FACTORS THAT INFLUENCE THE COMPOSITION OF MOUSE CHIMAERAS

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DECLARATION

The studies undertaken in this thesis were the unaided work of the author except where acknowledgement is made by reference. No part of this work has been previously accepted for another degree or is being submitted concurrently in candidature for another degree.

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ABSTRACT

By aggregating two cells or two embryos, which carry distinguishable markers, to make chimaeras, it has been possible to investigate the cell distribution to the different tissues in pre-implantation embryos or post-implantation conceptuses. In this thesis, several series of chimaeras were produced to give further insights into the effects of size regulation, cell size, ploidy, cell number and embryo stage on the contributions of two different cell populations to the various cell lineages in mouse chimaeric embryos.

The first study used electrophoretic variants of glucose phosphate isomerase (GPI1-A and GPI1-B) to quantify the contributions of the different cell populations in E12.5 chimaeric conceptuses in both genotypically unbalanced (abbreviated to "U") and balanced chimaeras (abbreviated to "B"), which were (BALB/c × BALB/c) ↔ (BF_1 × TGB) and (AAF_1 × AAF_1) ↔ (BF_1 × TGB) strain combinations, respectively. Four series of chimaeras were made by aggregating two whole 8-cell stage embryos or two half 8-cell stage embryos to test whether size regulation, that occurred in the aggregated chimaera to adjust the body size, provided an acute selection against BALB/c cells to cause the unbalanced composition of series U chimaeras. The results showed that even though size regulation had been inhibited by reducing the total cell numbers at aggregation, this strain combination remained unbalanced. This implied that size regulation did not play the major role in causing the low contribution of BALB/c cells, and it would not account for the genotypic imbalance. Also, this genotypic imbalance found in this strain combination is assumed to arise between E6.5 and E12.5.

In the second study, a β-globin transgene marker was used to analyse cell allocation in chimaeric blastocysts. Two sets of chimaeras were made by aggregating

one big diploid cell (produced by combination of micromanipulation and electrofusion of a 2-cell stage embryo) with one normal-sized cell from a 2-cell stage embryo. These experiments showed that bigger diploid cells (but not less developmentally advanced) made a greater contribution to the trophectoderm (TE) lineages than smaller diploid cells (not more developmentally advanced) at the blastocyst stage. This implied that the difference in cell size among blastomeres in mouse chimaeric blastocysts could underlie differences in cell allocation. Another two series of chimaeras were also produced by micromanipulation and electrofusion to make tetraploid ↔ diploid chimaeras, in which the cells with different ploidy were similar in size. These two series of chimaeras provided further insight of the effect of ploidy on cell allocation in mouse chimaeric blastocysts. The results suggested that differences in ploidy alone could cause non-random cell allocation of tetraploid cells, which resulted in their low contribution to the ICM.

In the third study, five series of chimaeras were produced: B(8+8), B(8+$^{1}/_{2}$8), B($^{1}/_{2}$8+8), B(8+4) and B(4+8), where B is designated as the strain combination described above and the numbers represent the embryo stage, *e.g.* $^{1}/_{2}$8 indicates a half 8-cell stage embryo. Results from this study showed that there was no significant difference in the composition of the epiblast derivatives analysed between series B(4+8) and B($^{1}/_{2}$8+8), or between B(8+$^{1}/_{2}$8) and B(8+4), and the cell populations reflected the cell numbers of contributing embryos at aggregation. It implied that cell number had a greater effect on cell allocation than other factors, *e.g.* embryo stage or cell size, but more detail was discussed in the thesis.

CONTENTS

TABLES

FIGURES

ABBREVIATION

$^1/_2$ cells, $^1/_4$ cells etc.	blastomeres from 2-cell, 4-cell stage embryos and so on
(8+8), ($^1/_2$8+8) etc.	one 8-cell or half 8-cell stage embryo, aggregated with one 8-cell stage embryo
B chimaera series	$(AAF_1 \times AAF_1) \leftrightarrow (BF_1 \times TGB)$
B2n	big diploid cells
B4n	big tetraploid cells
GPI	Glucose Phosphate Isomerase
ICM	Inner Cell Mass
mTE	mural Trophectoderm
pTE	polar Trophectoderm
S2n	small diploid
S4n	small tetraploid cells
Tg	mouse β-globin transgene
U chimaera series	$(BALB/c \times BALB/c) \leftrightarrow (BF_1 \times TGB)$
YsE	yolk sac endoderm
YsM	yolk sac mesoderm

CHAPTER 1

GENERAL INTRODUCTION

1.1 THE DEVELOPMENT OF MOUSE PRE-IMPLANTATION EMBRYOS

Developmental biology is concerned with the transformation from a one-cell fertilised egg to a multicellular and differentiated individual. The development of a fertilised egg involves a single cell dividing to produce millions of cells, which differentiate to form different organs, construct various systems and undertake many diverse functions. The developmental fates of these cells are not pre-determined in the cytoplasm of the fertilised mouse egg, but decided by other epigenetic factors, *e.g.* the position of cells in the embryo and the interactions between cells. In this section, a basic review of mouse embryonic development is considered.

1.1.1. *THE CLEAVAGE DIVISIONS*

The pre-implantation period starts with the first cleavage division of the fertilised egg and ends with the production of the blastocyst (see Fig. 1.1). The lengths of the first few cell cycles of cleavage in mouse pre-implantation embryos differ from each other (Smith and Johnson, 1986; see Table 1.1). It has also been shown that the length of the first cell cycle depends on the genetic background (Krishna and Generoso, 1977; Goldbard and Warner, 1982; Molls *et al.*, 1983; Howlett and Bolton, 1985), an effect influenced by both maternal and paternal genotypes (Shire and Whitten, 1980a, b). Strain differences in mice can cause the time of the first cleavage division to vary by as much as 4-6 hours (McLaren and Bowman, 1973; Niwa *et al.*, 1980; Shire and Whitten, 1980a, b). These differences in cleavage time may be the result of a variety of factors, including the time of:

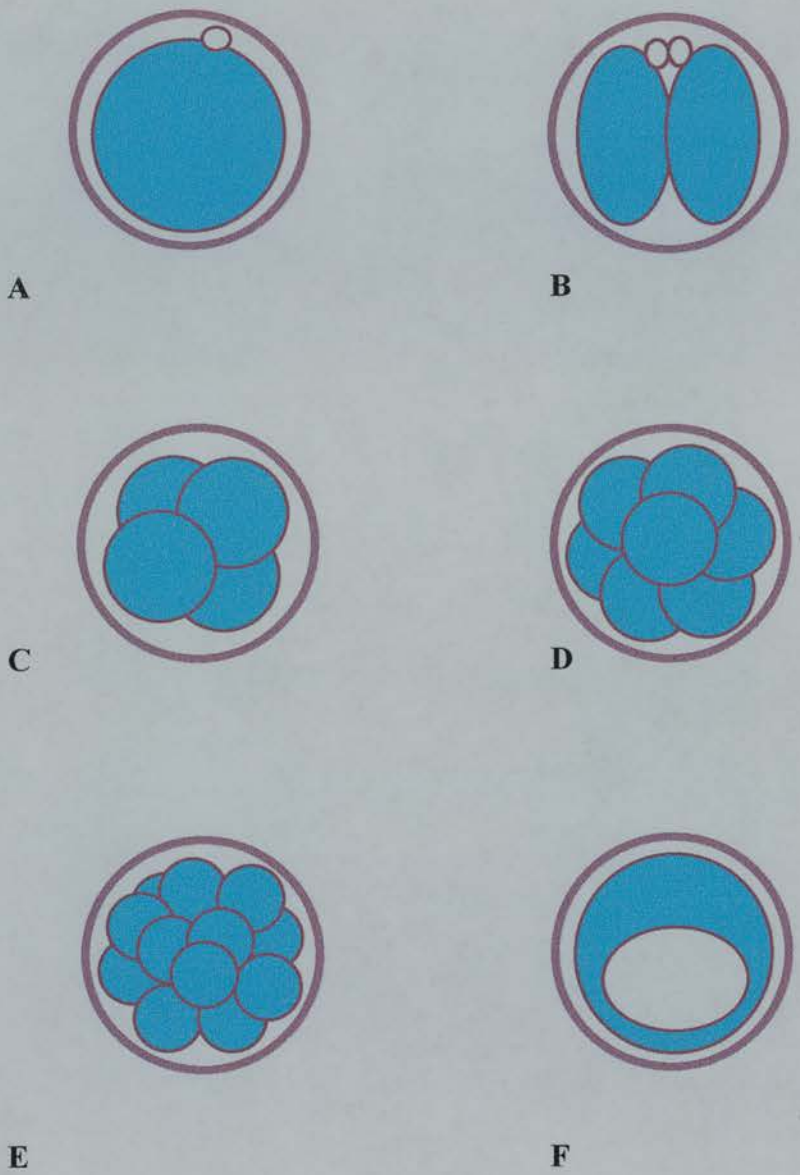

Fig. 1.1 Development of the pre-implantation mouse embryo. The grey rings outside these different stage embryos indicate the zonae pellucidae. **A.** fertilised egg, 1-cell stage; **B.** 2-cell stage; **C.** 4-cell stage; **D.** 8-cell stage; **E.** 16-cell stage; **F.** blastocyst stage.

Table 1.1 The lengths of the first four cell cycles in the mouse embryo

Cell cycle number	Length of phase (hours)		
	G1	S	G2 + M
First cell cycle	4.5 - 12.0	4.0 - 7.0	1.0 - 8.0
Second cell cycle	0.0 - 1.3	4.0 - 7.0	12.0 -18.0
Third cell cycle	1.0 - 1.5	7.0	0.5 - 5.0
Fourth cell cycle	2.0	7.0	1.0 -3.0

(from Smith and Johnson, 1986)

mating, ovulation, meiotic maturation and fertilisation (Iwamatsu and Chang, 1971; Fraser, 1977; Kaleta, 1977; Kasai *et al.*, 1978; Pedersen, 1986). In the first cell cycle, the G1 phase is particularly variable as it is the phase in which meiotic maturation is completed, and when the two pronuclei form near the periphery of the embryo, migrate and lie adjacent to each other in the centre of the embryo (Howlett and Bolton, 1985; Smith and Johnson, 1986; Polanski, 1997). This mouse strain-dependent cell-cycle length is also shown in the second cell cycle (Luthardt and Donahue, 1975; Molls *et al.*, 1983). For example, the second cleavage division in BALB/cGn embryos is 2.4 hours later than that in (BALB/cGn × 129/Rr)F_1 embryos (Whitten and Dagg, 1961). Over this period, the maternal mRNA is largely degraded and embryonic mRNA is synthesized and translated into proteins which are necessary and sufficient to carry development of the embryo to the late 8-cell stage. In contrast, the lengths of cell cycles are relatively invariant between the 4-cell and 64-cell stages and are approximately 10 hours (Pedersen, 1986).

Asynchronous cleavage divisions are not only observed between different embryos, but also within the embryo. From the second cell cycle onwards, blastomeres do not divide at the same time, yielding odd cell-number stage embryos, *i.e.* 3-cell, 5-cell stage embryos, etc. These asynchronous cleavage divisions continue to the blastocyst stage (Graham and Deussen, 1978; Kelly *et al.*, 1978; Graham and Lehtonen, 1979; Smith and Johnson, 1986). The asynchrony is caused by a different second cell cycle length in the two blastomeres of the 2-cell stage embryo. Subsequent cell cycle lengths are similar but the head start of the first-dividing blastomere at the 2-cell stage is retained by it descendants (Kelly *et al.*, 1978).

1.1.2. *THE DEVELOPMENTAL POTENTIAL OF BLASTOMERES*

The developmental capacity of single blastomeres from early cleavage stage embryos has been studied in several mammalian species, *e.g.* the rat, the mouse, the rabbit and the sheep (Nicholas and Hall, 1942; Tarkowski, 1959; Tarkowski and Wroblewska, 1967; Daniel and Takahashi, 1965; Moore *et al.*, 1968; Kelly, 1977; Rossant, 1976; Willadsen, 1981). Single blastomeres from 2-cell stage embryos (hereafter designated as $^1/_2$ cells) can give rise to whole organisms and are thus generally regarded as developmentally totipotent (Tarkowski, 1959; Willadsen, 1980; Tsunoda and McLaren, 1983; O'Brien *et al.*, 1984). It has also been reported that normal, live offspring can be born from $^1/_8$ rabbit blastomeres which had been transferred into foster mothers (Moore *et al.*, 1968). In the mouse, however, although it has been shown that blastocysts and pseudo-blastocysts[1] form *in vitro* from $^1/_4$ and $^1/_8$ blastomeres respectively (Tarkowski, 1959; Tarkowski and Wroblewska, 1967; Rossant, 1976), live offspring were not obtained after being transferred to foster mothers (Rossant, 1976). The cause of this failure was suggested to be insufficient cells in the ICM, since the formation of the mouse blastocyst is not related to the number of cells, but the number of nuclear divisions (Smith and McLaren, 1977). However, $^1/_4$ and $^1/_8$ mouse blastomeres have been shown to be totipotent in experiments in which the progeny of these cells contributed to both lineages of the ICM and trophectoderm (TE), when they were aggregated with a fertilised, parthenogenetic or tetraploid embryo. These experiments allowed the totipotency of a blastomere to be tested by increasing the number of the ICM cells at the blastocyst stage (Kelly, 1977; Tsunoda *et al.*, 1987; Tagami, 1993; Pinyopummin *et al.*, 1994).

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[1] either they are trophoblastic vesicles without the inner cell mass (ICM) or false blastocysts in which a group of cells that may imitate the ICM contributed to trophoblastic vesicles but were only slightly thicker than the trophoblastic vesicles

It has been suggested that the developmental potential of single blastomeres is progressively decreased during embryonic development (Tarkowski and Wroblewska, 1967; Moore *et al.*, 1968; O'Brien *et al.*, 1984). Unlike sea urchin eggs, that are polarised before fertilisation, the polarity of mouse blastomeres appears transiently at the 2-cell stage and develops finally at the 8-cell stage (Johnson and Ziomek, 1981; Handyside *et al.*, 1987). Two subpopulations of blastomeres, which differ in morphological and behavioural properties, appear at the 16-cell stage (Handyside, 1980; Johnson and Ziomek, 1981; Reeve and Ziomek, 1981; Ziomek and Johnson, 1981). Despite their morphological differences, experiments in which like-cells have been reaggregated have shown that these two subpopulations still contain totipotent cells capable of forming blastocysts and live born animals (Ziomek *et al.*, 1982). The totipotency of mouse 16-cell blastomeres was also demonstrated by injecting one outer cell from a 16-cell stage embryo into an 8-cell stage embryo. The descendants of the outer $^{1}/_{16}$ blastomere contribute to both ICM- and TE-derived tissues (Rossant and Vijh, 1980). Inner cells are also still thought to be totipotent. It has been suggested that mouse embryos have totipotent inner cells until the formation of the blastocyst cavity (Handyside, 1978; Hogan and Tilly, 1978; Spindle, 1978). From the blastocyst stage onwards, the totipotency is reduced to pluripotency (Gardner, 1968; Kato and Tsunoda, 1993, 1995). Therefore, cell commitment does not occur until relatively late in pre-implantation development, after cells have been allocated to different lineages and may involve various genes being switched off (Rossant, 1975a, b; Handyside and Johnson, 1978; Rossant and Lis, 1979).

It has been demonstrated that the nuclei of amphibian somatic cells (epithelial cells) can be re-programmed when transferred to enucleated oocytes. These "reconstituted cells" can then develop into a tadpole (Gurdon *et al.*, 1979). Although many studies have proved the possibility of re-programming nuclei from mammalian pre-implantation embryonic cells (Willadsen, 1986; Smith and Wilmut, 1989;

McLaughlin *et al.*, 1990; Cheong *et al.*, 1993; Keefer *et al.*, 1994; Takano *et al.*, 1997; Tsunoda and Kato, 1997), re-programming DNA from differentiated cells to support complete embryonic development was not successful in mammals until a big breakthrough made by Campbell *et al.* (1996). In these experiments, the donor nuclei came from a cell line derived from a day 9 sheep foetus. The cells were then forced to exit the growth cycle by serum starvation and enter the G0 phase of the cell cycle, a state of quiescence in which all genes are thought to be switched off. Lambs were produced by transferring the cells in this phase to the enucleated oocytes. After fusing the donor cell and enucleated oocyte, activation of the oocyte triggered the genes thus re-programming the DNA. One year later, the cloned sheep, Dolly was produced from an adult mammary gland cell, which had also been arrested at G0, proving the ability of DNA in mammalian somatic cells to be re-programmed (Wilmut *et al.*, 1997; Ashworth *et al.*, 1998; Signer *et al.*, 1998). More recently, mice have been cloned successfully from the nuclei of cumulus cells. This was possible because more than 90% of these cells are in the G0/G1 phases of the cell cycle (Wakayama *et al.*, 1998).

1.1.3. *DIFFERENTIATION*

Cells of the body, as a whole, are the descendants of a single cell, the fertilised egg. All these cells are genetically alike, but they exist in many different phenotypic forms. They are specialised for specific functions through the process of differentiation. Thus, cells switch on or off various genes encoding specific proteins. The inner cell mass (ICM) and trophectoderm (TE) are the first distinguishable cell types in embryonic development and are first seen at the blastocyst stage. After this stage, the fates of these blastomeres are committed, determined and cannot be reversed. The various tissues seen at later stages of development are derived from the four different cell lineages formed at late blastocyst stage: the primitive ectoderm

(pEct; epiblast), the primitive endoderm (pEnd; hypoblast), the polar trophectoderm (pTE) and the mural trophectoderm (mTE) (see Fig. 1.2).

Up to the 8-cell stage, all blastomeres in the mouse embryo are identical to each other (Kelly, 1977). Within a few hours of the formation of the 8-cell stage embryo, the blastomeres undergo the process of compaction. It has been revealed that the cell diversification to form the ICM and TE is influenced by these events (Johnson and Maro, 1985, 1986). During compaction, individual cells become polarised and junctional communication is formed (tight junctional and gap junctional complexes), blastomeres flatten and intercellular contact is maximised (Pratt *et al.*, 1982; Ziomek and Johnson, 1980). The process of compaction is shown in Fig. 1.3.

1.1.3.a. Polarisation

Visible polarisation of the cell surface is preceded by intracellular polarity (Johnson and Maro, 1986). This intracellular polarisation involves the reorganisation of organelles within the cell. This includes the elimination of endoplasmic reticulum in regions of cell:cell apposition and concentration of intracellular clathrin under the apical cell region (Maro *et al.*, 1985). Endocytotic vesicles come to lie in the apical region and the microtubule distribution becomes asymmetrical. This is achieved by decreasing cytoplasmic microtubules near cell contacts with the density of microtubules in the apical part being greater than in the basal half. Microfilaments containing actin concentrate beneath the apical surface (Reeve, 1981a, b; Pratt *et al.*, 1982; Johnson and Maro, 1984; Chisholm and Houliston, 1987; Houliston *et al.*, 1987; Maro *et al.*, 1991). Microvilli at the cell surface are restricted to the external facing pole (apical) which also exhibits an increased ligand-binding ability

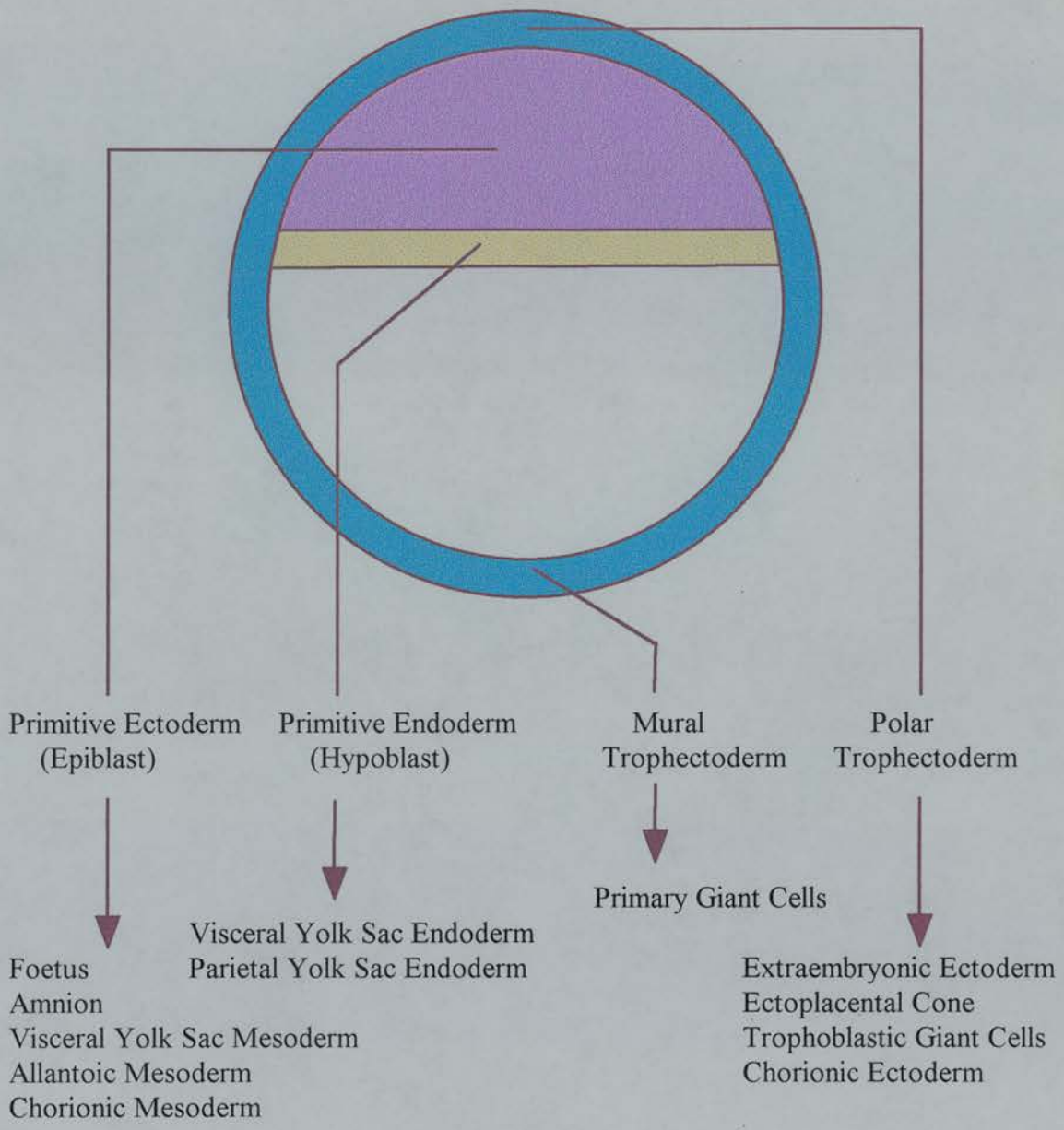


Fig. 1.2 Derivation of the tissues from the four primary cell lineages at the late blastocyst stage. (Adapted from Gardner and Papaioannou, 1975; Hogan *et al.*, 1994; West and Flockhart, 1994).

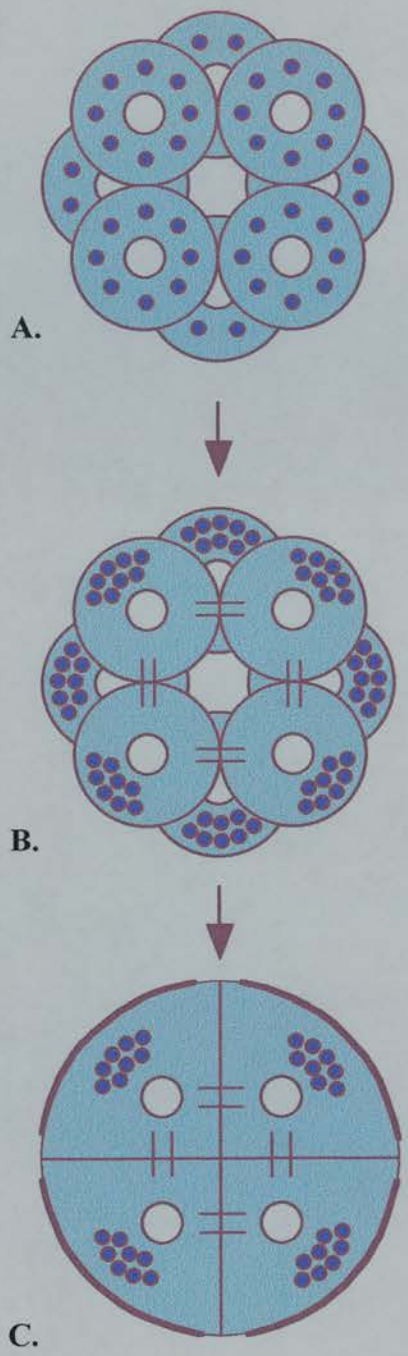


Fig. 1.3 Diagram of the changes during compaction. **A.** 8-cell stage mouse embryo before undergoing compaction, with identical cells; **B.** Intracellular polarisation and formation of junctions; **C.** Microvilli are concentrating on apical surface (cell surface polarisation); cells flatten upon each other to maximise cell contact and gap-junctional couplings form, which result in a polar phenotype cytocortically and cytoplasmically (Adapted from Johnson and Maro, 1986).

(Handyside, 1980; Ziomek and Johnson, 1980; Reeve and Ziomek, 1981).

By the end of the 8-cell stage, the distribution of surface features and the cytoplasmic components of blastomeres are no longer radially symmetrical, but polarised, with the axis of polarity such that the apical pole of each cell is at the most distant position from adjacent cells (Maro *et al.*, 1991). During the formation of the 16-cell stage mouse embryo, each 8-cell blastomere, divides in one of two ways, depending on the orientation of the division plane and the polar axis. It produces either two polar cells (by conservative cleavage) or one polar and one apolar cell (by differentiative cleavage). The cells derived from the apical region are always polar and occupy the outside position of the 16-cell stage embryo. In contrast, the inside cells at this stage are apolar and are derived from the basal region.

1.1.3.b. Flattening and junction formation

An important factor in distinguishing the basolateral surface from the apical surface of the 8-cell blastomeres is blastomere adhesiveness. The basolateral surface is much more adhesive than the apical surface (Kimber *et al.*, 1982). Differences in the intercellular adhesive forces and/or the involvement of intracellular microfilaments and microtubules cause the blastomeres to undergo a calcium-dependent change in shape and increase the area of intercellular contact, thus obscuring intercellular boundaries (Ducibella and Anderson, 1975; Ducibella and Anderson, 1979; Lehtonen, 1980; Hyafil *et al.*, 1981).

In the 2-cell stage mouse embryo, cytoplasmic exchange was achieved via cytoplasmic bridges as these early mouse embryos do not possess gap junctions. One feature of compaction is the formation of junctional communication. Early during compaction, the basal parts of the cells form gap junctions (Ducibella and Anderson,

1975; Lo and Gilula, 1979; Becker *et al.*, 1992). Also, a permeability barrier by tight junctions is formed at compaction, which is essential for blasotocoel cavitation and vectorial transport activity (Ducibella and Anderson, 1975; Ducibella *et al.*, 1975; Magnuson *et al.*, 1978; Fleming *et al.*, 1992).

1.1.3.c. Cell interactions - position effect

It has been suggested that the development of blastomeres follows the "inside-outside" hypothesis (Tarkowski and Wroblewska, 1967). In this hypothesis, cells surrounded by others mainly turn into cells of the ICM whereas descendants of the blastomeres occupying outside positions contribute greatly to the TE at the blastocyst stage. Thus, the developmental fate of blastomeres is determined by their relative position in the embryo. This epigenetic effect was also supported by studies in which a marker drop of silicon oil was injected into the cytoplasm of blastomeres (Wilson, *et al.*, 1972). The results suggested that blastomere development was not predetermined in the egg, but by physical-chemical positional effects. Several other studies also suggested that cells occupying an inner position tend to differentiate into the ICM (Hillman, *et al.*, 1972; Kelly, 1977; Graham and Lehtonen, 1979; Balakier and Pedersen, 1982; Ziomek and Johnson, 1982; Fleming, 1987).

As mentioned above (see 1.1.3.a), during compaction at the 8-cell stage, the blastomeres in the mouse embryo become polarised. At the 16-cell stage two cell subpopulations form: apolar cells in inner positions and polar cells on the outside of the embryo. Therefore, according to the "inside-outside" hypothesis, the fate maps of these two phenotypically different cell subpopulations are set up (Handyside, 1981; Johnson and Ziomek, 1981; Ziomek and Johnson, 1981). However, the final phenotypes and the determined developmental fates of the blastomeres at the 16-cell stage embryo are not yet fixed (Kimber *et al.*, 1982). The phenotype of a 16-cell blastomere can be altered by re-arranging its relative position in the embryo (Ziomek

and Johnson, 1981, 1982; Johnson and Ziomek, 1983). In this way, the original inside apolar cells may become polar and make a contribution to the trophectoderm after being removed to an outer position of the re-constructed 16-cell embryo. Nevertheless, the position effect seems to play a bigger role in guiding the fate of inside apolar cells than in determining the fate of the outside polar cells (Ziomek and Johnson, 1982). It was observed that only 11% of the progeny of a labelled polar cell, that had been enclosed inside the re-constructed 16-cell embryo, were found in both the ICM and TE lineages and 89% were still allocated solely to the TE lineage at the blastocyst stage, whereas 54% of the descendants of the apolar cell were found in the TE lineage after this apolar progenitor had been moved to an outside position in the re-constructed 16-cell embryo. This study implicated a position effect as an important factor in determining the developmental fate of apolar cells, while for polar cells, the phenotype is important (Ziomek and Johnson, 1982). Two possibilities were suggested for the recognition of the differences between the inner and outer cells: the cell surface properties and the cytoskeletal components or organisation. The reassortment may depend on the cell surface properties, such as adhesiveness. The more adhesive cells (inner cells) are predominantly engulfed by the less adhesive cells (outer cells), although the developmental potential of 16-cell blastomeres are dependent on the circumstances in which cells are placed. Also, the differences in cytoskeletal distribution may involve in reassortment. The outer cells may quickly respond to cell contact by flattening and it may prevent them from being engulfed (Surani and Handyside, 1983).

It has been suggested that the effect of position is accounted for by cell interactions (Graham and Lehtonen, 1979). The cell interactions, through the different cell contacts, appeared to be responsible for the allocation of cells to the ICM and the TE of the blastocyst. Cells with more cell contacts tended to be allocated into an inner position. It has been also shown that if a polar cell was

isolated, a high percentage of its next division would be differentiative, producing one polar and one apolar cell (Johnson and Ziomek, 1983). In contrast, if cell flattening has already occurred, the polar 16-cell blastomere shows no differentiative divisions. Therefore, during embryonic development, diversification of cell types is achieved by allocation and maintenance of cells in specific positions (Surani and Handyside, 1983). Also, continuing cell interactions are important for the cells to commit to a restricted developmental fate (Johnson and Ziomek, 1983). Gardner (1989) regarded the "polarisation" and "microenvironmental" hypotheses as complementary rather than opposing ways to account for the origin of the ICM and TE lineages. These two hypotheses indicate the importance of the events of polarisation at compaction and the interactions among cells in the embryo to determine the cell developmental fates.

In summary, the basis of later differentiation is formed by means of blastomere phenotype (*i.e.* adhesiveness), position (Kimber *et al.*, 1982), and enhanced variance between the environments of the cells buried inside and those exposed to the outside (Lo and Gilula, 1979). This differentiation gives rise to the ICM and TE, which are derived substantially from the apolar cells occupying inner positions and outer polar cells, respectively.

1.2 EXPERIMENTAL MOUSE CHIMAERAS AND MOSAICS

The definition of a chimaera is "an organism whose cells derive from two or more distinct zygote lineages" (Anderson *et al.*, 1951). Mammalian chimaeras can be divided into two classes: primary and secondary. The difference between them is that the former is formed at a very early embryonic stage and the latter is formed during later postimplantation or postnatal stages by tissue grafting or transplantation. Therefore, all tissues in the body are potentially chimaeric in primary chimaeras but

only one or a few tissues might be involved in the secondary chimaeras. In contrast, the different cell populations in mosaic animals are derived from a single zygote. Thus mosaics originate "as a result of irregularities during the cell cycle" (Ford, 1969).

In the context of developmental biology, experimentally-produced primary chimaera is a powerful tool for investigating events of development, at both the cell and molecular level. In the following sections, different methods of chimaeric embryo production are discussed.

1.2.1. *METHODS OF MAKING CHIMAERAS*

The first successful experimental production of mouse chimaeras was reported in 1961 by using aggregation method (Tarkowski, 1961). Since then other methods have been introduced including microinjection and co-culture (Bradley, 1987; Prather *et al.*, 1989; Peli *et al.*, 1996).

1.2.1.a. Aggregation

In this method, two cleavage stage mouse embryos, whose zonae pellucidae have been removed by mechanical means, enzyme digestion or acid treatment (Tarkowski, 1961; Mintz; 1962; Nicolson *et al.*, 1975), are placed in a small drop of culture medium and pushed together (see Fig. 1.4 A). The result is one double size cleavage embryo. Aggregated chimaeras have been used widely (Mintz, 1964; Mann and Stewart, 1991; Pinyopummin *et al.*, 1994). Adhesion of the embryos can be facilitated by exposure to phytohaemagglutinin (PHA) to make aggregation more efficient (Mintz *et al.*, 1973; McLaren, 1976; Pratt, 1987).

An attempt to introduce cultured cells into chimaeras by aggregation, rather than microinjection was performed by Stewart (1980) and Fujii and Martin (1980),

which is called "sandwiching". A clump of embryonal carcinoma (EC) cells or embryonic stem (ES) cells is placed between two cleavage stage zona-free embryos, as shown in Fig. 1.4 B. This method has been used to investigate the developmental potential of EC or ES cells in many studies (Stewart, 1980, 1982; Fujii and Martin, 1983; Bradley, 1987; Nagy *et al.*, 1990).

1.2.2.b. Microinjection

Microinjection of cells into the blastocyst cavity was first performed by Gardner (1968) and has since become a popular method to generate chimaeric animals (Gardner, 1972; Polzin *et al.*, 1986; Wilson *et al.*, 1993; Moens *et al.*, 1993). Under the microscope, the host-embryo is held firmly in a holding pipette. The injection pipette with the donor-cells is pushed through the zona pellucida into the cavity. The donor-cells are then expelled close to the inner cell mass (Fig. 1.5).

This microinjection method is an efficient way of producing chimaeras. However, the equipment is expensive and the method is difficult and time consuming, requiring an extended period to develop the skills necessary for successful manipulation.

1.2.2.c. Co-culture

By either sandwiching or injection, ES cells are introduced into other embryos. Alternatively, a high frequency of germ-line chimaeras can be produced by co-culturing the zona-free embryos with ES cells (Wood *et al.*, 1993a, b). The ES cells and feeder cells are trypsinised to obtain a single-cell suspension. The two cell types are separated either by their difference in adhesiveness to the plastic dishes or by gravity. The supernatant containing the ES cells is resuspended to an appropriate concentration. Five to ten zona-free embryos are then placed into droplets of this ES

cell suspension (15 μl) for 3-4 hours, as shown in Fig. 1.6 A. A modified method was reported by Khillan and Bao (1997). They used microwells, constructed by pressing a blunt end of a glass Pasteur pipette against the bottom of a 35-mm petri dish, to conduct the co-culture technique. Only one zona-free embryo was put into each well containing a few microliters of the ES cell suspension. This had the added benefit of preventing the embryos from forming a clump (see Fig. 1.6 B).

This method offers several advantages. Firstly, unlike aggregation or microinjection, it can handle massive numbers of embryos simultaneously and therefore reduce the manipulation time. Secondly, it does not require specific instruments or specialised skills.

1.2.2. *CELL MARKERS*

The previous section discussed how chimaeras are made from two distinct cell types. In order to make use of chimaeras as a tool, a cell marker to distinguish the different cell types in the chimaera is important later in analysis. For this reason, suitable cell markers are necessary and related to the purposes of making the chimaera or manipulated embryos.

It has been suggested that an ideal cell marker applied to distinguish cells in a chimaera should meet certain criteria (McLaren, 1976). These criteria require that marker: (1) is cell localised, *i.e.* will not be secreted from the cells; (2) is cell autonomous, *i.e.* will not transfer between cells or affect other cells; (3) is stable both within the first marked cells and all their descendants; (4) is distributed in all tissues throughout development; (5) is easy to detect, without elaborate processing; (6) is genetically polymorphic. In addition, it should be developmentally neutral, not causing cell selection or influencing developmental processes, such as cell mixing (Oster-Granite and Gearhart, 1981; West, 1984).

Fig. 1.4 Two methods of aggregation chimaera production. **A.** one pair of zona-free cleavage stage mouse embryos (normally at the 8-cell stage) are pushed together to form one complete embryo; **B.** a clump of cells, EC or ES cells, is sandwiched between two embryos.

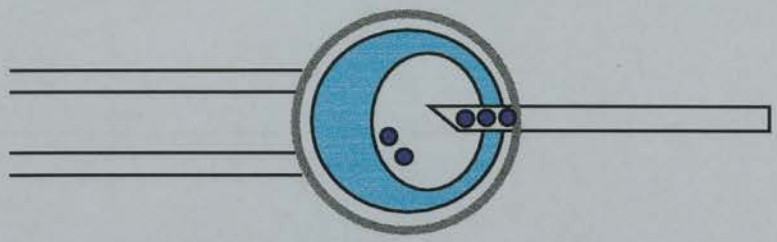


Fig. 1.5 Microinjection chimaera production. A host-blastocyst (the blue area) with zona pellucida (the grey ring) is held by a holding pipette and the donor cells (the purple circles) are injected into the blastocyst cavity (the white area).

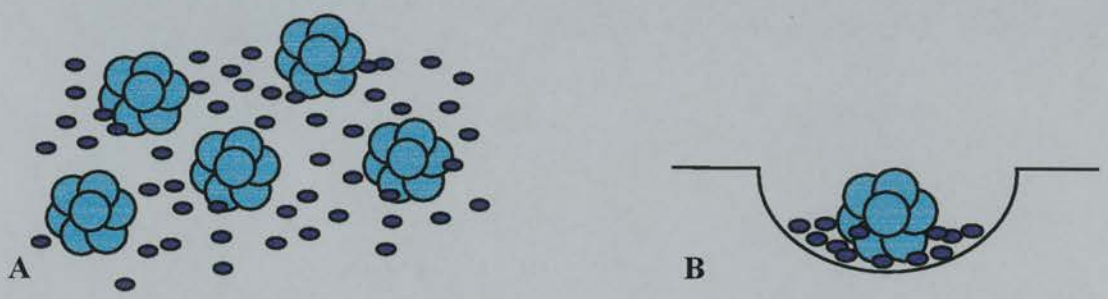


Fig. 1.6 Two methods of co-culture chimaera production. **A.** a few zona-free embryos are placed on the bed of ES cells (the purple circles); **B.** one zona-free embryo is placed into one well containing ES cells.

Some of the cell markers are suitable for using in non-chimaeric or re-constructed embryos, *e.g.* exogenous markers, to observe the development of the labelled cell within the embryos, whereas others are used in chimaera experiments. Several cell markers that have been applied in the past and the present will be discussed below.

1.2.2.a. Exogenous markers

Attempts at analysing cell fate in the intact early mouse embryo have made use of cell markers including silicone fluid (Wilson *et al.*, 1972), oil droplets (Graham and Deussen, 1978), horseradish peroxidase (HRP) (Balakier and Pedersen, 1982; Cruz and Pedersen, 1985; Winkel and Pedersen, 1988) and vital dyes such as DiI (Serbedzija *et al.*, 1991; Beddington, 1994). These have been injected into the cytoplasm to allow the development of the marked blastomere to be followed. The phagocytosis of melanin granules by the trophectoderm of blastocysts (Copp, 1979), or endocytosis of fluorescent latex microparticles by isolated blastomeres or intact zona-free embryos (Fleming and George, 1987) has also been used to label cells. Additionally, the developmental fate of blastomeres in re-constituted blastomeres and/or embryos can be investigated by combining untreated cells with tritiated thymidine ([^{3}H]thymidine) labelled cells (Hillman *et al.*, 1972; Garner and McLaren, 1974; Kelly *et al.*, 1978; Spindle, 1982), fluorescent stained cells, *e.g.* FITC (fluorescein isothiocyanate), (Ziomek, 1982; Ziomek and Johnson, 1982; Surani and Handyside, 1983; Surani and Barton, 1984) or nuclear stained cells (DAPI, 4:6-diamidino-2-phenylindole) (Johnson and Ziomek, 1983). Another reagent, trinitrobenzene sulphonic acid, was reported to label cells effectively (Surani and Handyside, 1983). This reagent results in the covalent binding of trinitrophenol (TNP) groups of membrane proteins and certain phospholipids, allowing the marked cells to be detected by an indirect method of immunohistochemistry.

Since it is essential to determine when the spatially distinct blastomeres are first established (the inner and outer cell groups) for understanding the mechanisms involved in the initiation of differentiation, a more accurate method to distinguish the number of cells in inner and outer position in the embryo is required (Handyside, 1981). The technique of immunofluorescence in which the rabbit anti-mouse species antiserum and fluorescein-conjugated goat anti-rabbit IgG were applied, can distinguish the cells in inner and outer positions at the morula stage. By labelling the zona-free morula embryo (which only the outer cells can be labelled) followed by disaggregation, the labelled outer cells and unlabelled inner cells can be separated. Also, by polynucleotide-specific fluorochromes cell positions *in situ* at the blastocyst stage have been visualised and estimates of cell number and position in the late pre-implantation mouse embryo have been made when the trophectoderm cells cannot be disaggregated easily (Handyside and Hunter, 1984).

However, some of the methods are limited in their application. For example, injected silicone fluid or oil droplets are not easily visible in the cytoplasm of later pre-implantation stage embryos and can not segregate into all the descendants of injected progenitor. Also, [^{3}H]thymidine has been reported to have a deleterious effect on embryonic development (Snow, 1973; Kelly and Rossant, 1976), but if the concentration of [^{3}H]thymidine is reduced to one which does not adversely effect development labelled cells can only be followed through two cell divisions without the levels of labelling becoming too weak (Garner and McLaren, 1974).

1.2.2.b. Histological markers

By means of differences in histological appearance, the proportions of distinguishable components in chimaeras can be estimated. These include coat/eye pigment (Gardner, 1968), T6 marker chromosomes (Ford *et al.*, 1975), sex chromosomes (Butler *et al.*, 1987), nuclear morphology of Ichthyosis mutant mice

(Goldowitz and Mullen, 1982), electrophoretic variants of glucose phosphate isomerase (GPI) (Chapman *et al.*, 1972; Gearhart and Mintz, 1972; Oster-Granite and Gearhart, 1981), β-glucuronidase (GUS) (Condamine *et al.*, 1971; West, 1976; Musci and Mullen, 1992) and H-2 antigens (Ponder *et al.*, 1983).

Not all these histological markers are suitable for experiments on every aspect of development. The analysis of spatial distribution of cells in chimaeric embryos or tissues cannot be performed by quantitative cell markers, such as GPI, T6 chromosomes or sex chromosomes. Also, some histological markers are only expressed or appear in specific tissues, *e.g.* the Ichtyosis nuclear marker has only been used to study the postnatal brain. In addition, detection of the cell markers is sometimes dependent on developmental stage, *e.g.* retinal pigmentation begins around E10.5 and is only therefore useful for later foetal analysis.

1.2.2.c. Transgenes

A suitable genetic cell marker which can be visualised in histological sections would be ideal to investigate pre-implantation and postimplantation development. Lo (1983) produced transgenic mice, strain 83, in which chromosome 3 has about 1000 tandem repeats of the mouse *β*-globin gene. This transgene can be detected by DNA-DNA *in situ* hybridisation on histological sections (Lo, 1986; Katsumata and Lo, 1988; Keighren and West, 1993), and has proved to fulfil most of the requirements of an ideal cell marker (West *et al.*, 1996). By aggregating transgenic and non-transgenic embryos together, cell distributions in the chimaeric embryos can be visualised *in situ* (Lo *et al.*, 1987; James *et al.*, 1995; Everett and West, 1996; West *et al.*, 1996).

Another exogenous gene, the *E. coli lac-Z* reporter gene, coding for *β*-galactosidase, has been introduced into embryonic stem cells (ES cells) (Lallemand

and Brulet, 1990) and the mouse embryo (Beddington *et al.*, 1989; Tsukui *et al.*, 1995; Tan *et al.*, 1995). This cytoplasmic marker can be used on whole mount embryos or tissues. The detection of this transgene is done by screening for β-galactosidase activity by X-Gal staining.

More recently, proteins derived from cnidaria, have been used as cell markers. One of these, green fluorescent protein (GFP), is derived from the jellyfish *Aequorea victoria*. GFP absorbs blue light and emits green fluorescence without exogenous substrates or cofactors, like β-galactosidase. It has become a useful marker for chimaeric analysis, because it can be observed directly in living cells. Transgenic mice carrying the GFP coding sequence has been reported (Ikawa *et al.*, 1995). Also, by injecting the RNA of a novel form of GFP, named MmGFP, into one blastomere of a 2-cell stage mouse embryo, the cell fate of this blastomere has been followed directly under a confocal microscope (Zernicka-Goetz *et al.*, 1997).

Although the GFP fluorescence persists in fixed samples (after treatment with formaldehyde) and it is envisaged that GFP can be used as a vital marker (Chalfie *et al.*, 1994). It has been shown that the *C. elegans* GFP fluorescence was interfered with by the chemical in nail polish which is often used to seal cover slips (notes in Chalfie *et al.*, 1994). The standard procedures for embedding and sectioning is thought to interrupt the GFP activity (Hadjantonakis *et al.*, 1998). A comparison among the transgenic markers was summarised by Rossant and Spence (1998) and is shown in Table 1.2.

