryo—There is no restriction for the contribution of mutant diploid cells, whereas the contribution of wild-type tetraploid cells is limited to the extraembryonic tissues (*see* Fig. 15.3). This will result in chimeras that have chimeric extraembryonic tissues and exclusively mutant-embryo-derived primitive ectoderm derivatives. Such an approach will be the choice to study embryonic phenotypes while rescuing the extraembryonic defects (14, 29).

1.2.4. Wild-Type ES Cells–Mutant Diploid Embryo—In this case, the extraembryonic tissue, that is, the trophoblast and primitive endoderm lineages, will be derived solely from the mutant embryo, while chimerism will be found in all other lineages (*see* Fig. 15.3).

If mutant embryos of a gene of interest show phenotypes in both the embryonic and extraembryonic lineages, this aggregation would be the choice to address whether the placental defects are cell autonomous or secondary to the embryonic defects. This will be possible as the aggregation with wild-type ES cells can rescue the embryonic phenotype depending on the degree of chimerism without having any ES cell contribution in the extraembryonic tissues.

1.2.5. Mutant ES Cell–Wild-Type Diploid Embryos—In chimeras resulting from this scenario, the contribution of mutant ES cells is restricted to primitive ectoderm derivatives (*see* Fig. 15.3). This aggregation combination is suitable for all uses described in Section 1.1.1. For the study of cell autonomy during the development of primitive ectoderm derivatives, mutant ES cell lines carrying haploinsufficient or X-chromosome-linked or dominant mutations, or homozygous for recessive mutations, are required. Chimeric embryos from this aggregation can also be used to investigate phenotypes resulting from the mutation depending on the degree of ES cell contribution in the primitive ectoderm cell lineages.

1.2.6. Wild-Type ES Cells–Mutant Tetraploid Embryos—In this case, the contribution of mutant cells is solely restricted to the trophoblast and primitive endoderm lineages in the resulting chimeras (*see* Fig. 15.3). If the mutation affects both lineages, such chimeras can allow for the extraembryonic phenotypes to be separated from embryonic ones (29). This is the clearest way to assess this question compared to other combinations, as there is no concern about the degree of chimerism due to the complementary distribution of ES cells and tetraploid embryo derivatives.

1.2.7. Mutant ES Cells–Wild-Type Tetraploid Embryos—If mutant ES cells are available, the aggregation with wild-type tetraploid embryos provides a powerful and quick way to analyze embryonic phenotypes without germline transmission. Normally, wild-type ES cells are capable of developing to form the primitive ectoderm derivatives (*see* Fig. 15.3) with help of wild-type tetraploid embryos, which provide functional placenta and yolk sac. In the case of mutant ES cells, the phenotype is manifested in the completely ES cell-derived embryo proper. This aggregation also makes it possible to assess embryonic phenotypes with no influence from the extraembryonic lineages.

2. Materials

1. ES cell medium Dulbecco's Modified Eagle's Medium (DMEM) High Glucose (Life Technologies 61965-026) supplemented with the following:
 - a. 0.1 mM Nonessential Amino Acids (NEAA) (100× stock, Life Technologies 11140-035)
 - b. 1 mM sodium pyruvate (100× stock, Life Technologies 11360-039)
 - c. 100 μ M β -mercaptoethanol (Sigma M 7522)
 - d. 2 mM L-Glutamine (100× stock, Life Technologies 25030-024)
 - e. 15% Fetal Bovine Serum (FBS). ES-cell tested.
 - f. Penicillin and streptomycin, final concentration 50 μ g/mL each (100× stock, Life Technologies 15140-122)
 - g. 1000–2000 U/mL Leukemia inhibitory factor (LIF) (Chemicon/Millipore ESG 1107)
2. 0.1% gelatin (Sigma G-2500).

3. PBS without calcium and magnesium. The solution is autoclaved and stored at 4°C.
4. Trypsin/EDTA (1 × solution, Life Technologies 25200).
5. M2 embryo culture medium (Specialty Media MR-015P-5F).
6. KSOM embryo culture medium (Specialty Media MR-020P-5F).

