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Mouse chimaeras revisited: recollections and reflections

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ABSTRACT This very personal and subjective article briefly describes the evolution of techniques used for obtaining mouse chimaeras from various sources (embryos, EC, ES and EG cells), summarizes studies on inter-specific chimaeras, mentions some of other applications ('rescuing' chimaeras), presents the contribution of Ralph Brinster to this area and tries to estimate whether the expectations I expressed in 1961 as to the usefulness of making and studying chimaeras turned out to be correct. Tribute is paid mainly to those, who as the first, contributed to various aspects of these studies.

KEY WORDS: *mammal, mouse, chimaera, interspecific, review*

The first steps

The first man-made mouse chimaera was born on the 6th of March 1961, i.e., exactly 37 years ago[1)], in the Department of Zoology, University College of North Wales, United Kingdom, where I was spending a year as the Rockefeller Foundation Fellow. At that time the idea of making one mammalian individual by aggregating two cleaving embryos[2)] must have looked rather preposterous and later I often wondered why Professor Rogers F.W. Brambell, under whose supervision I worked during that visit, had accepted this and other crazy projects which I proposed to carry out in his laboratory. In doing that experiment I was both lucky and unlucky. Lucky – because the experimental part of the study was completed in just two months [plus perhaps two more for overcoming embryo culture problems (this appears to be an eternal issue) and working out the technique of aggregating embryos (unnecessarily very difficult and tricky)]. Unlucky – because I was not able to confirm chimaerism in the only two experimental animals that had survived beyond weaning: one originated from two aggregated embryos of the same strain and in the other the unpigmented component was not observed. Fortunately the chimaerism of several other newborns that died at or within 3 days after birth was undoubtful (two populations of differently pigmented cells in the outer layer of the retina: Tarkowski, 1963,1964a). This experiment was soon described in *Nature* (Tarkowski, 1961).

In the early sixties there were not many experimental mammalian embryologists and I did not expect that in somebody else's mind a similar idea could have arisen. To my great surprise, however, there was another scientist doing exactly the same experiment –she was Beatrice Mintz of the Cancer Institute in Fox Chase, Philadelphia. As far as I remember she was equally amazed that the same idea had occurred to somebody else. In 1962, in two abstracts which appeared in *American Zoologist* she described successful attempts to produce chimaeric blastocysts (Mintz, 1962a,b), and in 1965 presented evidence that overtly chimaeric mice are viable and can survive till adulthood (Mintz, 1965).

This article is exactly what the title says: a few recollections and reflections on the subject of experimental chimaeras. This means that I have taken full liberty to choose some problems and not to mention others, and to quote some papers and not to quote others, even if they are really very important. I apologize to all of my colleagues whose work and publications have been not mentioned here and I beg them to believe that this does not mean my undervaluation of their contribution and achievements. In this article I wish to pay tribute mainly to those scientists who as the first

1) For an individual scientist the 37th anniversary of this kind means inevitably the descending days of his/her professional life, although optimists may argue that they are in the middle of the best period, perhaps going downhill but still close to the top. For man as a species the period of 37 years is equal to nearly two generations, assuming 20 years as the length of one generation. But for a laboratory mouse with a generation length of 3 months, 37 years mean nearly one hundred and fifty generations. In our own species 150 human generations mean three thousand years, which would take us back to the year 1000 B.C. Everything is a matter of perspective. A mouse historian as opposed to the human historian would have written on this subject a voluminous treatise rather than just a few pages, as I will do.

2) Nicholas and Hall (1942) claimed to produce by aggregation of two zona-free 1-cell rat zygotes a chimaeric foetus which, however, was dead when removed from the uterus by Caesarian section. No evidence of chimerism was presented and in view of the present knowledge of early mammalian development, the experimental protocol applied in the above study makes success a most unlikely event.

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made significant technical improvements or conceptual breakthroughs, because their effort and imagination deserve to be remembered. I do this deliberately because I observe that more and more often instead of quoting the real inventor or contributor, people refer to the review papers or to their own papers in which some time earlier they quoted once the original paper and from that moment on they feel justified to quote themselves for the rest of life for what they have never done. I object to this habit.

