

Chimaeras and mosaics for dissecting complex mutant phenotypes

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ABSTRACT Back at the first half of the 1980s, there was no mammalian experimental embryology in Hungary. One of us, AN, took up the challenge of establishing a small group in the field. In the absence of local information, AN and his former colleague, Andras Paldi (AP), used their tourist passport to visit several laboratories in Western Europe and collect information and advice. This is how AN and AP ended up one day sitting in Anne McLaren's office in the MRC Mammalian Development Unit at University College, London. They never forgot her endless enthusiasm and the way she clearly explained the important points of preimplantation embryo manipulation, chimaera making and embryo transfer. As well as the extremely useful suggestions, which were crucial to starting the lab in Hungary, they also took back her deep love for embryo development. They remember her telling them, 'never waste an embryo – there is always another unanswered question it can solve'. Many who have been lucky and experienced Anne's spirit and advice later realized how useful it was to generate 'new' ideas by following the 'not wasting' principle. Our views on chimaeras presented below definitely contain elements which grew out from this principle.

KEY WORDS:

Historical view

One of the special features of the human brain is the capacity of imagination – creating new combinations of existing elements. This capacity of the human brain is vital to many aspects of our culture: mythology, religion, technology, art and science. Imaginary creatures, called chimaeras, combining parts of different animals into one fierce beast, showed up independently in the ancient cradles of civilization of China, Assyria, Persia and the Roman Empire. The best known is the classic Roman 'chimaera', which has a serpent tail, lion head and a goat body. It is a beautiful physical representation of the chimaera from Greek mythology, who was the daughter of Typhon, the god of the hurricane, and Echidna, the snake mother. Similar imaginary creatures combining different parts of different organisms occur throughout recorded history, through the Middle Ages (see the paintings of the famous Dutch painter, Hieronymus Bosch) to our modern age of StarTrek movies. From such a rich cultural soil, it was no surprise to find that scientists were intrigued by the possibility of actually creating individuals from different embryonic origins. Mammalian embryonic chimaeras are one such manifestation of myth made reality. Originally fierce imaginary creatures have been harnessed and turned into a powerful and friendly tool for addressing important biological questions.

Chimaeras and mosaics

A definition of a chimaera is a single organism made up of cells of different embryonic origins. Mosaics are conceptually different from chimaeras, since in these individuals all the cells have the same embryonic origin but a random population of cells is genetically different from the rest.

Both chimaerism and mosaicism can occur naturally. In humans, conjoined twins can be considered as natural chimaeras. Frequently twin pregnancy in cattle leads to joint circulation of the two embryos though incomplete separation of their placentas. This results in a mixing of haematopoietic stem cells and chimaeric blood during their postnatal life. The best example of natural genetic mosaicism occurs in the females of placental mammals, where one X-chromosome undergoes random inactivation, leading to nonallelic expression of X-linked genes. Beside this obviously normal situation, mosaicism due to somatic chromosomal anomalies can be the underlying event in several human diseases (Mohrenweiser and Zingg, 1995). Mosaicism can now be produced at will in mice in a random, tissue-specific or inducible manner, by

Abbreviations used in this paper:

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use of the Cre-loxP technology to induce chromosomal alterations at will. This powerful technology is growing in use rapidly but has been reviewed extensively elsewhere (Nagy, 2000) and will not be further considered here. Instead, we will focus on embryonic chimaeras in all their different variations.

Mammalian embryonic chimaeras have been made since the 1960s, when Kristoph Tarkowski and Beatrice Mintz independently showed that two mouse embryos could be aggregated together at the 8-cell stage to generate one composite mouse that was a genetic mixture of the two parental cell lines. Those original experiments attested to the highly regulative nature of the mammalian preimplantation embryo; the embryo aggregate started at twice the normal size, but ended up as a normal-sized mouse with no abnormalities in patterning. Since that time, experimental manipulation of the early mouse embryo has been refined and modified in many different ways; cells can be removed, reoriented, added back at different stages; cells from sources other than the embryo can be added; embryos can be reconstituted from their constituent cell lineages; DNA, RNA and protein can be added; and still the embryo manages to survive and compensate for the indignities to which it has been exposed. Anne McLaren was a pioneer in the application of chimaera techniques to understanding the allocation of cell lineages in development and her monograph in 1976 entitled "Mammalian chimaeras" laid out the tenets of the field and formed the intellectual basis for much succeeding work on chimaeric analysis.

