

Aggregation Chimeras

Combining ES Cells, Diploid and Tetraploid Embryos

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1. Introduction

In recent years, chimeras have been providing a powerful way to study mouse development (1) in combination with invention and improvement of other techniques and materials, including embryonic stem (ES) cells (2) and tetraploid embryos (3,4). ES cells are pluripotent cell lines derived from late blastocyst-stage embryos, which are capable of differentiating into all derivatives of the primitive ectoderm (*see Fig. 1*) when aggregated with or injected into diploid embryos (5). In contrast, tetraploid embryos, which can be made by electrofusing two cell-stage diploid embryos (3,6,7), have been found to contribute preferentially to most of the extraembryonic cell lineages, i.e., the trophoblast (trophectoderm derivatives) and primitive endoderm derivatives (*see Fig. 1*) when aggregated with diploid embryos (3–8). Interestingly, ES cells show a deficiency in extraembryonic lineages, therefore these cells and tetraploid embryo derived cells have a complementary distribution in chimeras made between them. In such chimeras, the embryo proper, the amnion, the yolk sac mesoderm, the allantois and the chorionic mesoderm-derived part of the placenta are completely ES cell-derived, whereas the yolk sac endoderm and the trophoblast cell lineages are tetraploid embryo derived (3,7,9). It is certain that the 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 from developmentally competent cell lines (2–10). This feature is promoting their application in an increasing number of studies. In addition, chimeras between diploid cells (both embryo⇌embryo, and ES cell⇌diploid embryo chimeras) are going through a renaissance in addressing specific biological questions.

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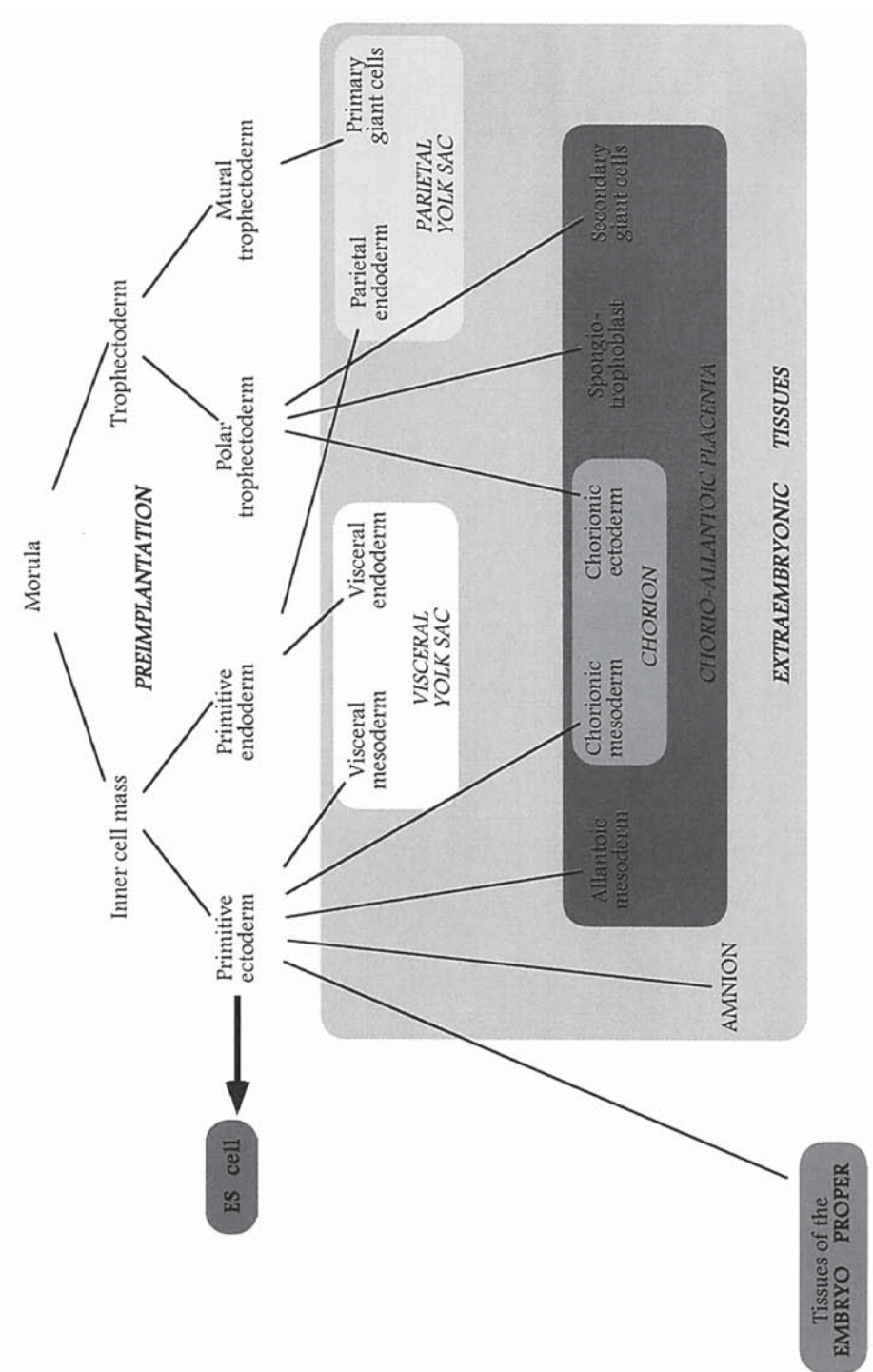


Fig. 1. Schematic representation of the various embryo proper and extraembryonic lineages and their relation to each other.

