J. Reprod. Fert., Suppl. **34** (1987), 251–259

Printed in Great Britain

Use of chimaeras to study development

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Introduction

In Greek mythology a chimaera was a fire-breathing she-monster having a lion's head, a goat's body and a serpent's tail. Chimaeras have been used extensively as models for research in developmental biology under the more general definition of a composite animal or plant in which different cell populations are derived from more than one fertilized egg, or the union of more than two gametes (McLaren, 1976). This paper is limited to chimaeras produced by combination of cells from two or more mammalian embryos. Characteristics of chimaeras, methods for production and uses in research are described. Effort has been made to include results of direct relevance to domestic animals. Excellent reviews on mammalian chimaeras and their uses in research are available from McLaren (1976) and Le Douarin & McLaren (1984).

Production of mammalian chimaeras

Manipulations to produce mammalian experimental chimaeras are commonly carried out early in embryonic development, which can lead to extensive chimaerism throughout the body. In some non-mammalian vertebrates, combination of embryos or embryonic cells can result in duplication of body parts while failure to replace completely embryonic cells that have been excised can lead to truncation of body parts. The early mammalian embryo has the ability to regulate its development in such a manner that foreign embryonic cells can be incorporated to produce a morphologically normal individual with two cell lines.

Mammalian experimental chimaeras are usually produced during preimplantation stages by aggregation of two or more embryos (aggregation chimaera) or injection of cells into a blastocyst (injection chimaera). Methods for production of aggregation chimaeras were first described by Tarkowski (1961) and Mintz (1962); these procedures have since been adapted for use in many laboratories. Aggregation procedures have been used to produce chimaeras from 2-cell through to morula-stage embryos. Eight-cell stage embryos are often used, however, because their geometric configuration allows greater contact between embryos than is achieved with earlier stage embryos, and by the morula stage tight junctions between blastomeres reduce the likelihood that aggregation will occur. Zona-free embryos are either placed in contact with one another and pushed together or placed in medium containing phytohaemagglutinin (Mintz *et al.*, 1973) or antibodies (Palmer & Dewey, 1983) to facilitate adhesion. Composite embryos are usually left in culture until they reach the blastocyst stage and then are transferred to the reproductive tracts of recipients for development to term. In domestic animals in-vitro culture systems for cleavage-stage embryos are not well developed and aggregated embryonic cells may be incubated for several days in a ligated oviduct of a temporary host before transfer to a recipient for development to term (Fehilly *et al.*, 1984a) or transferred immediately to a recipient (Brem *et al.*, 1984). Blastocysts produced by aggregation of embryos are larger than normal, but regulation of size occurs during gestation so that chimaeras have normal birthweights.

Production of injection chimaeras was first described by Gardner (1968). Simplified procedures for production of injection chimaeras have been published for use with embryos of the mouse

(Moustafa & Brinster, 1972), rabbit (Babinet & Bordenave, 1980) and sheep (Butler, 1986). Cells injected into blastocysts for production of chimaeras most often originate from the inner cell mass (ICM) of another blastocyst. Solter & Knowles (1975) developed an immunosurgical procedure for isolation of relatively intact ICMs. Other cell types that have been used to produce injection chimaeras include cleavage-stage blastomeres (Tucker *et al.*, 1974) and embryonal carcinoma cells (Brinster, 1974). A modification of the procedure for blastocyst injection has been described in which the ICM of one blastocyst is completely replaced with that of another blastocyst (Gardner *et al.*, 1973; Papaioannou, 1982), a procedure referred to as reconstitution of blastocysts. This procedure has been used for induction of successful interspecific pregnancy in which the fetus is surrounded by placental membranes of a different species (Rossant *et al.*, 1983a).