1.2.2.d. Different species/strains

In addition to those cell markers mentioned above, investigators have attempted to produce chimaeric embryos containing cells from two species and define the cell by genomic differences, *e.g.* goat-sheep chimaeras (Fehilly *et al.*, 1984; Ruffing *et al.*, 1993), rat-mouse chimaeras (Zeilmaker, 1973), or by differences in

Table 1.2 Comparison among transgenic markers[†]

	Multicopy transgene	β-Galactosidase	GFP
Ubiquitous	+	+	?
Neutral*	+	+	+
Cell autonomous	+	+	+
Detectable in intact embryos	?	+	+
Detectable in sections	+	+	?
Single-cell resolution	+	+	+
Simple detection system	No	+	+
Detectable in living cells	No	Partial	+

†: β-galactosidase and GFP are better markers for spatial analysis because they fill the cytoplasm whereas the multicopy transgenes only produce a hybridisation signal in the nucleus.

*: Developmental neutrality has not been tested properly for many transgenic markers.

(from Rossant and Spence, 1998)

satellite DNA sequences, *e.g. Mus caroli*↔*Mus musculus* interspecific chimaeras (Rossant *et al.*, 1983; Rossant and Chapman, 1983). A strain specific antibody which recognises cells derived from C3H strain mice has been also used as a marker in chimaeras (Kusakabe *et al.*, 1988; Yoshiki *et al.*, 1993).

Applications of interspecific chimaeras in lineage studies are limited, due to the incompatibility of growth patterns between species, although the interspecific chimaeras, *Mus musculus*↔*Mus caroli*, have been proved useful as a model for analysis of cell lineages (Rossant and Chapman, 1983). However, they have been used to study recipient-foetal incompatibility of the trophoblast. In addition, many of the techniques involved in detecting inter species or inter strain differences are very elaborate, *e.g.* detection of the C3H strain specific antigen involves fixation by cardiac perfusion.

1.3 APPLICATIONS OF CHIMAERAS AND OTHER EMBRYO MANIPULATIONS

By choosing an appropriate cell marker and method for making chimaeras, various investigations in developmental biology have been approached using experimental chimaeras. The following sections will discuss some of what we have learnt from them.

1.3.1. *CELL LINEAGE AND CELL ALLOCATION STUDIES*

Cell lineage analysis is important in order to determine how cells are allocated to various tissues and to understand the relationship between cell fate and cell determination. By marking cells, individual progenitors and their descendants can be recognised and followed. There has been a massive body of studies which have

investigated cell lineages of early mouse embryos and the origins of tissues by using chimaeras (for reviews see: Gardner, 1983, 1996b; Pedersen, 1986; Rossant, 1986). For example, the origin of cellular populations within the mouse placenta have been studied by this method. These studies involved injecting the ICM into TE vesicles (which showed different GPI activity), and transferring the reconstituted blastocysts to foster mothers (whose GPI activity was again different from both the ICM and TE). The results showed that about 70 % of the E13-15 placenta is trophectoderm-derived, whereas 30 % is maternal in origin and 4 % develops from the ICM (Rossant and Croy, 1985).

Furthermore, the mosaic arrays which form patches in chimaeric animals provide a useful analysis of organogenesis (Iannaccone, 1987; Beddington *et al.*, 1989). For instance, the formation of coat-colour patterns (Tachi *et al.*, 1991) and adrenal cortex (Iannaccone and Weinberg, 1987).

As discussed previously, cell position and/or cell interactions are responsible for cell diversification in mouse embryonic development, thus cell relative positions account for cell lineages. In studies of the relationship between cell position and cell lineages, it was observed that several factors influence cell allocation to the ICM and TE lineages. These are discussed below.

1.3.1.a. Cell division order - embryo stage

As mentioned above, asynchronous cleavage divisions occur from the second cell cycle onwards (Graham and Deussen, 1978; Kelly *et al.*, 1978; Graham and Lehtonen, 1979; Smith and Johnson, 1986). Long term observations have shown that most of the descendants of the first-dividing blastomere of the 2-cell stage embryo continue to divide ahead of those from the later-dividing blastomere. Also, they tended to be located internally (Kelly *et al.*, 1978), implying that the early

dividing cells make a better contribution to the ICM than those dividing later, as the result of a position effect (see 1.1.3. c.).

The influence of the embryo stage was also demonstrated by chimaeras, which were made by aggregating a single blastomere from a 2-cell stage embryo ($^1/_2$ cells) with two other cells from a 4-cell stage embryo ($^2/_4$ cells) to mimic the asynchronous cleavage division after the second cell cycle within the embryos (Surani and Barton, 1984). The resulting chimaeric mouse morulae showed that the descendants of $^2/_4$ cells made similar contributions to the inner and outer cells, but the progeny of $^1/_2$ cell made a significantly greater contribution to the outer cells than to the inner cells. Thus, the inner cells were predominantly the progeny of $^2/_4$ rather than of the $^1/_2$ cell, but there was no significant difference between them in the outer cells. That the more advanced cells greatly contributed to the inner cells was also supported by a further chimaeric study. By aggregating embryos from different stages (one 8-cell stage embryos and three 4-cell stage embryos), chimaeric blastocysts were obtained with a disproportionately higher contribution of the more advanced cells in the inner cell mass (Spindle, 1982).

One possible mechanism responsible for earlier-formed blastomeres contributing preferentially to the inside cell population than the later-formed blastomeres is that the "older" cells undergo the differentiative division more frequently than the "younger" cells do (Garbutt *et al.*, 1987). In this study, 4-cell stage embryos were disaggregated and the cleavage of the separate blastomeres was observed. The first or last pair of blastomeres to divide were labelled. Once all $^1/_4$ blastomeres had divided the embryo was re-aggregated and the labelled cells followed. The results revealed that, at the late 16-cell stage, 75% of inside cells were labelled when the first-formed pair had been labelled and only 36% when the last-formed pair had been labelled. Only the 8-cell blastomeres undergoing the

differentiative divisions can make a contribution to the inner apolar cells. Therefore, by means of preferentially undergoing this differentiative division, the difference in cell division order has an effect on cell allocation. Thus the more advanced cells can make a greater contribution to the inner cells than the less advanced cells.

1.3.1.b. Cell size

The divisions of blastomeres in the cleavage stage embryo differ from those of somatic cells in that there is no net growth in the blastomeres. Not increasing cell mass means that each blastomere halves its size at each cleavage division, until around 120-cell stage when the normal nucleo-cytoplasmic ratio is achieved (Smith and Johnson, 1986). Therefore, cell size is related to developmental stage or cell age. Thus, the more advanced the cell, the smaller the cell. In accordance with the effect of division order the smaller cells will be preferentially allocated into an inner position and make a good contribution to the inner cell mass (see 1.1.3. c. and 1.3.1.a.).

Additionally, in an intact embryo, it was reported that the inner cells at the 16-cell stage embryo are smaller than those in outer positions, even though they were produced from the same cleavage division (Handyside, 1981; Johnson and Ziomek, 1981; Surani and Handyside, 1983). Thus, without the asynchronous division, the difference in cell size occurs spontaneously at the 16-cell stage mouse embryo and the smaller cells tend to be allocated to an inner position. It implies that geometrical efficiency may result in the preferential allocation of the smaller cells to inside the embryo, because it is spatially more efficient to pack blastomeres within the zona pellucida by surrounding the smaller cells with the larger cells.

Therefore, in addition to cell flattening to maximise the cell contacts during compaction (see 1.1.3.b.), for the geometrical reasons, it may also increase cell contacts by pushing the smaller cell into an inner position. By the asynchronous

cleavage division among the blastomeres and the geometrical effect, the difference in cell size plays a role on the allocation of cell to the inner or outer position in the embryo and influences the developmental fate of the blastomeres.

1.3.1.c. Chromosomal abnormality

In mouse tetraploid/diploid mosaics, a disproportionate distribution of two cell populations was observed (Tarkowski *et al.*, 1977). Tetraploid cells were almost eliminated from the foetuses in four out of eight E9.5 and E12.5 mosaics, and in the rest, tetraploid cells also made a smaller contribution to the foetuses than to the extraembryonic membranes.

Also, tetraploid↔diploid mouse chimaeras showed that tetraploid cells were virtually absent from E12.5 foetuses but made a good contribution to the tissues derived from the trophectoderm (TE) and primitive endoderm (pEnd) lineages (Nagy *et al.*, 1990, 1993; James *et al.*, 1995). Two possibilities involved in the uneven distribution of tetraploid cells among the three primary lineages were suggested: tissue-specific selection against tetraploid cells (at the levels of the cell and conceptus) and unequal allocation of tetraploid cells to different developmental lineages (James *et al.*, 1995). It was found that tetraploid cells contributed to the embryonic ectoderm/mesoderm in 2 out of 12 E7.5 tetraploid↔diploid mouse chimaeras, and these two embryos were morphologically abnormal. However, selective death of chimaeras with tetraploid cells in the primitive ectoderm lineage is unlikely to be a major mechanism causing in the absence of tetraploid cells in the primitive ectoderm derivatives at later stages, because there was no evidence for massive embryonic losses (James *et al.*, 1995). Furthermore, the similarity of the distribution of tetraploid cells in E7.5 and E12.5 chimaeras implies that the restricted tissue distribution of tetraploid cells occurs before E7.5 stage.

To test if the restricted distribution of tetraploid cells was a result of tissue-specific cell selection or non-random allocation in the early development, as suggested by James *et al.* (1995), tetraploid↔diploid chimaeric blastocysts were made (Everett and West, 1996). The results showed that tetraploid cells were significantly more abundant in the mural trophectoderm (mTE) than the polar trophectoderm (pTE) region in E3.5 blastocysts and implied that a non-random cell allocation was partly the cause of the restricted tissue distribution of tetraploid cells. In addition, a further investigation, comparing E3.5 and E4.5 blastocysts, revealed that the proportion of tetraploid cells was reduced in the ICM (Everett and West, 1998). Thus, both cell selection and preferential allocation of tetraploid cells to the mTE of the chimaeric blastocysts could contribute to the restricted tissue distribution seen later in gestation.

1.3.1.d. Others

Although in a normal mouse embryo differences in genetic background play no role in cell allocation, in chimaeras genetic background has been shown to have an effect. Thus, the aggregation chimaeras of some strain combinations are consistently genotypically unbalanced in favour of one strain. For example, Mullen and Whitten (1971) showed that cells from the inbred C3HeB/FeJ strain made a poor contribution to the coats of C3HeB/FeJ ↔ (SJL/J × 129/Rr)F_1 aggregation chimaeras and that BALB/c cells tended to contribute poorly to the coats of C57BL/10GnDg ↔ BALB/cGnDgWt chimaeras. BALB/c cells were also poorly represented in mid-gestation BALB/c ↔ (C57BL × CBA)F_2 chimaeric conceptuses (West and Flockhart, 1994). The low contribution of BALB/c cells appears to be partly an effect of the BALB/c maternal genotype because (BALB/c × AF_1) embryos contributed less well to chimaeras than (AF_1 × BALB/c) embryos (West *et al.*, 1995). It implies that cell allocation could be influenced by genetic background. Several

mechanisms responsible for the consistent low contribution of BALB/c cells to the chimaeras were considered by West *et al.* (1995). Although it is possible that BALB/c cells are somehow preferentially allocated to the mural trophectoderm lineage, which contributes little to the mid-gestation conceptus and would have been undetected in the analysis, it seems more likely that generalised cell selection plays the most significant role. For instance, more BALB/c cells might die or their cell cycle might be lengthened preferentially. This system will be examined and discussed further in Chapter 3.

1.3.2. *MODELS FOR HUMAN DISEASE*

About 2% of human conceptuses show "confined placental mosaicism" (CPM) by chorionic villus sampling (CVS) (Ledbetter *et al.*, 1992; Wang *et al.*, 1992). CPM is defined as a difference in the chromosomal constitution between the placental tissues and the embryo or foetus (Kalousek and Dill, 1983). Thus, chromosomally normal and abnormal cells are in the chorionic villus samples but the foetuses are chromosomallly normal. Therefore, a false positive prenatal diagnosis of cytogenetic anomalies may be made by CVS. The reason for the chromosomally abnormal cells being confined to the placenta is unknown, but several possibilities have been proposed (Crane and Cheung, 1988). Abnormal cells that appear late during development may arise preferentially in the trophectoderm lineage. Alternatively, normal and abnormal cells may co-exist at an early developmental stage but by some mechanism abnormal cells may be preferentially allocated to the trophectoderm lineage or selected against in the primitive ectoderm.

In mouse chimaera experiments, combinations were found where one component was almost excluded from the foetus (West and Flockhart, 1994; James *et al.*, 1995). For example, analysis of E7.5 and E12.5 tetraploid↔diploid chimaeras has shown that tetraploid cells tended to be excluded from the foetuses but made a

contribution to the trophectoderm and primitive endoderm derivatives, as described previously (see 1.3.1.c.). The confined chimaerism in specific tissues in specific chimaeric combinations may provide an animal model for CPM (James and West, 1994, James *et al.*, 1995).

1.3.3. *DEVELOPMENTAL EVENTS*

As described in 1.1.2, the blastomeres from the early cleavage stage embryo are totipotent. The developmental potential, however, becomes more restricted gradually. The timing of the loss of blastomere developmental totipotency, and the progressive loss of developmental potential of cells from pre- and postimplantation embryos can be assessed by an analysis of chimaeras in which the progeny of blastomeres are followed (Rossant, 1975a, b; Rossant *et al.*, 1978; Gardner and Rossant, 1979; Cockroft and Gardner, 1987; Kato and Tsunoda, 1995).

During the embryonic development, cell movement in the mouse blastocyst has been demonstrated by labelling trophectoderm cells (Cruz and Pedersen, 1985). The results showed that during blastocyst expansion, the polar trophectoderm cells moved downward to replace some of the mural trophectoderm cells. A further study in which the inner cell mass cells were labelled showed that some of the polar trophectoderm cells were recruited from the inner cell mass cells and the origin of the primitive endoderm cells was from the inner cell mass, not from the trophectoderm lineage (Winkel and Pedersen, 1988). Also, cells were shown to reassort according to their differences in phenotypes, *e.g.* adhesiveness, polarity, etc., in the chimaeric embryos (Surani and Handyside, 1983). In these experiments, it showed that the apolar cell migrated into the inner position of the chimaeric embryo made by aggregating a labelled $^{1}/_{16}$ apolar mouse cell with a 8-10-cell stage mouse embryo after 3-6 hours of culture.

In addition, experimental chimaeras can be used to investigate how and when cells move and mingle together in the early developmental stages to result in the extensive cell mixing seen in the adult (Garner and McLaren, 1974; Kelly, 1979; Dvorak *et al.*, 1995; Gardner and Cockroft, 1998). For example, the study of the formation of spinal cord showed that cell mixing occurs when the spinal cord is forming and it involves a variety of movement and migration of neural plate cells (Musci and Mullen, 1992).

1.3.4 *GENE FUNCTION STUDY*

The importance of maternal and paternal genomes for normal development has been determined in studies of parthenogenetic, gynogenetic, and androgenetic embryos (Barton *et al.*, 1984; McGrath and Solter, 1984; Surani *et al.*, 1984). By aggregating a fertilised mouse embryo with an experimentally-produced parthenogenetic, gynogenetic or androgenetic embryo, the function and timing of gametic imprinting has been investigated (Surani *et al.*, 1977; Thomson and Solter, 1988; Mann and Stewart, 1991).

Analysis of chimaeras with a component which is mutant or carrying a lethal mutant gene provides an opportunity for understanding where a mutant gene is expressed, how it functions, which tissue(s) or cell type(s) are targetted, and where a functional gene is required (Maandag *et al.*, 1994; Quinn *et al.*, 1996; Ciruna *et al.*, 1997), because the mutant component can be rescued by the wild-type component in the chimaeric situation. The application of chimaeras in this type of mutant analysis has been reviewed recently (Rossant and Spence, 1998).

For example, disruption in the *Pax6* genes result in the small eye (*Sey*) mutation in the mouse (Hill *et al.*, 1991). Mice with homozygous *Sey/Sey* die at birth as a result of the failure to form eye and nasal cavities. It is, however, unclear what

developmental role *Pax6* plays to account for the *Sey* phenotype. By aggregating a wild-type embryo with a *Sey* mutant embryo from heterozygous crosses, it was found that mutant cells were excluded from lens and nasal epithelium, and were also eliminated from the retinal pigmented epithelium in the chimaeras. These experiments show that *Pax6* has effects on the nasal epithelium and the principal tissues of embryonic eye, suggesting that *Pax6* has multiple roles in eye and nasal development (Quinn *et al.*, 1996).

1.3.5. *PRODUCTION OF TRANSGENIC OR INBRED ANIMALS*

Microinjection of exogenous DNA into the mouse male pronucleus is the most common way to generate transgenic mice (Gorden *et al.*, 1980; Harbers *et al.*, 1981; Brinster *et al.*, 1985). However, the integration of the injected exogenous DNA into the genome is random and the integration site is not precise. By the technique of gene targeting, ES cells which have had a foreign DNA sequence introduced at a precise site or have an endogenous gene deleted/mutated, can be selected. A germ-line chimaera can then be produced by either microinjection or sandwiching the selected ES cells into or with preimplantation embryos. If the transgene or mutation stably transmits into the germ line, transgenic animals are produced (Gossler *et al.*, 1986; Robertson *et al.*, 1986; Price, 1987). The use of transgenic mice in the analysis of reproductive development and function has been recently discussed by Nishimori and Matzuk (1996).

Furthermore, Surani *et al.* (1977) indicated that the technique of making chimaeras which used parthenogenetic embryos could save time in producing inbred strains, if the parthenogenetic embryos contributed to the germ line. Anderegg and Markert (1986) also confirmed this opinion.

1.4 AIMS OF THIS STUDY

From previous studies of intact or chimaeric embryos, the descendants of the earlier-dividing blastomere tend to be allocated to the inside of the embryo (Graham and Deussen, 1978; Kelly *et al.*, 1978; Spindle, 1982). As a result of this position effect, according to the "inside-outside" hypothesis (Tarkowski and Wroblewska, 1967), the early-dividing cells occupy an inner position at the morula stage and will make greater contribution to the inner cell mass at the blastocyst stage than those occupying outside position. Also, it has been shown that tetraploid cells tend to be eliminated from the epiblast lineage by cell selection and non-random allocation at an early developmental stage (James *et al.*, 1995; Everett and West, 1996). Hence, cell size, timing of cleavage division, embryo stage, relative position in the embryo and chromosomal constitution can affect cell allocation in the preimplantation embryo.

However, the importance of each individual parameter has not been evaluated separately in previous studies. For instance, the more advanced cells were also smaller than the less advanced cells (embryo stage combined with cell size); the number of the descendants of the earlier-dividing cell could be greater than the number of progeny of the later-dividing cell in the embryo (embryo stage combined with cell number) and in experimental tetraploid↔diploid chimaeras, tetraploid embryos produced by electrofusion of two diploid cells were twice the size of diploid cells and had only about half as many as cells as the diploid embryos when they were aggregated together (ploidy combined with cell number).

In this thesis, several series of chimaeras were produced to give further insights into the effect of these parameters on the contributions of two different cell populations to the various cell lineages in mouse chimaeric embryos. In Chapter 3, the influence of the mechanisms, responsible for size regulation, on the unbalanced chimaeric combination was tested. Tetraploid↔diploid chimaeric blastocysts,

produced by micromanipulation and electrofusion, were considered in Chapter 4. The components in these chimaeras differed in either ploidy or cell size, but they had the same cell number and the same embryo stage at aggregation. This has made is possible to test whether ploidy and cell size play a role in the allocation of tetraploid cells in the blastocyst. In Chapter 5, two components which either differed in number of cells but from the same developmental stage (the embryos were disaggregated and different number of cells were chosen) or differed in developmental stage were aggregated together to examine the effect of the cell number and embryo stage. In the final Chapter, the interrelationship of all these factors will be discussed, overall conclusions drawn and possible future work suggested.

CHAPTER 2

MATERIALS AND METHODS

2.1 ANIMALS

2.1.1. *MOUSE STOCKS*

Details of the mouse strains used in these experiments are described in Table 2.1.

The original TgR(ROSA26)26Sor mice were kindly supplied from Babraham by Dr N. D. Allen. This stock carries a novel *lac*-Z reporter transgene encoding a protein, β-geo, with both β-galactosidase (β-gal) and neomycin phosphotransferrase (neo) activities (Friedrich and Soriano, 1991). Cells that carry this transgene turn a blue colour after staining with X-Gal due to the formation of a blue precipitate in the cytoplasm. TGB is a random-bred stock of predominantly (C57BL/Ola × CBA/Ca)F_1 genetic background which is homozygous for the reiterated β-globin transgenic sequence TgN(Hbb-b1)83Clo (Lo. 1983; 1986). This sequence can be detected by DNA-DNA *in situ* hybridisation (Keighren and West, 1993). The ROSA and TGB stocks used in this project had also been bred to select for homozygosity for *Gpi1-b*.

CBA/Ca males were obtained from the Institute of Cell, Animal and Population Biology, University of Edinburgh. BALB/c/Eumm and some BF_1 mice were purchased from the Department of Medical Microbiology, University of Edinburgh and A/J/Ola/Hsd mice were purchased from Harlan Olac Ltd (Bicester, UK). All other animals were bred and maintained, under conventional conditions in the Centre for Reproductive Biology, with a light dark cycle of 14 hours light (05:00h-19:00h) and 10 hours dark (19:00h-05:00h), unless otherwise stated (see

Table 2.1 Details of mouse stocks

Abbreviated stock names	Detail*	Genotype†			
		albino	*Tg*	*lac-Z*	*Gpi1*
AAF_1	(BALB/c × A/J)F_1 hybrid	c/c	-/-	-/-	*a/a*
A/J	A/J/Ola/Hsd	c/c	-/-	-/-	*a/a*
BALB/c	BALB/c/Eumm	c/c	-/-	-/-	*a/a*
BF_1	(C57BL/Ola × CBA/Ca)F_1 hybrid	C/C	-/-	-/-	*b/b*
C57BL	C57BL/OlaWs	C/C	-/-	-/-	*b/b*
CALB	BALB/c-*Gpi-1s*^{c}/Ws	c/c	-/-	-/-	*c/c*
CBA	CBA/Ca	C/C	-/-	-/-	*b/b*
CC	C57BL-*Gpi-1s*^{c}/Ws	c/c	-/-	-/-	*c/c*
CF_1	(CC × CALB) F_1 hybrid	c/c	-/-	-/-	*c/c*
ROSA	TgR(ROSA26)26Sor	C/C	-/-	+/+	*b/b*
TGB	derived from transgenic strain 83	C/C	+/+	-/-	*b/b*

*: The female parents are shown first in all crosses.

†: *Tg*, *β*-globin transgene; *lac-Z*, reporter transgene; *Gpi1*, encodes glucose phosphate isomerase activity.

5.2.1).

2.1.2. *SUPEROVULATION*

Adult female mice (5-7 weeks old) were superovulated by intraperitoneal injections of 5 I.U. pregnant mares' serum gonadotrophin (PMSG; Folligon, Intervet) at 12:00h, followed by 5 I.U. human chorionic gonadotrophin (HCG; Chorulon, Intervet) 48 hours later. If preimplantation embryos were required, the females were caged individually with stud males after the HCG injection. Mating was verified the following morning by the presence of a vaginal plug. This was designated 0.5 day *post coitum* (*p.c.*) or E0.5.

2.1.3. *PRODUCTION OF PSEUDOPREGNANT FEMALES*

A group of CF_1 females was examined and those in oestrus were selected and mated to vasectomised CF_1 males to produce pseudopregnant females. Mating was also verified the next morning by the presence of a vaginal plug and it was designated as 0.5 day of pseudopregnancy.

2.1.4. *EMBRYO TRANSFER*

Female mice on 2.5 days of pseudopregnancy were anaesthetised with 0.25 ml per 30 g body weight of a 1 : 1 (v/v) mixture of a 50% aqueous dilution of Hypnorm (Janssen Parmacenticals) and a 50% aqueous dilution Hypnovel (Roche). Pseudopregnancy was confirmed by the presence of corpora lutea. Only 5-8 embryos were transferred into each uterine horn. Resulting pregnancies were timed according to the pseudopregnant females.

2.2 EMBRYOS

2.2.1. *PREIMPLANTATION EMBRYO COLLECTION*

Pregnant mice were sacrificed by cervical dislocation. Preimplantation embryos were flushed from oviducts with M2 medium (Quinn *et al.*, 1982). For the collection of 8-cell stage and older embryos uteri were also flushed.

All the embryos flushed from the reproductive tract were washed several times in M2 medium, and kept in M2 medium before use.

2.2.2. *AGGREGATION AND DISAGGREGATION*

Before aggregation or disaggregation, the zonae pellucidae were removed by exposing the embryos to pre-warmed acidic Tyrode's solution (Nicolson *et al.*, 1975) for a few seconds at room temperature. Zona-free embryos were then washed several times in fresh M2 medium.

To separate blastomeres of the 2-cell stage embryos, the zona-free embryos were transferred to Ca^{++} and Mg^{++}-free M2 medium (see Appendix I. 1) for at least 15 minutes. These embryos were then disassociated by pipetting them through a fine bore flame-polished pipette (Kelly, 1977; O'Brien *et al.*, 1984). Disaggregation of 8-cell stage embryos was achieved by pipetting the zona-free embryos without exposure to Ca^{++} and Mg^{++}-free M2 medium.

Aggregation was facilitated by M2 medium containing phytohaemagglutinin (PHA, M-form, Gibco, cat. no. 10576-015; see 1.2.2.a.), which was made up by adding one part PHA to 19 parts M2 medium. Pairs of embryos or cells were pushed together in drops of this medium and monitored for 2 minutes at room temperature. These aggregates were then washed in M2 medium and cultured individually in drops

of pre-equilibrated M16 medium (Whittingham, 1971) at 37°C in 5% CO_2 in air under mineral oil (Sigma, M-8410). The oil used for culture had been tested for toxicity before use.

2.2.3. *EMBRYO DISSECTION*

In this project, E5.5, E6.5 and E12.5 postimplantation conceptuses were dissected to analyse the proportions of embryonic components in the chimaeras.

Female mice were sacrificed by cervical dislocation. The uteri were exposed and cut along the length. E5.5 and E6.5 embryos, which are at the pre- and primitive streak stage, were dissected out by tearing off the decidua in M2 medium. E12.5 conceptuses were removed whole by using watchmaker's forceps.

2.3 GLUCOSE PHOSPHATE ISOMERASE ELECTROPHORESIS

2.3.1. *SAMPLE COLLECTIONS*

Glucose phosphate isomerase (GPI) electrophoresis was performed on a variety of samples in this project. These included oocytes, E4.5 blastocysts, E6.5 embryos and various tissues of E12.5 conceptuses.

Five tissues from E12.5 were collected: foetus, amnion, yolk sac mesoderm, yolk sac endoderm and placenta. The mesoderm and endoderm layers of the visceral yolk sac of E12.5 conceptuses were separated by exposure to a trypsin/pancreatin solution (0.5 g trypsin and 2.5 g pancreatin in 100 ml phosphate buffered saline) at 4°C for approximately 3.5 hours (Levak-Svajger *et al.*, 1969), and then transferring to M2 medium for a further half hour. The yolk sac components were then easily dissected using watchmaker's forceps, as described by West and Flockhart (1994).

Oocytes were collected from superovulated females one day after HCG injection and exposed to hyaluronidase (100 I.U. per ml of PBS) to remove cumulus cells. E4.5 blastocysts were developed from E2.5 embryos cultured *in vitro* and E6.5 embryos were collected as described in 2.2.3.

2.3.2. *SAMPLE TREATMENTS*

The samples for GPI analysis were stored in 50% glycerol in water, with the volumes of this storage solution being dependent on the type of sample. These volumes are shown in Table 2.2.

All the samples were lysed by three cycles of freezing and thawing. The foetuses, placentas and the other tissues of E12.5 conceptuses were also disrupted mechanically during the freeze-thaw cycles.

2.3.3. *ELECTROPHORESIS*

The 60 × 76 mm cellulose acetate plates (Helena Laboratories, Titan III, cat. no. 3023) were soaked in GPI buffer (3g of Tris and 14.4g of glycine dissolved in 1000ml distilled water, pH 8.1) for at least 30 minutes, then dried with a paper towel and put on an aligning base (Helena Laboratories, cat. no. 4086) immediately before use. The samples of E12.5 conceptuses were loaded into the sample well plates (Helena Laboratories, super Z, cat. no. 4085) with or without dilution (see Table 2.3) and then transferred to acetate plates by using a sample applicator (Helena Laboratories, super Z, cat. no. 4084).

Due to the small volumes of storage solution of E6.5 embryos, (1 μl; see Table 2.1), a tiny drop of storage solution from each E6.5 embryo was transferred into the acetate plates by a pulled pipette. The blastocysts or oocytes were directly transferred to the acetate plates by blowing out the pipettes containing the samples.

Table 2.2 The volumes of storage solution for the samples undergoing GPI analysis

Individual sample	Volume of store solution	Container
oocyte	*M2 medium	pulled glass pipette
E4.5 blastocyst	*M2 medium	pulled glass pipette
E6.5 embryo	1 μl	96-well plate
E12.5 foetus	200 μl	1.5 ml eppendorf microfuge tube
E12.5 placenta	200 μl	1.5 ml eppendorf microfuge tube
E12.5 amnion	20 μl	96-well plate
E12.5 yolk sac endoderm	20 μl	96-well plate
E12.5 yolk sac mesoderm	20 μl	96-well plate

*: oocytes or blastocysts, in group of three or six, depending on the experimental design (see 3.2.2 and 3.2.3), were kept in minimal M2 medium in a pulled pipette sealed with a small amount of mineral oil in both ends.

Table 2.3 Dilution of the samples from E12.5 conceptuses

Individual sample	Dilution (storage solution : distilled water)
Foetus	2 μl : 8 μl
Placenta	2 μl : 8 μl
Amnion	10 μl : 0 μl
Yolk sac Endoderm	5 μl : 5 μl
Yolk sac Mesoderm	10 μl : 0 μl

The electrophoresis chamber (Helena Laboratories, cat. no. 1283) was filled with 50 ml of GPI buffer in each of the buffer reservoirs. Samples were run from anode to cathode with the power supply (Bioblock Scientific, E455) at 200 volts for one hour and then stained for GPI activity by stain reagents (see Appendix I. 2). The staining reaction was kept in the dark on a 37°C hotplate and the staining time was dependent on the enzyme activity, (from 3-20 minutes). The reaction was stopped by immersing the plates in water. The plates were then fixed in 5 % acetic acid for 5 minutes and rinsed in distilled water for 10 minutes. Plates were air dried overnight in the dark and a Helena Process-24 gel scanner was used to quantify the proportions of each GPI allozyme by densitometry as previously described (West *et al.*, 1986).

2.3.4. *THE SENSITIVITY OF GPI STAIN AND DENSITOMETRY*

In order to test the accuracy of GPI staining and densitometry, samples which were mixtures of known proportions of GPI1-A and GPI1-B were tested. Kidneys from mice that were homozygous for $Gpi1^a$ and $Gpi1^b$ respectively were homogenised with a final concentration of 100mg per ml distilled water. The proportions of GPI1-A and GPI1-B kidneys in the mixtures were 100 : 0, 99 : 1, 95 : 5, 90 : 10, 80 : 20, 70 : 30, 60 : 40, 50 : 50, 40 : 60, 30 : 70, 20 : 80, 10 : 90, 5 : 95, 1 : 99 and 0 : 100. These mixtures were prepared for GPI electrophoresis (see 2.3.3) and the plates were scanned for densitometry. This experiment was repeated three times and the mean observed percentages of GPI1-A were plotted against the expected percentages of GPI1-A in the mixtures (see Fig. 2.1).

The expected and the mean detected percentages of GPI1-A were positively correlated. However, a 1% level of GPI1-A or B was undetectable. The minor proportion in the mixture with various GPI activities tended to be overstained and it resulted in a slight overestimation of this small proportion. However, the GPI staining is still a quite sensitive method to detect the chimaerism of the sample.

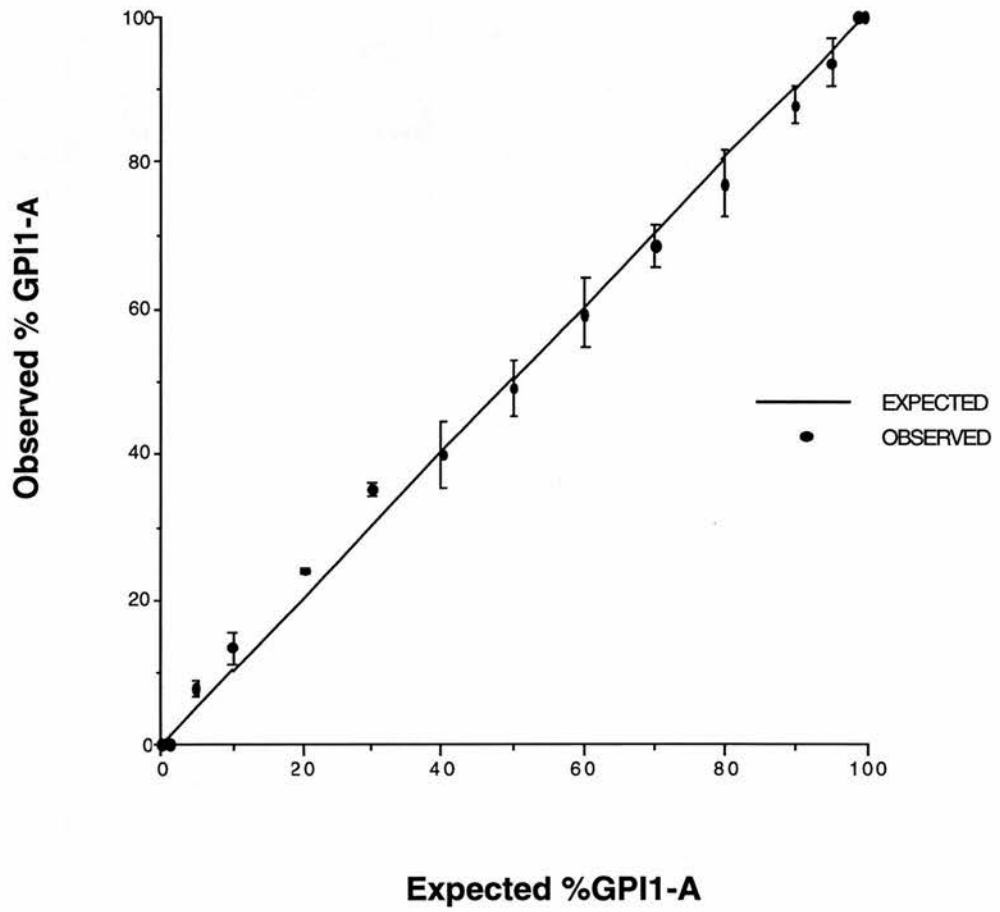


Fig. 2.1 Relationship between the expected percentage of GPI1-A and the mean detected percentage of GPI1-A (Mean ± SEM of three repeats) in the mixtures of homogenised kidneys.

2.4 *IN SITU* HYBRIDISATION

2.4.1. *WAX SECTIONS*

Samples for processing *in situ* hybridisation were placed in Tissue Tex cassettes and fixed in 3 : 1 (ethanol : acetic acid) for at least 6 hours at 4 °C. Samples were then transferred to 70 % ethanol and kept at 4 °C until processing. This was carried out by a tissue processor (Leica, TP1050). Procession included dehydration through graded alcohols into xylene and immersion in paraffin wax (see Table 2.4). After processing, the samples were embedded in paraffin wax (Bayer Diagnostic, tissue tek III embedding wax) using a Reichert-Jung tissue embedding centre and stored at 4 °C.

Table 2.4 Programme of tissue procession

70 % ethanol	1.5 h
80 % ethanol	1.5 h
90 % ethanol	1.5 h
95 % ethanol × 2	1.5 h × 2
100 % ethanol × 2	2 h × 2
Xylene × 3	1 h × 3
Paraffin wax × 2	1 h × 2
Paraffin wax	1.5 h

The 7 μm sections were cut with a microtome (Leica, Jung Biocut). Several ribbons of sections were placed on TESPA-coated slides (2% TESPA in acetone; 3-aminopropyltriethoxysilane, Sigma A-3648). The slides with the tissue sections were dried at 37 °C before further treatment.

2.4.2. *β-GLOBIN PROBE LABELLING*

The probe, pMβδ2, is derived from the plasmid pMJ that was inserted into the transgenic strain 83 mice (Lo, 1983; 1986). The TGB stock used in this project is derived from strain 83 and the *β*-globin probe labelled with digoxigenin is applied for *in situ* hybridisation.

The appropriate plasmid was linearised by the restriction endonuclease *Eco*RI (Boehringer, Mannheim). The denatured DNA digoxigenin-dUTP random primed labelling procedure and non-radioactive detection were carried out as previously described (Keighren and West, 1993). The final concentration of labelled DNA probe was 20μg/ml.

2.4.3. *PROCEDURE OF IN SITU HYBRIDISATION*

The wax sections were dewaxed in two changes of histoclear for 15 minutes at room temperature, then were placed in the following prior to the procedure of *in situ* hybridisation: in 100% ethanol for 5 minutes twice at room temperature; fresh 3% H_2O_2 in methanol for 15-30 minutes at room temperature; 70% ethanol for 5 minutes twice at room temperature; PBS for 5 minutes twice at room temperature; fresh 1mM NaOH for 3 minutes at 70 °C and PBS for 5 minutes twice at 4 °C. Thirty five μl of prehybridisation mixture (see Appendix I. 3) was then placed on sections under a glass coverslips. These slides were incubated in a pre-heated humidified with 2 × SSC chamber at 60 °C for 15 minutes.

The glass coverslips were removed. Forty μl of hybridisation mixture (see Appendix I. 3) were placed on the sample under a hydrophobic coverslip (GelBond, cat. no. 53745; FMC Bioproducts). Nail varnish was used to seal the coverslips.

After the nail varnish was dry, the slides were incubated overnight in a humidified chamber at 60 °C.

The next day, the hydrophobic coverslips and excess nail varnish were removed. The washing steps were then applied to remove the non-specifically binding probes, as described previously (Keighren and West, 1993).

The hybridised digoxigenin-labelled probes were visualised by horseradish peroxidase (HRP) immunocytochemistry. After the final post-hybridisation wash, the slides were soaked in buffer 1 for 5 minutes and then incubated with a 1 : 100 dilution of antibody (Anti-Digoxignin-POD) for 30 minutes in a humid box. The slides were washed twice in buffer 1 for 10 minutes, followed by a 5-minute wash in DAB buffer. The freshly prepared development reagent was placed on the slides. The reaction was kept in the dark for 40 minutes until the brown endpoint developed. All the slides were rinsed in water and counterstained with haematoxylin and eosin, immersed in histoclear and xylene, then mounted in Pertex (Cell Path). All the reagents for visualisation and counterstaining are shown in Appendix I. 3.

2.5 LAC-Z STAIN

Samples for β-galactosidase staining were washed in PBS before being put into fixative: fresh 4 % paraformaldehyde, at 4°C for 20 minutes. After being fixed, the samples were washed for 5 minutes in PBS three times at room temperature. Just before use, 5 ml of X-Gal stock stain (see Appendix I. 4) were added to 100 µl of X-Gal and filter sterilised. The samples were kept in this staining solution overnight at 30°C, then observed the next morning.

2.6 *STATISTICAL ANALYSIS*

Due to the characteristic of chimaera data, in which the chimaerism is always presented as percentages, and also, the resulting chimaerism is not normally distributed, non-parametric statistical tests were used for most of the analyses of the experiments present in this thesis. However, parametric tests were used to compare physical parameters, such as weights of conceptuses, foetuses and placentas and the foetal length.

The Kruskal-Wallis test (a non-parametric, 1-way analysis of variance type of test) was used to compare percentage data among three or more groups. If this revealed significant differences among the groups, multiple pairwise tests were performed using the Mann-Whitney U-test (a non-parametric equivalent of the unpaired Student's t-test) to identify the sources of the variation. Statistical tests were performed on an Apple Macintosh computer using the statistical packages 'StatView 4.0' (Abacus Concepts Inc., Berkley, USA). Also, the Multistat 1.12 software was used to calculate some Chi-square (χ^2) values. A routine established on the spreadsheet Microsoft Excel (Microsoft corporation) was also used.

CHAPTER 3

THE EFFECT OF SIZE REGULATION ON THE COMPOSITION OF UNBALANCED AGGREGATION CHIMAERAS

3.1 INTRODUCTION

Experimental chimaeras have been widely used in investigations of developmental biology and several methods of chimaera production have been introduced (see 1.2 and 1.3). To a varying extent, all the methods for making chimaeras increase the total number of cells in the pre-implantation embryo, compared with unmanipulated controls. However, normal-sized chimaeric offspring are born after transferring the aggregates to foster mothers. Hence, a regulatory mechanism must adjust the body size of these embryos during development (Buehr and McLaren, 1974; Lewis and Rossant, 1982).

Several experiments have shown that regulation of body size can occur in either a downward or an upward direction (Buehr and McLaren, 1974; Lewis and Rossant, 1982; Rands, 1986a, b; Power and Tam, 1993; Evsikov *et al.*, 1996). Compensatory growth may occur after implantation, if the number of cells is reduced at the pre-implantation stage (Snow and Tam, 1979; Rands, 1986a). In contrast, three possible mechanisms have been proposed for downward size regulation: enhanced cell death, an increase in the population of non-dividing cells and increasing the cell cycle length (Lewis and Rossant, 1982). Gardner (1996a) recently suggested that the size and composition of a chimaera would be simultaneously affected by whatever mechanisms cause size regulation. If extension of the cell cycle and/or cell death affected the two aggregated embryos unequally, size regulation would create

an acute selection pressure altering the balance of the two components in the chimaeras.

As described previously, BALB/c cells contribute poorly to chimaeras in which they are involved. Although several mechanisms have been proposed, it was thought likely that cell selection plays a significant role in the formation of such genotypically unbalanced aggregation chimaeras (see 1.3.1.d.). The low contribution of BALB/c cells may result from a continuous cell selection, which would reduce their contribution with time, or an acute selection at a specific "bottleneck" before mid-gestation. One possible "bottleneck" is when chimaeric embryos undergo size regulation. Whatever mechanism is involved in size regulation, it may act unequally on the two aggregated embryos and so create an acute selection pressure which could reduce the overall contribution of BALB/c cells in the chimaera, *e.g.* BALB/c cells might die more than other component cells or their cell cycle might be lengthened preferentially.

The aim of the experiments presented in this Chapter was to test if the mechanism(s) responsible for size regulation play a major role in causing the low contribution of BALB/c embryos in the genotypically unbalanced combination, (BALB/c × BALB/c) ↔ (BF_1 × TGB) (hereafter, this combination is abbreviated to "U" for unbalanced). By comparing the composition of chimaeras produced by aggregating two complete 8-cell stage embryos with that of chimaeras made by aggregating two half 8-cell stage embryos, the effect of size regulation on the genotypic imbalance was evaluated. A parallel experiment was also carried out with the genotypically balanced strain combination, (AAF_1 × AAF_1) ↔ (BF_1 × TGB) to act as a control (hereafter, this combination was abbreviated as "B" for balanced).

3.2 MATERIALS AND METHODS

3.2.1. *PRODUCTION OF CHIMAERAS*

8-cell stage embryos were flushed from the reproductive tracts of pregnant female mice at E2.5 with M2 medium (Quinn *et al.*, 1982). Four series of chimaeras were produced and are listed in Table 3.1. Disaggregation and aggregation were performed. The ($^1/_2$8+$^1/_2$8) aggregates were produced by pushing 4 cells, from the dissociated 8-cell stage embryos of each side of the combinations together in a drop of M2 medium containing PHA.

These aggregated embryos were cultured individually in drops of M16 medium (Whittingham, 1971) at 37°C in 5% CO_2 in air overnight. The following morning, well-developed embryos (see Fig. 3.1) were transferred into the uterine horns of CF_1 females at 2.5 days of pseudopregnancy, or cultured to the blastocyst stage (E4.5).

The techniques of embryo handling and transfer are described in Chapter 2.

3.2.2. *ANALYSIS OF CHIMAERAS*

Electrophoretic variants of GPI were used to distinguish the two cell populations in the chimaeras and the results are presented as the percentage of GPI1-A (%GPI1-A). The samples analysed in these chimaera experiments included E4.5 blastocysts, E6.5 foetuses and various tissues from E12.5 conceptuses (foetus, amnion, yolk sac mesoderm, yolk sac endoderm and placenta). Additionally, the percentage of the pigmented cells in the retinal epithelium of the E12.5 chimaeras was visually assessed and various physical parameters were also measured: weights of the whole conceptus, the foetus and placenta; the crown/rump length and the

Table 3.1 Description of the chimaera series made in these experiments

Series of chimaeras*	Combination	Detail	Stage of analysis
U(8+8)	(BALB/c × BALB/c) ↔ (BF_1 × TGB)	8-cell ↔ 8-cell	E4.5
			E6.5
			E12.5
B(8+8)	(AAF_1 × AAF_1) ↔ (BF_1 × TGB)	8-cell ↔ 8-cell	E4.5
			E6.5
			E12.5
U($^1/_2$8+$^1/_2$8)	(BALB/c × BALB/c) ↔ (BF_1 × TGB)	half 8-cell ↔ half 8-cell	E12.5
B($^1/_2$8+$^1/_2$8)	(AAF_1 × AAF_1) ↔ (BF_1 × TGB)	half 8-cell ↔ half 8-cell	E12.5

*: Embryos expressing GPI1-A activity are written first.