The embryo↔embryo chimeras are made by the aggregation of two blastomere-stage embryos. ES cell chimeras have traditionally been produced by injecting the cells into blastocyst-stage embryos using micromanipulators. However, recently we have learned that a more simple way of producing such chimeras can be performed by aggregating ES cells with an eight-cell stage embryo (*11*). This alternative method is simple and also gives a high efficiency in chimera production under optimal culture conditions. In this chapter, we discuss general use of aggregation chimeras.

1.1. Uses of Aggregation Chimeras

There are four combinations by which postimplantation chimeric embryos can be produced in vivo (*see Fig. 2*), these being “diploid embryo and diploid embryo” (*see Fig. 2A*), “diploid embryo and ES cells” (*see Fig. 2B*) and “diploid embryo and tetraploid embryos” (*see Fig. 2C*) and “tetraploid embryos and ES cells” (*see Fig. 2D*). A suitable aggregation combination should be chosen depending on the aim of the experiment. The general use of such aggregations will be described as follows.

1.1.1. Germline Transmission of ES Cells by Aggregation

To obtain germline transmission of an ES cell genome, ES cells should be aggregated with diploid embryos in order to create viable and fertile chimeras (*11*). Due to frequent X chromosomal instability in female ES cell lines almost exclusively male ES cells are used for this purpose. 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 (*12*). 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 negatively correlates with viability and sterility. The success of germline transmission also depends on the quality of ES cells. In almost all situations it is essential to perform any 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 resulting in sterile or nontransmitter chimeras.

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 (*13*).

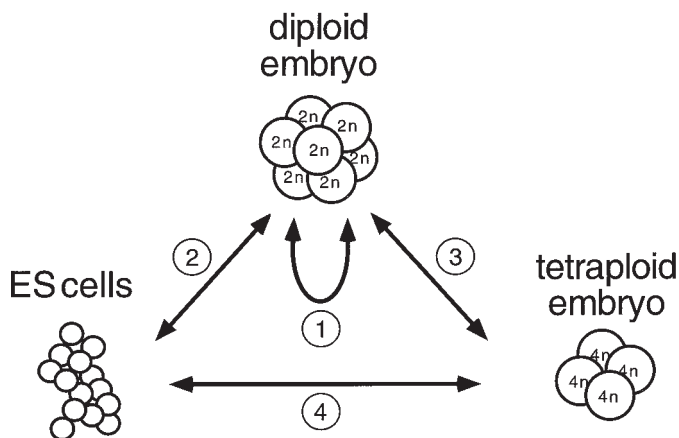


Fig. 2. The three components that can be combined to produce a chimera are diploid embryos, tetraploid embryos, are illustrated. 1. Diploid embryo \leftrightarrow diploid embryo, 2. diploid embryo \leftrightarrow ES cells, 3. diploid embryo \leftrightarrow tetraploid embryos, and 4. tetraploid embryos \leftrightarrow ES cells.

Other possible difficulties could arise if the mutation introduced into the ES cell line itself is the cause of the sterility resulted from for example a defect in spermiogenesis. A possible way to circumvent this problem can be provided by the occasional germline transmission through female chimeras (14). However, it is not clear what the status of the Y chromosome of these ES cells was in these cases. There is a possibility that the Y chromosome has to be lost or become nonfunctional in order to allow development through female gametogenesis.

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 consequences 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 ES cell \leftrightarrow tetraploid embryos (13).

Genomic imprinting could create a similar situation where 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 a viable progeny carrying the knockout allele of such a gene from a male chimera. In this case, again, female chimeras would be a choice to obtain germline transmission. The opposite, the knockout allele of a paternally imprinted gene, does not have germline transmission problem through male chimeras (15–17).

1.1.2. Determination of Cell Autonomy of Particular Mutations

Chimeric analysis has proven to be a powerful method for studying cell autonomous requirement of genes of interest (**18–20**). 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 **Subheading 1.2**.

1.1.3. Separation of Embryonic and Extraembryonic Phenotypes

Chimeras also provide an excellent way to separate the embryonic phenotype from any extraembryonic phenotype of a gene of interest (**21**). The complementary restricted developmental potential of ES cells and tetraploid embryos as mentioned in the introduction makes this feasible. For instance, if mutant diploid embryos die from placental failure or extraembryonic defect, one can rescue this defect by aggregating the mutant diploid embryo with wild-type tetraploid embryos. The latter provide functionally normal placentae to study the embryo proper phenotype of the mutation (**14,15,22,23**). It is also possible to rescue the embryonic phenotype to study the extraembryonic defect of the mutation by aggregating the mutant diploid embryo with wild-type ES cells that provide primitive ectoderm derivatives, but will never contribute to primitive endoderm or trophoblast cell lineages. The aggregation combination for this use will be discussed further in **Subheading 1.2**.

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 (**13**). In the case of recessive mutations, the production of ES cell lines homozygous for the mutation is required. The means to get homozygous ES cell lines for this use is reviewed in ref. (**24**).

1.2. Aggregation Combinations

Different aggregation combinations are required depending on the aim of experiments as discussed in the **Subheading 1.1**. In this section, all possible aggregation combinations using ES cells and diploid and tetraploid embryos (see **Fig. 3**) are listed and the expected contribution of mutant cells in resulting chimeras from each aggregation combination is discussed with examples of their practical use.