All embryo culture media should be stored at 4°C until use. The solution is equilibrated at 37°C/5% CO₂ overnight, just prior to use. M2 and KSOM can also be prepared from individual reagents according to Table 15.1. If this is done, care should be taken that the water is of the highest quality grade and embryo-tested. All chemicals should also be embryo-tested individually. The use of plastic pipettes and containers is recommended, since glassware often contains residues that can be deleterious for embryos. Concentrated stock solutions can be stored at −80°C for a few months. Ready-to-use medium should be filter-sterilized and stored at +4°C for no longer than 2 weeks.

7. 0.3 M mannitol
Mannitol (Sigma M4125) prepared in water with 0.3% BSA (Sigma A3311). Filter-sterilized and stored in aliquots at −20°C.
8. Light mineral oil, embryo-tested (Sigma, M8410)
9. Acid Tyrode's solution (Sigma T-1788)
10. Tissue-culture-treated plasticware (for cells and embryos): We routinely use Nunc, Corning, and Falcon plasticware.
11. Humidified incubators: Separate incubators for ES cell and embryo in vitro culture. Maintained at 37°C and 5% CO₂.
12. Upright stereo dissecting microscopes with illumination from below. These are required for preimplantation embryo work, such as flushing embryos from oviducts/uteri, setting up the aggregations, and for the transfer into recipient females.
13. Fine surgical instruments: Required for preimplantation-stage embryo recovery and embryo transfer into recipient females.
14. Darning needles: Suitable for making depressions in plates for aggregations. These should have the correct beveling such that a smooth depression is produced. Specially made needles can be purchased from BLS Ltd., H-1165 Budapest, Zsélyi Aladár u. 31, Hungary, <http://www.bls-ltd.com/>.
15. Pipette for handling embryos: Mouth pipette fitted with a drawn-out Pasteur pipette.
16. Electrofusion apparatus for tetraploid embryo production (BLS #CF-150).

3. Methods

3.1. Preparing the Aggregation Plate

1. Place four rows of drops of KSOM (approx. 3 mm in diameter) into a 35-mm tissue culture dish using a 1-mL syringe fitted with a 26G needle, with the first and fourth row comprising three drops and the second and third having five (*see Fig. 15.4; Note 1*).
2. Overlay the drops with mineral oil, so that they are totally submerged.

3. Sterilize the aggregation needle with ethanol, and immediately use it to make approximately six depressions per micro-drop (*see* Fig. 15.4, **Note 2**).
4. Put the plate into the incubator overnight, or at least 3 h (*see* **Note 3**).

3.2. Obtaining the Embryos

1. Remove both oviducts from 1.5 days post coitum (dpc) females (for tetraploids) and 2.5 dpc (for diploids), and transfer to a drop of M2 medium in a Petri dish (*see* **Note 4**).
2. Flush the oviducts by inserting a flushing needle attached to a 1-mL syringe filled with M2 into the infundibulum (*see* **Notes 5, 6, 7**).
3. Collect embryos and wash them free of any debris in several drops of M2 using a mouth pipette.
4. Wash embryos in several drops of KSOM. Transfer the embryos to an organ culture dish with KSOM medium, and place in the incubator until all embryos and cells are prepared.