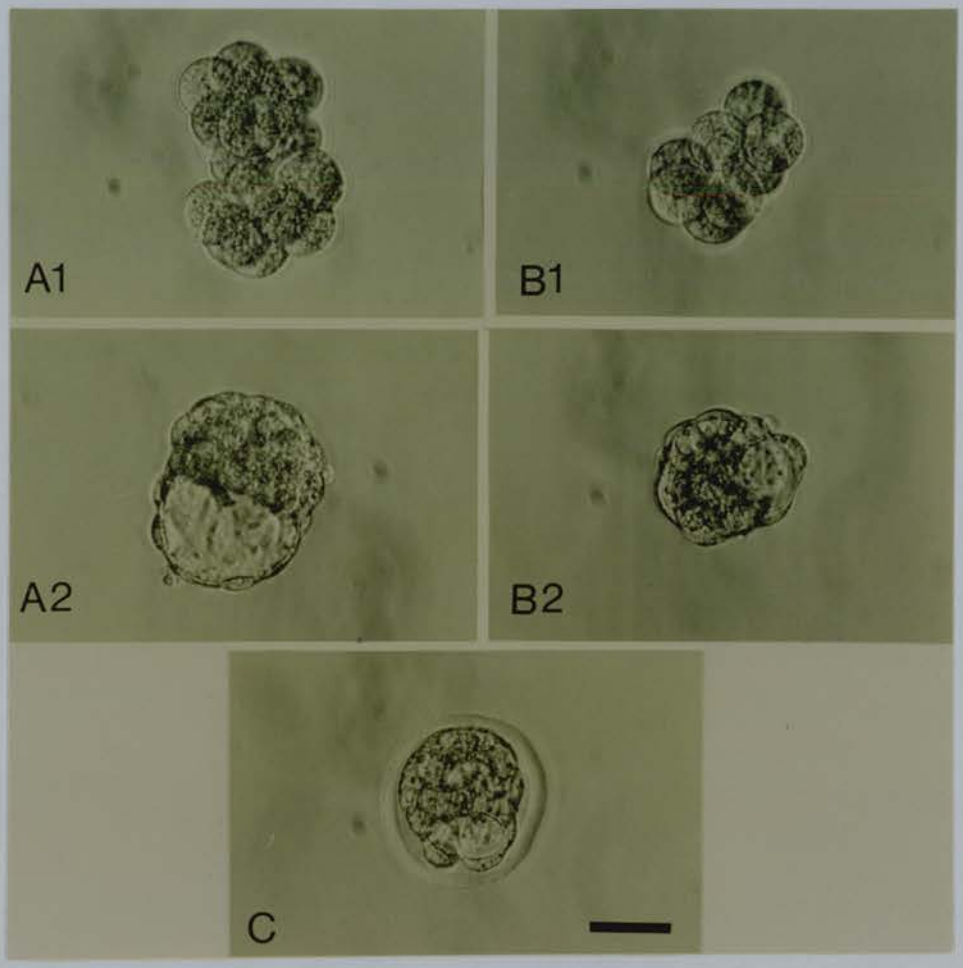


Fig. 3.1 Development of the aggregates cultured from E2.5 to E3.5. **A1.** One (8+8) aggregate made by pushing two 8-cell stage embryos together at E2.5; **A2.** the (8+8) aggregate at the early blastocyst stage (E3.5); **B1.** one ($^1/_2$8+$^1/_2$8) aggregate made by pushing two half 8-cell stage embryos together at E2.5; **B2.** the ($^1/_2$8+$^1/_2$8) aggregate at the early blastocyst stage (E3.5); **C.** an unmanipulated control embryo at E3.5 cultured *in vitro* from E2.5 (bar is 50μm).

morphological index (based on an assessment of hind limb development; McLaren and Buehr, 1990; Palmer and Burgoyne, 1991).

Due to weak GPI staining for single blastocysts, three chimaeric E4.5 blastocysts in groups were analysed in groups for total GPI activity. Sample treatment and the method for GPI staining are described in 2.3.

3.2.3. *CONTROL GROUPS*

Several control groups were produced for the E4.5 chimaeric blastocysts and are listed in Table 3.2. 8-cell stage embryos from (BALB/c × BALB/c), (AAF_1 × AAF_1) and (BF_1 × TGB) were collected and cultured individually in drops of M16 medium (37°C, 5% CO_2 in air) until blastocyst stage (E4.5). To compare GPI activity between chimaeric and non-chimaeric blastocysts, the component embryos in each strain combination were used as controls. Thus, 3 blastocysts from (BALB/c × BALB/c) and 3 blastocysts from (BF_1 × TGB) were grouped and formed the control group for three U(8+8) chimaeric blastocysts. Also, 3 blastocysts from (AAF_1 × AAF_1) and (BF_1 × TGB) formed the control for three B(8+8) chimaeric blastocysts. Additionally, to control for differences in GPI activity between BALB/c × BALB/c, BF_1 × TGB and AAF_1 × AAF_1 at the blastocyst stage, oocytes from superovulated BALB/c, BF_1 and AAF_1 females were collected and analysed for GPI activity in groups of six (three from each strain; see Table 3.2).

Additionally, some of the 8-cell stage embryos from each mating crosses, which had their zonae pellucidae removed, were cultured overnight and transferred to CF_1 females. These were analysed at E12.5 to obtain data of physical parameters to compare with those of the chimaeras.

Table 3.2 Description of the control groups used for GPI analysis

Control Combinations		Composition
Oocyte mixtures	U	BALB/c + BF_1
	B	AAF_1 + BF_1
Blastocyst mixtures	U	(BALB/c × BALB/c) + (BF_1 × TGB)
	B	(AAF_1 × AAF_1) + (BF_1 × TGB)

3.3 RESULTS

3.3.1. *E12.5 CONCEPTUSES*

3.3.1.a. The genotypically unbalanced and balanced strain combinations, U(8+8) and B(8+8)

Details of %GPI1-A for individual chimaeras in both series U(8+8) and B(8+8) are listed in Appendices III. 1 and 2. Thirty three chimaeric conceptuses were produced in the series U(8+8). Two of these embryos shared some membranes. There were 30 chimaeras in series B(8+8). In addition, there were 3 non-chimaeric conceptuses in this series; one was entirely (BF_1 × TGB) and two were (AAF_1 × AAF_1). There were also 4 non-chimaeric U(8+8) conceptuses; all of which were entirely (BF_1 × TGB). These non-chimaeric and twin conceptuses were not included in the analysis. Also, results of embryonic losses in the two chimaera series are shown in Table 3.3. There is no significant difference in the percentage of mole formation between the series U(8+8) and B(8+8) (6.6% vs. 12.9%, P=0.22).

The distributions of %GPI-1A in each tissues in the unbalanced and balanced series are shown in Fig. 3.2 A. All the tissues, except for the yolk sac endoderm, in series U(8+8) show a skewed towards low %GPI1-A. Thus, the contribution of (BALB/c × BALB/c) was very low in these tissues causing the skewed histograms of the unbalanced combination. The tissue of the yolk sac endoderm, however, shows that BALB/c cells contributed more to this tissue than to others, *i.e.* more yolk sac endoderm samples were composed of two cell populations. In contrast, the distributions of %GPI1-A in each tissue of series B(8+8) do not show the skewed distributions of %GPI1-A, and most of the tissues are chimaeric.

Table 3.3 A comparison of embryo losses between genotypically unbalanced and balanced strain combinations

Strain combination	Detail			Statistical significance*
	embryos transferred no.	implanted conceptuses no. (%)	moles no. (%)	χ^2 value
U(8+8)	122	36 (29.5%)	8 (6.6%)	
B(8+8)	70	33 (47.1%)	9 (12.9%)	1.48 (NS)
U($^1/_2$8+$^1/_2$8)	163	49 (30.1%)	10 (6.1%)	
B($^1/_2$8+$^1/_2$8)	109	43 (39.4%)	3 (2.8%)	0.98 (NS)

*: The percentage of moles of the total transferred embryos was tested for statistical significance between corresponding chimaera series. NS=not significant (P>0.05).

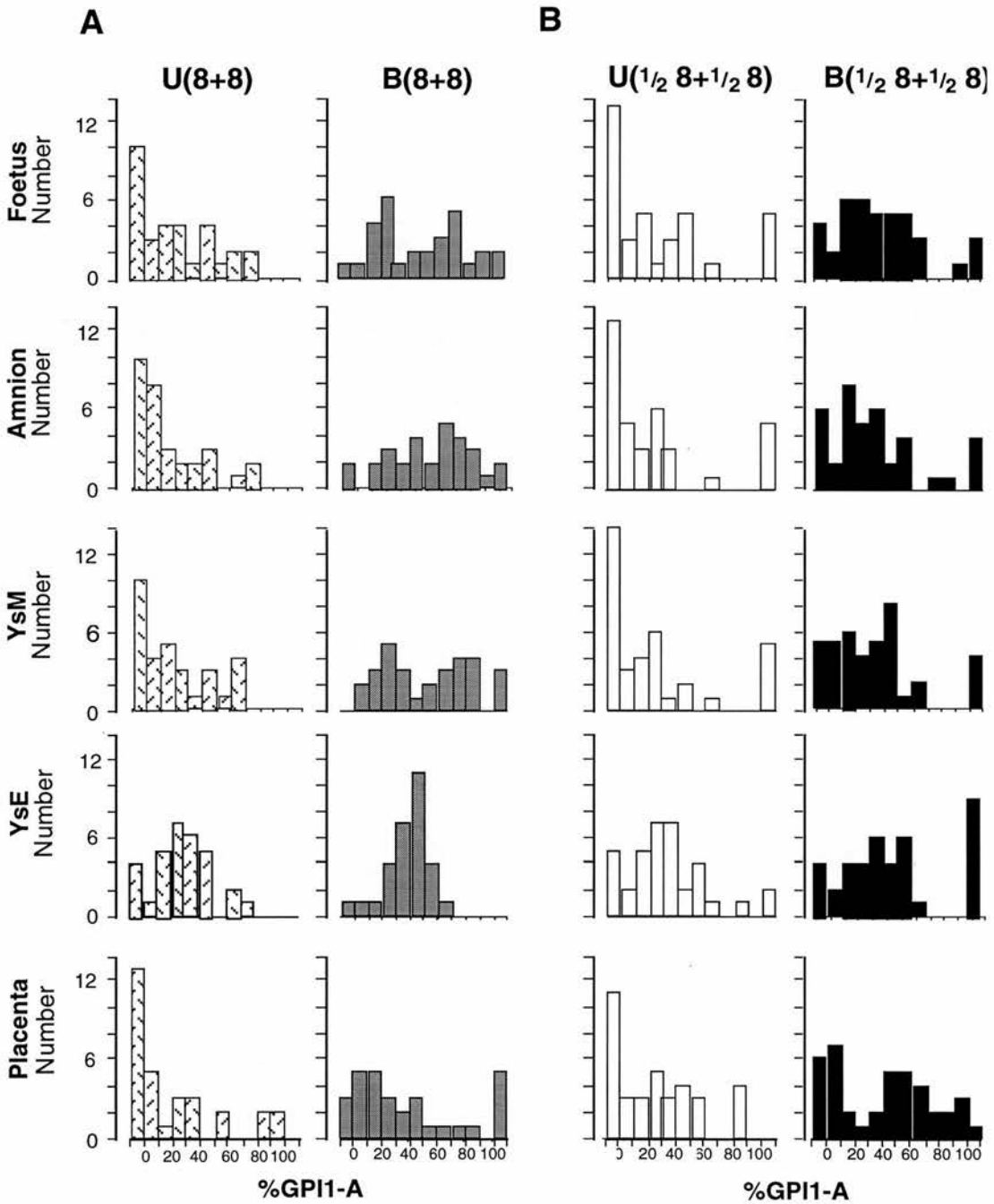


Fig. 3.2 A. Distribution of % GPI1-A in the five tissues analysed in the series of chimaeric conceptuses, U(8+8) and B(8+8) respectively; **B.** Distribution of % GPI1-A in the five tissues analysed in the series of chimaeric conceptuses, U($^1/_2$8+$^1/_2$8) and B($^1/_2$8+$^1/_2$8) respectively. Tissues with either 0 or 100% GPI1-A are shown separately at either end of the distributions.

The criteria used to clarify the distributions as balanced or unbalanced are similar to those described previously (Mullen and Whitten, 1971; West and Flockhart, 1994; West *et al.*, 1995). The first classification of GPI1-A distribution was modified from the original classes: < 30, 30-70 and > 70% in Mullen and Whitten (1971). To increase the discrimination between the bimodal distributions (typical of placenta) and the bell-shaped distributions (typical of foetus, amnion, yolk sac mesoderm and yolk sac endoderm), the thresholds were altered to < 25, 25-75 and > 75% (West *et al.*, 1995). Individual chimaeric conceptuses were then divided into these three classes by %GPI1-A. If the number of chimaeras with < 50% GPI1-A is statistically significantly different from those with > 50% GPI1-A, the combination would be considered unbalanced. Also, if the number of chimaeras in the class of 25-75% GPI1-A is not greater than, or equal to the numbers in the other two classes, the combination would be considered atypical.

In each of the five tissues in the series U(8+8), the genotypic imbalance is reflected by the significantly higher proportion of individual chimaeras with < 50% GPI1-A than > 50%. Also, for all tissues, but yolk sac endoderm, the skewed distributions are reflected by an atypical ratio where more samples had < 25% GPI1-A than 25-75% GPI1-A (see Table 3.4). On these grounds, series U(8+8) is therefore regarded as an unbalanced strain combination.

In contrast, the proportion of samples with < 50% GPI1-A is not significantly different from those with > 50% GPI1-A in series B(8+8), except for the yolk sac endoderm and placenta, where the ratios of < 50 : > 50% GPI1-A are 25 : 5 and 21 : 9 respectively (see Table 3.4). Inspection of individual samples, however, reveals that, for the yolk sac endoderm, this discrepancy can be accounted for by a high proportion of samples with 40-50% GPI1-A (see Fig. 3.2 A and Appendix III. 2). Overall, series B(8+8) shows the characteristics of a balanced strain combination.

Table 3.4 Chimaeric conceptuses in series U(8+8) and B(8+8) grouped according to % GPI1-A in each tissue

Chimaera series	Tissues	Classification of distribution of GPI-1A I < 25 : 25 -75 : > 75 %	II < 50 : > 50 %	Statistical significance P value*
Balanced distributions				
B(8+8)	Fetus	8 : 15 : 7	15 : 15	1
B(8+8)	Amnion	7 : 15 : 8	13 : 17	0.465
B(8+8)	YSM	7 : 13 : 10	14 : 16	0.715
Unbalanced but typical distributions				
B(8+8)	YSE	4 : 26 : 0	25 : 5	< 0.001
U(8+8)	YSE	11 : 19 : 1	28 : 3	< 0.001
Unbalanced and atypical distributions				
B(8+8)	Placenta	13 : 10 : 7	21 : 9	0.028
U(8+8)	Fetus	19 : 11 : 1	26 : 5	< 0.001
U(8+8)	Amnion	22 : 9 : 0	28 : 3	< 0.001
U(8+8)	YSM	21 : 10 : 0	26 : 5	< 0.001
U(8+8)	Placenta	21 : 6 : 4	25 : 6	< 0.001

* Tested against the expectation of equal proportions of < 50% and > 50% GPI-1A in classification II ($P<0.05$).

The mean %GPI1-A of each tissues in these two series are listed in Table 3.5. Five tissues analysed were grouped by their developmental origins. Mann-Whitney U tests were used to compare %GPI1-A between these two series of chimaeras. The results revealed that %GPI1-A of each tissue studied was significantly lower in the unbalanced series U(8+8) than in the balanced series B(8+8). This confirmed the basic difference in composition between these two chimaera series.

3.3.1.b. The genotypically unbalanced and balanced strain combinations, U($^1/_2$8+$^1/_2$8) and B($^1/_2$8+$^1/_2$8)

Detail of %GPI1-A of individual chimaeras in both the series U($^1/_2$8+$^1/_2$8) and B($^1/_2$8+$^1/_2$8) are listed in Appendices III. 3 and 4. Thirty six chimaeric conceptuses were produced in series U($^1/_2$8+$^1/_2$8) and 40 in series B($^1/_2$8+$^1/_2$8). There were 3 non-chimaeric B($^1/_2$8+$^1/_2$8) conceptuses; two were entirely (BF_1 × TGB) and one was (AAF_1 × AAF_1). There were also 13 non-chimaeric U($^1/_2$8+$^1/_2$8) conceptuses, all of which were entirely (BF_1 × TGB). Although these non-chimaeric conceptuses were not included in the analysis, the large number of non-chimaeric conceptuses without any (BALB/c × BALB/c) contribution suggested that series U($^1/_2$8+$^1/_2$8) was unbalanced. Comparing the proportion of embryonic losses in chimaera series U($^1/_2$8+$^1/_2$8) and B($^1/_2$8+$^1/_2$8) showed no significant difference from each other (6.1% vs. 2.8%, P=0.108; see Table 3.3).

Fig. 3.2 B also shows that the distribution of %GPI1-A in yolk sac endoderm in series U($^1/_2$8+$^1/_2$8) is unlike the skewed histograms shown in other tissues. This is because, like U(8+8), the minority (BALB/c × BALB/c) cell population made a

Table 3.5 Comparisons of the mean %GPI-1A among the tissues analysed in each series of chimaeras

Series of Chimaeras		pEct Lineage*			pEnd Lineage*	TE Lineage*
	N*	Foetus	Amnion	YsM	YsE	Placenta
U(8+8)	31	22.91 ± 4.34†	18.05 ± 4.06†	20.35 ± 4.17†	28.91 ± 3.56†	21.80 ± 5.61†
B(8+8)	30	49.98 ± 5.57	53.65 ± 5.11‡	53.02 ± 5.54‡	38.23 ± 2.65	38.68 ± 6.47
U($^1/_2$8+$^1/_2$8)	36	28.10 ± 5.65†	25.29 ± 5.67†	25.70 ± 5.66†	32.79 ± 4.45	27.00 ± 4.76†
B($^1/_2$8+$^1/_2$8)	40	38.01 ± 4.38	34.03 ± 4.81	33.84 ± 4.51	46.28 ± 5.41	40.62 ± 5.26

*: Abbreviation: pEct, primitive ectoderm; pEnd, primitive endoderm; TE, trophectoderm; YsM, yolk sac mesoderm; YsE, yolk sac endoderm; N, number of chimaeras analysed.

†: comparisons between series U(8+8) and B(8+8), or U($^1/_2$8+$^1/_2$8) and B($^1/_2$8+$^1/_2$8); $P<0.05$

‡: comparisons between series U(8+8) and U($^1/_2$8+$^1/_2$8), or B(8+8) and B($^1/_2$8+$^1/_2$8); $P<0.05$

better contribution to this tissue than to the others. The distribution of %GPI1-A in the series B($^1/_2$8+$^1/_2$8) shows a reasonable level of chimaerism in each tissue.

The analysis of series U($^1/_2$8+$^1/_2$8) shows that there are significantly more individual chimaeras with < 50% GPI1-A than with > 50% in the five tissues (see Table 3.6). These skewed distributions are also reflected in an atypical ratio where more samples had < 25% GPI1-A than 25-75%, except for the yolk sac endoderm, like the series U(8+8). These observations imply that halving the number of cells, to avoid the effects of size regulation, failed to prevent the unbalanced tissue composition.

In five U($^1/_2$8+$^1/_2$8) chimaeric conceptuses (PCTVa-4, 8, 23, 41 and 43), the foetus and other epiblast derivatives were entirely GPI1-A cells (from BALB/c embryos). In two of the five cases (PCTVa-4 and 41) the yolk sac endoderm was also entirely derived from the BALB/c component. This does not reflect a failure of selection against BALB/c cells, because it may be accounted for by allocation of entirely BALB/c cells to the epiblast or to the whole inner cell mass (which divides into the epiblast and primitive endoderm). If only BALB/c cells are allocated to the epiblast, even stringent selection against BALB/c cells cannot reduce the proportion below 100%. In a smaller embryo, fewer cells will be allocated to the epiblast and so an epiblast comprising entirely BALB/c cells may be produced more frequently than in a double-sized chimaeric aggregate. Despite these five chimaeras with entirely BALB/c epiblast tissues, the series U($^1/_2$8+$^1/_2$8) appeared to be unbalanced in the same way as U(8+8). In this series, there were also 11 chimaeric conceptuses with non-chimaeric epiblast derivatives which were entirely (BF_1 × TGB) (see Appendix III. 3).

There were also several chimaeric conceptuses with non-chimaeric epiblast derivatives in the series B($^1/_2$8+$^1/_2$8). Three chimaeras has epiblast derivatives which

Table 3.6 Chimaeric conceptuses in series U($^1/_2$8+$^1/_2$8) and B($^1/_2$8+$^1/_2$8) grouped according to % GPI1-A in each tissue

Chimaera series	Tissues	Classification of distribution of GPI-1A I < 25 : 25 -75 : > 75 %	II < 50 : > 50 %	Statistical significance P value*
Balanced distributions				
B($^1/_2$8+$^1/_2$8)	YSE	12 : 19 : 9	24 : 16	0.206
B($^1/_2$8+$^1/_2$8)	Placenta	16 : 18 : 6	23 : 17	0.343
Unbalanced but typical distributions				
B($^1/_2$8+$^1/_2$8)	Fetus	15 : 21 : 4	28 : 12	0.011
B($^1/_2$8+$^1/_2$8)	Amnion	16 : 18 : 5	29 : 10	0.002
B($^1/_2$8+$^1/_2$8)	YSM	17 : 19 : 4	33 : 7	< 0.001
U($^1/_2$8+$^1/_2$8)	YSE	15 : 18 : 3	28 : 8	< 0.001
Unbalanced and atypical distribution distributions				
U($^1/_2$8+$^1/_2$8)	Fetus	21 : 10 : 5	30 : 6	< 0.001
U($^1/_2$8+$^1/_2$8)	Amnion	24 : 7 : 5	30 : 6	< 0.001
U($^1/_2$8+$^1/_2$8)	YSM	23 : 8 : 5	30 : 6	< 0.001
U($^1/_2$8+$^1/_2$8)	Placenta	19 : 13 : 4	29 : 7	< 0.001

* Tested against the expectation of equal proportions of < 50% and > 50% GPI-1A in classification II (P<0.05).

were entirely (BF_1 × TGB) derived and one with (BF_1 × TGB) epiblast derivatives and an (AAF_1 × AAF_1)-derived yolk sac endoderm (PCTVId-12, 25, 28 and PCTVId-37, respectively; see Appendix III. 4). Also two chimaeras were observed with non-chimaeric epiblast derivatives which were entirely (AAF_1 × AAF_1) derived and one with (AAF_1 × AAF_1) epiblast derivatives and (BF_1 × TGB)-derived yolk sac endoderm (PCTVId-9, 34 and PCTVId-35, respectively; see Appendix III. 4). This supports the suggestion (see above) that mixed populations of cells are less frequently found in the epiblast and/or the primitive endoderm of chimaeras composed of fewer rather than more cells.

The proportions of samples, in the combination B($^1/_2$8+$^1/_2$8), with < 50% GPI1-A were not significantly different from those with > 50% GPI1-A in the yolk sac endoderm and placenta but there were significantly more chimaeras with < 50% GPI1-A in the foetus, amnion and yolk sac mesoderm (see Table 3.6). When the distributions of %GPI1-A in each tissues were classified into three groups (< 25 : 25-75 : > 75%), however, these three samples still remained typical. There were more samples with 25-75% GPI1-A than either < 25% or > 75% (see Table 3.6). Overall, the series B($^1/_2$8+$^1/_2$8) appeared to be slightly less balanced than the series B(8+8), and it seemed that halving the cell numbers of the embryos reduced the contribution of (AAF_1 × AAF_1) cells more than that of (BF_1 × TGB) cells to the chimaeras.

A comparison of the series U($^1/_2$8+$^1/_2$8) and B($^1/_2$8+$^1/_2$8) shows that %GPI1-A in each tissue, except for yolk sac endoderm, is significantly lower in series U($^1/_2$8+$^1/_2$8) than that in series B($^1/_2$8+$^1/_2$8) (see Table 3.5). The %GPI1-A of yolk sac endoderm is lower in the unbalanced series U($^1/_2$8+$^1/_2$8) than that in the balanced series B($^1/_2$8+$^1/_2$8), but this difference fails to reach statistical significance.

3.3.1.c. Comparisons between the series (8+8) and ($^1/_2$8+$^1/_2$8)

A comparison of U(8+8) and U($^1/_2$8+$^1/_2$8) reveals no significant differences in composition in any of the five tissues studied (see Table 3.5). This confirms that the effect of halving the number of cells in the aggregate did not convert the unbalanced series U(8+8) into a balanced one. However, a comparison between B(8+8) and B($^1/_2$8+$^1/_2$8) reveals significant differences in composition of the amnion and yolk sac mesoderm (see Table 3.5). This reflects the abundance of B($^1/_2$8+$^1/_2$8) amnion and yolk sac mesoderm with < 50% GPI1-A which may be partly attributable to the higher frequency of non-chimaeric tissues in chimaeras made with fewer cells, as discussed above.

One clear difference between the (8+8) and the ($^1/_2$8+$^1/_2$8) series is the frequency of non-chimaeric epiblasts and/or foetuses. The proportion of chimaeric conceptuses with non-chimaeric foetuses was 3/30 and 7/40 in series B(8+8) and B($^1/_2$8+$^1/_2$8), respectively (10.0% vs. 17.5%). For the unbalanced series, the equivalent proportions were 10/31 and 18/36 in the whole-embryo and half-embryo aggregates respectively (32.3% vs. 50.0%). If the number of non-chimaeric conceptuses are combined with the chimaeric conceptuses with non-chimaeric foetuses, the equivalent proportions are 6/33 and 10/43 for the balanced series (18.2% vs. 23.3%), 14/35 and 31/49 for the unbalanced series (40.0% vs. 63.3%).

Since some ($^1/_2$8+$^1/_2$8) chimaeric conceptuses have non-chimaeric epiblast because few cells were allocated to this lineage, statistical tests were applied again to analyse the data after the conceptuses with non-chimaeric epiblast derivatives have been removed. The results are shown in Table 3.7. The %GPI1-A of foetuses in series U(8+8) is no longer significantly different from that in series B(8+8) (33.82 vs. 48.26, P=0.074). Also, the %GPI1-A in the yolk sac mesoderm between

Table 3.7 Comparisons of the mean % GPI1-A among the conceptuses with chimaeric epiblast tissues analysed in each series of chimaeras

Series of Chimaeras		pEct Lineage*		
	N*	Foetus	Amnion	YsM
U(8+8)	21	33.82 ± 4.83	26.64 ± 5.01†	30.04 ± 4.90†
B(8+8)	29	48.26 ± 5.48	52.05 ± 5.03‡	51.41 ± 5.48‡
U($^1/_2$8+$^1/_2$8)	20	25.58 ± 3.82†	20.53 ± 3.64†	21.27 ± 3.68
B($^1/_2$8+$^1/_2$8)	33	36.98 ± 3.46	32.10 ± 4.11	31.92 ± 3.63

*: Mean ± SEM; N=sample size

†: Comparisons between the series U(8+8) and B(8+8) or U($^1/_2$8+$^1/_2$8) and B($^1/_2$8+$^1/_2$8), $P<0.05$

‡: Comparisons between the series U(8+8) and U($^1/_2$8+$^1/_2$8), or B(8+8) and B($^1/_2$8+$^1/_2$8), $P<0.05$

U($^1/_2$8+$^1/_2$8) and B($^1/_2$8+$^1/_2$8) is not significantly different (21.27 vs. 31.92, P=0.058; see Table 3.6). The conflicting results may be caused by the great numbers of non-chimaeric epiblast derivatives in the series U(8+8) and U($^1/_2$8+$^1/_2$8). These non-significant differences, which were different from the previous comparisons did not preclude U(8+8) and U($^1/_2$8+$^1/_2$8) being genotypically unbalanced. Statistical analysis still shows that there is a significant difference between the %GPI1-A in the amnion of series B(8+8) and B($^1/_2$8+$^1/_2$8), also in yolk sac mesoderm samples, as described previously.

3.3.1.d. Physical parameters of E12.5 chimaeras

The weights of E12.5 conceptuses whose yolk sacs were broken during dissection were excluded from analysis, because of fluid losses, but the other physical parameters in these conceptuses were still included. The mean weights of conceptuses, foetuses, placentas, the crown/rump length (foetal length) and hind limb morphological index of these four chimaeric series are shown in Table 3.8. The results consistently show that the weights of conceptuses, foetuses and placentas in the genotypically unbalanced strain combination, U(8+8), were significantly lighter than the balanced combination B(8+8). Also, the foetal length was shorter and development was more retarded in U(8+8) than in B(8+8). Overall, the chimaeras of the genotypically unbalanced strain combination, U(8+8), were smaller, lighter and more developmentally retarded than those in the series of B(8+8).

Similar results were also seen in a comparison within the other set of unbalanced and balanced experimental groups, U($^1/_2$8+$^1/_2$8) and B($^1/_2$8+$^1/_2$8). Chimaeric conceptuses in series B($^1/_2$8+$^1/_2$8) consistently appeared to be heavier, longer and more developmentally advanced than those in the series U($^1/_2$8+$^1/_2$8), although the mean length of foetus does not reach statistical significance between

Table 3.8 Physical parameters of E12.5 chimaeras

Physical parameters	U(8+8)		U($^1/_2$8+$^1/_2$8)		B(8+8)		B($^1/_2$8+$^1/_2$8)	
	Mean ± SEM	N*	Mean ± SEM	N*	Mean ± SEM	N*	Mean ± SEM	N*
Wt of conceptus (mg)	302.5 ± 9.9†‡	30	269.5 ± 5.3†	35	356.7 ± 7.7‡	30	303.2 ± 5.7	37
Wt of foetus (mg)	89.8 ± 3.5†	31	83.3 ± 2.3†	36	113.9 ± 3.7‡	30	92.4 ± 2.0	40
Wt of placenta (mg)	86.7 ± 3.0†‡	31	71.9 ± 1.4†	36	94.5 ± 2.2‡	30	82.9 ± 1.9	40
Foetal length (mm)	9.28 ± 0.15†‡	31	8.85 ± 0.10	36	9.95 ± 0.13‡	30	9.01 ± 0.08	40
Hind limb score	6.87 ± 0.17†	31	6.97 ± 0.11†	36	7.72 ± 0.12‡	30	7.38 ± 0.09	40

*: N=sample size

†: comparisons between the series U(8+8) and B(8+8) or U($^1/_2$8+$^1/_2$8) and B($^1/_2$8+$^1/_2$8), $P<0.05$

‡: comparisons between the series U(8+8) and U($^1/_2$8+$^1/_2$8), or B(8+8) and B($^1/_2$8+$^1/_2$8), $P<0.05$

these two series (see Table 3.8).

When comparing the groups of (8+8) with those of ($^1/_2$8+$^1/_2$8), halving the cell number in the aggregate was shown to result in the chimaeric conceptuses being shorter and lighter. According to hind limb scores, the B($^1/_2$8+$^1/_2$8) chimaeras are more developmentally retarded than B(8+8), although U(8+8) and U($^1/_2$8+$^1/_2$8) were not significantly different (see Table 3.8). In other words, the "double-size" aggregates were still larger than the "normal-sized" aggregates. However, they were not still twice as big.

The expected ratios of these various physical parameters between the groups of (8+8) and ($^1/_2$8+$^1/_2$8) is assumed to be 2 : 1, according to the number of contributing cells at aggregation, if no size regulation had occurred. The observed ratios of physical parameters between the series U(8+8) and U($^1/_2$8+$^1/_2$8), & B(8+8) and B($^1/_2$8+$^1/_2$8) were plotted and are shown in Fig. 3.3. The histograms show that the ratios are no longer 2 : 1, indicating that size regulation had occurred by E12.5. Nevertheless, the histograms also show that size regulation is not completed at E12.5 because some of the ratios are still more than 1 : 1.

Table 3.9 shows the mean physical parameters of E12.5 conceptuses of three different mouse matings: BF_1 × TGB, BALB/c × BALB/c and AAF_1 × AAF_1. The last two columns in Table 3.9 show the expected range of physical parameters of the chimaeric combinations. Comparing these expected values to the observed physical parameters in the four series of chimaeras (see Table 3.9), the observed weights of conceptuses, foetuses and placentas fell within the expected range for U(8+8), U($^1/_2$8+$^1/_2$8), and B($^1/_2$8+$^1/_2$8) but the B(8+8) chimaeras exceeded the range defined by the parental strains, these supporting evidence for vegetative heterosis in some series of chimaeras (Falconer *et al.*, 1981). The fact that heterosis was observed in

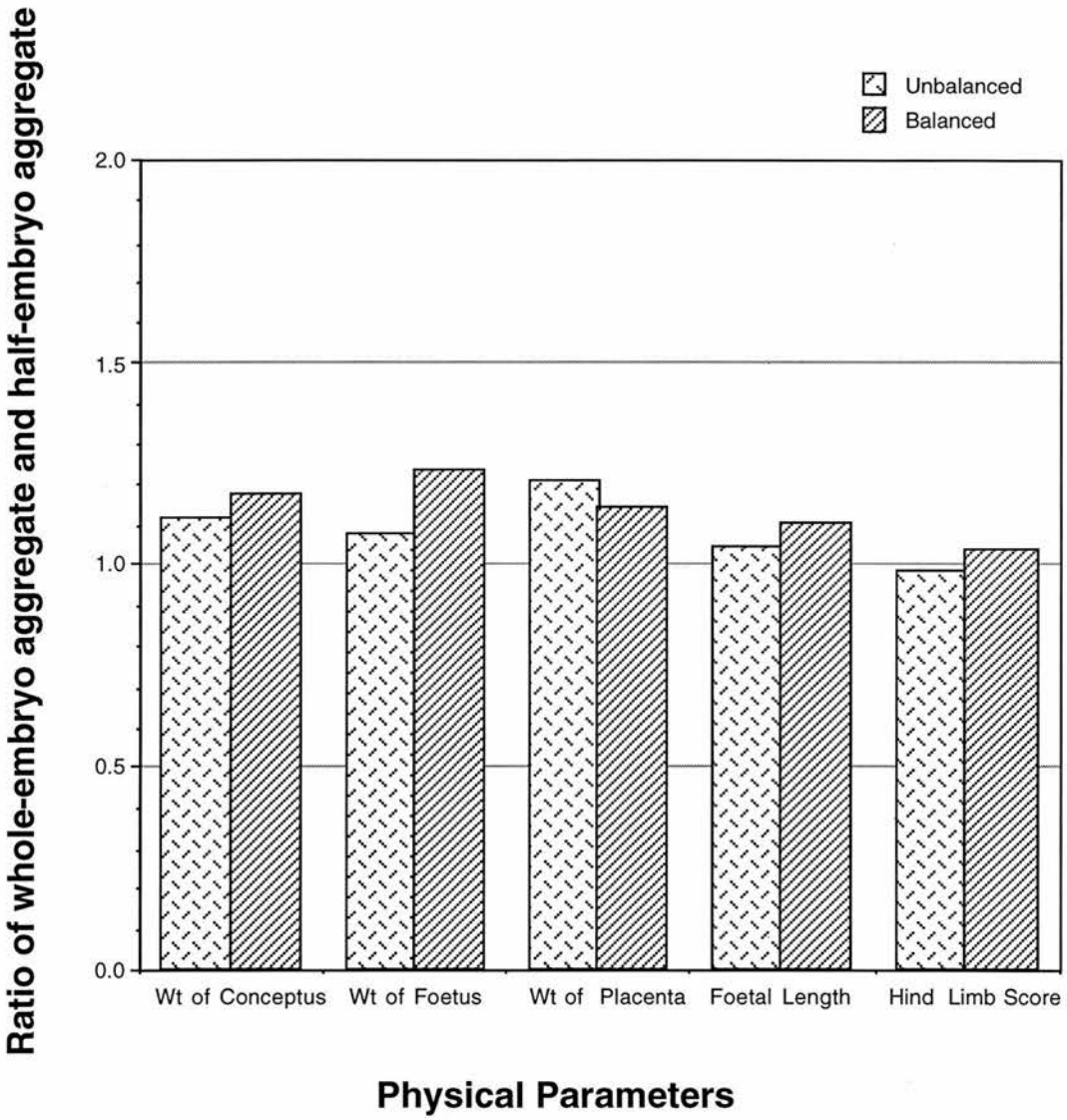


Fig. 3.3 The observed ratios of each physical parameter of "double-sized" aggregates and "normal-sized" aggregates

Table 3.9 The mean physical parameters of the control groups

Physical parameters	$BF_1 \times TGB$		BALB/c × BALB/c		$AAF_1 \times AAF_1$		Expected values in series U[§]	Expected values in series B[§]
	Mean ± SEM	N*	Mean ± SEM	N*	Mean ± SEM	N*	Range	Range
Wt of conceptus (mg) [†]	323.7 ± 11.1	19	244.8 ± 11.2	13	266.3 ± 7.7	31	245 - 324	266 - 324
Wt of foetus (mg)	110.1 ± 5.0	24	71.7 ± 4.5	14	80.1 ± 3.3	33	72 - 110	80 - 110
Wt of placenta (mg)	90.6 ± 3.5	24	71.0 ± 2.2	14	66.6 ± 1.6	33	71 - 91	67 - 91
Foetal length (mm)[‡]	9.63 ± 0.14	24	8.41 ± 0.19	14	8.81 ± 0.13	33	8.4 - 9.6	8.8 - 9.6
Hind limb score[‡]	7.46 ± 0.19	24	6.54 ± 0.25	14	7.13 ± 0.16	33	6.5 - 7.5	7.1 - 7.5

* : N=sample size.

[†]: Ignored the concepti whose yolk sac burst when dissected

[‡]: Some of the embryos were below the scale, and were consequently given the minimum score

[§]: Two ends of the ranges are from the values of the two strains that contributed to the chimaera

series B(8+8) but not B($^1/_2$8+$^1/_2$8) or genotypically unbalanced chimaeras suggests that cell numbers as well as interactions between different genotypes may be involved. It may also imply that size regulation is not complete, as suggested above.

3.3.2. *%GPI1-A IN OOCYTES, BLASTOCYSTS AND E6.5 FOETUSES IN THE CONTROLS AND SERIES U(8+8) AND B(8+8)*

Since halving the blastomere number in the (BALB/c × BALB/c) ↔ (BF_1 × TGB) chimaera combination did not cause a transformation from genotypically unbalanced to balanced, despite size regulation being avoided, a further study was performed to examine when the strain combination (BALB/c × BALB/c) ↔ (BF_1 × TGB) becomes unbalanced. E4.5 and E6.5 chimaeras were analysed for %GPI1-A.

It has been shown that oocyte GPI activity varies among strains of mice (Peterson and Wong, 1978; West and Fisher, 1984), so it is important to compare the GPI activity in BALB/c and AAF_1 oocytes before analysing chimaeras at stages when maternal GPI activity might persist. Therefore, E4.5 blastocysts and oocytes from BALB/c, AAF_1 and BF_1 female mice were also examined for GPI activity. Details of individual samples in each control and experimental group are listed in Appendices III. 5 and 6.

3.3.2.a. Non-chimaeric control-oocytes and blastocysts

The results of GPI analysis in the control oocyte mixture samples are listed in Table 3.10. No significant differences were found between the two strain combinations (43.50 vs. 43.26, P=0.39). Oocytes from BALB/c and AAF_1 strains therefore can be regarded to have similar GPI1-A activities. However, a comparison of blastocyst mixture controls showed a significant difference (P=0.03), implying that the timings of the degradation of maternal mRNA and/or the start of embryonic

Table 3.10 Comparisons of the mean %GPI1-A in U(8+8) and B(8+8) chimaera and control series

Stage	%GPI1-A[‡]	N[‡]
U(control oocyte mixtures)	43.50 ± 0.61	22
B(control oocyte mixtures)	43.26 ± 0.51	22
U(control blastocyst mixtures)	43.49 ± 1.06*	21
B(control blastocyst mixtures)	48.18 ± 1.62	20
U(8+8) E4.5	37.31 ± 2.15[†]	21
B(8+8) E4.5	41.40 ± 2.02[†]	24
U(8+8) E6.5	47.91 ± 4.58	32
B(8+8) E6.5	52.24 ± 4.73	41

[‡]: Mean ± SEM; N=sample size

*: Comparison between U(control blastocyst mixtures) and B(control blastocyst mixtures); $P<0.05$

[†]: Comparisons between control and chimaeric blastocysts in each combination; $P<0.05$

genome are not similar in these two strains of mice. It has been shown that the timing of GPI activity transition from oocyte-coded to embryo-coded is between E2.5 and E5.5 (West and Green, 1983). In the AAF_1 strain embryonic *Gpi1* gene expression may begin earlier or maternal enzyme activity may decrease later, since %GPI1-A of the blastocyst mixtures in this group is higher than in the group of U(control blastocyst mixtures) (48.18 vs. 43.49, P=0.03; see Table 3.10). Also, the difference between these two control blastocyst mixtures may be caused by the lagged development of BALB/c embryos. There is evidence that BALB/c embryos develop relatively slowly and the embryonic genome would be activated later in a retarded embryo. However, this delayed development was unlikely to play a role in the result presented here, since only embryos that had reached to the blastocyst stage were chosen to be analysed.

3.3.2.b. Series U(8+8) and B(8+8)- E4.5 and E6.5

The series U(8+8) and B(8+8) showed no significant differences in %GPI1-A at E4.5 (37.31 vs. 41.40, P=0.203; see Table 3.10), although the %GPI1-A in each of these two chimaeric blastocyst groups was significantly lower than in their control groups (37.31 vs. 43.49, P=0.006; 41.40 vs. 48.18, P=0.003; see Table 3.10). The chimaeric E6.5 foetuses also showed no significant difference between the series U(8+8) and B(8+8) (47.91 vs. 52.24, P=0.508; see Table 3.10). In both series of chimaeras, the mean %GPI1-A at E6.5 showed a higher value than at E4.5. The results seem to imply that the strain combination (BALB/c × BALB/c) ↔ (BF_1 × TGB) does not show the genotypic imbalance at an early postimplantation stage, although the mean composition of the chimaeras changes slightly during the E4.5 to E6.5 period.

3.4 DISCUSSION

The mechanism responsible for size regulation has been suggested as a possible cause of the genotypically unbalanced strain combination in which BALB/c cells are involved. However, in this study, the results have shown that despite aggregating two half 8-cell stage embryos (to avoid downward size regulation) BALB/c cells still form a disproportionately lower proportion of the tissues of the E12.5 chimaeric conceptuses analysed in these experiments. Also, a similar result was observed in the comparisons of B(8+8) and B($^1/_2$8+$^1/_2$8): the genotypically balanced combination ($AAF_1 \times AAF_1 \leftrightarrow BF_1 \times TGB$). Their typical distribution of %GPI1-A (contribution of $AAF_1 \times AAF_1$ cells) was not affected by reducing total number of cells in the aggregates.

The higher frequency of non-chimaeric foetuses (and other epiblast derivatives) in chimaeras made by aggregating two half embryos than two whole embryos in both genotypically balanced and unbalanced strain combinations is expected when the number of cells allocated to the epiblast lineage is low, because there would only be about half as many as cells in a blastocyst formed by two half embryos, so fewer cells will be allocated to each tissue, including the epiblast.

Because there was no significant difference in the comparisons of %GPI1-A in any of the five tissues from E12.5 conceptuses [between the series U(8+8) and U($^1/_2$8+$^1/_2$8)], this genotypic imbalance is probably not caused primarily by the mechanisms responsible for size regulation. The proportions of the two cell populations in some of the chimaeric tissues of mid-gestation chimaeric conceptuses were significantly different after size regulation had been avoided, *e.g.* the tissues of the amnion and yolk sac mesoderm in the series B(8+8) and B($^1/_2$8+$^1/_2$8). Hence, from the results presented in this study, it can be concluded that size regulation may

affect the composition of mouse chimaeras, but does not play a major role in producing consistently genotypically unbalanced chimaera combinations.

Some possibilities accounting for the low contribution of BALB/c cells in chimaeras have been proposed (West *et al.*, 1995). It is possible that chimaeras with a higher contribution of BALB/c embryos died. In this study, as shown in Table 3.3, it has been demonstrated that there is no significant difference in embryonic losses between the genotypically unbalanced and balanced chimaeras. Also, the results support the previous observation that no higher proportion of embryonic death occurred in the combination with BALB/c cells (West *et al.*, 1995). It implies that the poor contribution of BALB/c cells in the chimaera was not caused by embryonic losses, which the conceptuses with higher proportion of BALB/c cells may die. In addition, although both series U(8+8) and U($^1/_2$8+$^1/_2$8) showed that the proportion of BALB/c cells was higher in the yolk sac endoderm than in other tissues, %GPI1-A in the five tissues analysed were not significantly different from each other in these two genotypically unbalanced series (P=0.125 and P=0.269, respectively). Therefore, these results support the suggestion that high embryo losses do not cause the genotypic imbalance (West *et al.*, 1995).

Since downward size regulation is detectable before implantation in the quadruple aggregated embryo (Rands, 1986b) or soon after implantation in the "double-size" embryo (Buehr and McLaren, 1974; Lewis and Rossant, 1982), and that embryo size has been restored before the formation of the primitive streak. It is reasonable to examine the change of the composition of the chimaera from the late pre-implantation to the early postimplantation stage. According to the results from the E4.5 and E6.5 chimaeric embryos analysed in these experiments, no significant genotypic imbalance is apparent in the (BALB/c × BALB/c) ↔ (BF_1 × TGB) combination at E4.5 or E6.5. This implies that the mechanisms responsible for downward size regulation are not the main cause of production of the unbalanced

chimaeras. Moreover, the significant differences between chimaeric blastocysts and control blastocysts observed in both unbalanced and balanced strain combinations indicate that other mechanisms are involved in the composition of the chimaera.

