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A quantitative test for developmental neutrality of a transgenic lineage marker in mouse chimaeras

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Summary

The mouse transgene, provisionally designated TgN(Hbb-b1)83Clo, was produced by Dr C. Lo by pronuclear injection of the cloned ß-major globin gene and comprises a highly reiterated sequence that is readily detected by DNA *in situ* hybridization on histological sections. This fulfils many of the requirements of an ideal genetic cell marker and has been widely used for lineage studies with mouse chimaeras. However, it is not known whether it causes cell selection or influences developmental processes, such as cell mixing, in chimaeric tissues. In the present study, non-transgenic genetic markers (electrophoretic polymorphisms of glucose phosphate isomerase and differences in eye pigmentation) revealed no significant effect of the presence of hemizygous transgenic cells on the overall composition, size or gross morphology of 12½ d chimaeric foetuses, placentas or extraembryonic membranes. Also, a previously described maternal genetic effect on the composition of chimaeric tissues occurred in the presence or absence of the transgene. These tests have demonstrated that hemizygous cells are not at a significant selective disadvantage, when incorporated into mouse aggregation chimaeras with non-transgenic cells. Further studies are needed to test whether homozygous transgenic cells are also selectively neutral and to test whether hemizygous or homozygous transgenic cells influence developmental processes, such as cell mixing, that were not tested.

1. Introduction

The transgenic strain of mice originally known as 'strain 83' or 'ß83' was produced by Lo (1983, 1986) by pronuclear injection of the cloned ß-major globin gene, Hbb^{b1}. The transgene has been provisionally assigned the standardized name TgN(Hbb-b1)83Clo (West, Everett & Keighren, 1995*a*) and here it is abbreviated to *Tg*. It consists of approximately 1000 tandem repeats of plasmid pM$\beta\delta$2 (comprising plasmid pBR322 and a 7 kb insert of Hbb^{b1} DNA) on chromosome 3 (Lo, Diaz & Kirby, 1992; Everett, Keighren & West, 1994) and can be identified by DNA *in situ* hybridization on histological sections (Lo, 1986; Lo, Coulling & Kirby, 1987; Katsumata & Lo, 1988; Thomson & Solter, 1988*a*). This has provided a valuable lineage marker for many studies with mouse chimaeras (e.g. Thomson & Solter, 1988*a*, *b*, 1989; Nagy, Sass & Markkula, 1989; Palmer & Burgoyne, 1991; Boland & Gosden, 1994; James, Klerkx, Keighren, Flockart & West, 1995).

The use of DNA *in situ* hybridization with this reiterated transgene fulfils the six requirements of an ideal genetic cell marker for lineage studies that were originally listed by McLaren (1976). It is cell-localized, cell-autonomous, stable, universally distributed (in all nucleated cells), easy to detect (but with less than 100% efficiency), and genetically polymorphic (transgenic and non-transgenic strains). A lineage marker should also be developmentally neutral, so it does not cause cell selection (Oster-Granite & Gearhart, 1981) or influence developmental processes such as cell mixing (West, 1984). Although the reiterated transgene has been widely used as a lineage marker, it is not yet known whether it is developmentally neutral. The present study sets out to test whether cells that are hemizygous for the transgene ($Tg/-$) are selectively neutral or whether they are at a selective disadvantage, when incorporated into mouse aggregation chimaeras with non-transgenic ($-/-$) cells. This has been done by using additional (non-transgenic) genetic markers

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to compare the composition of chimaeras that contain the transgene with those that do not. Mid-gestation chimaeras were analysed to test whether transgenic cells contribute equally to embryonic and extra-embryonic tissues.

2. Materials and methods

(i) *Mice*

Three groups of F_1 mice were produced from the stocks shown in Table 1 as previously outlined (West & Flockhart, 1994). 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. All other animals were bred and maintained, under conventional conditions, in the Centre for Reproductive Biology.

Our original stock of TgN(Hbb-b1)83Clo animals was obtained from Dr Roger Gosden (Dept of Physiology, University of Edinburgh) in 1989 and was homozygous for *Gpi1-s*^{a} but had a mixed genetic background, probably with contributions from strains C57BL/6, SJL, C3H and CD1 (Keighren & West, 1994). Intercrosses between animals in our original stock produced albino offspring, which were maintained as strain '83' for this experiment. These mice tended to become obese and had a poor breeding performance, so another albino strain, homozygous both for *Tg* and *Gpi1-s*^{a}, was produced by crossing our original *Tg/Tg* stock and the albino, partly congenic strain C57BL/Ola.AKR-*Gpi1-s*^{a},*c*/Ws (name abbreviated to BC). Offspring were backcrossed to BC to obtain albino, *Tg*/− animals and intercrossed to produce *Tg/Tg* homozygotes. These animals were maintained as a closed, random-bred colony (stock CMA). Both homozygous *Tg/Tg* and hemizygous *Tg*/− CMA mice were used in the present study.