The laboratory mouse has been the species of choice for production of experimental chimaeras, but they also have been produced in the rat (Mayer & Fritz, 1974; Weinberg *et al.*, 1985), rabbit (Gardner & Munro, 1974; Babinet & Bordenave, 1980), sheep (Tucker *et al.*, 1974; Fehilly *et al.*, 1984a; Butler *et al.*, 1985), and cow (Willadsen, 1982; Brem *et al.*, 1984). Viable chimaeras have been produced also when embryos were combined from different species. *Mus musculus*←→*Mus caroli* chimaeras, produced from the domestic laboratory mouse and a South East Asian wild mouse (Rossant & Frels, 1980), will be discussed later. Embryos from *Bos taurus* and *Bos indicus*, two species of cattle that readily hybridize and produce fertile young, have been combined; although no overt chimaeras were produced, one individual had evidence of chimaerism of internal tissue (Summers *et al.*, 1983). Combination of embryos from sheep and goats, *Ovis aries*←→*Capra hircus*, has also lead to viable chimaeras (Fehilly *et al.*, 1984b; Polzin *et al.*, 1986). Chimaeric embryos have been produced from the mouse and bank vole (Mystkowska, 1975) and the mouse and rat (Gardner & Johnson, 1973; Stern, 1973; Zeilmaker, 1973). For these latter combinations normal post-implantation development failed when chimaeric embryos were transferred to the reproductive tracts of one of the parent species. Gardner & Johnson (1975) reported the birth of rat←→mouse chimaeras, all of which were dead at birth or died soon thereafter.

Characteristics of chimaeras

Perhaps the most consistent feature of experimental chimaeras produced from preimplantation mammalian embryos is their variability. Each chimaera is unique, which can be an advantage or a disadvantage for their use in research. Although some degree of control can be exercised over the genotype of the placenta relative to that of the fetus when embryonic cells are combined (Rossant *et al.*, 1982; Fehilly *et al.*, 1984b; Meinecke-Tillman & Meinecke, 1984), cell mixing that occurs during early development and allocation of cells to development of different tissues make it impossible to predict or control the exact cellular distribution of components in a chimaera. Falconer & Avery (1978) described a nearly flat distribution in the frequencies of chimaeras having various proportions of cells from two component lines. Furthermore, genotype of the component lines may influence the extent to which each line is represented in a chimaera (Mullen & Whitten, 1971), from relatively equal representation of the two lines over the population of chimaeras (balanced chimaeras) to the predominance of one line (unbalanced chimaeras). Even in balanced combinations, individual chimaeras may vary from an almost complete exclusion of one line to an almost complete exclusion of the other line. Chimaerism can occur in any and all tissues and organs of the body. In one series of chimaeras, Falconer *et al.* (1981) correlated the degree of chimaerism observed between organ pairs and found correlation coefficients that ranged from 0·37 to 0·89 and an average correlation coefficient of 0·73. For these chimaeras, there was a relatively strong relationship between the degree of chimaerism observed in different organs, even though each chimaeric individual may have differed markedly from another.

The sex ratio of a population of experimental chimaeras often deviates from 1:1. A combination of two female embryos produces a female chimaera; two male embryos yield a male chimaera. The

combination of a male and a female embryo, which is expected to occur approximately 50% of the time, is responsible for deviation from the normal sex ratio. In general, these sex chimaeras are phenotypically normal males, which results in a sex ratio in chimaeras of about 75% males and 25% females in 'balanced' strain combinations. However, a more normal 1:1 sex ratio has been reported to occur in 'unbalanced' strain combinations (Mullen & Whitten, 1971). Some XX/XY chimaeras do not differentiate as normal males, but rather as hermaphrodites or normal females (McLaren, 1984b). McLaren (1984b) summarized data on sex of known XX/XY chimaeras from various studies and reported that 22% were females, 7% were hermaphrodites and 71% were males.