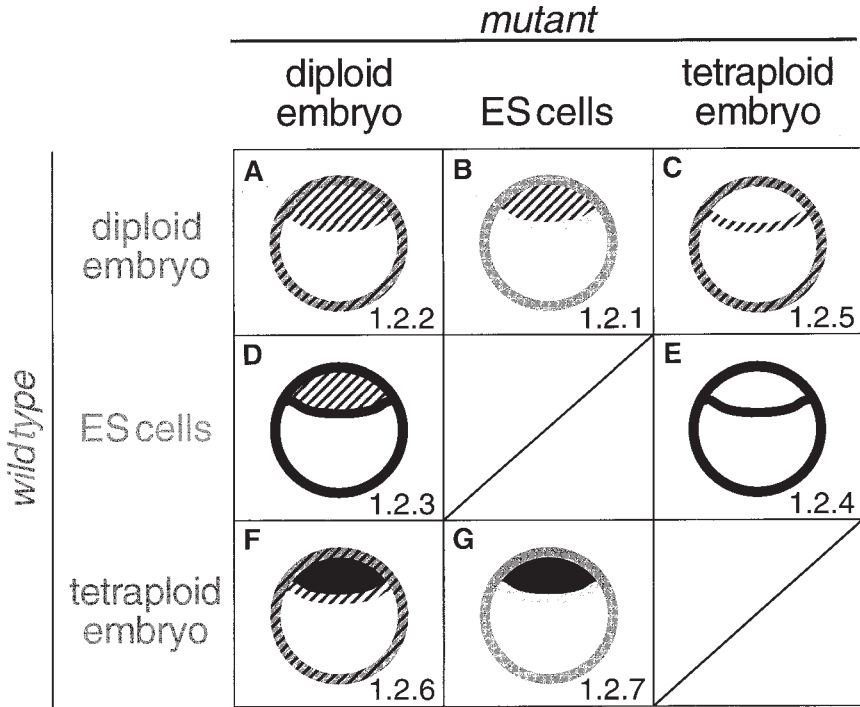


Fig. 3. Tissue contributions and lineage restrictions associated with the three components of chimeras.

1.2.1. Wild-Type Diploid Embryo⇌Mutant ES Cells

The contribution of mutant ES cells is restricted to primitive ectoderm derivatives in resulted chimeras (see Fig. 3B). This aggregation combination is suitable for all uses described in Subheading 1.1. To study 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 may also give phenotypes resulting from the mutation depending on the degree of ES cell contribution in the primitive ectoderm cell lineages.

1.2.2. Wild-Type Diploid Embryo⇌Mutant Diploid Embryo

The contribution of mutant diploid cells is expected to be in all cell lineages in chimeras made by this aggregation combination (see Fig. 3A) unless cells from the mutant embryo have developmental restrictions. This makes it possible to assess the question of cell autonomy of mutations of interest possibly in all cell lineages.

1.2.3. Wild-Type ES Cells \Leftrightarrow Mutant Diploid Embryo

The contribution of cells derived from mutant diploid embryo is expected to be in all cell lineages (*see Fig. 3D*) unless the mutation affects their developmental potential. The point of this aggregation combination is to make solely mutant embryo derived extraembryonic tissue, i.e. the trophoblast and primitive endoderm lineages, in combination with chimeric primitive ectoderm derivatives.

If mutant embryos of a gene of interest show phenotypes in both the embryonic and extraembryonic lineages, this aggregation is the choice to address whether the placental defects are cell autonomous or secondary to the embryonic defects, 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.

1.2.4. Wild-Type ES Cells \Leftrightarrow Mutant Tetraploid Embryos

The contribution of mutant cells is solely restricted to the trophoblast and primitive endoderm lineages in resulting chimeras (*see Fig. 3E*) so that the extraembryonic phenotypes can be separated from embryonic ones if the mutation affects both lineages. This is a clearer way to assess this question compared to other combinations, as there is no concern about the degree of chimerism because of the complementary distribution of ES cells and tetraploid embryo derivatives.

1.2.5. Wild-Type Diploid Embryo \Leftrightarrow Mutant Tetraploid Embryos

The contribution of mutant tetraploid cells is limited to the trophoblast and primitive endoderm derivatives (*see Fig. 3C*). The chimeras made by this aggregation are expected to have chimeric extraembryonic tissues with no contribution of mutant cells in the primitive ectoderm derivatives. 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 extraembryonic and embryonic lineages.

1.2.6. Wild-Type Tetraploid Embryos \Leftrightarrow Mutant Diploid 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. 3F*). This will result in chimeras that have chimeric extraembryonic tissues and exclusively mutant-embryo-derived primitive ectoderm derivatives. This aggregation will be the choice to study embryonic phenotype by rescuing the extraembryonic defects. The same question can be addressed by mutant ES cells \Leftrightarrow wild-type tetraploid embryos aggregation (*see Fig. 3G*), if such cells are available.

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 the quick way to analyze embryonic phenotype without germline transmission. Normally, wild-type ES cells are capable of developing to form the primitive ectoderm derivatives (*see Fig. 3G*) 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 make it possible to assess pure embryonic phenotype with no influence from the extraembryonic lineages.