The subject of experimental chimaerism has been thoroughly reviewed on several occasions, just to mention only the magnificent book by Anne McLaren '*Mammalian chimaeras'* published in 1976 and another book edited by her together with Nicole Le Douarin '*Chimaeras in Developmental Biology'* which appeared in 1984. The fact that twenty one scientists contributed to the latter book (as opposed to one author of the first monograph) was due not only to extending the scope of the subject to birds (and to a smaller extent to other animals as well) but to the very fast and multidirectional development of research in this field. In 1976 Anne McLaren cited 133 papers on experimental chimaeras, predominantly on mouse chimaeras but also few on chimaeras in other species and interspecific chimaeras. I failed to estimate the number of papers published on this subject since 1976, nor the dynamics of publications during the period of 37 years.

The very fact that the article concerned with chimaeras appears in this particular volume dedicated to Ralph Brinster implies that he has contributed to this field also: between 1972 and 1974 together with his collaborator Laila A. Moustafa, Ralph had published three papers on chimaeric embryos and mice produced by injecting cells into blastocysts.

Constructing chimaeras: the two components and the ways they can be 'put together'

Since 1961 the original technique of aggregating cleaving embryos has been largely simplified and a completely different microsurgical technique of producing chimaeric embryos and animals has been developed. What is more, chimaeras can be now produced not only by putting together two (several) embryos, but also by combining an embryo with embryonic cells of slightly asynchronous age, teratocarcinoma cells, and embryonic stem (ES) and embryonic germ (EG) cells.

Aggregation of cleaving embryos

My original technique has been simplified by: 1) removal of the zona pellucida by pronase (Mintz, 1962c) or acid Tyrode (Nicolson *et al.,* 1975) rather than by rupturing it mechanically in a micropipette. 2) Observation that simple apposition at physiological temperature is sufficient to bring about aggregation, making special tricks of squeezing partner embryos unnecessary (Mintz, 1962b). 3) Introducing phytohaemaglutinin as a useful (although not obligatory) chemical facilitating adhesion (Mintz *et al.,* 1973).

Injection of embryonic cells into blastocysts

This technique was developed by Gardner in 1968 and first used for transferring dissociated inner cell mass (ICM) cells. The original technique required usage of five instruments and permitted one to introduce into the blastocoel not only single cells but also a whole ICM (Gardner, 1971). By replacing five instruments with just two – a holding pipette and an injection pipette– Moustafa and Brinster (1972a,b) and Babinet (1980) considerably simplified this elegant technique. Moustafa and Brinster *(loc. cit.)* were the first to use it for transferring cells originating from advanced postimplantation embryos.

Combining an embryo with pluripotent or totipotent stem cells

Gardner's technique (Gardner, 1968) has been subsequently used by many investigators for producing chimaeras containing cells derived from embryonal carcinoma (EC) cells (Brinster, 1974; Mintz and Illmensee, 1975; Papaioannou *et al.,* 1975), from embryonic stem (ES) cells (Bradley *et al.,* 1984) and finally from EG cells, i.e. cells derived from primordial germ cells (Stewart *et al.,* 1994). Chimaeric embryos can be also produced by microsurgical introduction of cells under the zona pellucida (i.e., into the perivitelline space) of cleaving embryos: the injected cells eventually aggregate with blastomeres (Tokunaga and Tsunoda, 1992). Although this technique has been used with success by others until now (cf. Saburi *et al.,* 1997) it will probably 'lose' with a much simpler technique of aggregating zona-free cleaving embryos with ES cells (see below). Its advantage is that the number of introduced ES cells can be precisely controlled and that chimaerism can be achieved following transfer of just one cell.

Stewart was the first to show that chimaeric blastocysts (1980) and even viable chimaeric animals (1982) can be produced without using a sophisticated microsurgical technique, simply by sandwiching a group of EC cells between two cleaving embryos or just by aggregating a lump of such cells with one embryo. At present, the easiest way of producing chimaeras containing ES-derived cells is by short term culture of zona-free 8-cell embryos on a layer of ES cells followed by culture in the standard embryo culture medium until the blastocyst stage (Wood *et al.,* 1993).

Chimaeras produced with the help of the nuclear transfer technique

This smart technique differs from the other two techniques (i.e., aggregation and injection) in that it consists not in incorporating two types of cells into one embryo, but in replacing a nucleus in one blastomere at the 2-cell stage with a genetically different nucleus from another 2-cell (or 8-cell) embryo (Kono *et al.,* 1989). The manipulated blastomere is a nucleo-cytoplasmic hybrid and carries the mitochondrial DNA of the recipient embryo.

