Mouse embryonic chimeras: tools for studying mammalian development

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Development 130, 6155-6163
Published by The Company of Biologists 2003
doi:10.1242/dev.00893

Summary

Embryonic chimeras of the mouse are well-established tools for studying cell lineage and cell potential. They are also a key part of the analysis of complex phenotypes of mutant mice. By combining embryonic stem cell technology, molecularly tagged mutations and sensitive cell lineage markers, chimeras can provide invaluable insights into the tissue-specific requirement and the mode of action of many mouse genes.

Introduction

The imaginary creature of ancient mythology, the chimera, consisted of the head of a lion, the body of a goat and the tail of a serpent. How did it arise? Probably not by the modern techniques of chimera production, whereby cells of more than one embryonic origin are combined to make one genetically mixed animal. The first mouse embryonic chimeras were made in the 1960s by Kristoph Tarkowski and Beatrice Mintz by aggregating two eight-cell embryos. The result was one normal-sized mouse whose tissues were a mixture of cells derived from the two embryos.

The making of chimeras by injecting cells into blastocysts, devised by Richard Gardner and Ralph Brinster, opened up new possibilities for introducing foreign cells into the embryo. Pluripotent embryonic stem (ES) cells derived from the inner cell mass of the blastocyst (Fig. 1), can differentiate into all tissue types in chimeras, including the germline (Robertson, 1986). The demonstration by Oliver Smithies and Mario Capecchi that genes could be mutated by homologous recombination in ES cells (Doetschman et al., 1988; Thomas and Capecchi, 1989) ushered in a new era of targeted mutagenesis in the mouse. Chimera production using altered ES cells became a key tool for generating designer mice. However, chimeras are more than just a tool for making mouse mutants; they are crucial for analyzing the biological effects of genetic changes. It is this use we will discuss here, after describing the different approaches to generating chimeras and the types of markers used to distinguish the mixed population of cells.

The building blocks for chimera production

The mouse blastocyst just prior to implantation consists of three cell types: the epiblast and the primitive endoderm, both of which are both derived from the inner cell mass, and the outer trophectoderm layer (Fig. 1A). These three cell types have been shown by chimera analysis to give rise to distinct cell lineages in later development. The epiblast gives rise to the three germ layers (ectoderm, mesoderm and endoderm) that contain the progenitors of all the tissues of the fetus, and to the extra-embryonic mesoderm of the visceral yolk sac, the allantois and the amnion. The primitive endoderm gives rise to the endoderm of both the visceral yolk sac and the parietal yolk sac, which together constitute part of the Reichert's membrane that encloses the whole conceptus (Fig. 1B,C). The trophectoderm gives rise to trophoblasts of the Reichert's membrane and of the placenta.

Chimeras can be made by combining two whole eight-cell embryos (Fig. 2A, part I) or by combining subsets of blastomeres of two cleavage (two- to eight-cell) stage embryos. Because, at these stages, the early embryonic cells are not yet restricted in their lineage potency to contribute to the inner cell mass or the trophectoderm, they are equally capable of contributing to both lineages. When two diploid eight-cell embryos or blastomeres of two diploid embryos are aggregated together, chimerism can occur in the epiblast, the primitive endoderm and trophectoderm (Fig. 2A, parts II,III; Table 1). By contrast, when the inner cell mass (ICM) cells of a diploid blastocyst are used to make the chimera, whether injected microsurgically into morula or blastocyst, or aggregated with eight-cell diploid embryos, they contribute only to the epiblast and to the primitive endoderm, and not to the trophectoderm because of more restricted lineage potency of the ICM cells (Table 1). ES cells in the same situations behave more like epiblast cells (Table 1). They contribute only to germ layers that give rise to all the embryonic tissues and some extra-embryonic tissues (including the amnion, the mesoderm of the yolk sac, the allantois and the embryo-derived blood vessels in the placenta) (Beddington and Robertson, 1989) (Fig. 2B, parts II,III; Table 1), but not to trophectoderm or primitive endoderm, despite their ability to differentiate into the latter cell type in vitro. Trophoblast stem (TS) cells, which are permanent cell lines derived either from the trophectoderm of the blastocyst or from early postimplantation trophoblasts (Tanaka et al., 1998), contribute only to trophectoderm derivatives of the chimeras following injection into the blastocyst (Fig. 2D, parts II,III; Table 1).

The lineage potency of embryonic cells can be altered experimentally by the doubling of their DNA content, leading to tetraploidy (Box 1). Although tetraploid embryos can form blastocysts, postimplantation is poor because of the paucity of epiblast cells and the failure of embryos to survive beyond