(ii) *Production of chimaeras*

Preimplantation embryos were flushed from the reproductive tract of pregnant females at $2\frac{1}{2}$ d post coitum (usually between 09.00 and 11.00 h) and aggregated to produce chimaeras as previously described (West & Flockhart, 1994). The following day, the aggregated embryos were surgically transferred to the uteri of CF_1 pseudopregnant females (homozygous *Gpi1-s*^{c}/*Gpi1-s*^{c}), anaesthetized with Hypnorm and Hypnovel (West, Flockhart & Kissenpfennig, 1995*c*).

Chimaeras in series XF were produced by aggregating hemizygous transgenic (*Tg*/−) and non-transgenic (−/−) embryos. Series XL and XJ chimaeras were produced by aggregating either *Tg*/− or −/− embryos with genetically distinct (*C*/*C*, *Gpi1-s*^{b}/*Gpi1-s*^{b}) non-transgenic embryos. The *Tg*/−↔−/− and −/−↔−/− chimaeras were distinguished at the time of analysis (see below). Series XL and XJ were compared to our previous non-transgenic series XM and XN (West & Flockhart, 1994; West *et al.* 1995*c*). See Table 2 for details of strain combinations.

(iii) *Analysis of chimaeras*

Pregnant females were killed at $12\frac{1}{2}$ d gestation and the conceptuses were dissected as described by West & Flockhart (1994) to provide foetus, amnion, visceral yolk sac mesoderm, visceral yolk sac endoderm, parietal endoderm (Reichert's membrane), a sample of trophoblast (dissected from Reichert's membrane) and placenta. The weights of the total conceptus, the placenta and the foetus were recorded, along with the crown–rump length and morphological index based

Table 1. *Genotypes of the stocks of mice used to produce the five series of chimaeric conceptuses shown in Table 2*

Abbreviated stock name	Details	Genotype		
		Albino	*Gpi1*	β-globin transgene
C57BL	C57BL/OlaWs	*C*/*C*	*b*/*b*	−/−
CBA	CBA/Ca	*C*/*C*	*b*/*b*	−/−
BALB/c	BALB/c/Eumm	*c*/*c*	*a*/*a*	−/−
BC	C57BL/Ola.AKR-*Gpi1-s*^{a},*c*/Ws	*c*/*c*	*a*/*a*	−/−
CC	C57BL-*Gpi1-s*^{c},*c*/Ws	*c*/*c*	*c*/*c*	−/−
CALB	BALB/c-*Gpi1-s*^{c}/Ws	*c*/*c*	*c*/*c*	−/−
83	Albino derivative of strain 83 (Lo, 1983)	*c*/*c*	*a*/*a*	*Tg*/*Tg*
CMA	Derived from cross between strain 83 and BC	*c*/*c*	*a*/*a*	*Tg*/*Tg* or *Tg*/−
AF_1	(BC female × BALB/c male)F_1 hybrid	*c*/*c*	*a*/*a*	−/−
AF_2	(AF_1 female × AF_1 male)F_2 hybrid	*c*/*c*	*a*/*a*	−/−
BF_1	(C57BL female × CBA male)F_1 hybrid	*C*/*C*	*b*/*b*	−/−
BF_2	(BF_1 female × BF_1 male)F_2 hybrid	*C*/*C*	*b*/*b*	−/−
CF_1	(CC female × CALB male)F_1 hybrid	*c*/*c*	*c*/*c*	−/−

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Table 2. *Strain combinations of the five series of chimaeras*

Chimaera group	Embryos aggregated[a] GPIA embryo (female × male)	↔	GPIB embryo (female × male)	Transgenic genotype GPIA embryo ↔ GPIB embryo
XF	$AF_1 \times 83$	↔	$BF_1 \times BF_1$	$Tg/- \leftrightarrow -/-$
XL	$AF_1 \times$ CMA	↔	$BF_1 \times BF_1$	$Tg/- \leftrightarrow -/-$ or $-/- \leftrightarrow -/-$
XJ	BALB/c × CMA	↔	$BF_1 \times BF_1$	$Tg/- \leftrightarrow -/-$ or $-/- \leftrightarrow -/-$
XM[b]	$AF_1 \times AF_1$	↔	$BF_1 \times BF_1$	$-/- \leftrightarrow -/-$
XN[c]	BALB/c × AF_1	↔	$BF_1 \times BF_1$	$-/- \leftrightarrow -/-$