2. Materials

1. DMEM + (ES cell medium): Dulbecco's modified Eagle's (DMEM, Flow Labs, powder, cat. no. 430-1600) supplemented with the following:
 - a. 0.1 mM Nonessential amino acids (100X stock, Gibco, cat. no. 320-1140AG), 1 mM sodium pyruvate (100X stock, Gibco, cat. no. 320-1360).
 - b. 100 mM β -Mercaptoethanol (100X stock stored as aliquots at -20°C , Sigma, Cat. No. 600564AG).
 - c. 2 mM L-Glutamine (100X stock, stored as aliquots at -20°C , Gibco, cat. no. 320-5030AG).
 - d. 15% Fetal calf serum (FCS). We regularly test several batches from different FCS manufacturers for ES cell culture.
 - e. Penicillin and streptomycin (final concentration 50 $\mu\text{g}/\text{mL}$ each, Gibco, cat. no. 600-564AG).
 - f. Leukemia inhibitory factor (different sources, e.g., Gibco) 1000 U/mL.
2. 0.1% Gelatin: 1g (w/v) gelatin (Sigma or BDH) made up in 1 L water, autoclaved and stored at 4°C .
3. PBS (phosphate-buffered saline): Prepared without calcium and magnesium and used for all tissue culture work. one liter is made from 10 g NaCl, 0.25 g KCl, 1.5 g Na_2HPO_4 , 0.25 g KH_2PO_4 , pH 7.2. The solution is autoclaved and stored at 4°C .
4. Trypsin (0.1%): Dissolve 0.5 g trypsin powder (Gibco, cat. no. 0153-61-1) in 100 mL saline/ethylene diamine tetraacetic acid (EDTA) solution. Adjust the pH to 7.6, sterilize through a 0.22- μm filter and store at -20°C . This constitutes a 0.5% stock, which needs to be diluted to 0.1% on defrosting.
5. M2/M16 media for in vitro embryo culture: M2 and M16 media are prepared according to standard protocols (12).
6. KSOM: Our protocol for preparation of KSOM is modified from (25,26). It consists of the preparation of six stock solutions that are individually aliquoted and stored at -70°C . The six stocks are detailed in **Table 1**. Individual aliquots of stock solutions are thawed, then combined in the proportions detailed as follows. Bovine serum albumin (BSA) is then added, and the volume is made up using sterile water. The resulting solution is filter sterilized (0.2 μm filter), and stored at 4°C until use. The solution is equilibrated at $37^{\circ}\text{C}/5\% \text{CO}_2$ overnight, just prior to use.

Table 1
Stock Solutions for the Preparation of KSOM

Stock A' (100 mL) (10 mL aliquots)	10X	NaCl	950 mM	5.55 g
		KCl	25 mM	0.186 g
		KH ₂ PO ₄	3.5 mM	0.0476 g
		MgSO ₄ ·7H ₂ O	2.0 mM	0.0493 g
		Na-lactate	100 mM	1.87 g of 60% syrup
		D (+) glucose	2.0 mM	0.036 g
Stock B (100 mL) (10 mL aliquots)	10X	Penicillin-G	600 µg/mL	0.06g
		Streptomycin:sulfate	500 µg/mL	0.05 g
		NaHCO ₃	250 mM	2.10 g
		Phenol Red		0.001 g or 1 mL of 1% solution
Stock C' (1 mL aliquots)	100X	Na-pyruvate	20 mM	0.022 g
Stock D (10 mL) (1 mL aliquots)	100X	CaCl ₂ ·2H ₂ O	17.1 mM	0.25 g
Stock F (10 mL) (10 µL aliquots) (1 mL aliquots)	10,000X	Na ₂ EDTA·2H ₂ O	100 mM	0.372 g
	Or 100X	Na ₂ EDTA·2H ₂ O		0.372g
Stock G (0.5 mL aliquots)	200X	Glutamine	200 mM	(comes in solution)

Stock Solution	Volume
10X Stock A'	10 mL
10X Stock B	10 mL
100X Stock C'	1 mL
100X Stock D	1 mL
100X Stock F	1 mL
200X Stock G	0.5 mL
Solid BSA	0.10 g
ddH ₂ O	to make volume up to 100 mL

7. 0.3 M mannitol: Mannitol (Sigma, cat. no. M4125) prepared in water with 0.3% BSA (Sigma A4378). Filter sterilized and store in aliquots at -20°C.
8. Light mineral oil (embryo tested): Sigma, cat. no. M8410).
9. Acid Tyrode's solution: Sigma, cat. no. T1788.
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. Stereo dissecting microscopes such as Leica M3b, MZ8, or MZ12: 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. 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.
15. Pipet for handling embryos: For example a mouth pipet fitted with a drawn out Pasteur pipet.
16. Electrofusion apparatus for tetraploid embryo production: This specialized piece of equipment such as the CF-150 model available from BLS.

3. Methods

3.1. Preparing the Aggregation Plate

1. Place four rows of drops of M16/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. 4).
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 microdrop (see Fig. 4).
4. Put the plate into the incubator.