It has been shown that cell death occurs at blastocyst formation (Handyside and Hunter, 1986; Hardy *et al.*, 1989). In the "double-size" (8+8) embryos, cell death may occur somehow more actively than in the "normal-size" ($^1/_2$8+$^1/_2$8) embryos. This may influence the composition of the chimaera. However, since there are no significant differences in comparisons of %GPI1-A in E6.5 chimaeric embryos between these two strain combinations, cell death may cause a change in the composition of chimaeras, but cannot account for genotypic imbalance. This also seems to imply that genotypic imbalance does not appear until a later developmental stage.

Two other possible causes of the unbalanced strain combination were considered by West *et al.* (1995): cell selection against BALB/c cells during development and preferential allocation of BALB/c cells to the mural trophectoderm. As mentioned in the Introduction (see 3.1), Lewis and Rossant (1982) proposed that an extended cell cycle length in aggregated embryos could account for downward size regulation, and that this mechanism might be responsible for cell selection against BALB/c cells. Although size regulation appeared not to play a major role in causing the unbalanced distribution of BALB/c cells, it was observed that the development of (BALB/c × BALB/c) embryos lagged behind the other two strain embryos, (AAF_1 × AAF_1) and (BF_1 × TGB), at E2.5. There are several reports that highlight the influence of different genetic backgrounds on the development of mouse pre-implantation embryos (McLaren and Bowman, 1973; Niwa *et al.*, 1980; Shire and Whitten, 1980a, b; Du and Wales, 1993; Suzuki *et al.*, 1996). Although the series U(8+8) was produced by aggregating two 8-cell stage embryos, the (BALB/c × BALB/c) embryo could be at the very early 8-cell stage while the (BF_1 × TGB)

embryo could be at a mid or late 8-cell stage. In a study by Mystkowska *et al* (1979), it was observed that unbalanced chimaeras were caused by a difference in the rate of proliferation between the two cell populations. Hence, it is possible that cell selection against BALB/c embryos is mediated through a relative retardation of development.

A further possibility is that BALB/c cells are preferentially allocated to the mural trophectoderm. This might be achieved by a two-step process. The first step of which is that most of the BALB/c cells are left on the outside of the embryo and would therefore become trophectoderm. Also, allocation of BALB/c cells to the trophectoderm could possibly be achieved by cell movement away from the inner cell mass (see 1.3.3). Then, in the second step, the BALB/c cells could move preferentially towards the mural trophectoderm from the polar trophectoderm by cell movement (see 1.3.3). Therefore, by these two steps, the majority of the BALB/c cell population could colonise the mural trophectoderm, and subsequently only develop into giant cells which were not detectable in this study. Also, such a preferential allocation to the mural trophectoderm could not be shown in the analysis of E4.5 chimaeric embryos in this study, since a spatial cell marker was not used.

Apart from these mechanisms, BALB/c cells may be selected against during the process of cell mixing which occurs before E7.5. At the blastocyst stage, a comparison of cell mixing patterns showed no significant difference in the two strain combinations, (C3H/HeN)F_2 ↔ (BALB/cA)F_2 and (C3H/HeN)F_2 ↔ (C57BL/6N)F_2 (Dvorak *et al.*, 1995). In the E7.5 conceptuses, however, the strain combination (C3H/HeN)F_2 ↔ (BALB/cA)F_2 showed an intermingled cell mixing pattern but with C3H/HeN cells predominating, whereas in the other group, 82.6 % of the chimaeras had a higher proportion of C57BL/6N cells, with no specific and repeatable pattern. Therefore, BALB/c cells could be excluded during cell mixing. It was also suggested that changes in the cell ratio in aggregated chimaeras depends on the mouse strains

used and on the immune interactions between pseudopregnant foster mothers and foetuses. Since different foster animals were not used to test the pseudomaternal-foetal immune interaction in this study, there is no evidence to examine this suggestion. However, the interactions between cells and selection against some kinds of cells, resulting in different cell contributions in chimaeras, seems to imply a key role in arranging cell distribution. Cell-cell communication in chimaeras has been reviewed by Prather *et al.* (1989). However, whether the characters of BALB/c cells are recognised by other cells and selected against, as suggested by West *et al.* (1995), is not clear.

In this study, as previously described (West and Flockhart, 1994), it has been shown that both cell populations contribute to the yolk sac endoderm, or both yolk sac endoderm and the placenta, more frequently than to any other tissues in the genotypically unbalanced chimaera. There were more non-chimaeric derivatives of epiblast and trophectoderm lineages with 0 or 100% GPI1-A, than those in the primitive endoderm (see Fig. 3.1 A and B). By classifying the chimaeras with chimaeric or non-chimaeric derivatives of epiblast, hypoblast and trophectoderm, it was shown that approximately 10% of chimaeric conceptuses in the genotypically unbalanced strain combination, have confined chimaerism in the yolk sac endoderm (14.3% and 8.2% in series U(8+8) and U($^1/_2$8+$^1/_2$8), respectively; see Table 3.11) and only 3% in series B(8+8). Overall, chimaerism confined to the derivatives of hypoblast and trophectoderm occurs more frequently in the series of U(8+8) and U($^1/_2$8+$^1/_2$8) than in the series of B(8+8) and B($^1/_2$8+$^1/_2$8) (around 28-33% vs. 3-16%, respectively; see Table 3.11). It has also been demonstrated that chimaerism in tetraploid↔diploid chimaeras is confined to the primitive endoderm and trophectoderm lineages (James and West, 1994). Human chromosome mosaicism is also often confined to the placental trophoblast (Kalousek and Dill, 1983). The similarity of the confined distribution in both human and mouse conceptuses has

Table 3.11 Frequencies of chimaerism in the derivatives of three primary lineages at E12.5 conceptuses

GPI1 composition (mixed or single)			Number of conceptuses			
pEct Lineage*	pEnd Lineage*	TE Lineage*	Series U(8+8)	Series U($^1/_2$8+$^1/_2$8)	Series B(8+8)	Series B($^1/_2$8+$^1/_2$8)
			No. (%)	No. (%)	No. (%)	No. (%)
Chimaeric foetus						
(a) mixed	single	single	1 (2.9)	1 (2.0)	1 (3.0)	3 (7.0)
(b) mixed	single	mixed	0 (0.0)	1 (2.0)	0 (0.0)	8 (18.6)
(c) mixed	mixed	single	7 (20.0)	4 (8.2)	5 (15.2)	4 (9.3)
(d) mixed	mixed	mixed	13 (37.1)	12 (24.5)	21 (63.6)	18 (41.9)
Non-chimaeric foetus (chimaerism confined to extraembryonic tissues)						
(e) mixed	mixed	mixed	0 (0.0)	0 (0.0)	2 (6.1)	0 (0.0)
(f) mixed	mixed	single	0 (0.0)	2 (4.1)	0 (0.0)	0 (0.0)
(g) single	single	mixed	3 (8.6)	5 (10.2)	0 (0.0)	2 (4.7)
(h) single	mixed	single	5 (14.3)	4 (8.2)	1 (3.0)	0 (0.0)
(i) single	mixed	mixed	2 (5.7)	7 (14.3)	0 (0.0)	5 (11.5)
Non-chimaeric conceptuses						
(j) single	single	single	4 (11.4)	13 (26.5)	3 (9.1)	3 (7.0)
Total number of chimaeric conceptuses			31	36	30	40
Total number of conceptuses			4	13	3	3

*: Samples from epiblast lineage include: foetus, amnion, yolk sac mesoderm; from hypoblast lineage include: yolk sac endoderm; from trophectoderm lineage include: placenta

drawn attention to the possibility that human confined mosaicism may also occur in the yolk sac endoderm. This genotypically unbalanced mouse chimaera could therefore provide a useful animal model to investigate this human confined mosaicism (see 1.3.2).

Overall, this study has shown that size regulation alone cannot account for the genotypic imbalance which is observed in some strain combinations, and specifically does not play a major role in causing the low contribution of BALB/c cells to chimaeras. This study has also demonstrated that this genotypic imbalance arises sometime between E6.5 and E12.5.

CHAPTER 4

THE EFFECTS OF CELL SIZE AND PLOIDY ON CELL ALLOCATION OF THE CHIMAERIC BLASTOCYSTS

4.1 INTRODUCTION

It has been shown that ploidy plays a role in cell allocation in the mouse pre-implantation embryo. The restricted tissue distribution of tetraploid cells observed in postimplantation conceptuses, in which tetraploid cells contributed to the tissues derived from the trophectoderm and primitive endoderm lineages but were absent from the foetuses, could be the result of cell selection and preferential cell allocation of tetraploid cells to the mural trophectoderm at the blastocyst stage (Everett and West, 1996, 1998; see 1.3.1.c.).

As described in 1.3.1, several other factors can also cause cells to be preferentially allocated to different regions of the blastocyst. Embryo stage, timing of cleavage division and cell size can all affect cell allocation in pre-implantation embryos. In previous tetraploid↔diploid chimaeric experiments tetraploid embryos were produced by electrofusion of two diploid cells. This resulted in the tetraploid cells being twice the size of diploid cells (Nagy *et al.*, 1990; James *et al.*, 1995; Everett and West, 1996, 1998). Also, in both pre- and postimplantation chimaeric embryo studies (James *et al.*, 1995; Everett and West, 1996, 1998), tetraploid embryos had only about half as many cells as the diploid embryos at aggregation. Therefore, in these experiments, tetraploid and diploid embryos differed not only in ploidy, but also in cell number and cell size, but not embryo stage. Also, control chimaeras in these studies were made by aggregating embryos that differed in embryo

stage, cell size, cell number and ploidy. No experiments have yet adequately considered these factors separately.

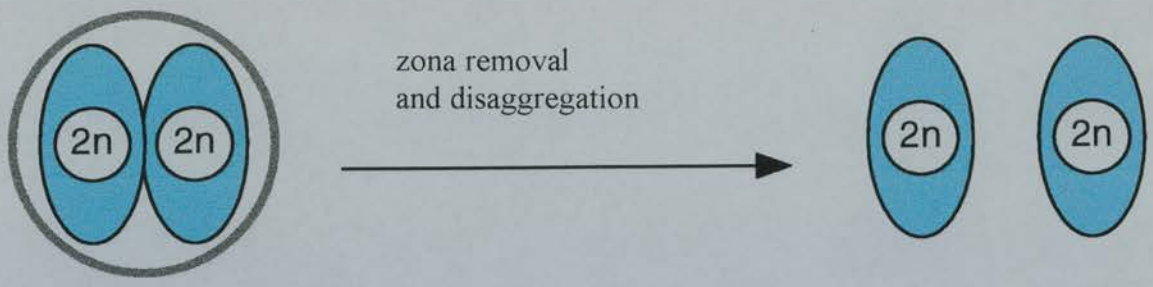
In this Chapter, by micromanipulation and electrofusion, cells from the 2-cell stage mouse embryo were produced which differed in cell size *or* ploidy. Several series of chimaeras were then made by aggregating pairs of cells, so that cell number and embryo stage were constant. The roles of cell size and ploidy in cell allocation among the tissues of chimaeric blastocysts were then examined individually. Also, by comparing with the results from these cell size experiments, the effect of embryo stage (which also affects the size of blastomeres) can be examined, as described in the next Chapter.

A summary of the production of these chimaera constituents is shown in Fig. 4.1.

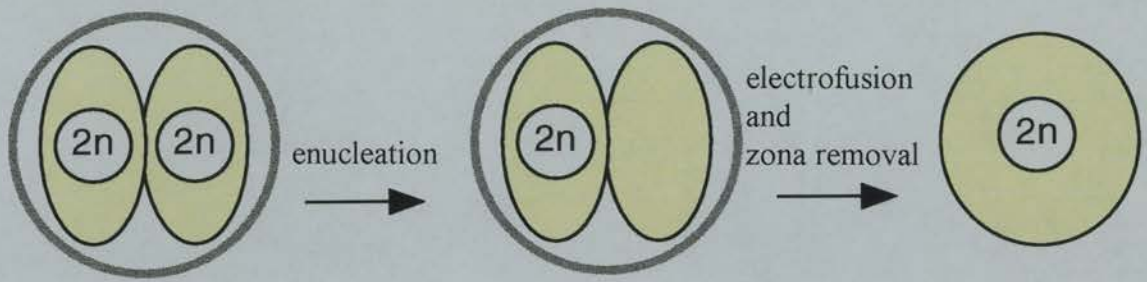
4.2 MATERIALS AND METHODS

4.2.1. *MOUSE STRAINS*

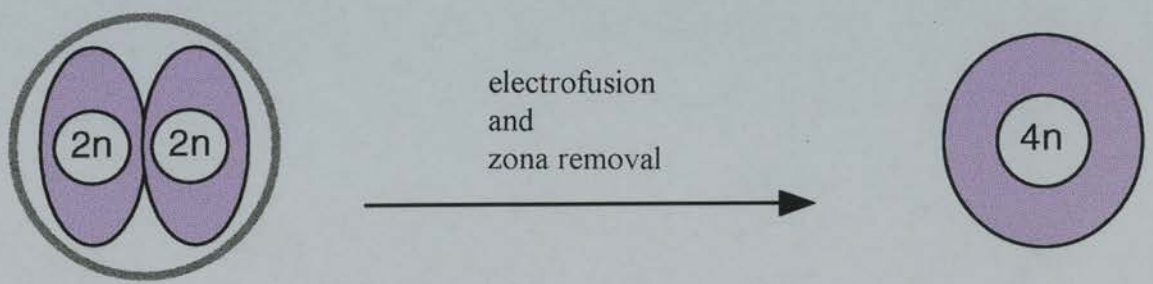
Details of mouse stocks used in these experiments are listed in Table 2.1. BF_1 female mice were superovulated, and mated to stud males. The chimaeric combination ($BF_1 \times BF_1$) $\leftrightarrow$ ($BF_1 \times$ ROSA) was used in preliminary experiments, and E5.5 chimaeras were analysed by X-Gal staining for β-galactosidase (see 2.5). However, due to the low implantation rate (see 4.3 Results), the chimaeric combination was altered to ($BF_1 \times BF_1$) $\leftrightarrow$ ($BF_1 \times$ TGB). Pre-implantation embryos, E4.5 chimaeric blastocysts, were analysed by DNA-DNA *in situ* hybridisation of the β-globin transgene (Keighren & West, 1993; see 2.4).



A. Production of S2n cells



B. Production of B2n cells



C. Production of B4n cells

Fig. 4.1 Production of cells used for making chimaeras in which the components differed in cell size or ploidy. **A.** S2n cells: small diploid cells were obtained from the disaggregated 2-cell stage embryos; **B.** B2n cells: big diploid cells were made by enucleating one blastomere of a 2-cell stage embryo and electrofusing the blastomeres; **C.** B4n cells: big tetraploid cells were made by electrofusing 2-cell stage embryos. Abbreviations: S=small; B=big; 2n=diploid and 4n=tetraploid.

4.2.2. *EMBRYO COLLECTION*

2-cell stage embryos were collected in M2 medium (Quinn *et al.*, 1982) from the oviducts of E1.5 pregnant mice. For technical reasons, the initial experiments were carried out at the Roslin Institute. During this period, the dissected oviducts were transported there in 1.5 ml eppendorf microfuge tubes containing M2 medium and the embryos were recovered approximate 2 hours later. Otherwise, all the 2-cell stage embryos were collected and manipulated in the Centre for Reproductive Biology (CRB).

4.2.3. *ENUCLEATION*

The micromanipulation systems used for enucleation are shown in Fig 4.2 and the methods for making micropipettes and manipulation chambers are described in Appendices II. 1 and 2.

Just before being attached to the micromanipulator (Leitz M, manual model), the inside of the enucleation pipette was washed with autoclave sterilised 1.25% Tween-80 (Sigma 4780) and coated with foetal bovine serum to prevent any cytoplasm sticking to the pipette. The whole manipulation system was free of air bubbles and full of fluorinert or mineral oil. Under the microscope, the holding pipette and enucleation pipette were positioned in the centre of manipulation chamber containing enucleation medium (M2 medium containing 10μg/ml cytochalasin B, 10mM nocodazole and 10% foetal bovine serum). A small amount of medium was sucked into the pipettes. Cytochalasin B (Sigma, C-6762) and nocodazole (Sigma, M-1404) were first dissolved in dimethyl sulfoxide (DMSO; Sigma, D-5879).

The 2-cell stage embryos were placed in enucleation medium at least 15 minutes before being transferred into the manipulation chamber and enucleation was

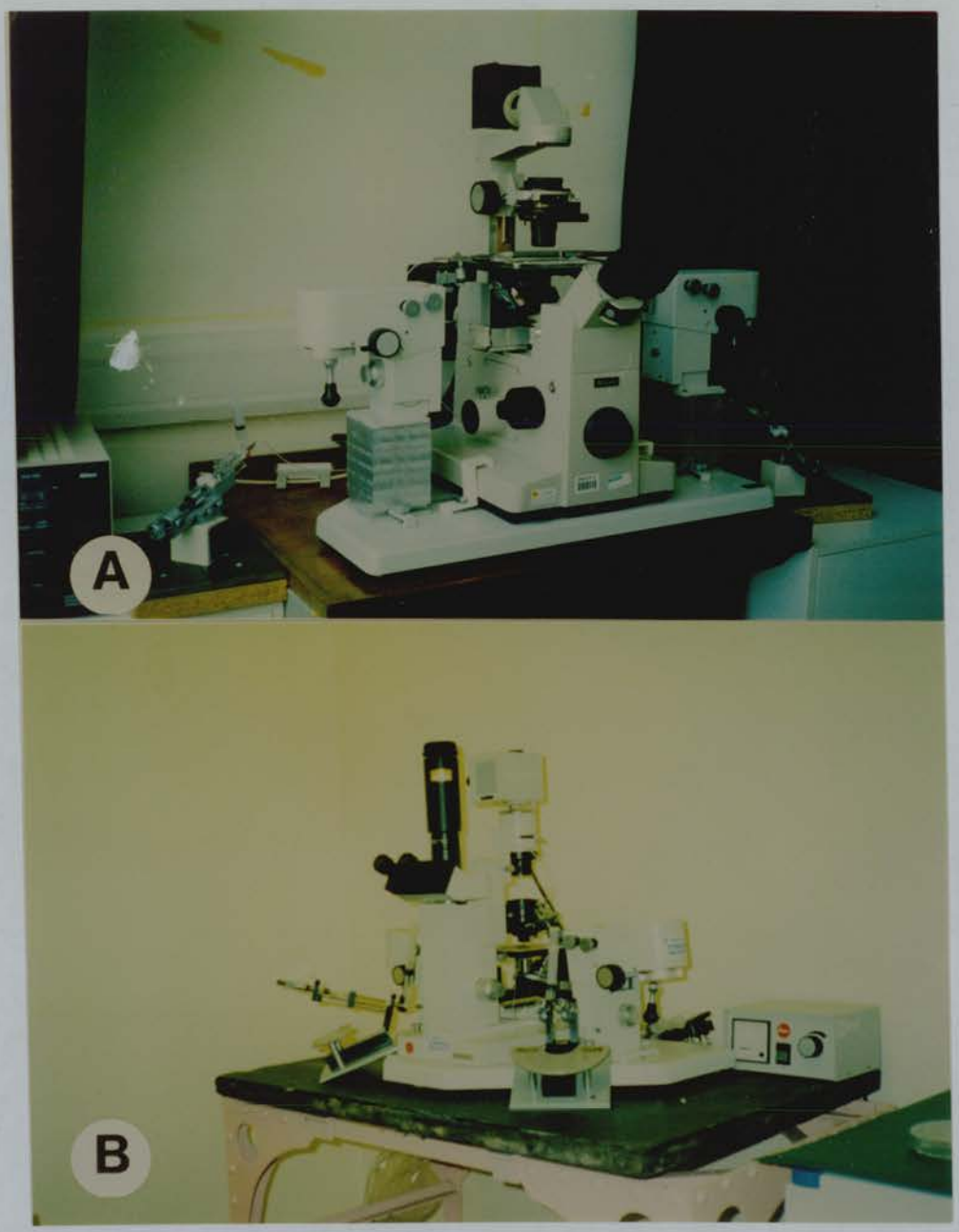


Fig. 4.2 The micromanipulation systems used in these experiments. **A.** The system used at Roslin Institute: a Nikon Diaphot TDM inverted microscope with Differential Interference Contrast (DIC) and epifluorescence, the Leitz M micromanipulators (manual model) associated with Narishige microinjectors (model IM-188) to hold two Hamilton (Sigma S-0142) syringes with a capacity of 500 µl, 250 µl for controlling the holding and enucleation pipettes respectively; **B.** The system used in the CRB: a Leitz Diavert microscope, the Leitz M micromanipulators (manual model) associated with RI microinjectors to hold one Hamilton syringe with a capacity of 100 µl for controlling the enucleation pipette and one 2 ml glass syringe (Weber Scientific) for controlling the holding pipette. All these manipulation syringes were connected to the tubes which go to the pipette holders with three-way taps that connect to another syringe containing fluorinert FC 77 (Sigma, F-4758; at Roslin Institute) or mineral oil (Sigma, M-8410; in the CRB) as a hydraulic reservoir.

performed in one of the blastomeres of the 2-cell stage embryo. The procedure is shown in Fig. 4.3. After being enucleated, the embryos were removed from the chamber and washed several times in fresh M2 medium.

4.2.4. *ELECTROFUSION*

An impulse generator and a corresponding electrode chamber were used in these experiments to electrofuse the two blastomeres of 2-cell stage embryos into single cells. The set used at the Roslin Institute was made by the institute workshop, while the one used at CRB was purchased from BLS Ltd (model CF-150 impulse generator and GPT-250 electrode chamber).

Two-cell embryos were transferred to the electrode chamber containing electrofusion solution (0.3M mannitol, 0.1mM $MgS0_4$, 0.05mM $CaCl_2$, 0.05mg/ml of BSA). One or two embryos were placed between the electrode filaments at a time. The embryos were then exposed to an alternating current (A.C.) pulse at 5.0 volts for 5 seconds (in order to orient the embryos and induce alignment of the blastomeres), followed by one direct current (D.C.) pulse at 20.0 volts for 80 μseconds (in order to cause the degradation of cell membrane and cell fusion).

After electrofusion, these embryos were washed several times in fresh M2 medium, then transferred into pre-equilibrated M16 medium (Whittingham, 1971) under mineral oil in an incubator (37°C, 5% CO_2 in air). Fifteen to thirty minutes later, those embryos, in which the two blastomeres had fused were used in chimaera production (see Fig. 4.4 A).

4.2.5. *PRODUCTION CHIMAERA SERIES*

Several series of chimaeras were produced. Details of chimaeras made in the

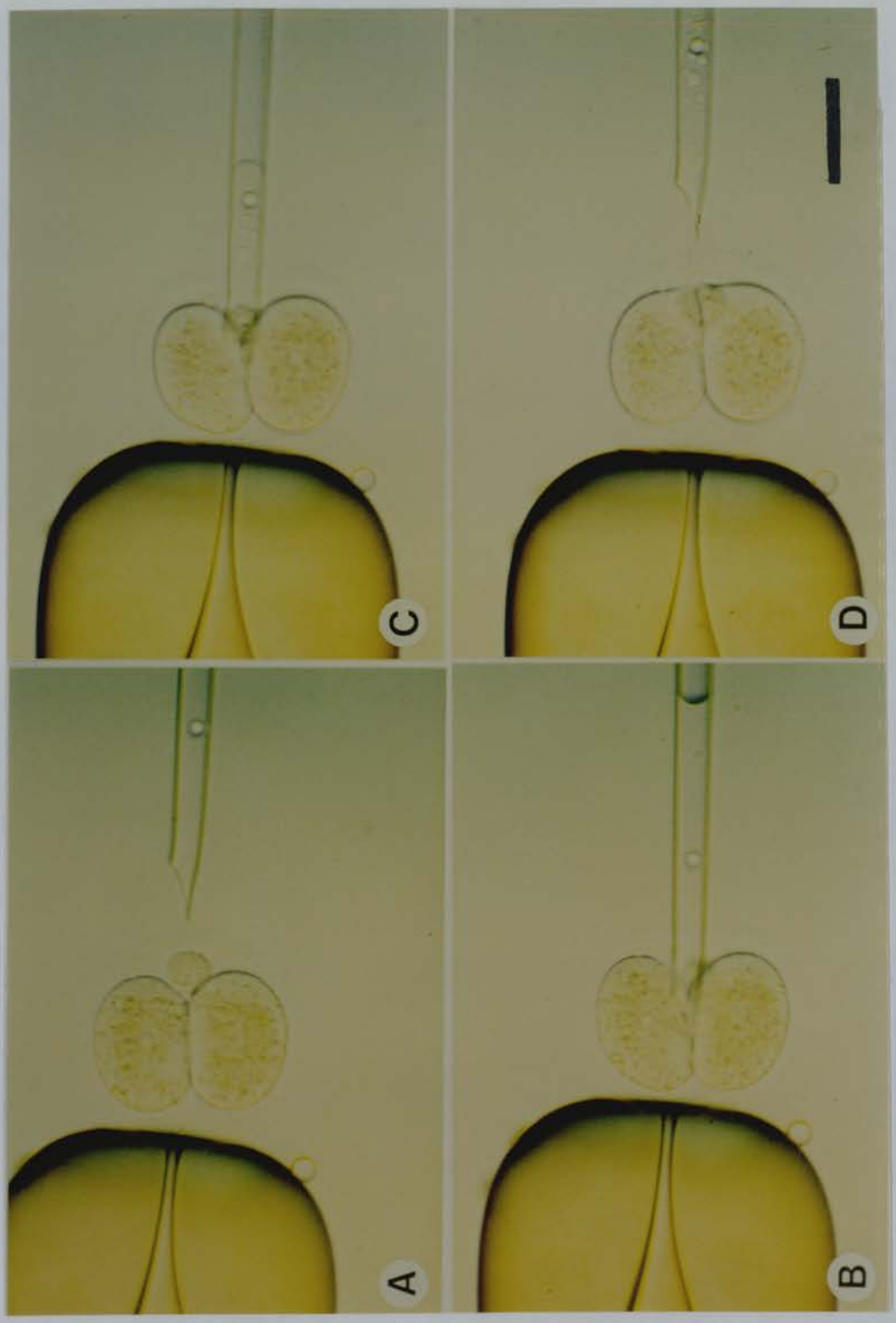


Fig. 4.3 The enucleation procedure. The 2-cell stage embryos were cultured in enucleation medium for 15 min before enucleation. **A.** Under the microscope one 2-cell stage embryo was picked up by the holding pipette. The embryo was then rotated with the enucleation pipette until two blastomeres were parallel to the pipettes; **B.** The enucleation pipette penetrated the zona pellucida and was pushed into the space between the cells; **C.** The enucleation pipette approached the nucleus of one blastomere and, without penetrating the cell, aspirated the nucleus with a small amount of cytoplasm; **D.** The pipette was withdrawn gently from the embryo and the nucleus was expelled (bar is 50 μm).

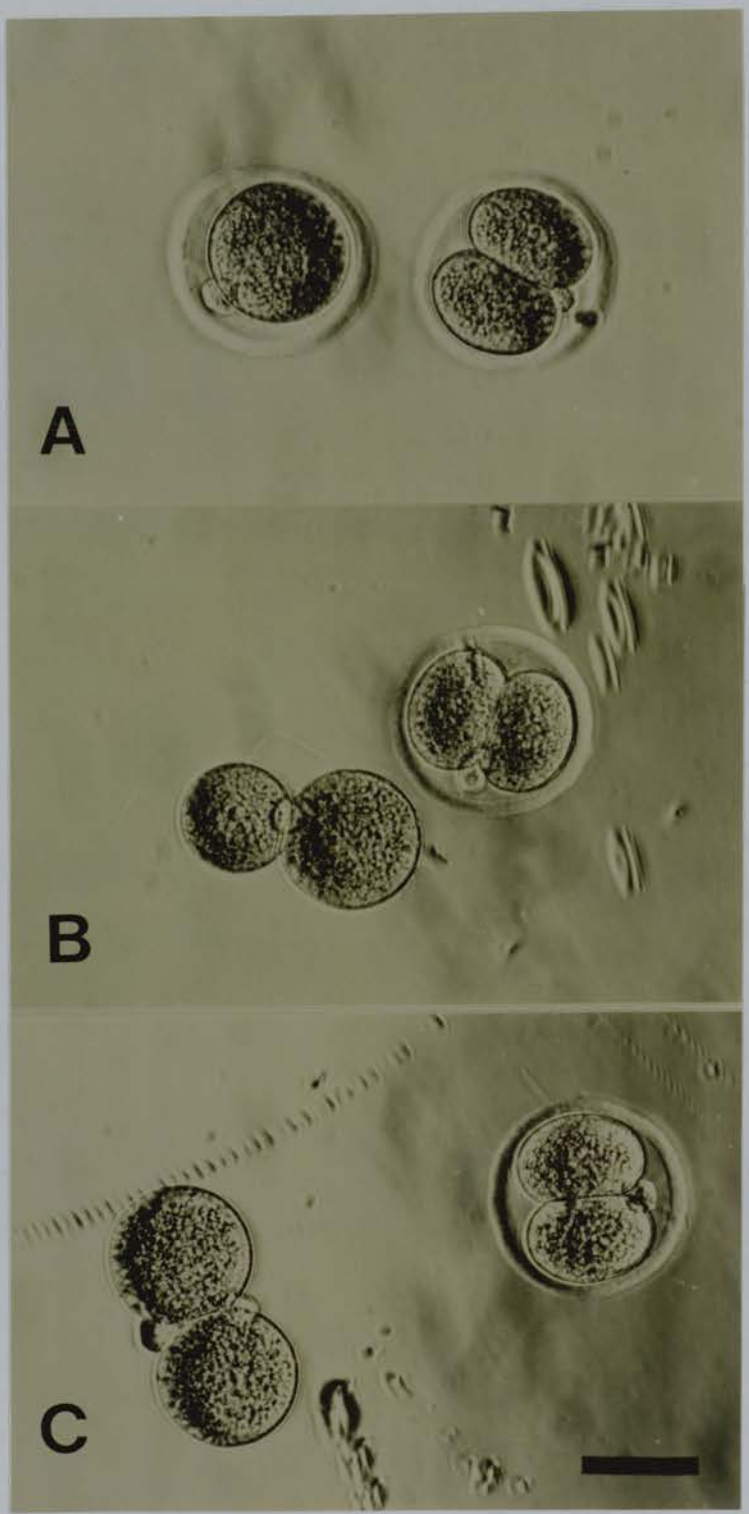


Fig. 4.4 Photos of manipulated cells and unmanipulated control 2-cell stage embryos with intact zonae pellucidae at E1.5. **A.** A big cell produced by electrofusion and a 2-cell stage embryo; **B.** An aggregate of one big diploid cell with one small diploid cell and a 2-cell stage embryo; **C.** An aggregate of one big tetraploid cell with one big diploid cell and a 2-cell stage embryo (bar is 50 μm).

preliminary and main experiments are shown in Tables 4.1 and 4.2 respectively. As shown in Fig. 4.1, the small diploid cells (S2n) were obtained from disaggregated 2-cell stage embryos; big diploid cells (B2n) were produced by a combination of enucleation and electrofusion and big tetraploid cells (B4n) were made by electrofusion of the 2-cell stage embryos. Disaggregation and aggregation were performed as described in 2.2.2. Pairs of cells (see Fig. 4.4 B and C) were then washed in fresh M2 medium and cultured individually in drops of pre-equilibrated M16 medium under mineral oil at 37°C in 5% CO_2 in air.

4.2.6. *EMBRYO TRANSFER*

The aggregated embryos made at the Roslin Institute were brought back to CRB the following day and were then further cultured over night. Initially, well-developed embryos were then transferred to the uterine horns of the pseudopregnant females at 2.5 days of pregnancy (see 2.1.4). However, due to the low implantation rate in the preliminary experiments (see 4.3.1), the analysis of aggregates was performed on pre-implantation embryos of strain combination of ($BF_1 \times BF_1$) $\leftrightarrow$ ($BF_1 \times$ TGB) by *in situ* DNA-DNA hybridisation of the β-globin transgene. Therefore, the embryos were cultured for three nights after being produced and those that reached the E4.5 blastocyst stage (see Fig. 4.5) were transferred into dissected β-globin transgene positive oviducts. These oviducts were not only used as blastocyst carriers to enable the chimaeric blastocysts to be processed, but also served as positive controls for the *in situ* hybridisation technique. The oviduct donors were given an intraperitoneal injection of 5 I.U. HCG one day before sacrifice to enlarge the space in the ampulla and prevent the transferred blastocysts becoming squashed. The oviducts, each containing 6-10 blastocysts, were wrapped in tissue paper and placed in Tissue Tex cassettes individually for the processing. The oviducts were subsequently embedded in paraffin wax and *in situ* hybridisation was performed on 7

Table 4.1 The chimaera series used in the preliminary experiments

Series of chimaeras[†]	Combination	Description	Stage of analysis
B2n ↔ *S2n	($BF_1 \times BF_1$) ↔ ($BF_1 \times$ ROSA)	big diploid *lac-Z*-negative cell ↔ small diploid *lac-Z*-positive cell	E5.5
S2n ↔ *B2n	($BF_1 \times BF_1$) ↔ ($BF_1 \times$ ROSA)	small diploid *lac-Z*-negative cell ↔ big diploid *lac-Z*-positive cell	E5.5

[†]: Abbreviations: B= big cells; S= small cells; 2n= diploid cells; 4n= tetraploid cells.

*: represents *lac-Z* reporter transgene-positive cells

Table 4.2 Detail of the control and main experimental series of chimaeras

Series of chimaeras†	Combination	Description	Stage of analysis
Control groups			
combination control:			
S2n ↔ *S2n	($BF_1 \times BF_1$) ↔ (BF_1 × TGB)	small diploid *Tg*-negative cell ↔ small diploid *Tg*-positive cell	E4.5
positive controls:			
*S2n ↔ *S2n	(BF_1 × TGB) ↔ (BF_1 × TGB)	small diploid *Tg*-positive cell ↔ small diploid *Tg*-positive cell	E4.5
*B2n ↔ *B2n	(BF_1 × TGB) ↔ (BF_1 × TGB)	big diploid *Tg*-positive cell ↔ big diploid *Tg*-positive cell	E4.5
*B4n ↔ *B4n	(BF_1 × TGB) ↔ (BF_1 × TGB)	big tetraploid *Tg*-positive cell ↔ big tetraploid *Tg*-positive cell	E4.5
Experimental groups			
B2n ↔ *S2n	($BF_1 \times BF_1$) ↔ (BF_1 × TGB)	big diploid *Tg*-negative cell ↔ small diploid *Tg*-positive cell	E4.5
S2n ↔ *B2n	($BF_1 \times BF_1$) ↔ (BF_1 × TGB)	small diploid *Tg*-negative cell ↔ big diploid *Tg*-positive cell	E4.5
B4n ↔ *B2n	($BF_1 \times BF_1$) ↔ (BF_1 × TGB)	big tetraploid *Tg*-negative cell ↔ big diploid *Tg*-positive cell	E4.5
B2n ↔ *B4n	($BF_1 \times BF_1$) ↔ (BF_1 × TGB)	big diploid *Tg*-negative cell ↔ big tetraploid *Tg*-positive cell	E4.5

†: Abbreviations as Table 4.1

*: represents β-globin transgene-positive cells

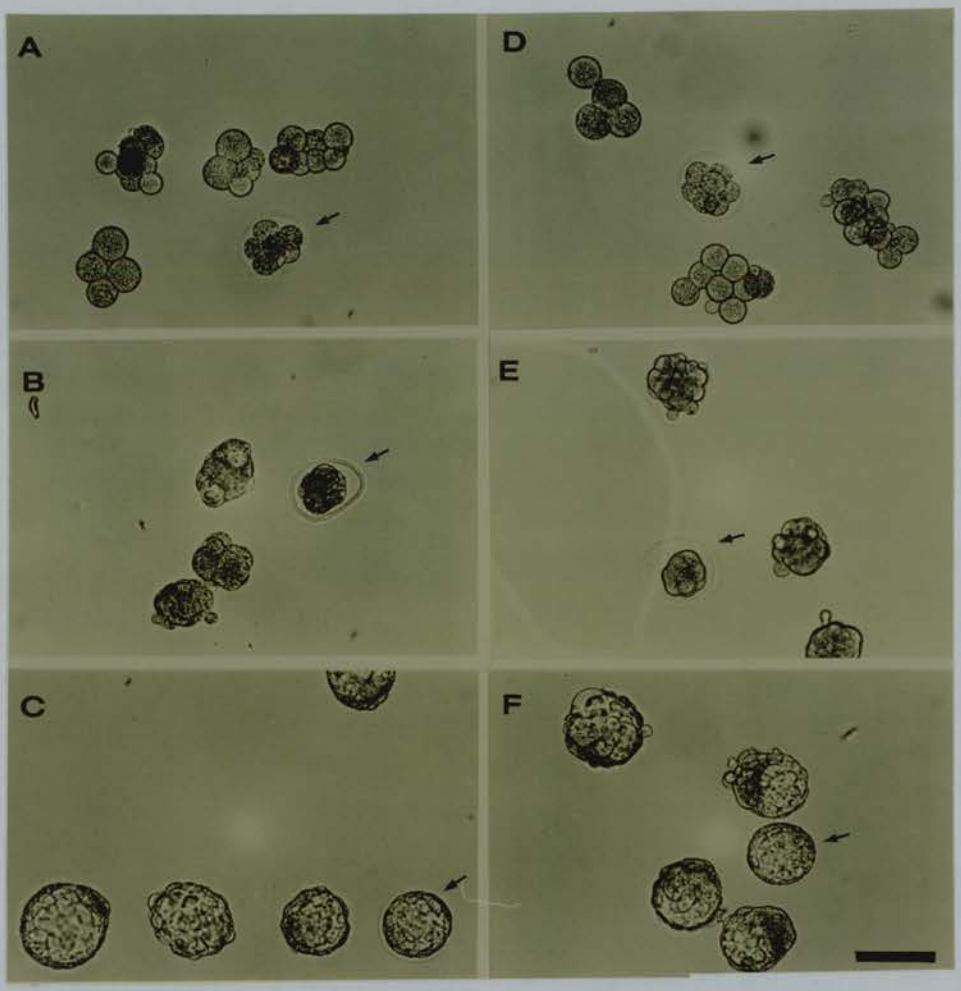


Fig. 4.5 The development of experimental aggregates and unmanipulated control embryos with intact zonae pellucidae. **A-C.** The aggregates of B2n ↔ S2n and the corresponding control embryos which are indicated with arrows. **A.** B2n ↔ S2n aggregates and one control embryo at E2.5; **B.** B2n ↔ S2n aggregates and one control embryo at E3.5; **C.** B2n ↔ S2n aggregates and one control embryo at E4.5. **D-F.** The aggregates of B4n ↔ B2n and the corresponding control embryos which are indicated with arrows. **D.** B4n ↔ B2n aggregates and one control embryo at E2.5; **E.** B4n ↔ B2n aggregates and one control embryo at E3.5; **F.** B4n ↔ B2n aggregates and one control embryo at E4.5 (bar is 100 μm).

μm sections.

4.2.7. *BLASTOCYST ANALYSIS*

In each section, the blastomeres were scored as *Tg*-positive or negative. They were also classified into different cell lineages: polar trophectoderm (pTE), mural trophectoderm (mTE) and inner cell mass (ICM). The percentage of *Tg*-positive cells (abbreviated as %*Tg*) in each lineage was calculated from the ratio of the total number of *Tg*-positive cells and the total number of cells in the serial sections of a whole blastocyst. To reduce the impact of technical problems (such as hybridisation failure and loss of sections), sections were only scored if the *in situ* hybridisation worked properly in the *Tg*-positive oviducts, and only blastocysts with more than 5 sections were included in the subsequent analysis, as described previously (Everett and West, 1996).

4.3 RESULTS

4.3.1. *PRELIMINARY EXPERIMENTS*

Several groups of E1.5 mouse embryos were treated to different conditions to assess whether transportation and manipulation had any influence on development. Considering the percentages of blastocyst formation at E4.5, transportation appears to have no adverse effect on the development of mouse embryos. 95.6% of the 2-cell stage embryos (control 1: which were flushed out from oviducts and cultured immediately) formed blastocysts at E4.5, compared with 95.9% which formed blastocysts in control 2, in which embryos had been transported to and from the Roslin Institute (see Appendix III. 7).

The S2n and B2n groups showed no significant difference in the proportions of embryos showing blastocyst formation at E4.5 (81.3% vs. 70.2%, χ^2=2.6501, P=0.1035; see Appendix III. 8). This implies that enucleation and electrofusion did not influence cell development and that because blastocyst formation is dependent on number of nuclear divisions, the cleavage rate of "bigger" cells is assumed to be similar to "normal-size" cells. The group of B2n ↔ S2n chimaeras also showed a comparable percentages of blastocyst formation to the group of control 2 (91.9% vs. 95.9%; see Appendices III. 7 and III. 8).

Two series of chimaeras were also produced at E1.5, as shown in Table 4.1, and the aggregated embryos which developed to the morula/early blastocyst stage at E3.5 were transferred to the uterine horns of females at 2.5 days of pseudopregnancy. The results are shown in Table 4.3. After 100 aggregated embryos had been transferred, however, only 3 embryos were found to have implanted at E5.5. These 3 embryos were of the S2n ↔ *B2n series in which the *lac-Z* positive cells were bigger. The results of X-Gal staining for *β*-galactosidase showed that one embryo turned entirely blue, one was white and the third one was only blue in part of the extraembryonic ectoderm. Due to such a small sample size, no conclusions can be drawn. Also, due to the low implantation rate, the effects of cell size and ploidy were subsequently performed in preimplantation chimaeric embryos.

4.3.2. *CONTROL GROUPS*

4.3.2.a. Positive control chimaeras

As described previously (Everett and West, 1996), nuclei may appear in several successive sections, but the *in situ* signal associated with each nucleus of the *Tg*-positive cell may not appear in each section. Therefore, the proportion of *Tg* in

Table 4.3 Development of the embryo made by aggregating a big diploid cell with a small diploid cells

Day	Stage	$(BF_1 \times BF_1) \leftrightarrow (BF_1 \times ROSA)$	
		B2n ↔ *S2n % (number)†	S2n ↔ *B2n % (number)*
E1.5	2-cell	100.0 (50)	100.0 (65)
E2.5	3-8-cell	100.0 (50)	93.8 (61)
	8-morula		3.1 (2)
	morula		3.1 (2)
E3.5	3-8-cell		3.1 (2)
	8-morula	8.0 (4)	
	morula	92.0 (46) †	96.9 (63)‡

*: represents the *lac-Z* transgene

*: %=(number of embryos at the developmental stage/total number of embryos)×100

†: 40 out of 46 embryos in the series B2n ↔ *S2n were transferred and none of them had implanted at E5.5

‡: 60 out of 63 embryos in the series of S2n ↔ *B2n were transferred and 3 implanted at E5.5

the chimaeric blastocysts may be underestimated. Hence, the ratios of the expected percentage of *Tg*-positive cells, (which would be 100% in positive controls), to observed percentage of *Tg*-positive cells in each cell lineages were used as correction factors. Details of scoring records of the positive controls are listed in Appendices III. 9, 10 and 11.

The results of observed %*Tg* in the three positive controls are shown in Table 4.4. As expected, the %*Tg* in each cell lineage is less than 100%. The big diploid positive control (*B2n ↔ *B2n) shows the lowest %*Tg* in the three lineages. It may be caused by more false negative sections due to the large cell size and only one copy of transgene in the nucleus. The other two positive controls, however, showed comparable %*Tg*. Three serial sections of *S 2n ↔ *S2n are shown in Fig. 4.6.

The fraction (expected %*Tg*/observed %*Tg*) from the appropriate positive control series was used to correct the observed %*Tg* in the experimental chimaeras. For example, the figures of 100/58.36, 100/57.84, 100/57.13, (derived from the series of *S2n ↔ *S2n shown in Table 4.4), would be the correction factors for the ICM, pTE and mTE, respectively, in the chimaeric combinations in which *Tg*-positive cells were small and diploid. Thus, the corrected %*Tg* in the three primary lineages in the series B2n ↔ *S2n and S2n ↔ *S2n can be obtained by multiplying the observed %*Tg* by these three figures respectively. The same strategy was applied to the other experimental groups and all subsequent data shown are corrected values.