Chimaeras in species other than the mouse

Although the mouse is the unquestionable leader and hero among experimental mammalian chimaeras, chimaeric animals were produced also in the rat (Mayer and Fritz, 1974), rabbit (Gardner and Munro, 1974; Moustafa, 1974), sheep (Tucker *et al.,* 1974; Fehilly *et al.,* 1984a) and cattle (Summer *et al.,* 1983; Brem *et al.,* 1984). Studies on chimaerism in farm animals, so far carried out on a limited scale, will explode as soon as embryonic stem (ES) cells become available in these species (see below).

Interspecific chimaeras

Creating embryos and occasionally even animals composed of cells derived from two different species is in a way a bow and a tribute paid by experimental embryology to ancient mythology which created monsters of dual, triple or even multiple origin,

without paying much attention to the taxonomic relationship between the 'contributing' species (cf. McLaren, 1976). However, the mammalian embryologist who uses preimplantation embryos for constructing interspecific chimaeras, produces embryos (individuals) in which cells of the two species are intermingled right from the beginning of development and, consequently, the resulting individual (embryo or animal) displays a patchy pattern of phenotypic traits of both species rather than being a conglomerate of complete parts of two adult bodies[3)].

For those who love experimenting in general, and in whom the childish curiosity and fantasy have not been yet completely ousted by logic and coolness of a respectful adult scientist, this is a wonderful experiment to do, but ... (see below).

Rat<->mouse chimaeric blastocysts were produced simultaneously and independently in 1973 by Mulnard, Stern and Zeilmaker, but the above authors did not study their postimplantation development. Mystkowska (1975) constructed bank vole<->mouse chimaeric embryos and studied their development both before and after implantation. The two most advanced embryos developing in mouse recipient females until the 9th and 10th day of pregnancy (respectively at the 4-somite and 12-somite stage) were alive and looked normal. Chimaerism of the 4-somite embryo was karyologically confirmed. Because the older embryo was investigated with ordinary histological rather than immunohistological techniques the coexistence of both bank vole and mouse cells in this embryo could not have been proved. All implantations examined between 11 and 17 day of pregnancy were resorbed.

More successful postimplantation development of interspecific chimaeric embryos was observed in rat<->mouse combinations by Gardner and Johnson (1973,1975), but the majority of newborn presumed chimaeric young were runted and dead at birth or died soon after, and the five mice killed at weaning appeared not to be chimaeric. The relatively long development was probably achieved due to the fact that these embryos were produced by injection of rat ICMs into mouse blastocysts rather than by aggregation of cleaving embryos as in Mystkowska's experiment (1975) (even though she sandwiched one bank vole embryo between two mouse embryos) or by aggregation of a rat ICM with a mouse morula as in Rossant's experiment (1976). Injection of an ICM into a blastocyst permits the creation of an embryo which displays chimaerism only in the ICM and its derivatives and whose trophectoderm is taxonomically concordant with the uterus of the recipient female. Incompatibility between the rat trophectoderm and mouse uterus (and vice versa) was shown long ago in interspecific transfers of embryos between these two species (Tarkowski, 1962).

Successful development of interspecific chimaeras till adulthood has been noted in *Mus musculus<->Mus caroli* (Rossant and Frels, 1980), sheep<->goat (Fehilly *et al.,* 1984b) and *Bos taurus<->Bos indicus* (Summer *et al.,* 1983). Some of these animals were fertile. Fehilly and Willadsen (1986) mention lambs with manifold malformations (and undoubtedly chimaeric) that were produced by aggregation of sheep blastomere(s) with a single bovine blastomere (unpublished observations of Willadsen, Miller and Lenn).

There is no doubt that the duration of survival of chimaeric embryos depends on the degree of the relationship between the two species; however, what really matters is the real, i.e. genetic, and not just taxonomic distance expressed by genders, families etc. The duration of survival may be additionally prolonged when the chimaeric embryo is enveloped by a trophectoderm of a species of the recipient female. This was elegantly shown by comparing the developmental success of aggregation *versus* injection chimaeras of *M. musculus<->M. caroli* (Rossant *et al.,* 1982).