3.2. Obtaining the Embryos

1. Remove both oviducts from 1.5 d post coitum (dpc) females (for tetraploids) and 2.5 dpc (for diploids), and transfer to a drop of M2 medium in a petri dish.
2. Flush oviducts by inserting a flushing needle attached to a 1-mL syringe filled with M2 into the infundibulum.
3. Collect embryos and wash free of any debris using a mouth pipet in several drops of M2.
4. Wash embryos in several drops of M16 or KSOM (referred to hereafter as M16/KSOM) and then transfer to M16 or KSOM medium and place in an organ culture dish in an incubator for in vitro culturing, while the aggregation plate and cells are prepared.
5. Once the plate is prepared, proceed to remove the zona pellucida from the embryos.
6. Place a few drops of M2 and KSOM/M16 and two of Tyrode's in a Petri dish.
7. Transfer a group of embryos into the first drop of Tyrode's, rinse briefly, and then transfer to the second drop.
8. Continually observe embryos, and note when their zona has dissolved. Immediately transfer them to a drop of M2, and subsequently wash in several drops of M2.
9. Wash embryos in M16/KSOM. Embryos are now ready for transfer to the aggregation plate.

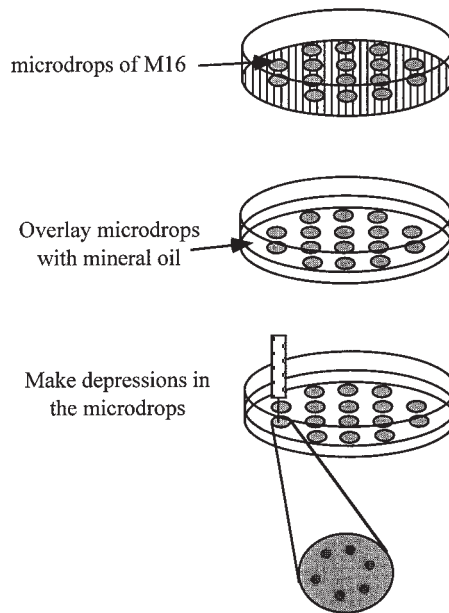


Fig. 4. Preparation of an aggregation plate involves making microdrops of media, overlaying them with mineral oil, followed by forging depressions for the placement of the aggregates. The plate once set up is placed in a temperature controlled humidified incubator overnight.

3.3. Electrofusion to Generate Tetraploid Embryos

1. Turn on the pulse generator about 1 h beforehand in order to warm it up.
2. Place the electrode in a 10-cm Petri dish, connecting the cables from the electrode to the pulse generator and adjust all the parameters; then put this arrangement under a dissecting stereomicroscope. We routinely use two pulses (<repeat> set to 2) of 100 V, and 40 μ s duration. These parameters, however, may vary between machines. Therefore, the optimum parameters should be experimentally determined for each.
3. Put a large drop of 0.3 M mannitol over the electrodes.
4. Place two drops of M2 medium and two drops of 0.3 M mannitol in a second 10 cm Petri dish placed under a second dissecting stereomicroscope.
5. Introduce 50–100 embryos to one drop of M2, from there take 20–25 embryos into a drop of the mannitol. After they have settled, place them between the electrodes of the chamber contained in the second Petri dish.
6. Carefully apply the orienting electric field. (If any embryos are not properly orientated, correct their orientation manually.)
7. When all embryos lie in the correct orientation, apply the pulse.
8. Then immediately transfer the embryos back to the other Petri dish into a drop of M2.
9. Repeat steps 5–8 until all the embryos are subject to the pulsing.

10. When all embryos have been treated, rinse them briefly in M2 and then in M16/KSOM, and transfer them to an organ culture dish containing M16/KSOM or microdrops under mineral oil, and place in the incubator.
11. After approximately 1 h, separate the fused embryos and continue overnight culture.

3.4. 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 (27,28). The protocol for the preparation of ES cells for aggregation is briefly detailed as follows.

1. Day 1: Thaw cells 4 d before the aggregation onto a feeder cell containing plate.
2. Day 2: Change the medium on the plate.
3. Day 3: Passage cells onto gelatinized plates but instead of the usual 1:5 ration, split them 1:50 or to an even higher dilution.
4. Day 4: Change the medium.
5. Day 5: Trypsinize the cells briefly, just until the colonies begin to detach from the plate. Stop the action of the trypsin by adding DMEM+ to the plate. Select clumps of 10–15 loosely attached cells for the aggregation, and transfer into M16 microdrops contained on the aggregation plate.

3.5. Setting Up the Aggregation

3.5.1. Diploid Embryo \Leftrightarrow Diploid Embryo Aggregation

1. Transfer the zona free embryos into a microdrop not containing any depressions.
2. From there, place individual embryos of the first genotype into the individual depressions of the central two rows of microdrops.
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. 5**), return the plate into the incubator and incubate overnight, thereby promoting the aggregation resulting in blastocyst formation by the following day.
5. The next day, most of the aggregates should have formed a single embryo that has progressed to the blastocyst stage and therefore be ready for transfer into recipient females.

3.5.2. Diploid Embryo \Leftrightarrow ES Cells Aggregation

1. Select several clumps of approx 10–15 ES cells (see **Fig. 5B**) and transfer them to the microdrops of the aggregation plate not containing depressions using a mouth pipet.
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 **Subheading 3.5.1**.

3.5.3. Tetraploid Embryo \Leftrightarrow Diploid Embryo Aggregation

1. First, place embryos into the microdrops of the aggregation plate not containing depressions, then take a single tetraploid embryo within a depression.