The utility of the original aggregation chimaeras for following the fate of genetically marked cells was somewhat restricted. First, aggregation at the 8-cell stage produced conceptuses in which all cell lineages, both embryonic and extraembryonic, were genetically mixed and, second, the markers used to follow lineage were not ubiquitous or detectable *in situ* at a single cell level. The first problem was alleviated in the late '60s when Richard Gardner in the UK and Ralph Brinster in the US developed techniques for introducing different cell types into the blastocyst stage of development. The second problem was intractable for a much longer time but now has been solved by the development of easily detectable *in situ* enzymatic markers and by the viable cell marker, Green Fluorescent Protein. In this review we will discuss the various kinds of embryonic chimaeras that can be generated and what different kinds of information can be obtained from them, using examples mostly from our own work.

The basic building blocks of chimaera production

In order to understand the lineage restrictions of the components that can be used for chimaera production, it is useful to review the restriction of the early cell lineages established in the blastocyst, since this is the latest stage at which cells can be added to form chimaeras before returning the embryo to the uterus to complete development. Chimaeras generated at different stages of preimplantation and with different cell types will end up with contributions in different lineages of the blastocyst. Three different cell types can be distinguished in the mouse blastocyst: trophoblast, primitive endoderm and primitive ectoderm (Fig. 1). These three lineages have separate fates. The trophoblast cells

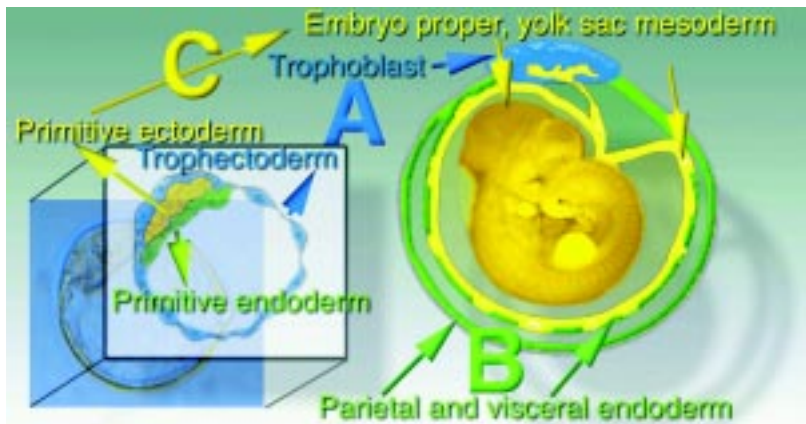


Fig. 1. Fate of the first embryonic lineages established in the blastocyst. (A) Trophoblast lineage; (B) primitive endoderm lineage; (C) primitive ectoderm lineage.

exclusively differentiate into the trophoblast layer of the placenta (Fig. 1, Lineage A), the primitive endoderm forms the parietal and visceral endoderm (Fig. 1, Lineage B) and the yolk sac mesoderm, amnion and the embryo proper are exclusively derived from the primitive ectoderm (Fig. 1, Lineage C). In the next sections, we delineate the potential of various components of chimaeras in terms of the three blastocyst cell lineages (A, B, C).

Diploid preimplantation stage embryos (D)

The developmental potential of any normal, diploid preimplantation embryo is complete. Their developmental potential is ABC (Fig. 1).

Tetraploid preimplantation stage embryos (T)

The experimental production of tetraploid embryos is very simple and efficient. Blastomeres of two cell stage embryos can be fused into one tetraploid cell by a simple electric pulse. These embryos go through preimplantation normally and implant, but rarely form proper embryonic structures beyond gastrulation. If they are aggregated with diploid embryos, they can contribute to extraembryonic compartments but they are efficiently selected against in the embryo proper (Tarkowski *et al.*, 1977). More specifically, they can contribute to the trophoblast and primitive endoderm lineage, but not very well to the primitive ectoderm derivatives (Nagy *et al.*, 1990). Their developmental potential is AB (Fig. 1).

Inner cell mass (ICM)

The ICM of the late stage blastocyst consists of two layers of cells: primitive endoderm and primitive ectoderm. Its isolation is relatively easy with immunosurgery, developed by Davor Solter (Solter and Knowles, 1975). Since these cells are no longer able to differentiate into trophoblast, the developmental potential of ICM is BC (Fig. 1).