Although creation of interspecific mammalian chimaeras is indeed a spectacular experiment, in the author's opinion its contribution to embryology and genetics of mammals has been rather limited and disappointing. Perhaps we have not perceived yet the real potential applications of this experimental system. One of the reasons for undertaking these experiments in the past was the possibility of exploiting gross antigenic differences between species as markers in cell lineage studies. However, for these particular studies the ideal experimental system is such in which the two cell populations differ as little as possible, preferably just by one neutral trait. This can be achieved now in intraspecific systems, by using cells (embryos) carrying a single genetic construct like LacZ or GFP (Green fluorescent protein).

'Rescuing' chimaeras

By 'rescuing' chimaeras I mean animals in which the normal diploid component enables prolonged survival and differentiation into manifold tissues including germ line of cells derived from another embryo which because of its wrong imprint, lethal mutation, aneuploidy or polyploidy, would not be able to develop into viable foetus or individual. A good example are diploid/diploid chimaeras composed of a normal (i.e., zygotic) component and a parthenogenetic (Stevens *et al.,* 1977; Surani *et al.,* 1977; Stevens, 1978), gynogenetic (Anderegg and Markert, 1986) or androgenetic constituent (Surani *et al.,* 1988; Mann and Stewart, 1991).

As another example of rescuing chimaeras I may mention diploid/triploid mouse chimaeras which we have produced recently in our laboratory (Tarkowski *et al.,* manuscript in preparation).

Ralph Brinster and his chimaeras

Although the contribution of Ralph and his collaborator Laila M. Moustafa to experimental chimaerism in mammals has been referred to above on one or two occasions, it deserves a separate mention and a comment. In two papers published in 1972 Moustafa and Brinster described investigations on the fate of chronologically older (5.5-, 8- and 12-day) cells injected into blastocysts. Chimaerism was evaluated on the basis of the pigmentation of eyes in 15-17-day foetuses and of eyes and coat in neonates. In the variant '5.5-day donor cells -> 3.5-day recipient blastocysts' the incidence of chimaerism was reasonably high (15.4% among foetuses and 12.6% among neonates, including both dead and alive embryos and animals), declined to 3.4% among foetuses when cells from 8-day embryos were injected into 4-day blastocysts, and dropped to nought when cells from 12-day embryos were transplanted. No chimaeric neonates were recorded following transfer of cells from 8 day embryos. The reliability of these results is strengthened by the large

[3)] However, bird embryologists can produce chimeras closely resembling the mythological creatures, for instance an embryo with a chick head and neck on a quail body (cf. Papaioannou and Dieterlen-Lievre, 1984, p. 17). At least in this particular case the adoption of the mythological term "chimera" for description of the product of the manipulations of contemporary embryologists finds full substantiation, and could not be challenged even by terminological purists.

number of operated blastocysts which varied among experimental group between 125 and 180. Unfortunately the precise origin of transplanted cells (part of the body, tissue) was not known, and such information would be interesting especially in the positive cases. So far this is the only observation that cells derived from embryos as old as 8-day can integrate with recipient's ICM and can give rise to cells that populate the eyes (and probably other tissues). According to Gardner and his colleagues (Gardner *et al.,* 1985) the oldest primitive ectoderm cells (i.e., cells which give rise to all tissues of the embryo proper) that contributed to chimaeric animals were from the 5th day implanting blastocysts, and this finding would be in agreement with the results of 5.5->3.5-day series in Moustafa and Brinster's experiments. However, not even one case of chimaerism was observed following transfer of the embryonic ectodermal cells from the delayed implanting blastocysts and from the 6- and 7-day implanted embryos (Gardner *et al., loc. cit.).*

In 1974 Ralph (Brinster, 1974) described his experiments aimed at producing chimaeras by injection of CBA-T6T6 bone marrow cells and 129 SvSl teratocarcinoma cells into Swiss albino blastocysts. In the first group (bone marrow) none of the 77 offspring showed pigmentation, in the second group (teratocarcinoma) one animal out of 60 offspring had several small patches of agouti hair. Animals from both groups showed significantly prolonged survival of skin grafts from each donor strain. However, cytogenetic studies did not reveal any CBA-T6T6 cells among ca 2000 chromosome spreads of peripheral lymphocytes (animals belonging to the first group), and the male with patches of agouti hair finally also rejected the graft of the 129 SvSl skin. Not being an immunologist I am not able to comment the prolonged survival of skin grafts in the experimental animals and the significance of this observation. At any rate, this was the first attempt to create chimaeras with a teratocarcinoma-derived component, with one successful case.

Have my expectations and predictions come true?