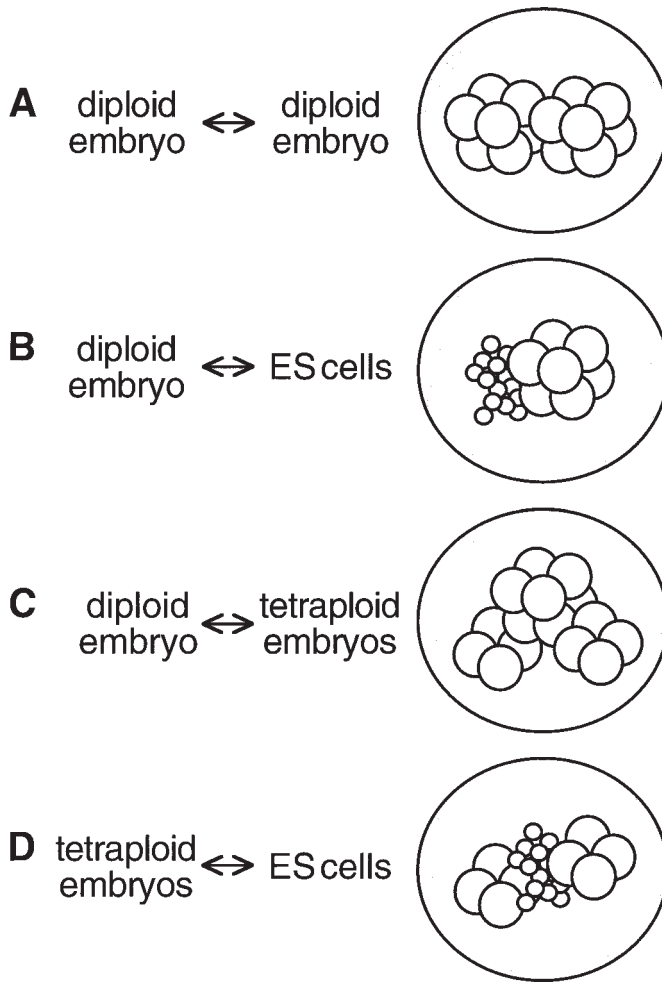


Fig. 5. Several different aggregate combinations can be set-up, comprising one or two of the three constituents of chimeras.

2. Following this, take the diploid embryos and transfer them to the microdrops of the aggregation plate not containing depressions (nor any tetraploid embryos!).
3. Carefully place a single diploid embryo next to the tetraploid embryo already positioned within the depression.
4. Follow **Steps 4** and **5** as in **Subheading 3.5.1**.

3.5.4. Tetraploid Embryo \leftrightarrow ES Cell Aggregation

1. First, place embryos into the microdrops of the aggregation plate free from depressions.

2. Then take a single tetraploid embryo and place it within a depression.
3. Following this, select several clumps of approx 10–15 ES cells and transfer them to the microdrops of the aggregation plate not containing depressions (nor any embryos!).
4. Take a loosely attached clump of cells, and place it carefully next to the embryo already positioned within the depression.
5. Introduce the second embryo into the depression so that it lies on the side of the cells opposing the first embryo. This is best done by gently rolling this embryo over the rim of the depression, at the proper site.
6. Repeat **Steps 2–5** until all the sandwiches have been set up.
7. Follow **Steps 4** and **5**, as in **Subheading 3.5.1**.

3.6. 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 recipient there are several solutions:
 - a. Fourteen embryos can be transferred per uterine horn (a total of 28).
 - b. Use recipients that are 3.5 dpc pregnant.
 - c. To culture the leftover, usually less advanced, embryos, which may still be at the morula stage for another overnight.
 - d. Transfer embryos into the oviduct of a 0.5–15 dpc pregnant female.
3. The embryo transfer procedure is detailed elsewhere (**12**).

4. Notes

4.1. Aggregation Details

4.1.1. Preparing the Aggregation Plate

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. Plates can be prepared a few hours in advance and kept in the incubator until use. This allows enough time to obtain the embryos and prepare the cells.
3. 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.

4.1.2. Obtaining the Embryos

4. Oviducts are removed by making incisions in 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 carried over.

6. Females are superovulated because of an outbred background such as CD1.
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. The zona pellucida is a glycoprotein coat that encapsulates the embryo. Late blastocyst stage embryos will usually hatch out of their zona prior to and in order to implant. The zona is refractory to aggregation, as an intimate contact cannot be made between the cells and/or embryos in the aggregation sandwich.
9. Even though it does not matter whether embryos are in M2 or M16/KSOM prior to their Tyrode's treatment, M2 is used right after as it has a superior buffering capacity.

4.1.3. Electrofusion to Generate Tetraploid Embryos

10. The adjustable AC field is applied in order to allow for the correct orientation of embryos. Only the minimal necessary voltage should be used. If the field is too high it can cause lysis of the cells.
11. 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 electrofused at the same time in addition to the ability of automatic orientation of the embryos with the high-frequency AC field.
12. It usually takes approximately half 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.
13. After overnight culture in M16/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.

4.1.4. Preparing the ES Cells

14. A highly diluted plating of single cells is required in order to produce the optimal size clumps (10–15 cells) required for the aggregation. Clumps in which cells are loosely connected are favored for setting up the aggregate.
15. Care should be taken so as not too disaggregate the cell clumps by pipetting too vigorously or with extensive trypsin treatment.

4.1.5. Setting Up the Aggregation

16. Aggregates should be set up in such a way that there is maximal contact made between the cells and embryo, or between embryos. Therefore it is important that the clump of cells is not too compact, it is best if cells are loosely connected.
17. 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.
18. 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.