[a] See Table 1 for full explanation of strain designations. All aggregated embryos were transferred to CF_1 pseudopregnant females.
[b] See West & Flockhart (1994).
[c] See West, Flockhart & Kissenpfennig (1995*c*).

on hind limb development (McLaren & Buehr, 1990; Palmer & Burgoyne, 1991). The morphological index values were converted to a numerical scale for statistical analysis. For example, stages 7 and late 7 were transformed to 7·0 and 7·5 respectively. Unless the foetus was too immature, the proportion of pigmented (*C/C*) cells in the retinal pigment epithelium of each eye was subjectively estimated and averaged. This is expressed as the % albino (*c/c*) cells (= 100-% pigmented) in the tables.

The foetal heads were fixed in acetic alcohol (3 parts ethanol: 1 part glacial acetic acid) and analysed by *in situ* hybridization to the transgene as described by Keighren & West (1993). Hybridized digoxygenin-labelled DNA probe was detected by diaminobenzidine (DAB) staining for peroxidase-labelled antibody. This was used to distinguish between $Tg/- \leftrightarrow -/-$ and $-/- \leftrightarrow -/-$ chimaeras in series XL and XJ, where the GPIA embryos (*Gpi1-s*^{a}/*Gpi1-s*^{a}) had $-/-$ mothers and either Tg/Tg or $Tg/-$ CMA fathers.

The foetal trunk and placenta were stored at −20 °C, in 100 μl of 50% glycerol in water, in 1·5 ml microtubes for glucose phosphate isomerase (GPI) electrophoresis. All other tissues were stored in 10 μl of 50% glycerol in microtest plates. Samples were lysed by three cycles of freeze/thawing with mechanical disruption of the foetal and placental tissues. Electrophoresis, staining for GPI activity and quantification of the %GPIA by scanning densitometry were carried out as previously described (West & Flockhart, 1994). Maternal tissue (e.g. in placentas) produced only the GPIC enzyme and was excluded from the analysis of electrophoresis bands. GPIAB heteropolymer was produced by some chimaeric placentas (West, Flockhart & Keighren, 1995*b*). Half of %GPIAB heteropolymer was added to both %GPIA and %GPIB values, so that the %GPIA was calculated as $(A + AB/2) \times 100/(A + AB + B)$. The raw data (as %GPIA allozyme given to one decimal percentage point) was used for statistical analysis and plotting the figures but they were rounded to the nearest integer for presentation in the Tables.

(iv) *Statistical analysis*

Statistical tests were performed on an Apple Macintosh computer using statistical packages 'StatView 4.1' (Abacus Concepts Inc., Berkeley, USA) and 'MultiStat' (Biosoft, Cambridge, UK) and a routine established on Microsoft Excel (Microsoft Corporation).

3. Results

In the first experiment, all of the chimaeras were $Tg/-$ *c,Gpi1-s*^{a}/*c,Gpi1-s*^{a} ↔ $-/-$ *C,Gpi1-s*^{b}/*C,Gpi1-s*^{b} and the overall contribution of the hemizygous transgenic ($Tg/-$) cell population was estimated by the %GPIA (or % albino in the eye). Although this analysis should reveal any strong selection pressure against $Tg/-$ cells it would not reveal more subtle effects because the strain combination affects the composition of the chimaeric tissues. Two series of chimaeras (XL and XJ) were analysed in the second experiment. As before, all chimaeras were *c,Gpi1-s*^{a}/*c,Gpi1-s*^{a} ↔ *C,Gpi1-s*^{b}/*C,Gpi1-s*^{b} but in each series, some chimaeras were $Tg/- \leftrightarrow -/-$ and some were $-/- \leftrightarrow -/-$. This allowed two sets of comparisons to be made. First, the composition of $Tg/- \leftrightarrow -/-$ and $-/- \leftrightarrow -/-$ chimaeras was compared within each series. Second, the overall composition of each series was compared to that of two previously published series. Previous studies (West & Flockhart, 1994; West *et al.* 1995*c*) have shown that embryos with BALB/c mothers tend to make a low contribution to chimaeras. In series XJ and XN, one of each pair of embryos aggregated had a BALB/c mother (Table 2). This comparison tests whether the presence of the transgene disrupts differences in chimaeric composition that are attributable to strain combination.