3.3. Electrofusion to Generate Tetraploid Embryos

1. Place the electrode in a plastic 10-cm Petri dish (*see* Fig. 15.5). Connect the cables from the electrode to the pulse generator, adjust all the parameters, and place on a dissecting stereomicroscope. We routinely use two pulses (“repeat” set to 2) of 100 V and 40 μ s duration with 250 μ m electrodes. These parameters, however, may vary between machines and genetic background of the embryos. Therefore, the optimum settings should be experimentally determined.
2. Put a large drop of 0.3 M mannitol over the electrodes.
3. Place two drops of M2 medium and one drop of 0.3 M mannitol on the surface of the Petri dish, outside the electrodes (*see* **Note 8**).
4. Introduce 50–100 embryos to one drop of M2. From there, take 20–25 embryos at a time into a drop of the mannitol. After they have settled, place them between the electrodes.
5. Carefully apply the orienting electric field. If some embryos do not properly align, correct their orientation manually (*see* **Note 9**).
6. When all embryos lie in the correct orientation, apply the pulse.
7. Immediately transfer the embryos back into a drop of M2. It is very important to keep the time the embryos spend in mannitol to a minimum. Until experience has been gained, it may be advisable to keep the number of embryos in each group lower.
8. Repeat Steps 5–8 until all the embryos have been subjected to the fusing pulses.
9. When all embryos have been treated, rinse them briefly in M2 and then in KSOM.
10. Transfer the embryos to an organ culture dish containing KSOM or microdrops under mineral oil, and place in the incubator (*see* **Note 10**).
11. After approximately 1 h, separate the fused embryos from those that are damaged or have remained diploid. This separation is a crucial point in the protocol. Only at this time-point is it possible to distinguish successfully fused (now tetraploid) embryos. Failing to carry out this step correctly could lead to the wrong interpretation of the chimera experiment. Embryos with a single round blastomere

and with no evidence of cell lysing (debris under the zona pellucida) are successfully fused and should be separated from all others (*see* Fig. 15.6a). Embryos with a single but small blastomere and debris present under the zona are not tetraploid; the second blastomere has lysed during the fusion process (*see* Fig. 15.6b). Some embryos will show an elongated appearance (*see* Fig. 15.6c). They are under the process of fusion and should be allowed to complete the process in the incubator for an additional 10min. Embryos that remain having two blastomeres are also still diploid and can be subjected to a second round of fusion (*see* Fig. 15.6d). If doing so, the same care should be taken to sort them after an additional hour in culture.

12. Incubate the embryos overnight.
13. The tetraploid embryos should now have developed to form four blastomeres, and are ready for aggregation (*see* **Note 11**). Embryos that only have two blastomeres can be left for a few more hours in the incubator as they may still divide.

3.4. Removing the Zona Pellucida from the Embryos

1. Place a few drops of M2, KSOM, and acid Tyrode's in a non-tissue culture-treated plastic Petri dish (if tissue culture dishes are used, it is important to use the lid so that the “naked” embryos will not stick to the surface).
2. Transfer a group of embryos into the first drop of acid, rinse briefly, and then transfer them to the second drop.
3. Continually observe the embryos, and note when their zona has dissolved (*see* **Note 12**). At that point, immediately transfer them to a drop of M2, and subsequently wash them in several drops of M2 (*see* **Note 13**).
4. Wash the embryos in KSOM. Embryos are now ready for transfer to the aggregation plate.

3.5. Preparing the ES Cells

Extensive protocols for the maintenance and culture of ES cells are beyond the scope of this chapter and are described elsewhere (36,37).

1. Day 1: Thaw the cells 3 days before the aggregation onto a feeder cell containing plate (*see* **Note 14**).
2. Day 2: Change the medium.
3. Day 3: Passage the cells onto gelatinized plates but instead of the usual 1:5 ratio, make 3–5 dishes with an increasingly higher dilution of 1:50–1:500 (*see* **Note 15**).
4. Day 4: Choose one of the dishes for preparation. It should be the one with a density showing small colonies with an average size of approximately 20 cells. Trypsinize the cells briefly, just until the colonies begin to detach from the plate. Stop the action of the trypsin by adding ES cell medium to the plate. Select clumps of 10–15 loosely attached cells for the aggregation, and transfer them into KSOM microdrops contained on the aggregation plate (*see* **Note 16**).

3.6. Setting Up the Aggregation

3.6.1. Diploid Embryo–Diploid Embryo Aggregation

1. Transfer the zona-free embryos to the aggregation plate and into a microdrop without depressions.

2. From there, place individual embryos with the first genotype into the individual depressions of the central two rows of microdrops (*see* **Notes 17, 18**).
3. Repeat Steps 1 and 2 with the embryos of the second genotype.
4. After all the embryos have been assembled into aggregates (*see* Fig. 15.7), return the plate into the incubator and incubate overnight (*see* **Note 19**).
5. The next day, most of the aggregates should have formed a single embryo that has progressed to the blastocyst stage and therefore ready for transfer into recipient females.