4.3.2.b. The genotypically balanced strain combination ($BF_1 \times BF_1$) ↔ ($BF_1 \times TGB$)

The chimaeric combination ($BF_1 \times BF_1$) ↔ ($BF_1 \times TGB$) was made to assess the genotypic balance of the strain combination. The results are shown in Table 4.5 and the scoring record is listed in Appendix III. 12. The proportions of the two cell

Table 4.4 Observed mean %*Tg* in the three positive control groups (expected %*Tg* is 100%)

	*S2n ↔ *S2n		*B2n ↔ *B2n		*B4n ↔ *B4n	
Lineages	%*Tg*†	N†	%*Tg*†	N†	%*Tg*†	N†
Inner cell mass	58.36 ± 2.47	17	44.44 ± 3.02	11	55.46 ± 4.38	11
Polar trophectoderm	57.84 ± 2.59	17	41.39 ± 2.85	11	51.13 ± 4.52	11
Mural trophectoderm	57.13 ± 2.44	19	40.95 ± 1.76	11	54.95 ± 2.19	14

†: Mean ± SEM; N=number of blastocysts

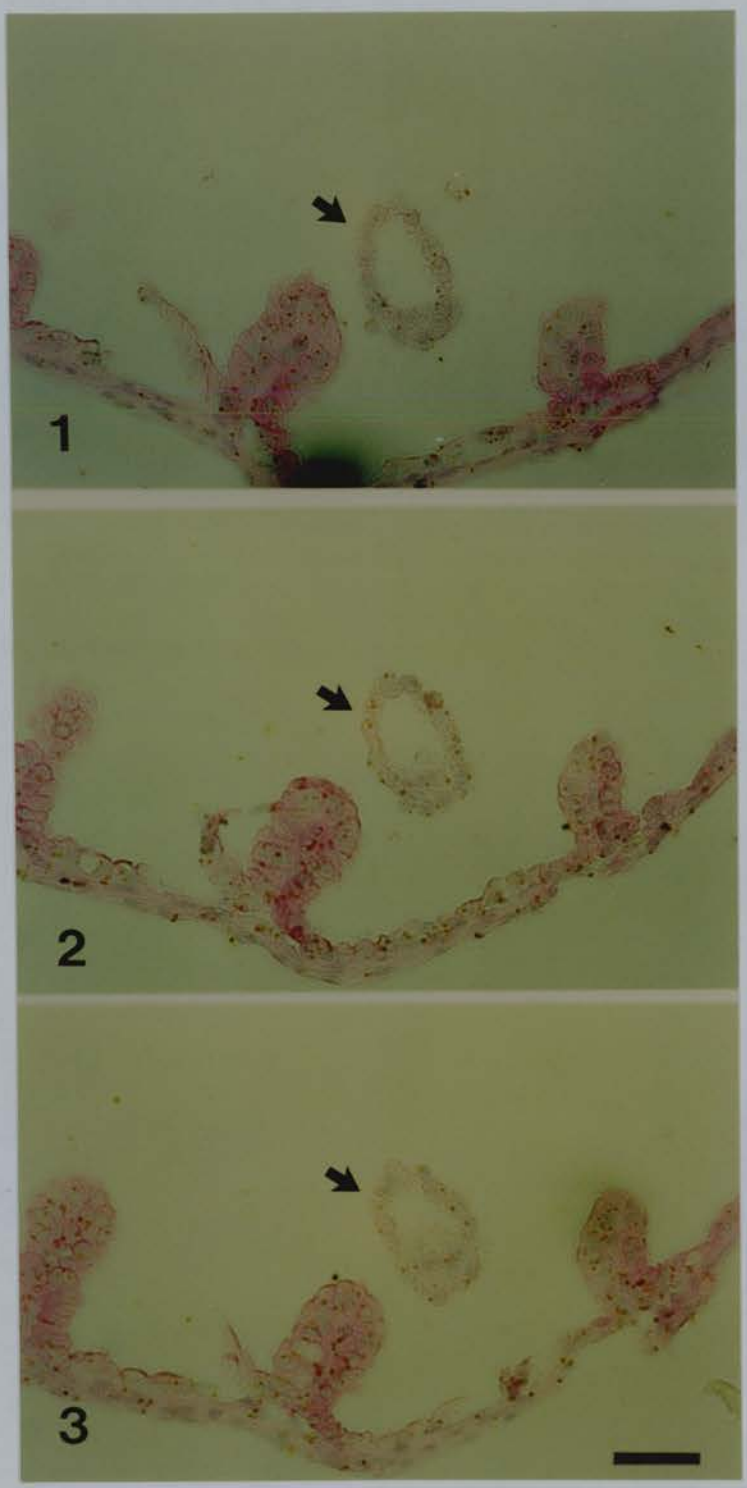


Fig. 4.6 Three serial sections (1-3) of the *S2n ↔ *S2n positive control. The brown spots are *in situ* hybridisation signals. Arrows indicate the blastocysts which are transferred into oviducts. As shown in the sections, not each nucleus in chimaeric blastocysts of the positive control shows an *in situ* signal (bar is 50 μm).

Table 4.5 The corrected mean %*Tg* in the three lineages of the combination control group ($BF_1 \times BF_1$) ↔ ($BF_1 \times$ TGB)

	S2n ↔ *S2n	
Lineages	%*Tg*†	N†
Inner cell mass	52.35 ± 4.69	35
polar Trophectoderm	57.04 ± 4.72	35
mural Trophectoderm	62.33 ± 2.69	38

†: Mean ± SEM; N=number of blastocysts

populations in the three lineages were not significantly different by the Kruskal-Wallis test for %*Tg*. Furthermore, when the numbers of chimaeras were plotted against the percentage of *Tg*-positive cells in the three cell lineages, most of the aggregated blastocysts analysed were shown to have 25-75% chimaerism (see Fig. 4.7), which implied that this combination is genotypically balanced (Mullen and Whitten, 1971; West and Flockhart, 1994; West *et al.*, 1995). Therefore, any significant difference in %*Tg* among these three lineages in the experimental groups could be assumed to be due to cell size or ploidy.

4.3.3. THE INFLUENCE OF CELL SIZE ON CELL DISTRIBUTION IN CHIMAERIC BLASTOCYSTS

Results of the two reciprocal series of chimaeras made to test the effect of cell size are shown in Table 4.6 and detail of scoring is listed in Appendices III. 13 and 14. In the series B2n ↔ *S2n, in which the *Tg*-positive cells were smaller than the other component of the chimaera, the corrected mean %*Tg* in the three lineages were significantly different. The smaller cells tended to make a significantly greater contribution to the inner cell mass than to the polar and mural trophectoderm lineages (61.48 vs. 35.33, P=0.0001 and 61.48 vs. 39.03, P=0.0002, respectively). However, there was no significant difference between two trophectoderm lineages (35.33 vs. 39.03, P=0.545). In the other series of chimaeras, in which *Tg*-positive cells were bigger than the *Tg*-negative cells, the %*Tg* in the mural trophectoderm was significantly greater than that in the polar trophectoderm and the inner cell mass (88.57 vs. 64.66, P=0.0014 and 88.57 vs. 48.76, P<0.0001, respectively). There was also a significant difference in %*Tg* between the polar trophectoderm and the inner cell mass (64.66 vs. 48.76, P=0.036). Apparently, the physical size influenced cell allocation in the blastocysts. Three sections of one of the series of B2n ↔ *S2n chimaeras are shown in Fig. 4.8.

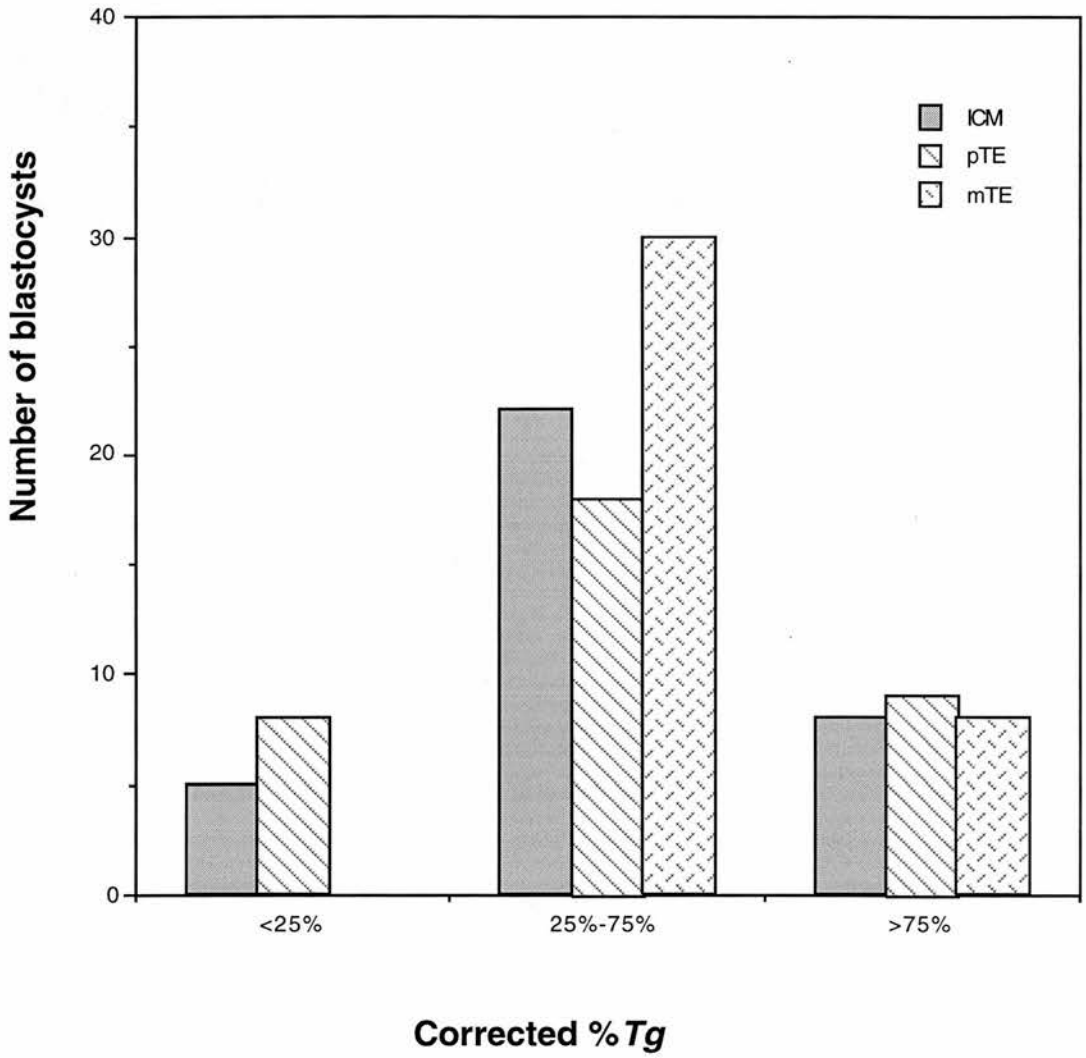


Fig. 4.7 Distribution of the *Tg*-positive cells in the three lineages of polar trophectoderm, mural trophectoderm and the inner cell mass in the combination control, S2n↔*S2n. It shows a balanced distribution, with most chimaeric blastocysts showing 25-75% chimaerism in all three lineages.

Table 4.6 The corrected mean %*Tg* of the three lineages from the experimental groups with components of different cell size in the aggregated blastocysts

	B2n ↔ *S2n		S2n ↔ *B2n	
Lineages	%*Tg*[†]	N[†]	% *Tg*[†]	N[†]
Inner cell mass	61.48 ± 4.57[a]	37	48.76 ± 5.25[c‡]	50
Polar trophectoderm	35.33 ± 4.26[b]	37	64.66 ± 5.91[d‡]	50
Mural trophectoderm	39.03 ± 3.28[b]	41	88.57 ± 3.77[e‡]	52

[†]: %*Tg*, corrected mean %*Tg* ± SEM (correction factors from the positive control combinations of *S2n ↔ *S2n and *B2n ↔ *B2n respectively); N=number of blastocysts.

[a-e]: There is a significant difference between any two means with a different letter in the same column by the Mann-Whitney U tests, $P<0.05$

[‡]: Comparisons between the two experimental groups, $P<0.05$

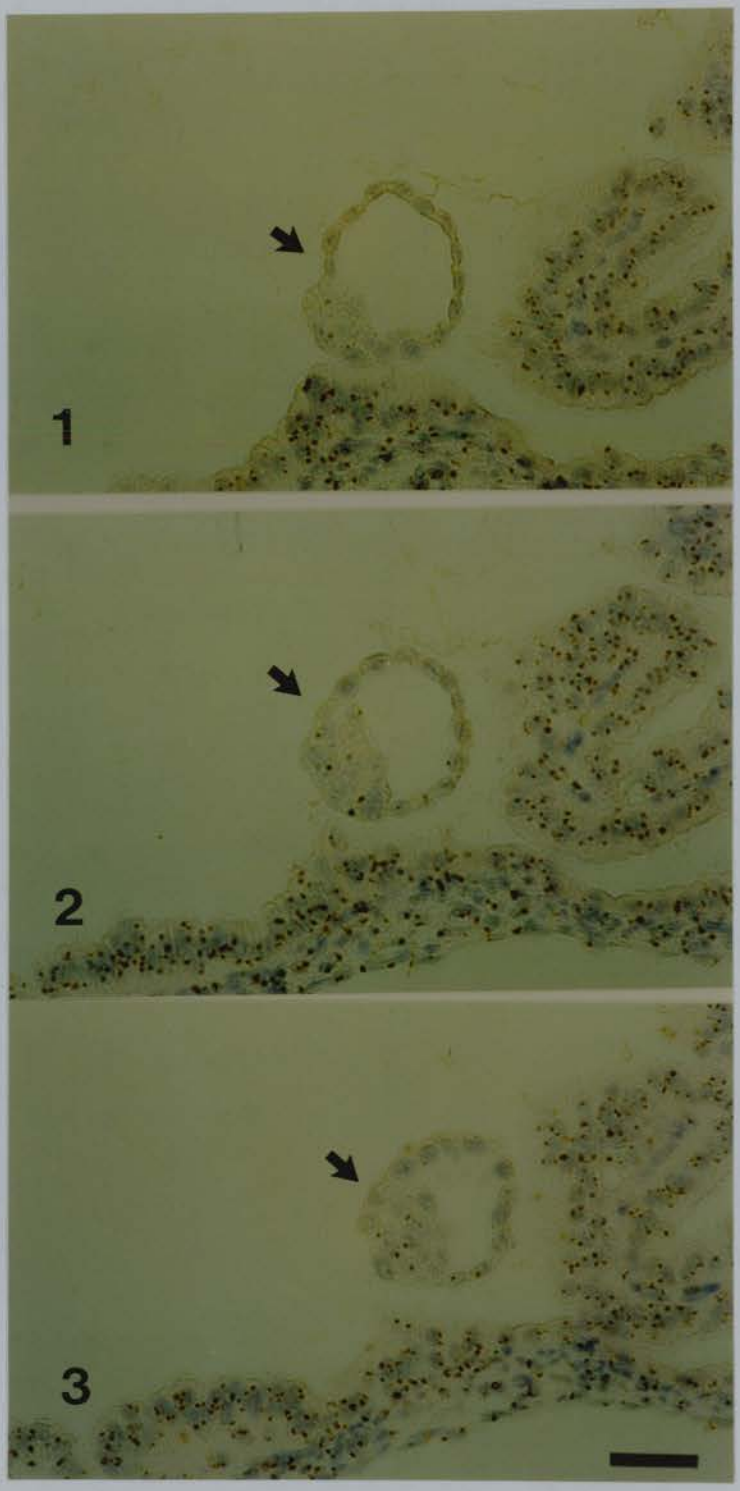


Fig. 4.8 Three serial sections (1-3) of a chimaeric blastocyst from the series B2n ↔ *S2n. Arrows indicate the blastocysts. The *in situ* signals (brown spots) are found more frequently in the inner cell mass rather than in the trophectoderm (bar is 50 μm).

Distributions of *Tg*-positive cells for each primary cell lineage in the two reciprocal series of chimaeras are shown in Fig 4.9 A. The distribution of %*Tg* is skewed towards a higher proportion in the inner cell mass (ICM) and a lower percentage in the trophectoderm lineages, when *Tg*-positive cells were small cells (in the series B2n ↔ *S2n). Whereas in S2n ↔ *B2n chimaeras, when *Tg*-positive cells were big , the distributions were skewed in the opposite direction: lower proportion in the ICM and a higher percentage in the trophectoderm lineages. Therefore these two reciprocal combinations showed the expected "mirror image" distributions for each of the three primary cell lineages.

4.3.4. THE INFLUENCE OF PLOIDY ON THE CELL DISTRIBUTION OF CHIMAERIC BLASTOCYSTS

Table 4.7 shows the results of the two series of chimaeras produced for testing the effect of ploidy when the cell size of each component was similar. Detailed scoring data are listed in Appendices III. 15 and 16.

Diploid cells in the B4n ↔ *B2n combination made a significantly greater contribution to the ICM than to the polar and mural trophectoderm lineages (56.69 vs. 34.50, P=0.0049 and 56.69 vs. 33.62, P=0.0041, respectively), but the proportions of diploid *Tg*-positive cells in the two trophectoderm lineages were not significantly different (34.5 vs. 33.62, P=0.917). In its reciprocal combination, B2n ↔ *B4n, in which the *Tg*-positive cells were tetraploid, the %*Tg* was significantly lower in the ICM than in the mural trophectoderm (49.47 vs. 60.70, P=0.0091), but there was no statistical difference between the ICM and polar trophectoderm (49.47 vs. 50.10, P=0.826) or between the two trophectoderm lineages (50.10 vs. 60.70, P=0.0508). Three serial sections of the chimaeric blastocyst from the series of B2n ↔ *B4n are shown in Fig. 4.10.

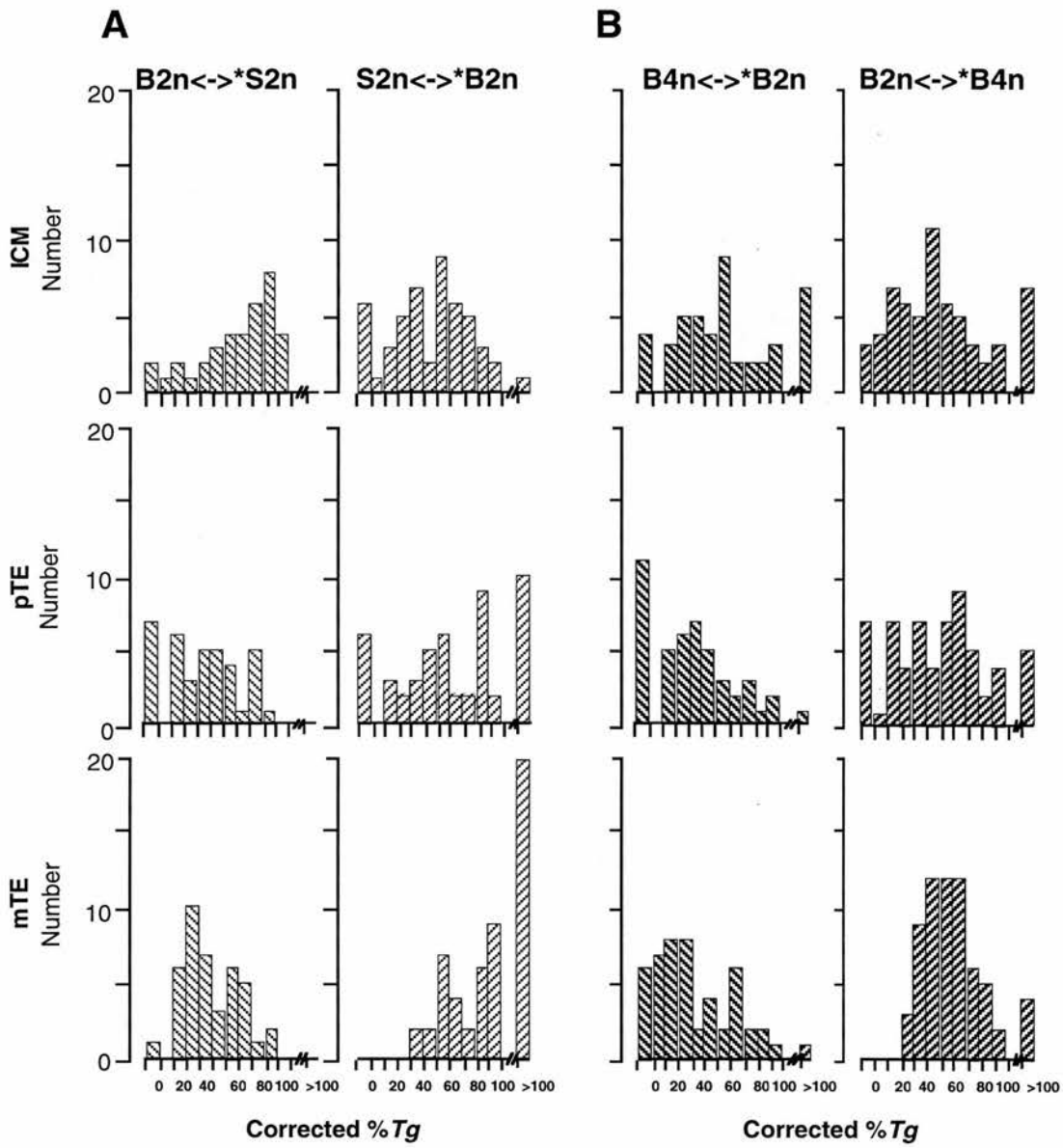


Fig. 4.9 **A.** The distribution of *%Tg* in the three lineages (polar trophectoderm, pTE; mural trophectoderm, mTE; inner cell mass, ICM) in the two reciprocal combinations in the cell size experiment; **B.** The distributions of *%Tg* in the three lineages in the two reciprocal combinations in the ploidy experiment.

Table 4.7 The corrected mean %*Tg* of the three lineages from the experimental groups in which the components in the chimaeric blastocysts differed in cell ploidy

	B4n ↔ *B2n		B2n ↔ *B4n	
Lineages	%*Tg*†	N†	%*Tg*†	N†
Inner cell mass	56.69 ± 5.93[a]	47	49.47 ± 4.32[c]	62
Polar trophectoderm	34.50 ± 4.41[b]	46	50.10 ± 4.38[cd‡]	62
Mural trophectoderm	33.62 ± 4.22[b]	49	60.70 ± 2.73[d‡]	65

†: %*Tg*, corrected mean %*Tg* ± SEM (correction factors from the positive control combinations of *B2n ↔ *B2n and *B4n ↔ *B4n respectively); N=number of blastocysts.

[a-d]: Significant difference occur where any two means are named with different letters in the same column, $P<0.05$

‡: Comparisons between the two experimental groups, $P<0.05$

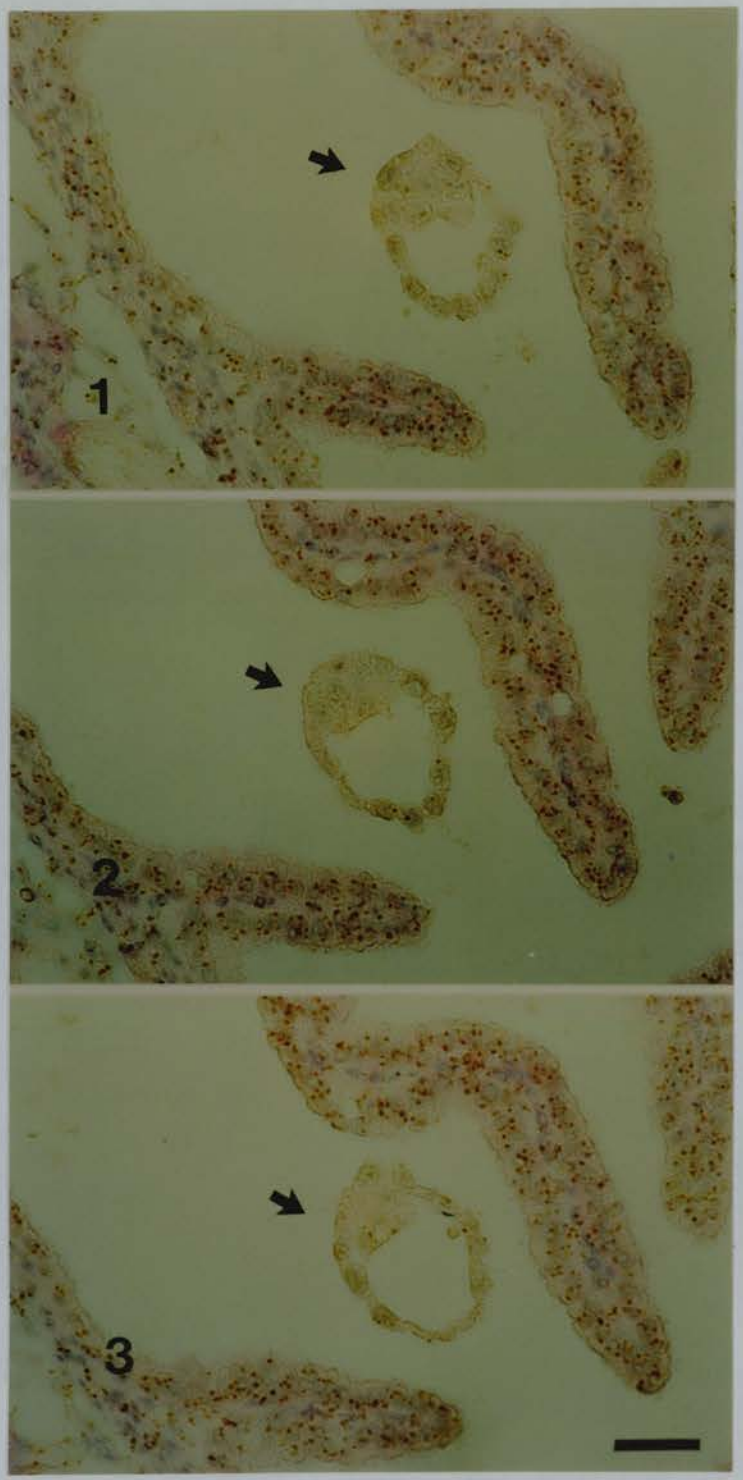


Fig. 4.10 Three serial sections (1-3) of a chimaeric blastocyst from the series B2n ↔ *B4n. Arrows indicate the blastocysts. The *in situ* signals (brown spots) are found more frequently in the trophectoderm than in the inner cell mass (bar is 50 μm).

The proportions of *Tg*-positive cells in the three lineages of these two ploidy reciprocal combinations are illustrated in Fig. 4.9 B. A higher number of chimaeras with a lower % *Tg* in the mural trophectoderm when the *Tg*-positive cells were diploid (series B4n ↔ *B2n), than when the *Tg*-positive cells were tetraploid (series B2n ↔ *B4n). The distribution of %*Tg* in the mural trophectoderm tended to be skewed towards a higher percentage. Although the expected "mirror image" did not show clearly in the other cell lineages (the inner cell mass and polar trophectoderm), the effect of ploidy is marked in the mural trophectoderm at the blastocyst stage embryo.

4.3.5. COMPARISONS BETWEEN RECIPROCAL COMBINATIONS

Comparisons were performed on each pair of reciprocal chimaeras. The two cell size experiments showed that the two reciprocal series differed significantly in %*Tg* in each of the three lineages (see Table 4.6). The small cells (in series B2n ↔ *S2n) made a statistically greater contribution to the inner cell mass than the big cells (series S2n ↔ *B2n) (P=0.0093) and there were more big cells in both trophectoderm lineages, (comparing S2n ↔ *B2n with B2n ↔ *S2n chimaeras; pTE, P=0.0007 ; mTE, P<0.0001). Since the aggregated embryos did not differ in ploidy, stage, a difference in physical cell size alone is sufficient to affect the allocation of cells to the ICM or trophectoderm.

Additionally, the two reciprocal chimaera series produced in the ploidy experiments were compared to test whether ploidy alone can affect the contribution of *Tg*-positive cells to each of the three lineages. The results show that the contribution of *Tg*-positive cells to the trophectoderm lineages differed significantly between the two series of chimaeras (B4n ↔ *B2n vs. B2n ↔ *B4n, pTE, 35.50 vs. 50.10, P=0.0153; mTE, 33.62 vs. 60.70, P<0.0001), indicating a predominance of

tetraploid cells in the trophectoderm lineages. The *%Tg* in the inner cell mass also differed in the expected direction. The contribution of tetraploid cells to the ICM is less in the series of B2n ↔ *B4n than in B4n ↔ *B2n although the difference was not statistically significant (see Table 4.7). It shows that ploidy alone, without any other influence, *e.g.* cell size, cell number or embryo stage, can cause a non-random cell distribution in chimaeric blastocysts.

4.4 DISCUSSION

This study supports a previous report that tetraploid cells were preferentially allocated to the mural trophectoderm of tetraploid↔diploid chimaeras (Everett and West, 1996) and has produced further insight into the effects of cell size and ploidy on cell allocation in chimaeric, preimplantation mouse embryos. In the previous study (Everett and West, 1996) tetraploid cells were found to be more abundant in the mural trophectoderm in both 4-cell stage tetraploid↔8-cell stage diploid and 4-cell stage tetraploid↔4-cell stage diploid chimaeras. Although diploid and tetraploid cells were of a similar size in the second series, they differed in developmental age. By using micromanipulation techniques it has been possible to evaluate ploidy and cell size separately without any confounding effects of stage differences. These results have shown that cell size alone (without the influences of differences in cell number, embryo stage or ploidy) can affect the cell distribution in chimaeric blastocysts. Larger cells made a greater contribution to the polar trophectoderm and mural trophectoderm than to the inner cell mass (ICM). Differences in ploidy had a similar effect so that larger cells and/or tetraploid cells tended to be allocated to the trophectoderm (especially to the mural trophectoderm) rather than to the ICM. The present results lead to the conclusion that differences in both cell size and ploidy can produce a non-random distribution of cells in chimaeric blastocysts and that both

factors probably contributed to the effects seen in the big tetraploid↔small diploid chimaeric blastocysts reported previously.

Big and/or tetraploid cells could be preferentially allocated to the mural trophectoderm in several ways. They may first be preferentially allocated to the trophectoderm rather than the ICM and later become more abundant in the mural trophectoderm than the polar trophectoderm. Alternatively, big and/or tetraploid cells may be specifically enriched in the mural trophectoderm in one step. A combination of both types of mechanisms is also possible.

According to the well-established 'inside-outside" hypothesis of Tarkowski and Wroblewska (1967), preferential allocation to the trophectoderm rather than the ICM means that big and/or tetraploid cells should segregate preferentially to the outer part of the embryo. In the chimaeric blastocysts of the present study and in both sets of tetraploid↔diploid chimaeras reported earlier (Everett and West, 1996), the proportion of big and/or tetraploid cells was significantly higher in the whole trophectoderm than in the ICM. This is consistent with preferential allocation to the outside of the embryo (trophectoderm) but the basis for this remains unclear. In principle, preferential segregation of cells to the outer layer could be influenced by various factors including timing of cleavage divisions, cell size or cell surface properties but, as discussed below, the first of these seems unlikely.

During mouse embryonic development, cells begin to divide asynchronously from the second cell cycle onwards and descendants of the earlier cleaving blastomeres are allocated preferentially to the inside and subsequently become ICM cells (Graham and Deussen, 1978; Kelly *et al.*, 1978; Graham and Lehtonen, 1979). This could be a simple geometrical effect of cell size or an effect of the timing of cleavage. Therefore, any difference in cleavage time between small and large cells, or between diploid and tetraploid cells, could underlie the non-random distribution

which was observed. If the big and/or tetraploid cells cleaved later they would tend to be left on the outside and so be preferentially allocated to the trophectoderm. The cleavage times of big and small diploid cells were not compared in this study but there is good evidence that big tetraploid cells do not cleave more slowly than small diploid cells (Eglitis and Wiley, 1981; Henery and Kaufman, 1991). Thus, it seems unlikely that tetraploid or big cells divided more slowly in our study. So, timing of cleavage divisions probably did not affect the allocation of cells to the inside or outside of the embryo.

These observations are important for another reason. The aim of this study was to dissociate differences in cell size and ploidy. This could have been undermined if the tetraploid cells had cleaved more slowly because they might have been one cell generation behind the diploid cells when the blastocyst was formed. The "big tetraploid cells" would then have been bigger than the "big diploid cells" and the two cell types would have differed in both size and ploidy. The evidence that tetraploid embryos do not cleave more slowly means that it is unlikely that these experiments were undermined in this way.

It is possible that big cells are allocated preferentially to the outside of the embryo on geometrical grounds. As described in 1.3.1.b., during the formation of a 16-cell stage embryo, apolar blastomeres are smaller and occupy an inner position (Ziomek and Johnson, 1982). It may be geometrically more efficient to surround smaller cells with bigger cells. Therefore, the smaller cells may tend to occupy the inner region while two aggregated embryos may broadly occupy two hemispheres in a chimaeric morula (Garner and McLaren, 1974; Kelly, 1979). In big diploid↔small diploid chimaeras this may involve "sorting-out" of cells (Curtis, 1961; Steinberg, 1970) based on cell size. Sorting-out might also occur in big tetraploid↔big diploid chimaeras if tetraploid and diploid cells had different cell surface properties.

Preferential allocation to the trophectoderm rather than the ICM (TE>ICM) does not, alone, explain the higher proportion in the mural trophectoderm than polar trophectoderm. However, this could occur if preferential allocation to the trophectoderm was followed by a second step, that caused big and/or tetraploid cells to become most abundant in the mural trophectoderm (mTE>pTE). This could occur if some polar trophectoderm cells were displaced by ICM cells, as suggested in another context by West and Flockhart (1994) and section 3.4. Several experiments indicate that ICM cells can displace polar trophectoderm cells to the mural trophectoderm (Handyside, 1978; Copp, 1979; Cruz and Pedersen, 1985; Rossant and Croy, 1985; Winkel and Pedersen, 1988). On this basis, the initially high proportion of big and/or tetraploid cells would be maintained in the mural trophectoderm but diluted in the polar trophectoderm. More recently Gardner and Nichols (1991) argued that this is not a normal feature of development, although even they occasionally found results consistent with ICM descendants in the polar trophectoderm (which they attributed to technical artefacts). Overall these results suggest that this mechanism is possible but may not occur frequently.

A mechanism that preferentially allocated big and/or tetraploid cells directly to the mural trophectoderm (mTE>other lineages) could also be involved and could either act alone or in concert with one or both of the mechanisms discussed earlier (TE>ICM and/or mTE>pTE). Such a mechanism was suggested by Everett and West (1996), based on evidence that cells do not mix extensively before implantation (Garner and McLaren, 1974; Kelly, 1979; Dvorak *et al.*, 1995; Gardner and Cockcroft, 1998). In big tetraploid↔small diploid chimaeric blastocysts, the tetraploid and diploid cells remained largely separated, with the tetraploid cells often associated with the blastocyst cavity (Everett and West, 1996; Everett *et al.*, 1996). Everett and West (1996) suggested that differences, in levels of gene expression, between tetraploid and diploid cells, might cause the blastocyst cavity to form

preferentially in the region where tetraploid cells predominated. Hence, tetraploid cells would mainly occupy the regions, surrounding the blastocyst cavity, that would become primitive endoderm (pEnd) and mural trophectoderm (mTE=pEnd>epiblast=pTE). This would also help explain why, in later stage tetraploid↔diploid chimaeras, tetraploid cells contributed more to the derivatives of the primitive endoderm than to the epiblast. This explanation may also apply to the big tetraploid↔big diploid chimaeras in the present study. However, it is a less plausible explanation of the observed preferential allocation of big cells to the polar trophectoderm in big diploid↔small diploid chimaeras. In this case, it would be necessary to invoke a mechanism whereby the blastocyst cavity tended to form nearer to big diploid cells than small diploid cells.

The mechanisms responsible for the observed non-random allocation of cells among the tissues of the chimaeric blastocysts remain unclear but the foregoing discussion suggests that a combination of factors could be involved. Big and/or tetraploid cells may preferentially sort to the outer (trophectoderm) layer. In some cases, cells in the polar trophectoderm may then be displaced to mural trophectoderm. The differential between mural and polar trophectoderm would be increased further if the blastocyst cavity tended to form preferentially among tetraploid cells. Regardless of the mechanisms involved, the present study demonstrates that differences in cell size, as well as ploidy, will have contributed to the previously observed non-random allocation of tetraploid cells among the tissues of tetraploid↔diploid chimaeric blastocysts.

CHAPTER 5

THE EFFECTS OF CELL NUMBER AND EMBRYO STAGE ON THE COMPOSITION OF AGGREGATION CHIMAERAS

5.1 INTRODUCTION

The descendants of the first-dividing cell of the 2-cell stage mouse embryo tend to be enclosed inside the embryo, whereas the progeny of the other blastomere tend to remain on the outside (Kelly, 1978; Graham and Lehtonen, 1979). According to the "inside-outside" hypothesis, the inner cells become the inner cell mass and the outer cells contribute to the trophectoderm at the blastocyst stage (Tarkowski and Wroblewska, 1967). It has also been observed in some chimaera experiments that the more advanced cells, (from a later developmental stage), make a better contribution to the inner cell mass than the less advanced cells (from an earlier developmental stage) (Spindle, 1982; Surani and Barton, 1984).

However, the significantly greater contribution of the later/older blastomeres to the inner cell mass was not observed consistently in all chimaeras made by aggregating embryos of different developmental stages. For instance, three different chimaera series were made in Spindle's experiments (1982). [^{3}H]thymidine labelling was used to distinguish the component embryos in the chimaera. Only one of these series, (made by aggregating an 8-cell stage embryo with three 4-cell stage embryos and hereafter abbreviated to 1×8c ↔ 3×4c), showed a significant contribution of more advanced embryos to the inner cell mass in the chimaeric blastocyst than less advanced embryos. In series 1×4c ↔ 3×8c, the [^{3}H]thymidine labelled 4-cell stage embryo was considered to fail to develop and the third chimaera series, 2×4c ↔ 2×8c was produced. However, a greater contribution of the component 8-cell stage

embryo to the inner cell mass was not observed. The mean percentage of the labelled cells (from the 4-cell stage embryos) in the isolated ICMs (by immunosurgery) was 30.76%, which reflected the ideal proportion of cell numbers of contributing embryos at aggregating [(4+4)/(4+4+8+8)].

A similar result was also shown in other chimaeric blastocyst experiment (1×8c ↔ 1×4c; Everett and West, 1996). The contributions of more advanced component embryos to the three primary tissues in the chimaeric blastocyst were close to the ideal proportion of 66.7% [8/(8+4)]. Similarly, the 8-cell embryos did not contribute better to the ICM derivatives of E12.5 conceptuses in another group of chimaeras made by the same method, 1×8c ↔ 1×4c (James *et al.*, 1995). Therefore, the better contribution of the later/older blastomeres to the inner cell mass than the earlier/younger blastomeres does not occur consistently and might also be influenced, at least in part, by a difference in the number of cells in the different stage embryos.

In this Chapter, one chimaeric combination: $(AAF_1 \times AAF_1) \leftrightarrow (BF_1 \times TGB)$ (abbreviated to "B" for balanced strain combination; see Chapter 3) was chosen to make several series of chimaeras (see Table 5.1; details of mouse stocks are in Table 2.1). By comparing the chimaeras, which were made by aggregating a 4-cell stage embryo with an 8-cell stage embryo [B(4+8) or B(8+4); see Table 5.1], with those produced by aggregating a half 8-cell stage embryo with a whole 8-cell stage embryo [B($^1/_2$8+8) or B(8+$^1/_2$8); see Table 5.1], the aim of this study was to distinguish the effects of cell number and embryo stage on cell allocation in the chimaera. The 4-cell stage embryo was predicted to make a poorer contribution to the ICM derivatives of E12.5 conceptuses than the half 8-cell stage embryo if embryo stage had a significant effect on cell allocation. Also, results from Chapter 4, which examined the effect of cell size on cell allocation (see 4.3.3), were considered when analysing series B(8+4) and B(4+8) to clarify the embryo stage effect.

Table 5.1 Descriptions of the chimaera series made

Series of chimaeras*	Combination	Detail
Control		
B(8+8)	$(AAF_1 \times AAF_1) \leftrightarrow (BF_1 \times TGB)$	8-cell ↔ 8-cell
Cell number experiment		
B($^1/_2$8+8)	$(AAF_1 \times AAF_1) \leftrightarrow (BF_1 \times TGB)$	half 8-cell ↔ 8-cell
B(8+$^1/_2$8)	$(AAF_1 \times AAF_1) \leftrightarrow (BF_1 \times TGB)$	8-cell ↔ half 8-cell
Embryo stage experiment		
B(4+8)	$(AAF_1 \times AAF_1) \leftrightarrow (BF_1 \times TGB)$	4-cell ↔ 8-cell
B(8+4)	$(AAF_1 \times AAF_1) \leftrightarrow (BF_1 \times TGB)$	8-cell ↔ 4-cell

*: Embryos expressing GPI1-A activity are shown first.

5.2 MATERIALS AND METHODS

5.2.1. *SUPEROVULATION AND EMBRYO COLLECTION*

In order to collect 4- and 8-cell stage embryos at the same time point, a second room with a different light-dark cycle was prepared. Mice, (males and females), providing the 4-cell stage embryos were acclimatised in this room for at least a fortnight prior to superovulation. In this second room the light period was from 24:00h to 14:00h and the females were injected with 5 I.U. PMSG and 5 I.U. HCG, 48 hours apart at 07:00h. The donors of the 8-cell stage embryos were kept under standard conditions and superovulated, as described in 2.1.1 and 2.1.2.

4-cell and 8-cell stage embryos were flushed from the reproductive tract of pregnant females 52-53 and 67-68 hours after HCG injections respectively.

5.2.2. *CHIMAERA SERIES*

By disaggregation and aggregation, several series of chimaeras were produced (see Table 5.1). 8-cell stage embryos were either aggregated with half 8-cell stage embryos or with 4-cell stage embryos. Each aggregate was cultured individually in a drop of M16 medium (Whittingham, 1971) at 37°C, in 5% CO_2 in air. The following morning, well-aggregated and well-developed embryos were transferred surgically to the uterine horns of CF_1 females at 2.5 days of pseudopregnancy, as described previously.

5.2.3. *ANALYSIS OF CHIMAERAS*

Conceptuses were dissected from CF_1 females at E12.5. Five tissues were collected, including: fetus, amnion, yolk sac mesoderm, yolk sac endoderm and placenta. They were analysed for the percentage GPI1-A (%GPI1-A). In addition to

the collection of these different tissues, physical parameters were also recorded (see 3.2.4).

5.3 RESULTS

5.3.1. *CHIMAERA PRODUCTION*

As is evident from Table 5.2, the sample size of E12.5 chimaeric conceptuses from the series B(4+8) and B(8+4) is not very large (15 and 3 respectively). This was due to technical difficulties.

Although there were many aggregated pairs produced at E2.5 in series B(4+8) and B(8+4) (171 and 177 respectively), after one day of culture, fewer of the contributing embryos formed single aggregated embryos in these two groups than in the others. The aggregation success rate (percentage of embryos transferred) was 55.9% in series B(8+4) and 76.0% in series B(4+8). Both figures were lower than the control and cell number experimental groups (87.5%, 96.5%, 87.7% respectively). In addition, embryos that appeared aggregated in these two series did not produce many chimaeric E12.5 conceptuses. In the series B(8+4), even though 24 conceptuses were recovered, only 3 of them were chimaeric (12.5%). The chimaeric conceptus production rate was higher in the reciprocal series [40.5% (15/37) in series B(4+8)], but the non-chimaeric E12.5 conceptus frequency in this series was higher than in other three chimaera series [59.5% (22/37) vs. 24.4% (11/45), 9.1% (3/33) and 10.0% (4/40) respectively].

Table 5.2 Chimaera series produced in these experiments

Chimaera series	E2.5	E 3.5	E12.5 conceptuses		
	Embryos aggregated no.	Embryos transferred no. (%)	E12.5 conceptuses recovered* no. (%)	Chimaeric E12.5 conceptuses no. (%)	Non-chimaeric E12.5 conceptuses no. (%)
B(4+8)	171	130 (76.0)	37 (28.5)	15 (40.5)	22 (59.5)
B($^1/_2$8+8)	85	82 (96.5)	45 (54.9)	34 (75.6)	11 (24.4)
B(8+8)	80	70 (87.5)	33 (47.1)	30 (90.9)	3 (9.1)
B(8+$^1/_2$8)	106	93 (87.7)	40 (43.0)	36 (90.0)	4 (10.0)
B(8+4)	177	99 (55.9)	24 (24.0)	3 (12.5)	21 (87.5)

*: Data not including moles

5.3.2. *THE PROPORTIONS OF GPI1-A IN TISSUES ANALYSED IN EACH CHIMAERA SERIES*

The data for each conceptus, analysed in the series B(4+8), B($^1/_2$8+8), B(8+8), B(8+$^1/_2$8), and B(8+4), are shown in Appendices III 17, 18, 2, 19 and 20 respectively. These data are plotted in the histograms in Fig. 5.1.

Fig. 5.1 shows that the distribution of %GPI1-A is similar among the five tissues analysed, both in series B($^1/_2$8+8) and series B(8+$^1/_2$8). The %GPI1-A in all tissues analysed was skewed towards a lower proportion in the series B($^1/_2$8+8) and towards a higher proportion in the reciprocal chimaera series, B(8+$^1/_2$8). None of the tissues was exceptional. Series B(8+4) is also consistent with this pattern, although two conceptuses are only chimaeric in the yolk sac endoderm and the total number of samples is only 3 (see Appendix III. 20). Distribution of %GPI1-A in the placenta in series B(4+8), however, is skewed more towards a lower proportion, compared to any other tissue in this series. Most placentas in this series were non-chimaeric (0% GPI1-A).