4.2. Genotyping F2 Diploid Embryos in Weight Chimeras

19. Genotyping of chimeras is sometime a problem because of the mixed cell populations between mutant and wild-type embryos. One can avoid dealing with this problem by isolating the tissue that is expected to be solely mutant in origin, e.g., the yolk sac endoderm in the case of “mutant diploid embryo \leftrightarrow ES cells” or “mutant tetraploid embryos \leftrightarrow ES cells,” and embryo proper in the case of “mutant diploid embryo \leftrightarrow tetraploid embryos.” In other cases, i.e., “mutant diploid embryo \leftrightarrow wild-type diploid embryo” and “mutant tetraploid embryos \leftrightarrow wild-type diploid embryo,” the contribution of mutant cells can be mixed with wild-type cells in tissues and that makes genotyping very difficult. Here, practical approaches to solve this problem are discussed.

4.2.1. Genotyping of Potentially Chimeric Tissues

20. In the case of recessive mutation, homozygotes for the mutation have to be obtained from a cross between heterozygous female and male. Genotyping of chimeras can be performed by preparing genomic DNA followed by genomic Southern or PCR. The problem occurs if mutant cells are mixed with wild-type cells in the tissue, as the result from genotyping will be indistinguishable between chimeras containing heterozygous cells and mutant cells (*see Fig. 6A*).

One solution for this problem is taking advantages of using two alleles for either wild-type alleles (*see Fig. 6B*) or mutant alleles (*see Fig. 6C*). In this way, one can distinguish chimeras made by mutant embryos from heterozygous littermates.

4.2.2. Isolation of Potentially Mutant Cells by Using Markers

21. It is possible to isolate potentially mutant cells (populations derived from het \times het cross) from wild-type cells if one can design the cross to introduce a gene as a ubiquitous marker into all progenies. For this purpose, markers such as *lacZ* or green fluorescent protein (GFP) are suitable. In the case of *lacZ*, genotyping is performed with *lacZ*-stained tissues and/or by PCR. GFP is more straightforward because one can detect GFP without the use of a chromogenic substrate, and GFP positive cells can be collected manually using a drawn-out glass pipet (A. N., unpublished observation) or fluorescence-activated cell sorting (FACS) (**29** and **30**). In general, the use of the ubiquitously expressed markers makes genotyping chimeras more feasible and reliable.

5. Conclusion

Chimeric studies have been a feature of modern mouse embryology since its inception almost 40 yr 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, this being mouse ES cells. To date the majority of ES cell chimeras have been made by the technically demanding and labor-intensive method of

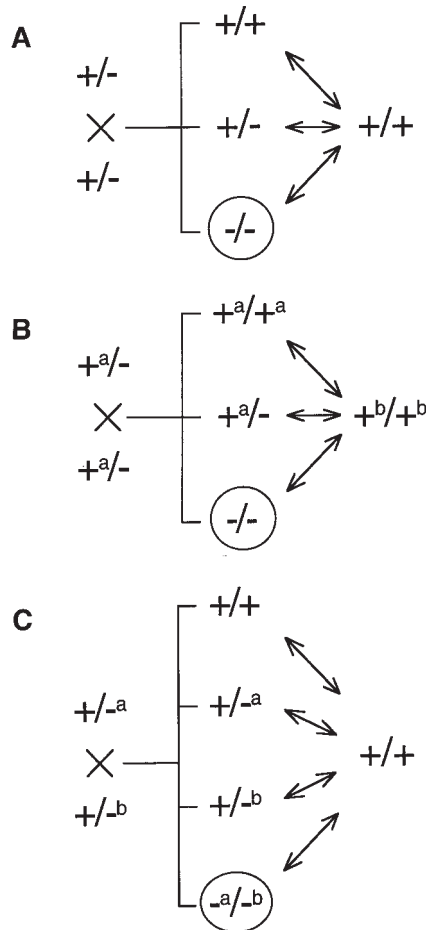


Fig. 6. If mutant cells are intermingled with wild-type cells in the tissues of a chimera (A) the result obtained from genotyping will be indistinguishable between chimeras containing heterozygous cells and mutant cells. To distinguish chimeras made by mutant embryos from heterozygous litter mates. One can employ two different tags for either wild-type alleles (B) or mutant alleles (C).

blastocyst injection and have been used for the introduction into mice of genome alterations made in ES cells. Even so, the 1990s bought back the classic use of chimeras as tools employed to answer biological questions, as the means for generating homozygous mutant ES cells became available, thereby a recessive phenotype would already be represented in cell culture.

We now have an ease of producing ES cell chimeras after revisiting and refining the aggregation chimera technology. We have also learned that ES

cells are developmentally restricted, so that they are not able to differentiate into trophoblast and primitive endoderm lineages, but have full potential in the primitive ectoderm lineage, e.g., in the embryo proper. A further component came into play after it was demonstrated that tetraploid embryos can 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 posed. As a consequence over the past few years there has been an upsurge in the number of such studies reported in the literature. It is therefore reasonable to expect that we will enter 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 and disease life.