3.6.2. Diploid Embryo–ES Cells Aggregation

1. Select several clumps of approx. 10–15 ES cells (*see* Fig. 15.7B) and transfer them to the microdrops without depressions.
2. From there, place individual clumps into the microdrops harboring the depressions.
3. Set up the aggregation either by first placing the embryo in the well and then overlaying the ES cell clump or by putting in the cells first and then the embryo.
4. Follow Steps 4 and 5, as in Section 3.6.1.

3.6.3. Tetraploid Embryo–Diploid Embryo Aggregation

1. Place embryos into the microdrops of the aggregation plate that do not contain depressions. From here, move two tetraploid embryos into each depression.
2. Place the diploid embryos into the microdrops of the aggregation plate that do not contain depressions (nor any tetraploid embryos).
3. Carefully place a single diploid embryo next to the tetraploid embryos that are already positioned in the depression.
4. Follow Steps 4 and 5 as in Section 3.6.1.

3.6.4. Tetraploid Embryo–ES Cell Aggregation

1. Place the tetraploid embryos in the drop without depressions in the aggregation plate.
2. From here, move individual tetraploid embryos and place each one in a depression.
3. Following this, select several clumps of approx. 10–15 ES cells and transfer them to a microdrop without depressions (nor any embryos).
4. Take a loosely attached clump of cells, and place it carefully next to the embryo already positioned within a depression.
5. Introduce the second tetraploid embryo into the depression so that it lies at the side of the cells opposing the first embryo. This is best done by gently rolling the second embryo over the rim of the depression.
6. Repeat Steps 2–5 until all the “sandwiches” have been set up.
7. Follow Steps 4 and 5, as in Section 3.6.1.

3.7. Transfer of Blastocyst-Stage Aggregates

On the day after aggregation, the embryos should have reached the blastocyst stage (corresponding to 3.5 dpc of development) and are ready for transfer to pseudopregnant recipient females.

1. Optimally, 8–10 embryos are transferred into each uterine horn of a 2.5 dpc pregnant female. We routinely use CD1 or ICR outbred mice as recipients.
2. In the event of a shortage of recipients, the number can be increased to 14 per uterine horn (a total of 28). Another alternative is to transfer the embryos into the oviduct of 0.5 dpc pregnant females.
3. The embryo transfer procedure is detailed elsewhere (38).

4. Notes

1. A few microdrops are usually left without depressions (upper and lower rows) so that they can be used to introduce and briefly rinse ES cells and/or embryos just before assembly of the aggregation.
2. The depression should have a clear smooth wall and be deep enough to hold the aggregates without a risk of disassembly of the aggregate or spilling over as the plate is placed in the incubator. They should however also not be made too deep, as this will make the recovery of the chimeric blastocysts difficult without damaging them.
3. The plates should be prepared at least a few hours in advance (better the day before) and kept in the incubator until use. This allows enough time for the media to equilibrate to the correct temperature and pH level.
4. Oviducts are removed by making incisions in the upper part of the uterus and right below the ovary.
5. Flushing is performed from the infundibulum, resulting in embryos being expelled from the short length of uterus.
6. Superovulation of female mice results in a much higher yield of embryos than can be expected from naturally ovulating animals. However, the hormone treatment has to be carefully adjusted to the genetic background of the mice as well as the light cycle in the vivarium. Detailed protocols for superovulation can be found elsewhere (38).
7. Flushing needles are made by cutting the tip off a 30 G1/2 needle, and then beveling the end with a sharpening stone.
8. Electrofusion can be performed in an electrolyte or nonelectrolyte solution. We favor – and have provided the protocol for – the nonelectrolyte method as it allows for multiple embryos to be fused at the same time, as well as the possibility to automatically orient the embryos with the high-frequency AC field.
9. The adjustable AC field is applied in order to allow for the correct orientation of the embryos. Only the minimal necessary voltage should be used. If the field is too high it can cause lysis of the cells.
10. It usually takes approximately half an hour to an hour for the blastomere fusion to occur. It is important to select only the fused (and therefore tetraploid embryos) approximately an hour after the pulsing. We recommend that embryos that have fused be transferred to a new organ culture dish or microdrop, and then cultured overnight.
11. After overnight culture in KSOM medium, tetraploid embryos will have developed to the four-cell stage, which is equivalent to the eight-cell stage of diploid embryos. Tetraploids should be aggregated at the four-cell stage, as it is at this time that they will initiate compaction.