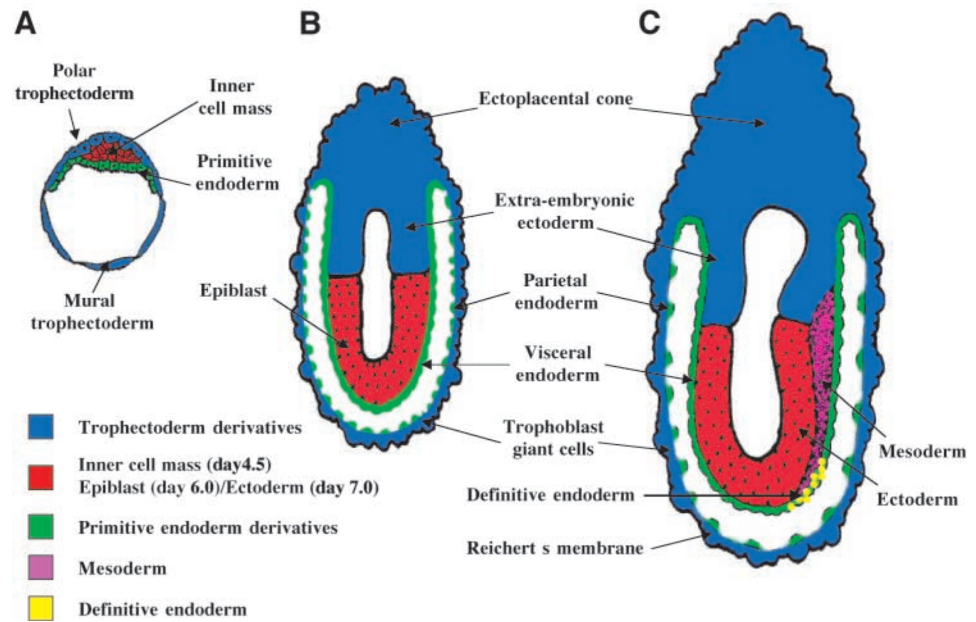

Fig. 1. Mouse embryos at the peri-implantation (A, day 4.5) and post-implantation (B, day 6.0; C, day 7.0) stages of development, showing the allocation of derivatives of the inner cell mass/epiblast, primitive endoderm and trophectoderm, to different tissue compartments of the conceptus. The inner cell mass is the precursor of the epiblast of day 6.0 embryos, and the transition between these two tissues is likely to be a progressive process (Rathjen et al., 2002). The epiblast gives rise to ectoderm, mesoderm and definitive endoderm. The primitive endoderm of the day 4.5 embryo is derived from the inner cell mass. It differentiates into parietal and visceral endoderm (both contribute to fetal extra-embryonic membranes). The trophectoderm gives rise to the ectoplacental cone, extra-embryonic ectoderm and the trophoblast giant cells. The Reichert's membrane is a composite layer of trophoblast giant cells, the basement membrane and the parietal endoderm.

Table 1. Contribution to three major tissue components of the conceptus by different types of cells used for constructing the chimera

	Cell types used to combined with the diploid embryo					
Tissues derived from	Blastomeres (two- to eight-cell stage)	Inner cell mass	Epiblast	Primitive endoderm	Embryonic stem cells	Trophectodermal stem cells
Epiblast	Yes	Yes	Yes	No	Yes	No
Primitive endoderm	Yes	Yes	No	Yes	No	No
Trophectoderm	Yes	No	No	No	No	Yes

mid-gestation (Kaufman and Webb, 1990). When the tetraploid embryos are used to make chimeras together with diploid cells, tetraploid cells rarely contribute to the embryo itself (which is derived from the epiblast) but contribute mainly to the primitive endoderm and the trophectoderm (Tarkowski et al., 1987). This distinctive bias in the lineage potency has been exploited to segregate diploid and tetraploid cells almost exclusively to two different compartments of the chimera. When diploid ES cells and tetraploid host embryos are used to make the chimera, the resultant conceptus will consist of an exclusively ES-cell derived fetus and extra-embryonic mesoderm, but will contain tetraploid trophectoderm and primitive endoderm derivatives (Fig. 2C, parts II,III). ES cell↔tetraploid embryo chimeras have been used extensively in the tetraploid complementation assay (Box 1) for testing the impact of altered gene function on the lineage potency and the functional interaction between embryonic and extra-embryonic tissues (see examples in later sections).

Lineage markers for chimera analysis

To analyze fully the development of chimeras, it is crucial that the fate of the two cellular components of a chimeric embryo can be followed using lineage markers (Table 2). Ideally, such a marker should have no effect on the cells that bear it, and it should be ubiquitous, cell-autonomous and detectable at the single-cell level in situ. Previously, the most common markers used in chimera analysis were the electrophoretic variants of housekeeping enzymes, such as glucose-6-phosphate isomerase (GPI). However, detecting GPI variants requires the destruction of tissues, which precludes detection of these variants at the spatial resolution needed to determine chimerism at the histological level. The first generation of genetic markers that were used for chimera analysis allowed cells of different origins to be distinguished by the presence of strain-specific DNA satellite markers (Rossant et al., 1983) or a large globin transgene insert (Lo et al., 1987). The use of these markers involves technically difficult DNA-DNA in situ hybridization of histological preparations of the embryo. At present, the most widely used marker is the β-galactosidase enzyme encoded by the *E. coli lacZ* gene. Its expression can be detected by simple histochemical staining of whole embryos and sectioned materials. The green fluorescent protein (GFP) of jellyfish, with all its spectral variants (Hadjantonakis et al., 2002), has great potential for chimera analysis, especially because its expression can be seen in living cells (Fig. 2, part II). However, the cellular resolution with which one can detect GFP activity is affected by standard histological processing, so its expression has to be detected by immunostaining instead.