In my paper published in 1961 I wrote in the introductory part: ... *'Work of this type may contribute,....., to the understanding of the mechanism of normal early development. It should offer, in addition, some new opportunities for research into genetics, developmental genetics and the factors controlling sexual differentiation'*. To be frank, I do not remember what I exactly meant by *'opportunities for research into genetics and developmental genetics'* but in general this prediction turned out to be correct. I do remember, however, what I meant by the rest of that statement. Firstly, evaluation of the regulative capacities of the mouse egg and embryo, and in particular getting insight into the mechanism of determination and differentiation of ICM and trophoblast. Secondly, investigating the effect of the co-existence in the individual of both genetically male and female cells on sexual differentiation (sex phenotype) and the capability of germ cells of one sex to undergo gametogenesis characteristic for the opposite sex. Further studies on mouse chimaeras carried out in our laboratory, and also by Beatrice Mintz and by many other workers have provided valuable and new information on these subjects.

Epigenetic versus preformationist mechanism of the differentiation of ICM and trophoblast

Great easiness with which two or even several embryos can unite even at later stages of cleavage (16-cell or more?) and develop into a perfectly normal giant blastocyst together with the evidence of the lack of cell mingling during the morula-blastocysts transition (Garner and McLaren, 1974) spoke against the idea of early and stable determination of the presumptive ICM and trophoblastic cells. These results were in agreement with the parallel studies on the development of blastomeres isolated at the 2-, 4- and 8-cell stage (rabbit: Seidel, 1952,1960; Moore *et al.,* 1968; mouse: Tarkowski, 1959a,b; Tarkowski and Wroblewska, 1967) and led to the conclusion that the fate of blastomeres during cleavage is labile and depends on their position in the morula (the concept referred to often as 'inside-outside hypothesis'). These first studies carried out mainly on the mouse (see also Mintz, 1964) have been later confirmed with the help of very sophisticated recombination experiments (isolation followed by aggregation) (Hillman *et al.,* 1972; Kelly, 1977) and substantiated also by similar studies in other mammalian species, mainly in the sheep (Willadsen, 1980,1981).

Sexual differentiation of sex chimaeras

The main conclusions of the studies carried out so far are the following: first, sex chimaeras develop mostly into fertile males, and true hermaphroditism is a surprisingly rare event (Tarkowski, 1961,1963,1964b; the results obtained by others up to 1975 are summarized in McLaren, 1976). Second, some XX/XY males (and probably the majority of them) pass during the foetal life through the hermaphroditic state with their gonads being ovotestes; the ovarian parts must disappear later and the normal male phenotype develops (Bradbury, 1987; Jankowska *et al.,* 1992). Third, genetically female germ cells are unable to undergo spermatogenesis (Mintz, 1968; Mystkowska and Tarkowski, 1968,1970) –this is a 'specialite' of mammals, as in non-mammalian vertebrates sex reversal permits the germ cells to undergo gametogenesis characteristic for the acquired sex. Fourth, XX/XY chimaeras develop occasionally into fertile females. Fifth, genetically male germ cells can undergo oogenesis and form functional oocytes (Ford *et al.,* 1975; Evans *et al.,* 1977). Although this is a very rare event, such a possibility has been confirmed on several occasions, most recently by Bronson *et al.,* (1995) in chimaeras produced by injection of genetically male ES cells into genetically female blastocysts.

These two areas of research, i.e., mechanisms of cell determination and differentiation in preimplantation development and sexual differentiation, have been already to a large extent explored and I do not expect that chimaeras can offer here spectacular discoveries, although interesting observations can certainly be made from time to time.

Contribution to genetics and developmental genetics

In recent years the interest in chimaeras boosted again when embryonic stem cells became available (until now mostly in the mouse) and when subtle genetic manipulations at the level of single genes enabled one to modify and produce genetically transformed ES cells. This way of modifying the genome is much more precise and predictable than injection of genes into pronuclei of zygotes, but requires transferring the transformed cells into 'carrier' embryos in order to introduce them (their descendants) into the germ line and to obtain gametes. The chimaeric animals thus produced are needed only as sires of an alien genotype and therefore are useless if they are only somatic and not germ line

chimaeras. In this (rather subservient) role chimaeras will continue to be an important tool in research, especially in genetics and developmental genetics, thus confirming my hopes expressed in 1961 (see above), although at that time I had not even the faintest idea of embryonic stem cells, homologous recombination, gene targeting, etc.

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