Just as chimaerism can occur readily in somatic tissues, germ cell chimaerism is often observed in XX/XX and XY/XY chimaeras. The use of genetic markers in breeding trials has demonstrated that XX/XX female chimaeras are capable of ovulating oocytes of both genetic lines; likewise, XY/XY males can produce both types of spermatozoa (reviewed by McLaren, 1984a). Germ cell chimaerism in XX/XY mammalian sex chimaeras usually does not occur. In some non-mammalian vertebrates differentiation of germ cells depends not on their genetic sex, but rather on the sex of the gonad into which they migrate (Blackler, 1965). In mammals, however, only XY germ cells appear to be capable of developing into spermatozoa; XX/XY chimaeric males are fertile, but produce spermatozoa only from the XY line. Whether or not XY germ cells can develop into viable oocytes is less clear. Offspring of XX/XY females usually develop from XX germ cells. One exception has been reported in which a mouse was born from an XY germ cell in an XX/XY chimaeric female (Ford *et al.*, 1975). A complete discussion of development of germ cells in chimaeras is presented by McLaren (1984a).

Uses of experimental chimaeras in research

The value of chimaeras in studying development and tissue interactions is based on the ability to distinguish between the two genetically different cell populations within the animal. McLaren (1976) described the ideal cell marker to distinguish one chimaeric component from the other as being "cell-localized, cell-autonomous, stable, distributed universally among both the internal and external tissues of the body, and easy to detect, both grossly and in histological sections, without elaborate processing. No such marker exists." Despite the lack of an ideal marker, many useful markers are available. For a detailed review of available markers for use with chimaeras the reader is referred to West (1984).

Studies of normal development

Chimaeras have been used extensively to study the course of normal embryogenesis and several examples are presented here. Genetically marked cells introduced into an embryo and followed through development have been used in the analysis of cell lineage. For example, the fates of ICM and trophectoderm have been examined using reconstituted murine blastocysts in which an ICM was injected into a trophoblastic vesicle (Papaioannou, 1982), rat←→mouse chimaeras (Gardner & Johnson, 1975; Rossant, 1976) and *M. musculus*←→*M. caroli* chimaeras (Rossant *et al.*, 1983b). Chimaeras have also been used to determine the minimum number of clones responsible for development of various tissues. In 1976, McLaren (p. 33) stated, "No instance has been found of a tissue or organ, however small, which in a chimaeric animal is always formed of one component only. This implies that it is groups of cells, rather than single cells, which are directed towards particular developmental pathways and this gives rise to particular tissues and organs." Chimaerism has been found in essentially all tissues studied, which demonstrates that at least two embryonic cells contribute to the formation of most tissues and organs of the body. Analysis of results from chimaeras has also led to the conclusion that only a few cells of the blastocyst contribute

to formation of the embryo proper; all other cells contribute to extraembryonic structures. Circumstantial evidence to support this hypothesis comes from observations that injection of only a single cell into another embryo can produce extensive chimaerism in the resulting offspring (Ford *et al.*, 1975; Illmensee & Mintz, 1976). Mintz (1970) proposed that the number of cells in the blastocyst that form the embryo is only three. This hypothesis was based on the observation that, in about 25% of the individuals produced by aggregation of two embryos, only one component cell line is expressed. Markert & Petters (1978) demonstrated that at least 3 cells are allocated to produce the embryo when they produced a triply chimaeric mouse by aggregation of 3 embryos. They pointed out that their results set a lower but not an upper limit on the number of cells that originally contribute to the embryo. The same authors (Petters & Markert, 1980) subsequently reported the production of an aggregation chimaera that expressed four different genotypes, which might argue that 4, not 3, cells of the blastocyst contribute to the embryo. It may be argued also, however, that allocation of cells in an embryo that is four times normal size may be different from that in a single embryo. Recent results have set at 8 the maximum number of cells from which the embryo is derived (Soriano & Jaenisch, 1986).

Another interesting question raised from research with experimental chimaeras is the origin of primordial germ cells that colonize the gonad during early development and ultimately give rise to gametes. From work with non-mammalian species primordial germ cells have been shown to develop from yolk sac endoderm and a similar origin has been assumed for mammalian primordial germ cells. Two lines of evidence indicate that these cells are derived from ectoderm in mammals rather than from endoderm. Falconer & Avery (1978) showed that germ cell chimaerism is positively correlated with chimaerism of somatic tissues, which suggests a similar origin. More conclusively, Gardner & Rossant (1979) showed that ectoderm but not endoderm that is injected into blastocysts contributes to germ cell lineage of the resulting chimaeras.