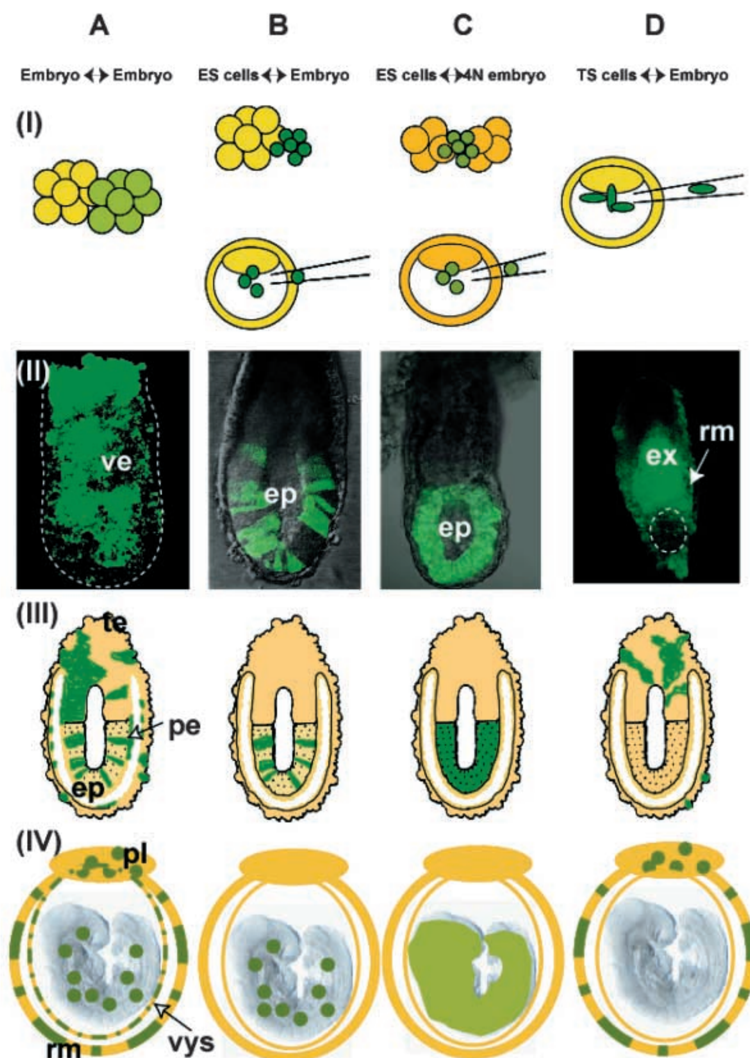

Fig. 2. Lineage contributions of different types of cells used in the construction of four types of embryonic chimeras: (A) embryo↔embryo chimera; (B) ES (embryonic stem) cells↔embryo chimera; (C) ES cells↔4N (tetraploid) embryo chimera; and (D) TS (trophectodermal stem) cells↔embryo chimera. (I) The production of chimeras. (IA) embryo↔embryo chimera produced by the aggregation of two eight-cell embryos. (IB) ES cells↔embryo chimera produced by the aggregation of ES cells with an eight-cell embryo (top) or by injecting ES cells into the blastocyst (bottom). (IC) ES cells↔4N (tetraploid) embryo chimera produced by the aggregation of ES cells with two tetraploid four-cell embryos (top) or by injecting ES cells into a tetraploid blastocyst (bottom). (ID) TS cell↔embryo chimera produced by injecting TS cells into a blastocyst. Introduced cells are dark green in order to represent green fluorescent protein (GFP) expression and the host cells are pale yellow. Cells of tetraploid embryos are dark yellow. (II) The different patterns of contribution by the GFP-expressing cells to different tissue compartments in the four types of chimeras developed to the early postimplantation stage (days 5.5 to 6.5) after transfer to pseudopregnant female mice for implantation and further development. (IIA) Day 6.5 embryo: GFP-expressing cells from the eight-cell embryos are found in the visceral endoderm (ve), which covers the extra-embryonic ectoderm and the epiblast. The epiblast also contains GFP-expressing cells. The broken line traces the primitive endoderm layer of the embryo. (IIB) Day 6.5 embryo: GFP-expressing ES-derived cells are found only in the epiblast (ep). (IIC) Day 5.5 embryo: exclusive contribution of GFP-expressing cells to the epiblast (ep). (IID) Day 5.5 embryo: exclusive contribution of GFP-expressing cells to the trophectoderm derivatives: extra-embryonic ectoderm (ex), Reichert's membrane (rm). The broken line marks the epiblast, which is not populated by GFP-expressing cells. (III) Day 6.0 pre-streak stage embryo illustrating the pattern of distribution of the GFP-expressing cells (shown in II) in the epiblast (ep), primitive endoderm (pe) and trophectoderm (te) derivatives. (IV) The different pattern of chimerism in the fetus proper, extra-embryonic membranes (rm, Reichert's membrane; vys, visceral yolk sac) and the placenta (pl) of the mouse conceptus at early organogenesis stage (day 9.5). For clarity, chimerism of the derivatives of extra-embryonic mesoderm, which is concordant with that of the fetus proper, is not shown.