12. The zona pellucida is a glycoprotein coat that encapsulates the embryo. Late blastocyst-stage embryos will usually hatch out of their zona prior to implantation in the uterine wall. The zona is refractory to aggregation, as an intimate contact is needed between the cells and/or embryos.
13. Even though it does not matter whether embryos are in M2 or KSOM prior to their Tyrode's treatment, M2 is used right after as it has a superior buffering capacity.
14. Some ES cell clones may exhibit a lower than usual grow rate. These can be thawed one or two additional days earlier and stated in the protocol.
15. A highly diluted plating of single cells is required in order to produce optimally sized clumps (10–15 cells). Clumps in which cells are loosely connected are favored for setting up the aggregate. If clumps of the right size cannot be obtained, larger clumps can be reduced in size by careful pipetting. If the clumps are too small, two or three clumps can be used instead of one.
16. Care should be taken so as not to disaggregate the cell clumps by pipetting too vigorously or by using extensive trypsin treatment.
17. Aggregates should be set up in such a way that there is maximal contact between the cells and embryos.
18. Aggregations involving tetraploid embryos are set up as a “sandwich,” where two tetraploid embryos are used to flank either the ES cells or the diploid embryo. However, if there is a shortage of tetraploid embryos, a one-to-one ratio can also be used.
19. When setting up the aggregates, especially if they are tetraploid “sandwich” types, take care not to jolt the plate and dissociate the intimately contacted embryos and/or cells.

4.1. Genotyping Segregating Embryo Components in Chimeras

Genotyping of chimeras can sometimes be a problem because of the mix of mutant and wild-type cell populations. One can avoid dealing with this issue by isolating the tissue that is expected to be solely mutant in origin, for example, the yolk sac endoderm in the case of “mutant diploid embryo–ES cells” or “mutant tetraploid embryos–ES cells,” and embryo proper in the case of “mutant diploid embryo–tetraploid embryos.” In other cases, that is, “mutant diploid embryo–wild-type diploid embryo” and “mutant tetraploid embryos–wild-type diploid embryo,” the contribution of mutant cells are mixed with wild-type cells and that makes genotyping very difficult. Here, practical approaches to solving this problem will be discussed.

4.1.1. Genotyping of Potentially Chimeric Tissues—In the case of recessive mutations, homozygotes for the mutation have to be obtained from a cross between heterozygous females and males. Genotyping chimeras can be performed by preparing genomic DNA followed by genomic Southern or PCR. Problem occurs if mutant cells are mixed with wild-type cells. As a result, phenotyping will be indistinguishable between chimeras containing heterozygous cells and mutant cells (*see* Fig. 15.8).

One solution for this problem is taking advantage of using two alleles for either wild-type alleles (*see* Fig. 15.8) or mutant alleles (*see* Fig. 15.8). In this way, one can distinguish chimeras made by mutant embryos from heterozygous litter mates.

4.1.2. Isolation of Potentially Mutant Cells by the Use of Markers—It is possible to isolate potentially mutant cells (populations derived from het × het crosses) from wild-

type cells if one can design the cross to introduce a gene as an ubiquitous reporter. For this purpose, markers such as lacZ or a fluorescent protein (GFP, YFP, CFP or RFP) are suitable. In the case of lacZ, genotyping is performed with lacZ-stained tissues and/or by PCR. The use of fluorescent proteins (FPs) is more straightforward because one can detect them without the use of a chromogenic substrate. FP-positive cells can be collected manually using a drawn-out glass pipette (A. N., unpublished observation) or by the use of fluorescence-activated cell sorting (FACS) (39,40). In general, the use of the ubiquitously expressed reporters makes genotyping chimeras more feasible and reliable.