Study of abnormal development

Experimental chimaeras have also been useful in the study of certain developmental anomalies. Again, several examples will be given to illustrate how the model is used. When mouse chimaeras were produced from normal embryos and embryos from a strain affected by muscular dystrophy, their phenotypes were normal despite the presence of defective genes within multinucleate muscle cells (Peterson, 1974). Similar results have been obtained with bovine chimaeras in which one embryo was homozygous for the double-muscling gene (R. B. Church, personal communication). Normal embryonic cells are also capable of directing the development of parthenogenetically activated ova. While parthenogenesis can be readily induced in mammals, in no case has development proceeded to term. Normal murine aggregation and injection chimaeras containing cells of parthenogenetic origin have been produced, however, when parthenogenetically activated embryos were combined with normally fertilized embryos (Surani *et al.*, 1977; Stevens, 1978). Chimaeras produced by Stevens (1978) produced germ cells from both the normal and parthenogenetic line, which demonstrated that parthenogenetically activated ova retain the ability to contribute to both somatic and germ cells.

An extreme example of the ability of normal cells to direct development of abnormal cells in a chimaera is the reversal of malignancy of embryonal carcinoma cells, which are stem cells derived from a teratocarcinoma. Embryonal carcinoma cells show morphological and biochemical similarities to pluripotent embryonic cells, possess the ability to proliferate indefinitely in the undifferentiated state and, under the appropriate conditions, will differentiate in a more or less orderly fashion (Rossant & Papaioannou, 1984). The most dramatic examples of their differentiation are injection into blastocysts (Brinster, 1974; Mintz & Illmensee, 1975; Papaioannou *et al.*, 1975; Stewart & Mintz, 1981) and aggregation with embryos (Stewart, 1982). Under these conditions embryonal carcinoma cells have the ability to contribute to development of normal tissues and organs of a chimaeric individual. Furthermore, colonization of the germ cell line can occur so that

the resulting chimaeras produce gametes that contain haploid genotypes from the embryonal carcinoma cell line. Recently, pluripotential cell lines have been derived in culture from embryos (Martin, 1981; Magnuson *et al.*, 1982; Kaufman *et al.*, 1983; Axelrod, 1984; Wobus *et al.*, 1984a). These cell lines are usually referred to as embryonic stem cells and resemble stem cells derived from teratocarcinomas, but in many respects may be more like pluripotential cells from morulae or early inner cell masses than they are like teratocarcinoma stem cells (Rossant & Papaioannou, 1984). Embryonic stem cells will readily be incorporated into chimaeras and will colonize the germ line at substantially greater frequency than will teratocarcinoma stem cells (Bradley *et al.*, 1984). Of particular interest in domestic livestock is development of embryonic stem cells for incorporation of foreign DNA. The feasibility of DNA transformation of embryonic stem cells has been demonstrated (Wobus *et al.*, 1984b). Theoretically, it may be possible to insert foreign DNA into a stem cell, perhaps screen the transformed cell line for appropriate incorporation of the gene, and introduce the desired gene into the germ cell line of a chimaera (reviewed by Stewart, 1984).