Kruskal-Wallis statistical analyses were performed to identify whether cell number affected the proportion of GPI1-A differently in the various tissues in each chimaera series. The series B(8+8) was used as a control because both components of the combination were equal in cell number and embryo age. The results from this chimaera series showed that the contribution of (AAF_1 × AAF_1) embryos to the derivatives of the three primary cell lineages are not significantly different from each other (P=0.0931; see Table 5.3). When either the cell number of the embryos from (AAF_1 × AAF_1) or (BF_1 × TGB) was halved, the contribution was reduced but none of the tissues studied was specifically affected, *i.e.* all tissues showed similar %GPI1-A in either series B($^1/_2$8+8) or B(8+$^1/_2$8), with no significant difference among the

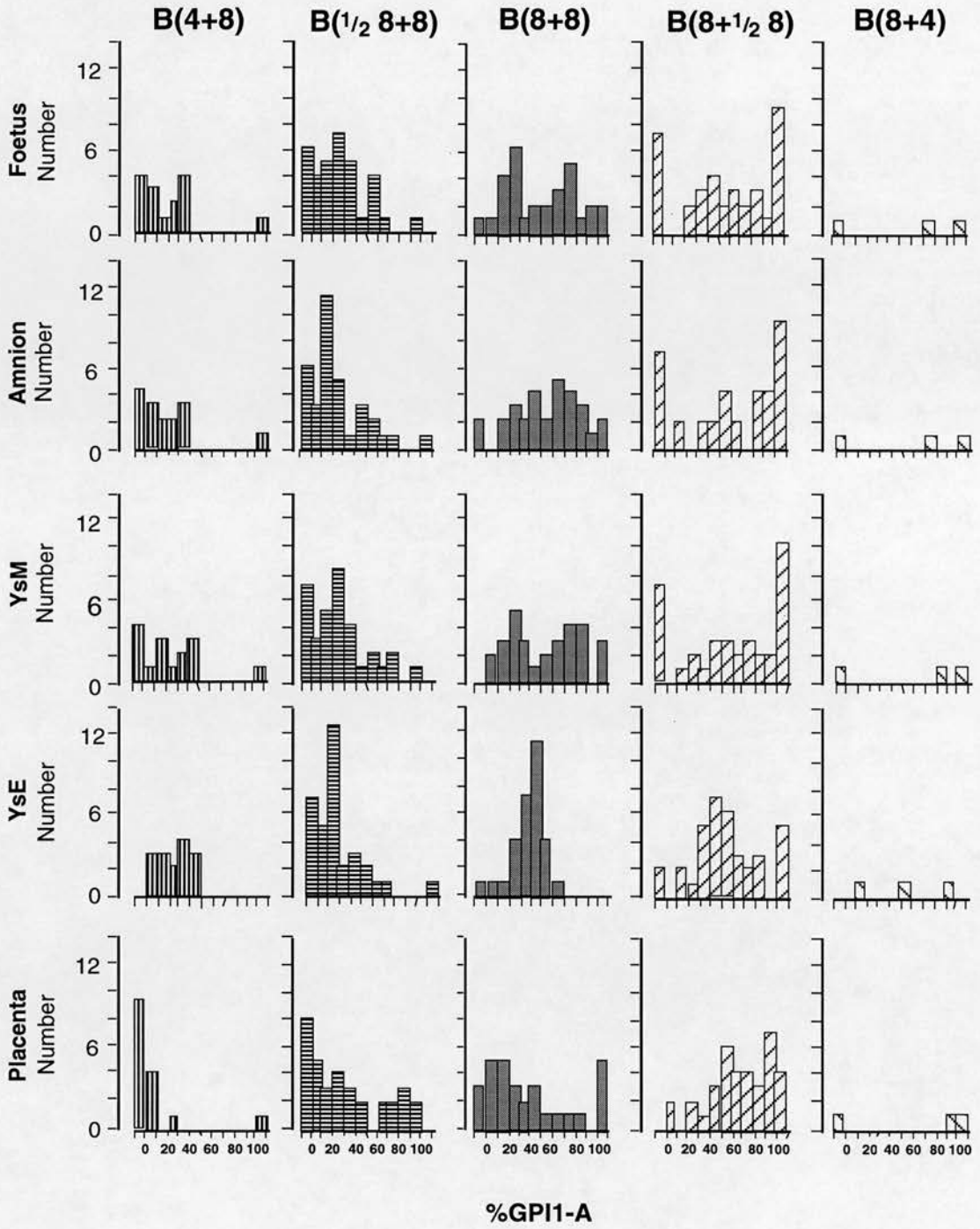


Fig. 5.1 Distribution of the percentages of GPI1-A of the different tissues analysed in the series B(4+8), B($^1/_2$8+8), B(8+8), B(8+$^1/_2$8) and B(8+4). Tissues with either 0 or 100% GPI1-A are shown separately at either end of the distribution.

Table 5.3 Comparisons of the mean %GPI-1A (Mean ± SEM) among the tissues analysed in each chimaera series

Series of		pEct Lineage*			pEnd Lineage*	TE Lineage*
Chimaeras	N*	Foetus	Amnion	YsM	YsE	Placenta
B(4+8)	15	$21.89 \pm 6.74^{\#\dagger}$	$18.45 \pm 6.62^{\#\dagger}$	$24.39 \pm 6.94^{\#\dagger}$	$26.72 \pm 3.75^{\#\dagger}$	$9.22 \pm 6.63^{\#\dagger}$
B($^1/_2$8+8)	34	$25.22 \pm 3.81^{\dagger}$	$24.71 \pm 4.22^{\dagger}$	$26.55 \pm 4.20^{\dagger}$	$18.96 \pm 3.72^{\dagger\ddagger}$	$31.42 \pm 5.47^{\ddagger}$
B(8+8)[§]	30	49.98 ± 5.57	53.65 ± 5.11	53.02 ± 5.54	38.23 ± 2.65	38.68 ± 6.47
B(8+$^1/_2$8)	36	56.79 ± 6.16	59.74 ± 6.48	58.69 ± 6.36	$54.84 \pm 4.53^{\dagger}$	$67.22 \pm 4.50^{\dagger}$
B(8+4)	3	58.93 ± 30.22	59.57 ± 30.41	60.73 ± 30.80	56.43 ± 22.24	66.03 ± 33.02

*: Abbreviations: pEct, primitive ectoderm; pEnd, primitive endoderm; TE, trophectoderm; YsM, yolk sac mesoderm; YsE, yolk sac endoderm; N=number of chimaeras analysed.

§: Data from Chapter 3

#: Significant differences among the five tissues by the Kruskal-Wallis test (P=0.0045)

†: Significantly different from B(8+8); P<0.05

‡: comparisons between series B(4+8) & B($^1/_2$8+8) and B(8+$^1/_2$8) & B(8+4); P<0.05

five tissues studied (P=0.5544 and P=0.6028 respectively; see Table 5.3). Therefore, halving the cell number of the contributing embryo in chimaeras reduced substantially their overall contribution to the various cell lineages, but did not specifically affect any particular tissue. This implies that cell number has a similar effect on the derivatives of the inner cell mass and trophectoderm lineages, that were analysed, when the cell number of one component is reduced.

Results from series B(8+4) show that there is no significant difference among the tissues in this series (P=0.9876). However, due to a high standard deviation and small sample size, the results from this series might not be reliable (see Table 5.3). In the series, B(4+8), the proportions of GPI1-A among these tissues were shown to be significantly different by the Kruskal-Wallis test (P=0.0045; see Table 5.3). The contribution of ($AAF_1 \times AAF_1$) cells (the 4-cell component of the aggregate) to the placenta was significantly lower than in any other tissue by the Mann-Whitney U test (placenta vs. foetus, P=0.0225; vs. amnion, P=0.0465; vs. yolk sac mesoderm, P=0.0225; vs. yolk sac endoderm, P=0.0002). Additionally, the %GPI1-A in the amnion and yolk sac endoderm were statistically different (18.45 vs. 26.72, P=0.0344). Thus, although this appears to show a difference in composition of the inner cell mass and trophectoderm derivatives, it is the opposite of the prediction, because the 4-cell component embryo contributed more to the inner cell mass than to the trophectoderm lineages.

5.3.3. *THE EFFECT OF CELL NUMBERS*

Compared with the series B(8+8), the proportion of GPI1-A in the series B($^1/_2$8+8) is significantly lower in each tissue, (except for the placenta), when the cells from ($AAF_1 \times AAF_1$) were halved at aggregation (foetus, 25.22 vs. 49.98, P=0.0013; amnion, 24.71 vs. 53.65, P<0.0001; yolk sac mesoderm, 26.55 vs. 53.03,

P=0.0006; yolk sac endoderm, 18.96 vs. 38.23, P<0.0001). Additionally, in this series, the observed %GPI1-A in each tissue was lower than the predicted ideal proportion of 33.3% [4/(8+4)].

In addition, the series B(8+$^{1}/_{2}$8), in which the cells from (BF_1 × TGB) embryos were halved at aggregation, shows that the %GPI1-A in each chimaeric tissue is increased, compared with series B(8+8). The proportions of GPI1-A in yolk sac endoderm and placenta between these two series of chimaeras reached statistical significance (54.84 vs. 38.23, P=0.0053 and 67.22 vs. 38.68, P=0.0017, respectively), although the other tissues did not (see Table 5.3). The observed %GPI1-A of each tissue, except for the placenta, was also lower than the ideal proportion of 66.7% [8/(8+4)].

Many chimaeric conceptuses of series B($^{1}/_{2}$8+8) and B(8+$^{1}/_{2}$8) (6/34 and 15/36 respectively) had chimaeric primitive endoderm and trophectoderm derived tissues, but did not have chimaeric epiblast derivatives (see Appendices III. 18 and III. 19 for detail of %GPI1-A in individual conceptuses). Table 5.4 shows the mean percentages of GPI1-A in the series in which chimaeras with non-chimaeric epiblast derivatives have been excluded. This table shows that, excluding these non-chimaeric tissues brings the observed proportions of GPI1-A in epiblast derivatives in series B($^{1}/_{2}$8+8) and B(8+$^{1}/_{2}$8) closer to the ideal proportions of 33.3% and 66.7% respectively. The results of statistical comparisons with B(8+8), however, remained as before.

In addition, it was observed that halving the cell number of the embryos from (AAF_1 × AAF_1) seemed to have a more negative effect on their contribution to chimaeras, compared with the aggregates in which the cell number of the embryos from (BF_1 × TGB) had been halved. A similar phenomenon was also observed in

Table 5.4 Mean %GPI1-A in chimaeras whose derivatives of epiblast were chimaeric★

Series of		pEct Lineage*			pEnd Lineage*	TE Lineage*
Chimaeras	N*	Foetus	Amnion	YsM	YsE	Placenta
B(4+8)	10	22.84 ± 4.10†	17.68 ± 3.62†	26.58 ± 4.68†	29.35 ± 4.33	3.83 ± 1.96†
B($^1/_2$8+8)	28	30.63 ± 3.92†	30.01 ± 4.53†	32.24 ± 4.41†	19.15 ± 4.18†‡	30.86 ± 6.06‡
B(8+8)§	29	48.26 ± 5.48	52.05 ± 5.03	51.41 ± 5.48	37.36 ± 2.59	36.57 ± 6.33
B(8+$^1/_2$8)	21	59.26 ± 4.94	64.32 ± 5.87	62.51 ± 5.53	58.88 ± 5.18†	66.69 ± 5.40†
B(8+4)	1	76.80	78.70	82.20	59.90	98.10

★: B(8+4) was excluded from the statistical analysis because only one chimaera met these criteria

§: Data from Chapter 3

*: MEAN ± SEM; N=number of chimaeras

†: compared with B(8+8); $P<0.05$

‡: comparisons between B(4+8) & B($^1/_2$8+8); $P<0.05$

the study reported in Chapter 3 where it was suggested that genetic background may account for any differences observed.

5.3.4. *THE EFFECT OF EMBRYO STAGE COMBINED WITH CELL NUMBER AND CELL SIZE*

In this section, the composition of the B(8+4) and B(4+8) chimaeras were compared first to series B(8+8) and then to either series B(8+$^1/_2$8) or B($^1/_2$8+8) as appropriate. The aim of these comparisons was to distinguish the effects of embryo stage and cell size from the effects of cell numbers and test the prediction that younger embryos (with larger cells) would contribute less to the ICM derivatives but more to the trophectoderm derivatives.

There was no significant difference in %GPI1-A between series B(8+8) and series B(8+4) for any of the tissues analysed, although the proportions of GPI1-A in each tissue are slightly increased in the series B(8+4) (see Table 5.3). This seems to imply that when the more advanced embryos had twice the cell number of the other component in the chimaera, the proportions contributed by those embryos to the epiblast derivatives analysed were not (as predicted) significantly greater than the ideal proportion (66.7%). Also, it was observed that the contribution of more advanced embryos to the placenta was close to the ideal proportion (66.03% vs. 66.7% respectively), and not (as predicted) significantly less than the contribution to the epiblast derivatives. However, no firm conclusions can be drawn from these observations due to the small sample size.

In addition, in a comparison of B(8+8) and B(4+8), an increased contribution to the placenta from the less advanced embryos was not evident with the %GPI1-A in series B(4+8), being significantly lower than in series B(8+8) (9.22 vs. 38.68, P=0.0002). The epiblast- and hypoblast-derived tissues, however, showed a

significantly higher proportion of GPI1-A in series B(8+8) compared with series B(4+8) (foetus, 49.98 vs. 21.89, P=0.0042; amnion, 53.65 vs. 18.45, P=0.0002; yolk sac mesoderm, 53.03 vs. 24.39, P=0.0034; yolk sac endoderm, 38.23 vs. 26.72, P=0.0141). This suggests that the more advanced embryo made a better contribution than the less advanced embryo to these ICM-derived tissues. Thus, the 4-cell embryo contributed poorly to both ICM and trophectoderm derivatives.

Again, the chimaeras with non-chimaeric epiblast derivatives were excluded for further analysis. Results are shown in Table 5.4. In series B(4+8), although the less advanced embryo made a lower contribution to the derivatives of epiblast than the more advanced embryo, they also made a poorer contribution to the placenta. The series B(8+8) and B(4+8) were significantly different in each tissue, except the yolk sac endoderm (foetus, P=0.0244; amnion, P=0.0007; yolk sac mesoderm, P=0.0244; yolk sac endoderm, P=0.0668; placenta, P=0.0004; see Table 5.4). However, these comparisons showed more or less similar results to the previous ones. On the other hand, it was impossible to compare series B(8+8) and B(8+4), because only one chimaera remained in series B(8+4) after the chimaeras with non-chimaeric epiblast derivatives had been excluded.

In addition, the effect of embryo stage on the composition of chimaeras was evaluated by comparing the groups, B($^1/_2$8+8) with B(4+8) and B(8+$^1/_2$8) with B(8+4). The percentage of GPI1-A in the series B(8+4) was not significantly different from that in the series B(8+$^1/_2$8) for each tissue. This implies that the different embryo stages are unimportant in the allocation of cells to particular lineages. But this conclusion, again, was not based on a large sample size and may not be very reliable. However, a comparison of series B(4+8) and B($^1/_2$8+8) showed more clearly that the earlier stage/younger embryos, (4-cell stage embryo component) did not make a greater contribution to the trophectoderm lineage either. Moreover,

the proportion of GPI1-A in the placenta was significantly lower in series B(4+8) than in series B($^1/_2$8+8) (9.22 vs. 31.42, P=0.0038; see Table 5.3).

Results from the comparison of B(4+8) and B($^1/_2$8+8) did not show what was predicted: a greater %GPI1-A in the placenta and a lower %GPI1-A in the ICM derivatives of the series B(4+8). In fact, the observation was opposite to the prediction. It was observed that the 4-cell component embryo in this series, B(4+8), made a significantly poorer contribution to the placenta and a higher contribution to the yolk sac endoderm, compared to the series B($^1/_2$8+8). Similarly, a prediction of a higher %GPI1-A in the ICM derivatives and a lower %GPI1-A in the placenta of the series B(8+4) was not shown. There was no significant differences between series B(8+$^1/_2$8) and B(8+4). However, due to the sample size in series B(8+4), a firm conclusion cannot be drawn.

The distributions of %GPI1-A in the five E12.5 tissues of the two series B(4+8) & B($^1/_2$8+8) are shown in Fig. 5.1. As discussed above, B(8+4) and B(8+$^1/_2$8) are more difficult to compare. The patterns of %GPI1-A distributions in the epiblast-derived tissues in series B(4+8) is similar to those in the series B($^1/_2$8+8). This indicates that the composition of the chimaeric derivatives of the epiblast lineage under the effects of embryo stage, cell number and cell size is similar to that under the influence of cell number only. The low %GPI1-A in the placenta in series B(4+8) reflected that most of the chimaeric conceptuses had 0 or less 10% GPI1-A in this tissue.

5.3.5. *PHYSICAL PARAMETERS OF E12.5 CHIMAERAS*

The mean physical parameters of each series of chimaeras are shown in Table 5.5.

Table 5.5 The mean physical parameters of each series of chimaeras

Physical Parameters	B(4+8)		B($^1/_2$8+8)		B(8+8)[§]		B(8+$^1/_2$8)		B(8+4)	
	Mean ± SEM	N[‡]	Mean ± SEM	N[‡]	Mean ± SEM	N[‡]	Mean ± SEM	N[‡]	Mean ± SEM	N[‡]
Wt of conceptus (mg)	313.6 ± 9.3*	15	316.6 ± 5.1*	34	356.7 ± 7.7	30	291.7 ± 5.7*	34	261.6 ± 12.1*	3
Wt of foetus (mg)	94.4 ± 4.1*	15	101.0 ± 2.1*	34	113.9 ± 3.7	30	87.9 ± 2.7*	36	81.0 ± 5.8*	3
Wt of placenta (mg)	88.4 ± 2.8	15	82.3 ± 1.9*	34	94.5 ± 2.2	30	80.2 ± 1.7*	36	65.4 ± 1.7* [†]	3
Length of foetus (mm)	9.16 ± 0.12*	15	9.40 ± 0.07*	34	9.95 ± 0.13	30	9.48 ± 0.10*	36	8.68 ± 0.12* [†]	3
Morphological score	7.37 ± 0.15	15	7.58 ± 0.07	34	7.72 ± 0.12	30	7.01 ± 0.13*	36	7.08 ± 0.22*	3

[§]: Data from Chapter 3

[‡]: N=number of chimaeras

[†]: Comparisons between B(8+$^1/_2$8) & B(8+4); $P<0.05$

* : Compared with B(8+8); $P<0.05$

There were no significant differences between one of the corresponding series of chimaeras with equal cell number, *i.e.* B(4+8) and B($^1/_2$8+8), whereas the other two series, B(8+4) and B(8+$^1/_2$8) showed significant differences in the weight of placenta and length of foetus (P=0.0121 and P=0.0351 respectively; see Table 5.5). However, the control chimaeras, B(8+8), were consistently longer, heavier and more developmentally advanced than all the experimental chimaeras. Also, the ratios of the weights of conceptuses, foetuses and placentas between the series of B(8+8) and B(8+4) were greater than 16 : 12 [the initial ratio of the cell numbers in the series of B(8+8) and B(8+4) aggregates; see Fig. 5.2]. The ratio of the weights of foetuses between the series of B(8+8) and B(8+$^1/_2$8) was also close to 16 : 12 (1.3 : 1). However, other comparisons of the half 8-cell stage or 4-cell stage embryos from ($AAF_1 \times AAF_1$) have shown that none of the ratios of physical parameters between the series B(8+8) and B(4+8) or B($^1/_2$8+8) maintains the 1.3 : 1 ratio (see Fig. 5.2). These differing ratios may reflect their different genetic contributions.

5.4 DISCUSSION

In the chimaera experiments presented here, cell number has been shown to have an effect on the composition of the chimaeric conceptus. The proportions of GPI1-A in the E12.5 chimaeric tissues derived from the epiblast lineage reflected the numbers of cells in the contributing embryos at aggregation. Thus, the %GPI1-A in these tissues varied, as predicted by the expected ideal proportions, 33.3%, 50.0% and 66.7% [in the series B($^1/_2$8+8), B(8+8) and B(8+$^1/_2$8) respectively].

It has been suggested that the more developmentally advanced cells make a greater contribution to the inner cell mass or inner cells than the less advanced cells in the embryo (see 1.3.1.a). If this preferential allocation was represented in the

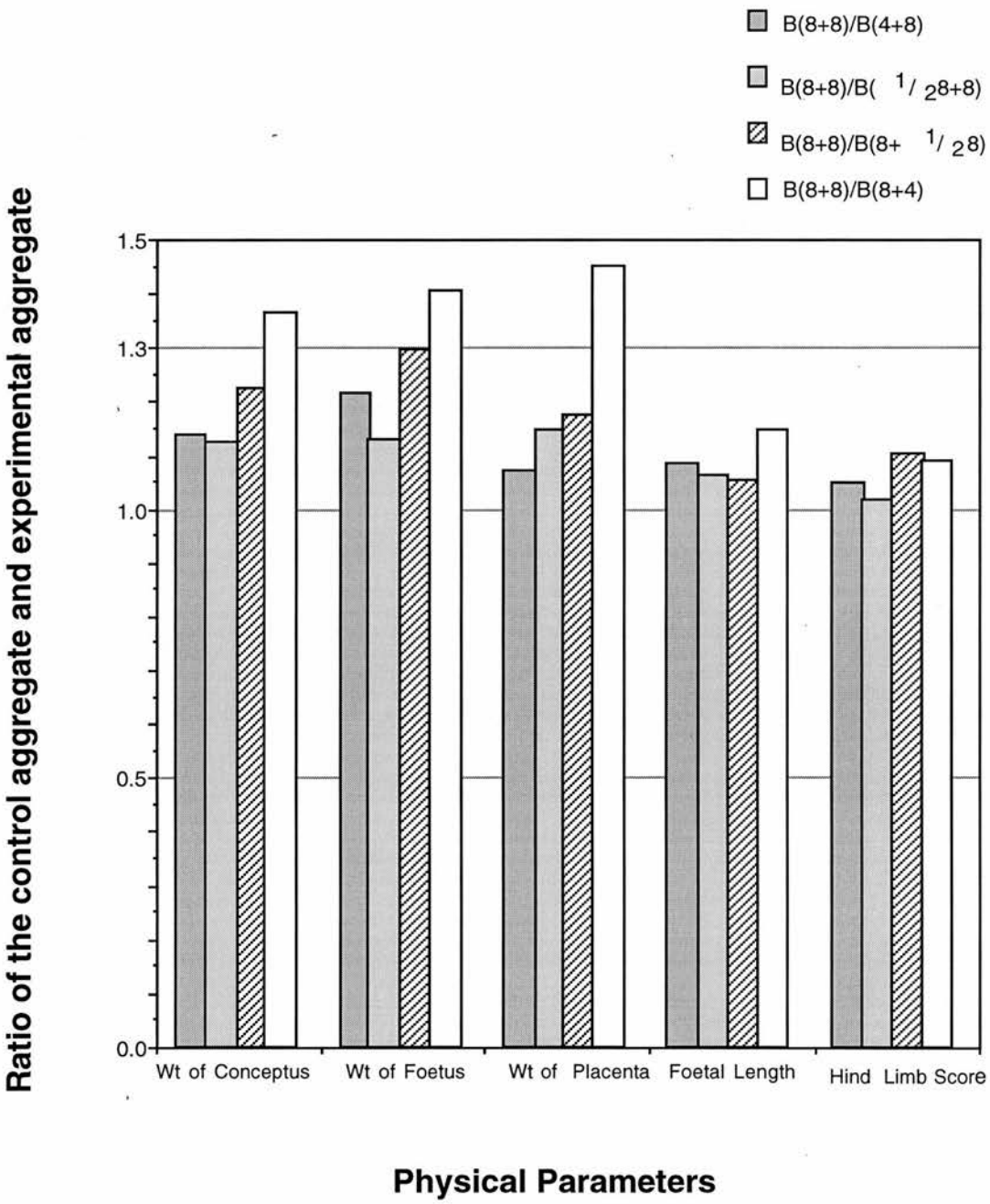


Fig. 5.2 The ratios of physical parameters of the control series B(8+8) to the experimental chimaera series produced in these studies.

chimaera series B(4+8) and B(8+4), a significantly higher contribution of the (AAF_1 × AAF_1) embryo (a higher %GPI1-A than ideal proportion 66.7%) to the ICM-derived samples than the (BF_1 × TGB) embryo would be expected in series B(8+4) and lower %GPI1-A (less than the ideal proportion 33.3%) in the ICM-derived tissues would be presented in chimaera series B(4+8). It was observed that %GPI1-A in the epiblast tissues of the series B(4+8) was lower than the ideal proportion 33.3%. However, the experimental groups, B($^1/_2$8+8) and B(4+8), showed a similar distribution of %GPI1-A in the E12.5 chimaeric epiblast-derived tissues. Surprisingly, the %GPI1-A in the yolk sac endoderm and the placenta showed an opposite proportion, which the %GPI1-A was significantly higher in the yolk sac endoderm and lower in the placenta in series B(4+8). Also, no significant difference in %GPI1-A in the five tissues analysed was found between series B(8+4) and B(8+$^1/_2$8), although the sample size was only 3 in series B(8+4). This suggests that the better contribution to the derivatives of epiblast lineage made by the more advanced embryos in group B(4+8) is probably due to differences in cell number rather than developmental stage. These observations in the epiblast-derived tissues in this study are similar to other chimaera experiments, in which the contribution of the descendants of less developmentally advanced embryos in the inner cell mass still remained close to the ideal proportion, 33.3%, in the chimaeric blastocyst (2×8c ↔ 2×4c in Spindle, 1982; 1×8c ↔ 1×4c in Everett and West, 1996).

The constituent embryos in series B(8+4) and B(4+8) differed in cell size, cell number and embryo stage. Comparison of the observations in this study with the results in Chapter 4 (see Table 4.6), provides further insight into the effect of embryo stage on chimaera composition. Results from Chapter 4 showed that bigger cells made a greater contribution to the polar and mural trophectoderm than smaller cells at the blastocyst stage. In series B(8+4), however, the proportion of GPI1-B cells

(from the 4-cell stage embryo component) was not specifically higher in the placenta than in other tissues. This suggests that the bigger cells (4-cell stage embryos) did not make a significantly greater contribution than the smaller cells (8-cell stage embryos) to the polar trophectoderm lineage, but the sample size was small. However, a similar result was obtained from the comparison of B(4+8) and cell size experiments (see Chapter 4 Table 4.6), in which bigger cells in series B(4+8) did not show a greater contribution to the trophectoderm lineage. Similarly, the smaller cells did not make a greater contribution to the inner cell mass lineages. In each of these comparisons cell size has been shown not to be a major influence in cell allocation at the blastocyst stage and subsequently influence the composition of the chimaera.

In one experiment (Surani and Barton, 1984), chimaeric mouse morulae were made by aggregating a single blastomere from a 2-cell stage embryo ($^{1}/_{2}$ cells) with two other cells from a 4-cell stage embryo ($^{2}/_{4}$ cells) to mimic asynchronous cleavage divisions. The mean cell numbers of the descendants from the $^{1}/_{2}$ cell to the outer and inner cells were 6.58 vs. 2.63, whereas from the $^{2}/_{4}$ cells were 8.0 vs. 7.32 respectively. It showed that the descendants of the $^{1}/_{2}$ cells made a significantly greater contribution to the outer cells than to the inner cells and the proportion of the progeny from the $^{2}/_{4}$ cells in the inner cells was slightly higher but close to the ideal proportion (7.32/9.95 ; 2/3). The results seem to conflict with the observations in this study, in which the less advanced cell did not make a greater contribution to the placenta in series B(4+8) and B(8+4). However, in this study, no mural trophectoderm derivatives could be analysed. Also in Surani and Barton's experiments, no data was shown for the later stage embryos, therefore it is unclear whether less advanced cells/embryos made a greater contribution to the mural trophectoderm lineages than to the polar trophectoderm.

In this study, the contribution of later-stage embryos [8-cell component embryo in the series B(4+8) and B(8+4)] to the epiblast-derived tissues was not significantly higher than to the placenta, which conflicts with some previous observations that the descendants of 8-cell stage embryos made a significantly higher contribution to the inner cell mass in the chimaeric blastocyst produced by aggregating one 8-cell stage embryos with three 4-cell stage embryos (Spindle, 1982). The possibilities causing the different results between Spindle's experiment and this study are discussed below.

Asynchronous cleavage divisions occur among blastomeres in the embryo from the second cell cycle onwards, due to a different second cell cycle length (Kelly *et al*, 1978). With the descendants of the first-dividing cell still retaining their initial head start, there may be a higher number of cells in the embryo from this first-dividing cell than from the last-dividing cell. However, to what extent this is true, is unclear. From the observations of Kelly *et al.* (1978), the ratio of average cell numbers between two half blastocysts each formed from a single blastomere, the first-dividing and the last-dividing cell, of a 2-cell stage embryo was much closer to 1 : 1 than 2 : 1 (33.7 : 31.2; Kelly *et al.*, 1978). This implies that the number of descendants from the first-dividing cell, at the 2-cell stage, was not twice that from the last-dividing cell. This may explain Spindle's observation. In her experiment, the embryos were flushed out from the same group of donors. Therefore, the embryos were at the same age and just divided asynchronously. Aggregating these embryos together may mimic the asynchronous dividing blastomeres in the embryo. The descendants from the 8-cell embryos are able to colonise a large proportion of the inner cell mass in the chimaeric blastocyst as observed in an intact embryo.

The chimaera series made by aggregating two embryos of different developmental stages in this study were different from 1×8c ↔ 3×4c chimaeras in Spindle's experiments (1982). Firstly, the 8-cell stage embryos and 4-cell stage

embryo were collected from the donor females which were superovulated at different time and these embryos were different in developmental age and stage. The late 8-cell stage embryos and early 4-cell stage embryos may be collected due to the time point of embryo collection (67-68 hours and 52-53 hours after HCG injection respectively). Therefore, the components for the 8c ↔ 4c aggregates in this study may be actually nearly two cell cycles apart. It has been shown that the stage-specific protein was delayed to express in the chimaeras made by highly asynchronous blastomeres (a single blastomere from an 8-cell stage embryo aggregated with a 2-cell stage embryo; Prather and First, 1988). Hence, the development of the 8-cell stage embryo in the chimaera may be affected and could not make a greater contribution as predicted.

Secondly, the chimaera produced in this study were composed of only two embryos, not four embryos as 1×8c ↔ 3×4c chimaeras. It has been shown that continuing cell interactions are important for cells to commit to a restricted developmental fate (Johnson and Ziomek, 1983; see 1.1.3.c). Although 1×8c ↔ 3×4c chimaeras were produced by arranging these 4 embryos to expose equally to the outside environment, the 8-cell component may have more cell contacts in this arrangement and be surrounded by others better than that in the aggregates made by two embryos only. Therefore, the 8-cell embryos in 1×8c ↔ 3×4c chimaeras may be easily enclosed and subsequently made a greater contribution to the inner cell mass by geometrical effect.

In this study, the placenta and sometimes the yolk sac endoderm showed a different chimaeric pattern than that seen elsewhere in the conceptus. Previous studies have also shown that the individual placenta of chimaeras tends to be skewed in favour of one or other component (James *et al.*, 1993; West and Flockhart, 1994). In the series B(4+8), the placentas of 9/15 chimaeras were derived completely from the embryos of (BF_1 × TGB). The low mean %GPI1-A in the placenta might

therefore be caused by a skewed distribution in this tissue. Also, it was observed that two reciprocal combinations, (in which the different mouse strains underwent a similar experimental manipulation), did not show exactly reciprocal results, despite being an apparently genotypically balanced strain combination, ($AAF_1 \times AAF_1$) $\leftrightarrow$ ($BF_1 \times$ TGB) (see Chapter 3). For instance, the halving procedure reduced the %GPI1-A in the epiblast derivatives to 30-32% in the series B($^1/_2$8+8), but the contribution of %GPI1-B in the series B(8+$^1/_2$8) was 36-41%. In addition, the lowest physical parameters were found in the series B(8+$^1/_2$8) and B(8+4), in which the embryos from ($BF_1 \times$ TGB) had been manipulated. This implies that the physical parameters of chimaeras made by aggregating one whole 8-cell stage embryo with one half 8-cell or whole 4-cell stage embryo from ($BF_1 \times$ TGB) were affected more than in the reciprocal chimaeras produced by aggregating one whole 8-cell stage embryo with one half 8-cell or whole 4-cell stage embryo from ($AAF_1 \times AAF_1$). These different responses to experimental treatment may be caused by a genetic background effect. Possibly the strain combination is not completely genotypically balanced.

In conclusion, this study has shown that cell number should be considered as a major factor influencing the composition of chimaeras. Also, the predominance of the more developmentally advanced cells in the epiblast lineage may be caused by a difference in cell number, rather than solely the result of embryo stage or cell size.

CHAPTER 6

GENERAL DISCUSSION

Results from Chapters 3 to 5 have demonstrated several features concerning the development of mouse chimaeric embryos.

In Chapter 3, genotypic imbalance was shown in a strain combination in which BALB/c cells made a poor contribution to the E12.5 conceptus. The influence of the number of cells aggregated and/or size regulation was reflected by the significant difference in the cell contributions to some chimaeric tissues analysed. For example, the composition of the amnion and yolk sac mesoderm differed significantly between chimaera series B(8+8) and B($^1/_2$8+$^1/_2$8). However, the hypothesis that size regulation may have played a major role in the production of unbalanced chimaeras was tested and rejected, because the genotypically unbalanced strain combination still remained unbalance after size regulation was avoided. In addition, the timing of the origin of the genotypic imbalance was narrowed down to between E6.5 and E12.5.

In Chapter 4, it was demonstrated that the preferential distribution of tetraploid cells to the mural trophectoderm in the tetraploid↔diploid chimaeric blastocyst can be caused by cell ploidy alone, without any additional geometric effect, *i.e.* cell size. Also, diploid cells of different physical size (but at the same developmental stage) were allocated non-randomly within the blastocyst, with the bigger cells tending to make a significantly greater contribution to the mural trophectoderm than the smaller cells.

In contrast, in Chapter 5, results showed that cell size had no effect on cell allocation in the chimaeras produced by aggregating two embryos of different

developmental stages as well as cell size and cell number (8-cell ↔ 4-cell). The contribution made by the constituent embryos to the chimaeric foetus in all experimental groups was demonstrated to reflect the cell number of contributing embryos at aggregation, *i.e.* cell number at aggregation was the major factor influencing cell allocation.

The implications of these experiments are discussed in this Chapter and possible future work is suggested.

6.1 THE DEVELOPMENT OF MOUSE CHIMAERIC EMBRYOS

6.1.1. *GENOTYPIC IMBALANCE AND 8-CELL ↔ 4-CELL AGGREGATES FROM A BALANCED STRAIN COMBINATION*

Several possible mechanisms have been discussed to account for the production of the unbalanced chimaera series, (BALB/c × BALB/c) ↔ (BF_1 × TGB): cell selection against BALB/c cells (probably during blastocyst formation or the cell mixing process) and/or preferential allocation of BALB/c cells to the mural trophectoderm lineage. Although the cell marker (GPI) used in these experiments cannot provide a view of the spatial distribution of BALB/c cells in the embryo, a further comparison between the two chimaera series U(8+8) and B(4+8) (from Chapter 3 and Chapter 5 respectively) suggests a possible mechanism whereby BALB/c cells may be preferentially allocated to the mural trophectoderm.

The %GPI1-A in each of five tissues of the series U(8+8) and B(4+8) are shown in Table 6.1. No significant difference in %GPI1-A was shown between the series for any of the tissues analysed, indicating that both (BALB/c × BALB/c) 8-cell stage embryos and (AAF_1 × AAF_1) 4-cell stage embryos made a similar contribution

Table 6.1 Comparisons of the mean %GPI-1A among the tissues analysed in two series of chimaeras

Series of Chimaeras*	N†	pEct Lineage‡			pEnd Lineage‡	TE Lineage‡
		Foetus	Amnion	YsM	YsE	Placenta
U(8+8)	31	22.91 ± 4.34	18.05 ± 4.06	20.35 ± 4.17	28.91 ± 3.56	21.80 ± 5.61
B(4+8)	15	21.89 ± 5.57	18.45 ± 6.62	24.39 ± 6.94	26.72 ± 3.75	9.22 ± 6.63

*: Series U(8+8) is made by aggregating one 8-cell stage embryo from (BALB/c × BALB/c) with one 8-cell stage embryo from (BF_1 × TGB); series B(4+8) is made by aggregating one 4-cell stage embryo from (AAF_1 × AAF_1) with one 8-cell stage embryo from (BF_1 × TGB)

†: N=number of chimaeras analysed.

‡: Mean ± SEM

to each other in the resulting chimaera when aggregated with an 8-cell stage (BF_1 × TGB) embryo. Distributions of %GPI1-A in each tissue were plotted as histograms which are shown in Fig. 6.1. The skewed distributions of %GPI1-A for each tissue in the series B(4+8), (a genotypically balanced strain combination), were similar to those in the genotypically unbalanced series U(8+8). This implies that there may be a preferential allocation of BALB/c cells to the mural trophectoderm mediated through their delayed development.

It has been observed that (BALB/c × BALB/c) embryos are more developmentally retarded than the embryos from (AAF_1 × AAF_1) or (BF_1 × TGB) during this study period. As mentioned in Chapter 1 (see 1.1.1), the different genetic background can influence the length of cell cycles in the embryo. For example, the second cleavage division occurred later in (BALB/cGn × BALB/cGn) inbred pre-implantation embryos than that in (BALB/cGn × 129/Rr)F_1 hybrid embryos (Whitten and Dagg, 1961). Also, the mean cell numbers per embryo at 89 hours after HCG injection are 39.1 and 22.0 for B10.D2 (on C57BL/10Sn background) and BALB/c (on BALB/c background) mouse strains, respectively (Goldbard and Warner, 1982). In addition to the different background gene which can attribute to the difference of embryonic development, one gene that controls the rate of cleavage division was discovered in 1980s in Warner's laboratory (reviewed by Warner *et al.*, 1998a, b). One gene, named *Ped* (preimplantation embryo development), was identified, which does not influence ovulation time, but influences the time of the first cleavage division and the subsequent rate of embryonic cleavage (Verbanac and Warner, 1981; Goldbard *et al.*, 1982). The *Ped* gene phenotype is controlled by two alleles, *fast* and *slow*, and the *fast* allele is dominant. It was found that *slow Ped* gene phenotype mouse strains, such as CBA and C3H are H-2^k haplotype. Although BALB/c strain embryos develop slowly, they have the *fast Ped* allele and are H-2^d.

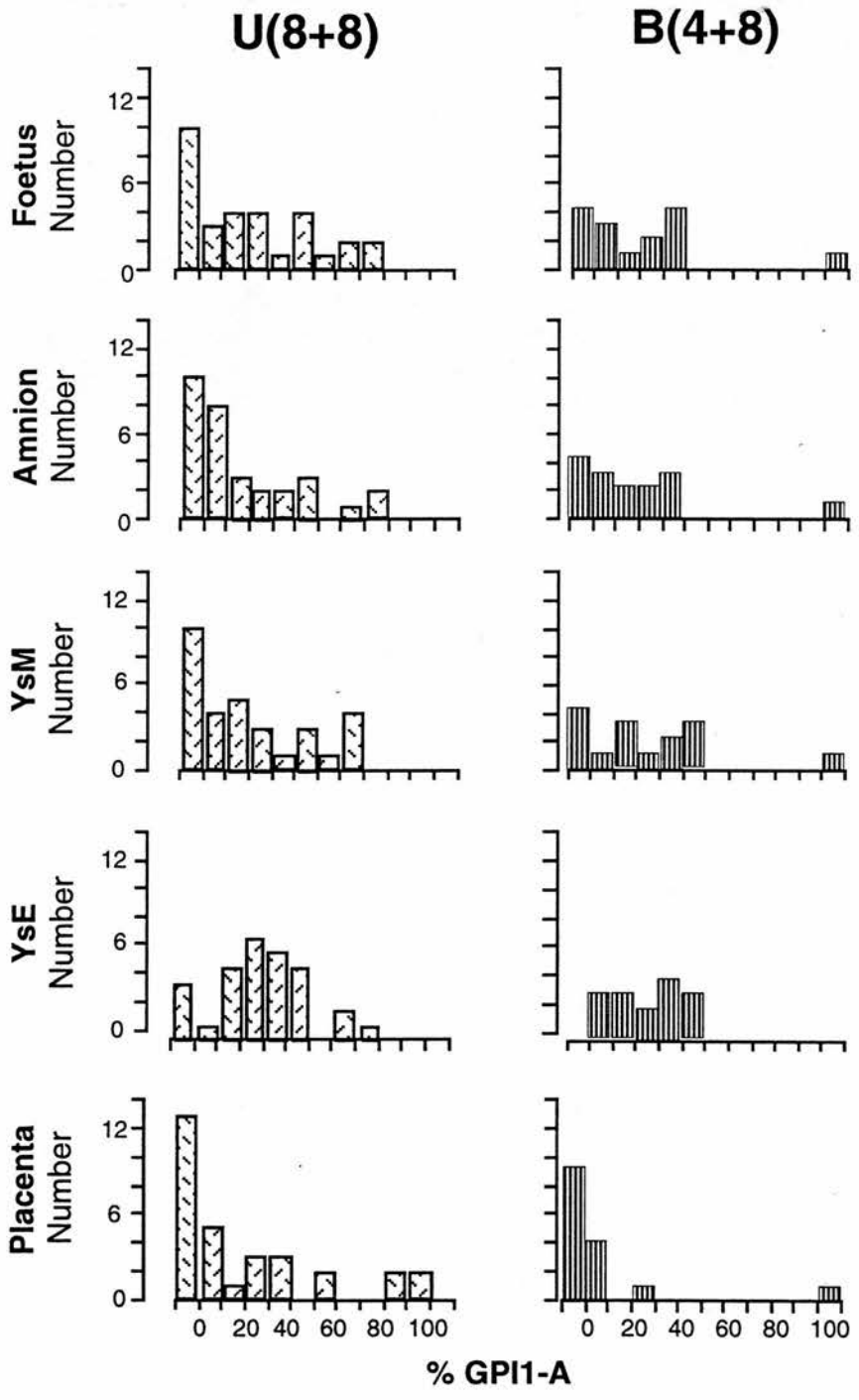


Fig. 6.1 The distribution of %GPI1-A in the five tissues analysed in chimaeric conceptuses, from series U(8+8) and B(4+8). Tissues with either 0 or 100% GPI1-A are shown separately at either end of the distribution. The skewed distributions for each tissue in both series show no significant differences.

They develop slowly because of other genetic background effects. Therefore, a "superslow" strain (BALB.K) was produced, by combining the slow background genes from the BALB/c strain and the H-2^K haplotype (Goldbard and Warner, 1982). BALB.K embryo developed more slowly than either BALB/c or standard *Ped slow* embryos.

Ped gene is located in the Qa subregion of the *H-2* complex (the MHC, major histocompatibility complex). The linkage of *Ped* gene phenotypes and Qa-2 antigen expression strongly suggested that Qa-2 antigen is the *Ped* gene protein product (Warner *et al.*, 1987a, b; Warner *et al.*, 1988; Warner *et al.*, 1991). More recently, several studies have demonstrated that *Q7/Q9* genes were responsible for the *fast Ped* gene phenotype (Xu *et al*, 1993; 1994). Eventually, it has been shown that in the *Ped fast* mouse strain, C57BL/6, only *Q7* and *Q9* were transcribed in the blastocyst. In contrast, the mouse strain, CBA/Ca, expressing the *slow Ped* gene phenotype had a deletion for these genes on blastocysts (Cai *et al.*, 1996; Wu *et al.*, 1998). Some fast developing mouse strains, such as C57BL/6, B10, B3, expressed both *Q7* and *Q9*, while other *Ped fast* strains only express one of these. For example, DBA/1 only expresses *Q7* whereas B6.K2 only expresses *Q9*. However, those "double-positive" strains do not have a faster *Ped* phenotype than the "single-positive" strains, suggesting that the level of Qa-2 antigen in preimplantation mouse embryos might be similar in both double- and single-positive strains (Wu *et al.*, 1998).

Although it has not been determined whether the mouse strains used in this study were *Ped fast* or *Ped slow* strains of mice, BALB/c cells were believed to be less developmentally advanced than (BF_1 × TGB) cells in the chimaera. For example, a recent sample of embryos at E2.5 in our laboratory showed that (BALB/c × BALB/c) embryos (5 2-cell, 31 3-4-cell and 52 5-8-cell) lagged behind (BF_1 × TGB) embryos (all 96 had reached the 5-8-cell stage and 32 of these were beginning to

compact). Although at the time of aggregation both contributing embryos were chosen to be at the 8-cell stage, (BALB/c × BALB/c) embryos may be at the early 8-cell stage whereas (BF_1 × TGB) embryos may be at the late 8-cell stage. As described in 1.3.1.a., some previous studies have shown that less developmentally advanced cells tended to remain on the outside (Graham and Deussen, 1978; Kelly *et al.*, 1978; Graham and Lehtonen, 1979; Spindle, 1982; Surani and Barton, 1984) and subsequently, mainly differentiate into the trophectoderm lineages.

The results imply that the lagging development of BALB/c cells may be responsible for the genotypic imbalance. Thus, BALB/c cells may be preferentially allocated to the trophectoderm due to their delayed development and the inner cell mass region would be predominantly occupied by (BF_1 × TGB) cells initially. The BALB/c cells allocated to the polar trophectoderm lineage may be then displaced to the mural trophectoderm lineage by cells (mainly non-BALB/c cells) migrating from the inner cell mass to the polar trophectoderm (see 1.3.3). By these two steps, most of the BALB/c cells may be pushed away to the mural trophectoderm lineage where they would not be detected in E12.5 conceptuses, since this lineage makes little contribution at later stages. Therefore, the genetic background responsible for slower preimplantation development may play an important role in causing unbalanced chimaeras.

In addition, it is known that sub-optimal culture conditions are detrimental to the development of preimplantation embryos. For instance, the block to development *in vitro* occurs at the cleavage stages in most of mammalian embryos, e.g. at the 2-cell stage in the mouse, at 8-16-cell stage in the sheep and cattle, at the 4-cell stage in the pig and at the 4-8-cell stage in human (reviewed by Bavister, 1995). It has been shown that modification of culture media and optimisation of culture conditions can overcome the block to development (Abramczuk *et al.*, 1977; ; Schini and Bavister, 1988; Chatot *et al.*, 1989; 1990a, b; Ellington *et al.*, 1990; Gardner and Lane, 1996;

Leese *et al.*, 1998). Nevertheless, the beneficial effect of some modifications of culture media on the embryonic development is variable, thus, strain-dependent.