Trophoblastic vesicles (TV)

With skilled micromanipulation, it is possible to isolate pure trophoblast vesicles from blastocyst stage embryos (Gardner, 1972). Just like ICM this is also a primary component of a blastocyst, therefore its developmental potential is the corresponding A (Fig. 1).

Embryonic stem (ES) cells

ES cells are permanent cell lines derived from the blastocyst with properties that suggest they are closest to the embryonic ectoderm

lineage of the blastocyst. Chimaera studies showed that their developmental potential is C, with no capacity to contribute to the primitive endoderm and trophoctoderm (Nagy *et al.*, 1990) (Fig. 1).

Trophoblast stem (TS) cells

The recently established TS cells are derived from the trophoctoderm lineage and just like ES cells they retain many features of their origin, including developmental potential (Tanaka *et al.*, 1998), which is now in this case A (Fig. 1).

Somatic stem cells

This is an emerging and very exciting area of exploration and discovery, which is challenging the dogma of renewal and regeneration. Many adult tissues contain stem cells, which have been considered to have self-renewal capacity but restricted developmental potential. However, recent studies have shown that adult restricted stem cells can have much broader differentiation capacity than originally thought. For example, haematopoietic stem cells can contribute to muscle lineages, neuronal stem cells can apparently contribute to multiple cell types, and muscle stem cells can make haematopoietic lineages (Clarke *et al.*, 2000; Gussoni *et al.*, 1999; Jackson *et al.*, 1999). Identifying and isolating these stem cells is not easy due to their small number and more are expected to be discovered. At this stage not many have been tested for their capacity to contribute to embryonic lineages in chimaeras. However, this is a critical test for multipotentiality and will be an important area of research for the future.

Legitimate combinations and component allocations

If the possible components of chimaera production are put together in a single matrix, where the rows are the three primary lineages of development (A, B and C, as above) and the columns are the components (Fig. 2), it is immediately obvious that not all the possible combinations of two components could cover all the three lineages, which is a minimal requirement for an embryo to develop. Those combinations where the two components together cover all the lineages and, therefore, allow embryonic development are

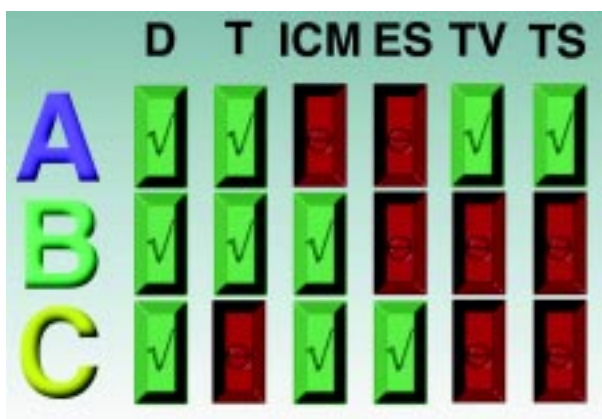


Fig. 2. Developmental potential (green = full, red = restricted) of the possible chimaera components in the three primary embryonic lineages shown in Fig. 1. *D*, diploid embryo; *T* tetraploid embryo; *ICM*, inner cell mass; *ES*, embryonic stem cell; *TV*, trophoblastic vesicle; *TS*, trophoblast stem cell.

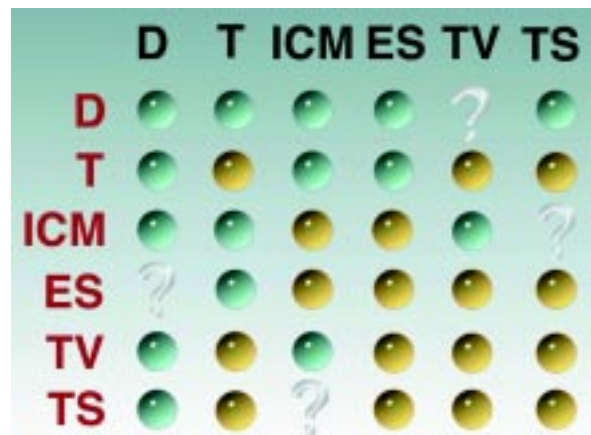


Fig. 3. Legitimate (blue) and illegitimate (yellow) chimaeric combinations between pairs of components. *D*, diploid embryo; *T*, tetraploid embryo; *ICM*, inner cell mass; *ES*, embryonic stem cell; *TV*, trophoblastic vesicle; *TS*, trophoblast stem cell.

shown in Fig. 3. Untested but potentially legitimate combinations are shown with a question mark.