Study of expression of quantitative genetic traits

Most production traits of economic importance in livestock are under the control of many genes, the contribution of each being difficult to identify. Improvement of production traits has resulted from selective breeding, but the physiological basis for genetic differences is not always clear. Experimental chimaeras have been used on only a limited basis in the study of expression of quantitative production traits. Falconer *et al.* (1981) produced aggregation chimaeras from strains of mice selected for large and small body size and unselected controls. Body weight was linearly related to the mean cell proportions of each cell line within an individual chimaera. Correlation coefficients of body weight to chimaerism in blood, liver, lung, spleen, spinal cord, brain, pituitary gland, kidney, adrenal gland and testis indicated that none of these organs had a predominant influence on growth. In our laboratory male aggregation chimaeras from a line of mice selected for rapid post-weaning growth and a line of mice with a normal rate of growth exhibited a linear relationship between rate of growth and proportion of cells from the rapid-growth line (estimated from coat-colour chimaerism) only up to 50% contribution of the rapid-growth line. Animals with greater than 50% contribution of the rapid-growth line had a mean growth rate similar to that of the selected rapid-growth line (unpublished observations), suggesting that diffusible gene product(s) from the rapid-growth line cells affected growth of tissues of both genotypes. Reproductive performance of experimental chimaeras produced from genetic lines of mice that differ in litter size has also been studied (Craig-Veit & Anderson, 1985). Reproductive characteristics were compared for line crosses having half the genetic information of each line in all cells and chimaeras having the full genetic complement of the two lines in different cells of the body. For most traits, means for crossbreds and chimaeras were similar, regardless of whether means were at or above the mid-parent average. In contrast, for ovulation rate and body weight, genetic crossbreds and chimaeras clearly differed, with chimaeric females being similar to the high litter size line and crossbred females exhibiting additive inheritance. Results from studies of the cell types found in ovarian follicles of chimaeras produced from selected lines that differ in ovulation rate suggested that follicles composed of cells of a high-ovulation line may be preferentially recruited for growth and ovulation over follicles composed of cells of the unselected line (unpublished observations). These results may explain why chimaeric females produced from two lines that differ in ovulation rate have an ovulation rate characteristic of the high-ovulation line (Craig-Veit & Anderson, 1985) and may further suggest that increased ovulation rate resulting from selection for large litter size was due to changes at the level of the ovary.

Barriers to interspecies reproduction

An extensively studied model for interspecific pregnancy has been the *M. musculus*$\leftarrow\rightarrow$*M. caroli* chimaera (reviewed by Rossant *et al.*, 1983a). *M. musculus* and *M. caroli* do not readily produce

viable hybrids (West *et al.*, 1977, 1978; Frels *et al.*, 1980). Likewise, *M. caroli* embryos fail to develop in the *M. musculus* uterus, even when accompanied by *M. musculus* embryos that develop normally (Croy *et al.*, 1982, 1985). Immunization of *M. musculus* recipients with *M. caroli* lymphocytes promoted failure of interspecific pregnancy, but transfer of *M. caroli* embryos to T cell-deficient or NK cell-deficient *M. musculus* recipients or treatment of *M. musculus* recipients with cyclosporin A or anti-Ia antiserum failed to prolong survival (Croy *et al.*, 1985). *M. caroli* embryos can be protected from the *M. musculus* uterus, however, by construction of *M. musculus*←→*M. caroli* chimaeric embryos. When *M. caroli* ICM were injected into *M. musculus* blastocysts that were then transferred to the uteri of *M. musculus* recipients, viable interspecific chimaeras were produced (Rossant & Frels, 1980). *M. musculus* ICM transplanted to *M. caroli* blastocysts failed to survive in the *M. musculus* uterus (Rossant *et al.*, 1982). Final proof of the protection of *M. caroli* fetuses by *M. musculus* trophoblast was provided by Rossant *et al.* (1983a) who transferred *M. caroli* ICM into *M. musculus* trophoblastic vesicles that contained no *M. musculus* ICM cells; a *M. caroli* fetus was carried to term in a *M. musculus* uterus. The importance of trophoblast genotype in maintenance of pregnancy has also been demonstrated in production of sheep←→goat chimaeras and sheep/goat interspecific pregnancies. Aggregation chimaeras have been used to construct ovine fetuses with caprine placentas carried to term in does (Fehilly *et al.*, 1984b) and caprine fetuses with ovine placentas carried to term in ewes (Fehilly *et al.*, 1984b; Meinecke-Tillman & Meinecke, 1984). Injection chimaeras produced by transplanting the ICM of one species into the blastocyst of the other species have also resulted in successful interspecific pregnancies and chimaeras between sheep and goats when genotypes of the trophoblast and recipient uterus were the same (Fehilly *et al.*, 1984b; Polzin *et al.*, 1986). Pregnancies of horse←→donkey chimaeras have also been established (R. L. Pashen, personal communication), but none survived to term.