Making chimeras of different tissue constitutions

Diploid mutant embryo↔diploid wild-type embryo chimeras

This type of chimera is made by aggregating mutant and wild-type eight-cell embryos. All tissue lineages will potentially be mosaic, and the ability of mutant cells to colonize any tissue during pre- or postnatal development can be assessed. The exclusion of mutant cells from a particular lineage or tissue, or their abnormal differentiation in a given tissue, can pinpoint cell-autonomous primary defects that are caused by the mutation. A major drawback of this technique is the difficulty in identifying informative chimeras. Only 25% of the chimeric embryos derived from heterozygous crosses will be homozygous mutant. Unless embryos are genotyped before aggregation, a genotyping strategy is required to distinguish the following three possible types of chimeras: wild type↔wild type, heterozygous mutant↔wild type and homozygous mutant↔wild type, before a proper analysis of the experimental outcome could be made. This can be accomplished by tracking strain-specific polymorphisms or, more commonly, two molecularly distinctive mutant alleles of the gene of interest. For example, the genotyping of embryos obtained from the crossing of heterozygous mutant mice that are used to make the chimera may be facilitated by the availability of two different targeted alleles, e.g. one with and other without an insertion of a neomycin-resistant gene (Hart et al., 2002). Using allele-specific PCR analysis, the genotype is determined by the presence of both targeted alleles in the homozygote↔wild-type chimera, only one of the two alleles in the heterozygote↔wild-type chimera and the absence of both targeted alleles in the wild-type↔wild-type chimera.

ES cell↔diploid embryo chimeras

Currently, the most common tool for studying mutant phenotypes is the ES cell↔embryo chimera, which is generated by introducing wild-type ES cells into mutant embryos or vice versa. In both cases, the genetic constitution of the epiblast derivatives (the mouse itself) will be a mixture of mutant and wild-type cells. However, ES cells do not contribute to the primitive endoderm or trophectoderm, so the

Table 2. Properties of markers used in tracking cells for chimera analysis

	Cell markers				
Properties of markers	Glucose phosphate isomerase: electrophoretic variants*	Species-specific satellite DNA (*Mus musculus* versus *M. caroli*)[†]	Transgene insert (multiple copies of β-major globin)[‡]	β-Galactosidase encoded by *E. coli lacZ* and alkaline phosphatase encoded by *Akp2* or *Plap*[§,¶]	Fluorescent proteins (spectral variants: green, yellow, cyan and red)**
Present or expressed ubiquitously in all cell types	Housekeeping enzyme expressed in all cells	Inherent to the genome, but detectable only in nucleated cells	Permanent and inheritable integration in the genome, but detectable only in nucleated cells	Expressed in every cells if driven by a universally active promoter/enhancer **or** restricted to specific cell types if driven by lineage- or temporal-specific regulatory elements	Expressed in every cells if driven by a universally active promoter/enhancer **or** restricted to specific cell types if driven by lineage- or temporal-specific regulatory elements
Detectable at single-cell resolution	No, detection requires tissue destruction	Yes	Yes	Yes	Yes
Detectable in whole mount preparation of the specimen	No, detection requires tissue destruction	No	No	Yes	Yes
Detectable after histological processing	No, detection requires tissue destruction	Yes, by in situ DNA-DNA hybridization	Yes, by in situ DNA-DNA hybridization	Yes	Yes, visualized by fluorescence microscopy of wax-embedded sections, or by immunostaining of the protein
Detectable in living cells or whole organism	No, detection requires tissue destruction	No	No	No	Yes

*Buehr and McLaren (1981).
†Rossant et al. (1983).
‡Lo et al. (1987).
§Soriano (1999).
¶Lobe et al. (1999).
**Hadjantonakis et al. (2002).

composition of the extra-embryonic primitive endoderm and trophectoderm will differ depending on the genotype of the ES cells and embryo used. In a wild-type ES cell↔mutant embryo chimera, the primitive endoderm and trophectoderm will be mutant; whereas in a mutant ES cell↔wild-type embryo chimera, the primitive endoderm and trophectoderm will be wild type. ES cell↔embryo chimeras can be used to distinguish between the embryonic and extra-embryonic effects of genetic mutations, as well as to determine lineage-specific effects of mutations in the embryo proper. If homozygous mutant ES cells are used, the extra-embryonic tissues will not contain the mutation, all chimeras will be informative and no genotyping will be necessary. However, if wild-type ES cells are used with mutant embryos, the extra-embryonic tissues, as well as some tissues of the embryo, may contain the mutation. Hence, interpreting the impact of mutation on the development of the chimera will require knowledge of the genotype of the embryo into which ES cells are introduced. This is accomplished by sampling extra-embryonic tissues (either visceral endoderm or trophoblasts) of the chimera to determine which chimeras contain cells from the heterozygous or homozygous mutant embryos. In contrast to the situation of the embryo↔embryo chimera, genotyping using the extra-embryonic tissue of a wild-type ES cell↔mutant embryo chimera is not complicated by the presence of wild-type cells originating from the ES cells. A simpler assay for the presence of the wild-type and a mutant allele will suffice to distinguish all possible genotypes.

ES cell´tetraploid embryo chimeras

In an ES cell↔tetraploid embryo chimera, the ES cells contribute primarily to the epiblast-derived tissues, whereas cells of the tetraploid embryo mainly give rise to the extra-embryonic primitive endoderm and trophectoderm. The almost complete segregation of descendants of the ES and tetraploid cells provides a powerful means for revealing the effect of the mutation on the embryonic versus the extra-embryonic tissues (Box 1). In addition, as the fetus proper is constituted exclusively by the ES cells in this type of chimeras, ES cell-derived embryos and adult mice of the same genotype as the ES cells can be produced immediately for phenotypic studies (Box 1). Large numbers of embryos of the genotype of the ES cells can be made by this means, bypassing the tedious process of breeding mutant mice. Finally, ES cell↔tetraploid embryo chimeras may provide an innovative means for high-throughput analysis of gene function. In addition to the conventional genomic modification by transgenesis and gene targeting in the ES cells, the function of single or multiple genes could be altered efficiently by knocking down the transcriptional activities in the ES cells using RNAi reagents. Using the ES cell↔tetraploid embryo chimeras as the experimental tool, a substantial number of ES cells can be

Box 1. ES cell↔tetraploid embryo chimeras

Producing the tetraploid embryo
Tetraploid mouse embryos are produced by the electrofusion of the blastomeres of a two-cell stage preimplantation embryo together into one cell by the application of the direct electrical current. At the cell division that follows the replication of the combined set of genetic materials of the fused cell, the nuclei of the resultant two-cell embryo will contain double the diploid content of DNA, i.e. it will be tetraploid. The tetraploid embryo will develop normally to the blastocyst stage, although with a significant reduction in the cell number. However, it will implant normally, because the tetraploid trophectoderm and the trophoblasts derived from it are functionally competent for postimplantation development.