(i) *$Tg/-$ cells were not deficient in the ($AF_1 \times 83$) ↔ BF_2 series of* $Tg/- \leftrightarrow -/-$ *chimaeras*

Electrophoresis of GPI was used to estimate the contribution of each of the original 8-cell stage

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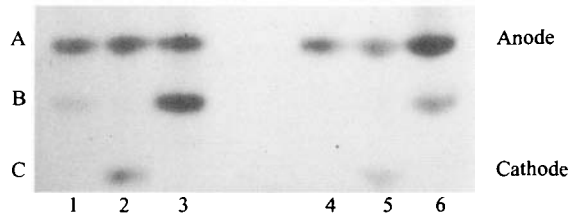

Fig. 1. GPI electrophoresis plate; samples were loaded with a 0·25 μl applicator. GPI allozymes (A, B and C) are indicated at the side; migration was towards the cathode. Lanes 1–3, foetus, placenta and yolk sac endoderm from conceptus XF5. Lanes 4–6, foetus, placenta and yolk sac endoderm from conceptus XF1. Maternal GPIC was present in the placenta samples (lanes 2 and 5).

embryos to the foetus, placenta and other extra-embryonic tissues of each $12\frac{1}{2}$ d chimaeric conceptuses as shown in Fig. 1. The estimated contribution of the transgenic *Tg*/− ($AF_1 \times 83$) cells (% albino in the eye or % GPIA in the other tissues) is shown in Table 3. The mean contribution of transgenic cells was at least 50% in all tissues analysed, indicating that there was no overall selection pressure against *Tg*/− cells in the foetus or extraembryonic tissues.

For each of the eight tissues analysed, the chimaeras were grouped into two sets of classes to test whether the series of chimaeras was genotypically unbalanced, as previously described (West & Flockhart, 1994; West *et al.* 1995*c*). In the first classification, shown in Table 3, the strain combination was considered to be unbalanced if the number of individuals with < 50% GPIA (or albino in the eye) was statistically significantly different from those with > 50% GPIA. In the second classification, individual samples were divided into three classes according to the %GPIA: < 25%, 25–75% and > 75%. If the number of individuals in the 25–75% GPIA class was not the highest or joint highest of the three classes, the strain combination was considered to be atypical. Using these two criteria, the distributions of %GPIA (or % albino) in different tissues were classified as (1) balanced and typical, (2) balanced but atypical or (3) unbalanced. None of the tissues in this series showed

Table 3. *Percentage GPIA (or % albino in the eye) in tissues of $12\frac{1}{2}$ d chimaeric conceptuses in series XF ($AF_1 \times 83 \leftrightarrow BF_2$), ranked by % GPIA in the foetus*

	Primitive ectoderm lineage[a]				Primitive endoderm		Trophectoderm	
Chimaera ref.	Eye % albino	Foetus	Amnion	YS mes	YS end	P. end	Troph	Placenta
XF21	20	29	28	33	22	22	0	2
XF14	25	36	40	48	40	27	93	92
XF3	40	48	40	24	47	41	100	92
XF18	40	52	26	41	18	28	33	19
XF8	50	54	56	54	45	52	3	6
XF23	65	60	44	48	77	82	100	96
XF12	65	64	60	59	80	91	100	97
XF16	90	67	69	73	43	54	100	90
XF26	68	80	71	65	0	0	80	92
XF5	83	81	70	69	36	67	100	96
XF10	88	85	57	54	87	89	100	99
XF22	85	88	90	69	28	40	85	75
XF11	85	88	80	79	93	72	73	83
XF20	99	91	78	78	67	67	90	90
XF7	99	95	92	91	82	41	100	92
XF9	97	97	100	100	41	39	100	98
XF6	100	100	89	97	51	100	100	94
XF17	100	100	100	100	81	68	100	98
Mean	72·06	73·06	66·02	65·62	52·11	54·39	81·03	78·36
S.E.	6·31	5·26	5·60	5·34	6·34	6·37	7·83	7·66
n	18	18	18	18	18	18	18	18
< 50: > 50%	4·5:13·5*	3:15**	5:13	5:13	10:8	8:10	3:15**	3:15**
< 25:25–75: > 75%	1:7:10[b]	0:8:10[b]	0:11:7	1:11:6	3:9:6	2:12:4	2:2:14[b]	3:1:14[b]