References

1. Lobe, C. and Nagy, A. (1998) Conditional genome alterations. *BioEssay* **20**, 200–208.
2. Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J. (1993) Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **90**, 8424–8428.
3. Nagy, A., Gocza, E., Diaz, E. M., Prideaux, V. R., Ivanyi, E., Markkula, M., and Rossant, J. (1990) Embryonic stem cells alone are able to support fetal development in the mouse. *Development* **110**, 815–821.
4. Kaufman, M. H. and Webb, S. (1990) Postimplantation development of tetraploid mouse embryos produced by electrofusion. *Development* **110**, 1121–1132.
5. Martin, G. R. (1981) Isolation of pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA* **78**, 7634–7638.
6. Kubiak, J. Z. and Tarkowski, A. K. (1985) Electrofusion of mouse blastomeres. *Exp. Cell Res.* **157**, 561–566.
7. Nagy, A. and Rossant, J. (1999) Production of ES-cell aggregation chimeras. *Gene Targeting: A Practical Approach* (Joyner, A., ed.) IRL Press at Oxford University, Oxford, UK, pp. 177–205.
8. Lu, T.-Y. and Markert, C. L. (1980) Manufacture of diploid/tetraploid chimeric mice. *Proc. Natl. Acad. Sci. USA* **77**, 6012–6016.
9. Tanaka, M., Gertsenstein, M., Rossant, J., and Nagy, A. (1997) Mash2 acts cell autonomously in mouse spongiotrophoblast development. *Dev. Biol.* **190**, 55–65.
10. Ueda, O., Jishage, K., Kamada, N., Uchida, S., and Suzuki, H. (1995) Production of mice entirely derived from embryonic stem (ES) cell with many passages by coculture of ES cells with cytochalasin B induced tetraploid embryos. *Exp. Anim.* **44**, 205–210.

11. Wood, S. A., Allen, N. D., Rossant, J., Auerbach, A. and Nagy, A. (1993) Non-injection methods for the production of embryonic stem cell-embryo chimeras. *Nature* **365**, 87–89.
12. Hogan, B., Beddington, R., Constantini, F. and Lacy, E. (1994) *Manipulating the Mouse Embryo*, Cold Spring Harbor Laboratory Press .
13. Carmeliet, P., Ferreira, V., Breier, G., et al. (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380**, 435–439.
14. Riley, P., Anson-Cartwright, L., and Cross, J. C. (1998) The Hand1 bHLH transcription factor is essential for placentation and cardiac morphogenesis. *Nat. Genet.* **18**, 271–275.
15. Guillemot, F., Nagy, A., Auerbach, A., Rossant, J., and Joyner, A. L. (1994) Essential role of *Mash-2* in extraembryonic development. *Nature* **371**, 333–336.
16. Zhang, P., Liegeois, N. J., Wong, C., et al. (1997) Altered cell differentiation and proliferation in mice lacking p57^{KIP2} indicates a role in Beckwith-Wiedemann syndrome. *Nature* **387**, 151–158.
17. Yan, Y., Frisen, J., Lee, M. H., Massagué, J., and Barbacid, M. (1997) Ablation of the CDK inhibitor p57Kip2 results in increased apoptosis and delayed differentiation during mouse development. *Genes Dev.* **11**, 973–983.
18. Partanen, J., Puri, M. C., Schwartz, L., et al. (1996) Cell autonomous functions of the receptor tyrosine kinase TIE in a late phase of angiogenic capillary growth and endothelial cell survival during murine development. *Development* **122**, 3013–3021.
19. Shalaby, F., Ho, J., Stanford, W. L., et al. (1997) A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. *Cell* **89**, 981–990.
20. Varlet, I., Collignon, J., and Robertson, E. J. (1997) nodal expression in the primitive endoderm is required for specification of the anterior axis during mouse gastrulation. *Development* **124**, 1033–1044.
21. Rossant, J. (1996) Mouse mutants and cardiac development: new insights into cardiogenesis. *Circ. Res.* **78**, 349–353.
22. Duncan, S. A., Nagy, A., and Chan, W. (1997) Murine gastrulation requires HNF-4 regulated gene expression in the visceral endoderm: tetraploid rescue of *Hnf-4*^{-/-} embryos. *Development* **124**, 279–287.
23. Rossant, J., Guillemot, F., Tanaka, M., et al. (1998) Mash2 is expressed in oogenesis and preimplantation but is not required for blastocyst formation. *Mechanisms Dev.* **73**, 183–191.
24. Nagy, A. (1996) The power of the new mouse genetics [meeting abstract]. *J. Neurochem.* **66**(Supp 1), 76.
25. Lawitts, J. A. and Biggers, J. D. (1993) *Culture of Preimplantation Embryos* **1**, 153–165.
26. Erbach, G. T., Lawitts, J. A., Papaioannou, V. E., and Biggers, J. D. (1994) Differential Growth of the mouse preimplantation embryo in chemically defined media. *Biol. Reprod.* **50**, 1027–1033.
27. Wurst, W. and Joyner, A. (1993) Embryonic stem cells, creating transgenic animals. *Gene Targeting: A Practical Approach* (Joyner, A., ed.) IRL Press at Oxford University, Oxford, UK, pp. 33–62.

28. Pirity, M., Hadjantonakis, A.-K. and Nagy, A. (1998) *Cell Culture for Cell and Molecular Biologists* (Mather, J. P. and Barnes, D., eds.) Academic Press, San Diego, pp. 279–293.
29. Gagnet, S., Le, Y., Miller, J., and Sauer, B. (1997) Brief expression of a GFP cre fusion gene in embryonic stem cells allows rapid retrieval of site-specific genomic deletions. *Nucleic Acids Res.* **25**, 3326–3331.
30. Hadjantonakis, A.-K. and Nagy, A. (2000) FACS for the isolation of individual cells from transgenic mice harboring a fluorescent protein marker. *GeneSys* (in Press).