The differing developmental potential of the different chimaera components means that it is possible to generate chimaeras with chimaerism restricted to different embryonic lineages. This capacity has been extremely useful in both cell lineage analysis and dissection of complex mutant phenotypes, as described below.

Applications

Diploid embryo ↔ Diploid embryo chimaeras

This type of chimaera is one of the easiest to produce by aggregating 8-cell stage embryos. It is very useful for examining mutant vs. wild-type cell behaviour in a competitive situation and addressing the question of cell autonomous phenotypes (Rossant and Spence, 1998). Since chimaeras are made by aggregating the embryos from a heterozygous cross with wild-type embryos, it is necessary to be able to distinguish the three possible classes of chimaeras. This can be achieved by using two different mutant alleles to generate homozygous embryos, or by use of linked markers. In the case of imprinted genes, chimaera analysis is made easy by the fact that heterozygous embryos will have the mutant phenotype if they inherit the mutant allele on the non-imprinted chromosome. We took advantage of this in chimaeric analysis of *Mash2* mutants. Earlier we had shown that a targeted null mutation in the *Mash2* gene resulted in a midgestational embryonic lethality (Guillemot *et al.*, 1994). The dying embryos had a severe placenta phenotype characterized by the lack of spongiotrophoblast and defective labyrinth layers. In order to address the cell autonomous nature of the phenotype we performed a chimaeric study (Tanaka *et al.*, 1997). One component of the chimaeras was wild-type and the other was *Mash2* deficient, and tagged by ubiquitous *lacZ* expression of the *Rosa26* transgene. In the chimaeras, the *Mash2* deficient cells were completely excluded from the spongiotrophoblast lineage, strongly supporting the cell autonomous nature of the *Mash2* deficiency in this lineage. On the other hand, *Mash2* deficient cells could contribute at very high levels to all other layers of the placentas, including the labyrinthine layer, without showing any signs of abnormality. Therefore, the labyrinthine phenotype observed in the *Mash2*

deficient animals was presumably secondary to the spongiotrophoblast defect. This kind of study is important in focussing the search for downstream effectors of a gene's action on a particular cell lineage.

Diploid embryo ⇌ Tetraploid embryo chimaeras

The skewed distribution of the different embryonic lineages achieved in diploid embryo ⇌ tetraploid embryo chimaera, can be used to advantage to distinguish primary placental or embryonic phenotypes. We first used this technique with the *Mash2* mutants, where we suspected that the embryonic lethal phenotype was due solely to a placental function for *Mash2*. We aggregated wild-type tetraploid embryos with diploid embryos from a cross of *Mash2* +/- heterozygotes. With the tetraploid embryos providing cells in the placenta, *Mash2* homozygous embryos were capable of surviving pre- and perinatal development. Adult *Mash2* homozygotes were normal and fertile, clearly demonstrating that *Mash2* gene function is dispensable in the embryo proper and for postnatal life and it is solely required for placental development (Guillemot *et al.*, 1994).

We have gone on to use the same approach to rescue the placental phenotypes of a number of mouse mutations, including *ERR* and *Brca1* (Hakem *et al.*, 1996; Luo *et al.*, 1997). The approach is generally useful for revealing later phenotypes obscured by early placental defects, e.g. *PPARγ* (Barak *et al.*, 1999). So far *Mash2* is unique in showing complete phenotypic rescue by this approach; most genes involved in placental function appear to have some later embryonic or postnatal function. This type of chimaera is similar to the ES cell ⇌ tetraploid embryo chimaera in outcome. Its main advantage is that viable mutant offspring may be born if the extraembryonic phenotype can be fully rescued, whereas such an outcome is rare when ES cells are used. Its main disadvantage is that the skewing of distribution between embryonic and extraembryonic lineages is not as complete as for ES cell ⇌ tetraploid embryo chimaeras.

Diploid embryo ⇌ ICM chimaeras

ICMs can be aggregated with 8-cell embryos or injected into blastocysts, and will contribute to all lineages except the trophoblast. Chimaeras of this sort were fundamental to the delineation of the fate of early cell lineages in the mouse embryo and to the establishment of the time at which different lineages became committed (Rossant, 1987). In addition, it was the observation that a single ICM cell could contribute to all somatic cell lineages including the germ line (Gardner, 1985), that led to the drive to develop permanent cell lines from the ICM as a vehicle for introducing genetic changes into the germ line.