It has been demonstrated that the development of 1-cell embryos cultured in a chemically defined medium (Whitten's medium, WM, containing glucose, lactate and BSA) from the blocking inbred and random-bred strains of mice (C57BL/6 and ICR, respectively) into the blastocyst stage can be improved by the addition of EDTA (Abramczuk *et al.*, 1977). However, the response to the presence of EDTA was dependent on the mouse strain (Abramczuk *et al.*, 1977; Nasr-Esfahani *et al.*, 1992; Du and Wales, 1993). It has been demonstrated that the proportion of inbred C57BL/6 1-cell embryos developing to blastocysts in the medium with EDTA was higher than that of inbred BALB/c 1-cell embryos in the same medium (Abramczuk *et al.*, 1977). In addition, 1-cell embryos, from outbred CF-1 females × B6SJLF1/J males, could not overcome the 2-cell block in EBSS (Earles balanced salt solution) with EDTA (containing glucose, pyruvate and lactate; Chatot *et al.*, 1989). Also, the beneficial effect of supplementation of glutamine in the culture medium of mouse embryos, from the zygote to the blastocyst stage, was also shown to be strain-dependent. In two studies, addition of glutamine to the medium had no significant effect on the frequency of blastocysts produced, either blocking (Qs outbred) or nonblocking [F_1(C57BL/6 × CBA/Ca)] strains of mice (Du and Wales, 1993; Devreker and Hardy, 1997). Furthermore, the presence of glutamine in CZB and KSOM media was inhibitory for cell division between the 8-cell stage and the blastocyst stage during *in vitro* development of embryos from a hybrid strain [F_1(C57BL/6 × CBA/Ca)] (Devreker and Hardy, 1997). In contrast, different blocking (CF_1 × B6SJLF_1/J; DBA/2J × B6SJLF_1/J) and nonblocking (B6D2F_1/J × B6SJLF_1/J; CD1 × B6SJLF_1/J) mouse strains the proportion of embryos developing to the blastocyst stage was improved with glutamine in the culture medium (Chatot *et al.*, 1989; 1990b).

Results from the experiment of culture media mentioned above implicated that the culture medium used in this study might have different impacts on the development of embryos from (BALB/c × BALB/c), (AAF_1 × AAF_1) and (BF_1 × TGB). If this culture medium (i.e. M16 medium) is less optimal for BALB/c embryos than for (BF_1 × TGB) embryos, then the non-BALB/c cells might dominate in the chimaeric embryo during the culture period *in vitro*. However, due to the 2-cell block, the development of preimplantation mouse embryos may be greatly influenced by culture media if they were cultured from the zygote stage. In this study, all the embryos were collected and cultured from the 8-cell stage and BALB/c embryos appeared to develop normally in M16 medium. It seems unlikely that the culture medium would affect the composition of (BALB/c × BALB/c) ↔ (BF_1 × TGB) chimaeras. Nevertheless, the culture medium might affect the embryonic development in a subtle way and, subsequently, the contribution of the component embryos in the chimaeras could be affected slightly.

6.1.2. *THE EFFECTS OF CELL SIZE AND PLOIDY*

It has been shown that larger, outer, polar cells divide approximately two hours earlier than smaller, inner, apolar cells while blastomeres are undergoing the fifth cell cycle in the embryo (MacQueen and Johnson, 1983). If bigger cells tended to divide earlier than smaller cells, a greater contribution of bigger cells (which were then assumed to be "more developmentally advanced") would be expected in the inner cell masses of the B2n ↔ S2n chimaeric blastocysts. The results from the observations in Chapter 4, however, have been demonstrated that bigger diploid cells made a poorer contribution to the inner cell mass than smaller diploid cells and, moreover, they contributed greatly to the mural trophectoderm. Comparing these two studies: in the experiments performed in this thesis, the cells were from the 2-cell stage embryos and were thought to be identical, since cell differentiation starts at 8-

cell stage (see 1.1.3). Also, in the test of culture conditions (see Appendix III. 8), it showed that there was no significant difference in the percentage of blastocyst formation between the B2n and S2n cells. Therefore, the difference in the division time found in the intact later stage embryos of MacQueen and Johnson (1983) may possibly be accounted for by the start of cell diversification at the 16-cell stage. In the chimaeras the large and small cells were present earlier and so had the opportunity to influence allocation. In addition, results from Chapter 5 showed that cell size had no detectable effect on cell allocation in the chimaeras made by aggregating two embryos of different developmental stages. It implies that the physical difference in blastomere size is an important factor on cell allocation in the embryo only if the cells are the same age and only before blastomere differentiation.

Possible mechanisms responsible for the abundant tetraploid cells found in the mural trophectoderm in the tetraploid↔diploid chimaeric blastocyst have been discussed in Chapter 4. In the later developmental stage, mural trophectoderm cells will cease division and become giant cells (Copp, 1978, 1979). From observations of the late mouse morula stage, it has been shown that some binucleate cells consistently occupy an outer position (Soltynska *et al.*, 1985). These cells were assumed to present a normal developmental phenomenon and were suggested to represent stem cells of trophectoderm which subsequently became the polyploid giant cells. In addition, Ilgren (1981) suggested that giant cells derived from trophectoderm in mice must go through the "binucleate phase". However, no evidence has been reported that binucleate cells are found at the blastocyst stage and the proportion of "spontaneously" formed binucleate cells found in the trophectoderm lineages is unknown. Also, it has been demonstrated by *in situ* hybridisation that the giant cells are polytene rather than polyploid (Varmuza, *et al.*, 1988). Results from Chapter 4 showed consistently that there was a significant difference in the $\%Tg$ for the mural trophectoderm lineage between each reciprocal groups, *i.e.* B2n ↔ *S2n & S2n ↔

*B2n and B2n ↔ *B4n & B4n ↔ *B2n. Even if trophectoderm cells do undergo a binucleate phase, it is unlikely that it would influence the effect of ploidy on cell allocation because there is a significant difference between the trophectoderm and the inner cell mass.

More recently, Everett and West (1998) have demonstrated that tetraploid cells were selected against during the formation of blastocysts. Analysis of the early and late stages of tetraploid ↔ diploid chimaeric blastocysts (E3.5 and E4.5, respectively) showed that the proportion of tetraploid cells decreased during this period. In agreement with the results presented in this thesis, Everett and West (1998) found that tetraploid cells tended to be preferentially allocated to the mural trophectoderm lineage. Several possible mechanisms for this were discussed in Chapter 4, section 4. In addition, if the gradual loss of tetraploid cells in tetraploid ↔ diploid chimaeric blastocysts was caused by apoptosis, it might occur preferentially in tetraploid cells allocated to the inner cell mass.

It has been showed that programmed cell death (PCD) with typical features of apoptosis is a normal developmental process, functioning in tissue sculpting (such as formation of digits), deleting structures that were no longer required, adjusting cell numbers or eliminating dangerous or injured cells (Jacobson *et al.*, 1997). Apoptosis is under genetic control and it affects single cells in isolation, in which chromatin aggregates together on the inner nuclear membrane and nucleus fragments resulting in degradation of the DNA into oligonucleosomal fragments, which give the appearance of a DNA ladder after electrophoresis. (Jurisicova *et al.*, 1995; Wolpert *et al.*, 1998). During the formation of mouse blastocysts, cell death has been also described (Potts and Wilson, 1967; El-Shershaby and Hinchliffe, 1974; Copp, 1978; Handyside and Hunter, 1986; Hardy *et al.*, 1989;). The development of TUNEL (TdT-mediated dUTP nick-end labelling) assay, which labels the 3' end of oligonucleosomal fragments, allows the assessment of DNA fragmentation on

specimens with only a few cells *in situ*. TUNEL was used to demonstrate that PCD occurs predominantly in the ICM of mammalian blastocysts (reviewed by Jurisicova *et al.*, 1995; reviewed by Hardy, 1997; Brison and Schultz, 1997; 1998). Also, results from injection of different embryonal carcinoma cell lines into the blastocoele led to the postulate that PCD is designed to eliminate redundant inner cell mass cells with trophectodermal potential (Pierce *et al.*, 1989).

It has been shown that the genes for cell death suppressers (*Bcl-2*, *Bcl-w* and *Bcl-X_L*), cell death inducers (*Bax* and *Bad*) and activators & executors of cell death (*p53*, *MA-3*, *Ttg*) were all detectable in mouse oocytes and normal embryos (up to the blastocyst stage) (Jurisicova *et al.*, 1998; Warner *et al*, 1998b). The expression pattern of genes controlling apoptosis may be altered in embryos undergoing fragmentation (Jurisicova *et al.*, 1998; Moley *et* al., 1998). For example, the expression of cell death inducer gene, *Bax*, was increased, in both mRNA and protein levels, when mouse embryos cultured from the 2-cell stage for 72 hours in 30mM D-glucose, in which apoptosis was induced (Moley *et* al., 1998). Also, barely detectable expression of mRNA for *Bcl-2* in fragmented C57BL/6 zygotes (thus undergoing apoptosis), which is much lower than normal zygotes (Jurisicova *et al.*, 1998). Also, epigenetic factors may be important. It has been shown that hydrogen peroxide is present in the late blastocoele fluid (Pierce *et al.*, 1991). The survival from the toxic activity of H_2O_2 is glutathione-dependent. Glutathione effectively protects against reactive oxygen radicals and decreases by 10-fold during fertilisation and cleavage to the blastocyst stage.

If the preferential loss of tetraploid cells in tetraploid $\leftrightarrow$ diploid chimaeras is caused by apoptosis, it is possible that tetraploid cells overexpress the cell death genes, such as *Bax*, or they have an altered balance of cell death genes and cell survival genes. Alternatively, they may have lower levels of protective glutathione. However, further work is needed to test these possibilities.

6.1.3. *CHIMAERAS MADE BY COMBINING TWO EMBRYOS THAT DIFFERED IN DEVELOPMENTAL STAGE*

Series B(8+4) and B(4+8) not only showed low aggregation success rates but also low implantation rates. As shown in Table 6.2, the average implantation rates are lower in these two series than the other groups of this balanced strain combination (24.2-28.5% vs. 43.0-54.9%). Also, low implantation rates were observed in the genotypically unbalanced series U(8+8) and U($^1/_2$8+$^1/_2$8) (29.5% and 30.1% respectively).

Previous experimental data has shown that the development of blastomeres can be altered by adjacent cells, *e.g.* the timing of the formation of the blastocyst cavity is altered when two embryos of different stages are aggregated (Prather and First, 1986). Additionally, it has been observed that the expression of certain proteins in a chimaera made by aggregating highly asynchronous blastomeres ($^1/_8$ cell $\leftrightarrow$ 2-cell embryo) is altered either quantitatively or qualitatively (Prather and First, 1988). A stage-specific protein was also found to have delayed expression in this chimaera series. It is possible that the low implantation rates observed in series B(4+8) and B(8+4), also in the unbalanced series, may be caused by unsuitable pseudopregnant foster mothers. Thus, the uterus of the female mice at 2.5 days of pseudopregnancy may be too advanced for the (8+4) aggregates of balanced strain combination as well as the unbalanced series, as the development of these chimaeras may be retarded.

6.2 EXTRAPOLATING FROM CHIMAERAS TO NORMAL DEVELOPMENT OF MOUSE EMBRYOS

Although the development of chimaeric embryos may not completely reflect the development of normal embryos, the profile of mouse embryonic development

Table 6.2 Implantation rate in different chimaera series

Chimaera series	number of transferred embryos at E3.5	number (%) of implanted embryos at E12.5*
B(8+4)	99	24 (24.0)
B(4+8)	130	37 (28.5)
U(8+8)	122	36 (29.5)
U($^1/_2$8+$^1/_2$8)	163	49 (30.1)
B($^1/_2$8+$^1/_2$8)	109	43 (39.5)
B(8+$^1/_2$8)	93	40 (43.0)
B(8+8)	70	33 (47.1)
B($^1/_2$8+8)	82	45 (54.9)

*: Data ranked by the percentage of implantation rate.

can still be revealed through chimaera experiments.

As described in Chapter 1 (1.1.3.c and 1.3.1), cell allocation and the developmental fates of blastomeres in the embryo are related. From the results presented in this study, cell number seems to play a major role in allocating blastomeres. Thus, the more developmentally advanced cells may make a greater contribution to the inner cell mass than the less advanced cells, presumedly due to their higher cell number. However, it is unclear in an intact embryo how different the cell number is between the two descendants of the first- and last-dividing cell at the 2-cell stage embryo. Also, in the study of cell allocation in twin half mouse embryos bisected at the 8-cell stage (Hardy and Handyside, 1993), it has been shown that the cell number of the trophectoderm added from the two half blastocysts was significantly higher than the number of trophectoderm cells of the intact blastocyst, although there was no significant difference between the total cell number or the number of the inner cell mass cells of two half blastocysts and the intact blastocyst. It implicated that the increased trophectoderm cell number might be resulted from a larger surface area : volume ratio, which made the trophectoderm cells more spread and less crowded and divide more. Therefore, the division rate and the number of the trophectoderm cells in the chimaeric blastocysts might also be influenced by the surface area : volume ratio, and the conclusion adapted from the chimaera experiments for the normal mouse development remains uncertain in some extent.

The effect of relative cell position in the embryo is undoubtedly important in determining the developmental fate of blastomeres. Although chimaera experiments are useful for investigating the effect of position, they may also introduce additional factors such as relative cell position to the results from the chimaera because of the alteration of relative position. Thus, when applying the observations from the chimaera experiments to the normal development of mouse embryos, a possible bias

resulted from the altered relative cell positions should bear in mind. Experiments have shown that changes in cell properties occur after their position is altered (see 1.1.3.c). However, other methods of making chimaeras may enable the original cell contacts to be retained (*e.g.* see Kelly, 1979).

6.3 FUTURE WORK

The aim of this thesis was to understand the factors influencing cell allocation during the development of mouse chimaeras. However, some aspects of how these factors affect the developmental fates of blastomeres in the embryo and when these influences occur remain unclear. The following are suggested further studies which would provide a further insight into the interactions of these factors during development.

Firstly, as mentioned in 6.1.1, a sub-optimal culture condition can affect the development of preimplantation embryos *in vitro* and subsequently it might affect the interpretation of the experimental result. More recently, the effect of growth factors on embryonic development has been reviewed (Kane, 1997; Kaye, 1997). By using techniques of RT-PCR (reverse transcription-polymerase chain reaction) and immunohistochemical staining, it has been shown that the preimplantation embryo and maternal reproductive tract express mRNA encoding a diversity of growth factors and growth factor receptors (Rappolee *et al.*, 1988; Pampfer *et al.*, 1991; Rappolee *et al.*, 1992; Schultz and Heyner, 1993; Doherty *et al.*, 1994; Roelen *et al.*, 1998). Hence, supplementation of the culture medium with growth factors, such as insulin-like growth factors (IGF-I, IGF-II), TGF-α (transforming growth factor-α), EGF (epidermal growth factor), can improve the proportion of blastocyst formation and the cell number in blastocysts through the autocrine, paracrine and juxtacrine circuits (Schultz and Heyner, 1993; Kane, 1997; Kaye, 1997). Furthermore, IGFs

and TGF-α can act as survival factors, preventing cell death (Stewart and Rotwein, 1996; Brison and Schultz, 1997; 1998). Therefore, it might be necessary to evaluate the influence of the culture system used in this study on the development of preimplantation embryos from different strains of mice. Subsequently, the improvement of culture media will supply a more optimum culture environment for the development of different strain combinations of chimaeric embryos and to obtain more precise information from chimaera experiments.

In the study of the genotypically unbalanced strain combination, (BALB/c × BALB/c) ↔ (BF_1 × TGB), it was suggested that E6.5 to E12.5 period may be crucial for the origin of genotypic imbalance, since there was no significant difference in %GPI1-A between the genotypically balanced and unbalanced chimaera series at E6.5, but was at E12.5 (see 3.4). Therefore, an analysis of E7.5 or E8.5 chimaeras would be beneficial in another understanding of this genotypic imbalance by GPI. This study has now been carried out and completed. E7.5 and E8.5 chimaeric conceptuses from these two series of chimaeras were analysed. The comparison between the genotypically balanced and unbalanced chimaera series starts to show significant difference at E8.5. Thus, the genotypic imbalance appears after size regulation has occurred. The results are consistent with the idea that a gradual selection against BALB/c cells plays a major role in the genotypic imbalance.

Additionally, use of a quantitative cell marker (GPI) did not provide data of the spatial cell distributions (see 1.2.2.b, c). Without direct evidence showing this spatial distribution, it was not possible to assess if a preferential allocation of BALB/c cells to the mural trophectoderm was responsible for the genotypic imbalance. Therefore, use of another cell marker, *e.g.* an *in situ* marker, *lac-Z* or GFP transgene (see Chapter 1), would enable the investigation of cell spatial distribution in the embryo to be carried out. In fact, another chimaera experiments, which were not included in this thesis, had been performed by using *lac-Z* transgene marker to

investigate the cell distribution in E5.5 chimaeras. However, due to the variable staining of E5.5 control embryos, these experiments were discontinued. Nevertheless, in both the genotypically balanced and unbalanced chimaera series produced in this study, another cell marker, the β-globin, carried by the transgenic mouse strain, TGB, can provide spatial information on cell distributions in the embryo. Therefore, it would be worthwhile to analyse the cell distribution in chimaeric embryos with this cell marker to test whether BALB/c cells are preferentially allocated to the mural trophectoderm at the blastocyst stage.

Secondly, analysis of apoptosis by TUNEL assay in the chimaera series of tetraploid ↔ diploid and genotypically unbalanced strain combination might give a further insight of the underlying mechanisms.

Recently, it has been reported that the transcription factor, Oct-4, (which is present exclusively in the oocyte, blastomeres of the cleavage embryos, the ICM of the blastocyst and primordial germ cells), is essential for the establishment of pluripotency in the ICM (Abdel-Rahman *et al.*, 1995; Nichols *et al.*, 1998; Schöler *et al*, 1990a, b; Schöler , 1991). The initial formation of blastocysts in the *Oct-4*-deficient embryo was observed but the ICMs fail to differentiate into epiblasts at the late blastocyst stage and the cultured whole mutant blastocyst, or internal cells, can only yield trophoblast giant cells. Furthermore, by analysis of immunoreaction with antibody Troma-1, which reacts with intermediate filaments first expressed in nascent TE but not in the ICM cells, the inner cells of *Oct-4*-deficient blastocysts has shown to divert into the trophoblast lineage and begun to differentiate while still in an internal location (Nichols *et al.*, 1998). It might be interesting to investigate the expression of Oct-4 in the component embryos of aggregation chimaeras. For example, various embryo types might express different levels of Oct-4 and those with higher level might contribute more readily to the inner cell mass.

Finally, since the cell numbers of the descendants from the first- and last-dividing cell at the 2-cell stage are unknown, use of a marker allowing the progeny of one of these two blastomeres to be traced is essential for understanding the difference in cell numbers between the two different descendants. The results then can be analysed to give a further insight into the effect of cell number and developmental age on cell allocation in the embryo. Alternatively, the effect of developmental stage may be investigated by making another chimaera series, in which embryos of different embryo stages are aggregated, but keeping the cell number of the contributing embryos consistent by disaggregation and re-aggregation. In this way, the role of the more developmentally advanced cells in embryonic development can be assessed.

Results obtained from this study have clarified several factors that influence the development and the composition of mouse chimaeric embryos. This information could be used to optimise the composition of chimaeras produced for other experimental purposes, such as analysis of cell mixing during organogenesis (Ng and Iannaccone, 1992), or phenotypic analysis of mutant genes by chimaeric rescue analysis (Rossant and Spence, 1998). Also, an understanding of the factors influencing the composition of chimaeric embryos may provide some insights into how cell allocation and interactions occur in intact (non-chimaeric) mouse embryos. For example, the higher proportion of more developmentally advanced blastomeres in the inner cell mass may be caused by the difference in cell numbers of the descendants from the asynchronously dividing cells at the 2-cell stage embryo.

APPENDIX I.

I. 1 Recipe for Ca^{++}, Mg^{++}-free M2 medium

Mg^{++}, Ca^{++}-free M2 medium (pH 7.4): 10 ml

Stock Mg^{++}-free A	1.00 ml
Stock B	0.16 ml
Stock C	0.10 ml
15 mg/ml NaCl	0.10 ml
Stock E	0.84 ml
double distilled H_2O	7.80 ml
Bovine Serum Albumin (Sigma, A4378)	6 mg/ml

After pH adjustment, M2 medium is filter sterilised (0.22 μm pore size) and stored in aliquots at 4°C. It is kept for 2 weeks only.

Stock solutions:

Stock	Component	Final concentration	Amount (g/100 ml)
Mg^{++}-free A	NaCl	1 M	5.534
	KCl	0.05 M	0.356
	KH_2PO_4	1.2 M	0.162
	Na lactate (60% syrup)	0.23 M	3.3 ml
	Glucose	5.5 mM	1.000
	Penicillin	10^5 units	0.060
	Streptomycin	3.75×10^4 units	0.050
B	$NaHCO_3$	0.25 M	2.106
	Phenol red	0.01 % (w/v)	0.010
C	Na pyruvate	0.33 M	0.360
E (pH 7.4)	HEPES	0.25 M	5.957
	Phenol red	0.01 % (w/v)	0.010

All the stock solutions are made up in BDH analysing water (cat. no. 10292), then filter sterilised (0.22 μm pore size) and stored in aliquots at 4°C. Stock A and E will keep for 3 months, stock B and C for two weeks.

I. 2 Reagents for GPI staining

GPI staining: for one plate

Glycerol / 0.2% $MgCl_2$ solution (1:1 v/v mixture)	1.5 ml
Tris citrate (pH 8.0)	170 µl of stock I
F6P (fructose-6-phosphate)	170 µl of stock II-1
NBT (nitro blue tetrazolium)	170 µl of stock II-2
NADP (β-nicotinamide adenine dinucleotide phosphate)	170 µl of stock II-3
G6PD (glucose-6-phosphate dehydrogenase)	3 µl* of stock II-4
PMS (phenazine methosulfate)	20 µl of stock II-5

*: various; it depends on the stock, as long as it is 4 units per plate.

Stock I (Buffer)

Tris citrate (pH 8.0): 500 ml	
Tris	20.1 g
Na citrate (BDH, 10242)	8.0 g

The stock is made up in distilled water and kept at 4°C.

Stocks II-1 to II-5

II-1	F6P	(Sigma, F3627)	20.0 mg/ml
II-2	NBT	(Sigma, N6876)	2.7 mg/ml
II-3	NADP	(Sigma, N0505)	2.7 mg/ml
II-4	G6PD	(Sigma, G8878)	various concentration in every batch
II-5	PMS	(Sigma, P9625)	2.5 mg/ml

All the stocks are made up in distilled water. Keep G6PD and PMS at 4°C and F6P, NBT and NADP at -20°C.

I. 3 DNA-DNA *in situ* hybridisation reagents

Prehybridisation mixture*: (for 10 slides)

50 × Denhardt's	40 µl
deionised formamide	180 µl
filter sterilised 20 × SSC	120 µl
sonicated salmon sperm DNA	20 µl
filter sterilised distilled H_2O	40 µl

*: Details are listed below.

Hybridisation mixture*: (for 10 slides)

denatured digoxigenin labelled probe†	10-20 µl‡
sonicated salmon sperm DNA	20 µl
filter sterilised 20 × SSC	100 µl
filter sterilised distilled H_2O	60-70 µl
20 % dextran sulphate	200 µl

*: Details are listed below.

†: The appropriate volume of digoxigenin-labelled probe was put into an eppendorf microfuge tube and boiled at 100 °C to denature DNA for at least 10 minutes, then cooled on ice and the vapour inside of the tube spun down before use.

‡: The volume used depends upon the labelling result. Normally, the final concentration of the labelled probe is 20 ng/slide.

Details of reagents used for pre-treatment and hybridisation

Reagents	Descriptions
Histoclear	non-toxic
10 × PBS	made up from tablets (Oxoid Code BR 14a)
50 × Denhardt's	bovine serum albumin 0.5 g polyvinyl pyrrolidone 0.5 g ficoll 0.5 g distilled H_2O 50.0 ml (stored in aliquots in the freezer)
20 × SSC, pH 7-7.4	NaCl 3.0 M Na_3 citrate 0.3 M (kept at 4 °C; filter sterilised for pre- and hybridisation)
Salmon sperm DNA	stock is 10 mg/ml in TE buffer sonicated and stored at 4 °C
TE buffer, pH 7.5	Tris 10 mM EDTA 1 mM

List of *in situ* washes and visualisation reagents

Reagents	Descriptions
Buffer 1	Tris 0.10 M M NaCl 0.15 M pH 7.5
Anti-Digoxignin-POD (HRP-antibody; Boehringer, 1 207 733)	polyclonal sheep anti-digoxigenin Fab fragments conjugated to horse radish peroxidase the stock concentration of this antibody is 150 U/ml
DAB buffer	Tris 50 mM pH 7.3 (adjusted with HCl) (kept at 4 °C)
Development reagent (for HRP)	stock diaminobenzidine (DAB; Sigma, D-5637): 50 mg/ml DAB buffer; 100 µl of aliquots kept at -20 °C. for 10-slide development reaction: DAB buffer 10 ml DAB stock 100 µl H_2O_2 3 µl (just before use)

List of counterstaining reagents

Reagents	Descriptions
Harris's haematoxylin	bought from Pioneer Research Chemicals Ltd (Cat. no. PRC/R/51)
Acid alcohol	70% alcohol 99 ml concentrated HCl 1 ml
Scott's tap water	$KHCO_3$ 2 g $MgSO_4$ 20 g distilled water 1000 ml
Eosin (Eosin yellowish; BDH cat. no. 3419720)	1 % aqueous eosin : 1 % alcohol eosin = 3 : 1 add 0.05 % acetate before use

I. 4 Solutions for Lac-Z stain

X-Gal stock stain: 25 ml

Component	Amount
spermidine	6.0 mg
$K_3Fe(CN)_6$	41.0 mg
$K_4Fe(CN)_6$	52.5 mg
0.085 % (w/v) NaCl	0.4 ml
X-Gal wash	25.0 ml

X-Gal wash: 50 ml

Component	Amount	Final concentration
phosphate buffer* (pH 7.3)	45 ml	
20 mM $MgCl_2$	5 ml	2 mM
10 % sodium desoxycholate	50 µl	0.01 %
10 % NP40	100 µl	0.02 %
BSA	25 mg	0.05 %

*: made up by 0.1 M Na_2HPO_4 : 0.1 M NaH_2PO_4=21 : 4

X-Gal (Sigma, B-4252):

25 mg X-Gal (5-bromo 4-chloro 3-indolyl β-D galactopyranoside) dissolved in 0.5 ml dimethyl formamide.

APPENDIX II.

II. 1 Micromanipulation pipettes

Two sets of micromanipulation pipettes including holding pipettes and enucleation pipettes were made to suit the two different micromanipulators at the Roslin Institute and CRB. The methods were described as below.

Micropipettes used at the Roslin Institute

Holding pipettes were made from thick-wall without filament glass capillaries (Clark Electromedical Instruments GC100-10), whose outside diameter is 1.0 mm and inside diameter is 0.58 mm. The glass was softened on a very small flame and the capillary was pulled by hand to give a diameter of 100-150 μm. The capillary was mounted on the microforge (Research Instruments MF1) to make four bends at an angle of 45° by rotating the pipette holder at the desired angle. Each sections were parallel to one another (see Fig. II.1). The end of the pipette was cut with a diamond pen and the break was straight and vertical to the pipette. The pipette tip was heated above the glass bead on the filament of the microforge to blunt the tip and close up the hole to about 20 μm of inside diameter.

Thin-wall glass capillaries without filament (Clark Electromedical Instruments GC100-T15), with an outside diameter of 1.0 mm and an inside diameter of 0.78 mm, were used for making enucleation pipettes. The glass capillary was pulled by a pipette puller (Campden Instruments Moving Coil Microelectrode puller Model 753) to produce a taper. The taper was mounted on the microforge. A straight snap was made to give a diameter of 15-20 μm. The snap was achieved when the taper started to stick to the glass bead on the filament which was heated at low temperature, and then switched off the heater. The contact point was broken by the glass bead retracting. The tip of enucleation pipette was ground at an angle of 45° on a piece of plastic (grit size of 3 μm) which was placed on a turntable. After grinding, the

outside of pipette was cleaned by dipping in 20% hydrofluoric acid for 20-30 seconds, then washed in distilled water to remove the acid. To avoid the glass dirt from the grinding and hydrofluoric acid from washing coming into inside of the pipette, a continuous flow of air blowing through the pipette was necessary, which was achieved by pressing a syringe connected to the pipette. After washing, the cleaned pipette was then mounted on the microforge again but horizontally. The pipette tip was moved toward to the glass bead, which was on the heating filament at a low temperature and stuck to it, then withdrawn from the glass bead in a quick movement. This should result in a spike on the tip. Diagrams of the pipettes are shown in Fig. II.2.

Micropipettes used in the CRB

The pipette-making equipment was located in the Assisted Conception Unit in the Royal Infirmary of Edinburgh. Holding pipettes were made from the same capillaries as above, but pulled by a pipette puller [Research Instruments (RI) puller], which was set as follows: right hand carriage at mark 10, tension at position 6, left hand stop at 50. The capillaries were pulled at high heat and the heater was switched off soon after the carriage reached the stop. This could produce a thin section (outside diameter is about 50-100 μm) in the middle of the capillary which was long enough to break in the middle into two tapers. Two holding pipettes could be made from each section. One of these pieces was mounted on the RI microforge, and a square break at 100-150 μm diameter was made by using the same way as snapping the enucleation pipette described previously. The tip of the capillary was polished by using high heat and make the inside diameter is about 20 μm. A 2-3 mm bend was made at an angle of 35° (see Fig. II.3).

The thin-wall capillaries were pulled by a Sutter puller (Model P-97) for making enucleation pipettes. One pulled capillary was mounted on a Narishige

microforge (MF-90), and broken straight at 20 μm diameter as described above. The snapped end was ground by a microbeveller (RI, MB-3), in which the waterbath underneath the grinder wheel was filled with a flow of 100 % ethanol which helped remove the glass dirt. Additionally, a syringe was connected to the pipette holder of the microbeveller and the air pressure was produced to prevent dirt going into the pipette. Spiking was done by the Narishige microforge in the same way as done on the RI microforge previously. However, the enucleation pipettes had to be bent with regard to the chamber used in the CRB. A 35° bend was made on the microforge while the aperture of the pipette was in view (see Fig. II.4).

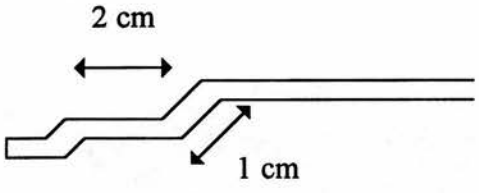


Fig. II. 1 Diagram of the holding pipette made at the Roslin Institute

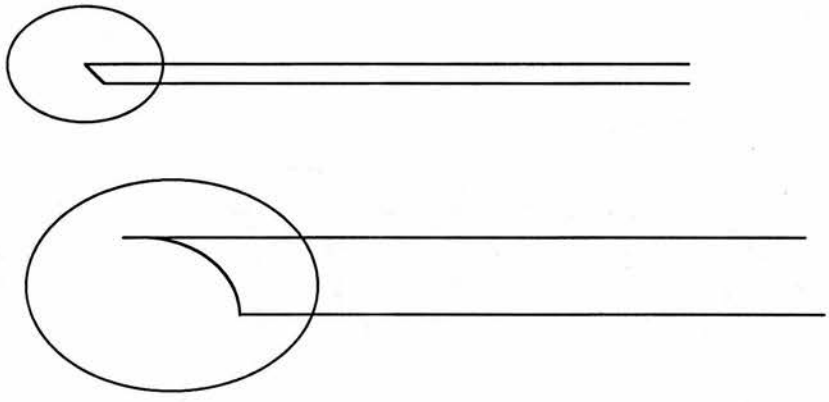


Fig. II. 2 Diagram of the enucleation pipette used at the Roslin Institute

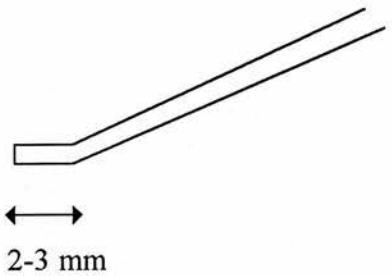


Fig. II. 3 Profile of the holding pipette made in the ACU

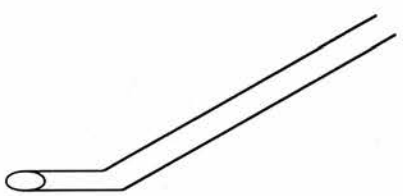


Fig. II. 4 Profile of the enucleation pipette made in the ACU

II. 2 Manipulation chambers

Different chambers were used to suit the different manipulation systems, as described below.

Manipulation chamber used at the Roslin Institute

The slides for making the base of the chambers were treated by the following procedure:

a. glass slides were washed and then soaked in Decon (BDH, cat. no. 56022) for 2-24 hours;

b. rinsed with hot tap water and distilled water for the final wash;

c. dried at 37 °C oven over night

d. siliconized by dipping these clean slides in Sigmacote (Sigma SL-2)

e. dried at 37 °C oven

4 × 25 mm glass chips, which were used as 22 × 22 mm coverslip supports, were cut from 2 mm thick glass. Just before making a manipulation chamber, slides, coverslip and glass chips were cleaned with 70% ethanol and dried. A mixture of petroleum jelly and 10% hard paraffin wax was put on the longer sides of the slide, and two glass chips were stuck on the slide. The jelly mixture was then applied to the tops of the glass chips. 300μl of enucleation medium was placed in the middle between these two chips, and a coverslip was then put on the top. The coverslip was pushed down in order to seal the chamber completely. 200μl of mineral oil was loaded in the two ends of the chamber (see Fig. II. 5).

Manipulation chamber used in the CRB

The chambers used in CRB were made of the bases cut from 30 ml plastic universal container (Sterilin). The ring-like bases and the 36 × 64 mm glass

coverslips were soaked in 70 % ethanol. Before use, a ring and one coverslip were dried by paper tissue, then the ring was stuck on the coverslip by applying jelly mixture. Around 500 μl of enucleation medium was placed inside of the ring and the surface was covered by mineral oil (see Fig. II. 6).

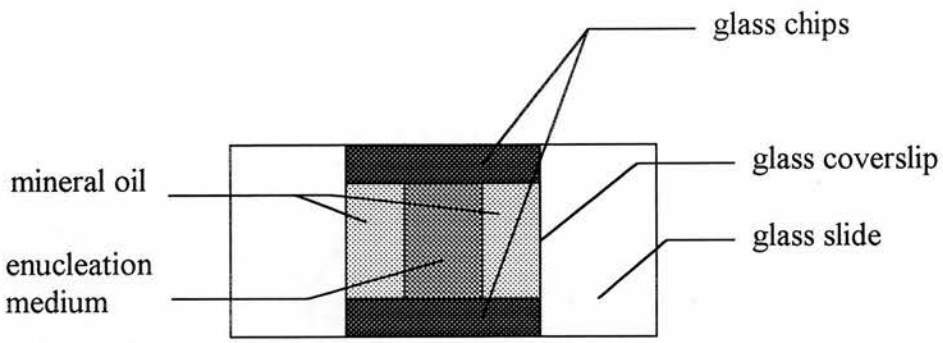


Fig. II. 5 Diagram of the manipulation chamber used at the Roslin Institute

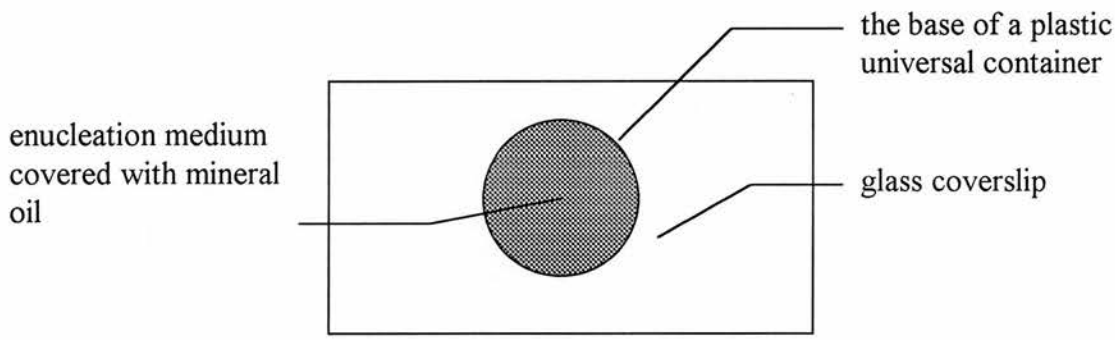


Fig. II. 6 Diagram of the manipulation chamber used in the CRB.

APPENDIX III.

III. 1 Chimaera series U(8+8)

(data ranked by the % GPI1-A of foetus)

(BALB/c × BALB/c)↔(BF$_1$ x TGB): 8-cell ↔ 8-cell					
	% GPI1-A				
number	foetus	amnion	yolk sac mes	yolk sac end	placenta
PCTV-6	0.00	0.00	0.00	33.00	0.00
PCTV-7	0.00	0.00	0.00	45.10	0.00
PCTV-8	0.00	0.00	0.00	79.10	0.00
PCTV-14	0.00	0.00	0.00	30.50	59.40
PCTV-16	0.00	0.00	0.00	15.00	0.00
PCTV-19	0.00	0.00	0.00	13.90	0.00
PCTV-22	0.00	0.00	0.00	0.00	3.70
PCTV-25	0.00	0.00	0.00	0.00	23.00
PCTV-28	0.00	0.00	0.00	0.00	53.20
PCTV-34	0.00	0.00	0.00	29.70	2.60
PCTV-11	6.60	4.90	6.10	11.90	3.50
PCTV-12	7.60	5.00	4.50	28.30	0.00
PCTV-26	8.50	9.10	9.70	40.30	0.00
PCTV-5	10.90	6.40	11.10	27.80	0.00
PCTV-30	11.10	8.50	15.50	8.90	19.30
PCTV-2	12.30	5.90	4.50	0.00	0.00
PCTV-27	17.90	12.40	22.80	31.80	0.00
PCTV-9	23.80	1.60	11.40	31.70	0.00
PCTV-13	23.90	18.90	11.60	21.90	32.50
PCTV-32	27.80	9.50	14.50	29.60	0.00
PCTV-17	29.60	14.10	20.70	10.90	33.80
PCTV-36	30.20	23.50	25.60	43.20	2.30
PCTV-15	40.30	29.30	32.30	28.70	31.80
PCTV-29	40.50	37.30	40.30	13.10	81.35
PCTV-20	44.70	32.70	40.10	35.10	0.00
PCTV-18	47.60	43.60	40.80	37.60	88.80
PCTV-24	52.60	65.90	58.40	25.20	20.85
PCTV-33	62.70	75.00	64.60	41.00	93.70
PCTV-1	64.60	41.00	63.40	68.00	27.45
PCTV-31	70.60	70.70	69.90	49.60	95.00
PCTV-35	76.40	44.10	63.00	65.30	3.50
Sample Size: 31					
Mean	22.91	18.05	20.35	28.91	21.80
SD	24.18	22.62	23.22	19.82	31.24
SEM	4.34	4.06	4.17	3.56	5.61
Twin					
PCTV-21a	13.60	15.90	17.30	28.80	40.50
PCTV-21b	20.10	24.10	25.70	10.40	-
Non-Chimaeras					
PCTV-3	0.00	0.00	0.00	0.00	0.00
PCTV-4	0.00	0.00	0.00	0.00	0.00
PCTV-10	0.00	0.00	0.00	0.00	0.00
PCTV-23	0.00	0.00	0.00	0.00	0.00

III. 2 Chimaera series B(8+8)

(data ranked by the % GPI1-A of foetus)

$(AAF_1 \times AAF_1) \leftrightarrow (BF_1 \times TGB)$: 8-cell $\leftrightarrow$ 8-cell

number	% GPI1-A foetus	amnion	yolk sac mes	yolk sac end	placenta
PCTVI-22	0.00	0.00	5.50	7.50	25.60
PCTVI-6	8.50	0.00	5.90	11.60	25.20
PCTVI-17	12.80	27.90	14.70	43.90	6.50
PCTVI-20	13.40	36.10	17.30	29.50	25.95
PCTVI-24	15.70	13.10	16.50	45.10	80.10
PCTVI-9	17.80	23.70	20.10	44.10	12.90
PCTVI-14	22.70	42.00	34.80	40.90	44.00
PCTVI-32	24.00	34.80	29.00	43.80	3.80
PCTVI-3	25.10	42.90	34.30	0.00	0.00
PCTVI-25	28.00	19.50	29.10	33.10	7.05
PCTVI-21	28.40	21.50	24.80	30.70	18.60
PCTVI-1	28.70	40.40	30.85	25.50	0.00
PCTVI-12	33.10	47.30	26.50	38.60	68.20
PCTVI-15	41.70	51.30	49.70	39.00	0.00
PCTVI-30	43.00	54.60	56.50	56.70	47.40
PCTVI-2	57.50	63.70	57.30	36.30	6.70
PCTVI-33	58.60	64.40	65.40	40.70	9.30
PCTVI-8	62.40	62.00	82.30	43.00	32.95
PCTVI-26	63.40	71.40	63.60	45.80	100.00
PCTVI-28	67.70	65.10	75.60	31.30	13.40
PCTVI-19	72.10	80.50	72.60	54.10	100.00
PCTVI-18	72.40	85.30	75.90	54.10	51.00
PCTVI-13	73.20	63.50	79.40	47.40	44.10
PCTVI-4	77.40	70.90	67.50	49.30	100.00
PCTVI-11	79.50	77.00	81.20	21.50	12.50
PCTVI-31	86.90	77.50	87.60	28.60	34.30
PCTVI-10	90.00	82.90	86.80	49.90	13.10
PCTVI-27	95.50	100.00	100.00	39.80	100.00
PCTVI-16	100.00	100.00	100.00	63.60	100.00
PCTVI-23	100.00	90.20	100.00	51.60	77.80
Sample Size: 30					
Mean	49.98	53.65	53.03	38.23	38.68
SD	30.49	28.01	30.34	14.52	35.45
SEM	5.57	5.11	5.54	2.65	6.47
Non-Chimaeras					
PVTVI-5	0.00	0.00	0.00	0.00	0.00
PCTVI-7	100.00	100.00	100.00	100.00	100.00
PCTVI-29	100.00	100.00	100.00	100.00	100.00

III. 3 Chimaera series U($^1/_2$8+$^1/_2$8)

(data ranked by the % GPI1-A of foetus)

(BALB/c × BALB/c) ↔ (BF$_1$ × TGB): $^1/_2$ 8-cell ↔ $^1/_2$ 8-cell					
number	% GPI1-A foetus	amnion	yolk sac mes	yolk sac end	placenta
PCTVa-3	0.00	0.00	0.00	10.50	0.00
PCTVa-5	0.00	0.00	0.00	57.00	51.60
PCTVa-6	0.00	5.00	0.00	25.10	0.00
PCTVa-7	0.00	0.00	0.00	36.10	6.80
PCTVa-13	0.00	0.00	8.60	23.70	0.00
PCTVa-16	0.00	0.00	0.00	53.50	0.00
PCTVa-20	0.00	0.00	0.00	47.90	52.65
PCTVa-27	0.00	0.00	0.00	25.20	0.00
PCTVa-29	0.00	0.00	0.00	0.00	1.60
PCTVa-30	0.00	0.00	0.00	13.00	12.20
PCTVa-32	0.00	0.00	0.00	0.00	40.10
PCTVa-42	0.00	0.00	0.00	20.70	0.00
PCTVa-45	0.00	0.00	0.00	0.00	20.20
PCTVa-17	5.60	6.90	7.90	9.80	0.00
PCTVa-39	5.90	0.00	0.00	87.30	0.00
PCTVa-10	9.20	23.50	10.90	45.10	86.40
PCTVa-22	17.20	26.30	24.60	0.00	87.90
PCTVa-15	17.30	10.70	8.70	30.90	25.15
PCTVa-49	17.40	9.70	13.70	69.50	87.00
PCTVa-28	17.90	6.60	17.90	28.70	0.00
PCTVa-1	19.20	6.80	0.00	5.60	35.50
PCTVa-40	27.90	18.40	26.40	11.50	41.10
PCTVa-38	30.10	22.40	26.40	0.00	0.00
PCTVa-11	35.00	37.60	29.20	31.80	0.00
PCTVa-48	39.40	26.90	27.40	58.10	20.00
PCTVa-34	40.00	35.00	37.70	18.00	7.10
PCTVa-46	40.90	36.20	42.40	35.70	87.70
PCTVa-12	41.50	19.90	20.05	56.20	48.70
PCTVa-37	42.30	26.30	41.60	38.20	29.80
PCTVa-2	43.50	23.90	18.20	29.20	49.40
PCTVa-31	61.30	68.40	63.70	23.10	28.15
PCTVa-4	100.00	100.00	100.00	100.00	59.55
PCTVa-8	100.00	100.00	100.00	32.10	11.40
PCTVa-23	100.00	100.00	100.00	18.10	31.35
PCTVa-41	100.00	100.00	100.00	100.00	14.60
PCTVa-43	100.00	100.00	100.00	39.00	35.95
Sample Size: 36					
Mean	28.10	25.29	25.70	32.79	27.00
SD	33.89	33.99	33.97	26.68	28.56
SEM	5.65	5.67	5.66	4.45	4.76
Non-chimaeras					
PCTVa-9	0.00	0.00	0.00	0.00	0.00
PCTVa-14	0.00	0.00	0.00	0.00	0.00
PCTVa-18	0.00	0.00	0.00	0.00	0.00
PCTVa-19	0.00	0.00	0.00	0.00	0.00
PCTVa-21	0.00	0.00	0.00	0.00	0.00
PCTVa-24	0.00	0.00	0.00	0.00	0.00
PCTVa-25	0.00	0.00	0.00	0.00	0.00
PCTVa-26	0.00	0.00	0.00	0.00	0.00
PCTVa-33	0.00	0.00	0.00	0.00	0.00
PCTVa-35	0.00	0.00	0.00	0.00	0.00
PCTVa-36	0.00	0.00	0.00	0.00	0.00
PCTVa-44	0.00	0.00	0.00	0.00	0.00
PCTVa-47	0.00	0.00	0.00	0.00	0.00

III. 4 Chimaera series B($^1/_2$8+$^1/_2$8)

(data ranked by the % GPI1-A of foetus)