Constructing the ES cell↔tetraploid embryo chimera
Chimeras are made by aggregation of clumps of ES cells with a zona pellucida-free (zona pellucida is removed by pronase digestion) tetraploid morula stage embryo. Because of the electrofusion that results in the reduction of two cells to one cell, the cell number of the tetraploid morula will be reduced by approximately half at all stages of preimplantation development. To compensate for the smaller cell number per embryo, two tetraploid embryos are used for the aggregation with ES cells.

Tetraploid complementation assay
The basis of this assay is that the two cellular constituents of the ES cell↔tetraploid embryo chimera display different lineage potency such that the ES cells give rise to all structures in the fetus, while the descendants of the tetraploid cells contribute to the extra-embryonic primitive endoderm and the trophectoderm. If the ES cells and the tetraploid embryos are of different genotypes, for example mutant versus wild type, there will be a complete segregation of mutant cells from wild-type cells into two distinctive tissue compartments of the conceptus. The ES cell↔tetraploid embryo chimera therefore provides a useful experimental tool with which to test the crucial requirement of gene function in the embryonic versus extra-embryonic tissues for the development of the conceptus. If the development of the embryo is abnormal in the presence of mutant tetraploid cells in the extra-embryonic tissues, despite the presence of wild-type ES cells in the epiblast-derived tissues, then the mutant phenotype is likely to be caused by the mutation of the extra-embryonic endoderm and the trophectoderm. Conversely, if the development of the embryo is abnormal in the presence of mutant ES cells in the epiblast-derived tissues, despite the presence of wild-type tetraploid cells in the extra-embryonic tissues, the primary defects that lead to the mutant phenotype must lie in the embryonic tissues or the extra-embryonic mesoderm.

Completely ES cell-derived embryos
As the fetus proper is derived entirely from the epiblast, to which ES cells contribute exclusively, the ES cell↔tetraploid embryo chimera offers a unique means with which to produce an embryo that is completed derived from ES cells (Nagy et al., 1993). Therefore, when genetically altered ES cells are used to produce ES cell↔tetraploid embryo chimeras, the phenotypic effects of the mutation can be examined directly in the ES cell-derived embryo. This removes the necessity of generating an ES cell↔diploid wild-type embryo chimera for transmitting the mutation through the germline. Previously, it was found that embryos that were derived completely from ES cells are not viable after birth, but judicious selection of robust ES cell lines has overcome this problem. Currently, ES cell lines made from F1 hybrid strains of mice are used routinely to make live mice by this approach, even after multiple rounds of genetic alterations (Eggan et al., 2001). The ability to analyse the phenotype in completely ES cell-derived embryo is particularly useful in situation where the heterozygous mutation is lethal and where the homozygous mutant mice display fertility problems. The use of ES cell↔tetraploid embryo chimeras enables both the heterozygous and the homozygous phenotypes of the mutation can be studied using ES cells of the appropriate genotype (Carmeliet et al., 1996). A further application of the ES cell↔tetraploid embryo chimeras is the production of embryos that harbor mutations of multiple genes. For example, by using ES cells with null mutations in two or more genes, it is possible to produce embryos with complex genotypes much more efficiently than by the conventional strategy of breeding multiple lines of mutant mice.

screened efficiently for the phenotypic effects of the RNAi treatment (Kunath et al., 2003).

Application of chimera analysis in dissecting complex phenotypes

The conventional approach for assessing gene function is the analysis of phenotypes of mutant embryos and adult mice. However, the interpretation can be confounded by lethality of the mutant during development and the manifestation of complex malformations or multiple functional deficits, which preclude a thorough elucidation of the full impact on of the mutation. As a result, important functional roles and the primary cellular targets of the gene may be missed. Chimera analysis can offer a complementary experimental approach to the straightforward assessment of phenotype. The examples described in this section, which are drawn mainly from studies of early embryonic development, illustrate how chimera analysis may achieve some of the goals in the quest of knowledge of specific aspects of gene function.

Goal 1: determining lineage-specific gene function

Chimeras that contain normal and mutant cells have proven to be highly effective for determining the function of specific genes essential for the specification or differentiation of certain tissue lineages. This is because the impact of gene function on a specific lineage can be revealed by the abnormal differentiation of mutant cells from the lineage in question or, in extreme cases, by a reduced contribution to, or the exclusion of mutant cells from, specific types of tissues in the chimera.