The importance of genotype of the placenta in maintenance of successful interspecific and chimaeric pregnancies has been clearly established, but the degree of chimaerism that will be tolerated in the placenta is not known. Rossant *et al.* (1982) reported that aggregation *M. musculus*←→*M. caroli* chimaeras, which are expected to have chimaeric trophoblast, survive in the *M. musculus* uterus. Furthermore, participation by *M. caroli* cells in formation of the placenta was confirmed in viable Day-9·5 conceptuses. Unfortunately, chimaeric trophoblast in term pregnancies was not identified.

Germ cell chimaerism has been confirmed in *M. musculus*←→*M. caroli* chimaeras (Rossant & Chapman, 1983). When these chimaeras were mated to *M. musculus* males, some litters contained both *M. musculus* and hybrid offspring, which confirmed that chimaeric females were ovulating both *M. musculus* and *M. caroli* oocytes. Intraspecific chimaeras are known to have allogeneic tolerance of the component lines (reviewed by Matsunaga, 1984) and since *M. musculus*←→*M. caroli* and sheep←→goat chimaeras remain generally healthy, this tolerance apparently extends to interspecific chimaeras. (An interesting exception appears to be the chick←→quail spinal cord chimaera, in which rejection of the grafted tissue later extends to that of the host (Kinutani *et al.*, 1986). Allografts of spinal cord performed between two non-histocompatible strains of chickens, however, produced viable chimaeras in which the foreign neural tissue was permanently tolerated (Kinutani & Le Douarin, 1985). Chick←→quail chimaeras represent more localized grafts of foreign tissue with less subsequent cell mixing than occurs in mammalian aggregation and injection chimaeras.) One might suggest that interspecific mammalian chimaeras are able to carry to term pregnancies of either species. As already mentioned, *M. musculus*←→*M. caroli* chimaeric females mated to *M. musculus* males produced litters of both *M. musculus* and hybrid offspring (Rossant & Chapman, 1983). Somewhat surprisingly, pregnancies were not carried to term when chimaeric females were inseminated with *M. caroli* spermatozoa, suggesting that tolerance may be unidirectional. When embryos of the two species were transferred to *M. musculus*←→*M. caroli* chimaeric recipients, *M. musculus* embryos survived but *M. caroli* embryos did not. Further complicating interpretation of these results is the observation that *M. musculus* embryos can survive in the *M. caroli* uterus. The authors concluded that the primary event in *M. caroli* embryo failure in the *M. musculus* uterus is

incorrect interaction between *M. caroli* trophoblast and *M. musculus* uterine tissue. Support for this hypothesis comes from results in our laboratory with transfer of ovine and caprine embryos to a female sheep← →goat chimaera. Pregnancy was confirmed by ultrasonography at approximately 70 days of gestation and two fetuses were thought to exist. At about 4 months gestation, however, a single ovine fetus was aborted. The fetus appeared normal except for being small for age. Only about 15 cotyledons were present on the allantochorion (unpublished observations). Abnormal placental formation may have resulted from inappropriate interaction between ovine allantochorion and caprine uterine cells. A similar observation was made when *Bos gaurus* embryos were transferred to *B. taurus* recipients (Stover *et al.*, 1981). Two fetuses that went to term in the domestic recipients were reported to have small placentomes and a reduced number of cotyledons. Horse embryos develop to term in donkey recipients more frequently than the reciprocal transfer in which the donkey chorionic girdle fails to invade the endometrium of the mare to form endometrial cups (Allen, 1982). On the other hand, failure of sheep/goat hybrid pregnancies in ewes has been attributed to maternal cell-mediated attack against trophoblastic tissue in the caruncular areas (Dent *et al.*, 1971). Chimaeras with complete genotypes of two different species will be useful for studies of maintenance of fetal allografts.

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