[a] The tissues are arranged according to their developmental origin from the primitive ectoderm (epiblast), primitive endoderm (hypoblast) or trophectoderm lineage. Abbreviations: YS mes, yolk sac mesoderm; YS end, yolk sac endoderm; P. end, parietal endoderm; Troph, trophoblast overlying Reichert's membrane. Another two conceptuses were non-chimaeric: XF4 was 100%GPIA and XF15 was 100%GPIB. Three pairs of chimaeras had fused placentas (XF1 with XF2, XF24 with XF25 and XF13 with XF13B) and three conceptuses were dead (XF13B, XF19 and XF27).

[b] Distributions were classified as 'atypical' because there were fewer individuals with 25–75% GPIA than in one of the other categories (< 25 or > 75%).

* $P \leqslant 0{\cdot}05$, ** $P \leqslant 0{\cdot}01$: significantly unbalanced distributions, when tested against the expectation of a 1:1 ratio of conceptuses with < 50 and > 50% GPIA by χ^2 test.

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Table 4. *Percentage GPIA (or % albino in the eye) in tissues of 12½ d chimaeric conceptuses in series XL ($AF_1 \times CMA \leftrightarrow BF_2$), ranked by % GPIA in the foetus*

Chimaera ref.	Tg?[a]	Primitive ectoderm lineage: Eye % albino	Foetus	Amnion	YS mes	Primitive endoderm: YS end	P. end	Trophectoderm: Troph	Placenta
XL15	N	5	13	17	18	84	100	0	7
XL24	N	15	19	29	15	18	18	77	69
XL33	N	20	25	32	33	27	29	7	40
XL36	N	10	26	15	19	22	42	10	0
XL17	N	20	33	51	46	87	73	94	71
XL32	N	20	38	57	44	21	35	0	2
XL13	N	—	41	41	39	40	41	38	35
XL9	N	60	68	74	75	39	41	18	39
XL21	N	60	69	71	67	51	75	74	71
XL6	N	75	71	85	67	21	11	12	36
XL39	N	55	73	81	65	97	100	100	100
XL12	N	80	95	86	89	54	71	0	6
XL22	N	100	100	100	100	68	88	100	93
XL11	N	100	100	100	100	93	87	100	100
XL1	Y	8	23	25	12	55	91	0	4
XL14	Y	15	31	42	30	73	74	95	96
XL4	Y	15	33	30	24	69	63	25	42
XL37	Y	40	36	56	36	5	61	0	3
XL34	Y	25	36	43	35	48	26	14	37
XL5	Y	60	36	29	30	23	32	29	6
XL44	Y	30	36	36	34	22	42	39	25
XL7	Y	35	38	37	23	34	55	—	5
XL3	Y	50	39	13	15	40	9	0	12
XL30	Y	25	39	49	41	75	81	57	77
XL41	Y	25	42	45	35	43	42	38	71
XL43	Y	45	49	55	46	83	68	52	15
XL42	Y	50	53	51	53	58	74	72	88
XL31	Y	55	53	39	35	11	42	0	2
XL35	Y	40	59	53	52	47	43	100	98
XL29	Y	50	68	53	57	63	80	0	6
XL18	Y	80	88	89	91	30	47	100	98
XL2	Y	95	88	85	69	59	54	17	31
XL23	Y	88	91	79	85	55	70	43	70
XL27	Y	90	96	89	88	64	89	100	90
XL40	Y	85	98	94	100	70	68	90	88
Mean		47·79	54·30	55·11	50·46	49·86	57·71	43·99	46·58
S.E.		5·03	4·50	4·32	4·59	4·16	4·22	6·75	6·24
n		34	35	35	35	35	35	34	35
< 50: > 50%		18·5:15·5	19:16	16:19	20:15	17:18	15:20	20:14	20:15
< 25:25–75: > 75%		9:17:8	3:24:8	3:22:10	7:20:8	8:22:5	3:23:9	15:9:10[b]	12:13:10

[a] Y = transgene present in foetus; N = transgene absent from foetus. Other abbreviations as in Table 3. Another five conceptuses were non-chimaeric: XL10, XL28 and XL38 were 100% GPIA and XL8 and XL16 were 100% GPIB. Two pairs of chimaeras had fused placentas (XL19 with XL20 and XL25 with XL26).