Diploid embryo ⇌ ES cell chimaeras

The development of ES cell lines did indeed allow the power of chimaeras to be applied to the generation of many kinds of genetically altered mice. ES cells can be manipulated genetically *in vitro*, mostly notably by various homologous and site-specific recombination approaches (Rossant and Nagy, 1995), and then used to generate chimaeras for transmission of the altered genotype into the germ line.

However, embryo-ES cell chimaeras are more than just a vehicle for germ line transmission of mutations. ES cells, homozygous for a mutation of interest, are extremely useful for analyzing the lineage-specificity of mutant defects. Although mutations generated in ES cells are generally only at one allele, the cells can be made homozygous by a variety of means. These include loss of heterozygosity

induced by increased drug selection, gene targeting at the second allele, or generation of new homozygous lines from homozygous embryos (Rossant and Nagy, 1995). In all cases, it is also possible to introduce markers to help follow mutant cells in chimaera

Having homozygous ES cells makes the task of generating large numbers of mutant ⇌ wild-type chimaeras much easier, and this approach is now widely used for chimaeric analysis of mutants. We have used it in a number of studies, including study of the complex gastrulation phenotype of *Fgfr1* mutants (Ciruna *et al.*, 1997). Chimaeric analysis revealed that the phenotypic effects appeared to be separable into an effect on cell adhesion, manifested by the failure of mutant cells to migrate through the streak, and an effect on mesoderm cell-type specification. Rescue of the early gastrulation phenotype in the chimaeras also revealed later tissues where *Fgfr1* mutant cells were excluded, which thus were likely to be targets of *Fgfr1* action. These included the progress zone of the limb buds (Saxton *et al.*, 2000), the branchial arch mesenchyme and the gut.

Because ES cells do not contribute to the trophoctoderm and primitive endoderm lineage, diploid embryo ⇌ ES cell chimaera can help delineate the primary site of action of mutations causing early patterning defects. If chimaeras made by mixing wild-type ES cells with mutant embryos always show the mutant phenotype whatever the extent of ES cell contribution, then it is likely that the phenotypic effect resides in the trophoblast or primitive endoderm. In the reverse experiment, rescue of the phenotype by introduction of mutant ES cells into wild-type embryos also implies an extraembryonic effect. Experiments of this sort have been critical in establishing the role of the visceral endoderm in anterior-posterior patterning, via the action of the nodal-Smad2 signaling pathway (Varlet *et al.*, 1997; Waldrip *et al.*, 1998).

Diploid embryo ⇌ TS cell chimaeras

TS cells, which derive from the trophoctoderm of the blastocyst, produce chimaeras in which only the trophoblast lineages are genetically mixed (Tanaka *et al.*, 1998). This combination of lineage contributions is not available from any other chimaeric situation and is potentially very powerful for trophoblast-specific rescue of mutants. However, to date, the extent of chimaerism achieved by injection of TS cells into blastocysts is not consistently high, making mutant rescue difficult. We have attempted to rescue *Mash2* mutants by injection of wild-type TS cells without success so far (unpublished results). However, improved contribution of TS cells to chimaeras should be possible by introduction of TS cells into earlier embryos. Clearly more technical work is needed before these chimaeras can be used to their full potential.

Tetraploid embryo ⇌ ICM chimaeras

This particular combination is rarely used but can generate mice entirely derived from the ICM genotype very efficiently. We used this as a control when trying to generate ES-cell derived offspring from tetraploid embryo ⇌ ES cell aggregates (Nagy *et al.*, 1990). ICM-derived offspring from tetraploid embryo aggregates survive at a much higher rate than ES-derived offspring, showing that the neonatal problems of ES-derived mice stem from their ES cell origin not the mode of chimaera production.

Tetraploid embryo ⇌ ES cell chimaeras

This combination gives a very clear separation of contributions to the primitive ectoderm (from the ES cells) and the primitive endoderm

and trophectoderm lineages (from the tetraploid cells) (Nagy *et al.*, 1990). We used this to show that it was possible to produce viable entirely ES-derived mice, at least from early passage ES cells (Nagy *et al.*, 1993). This demonstrated unequivocally the pluripotential nature of ES cells and is a critical underpinning of the current interest in the derivation of human ES cells for possible use in tissue repair of all cell types.