5. Conclusion

Chimeric studies have been a feature of modern mouse embryology since its inception more than 50 years ago. During the first half of this period, embryo–embryo chimeras answered many questions about basic events during embryogenesis, such as cell movement and clonality. The 1980s brought a new component to chimera analyses: mouse ES cells. A vast knowledge about gene function has since been generated by the introduction of mutations in ES cells and subsequent passage of the mutant phenotype through the germline of ES cell chimeras. In the 1990s, the classic use of chimeras as tools employed for answering biological questions re-entered the scientific scene. As the means for generating homozygous mutant ES cells became available, recessive phenotypes could be represented in cell culture and the resulting chimeras.

During the past decade, we have learned that ES cells are developmentally restricted, so that they are not able to differentiate into trophoblast or primitive endoderm lineages, but have full potential in the primitive ectoderm lineage, for example, in the embryo proper. A further component came into play after it was demonstrated that tetraploid embryos could provide a normal extraembryonic environment to an ES cell-derived embryo, such that tetraploid cells are selected against in the embryo proper if diploid cells (ES or embryo) are present.

Thus, today the three chimera components, diploid embryos, tetraploid embryos, and ES cells, whether mutant or wild type, open up a variety of possible combinations for creating specific chimeras, each tailor-made to address any relevant biological question. As a consequence, over the past few years, there has been an upsurge in the number of such studies reported in the literature. We have now entered the twenty-first century with a fully updated version of a classical tool that can be applied in many laboratories using genetic technologies in order to understand normal development, adult life and disease. This approach is going to have a particular importance in the massive effort of functionally annotating the mammalian genome, particularly when all the mouse genes will be mutated by the ongoing international efforts.

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Fig. 15.1. Schematic presentation of the various embryo proper and extraembryonic lineages, as well as their relation to each other.

Fig. 15.2.

The three components that can be combined to produce chimeras: diploid embryos, tetraploid embryos, and ES cells. (1) Diploid embryo-diploid embryo, (2) diploid embryo-ES cells, (3) diploid embryo-tetraploid embryos, and (4) tetraploid embryos-ES cells.

Fig. 15.3. Tissue contributions and lineage restrictions associated with the three components of chimeras.

Fig. 15.4.

Preparation of an aggregation plate involves making microdrops of media, overlaying them with mineral oil, followed by forging depressions for the placement of the aggregates. The plate once set up is placed in a temperature-controlled humidified incubator with 5% CO₂ content.

Fig. 15.5.

Dish setup for electrofusion. The electrodes are placed in the middle. Drops of M2 media are placed on the dish for washing embryos. Mannitol solution is placed on the electrodes for the fusion process.

Fig. 15.6.

Embryos following the fusion procedure. (A) Correctly fused, tetraploid embryo, (B) one blastomere has lysed, the embryo remains diploid, (C) embryo that has not yet completed the fusion, and (D) non-successful fusion, the embryo remains diploid.

Fig. 15.7.
Different aggregate combinations.

Fig. 15.8.

If mutant cells are intermingled with wild-type cells in the tissues of a chimera (**A**) the result obtained from genotyping will be indistinguishable between chimeras containing heterozygous cells and mutant cells. To distinguish chimeras made by mutant embryos from heterozygous littermates, one can employ two different tags for either wild-type alleles (**B**) or mutant alleles (**C**).

Table 15.1
Composition of embryo culture media

	KSOM	M2
Component	Concentration (g/L)	Concentration (g/L)
NaCl	5.55	5.53
KCl	0.186	0.356
KH ₂ PO ₄	0.0476	0.162
MgSO ₄	0.0493	0.293
Nalactate	1.12 or 1.87 g of 60% syrup	4.349 g of 60% syrup
D(+)-glucose	0.036	1.00
Na ₂ pyruvate	0.022	0.036
NaHCO ₃	2.10	0.349
CaCl ₂ (dihydrous)	0.251	0.252
L-glutamine (Gibco, 25030)	0.146	–
EDTA (tetrasodium salt)	0.0038	–
BSA (Sigma, A3311)	1.0	4.00
Penicillin-G	0.060	0.060
Streptomycinsulfate	0.050	0.050
Phenol Red	–	0.01
HEPES Buffer	–	4.969