[b] Atypical distribution; none was considered unbalanced (see Table 3).

an overall imbalance against the transgenic, cell type (GPIA or albino) but four tissues (eye, foetus, trophoblast and placenta) were unbalanced in the opposite direction with significantly more chimaeras having > 50% *Tg/−* cells than < 50% *Tg/−* cells (Table 3). Without a control series, with no transgenic cell population, the cause of the imbalance in favour of ($AF_1 \times 83$) cells cannot be determined. However, it is clear that there was no gross deficiency of *Tg/−* cells in any of the tissues of the mid-gestation chimaeric conceptuses.

(ii) *The composition of Tg/− ↔ −/− and −/− ↔ −/− chimaeras is similar within series XL and XJ*

Tables 4 and 5 show the tissue composition of the (AF_1 female × CMA male) ↔ BF_2 and (BALB/c female × CMA male) ↔ BF_2 chimaeras (series XL and XJ, respectively). Some of the CMA strain males were homozygous (*Tg/Tg*) while others were hemizygous (*Tg/−*), so each series was a mixture of *Tg/− ↔ −/−* and *−/− ↔ −/−* chimaeras. These

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Table 5. *Percentage GPIA (or % albino in the eye) in tissues of 12½ d chimaeric conceptuses in series XJ (BALB/c × CMA ↔ BF$_2$), ranked by % GPIA in the foetus*

Chimaera ref.	Tg?[a]	Primitive ectoderm lineage: Eye % albino	Foetus	Amnion	YS mes	Primitive endoderm: YS end	P. end	Trophectoderm: Troph	Placenta
XJ7	NK	0	0	0	0	0	0	11	0
XJ4	NK	0	0	0	0	6	12	16	11
XJ20	NK	0	0	0	0	12	—	0	4
XJ34	NK	0	0	0	0	12	6	72	58
XJ1	NK	0	0	0	0	34	0	10	0
XJ9	NK	0	0	0	0	36	54	14	0
XJ26	N	0	5	10	15	72	100	—	4
XJ30	N	8	14	9	21	9	7	0	6
XJ13	N	73	54	55	48	24	56	53	47
XJ29	N	40	62	61	60	57	92	17	19
XJ33	N	100	100	100	100	91	86	33	95
XJ12	Y	0	9	4	6	19	9	26	35
XJ8	Y	5	12	17	18	12	27	22	54
XJ2	Y	10	19	16	29	39	44	20	10
XJ21	Y	3	19	29	16	39	27	16	71
XJ31	Y	13	22	31	32	19	5	80	65
XJ32	Y	15	28	28	33	38	62	38	55
XJ17	Y	15	40	37	30	98	84	5	15
XJ24	Y	75	46	31	34	65	13	100	87
XJ10	Y	65	47	50	36	38	22	24	56
XJ16	Y	15	54	61	41	10	22	100	91
XJ15	Y	40	58	40	28	43	47	10	13
XJ19	Y	30	59	36	41	56	45	5	12
XJ3	Y	80	77	95	76	45	57	39	76
XJ11	Y	100	100	100	100	100	100	78	100
XJ18	Y	100	100	100	100	88	91	100	87
Mean		30·21	35·52	34·90	33·32	40·82	42·60	35·45	41·20
S.E.		7·11	6·56	6·64	6·17	5·85	6·81	6·64	6·85
n		26	26	26	26	26	25	25	26
< 50: > 50%		19:7*	17:9	19:7*	21:5**	18:8*	15:10	18:7*	14:12
< 25:25–75: > 75%		16:6:4[b]	13:9:4[b]	11:11:4	11:11:4	10:12:4	10:9:6[b]	14:6:5[b]	12:8:6[b]

[a] Y = transgene present; N = transgene absent; NK = presence of transgene in conceptus unknown because (BALB/c × CMA) genotype was absent from foetus. Other abbreviations as in Table 3. Another four conceptuses were non-chimaeric: XJ22, XJ23, XJ25 and XJ27 were all 100%GPIB. Two pairs of chimaeras had fused placentas (XJ5 with XJ6 and XJ28A with XJ28B) and one pair (XJ14A and XJ14B) were dead conceptuses with a shared placenta and Reichert's membrane.

[b] Atypical distribution (see Table 3).

* $P \leqslant 0{\cdot}05$, ** $P \leqslant 0{\cdot}01$: significantly unbalanced distributions.

two types of chimaeras were distinguished by *in situ* hybridization to detect the transgene in histological sections of the foetal heads (Fig. 2). Presence or absence of the transgene is indicated in Tables 4 and 5. The transgenic status of six (BALB/c × CMA) ↔ BF$_2$ chimaeras was unknown because (BALB/c × CMA) cells did not contribute to the foetus.