However, the tetraploid embryo↔ES cell chimaera has much wider uses. It is the cleanest way to assess the relative roles of a particular gene in embryonic versus extraembryonic lineages. For example, we have used tetraploid embryo-ES cell aggregates to show that early gastrulation effects of the *HNF3β* mutation reside in the primitive endoderm, but that later defects in the node and notochord are due to defects in the primitive ectoderm lineage (Dufort *et al.*, 1998). Wild-type tetraploid embryo↔mutant ES chimaeras showed normal gastrulation but absence of node and notochord, while mutant tetraploid embryo↔wild-type ES cell chimaeras produced the mutant gastrulation defects.

If the effect of a particular mutation is confined to the primitive ectoderm lineage, tetraploid embryo↔mutant ES cell chimaeras can be a very useful way of generating large numbers of mutant embryos for analysis. For example, we use this routinely to generate *Fgfr1* mutant embryos for analysis, since this mutation shows no trophoblast or primitive endoderm defects.

In the previous example, the homozygous mutant phenotype was known from standard breeding of heterozygotes first and then duplicated in tetraploid embryo aggregates. However, it is also possible to analyze the primary mutant phenotype in tetraploid embryo chimaeras and thus avoid germline transmission. We examined both the heterozygous and homozygous phenotype of deletion of the *Vegf-A* gene by this approach (Carmeliet *et al.*, 1996). Embryos heterozygous for loss of *Vegf-A* showed embryonic lethality due to reduced vascularity. Thus, no live heterozygotes could even be produced to assess the homozygous phenotype. We generated homozygous mutant ES cell lines and used them to generate homozygous embryos by tetraploid embryo aggregation. These embryos showed an even more severe vascular defect similar to the mutant defect in the knock-out of the VEGF receptor, *Fk1* (Shalaby *et al.*, 1995). In this particular instance, analysis of the homozygous phenotype by tetraploid embryo aggregation was the only possible route. However, because it speeds up considerably the time from identification of targeted cell line to analysis of mutant phenotype, it can also be used as a means of gaining a quick insight into mutant phenotype. This is useful for early embryonic lethal phenotypes but should not be relied on for late gestation defects onwards. Even wild-type ES cell↔tetraploid embryo chimaeras tend to show reduced viability close to term and so it becomes difficult to separate mutant effects from generalized ES cell defects.

ICM↔Trophectoderm vesicle chimaeras

This masterpiece of micromanipulation involves injection of ICMs into microsurgically prepared trophectoderm vesicles. This generates a reconstituted blastocyst with ICM and trophectoderm of separate genotypes. This was the original tool used to determine the later lineage derivatives of ICM and trophectoderm (Gardner *et al.*, 1973). It was also used to rescue trophoblast defects before the development of the tetraploid approach. For example, we showed that embryos from the divergent mouse species, *Mus caroli*, fail to develop in the *M. musculus* uterus. However, *M. caroli* ICMs in *M.*

musculus trophectoderm vesicles can implant and develop normally (Rossant *et al.*, 1983). This implies that species-specific trophoblast-uterine interactions may be a critical component of speciation. Rescue of endangered species by interspecific transfer may be enhanced by provided trophoblast of host genotype by either this microsurgical approach or the easier tetraploid embryo aggregation method. Although tetraploid embryo aggregation has tended to supercede the reconstituted blastocyst approach, because of its simplicity, tetraploid embryo aggregation does not lead to the immediate separation of the lineage components. Thus, the reconstituted blastocyst approach may still be necessary to dissect apart very early lineage defects (Gardner *et al.*, 1990).

Addendum

It is clear that the chimaera is more than a mythological curiosity, but a living, breathing manifestation of our curiosity about the origins of development from egg to embryo to adult. Manipulation of the preimplantation mouse embryo brings together the power of both experimental embryology and genetics to further our understanding of our own origins. Anne McLaren has throughout her career recognized the importance of combining embryology and genetics and has led the way in promoting their convergence. Her early work on defining the parameters necessary for successful embryo transfer was critical for all succeeding work in the field. Her recognition of the power of chimaeras was critical for her own studies on sex determination, and her lucid exposition of the questions and problems in the field in many different publications was also the catalyst for many of the rest of us to pursue the questions she raised. Anne has been a wise and supportive presence in the field of mammalian developmental biology and her influence continues to be felt worldwide. It is always a pleasure to meet with Anne, discuss science and respond to her questions, which always get to the critical issues of the day. When giving a talk, she is scrupulous in her acknowledgement of other people's contributions, and so it is a pleasure to contribute to a whole issue where we in turn acknowledge and applaud her major contributions to our research programs.

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