In an example of this, loss of function of a multitude of genes, such as *Madh2* (previously *Smad2*), *Tcf2* (previously *Hnf1*), *Foxh1*, β-catenin, *Mixl1* and *Sox17*, results in different phenotypes of the mutant embryo, ranging from arrested gastrulation to abnormal formation of the head or trunk, which superficially have little in common and would not have revealed any specific function in endoderm development. However, in ES cell↔embryo chimeras, ES cells that lack the function of any one of these genes are consistently under-represented in or excluded from the gut endoderm of the embryo (Coffinier et al., 1999; Hart et al., 2002; Hoodless et al., 2001; Kanai-Azuma et al., 2002; Lickert et al., 2002; Tremblay et al., 2001; Yamamoto et al., 2001). The impact of these mutations on the endoderm potency of the ES cells therefore reveals the heretofore unrecognized requirement of the function of these genes for endoderm specification, differentiation and maintenance.

An innovative approach was recently undertaken to test the role of *Egr2* (previously *Krox20*) in the sorting properties of cells in the rhombomeres of the embryonic hindbrain (Voiculescu et al., 2001). In this approach, the chimera analysis was performed by aggregating wild-type and *Egr2*$^{lacZ/Cre}$ *R26R* embryos. R26R is a Cre-dependent *lacZ* reporter transgene. In ES cells derived from *Egr2*$^{lacZ/Cre}$ *R26R* embryos, expression of Egr2 from both *Egr2* alleles is lost. This is due to the targeted mutations caused by the integration of the *lacZ* and the Cre-recombinase genes. When the *Egr2* gene is activated in the descendants of these cells in the hindbrain, the cells will express the *lacZ* and the *Cre* transgene, but only as far as the *Egr2* locus remains active. However, the transient expression of *Cre* recombinase in the *Egr2*$^{lacZ/Cre}$ cells will permanently activate the *lacZ* reporter of the *R26R* locus. This allows the fates of the *Egr2*-null cells to be tracked by the *R26R lacZ* expression for an extended period of development long after the expression of the *Egr2*^{lacZ} allele has ceased. Results of this study showed that *Egr2*-null cells fail to mix normally with wild-type cells in rhombomere 5, but are able to mix with the adjacent even-number rhombomeres, demonstrating compellingly that *Egr2* function is essential for the maintenance of segment identity (Voiculescu et al., 2001).

Goal 2: dissecting gene functions in extra-embryonic versus embryonic tissues for embryonic patterning

Embryological and gene expression studies have shown that the early patterning of the anteroposterior axis of the mouse embryo at around the time of gastrulation requires signalling and transcriptional activity in both the extra-embryonic and embryonic tissues (Beddington and Robertson, 1999). The analysis of mutations in candidate patterning genes in chimeric mice has played a key role in elucidating the importance of extra-embryonic tissues as sources of patterning signals in the early mouse embryo. ES cell↔diploid chimeras or, more commonly, ES cell↔tetraploid chimeras can be used to distinguish whether a patterning defect is caused by the embryonic or extra-embryonic effects of a mutation.

Three possible outcomes may be obtained from these ES cell↔diploid and ES cell↔tetraploid chimera experiments.

The first possible outcome is that the loss of gene function in the extra-embryonic tissues alone results in a phenocopy of (i.e. similar phenotype to) the null mutation and wild-type extra-embryonic tissues can rescue this mutant phenotype. For example, *Hnf4*$^{-/-}$ embryos fail to undergo gastrulation, and have an abnormal epiblast. However, the null phenotype is rescued by wild-type primitive endoderm and trophectoderm in *Hnf4*$^{-/-}$ ES cell↔tetraploid chimeras (Duncan et al., 1997). This outcome indicates that *Hnf4* function in the extra-embryonic tissues is crucial for normal gastrulation. Similarly, a null mutation of the *Tcfap2c* gene that encodes the AP2γ factor is embryonic lethal, but the presence of wild-type extra-embryonic tissues allows mutant embryos to survive. By contrast, wild-type ES cells could not rescue the lethality of *Tcfap2c*$^{-/-}$ embryos because of the defective extra-embryonic tissues (Auman et al., 2002). Defects in early embryonic patterning that are associated with the abnormal function of the primitive streak (such as those caused by mutations of *Amn* or *Nodal*), or with the abnormal function of the gastrula organizer (such as those caused by mutations of *Akd* or *Foxa2*), can be partially rescued by the presence of wild-type extra-embryonic tissues. Therefore, these studies showed that *Amn*, *Nodal*, *Akd* and *Foxa2* function is essential in the extra-embryonic tissues for normal embryogenesis and that early patterning events must involve a complex interplay between embryonic and extra-embryonic tissues (Dufort et al., 1998; Episkopou et al., 2001; Kalantry et al., 2001; Varlet et al., 1997).

In these chimeras, the extra-embryonic tissues consist of cells of two different lineages: the trophectoderm and the primitive endoderm. Thus, without knowing the cell types in which the gene of interest is expressed, it is not possible to conclude from such experiments whether gene function is specifically required in the derivatives of the trophectoderm or the primitive endoderm or in subsets of cells in both tissues. The following example, however, shows that this ambiguous aspect of chimera analysis may be overcome if it is known which tissue expresses the gene of interest. *Otx2*$^{-/-}$ embryos display abnormal development of the fore- and midbrain. In *Otx2*$^{-/-}$ ES cell↔diploid chimeras, the anterior neural plate initially develops normally, even though over 90% of the embryo consists of *Otx2*$^{-/-}$ cells, suggesting that normal Otx2 function in the extra-embryonic tissue is sufficient and essential for the for early morphogenesis of the anterior neural primordium. As *Otx2* is not expressed in the trophectoderm derivatives, its function is therefore likely to be required only in the visceral endoderm (Rhinn et al., 1998).