The composition of *Tg*/− ↔ −/− and −/− ↔ −/− chimaeras was compared within each series. Figure 3 shows similar distributions of the % GPIA in the seven tissues analysed for *Tg*/− ↔ −/− and −/− ↔ −/− conceptuses. Moreover, Mann–Whitney *U*-tests (Table 6) revealed no significant differences in the composition of *Tg*/− ↔ −/− and −/− ↔ −/− conceptuses. These results again imply that there was no pronounced selection against survival of *Tg*/− cells in the foetus or extraembryonic tissues.

(iii) *The influence of the strain combination on the chimaeric composition is unaffected by the transgene*

Previous studies have shown that embryos with BALB/c mothers tend to make a low contribution to chimaeras so that the mean % GPIA in a series of (BALB/c × AF$_1$) ↔ BF$_2$ chimaeras was lower than in a series of (AF$_1$ × AF$_1$) ↔ BF$_2$ chimaeras (West & Flockhart, 1994; West *et al.* 1995*c*). If the presence of the transgene affected the composition of chimaeras it might interfere with the ability of the strain combination to influence the composition of the chimaeric

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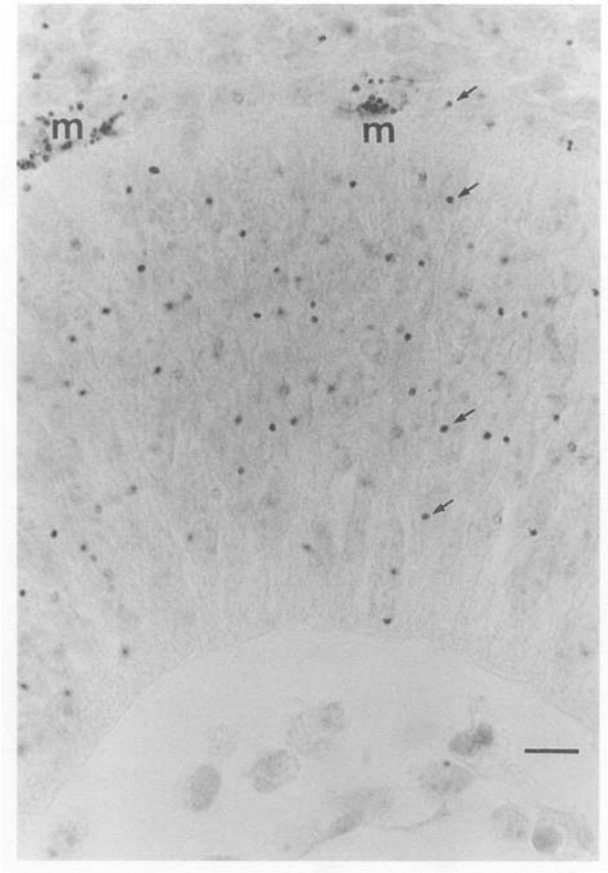

Fig. 2. A 7 μm histological section through one eye of foetal chimaera XF8 after *in situ* hybridization to detect the reiterated transgene (see Materials and methods). Clusters of dark melanin granules (m) can be seen in some retinal pigmented epithelium cells (from the BF_2 embryo) and single dark hybridization signals (some shown by arrows) identify hemizygous *Tg*/− nuclei (from the $AF_1 \times 83$ embryo). Scale bar = 10 μm.