$(AAF_1 \times AAF_1) \leftrightarrow (BF_1 \times TGB)$: $^1/_2$ 8-cell $\leftrightarrow$ $^1/_2$ 8-cell

number	% GPI1-A foetus	amnion	yolk sac mes	yolk sac end	placenta
PCTVId-12	0.00	0.00	0.00	21.60	16.65
PCTVId-25	0.00	0.00	0.00	34.10	53.30
PCTVId-28	0.00	0.00	0.00	50.30	43.25
PCTVId-37	0.00	0.00	0.00	100.00	7.50
PCTVId-4	8.10	0.00	0.00	27.60	0.00
PCTVId-36	8.80	5.20	6.00	11.60	0.00
PCTVId-30	10.60	10.10	13.20	15.90	69.30
PCTVId-1	14.80	10.30	17.30	37.70	41.65
PCTVId-20	15.40	miss	8.80	12.80	3.90
PCTVId-38	16.50	14.60	18.60	100.00	5.50
PCTVId-5	18.10	16.10	17.00	49.90	72.85
PCTVId-23	19.80	7.80	7.50	4.20	0.00
PCTVId-40	20.70	31.00	25.30	100.00	65.60
PCTVId-24	22.80	0.00	9.90	10.00	0.00
PCTVId-14	24.40	29.00	18.40	29.90	56.75
PCTVId-13	25.00	16.70	16.30	0.00	0.00
PCTVId-39	26.50	17.20	9.40	7.80	64.90
PCTVId-33	28.00	10.40	26.00	49.00	21.40
PCTVId-32	35.20	29.20	35.40	46.50	18.50
PCTVId-42	35.80	38.70	36.40	100.00	45.60
PCTVId-10	36.30	32.10	34.60	59.00	5.80
PCTVId-26	36.50	39.30	37.10	100.00	100.00
PCTVId-3	38.80	26.90	32.60	100.00	30.40
PCTVId-29	40.20	25.80	41.40	100.00	55.35
PCTVId-17	45.00	31.30	29.70	21.60	57.30
PCTVId-41	45.10	40.70	42.90	34.30	40.00
PCTVId-8	46.00	16.40	22.40	0.00	0.00
PCTVId-21	46.30	53.50	43.20	36.70	89.80
PCTVId-22	50.10	59.00	61.60	50.10	35.00
PCTVId-7	52.50	28.40	44.00	57.80	7.00
PCTVId-16	53.30	55.10	45.50	31.20	44.80
PCTVId-19	53.90	42.50	43.60	100.00	94.90
PCTVId-15	58.70	53.00	43.00	62.20	4.20
PCTVId-11	61.20	32.90	40.20	0.00	4.70
PCTVId-43	65.20	81.50	66.80	56.70	96.10
PCTVId-6	66.60	72.60	59.40	40.80	52.35
PCTVId-2	94.30	100.00	100.00	100.00	88.20
PCTVId-9	100.00	100.00	100.00	38.60	61.15
PCTVId-34	100.00	100.00	100.00	53.10	74.85
PCTVId-35	100.00	100.00	100.00	0.00	96.40
Sample Size: 40					
Mean	38.01	34.03	33.84	46.28	40.62
SD	27.71	30.39	28.54	34.24	33.29
SEM	4.38	4.81	4.51	5.41	5.26
Non-chimaeras					
PCTVId-27	0.00	0.00	0.00	0.00	0.00
PCTVId-31	0.00	0.00	0.00	0.00	0.00
PCTVId-18	100.00	100.00	100.00	100.00	100.00

III. 5 Chimaera series U

(BALB/c × BALB/c) ↔ (BF_1 × TGB): 8-cell ↔ 8-cell (% of GPI-A)						
number	oocyte control	E4.5 control	E4.5 embryo	E6.5 embryo		
U1	40.30	51.00	58.54	88.20		
U2	44.50	46.00	29.16	4.10		
U3	46.80	51.00	33.03	85.60		
U4	42.70	39.00	34.68	85.10		
U5	44.90	42.30	43.60	42.20		
U6	44.20	40.70	38.65	52.30		
U7	45.20	45.60	23.80	26.70		
U8	43.70	39.60	31.60	55.40		
U9	47.30	36.90	31.60	34.40		
U10	42.70	43.80	31.40	69.90		
U11	43.30	50.20	26.80	30.20		
U12	44.10	41.70	45.90	36.00		
U14	48.80	42.90	37.80	61.40		
U15	46.00	43.20	35.50	6.70		
U16	42.20	44.20	31.40	39.40		
U17	37.20	44.20	41.10	31.10		
U18	44.90	40.70	40.00	80.90		
U19	42.50	32.40	58.70	52.50		
U20	44.60	44.70	42.10	28.10		
U22	40.90	41.30	46.90	7.60		
U24	43.10	51.90	21.30	84.90		
U25	37.10			23.90		
U26				27.70		
U27				68.20		
U28				72.60		
U29				56.30		
U30				17.80		
U31				60.00		
U32				77.90		
U33				54.30		
U34				64.50	Non-chimaeric: E6.5	
U36				7.10	U13	100.00
					U21	0.00
sample size	22	21	21	32	U23	0.00
Mean	43.50	43.49	37.31	47.91	U35	0.00
SD	2.86	4.84	9.85	25.88	U37	0.00
SEM	0.61	1.06	2.15	4.58	U38	0.00

III. 6 Chimaera series B

($AAF_1 \times AAF_1$) ↔ ($BF_1 \times TGB$): 8-cell ↔ 8-cell (% of GPI-A) number	oocyte control	E4.5 control	E4.5 embryo	E6.5 embryo
B1	43.20	40.90	37.20	91.50
B2	45.40	40.80	42.80	29.60
B3	47.20	36.70	42.00	73.90
B4	40.50	41.40	36.30	77.70
B5	48.10	46.00	29.10	29.10
B6	44.30	49.90	23.50	84.50
B7	43.20	43.70	32.20	66.20
B8	48.30	49.20	57.50	54.90
B9	43.90	69.10	37.30	22.60
B11	45.20	46.90	51.80	6.30
B12	42.70	50.70	36.10	4.00
B13	41.10	52.10	50.30	96.70
B14	42.30	50.10	41.40	31.10
B15	43.30	51.90	41.00	21.30
B16	41.00	47.70	33.50	47.80
B17	41.70	45.10	40.20	19.30
B18	41.80	45.00	31.30	92.50
B20	42.00	42.60	55.30	88.10
B22	40.90	57.50	26.60	82.60
B23	39.30	56.30	37.70	6.60
B24	43.90		52.20	57.60
B25	42.40		47.90	8.70
B26			52.10	80.40
B27			58.40	56.10
B28				76.10
B29				82.70
B30				63.10
B31				74.20
B32				93.00
B33				43.20
B34				43.00
B35				84.10
B36				39.20
B39				28.50
B40				86.10
B41				20.30
B42				18.30
B43				39.10
B44				23.00
B45				89.80
B46				9.00
sample size	22	20	24	41
Mean	43.26	48.18	41.40	52.24
SD	2.40	7.24	9.89	30.29
SEM	0.51	1.62	2.02	4.73

Non-chimaeric: E6.5	
B10	100.00
B19	0.00
B21	0.00
B37	0.00
B38	100.00

III. 7 The development of control embryos and embryos transported

Day	Stage	Control 1* Not transported % (number)	Control 2* Transported % (number)
E1.5	2-cell	100.0 (91)	100.0 (96)
E2.5	under 8-cell	18.7 (17)	69.8 (67)
	8-morula	13.2 (12)	9.4 (9)
	morula	67.0 (61)	20.8 (20)
	blastocyst†	1.1 (1)	
E3.5	under morula	1.1 (1)	
	morula	16.5 (15)	38.6 (37)
	blastocyst†	80.2 (73)	61.4 (59)
	vesicle	1.1 (1)	
	dead	1.1 (1)	
E4.5	morula		4.1 (4)
	blastocyst†	95.6 (87)	95.9 (92)
	vesicle	1.1 (1)	
	dead	3.3 (3)	
E5.5	blastocyst†	95.6 (87)	97.9 (94)
	vesicle	1.1 (1)	
	dead	3.3 (3)	2.1 (2)

*: control 1, 2-cell stage embryos were flushed and cultured in the CRB from E1.5; control 2, 2-cell stage embryos were flushed out 2 hours after oviducts were excised and cultured overnight in Roslin Institute and carried back to the CRB to continue culture from E2.5.

†: This category includes the early blastocyst, expanded blastocyst stage and hatched embryos.

III. 8 The development of control embryos and embryos treated with various manipulations

Day	Stage	S2n† % (number)	B2n† % (number)	B2n↔S2n† % (number)
E1.5	1-cell	100.0 (64)	100.0 (128)	
	2-cell			100.0 (37)
E2.5	1-cell	10.9 (7)	5.5 (7)	
	2-cell	34.4 (22)	39.0 (50)	5.4 (2)
	3-8-cell	54.7 (35)	54.7 (70)	89.2 (33)
	morula		0.8 (1)	5.4 (2)
E3.5	under morula	15.7 (10)	10.9 (14)	10.8 (4)
	morula	70.3 (45)	78.9 (101)	89.2 (33)
	blastocyst*	10.9 (7)	9.4 (12)	
	vesicle		0.8 (1)	
	dead	3.1 (2)		
E4.5	under morula	7.8 (5)	7.1 (9)	2.7 (1)
	morula	1.6 (1)	18.8 (24)	5.4 (2)
	blastocyst*	81.3 (52)	70.2 (90)	91.9 (34)
	vesicle	3.1 (2)	0.8 (1)	
	dead	6.2 (4)	3.1 (4)	

*: This category includes the early blastocyst, expanded blastocyst stage and hatched embryos.

†: The 2-cell stage embryo used in these experiments were flushed out 2 hours after oviducts were excised. S2n was produced by disaggregating 2-cell stage embryos; B2n was produced by enucleation of one blastomere of 2-cell stage and electrofusion these 2-cells; B2n↔S2n was produced by aggregating one B2n cell with one S2n cell.

III. 9 Positive control *S2n ↔ *S2n (raw data without correction)

$(BF_1 \times TGB) \leftrightarrow (BF_1 \times TGB)$: *S2n ↔ *S2n	TE		ICM	TE				ICM	
	pTE *%Tg*	mTE *%Tg*	ICM *%Tg*	pTE + cell no	total no	mTE + cell no	total no	pEct + cell no	total no
PCT III-145	46.43	48.74	50.00	13	28	58	119	22	44
	57.14	61.32	75.00	20	35	65	106	12	16
	78.26	73.81	57.14	36	46	62	84	24	42
	56.25	57.69	70.00	9	16	45	78	14	20
PCT III-146	62.07	54.00	54.84	18	29	27	50	17	31
	45.83	74.11	48.15	11	24	83	112	13	27
	62.79	56.25	52.94	27	43	36	64	9	17
	78.95	77.22	62.50	15	19	61	79	10	16
	51.72	69.32	56.25	15	29	61	88	18	32
PCT III-148	-	66.18	-	-	-	45	68	-	-
	51.85	43.24	53.49	14	27	32	74	23	43
	66.67	49.09	57.14	6	9	27	55	8	14
	66.67	42.11	54.55	8	12	32	76	6	11
PCT III-161	45.45	47.52	64.29	5	11	48	101	9	14
	61.70	52.94	47.50	29	47	18	34	19	40
PCT III-164	44.44	48.28	41.67	4	9	42	87	5	12
PCT III-165	50.00	60.53	66.67	2	4	46	76	2	3
	57.14	50.65	80.00	8	14	39	77	8	10
	-	52.38	-	-	-	44	84	-	-
N	17	19	17	17	17	19	19	17	17
Mean	57.84	57.13	58.36	14.1	23.6	45.8	79.6	12.9	23.1
SD	10.67	10.66	10.19	9.4	13.5	16.1	21.0	6.6	13.2
TOTAL				240	402	871	1512	219	392

III. 10 Positive control *B2n ↔ *B2n (raw data without correction)

$(BF_1 \times TGB) \leftrightarrow (BF_1 \times TGB)$: *B2n ↔ *B2n	TE		ICM	TE				ICM	
	pTE *%Tg*	mTE *%Tg*	pEct *%Tg*	pTE + cell no	total no	mTE + cell no	total no	pEct + cell no	total no
PCT III-158	50.00	41.82	50.00	9	18	23	55	15	30
PCT III-159	30.77	41.25	50.00	8	26	33	80	18	36
	31.25	50.00	45.00	5	16	26	52	9	20
PCT III-166	33.33	38.10	37.50	18	54	24	63	15	40
	52.94	32.89	47.83	9	17	25	76	11	23
	46.15	33.91	38.46	6	13	39	115	5	13
	36.84	35.24	29.17	7	19	37	105	7	24
	36.00	43.62	40.74	9	25	41	94	11	27
	34.62	38.54	40.00	9	26	37	96	8	20
	57.89	46.36	68.42	11	19	70	151	13	19
	45.45	48.68	41.67	5	11	37	76	5	12
N	11	11	11	11	11	11	11	11	11
Mean	41.39	40.95	44.44	8.7	22.2	35.6	87.5	10.6	24.0
SD	9.46	5.83	10.03	3.61	11.67	13.17	29.06	4.30	8.76
TOTAL				96	244	392	963	117	264

III. 11 Positive control *B4n ↔ *B4n (raw data without correction)

$(BF_1 \times TGB) \leftrightarrow (BF_1 \times TGB)$: *B4n ↔ *B4n	TE		ICM	TE				ICM	
	pTE %Tg	mTE %Tg	pEct %Tg	pTE + cell no	total no	mTE + cell no	total no	pEct + cell no	total no
PCT III-153	-	55.32	-	-	-	52	94	-	-
	30.77	56.86	47.06	4	13	29	51	8	17
	58.33	56.10	70.00	7	12	69	123	7	10
	33.33	62.73	55.56	5	15	69	110	15	27
	57.14	63.04	63.64	12	21	58	92	21	33
PCT III-154	31.82	57.14	62.50	7	22	32	56	20	32
	68.75	59.04	53.85	11	16	49	83	7	13
	75.00	72.86	69.23	15	20	51	70	18	26
PCT III-167	58.33	48.33	70.59	7	12	29	60	12	17
	44.44	50.00	56.25	8	18	43	86	9	16
	-	45.28	-	-	-	24	53	-	-
PCT III-168	58.33	47.83	36.36	7	12	22	46	4	11
	-	41.38	-	-	-	24	58	-	-
	46.15	53.33	25.00	6	13	16	30	1	4
N	11	14	11	11	11	14	14	11	11
Mean	51.13	54.95	55.46	8.1	15.8	40.5	72.3	11.1	18.7
SD	14.99	8.19	14.54	3.27	3.84	17.71	26.44	6.64	9.49
TOTAL				89	174	567	1012	122	206

III. 12 Combination control S2n ↔ *S2n (raw data without correction)

$(BF_1 \times BF_1) \leftrightarrow (BF_1 \times TGB)$: S2n ↔ *S2n	TE		ICM	TE				ICM	
	pTE *%Tg*	mTE *%Tg*	pEct *%Tg*	pTE + cell no	total no	mTE + cell no	total no	pEct + cell no	total no
PCT III-9	23.08	33.33	27.27	6	26	8	24	9	33
	36.36	45.71	15.38	8	22	16	35	2	13
	42.31	32.76	34.38	11	26	19	58	11	32
PCT III-10	34.78	40.35	0.00	8	23	23	57	0	15
	40.74	53.85	23.81	11	27	28	52	5	21
	25.93	45.71	43.75	7	27	16	35	14	32
	33.33	45.00	60.00	4	12	27	60	6	10
PCT III-20	30.77	31.58	33.33	12	39	12	38	8	24
	62.86	40.85	50.00	22	35	29	71	14	28
PCT III-149	57.14	38.24	11.11	4	7	26	68	1	9
	45.00	30.95	27.59	9	20	13	42	8	29
	33.33	24.64	20.00	1	3	17	69	1	5
PCT III-150	31.58	35.29	21.74	6	19	30	85	5	23
	15.00	36.14	9.09	3	20	30	83	2	22
	52.63	41.84	48.15	10	19	41	98	13	27
	33.33	30.77	31.58	5	15	20	65	6	19
	50.00	25.24	41.67	4	8	26	103	5	12
	25.71	47.37	16.13	9	35	18	38	5	31
PCT III-151	33.33	34.78	60.87	8	24	32	92	14	23
	41.94	42.11	48.15	13	31	32	76	13	27
	44.44	23.08	27.78	8	18	15	65	5	18
	64.29	22.45	27.78	18	28	22	98	5	18
	38.89	34.82	50.00	7	18	39	112	7	14
	14.29	45.71	20.00	4	28	32	70	6	30
	9.09	25.00	5.56	1	11	20	80	1	18
	14.29	41.07	27.27	2	14	23	56	3	11
PCT III-152	12.50	38.24	18.18	1	8	26	68	2	11
	50.00	29.82	50.00	4	8	17	57	3	6
	50.00	50.91	25.00	10	20	28	55	3	12
	33.33	34.04	32.35	10	30	16	47	11	34
PCT III-155	-	36.67	-	-	-	22	60	-	-
	14.29	41.82	42.86	1	7	23	55	3	7
	14.29	35.42	14.29	1	7	17	48	1	7
	8.33	26.04	25.00	1	12	25	96	3	12
	0.00	17.65	16.67	0	8	12	68	1	6
PCT III-156	-	23.81	-	-	-	10	42	-	-
	-	16.67	-	-	-	6	36	-	-
PCT III-157	37.50	53.33	62.50	3	8	24	45	5	8
N	35	38	35	35	35	38	38	35	35
Mean	32.99	35.61	30.55	6.6	18.9	22.1	63.3	5.7	18.5
SD	16.18	9.48	16.21	4.99	9.57	8.10	21.51	4.26	9.19
TOTAL				232	663	840	2407	201	647

III. 13 Chimaera series B2n ↔ *S2n (raw data without correction)

$(BF_1 \times BF_1) \leftrightarrow (BF_1 \times TGB)$: B2n ↔ *S2n

	TE		ICM	TE				ICM	
	pTE	mTE	pEct	pTE		mTE		pEct	
	%Tg	*%Tg*	*%Tg*	+ cell no	total no	+ cell no	total no	+ cell no	total no
PCT III-3	0.00	16.67	41.67	0	9	6	36	5	12
PCT III-11	0.00	17.39	25.00	0	12	24	138	4	16
	43.48	36.36	47.62	10	23	20	55	10	21
	0.00	0.00	50.00	0	12	1	47	2	4
	9.52	13.79	15.38	2	21	8	58	2	13
	0.00	14.08	0.00	0	8	10	71	0	8
	40.91	22.58	48.00	9	22	21	93	12	25
	-	6.67	-	-	-	5	75	-	-
PCT III-12	21.43	16.39	36.84	3	14	10	61	7	19
	50.00	42.19	54.14	5	10	27	64	4	7
PCT III-21	20.00	46.67	40.00	3	15	28	60	6	15
	8.30	23.81	11.11	1	12	20	84	1	9
PCT III-110	13.79	25.00	50.00	4	29	7	28	7	14
	-	7.50	-	-	-	6	80	-	-
	0.00	13.11	0.00	0	4	8	61	0	5
PCT III-111	-	11.25	-	-	-	9	80	-	-
	0.00	8.96	26.67	0	9	6	67	4	15
	30.43	39.19	40.91	7	23	29	74	9	22
PCT III-116	10.71	28.81	57.58	3	28	15	63	19	33
PCT III-120	-	17.54	-	-	-	10	57	-	-
	35.71	15.79	39.13	10	28	15	95	9	23
	22.58	18.75	25.71	7	31	12	64	9	35
PCT III-121	33.33	30.19	51.85	10	30	16	53	14	27
	10.00	37.14	4.55	3	10	26	70	1	22
	24.39	5.88	22.73	10	41	1	17	5	22
	16.67	32.61	46.67	4	24	15	46	7	15
PCT III-126	8.70	17.50	11.11	2	23	14	80	3	27
	7.50	20.63	31.43	3	40	13	63	11	35
PCT III-127	27.78	13.04	40.91	5	18	3	23	9	22
	30.56	11.11	29.41	11	36	5	45	10	34
	16.67	13.56	50.00	1	6	8	59	3	6
PCT III-129	24.00	13.51	22.86	12	50	10	74	8	35
	24.39	16.22	45.00	10	41	18	111	18	40
	42.31	38.89	51.85	11	26	42	108	14	27
PCT III-138	45.45	50.00	53.85	10	22	14	28	7	13
	23.91	32.61	33.33	11	46	15	46	13	39
	0.00	17.14	40.00	0	10	12	70	4	10
PCT III-139	20.00	35.53	50.00	1	5	27	76	3	6
	18.75	29.58	33.33	3	16	21	71	4	12
	30.43	27.42	57.14	7	23	17	62	12	21
	44.44	29.23	41.67	4	9	19	65	5	12
N	37	41	37	37	37	41	41	37	37
Mean	20.44	22.30	35.88	4.9	21.2	14.5	65.3	7.1	19.5
SD	15.00	11.98	16.21	4.09	12.18	8.77	23.50	4.79	10.29
TOTAL				182	786	593	2678	261	721

III.14 Chimaera series S2n ↔ *B2n (raw data without correction)

$(BF_1 \times BF_1) \leftrightarrow (BF_1 \times TGB)$: S2n ↔ *B2n	TE		ICM	TE				ICM	
	pTE	mTE	pEct	pTE		mTE		pEct	
	%Tg	*%Tg*	*%Tg*	+ cell no	total no	+ cell no	total no	+ cell no	total no
PCT III-1	21.05	37.04	10.00	8	38	10	27	2	20
	35.48	47.06	22.22	11	31	8	17	4	18
PCT III-2	35.00	52.94	26.67	7	20	9	17	4	15
	36.36	55.00	17.39	8	22	22	40	4	23
	20.00	28.57	16.67	5	25	8	28	4	24
PCT III-6	23.08	38.30	15.79	3	13	18	47	3	19
PCT III-15	-	33.33	-	-	-	25	75	-	-
	22.22	44.09	11.11	2	9	41	93	1	9
PCT III-16	28.57	22.92	0.00	2	7	11	48	0	8
	50.00	47.52	100.00	4	8	48	101	5	5
	23.33	47.25	33.33	7	30	43	91	13	39
	0.00	16.00	2.70	0	30	8	50	1	37
PCT III-24	4.88	44.44	11.90	2	41	24	54	5	42
	61.54	44.64	26.67	16	26	50	112	8	30
	18.18	37.25	30.00	2	11	19	51	3	10
	32.00	20.59	23.08	8	25	7	34	6	26
	55.56	33.72	31.03	10	18	29	86	9	29
PCT III-112	-	37.65	-	-	-	32	85	-	-
	10.53	30.77	5.00	2	19	16	52	1	20
	26.32	43.59	34.48	5	19	51	117	10	29
PCT III-113	0.00	32.50	25.00	0	3	13	40	1	4
	42.86	26.32	0.00	6	14	15	57	0	12
	36.84	26.87	32.26	7	19	18	67	10	31
PCT III-114	13.79	46.72	36.00	4	29	57	122	9	25
	50.00	46.81	38.71	18	36	22	47	12	31
	4.35	16.67	8.33	1	23	10	60	2	24
	0.00	21.21	0.00	0	4	14	66	0	6
	11.11	22.00	13.64	2	18	11	50	3	22
	36.36	50.00	12.50	8	22	19	38	2	16
PCT III-117	17.95	45.65	21.95	7	39	21	46	9	41
	37.14	22.00	12.77	13	35	11	50	6	47
	41.18	26.32	23.53	7	17	10	38	4	17
PCT III-118	33.33	35.42	25.00	12	36	17	48	4	16
	56.76	33.33	0.00	21	37	25	75	0	11
PCT III-122	21.43	18.60	27.78	3	14	8	43	5	18
	15.38	12.50	0.00	2	13	8	64	0	14
	14.71	22.22	37.93	5	34	14	63	11	29
	29.03	22.89	41.67	9	31	19	83	5	12
PCT III-123	21.21	37.04	19.35	7	33	30	81	6	31
	18.75	42.65	28.57	3	16	29	68	4	14
PCT III-124	0.00	34.21	7.69	0	6	13	38	1	13
	0.00	46.43	13.79	0	18	26	56	4	29
	0.00	39.53	0.00	0	3	17	43	0	3
	6.06	35.96	31.11	2	33	41	114	14	45
PCT III-133	47.50	50.00	25.64	19	40	11	22	10	39
	45.45	53.73	26.32	10	22	36	67	10	38
	48.48	38.71	24.00	16	33	24	62	6	25
	37.93	39.39	22.22	11	29	13	33	6	27
	57.58	45.45	42.86	19	33	20	44	9	21
PCT III-143	20.00	37.04	33.33	4	20	10	27	5	15
	33.33	51.16	16.67	1	3	22	43	1	6
	35.29	43.86	16.67	6	17	25	57	2	12
N	50	52	50	50	50	52	52	50	50
Mean	26.76	36.27	21.67	6.5	22.4	21.3	58.4	4.9	21.9
SD	17.29	11.12	16.50	5.59	10.96	12.66	25.71	3.80	11.44
TOTAL				325	1122	1108	3037	244	1097

III. 15 Chimaera series B4n ↔ *B2n (raw data without correction)

$(BF_1 \times BF_1) \leftrightarrow (BF_1 \times TGB)$: B4n ↔ *B2n

	TE		ICM	TE				ICM	
	pTE	mTE	pEct	pTE		mTE		pEct	
	%Tg	*%Tg*	*%Tg*	+ cell no	total no	+ cell no	total no	+ cell no	total no
PCT III-51	0.00	18.37	20.00	0	17	9	49	4	20
	17.65	16.67	25.00	3	17	11	66	5	20
	15.38	11.36	17.07	4	26	5	44	7	41
PCT III-63	12.50	4.35	12.24	4	32	1	23	6	49
	19.23	8.18	3.85	5	26	9	110	1	26
	7.69	0.00	21.05	2	26	0	33	4	19
PCT III-64	13.64	0.00	8.16	6	44	0	49	4	49
	20.69	2.17	23.68	6	29	1	46	9	38
PCT III-65	6.67	4.55	14.29	1	15	1	22	5	35
PCT III-67	0.00	0.00	0.00	0	35	0	34	0	42
	8.33	10.34	11.32	3	36	3	29	6	53
	0.00	3.57	0.00	0	8	1	28	0	22
	0.00	0.00	5.26	0	28	0	29	1	19
PCT III-68	0.00	7.02	18.18	0	9	4	57	2	11
	16.00	4.90	17.24	4	25	5	102	5	29
	11.11	5.75	25.00	2	18	5	87	4	16
	21.43	7.59	11.76	3	14	6	79	2	17
PCT III-69	0.00	2.94	10.00	0	24	2	68	5	50
PCT III-71	0.00	3.03	15.38	0	4	1	33	2	13
	15.91	10.00	37.70	7	44	1	10	23	61
	36.36	27.27	43.75	12	33	15	55	14	32
	26.32	15.00	29.17	5	19	6	40	7	24
PCT III-72	12.90	25.00	18.37	8	62	1	4	9	49
PCT III-73	0.00	10.11	14.29	0	22	9	89	3	21
	9.09	10.81	56.00	2	22	12	111	14	25
PCT III-74	21.43	18.52	44.83	6	28	10	54	13	29
	10.00	2.13	9.09	1	10	1	47	2	22
	6.67	0.00	26.67	1	15	0	34	8	30
PCT III-75	8.11	0.00	23.64	3	37	0	5	13	55
PCT III-78	9.52	0.88	25.93	2	21	1	113	7	27
	14.29	10.42	23.08	2	14	10	96	6	26
	11.11	7.87	0.00	1	9	7	89	0	13
PCT III-79	46.15	29.36	47.62	12	26	32	109	20	42
	-	8.33	77.78	-	-	4	48	7	9
	16.67	30.00	40.00	4	24	15	50	8	20
	0.00	3.16	0.00	0	4	3	95	0	5
	31.25	25.00	63.64	5	16	25	100	14	22
	40.00	27.91	60.00	10	25	12	43	12	20
PCT III-80	17.24	35.00	26.47	5	29	21	60	9	34
	-	22.86	-	-	-	16	70	-	-
	-	18.64	-	-	-	11	59	-	-
PCT III-81	4.76	12.15	24.14	1	21	13	107	7	29
PCT III-83	31.82	26.42	39.39	7	22	28	106	13	33
	29.41	48.15	25.00	5	17	26	54	3	12
PCT III-84	41.38	23.08	53.33	12	29	12	52	16	30
PCT III-85	0.00	28.57	33.33	0	2	18	63	1	3
	0.00	14.29	7.14	0	16	3	21	1	14
PCT III-86	21.05	35.29	40.74	4	19	6	17	11	27
	25.00	37.62	33.33	5	20	38	101	8	24
N	46	49	47	46	46	49	49	47	47
Mean	14.28	13.77	25.19	3.54	22.59	8.57	58.98	6.83	27.81
SD	12.37	12.09	18.09	3.38	11.36	9.15	31.52	5.40	13.71
TOTAL				163	1039	420	2890	321	1307

III. 16 Chimaera series B2n ↔ *B4n (raw data without correction)

$(BF_1 \times BF_1) \leftrightarrow (BF_1 \times TGB)$: B2n ↔ *B4n	TE		ICM	TE				ICM	
	pTE *%Tg*	mTE *%Tg*	pEct *%Tg*	pTE + cell no	total no	mTE + cell no	total no	pEct + cell no	total no
PCT III-49	60.00	69.23	65.38	9	15	27	39	17	26
	32.14	22.22	58.82	9	28	10	45	20	34
	43.75	39.64	80.95	7	16	44	111	17	21
	15.79	21.57	25.00	3	19	11	51	5	20
PCT III-50	35.71	40.74	21.88	5	14	11	27	7	32
	12.50	43.66	58.06	3	24	31	71	18	31
PCT III-60	47.62	69.77	50.00	10	21	30	43	5	10
	70.00	33.68	41.67	7	10	32	95	5	12
PCT III-89	29.41	57.41	56.52	5	17	31	54	13	23
	29.41	21.67	52.17	5	17	26	120	12	23
PCT III-90	40.00	40.43	25.00	4	10	38	94	3	12
	0.00	35.56	14.29	0	8	32	90	1	7
	-	29.17	-	-	-	21	72	-	-
	0.00	18.57	0.00	0	15	13	70	0	12
PCT III-92	40.00	20.00	4.76	8	20	9	45	1	21
PCT III-95	37.50	29.73	9.52	12	32	11	37	2	21
	6.25	26.92	6.25	1	16	14	52	1	16
	11.11	26.73	35.71	1	9	27	101	5	14
	18.18	20.51	34.48	4	22	16	78	10	29
	36.84	46.58	31.58	7	19	34	73	6	19
	0.00	24.53	0.00	0	10	13	53	0	18
PCT III-96	48.15	32.94	40.74	13	27	28	85	11	27
	6.25	53.33	16.67	1	16	32	60	2	12
	10.00	20.97	10.00	1	10	13	62	1	10
	28.13	35.53	12.12	9	32	27	76	4	33
	32.56	26.32	26.67	14	43	5	19	8	30
	42.86	26.00	58.06	9	21	26	100	18	31
PCT III-97	31.25	45.12	23.08	5	16	37	82	3	13
	0.00	31.51	33.33	0	3	23	73	1	3
	10.34	29.17	3.33	3	29	21	72	1	30
PCT III-98	18.42	31.82	8.33	7	38	7	22	1	12
	34.21	25.00	48.00	13	38	10	40	12	25
PCT III-99	25.00	26.21	26.83	8	32	27	103	11	41
	9.09	28.43	25.00	1	11	29	102	4	16
	33.33	26.56	31.82	14	42	17	64	7	22
	7.69	48.57	37.93	1	13	17	35	11	29
	58.33	19.05	66.67	7	12	16	84	2	3
	46.15	37.36	13.64	12	26	34	91	3	22
PCT III-100	20.00	34.04	15.38	6	30	16	47	6	39
PCT III-101	-	35.85	-	-	-	19	53	-	-
	0.00	26.32	0.00	0	9	10	38	0	10
	5.26	19.57	5.88	1	19	18	92	1	17
PCT III-104	29.41	40.00	39.29	5	17	42	105	11	28
	20.00	35.48	23.26	12	60	11	31	10	43
	0.00	31.71	2.63	0	32	26	82	1	38
	12.50	50.00	14.63	5	40	29	58	6	41
PCT III-105	6.67	30.86	20.00	1	15	50	162	3	15
	0.00	32.61	8.57	0	32	30	92	3	35
	-	33.33	-	-	-	34	102	-	-
	23.08	48.28	25.71	6	26	42	87	9	35
PCT III-106	58.62	44.90	29.03	17	29	22	49	9	31
	25.93	36.84	37.25	14	54	7	19	19	51
	15.91	26.09	31.82	7	44	18	69	14	44
	32.86	16.00	29.33	23	70	4	25	22	75
	21.57	30.77	18.92	11	51	4	13	7	37
	58.06	21.43	23.40	18	31	15	70	11	47
	47.06	24.62	14.81	8	17	32	130	4	27
	31.11	12.24	27.59	14	45	6	49	16	58
PCT III-107	38.10	57.14	23.64	16	42	20	35	13	55
	28.13	38.04	51.85	9	32	35	92	14	27
	3.22	12.33	2.50	1	31	9	73	1	40
	21.43	41.46	10.81	6	28	17	41	4	37
	30.95	37.04	16.67	13	42	10	27	4	24
	30.30	31.03	45.95	10	33	36	116	17	37
	20.00	37.84	28.00	6	30	14	37	7	25
N	62	65	62	62	62	65	65	62	62
Mean	25.62	33.35	27.44	6.89	25.97	21.94	67.46	7.42	27.03
SD	17.61	12.089	18.84	5.36	13.81	11.19	30.72	6.01	13.91
TOTAL				427	1610	1426	4385	460	1676

III. 17 Chimaera series B(4+8)

(data ranked by the % GPI1-A of foetus)

$(AAF_1 \times AAF_1) \leftrightarrow (BF_1 \times TGB)$: 4-cell $\leftrightarrow$ 8-cell

number	% GPI1-A foetus	amnion	yolk sac mes	yolk sac end	placenta
PCTVIb-17	0.00	0.00	0.00	20.00	0.00
PCTVIb-22	0.00	0.00	0.00	12.80	0.00
PCTVIb-27	0.00	0.00	0.00	9.60	0.00
PCTVIb-36	0.00	0.00	0.00	15.20	0.00
PCTVIb-28	6.00	10.30	13.80	24.10	0.00
PCTVIb-26	6.60	4.30	4.70	9.20	0.00
PCTVIb-19	7.60	3.20	11.00	19.10	0.00
PCTVIb-21	18.00	31.70	32.40	36.80	20.15
PCTVIb-13	21.50	9.90	21.30	35.60	0.00
PCTVIb-8	24.80	22.70	30.50	6.30	0.00
PCTVIb-15	34.00	11.00	19.20	38.30	4.40
PCTVIb-9	35.30	31.80	46.60	43.10	3.60
PCTVIb-11	35.30	31.90	41.20	39.20	3.80
PCTVIb-20	39.30	20.00	45.10	41.80	6.30
PCTVIb-5*	100.00	100.00	100.00	49.70	100.00
Sample Size:15					
Mean	21.89	18.45	24.39	26.72	9.22
SD	26.11	25.63	26.88	14.51	25.66
SEM	6.74	6.62	6.94	3.75	6.63
Non-chimaeras					
PCTVIb-1	0.00	0.00	0.00	0.00	0.00
PCTVIb-2	0.00	0.00	0.00	0.00	0.00
PCTVIb-3*	0.00	0.00	0.00	0.00	0.00
PCTVIb-4*	0.00	0.00	0.00	0.00	0.00
PCTVIb-6*	0.00	0.00	0.00	0.00	0.00
PCTVIb-7*	0.00	0.00	0.00	0.00	0.00
PCTVIb-10	0.00	0.00	0.00	0.00	0.00
PCTVIb-12	0.00	0.00	0.00	0.00	0.00
PCTVIb-14	0.00	0.00	0.00	0.00	0.00
PCTVIb-16	0.00	0.00	0.00	0.00	0.00
PCTVIb-18	0.00	0.00	0.00	0.00	0.00
PCTVIb-23	0.00	0.00	0.00	0.00	0.00
PCTVIb-24	0.00	0.00	0.00	0.00	0.00
PCTVIb-29	0.00	0.00	0.00	0.00	0.00
PCTVIb-30	0.00	0.00	0.00	0.00	0.00
PCTVIb-31	0.00	0.00	0.00	0.00	0.00
PCTVIb-32*	0.00	0.00	0.00	0.00	0.00
PCTVIb-33*	0.00	0.00	0.00	0.00	0.00
PCTVIb-34	0.00	0.00	0.00	0.00	0.00
PCTVIb-35	0.00	0.00	0.00	0.00	0.00
PCTVIb-37	0.00	0.00	0.00	0.00	0.00
PCTVIb-25	100.00	100.00	100.00	100.00	100.00

*: the total number of conceptus dissected from the female is more than the one of transferred

III. 18 Chimaera series B($^1/_2$8+8)

(data ranked by the % GPI1-A of foetus)

$(AAF_1 \times AAF_1) \leftrightarrow (BF_1 \times TGB)$: $^1/_2$ 8-cell $\leftrightarrow$ 8-cell

number	% GPI1-A foetus	amnion	yolk sac mes	yolk sac end	placenta
PCTVIe-5	0.00	0.00	0.00	6.80	10.00
PCTVIe-9	0.00	0.00	0.00	15.70	0.00
PCTVIe-27	0.00	0.00	0.00	60.80	64.05
PCTVIe-33	0.00	0.00	0.00	12.10	42.80
PCTVIe-38	0.00	0.00	0.00	11.30	81.60
PCTVIe-40	0.00	0.00	0.00	1.70	5.70
PCTVIe-23	2.70	9.50	0.00	0.00	32.00
PCTVIe-19	3.70	11.50	4.60	0.00	0.00
PCTVIe-22	4.30	21.60	18.40	11.00	74.30
PCTVIe-29	6.00	5.30	4.70	5.30	0.00
PCTVIe-28	12.00	11.10	6.40	47.50	26.85
PCTVIe-20	12.10	15.30	19.30	0.00	81.00
PCTVIe-7	12.70	18.10	19.40	11.90	5.80
PCTVIe-13	17.30	8.70	25.60	0.00	4.60
PCTVIe-16	18.00	12.90	19.30	0.00	0.00
PCTVIe-35	24.10	16.40	21.00	7.60	85.40
PCTVIe-3	24.20	13.00	14.30	14.80	0.00
PCTVIe-14	24.50	27.20	31.00	0.00	16.60
PCTVIe-6	25.70	24.90	21.80	0.00	23.90
PCTVIe-4	27.40	10.60	23.00	12.10	0.00
PCTVIe-39	28.10	12.50	28.20	12.00	33.80
PCTVIe-31	28.50	14.60	20.30	54.10	0.00
PCTVIe-34	30.10	54.30	38.00	44.30	64.00
PCTVIe-18	30.60	27.20	28.80	17.20	7.90
PCTVIe-26	34.20	19.20	22.80	22.80	0.00
PCTVIe-8	36.60	42.60	37.30	13.00	6.30
PCTVIe-30	38.20	24.10	38.60	30.90	24.55
PCTVIe-25	45.80	39.00	50.60	7.70	27.65
PCTVIe-1	50.00	49.10	42.30	20.60	43.25
PCTVIe-24	53.20	69.10	74.00	100.00	16.40
PCTVIe-36	55.30	57.70	68.00	14.70	91.40
PCTVIe-32	55.70	45.50	54.30	31.50	72.15
PCTVIe-37	65.60	79.30	76.70	38.20	31.50
PCTVIe-11	91.00	100.00	93.90	19.10	94.80
Sample Size: 34					
Mean	25.22	24.71	26.55	18.96	31.42
SD	22.21	24.62	24.51	21.67	31.89
SEM	3.81	4.22	4.20	3.72	5.47
Non-chimaeras					
PCTVIe-2	0.00	0.00	0.00	0.00	0.00
PCTVIe-10	0.00	0.00	0.00	0.00	0.00
PCTVIe-12	0.00	0.00	0.00	0.00	0.00
PCTVIe-15	0.00	0.00	0.00	0.00	0.00
PCTVIe-17	0.00	0.00	0.00	0.00	0.00
PCTVIe-21	0.00	0.00	0.00	0.00	0.00

III. 19 Chimaera series B(8+$^{1}/_{2}$8)

(data ranked by the % GPI1-A of foetus)

$(AAF_1 \times AAF_1) \leftrightarrow (BF_1 \times TGB)$: 8-cell $\leftrightarrow$ $^{1}/_{2}$ 8-cell

% GPI1-A

number	foetus	amnion	yolk sac mes	yolk sac end	placenta
PCTVIa-11	0.00	0.00	0.00	59.80	25.40
PCTVIa-20	0.00	0.00	0.00	34.30	51.10
PCTVIa-32	0.00	0.00	0.00	0.00	54.80
PCTVIa-33	0.00	0.00	0.00	52.00	91.80
PCTVIa-35	0.00	0.00	0.00	38.10	6.30
PCTVIa-37	0.00	0.00	0.00	0.00	61.40
PCTVIa-40	0.00	0.00	0.00	39.10	92.00
PCTVIa-34	27.30	12.30	14.10	39.50	87.90
PCTVIa-25	29.30	19.70	28.10	46.50	64.30
PCTVIa-10	30.30	53.60	30.50	46.50	85.10
PCTVIa-39	31.70	32.00	27.70	14.90	73.90
PCTVIa-21	34.30	57.20	43.80	74.70	55.70
PCTVIa-38	43.60	58.60	43.50	61.80	56.60
PCTVIa-13	45.40	45.60	48.70	68.20	42.00
PCTVIa-16	46.60	61.50	58.30	42.10	78.60
PCTVIa-18	48.40	32.00	55.00	51.60	22.80
PCTVIa-7	54.70	53.50	52.70	100.00	95.70
PCTVIa-1	55.70	44.30	70.40	59.10	81.40
PCTVIa-3	65.90	83.30	75.40	100.00	52.20
PCTVIa-9	67.20	84.50	69.50	42.20	96.20
PCTVIa-4	68.90	81.60	69.70	100.00	91.50
PCTVIa-24	72.60	89.40	84.20	46.40	47.70
PCTVIa-19	76.80	64.10	72.30	27.00	2.70
PCTVIa-8	82.00	97.60	100.00	82.90	100.00
PCTVIa-31	85.10	92.20	94.80	50.10	64.70
PCTVIa-36	87.00	90.40	81.40	49.10	74.60
PCTVIa-17	91.70	97.30	100.00	83.70	56.80
PCTVIa-2	100.00	100.00	100.00	80.80	100.00
PCTVIa-14	100.00	100.00	92.60	50.10	70.00
PCTVIa-15	100.00	100.00	100.00	17.70	90.80
PCTVIa-22	100.00	100.00	100.00	63.40	100.00
PCTVIa-23	100.00	100.00	100.00	100.00	33.00
PCTVIa-26	100.00	100.00	100.00	32.80	97.20
PCTVIa-27	100.00	100.00	100.00	75.50	66.40
PCTVIa-28	100.00	100.00	100.00	44.50	100.00
PCTVIa-29	100.00	100.00	100.00	100.00	49.30
Sample Size:36					
Mean	56.79	59.74	58.69	54.84	67.22
SD	36.99	38.87	38.14	27.16	27.02
SEM	6.17	6.48	6.36	4.53	4.50
Non-chimaeras					
PCTVIa-5	100.00	100.00	100.00	100.00	100.00
PCTVIa-6	100.00	100.00	100.00	100.00	100.00
PCTVIa-12	100.00	100.00	100.00	100.00	100.00
PCTVIa-30	100.00	100.00	100.00	100.00	100.00

III. 20 Chimaera series B(8+4)

(data ranked by the % GPI1-A of foetus)

$(AAF_1 \times AAF_1) \leftrightarrow (BF_1 \times TGB)$: 8-cell $\leftrightarrow$ 4-cell

number	% GPI1-A foetus	amnion	yolk sac mes	yolk sac end	placenta
PCTVIc-5	0.00	0.00	0.00	16.30	0.00
PCTVIc-6	76.80	78.70	82.20	59.90	98.10
PCTVIc-16	100.00	100.00	100.00	93.10	100.00
Sample Size: 3					
Mean	58.93	59.57	60.73	56.43	66.03
SD	52.34	52.67	53.34	38.52	57.19
SEM	30.21	30.41	30.80	22.24	33.02
Non-chimaeras					
PCTVIc-1	100.00	100.00	100.00	100.00	100.00
PCTVIc-2	100.00	100.00	100.00	100.00	100.00
PCTVIc-3	100.00	100.00	100.00	100.00	100.00
PCTVIc-4	100.00	100.00	100.00	100.00	100.00
PCTVIc-7	100.00	100.00	100.00	100.00	100.00
PCTVIc-8	100.00	100.00	100.00	100.00	100.00
PCTVIc-9	100.00	100.00	100.00	100.00	100.00
PCTVIc-10	100.00	100.00	100.00	100.00	100.00
PCTVIc-11	100.00	100.00	100.00	100.00	100.00
PCTVIc-12	100.00	100.00	100.00	100.00	100.00
PCTVIc-13-1*	100.00	100.00	100.00	100.00	100.00
PCTVIc-13-2*	100.00	100.00	-	-	-
PCTVIc-14*	100.00	100.00	100.00	100.00	-
PCTVIc-15	100.00	100.00	100.00	100.00	100.00
PCTVIc-17	100.00	100.00	100.00	100.00	100.00
PCTVIc-18	100.00	100.00	100.00	100.00	100.00
PCTVIc-19	100.00	100.00	100.00	100.00	100.00
PCTVIc-20	100.00	100.00	100.00	100.00	100.00
PCTVIc-21	100.00	100.00	100.00	100.00	100.00
PCTVIc-22	100.00	100.00	100.00	100.00	100.00
PCTVIc-23	100.00	100.00	100.00	100.00	100.00
PCTVIc-24	100.00	100.00	100.00	100.00	100.00

*: placenta with two yolk sacs; one yolk sac with two fetuses

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