The second possible outcome is that the loss of gene function in the extra-embryonic tissues does not cause the mutant phenotype. For example, *Dkk1*, *Lhx1* (previously *Lim1*), *Hhex* (previously *Hex*) and *Hesx1* are expressed in the visceral endoderm of the mouse embryo prior to and at early gastrulation and later in the mesoderm or the ectoderm of the embryo (Martinez-Barbera et al., 2000a; Martinez-Barbera et al., 2000b; Mukhopadhyay et al., 2001; Shawlot et al., 1999). Chimeras that contain mutant extra-embryonic tissues harboring mutations in one of these genes display normal gastrulation, even when a mixture of wild-type and mutant cells is present in the embryo. By contrast, chimeras that contain wild-type extra-embryonic tissues and mutant embryonic cells form abnormal head structures like the null mutant (Martinez-Barbera et al., 2000a; Martinez-Barbera et al., 2000b; Mukhopadhyay et al., 2001; Shawlot et al., 1999). These findings indicate that the function of these genes is required in the embryonic tissues and not the extra-embryonic tissues.

In the third scenario, loss of gene function in either embryonic or extra-embryonic tissues results in a mutant phenotype. This suggests that the function of a mutated gene is essential for both tissue types. For example, in chimeras that lack *Bmp4* activity in the extra-embryonic tissue, primordial germ cells are not formed from the proximal epiblast. In *Bmp4*-null ES cell↔tetraploid chimeras, the lack of *Bmp4* activity in the ES cell-derived extra-embryonic mesoderm does not affect germ cell formation but does disrupt the localization of the germ cells, the formation of the allantois and the establishment of left-right asymmetry (Fujiwara et al., 2001; Fujiwara et al., 2002). *Sox2* is another example of a gene that is required in both embryonic and extra-embryonic tissues. Chimeric analysis of *Sox2* function has revealed that the viability of the epiblast in *Sox2*$^{-/-}$ mutant embryos can be restored by wild-type ES cells. However, such chimeras fail to survive beyond gastrulation, even when the embryo proper consists of predominantly wild-type ES cells. Chimeras formed by

aggregating *Sox2*$^{-/-}$ ES cells with tetraploid embryos, however, survive for much longer, suggesting that the failure of Sox2 mutant embryos to develop beyond gastrulation is because of an extra-embryonic, rather than an embryonic, defect (Avilion et al., 2003). These chimera studies show that *Bmp4* and *Sox2* functions are required in both extra-embryonic and embryonic tissues to sustain development.

Goal 3: identifying cell-autonomous and non-cell-autonomous gene function

The effect of the mutation may affect only the cells that are expressing the gene and not other genotypically mutant cells in the same animal. The restriction of phenotypic effects reflects the cell-autonomous requirement of gene function. Alternatively, a mutant phenotype could arise by a mutation that impacts not only on the cells expressing the genetic activity but also on other cells that do not express the gene. The non-cell-autonomous action of the gene will mean that the normal function of the gene is not restricted to any cell population. Analysis of phenotype in an embryo containing only mutant cells is therefore insufficient for distinguishing between these two modes of gene action. However, chimera analysis can reveal whether the gene of interest functions in a cell-autonomous or non-cell-autonomous manner.

In the chimera, cell-autonomous gene function may be revealed by the exclusion of mutant cells from a certain tissue lineage or by the expression of an abnormal phenotype in only those cells with the mutant genotype. For example, in a chimera that lacks either *Fgfr1* or *T* function, cells fail to ingress properly through the primitive streak, resulting in the accumulation of the mutant cells in the posterior region of the chimera (Ciruna et al., 1997; Wilson et al., 1995). These defects in cell movement persist in the presence of wild-type cells in the chimera, suggesting that each of these genes function in a cell-autonomous manner. An example of non-cell-autonomous gene action is that of *Foxd3*. Loss of *Foxd3* function results in a poorly formed epiblast, an absent primitive streak, abnormal extra-embryonic endoderm and the demise of embryos shortly after gastrulation. The presence of a small number of wild-type cells in the embryo proper of a *Foxd3*$^{-/-}$ ES cell↔diploid chimera rescues these developmental defects. *Foxd3* therefore seems to act in a non-cell-autonomous manner, presumably by regulating cell-cell signalling activity (Hanna et al., 2002).

Some genes act in either cell-autonomous or cell-non-autonomous mode in different tissue lineages. *Ascl2*$^{-/-}$ (previously *Mash2*) mutant embryos die early in development because of a placental deficiency that is associated with a lack of spongiotrophoblasts and labyrinthine trophoblasts. In the chimeric placenta, *Ascl2*-deficient cells are excluded from the spongiotrophoblasts but not the labyrinthine trophoblasts, suggesting that *Ascl2* activity is required cell-autonomously in the formation of the spongiotrophoblasts but non-cell-autonomously for that of the labyrinthine trophoblasts (Tanaka et al., 1997). Similarly, *Eed* function is required in a non-cell-autonomous manner as *Eed*$^{-/-}$ cells can participate in gastrulation when wild-type cells are present, but not in their absence. However, *Eed* is required autonomously in cells to enable their differentiation to forebrain tissues and somites, as *Eed*$^{-/-}$ cells are excluded from these tissues of the chimera. Tetraploid rescue experiments have shown that the defective morphogenesis of *Eed* null embryos cannot be rescued by wild-type extra-embryonic tissues because *Eed* acts cell-autonomously in the extra-embryonic tissues (Morin-Kensicki et al., 2001).

Goal 4: distinguishing between primary versus secondary defects

The mutant phenotype is a culmination of the loss of gene function in the primary target cells (the primary effect), as well as additional effects that are elicited in other tissues by changes in the function of the primary target cells (the secondary effect). In chimeras, the primary effects of a mutation can be distinguished from the secondary effects by the ability to tightly associate a phenotype with the preponderance of mutant cells in the primary target tissues.

Using ES cell↔diploid embryo chimeras, it has been possible to show that the definitive (gut) endoderm is the primary target tissue of *Mixl1* and *Sox17* function. In chimeras with extensive *Mixl1* or *Sox17* mutant contributions, only the gut is populated by wild-type cells. Another significant feature is that the development of the head (of the *Mixl1*$^{-/-}$ ES cell↔embryo chimera) and of the trunk (of the *Sox17*$^{-/-}$ ES cell↔embryo chimera) is significantly more advanced than in embryos null for either gene. These results indicate that, in addition to the primary function of these two genes in endoderm formation, they also have a secondary role in the provision of morphogenetic activity by the gut endoderm for the development of specific body parts (Hart et al., 2002; Kanai-Azuma et al., 2002).

Loss of the function of the tumor suppressor retinoblastoma (*Rb*) gene in the mouse embryo leads to neural and erythroid tissue defects, neuronal apoptosis and early embryonic lethality. However, chimeras produced by the aggregation of *Rb*$^{-/-}$ ES cells with tetraploid wild type embryos are viable and free from neural and blood abnormalities, suggesting that these features of the null phenotype are caused secondarily by extra-embryonic dysfunction, most likely of the placenta. Nevertheless, rescued mice still have abnormal lens development and show elevated cell proliferation in the nervous tissues, which are typical of *Rb* mutant mice, implying that these may occur as a result of the loss of *Rb* in these tissues, an effect that is unrelated to the placental deficiency (de Bruin et al., 2003; Wu et al., 2003). Secondary defects in an embryo that arise as a result of placental dysfunction are fairly common. Chimera analysis should thus be a standard part of phenotypic investigation of mutant embryos in cases of mid-gestation lethality, even where the embryonic defects appear to be fairly specific.

Goal 5: expediting phenotypic analysis

The early death of a mutant embryo is a major factor that can confound the assessment of all later aspects of gene function. In chimeras, the incorporation of wild-type cells into the embryo or the extra-embryonic tissues can rescue the embryo from this early lethality. If gene function becomes crucial later in development, the resultant chimeric embryo may develop an abnormal phenotype that is not found in the null mutant embryo. For example, chimeras containing PLCγ-deficient cells are viable due to the restoration of the hematopoietic function by wild-type cells. However, the development of renal abnormalities in the mutant embryo has revealed an otherwise

undetected cell-autonomous function of PLCγ in kidney formation (Shirane et al., 2001). Subtle variations in the requirement of gene function by cells in different parts of an organ can also be revealed by chimera analysis. For example, in mutant ES cell↔diploid chimeras with an overwhelming presence of the mutant cells in the embryo proper, *Sox17*$^{-/-}$, *Mixl1*$^{-/-}$ and *Madh2*$^{-/-}$ cells are excluded from the mid- and hindgut but, conversely, *Foxa2*$^{-/-}$ and *Foxh1*$^{-/-}$ cells are excluded from the fore- and midgut (Hart et al., 2002; Hoodless et al., 2001; Kanai-Azuma et al., 2002; Tremblay et al., 2000; Yamamoto et al., 2001). The loss of the potency (hence the exclusion) of the mutant cells to populate specific segments of the embryonic gut implies that the endoderm in different gut segments requires the activity of different genes for its formation or maintenance

Future perspectives

The embryonic chimera has proven to be very useful for elucidating gene function in the mouse beyond the simple characterization of mutant phenotypes. Its application is not limited to the embryological study of early development but also to the analysis of organogenesis, postnatal maturation and bodily function. When used in combination with molecular tools that can modify genetic activity in a time- and lineage-specific manner in the cell population under scrutiny, the chimera offers practically unlimited options for a precise, in-depth and large-scale analysis of gene function. Genetic activity can be modified by incorporating the means with which to alter gene expression conditionally [i.e. by gene-driven and inducible Cre recombinase activity (Nagy, 2000)], or to modulate (i.e. up- or downregulate) gene function (by applying molecular reagents such as morpholinos, antisense oligonucleotides and RNAi). Current effort in the evaluation of the developmental potency of stem cells from adults and embryos focuses on studying cell differentiation in vitro and the profiling of transcriptional activities of these cells to identify the signature molecules that reflect the level of pluripotency. A unique feature of the embryonic chimera is that foreign cells introduced to the embryonic environment may be provided with all the possible lineage options available normally to the cells during development. As a result, the cells are subject to a test of the full range of lineage potency, which may reveal the true extent of pluripotency (Clarke et al., 2000; Jiang et al., 2002) Chimera analysis is expected to become an essential tool for the most comprehensive and stringent in vivo assessment of the characteristics of embryonic and adult tissue stem cells of mouse and other mammalian species.

P.P.L.T. is a Senior Principal Research Fellow of the NHMRC of Australia and J.R. is a CIHR Distinguished Investigator.

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