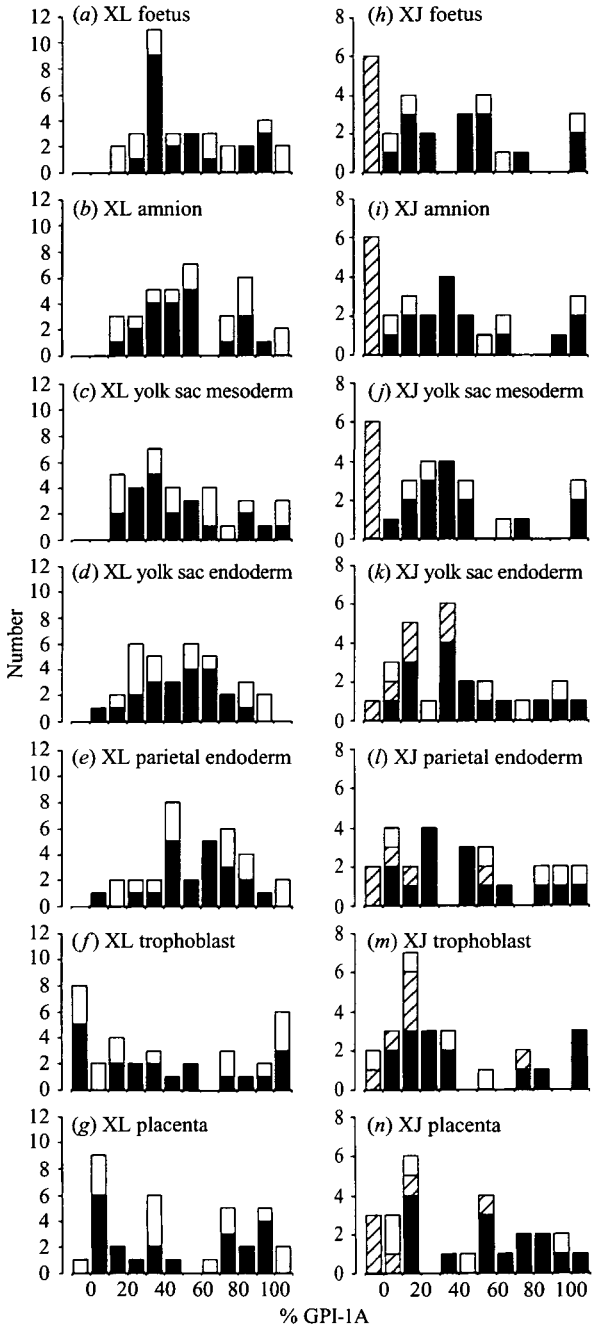

Fig. 3. Distributions of % GPIA in seven tissues analysed in chimaeric conceptuses from series XL and XJ. Tissues with either 0 or 100% GPIA are shown separately at either end of the distributions. Shading distinguishes between conceptuses that had a *Tg*/− cell population in the foetal head (identified as shown in Fig. 2; shaded black), those with a non-transgenic (−/−) GPIA cell population (open) and those with no GPIA cell population in the foetus (transgenic status not known; hatched shading).

tissues. Figure 4 shows that the mean contributions of $(BALB/c \times AF_1)$ and $(BALB/c \times CMA)$ embryos to chimaeras (in series XN and XJ respectively) were lower than for $(AF_1 \times AF_1)$ and $(AF_1 \times CMA)$ embryos to chimaeras (in series XM and XL respectively). The composition of the $(AF_1 \times 83) \leftrightarrow BF_2$ chimaeras, discussed in (i) above, is also shown in Fig. 4.

Figure 3 also indicates that the distributions of % GPIA were more skewed towards lower GPIA contributions in the $(BALB/c \times CMA) \leftrightarrow BF_2$ chimaeras than in the $(AF_1 \times CMA) \leftrightarrow BF_2$ chimaeras. According to the ratio of individuals with $< 50\%$ GPIA to those with $> 50\%$ GPIA, none of the tissues in the latter series was classified as unbalanced (Table 4). In contrast, five tissues were classified as unbalanced (with $< 50\%$ GPIA predominating) in the $(BALB/c \times CMA) \leftrightarrow BF_2$ series (Table 5).

Table 7 shows that there are statistically significant differences in chimaeric composition among series XM, XN, XL and XJ. Pairwise comparisons show that $(BALB/c \times AF_1) \leftrightarrow BF_2$ chimaeras differed from $(AF_1 \times AF_1) \leftrightarrow BF_2$ chimaeras in six of the eight tissues studied. Neither series contains the transgene and these differences are attributable to differences in strain combination. The $(BALB/c \times CMA) \leftrightarrow BF_2$ chimaeras show a similar difference from $(AF_1 \times CMA) \leftrightarrow BF_2$ chimaeras. This indicates that differences attributable to BALB/c versus AF_1 maternal genotype occur with either CMA or AF_1 as the paternal strain and are retained when the majority of

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Table 6. *Comparison of % GPIA in chimaeric conceptuses with and without the transgene in series XL and XJ*

Sample	Series XL: $(AF_1 \times CMA \leftrightarrow BF_2)$ Mean $Tg/- \leftrightarrow -/-$ $n = 21$	Mean $-/- \leftrightarrow -/-$ $n = 14$	P-value[a]	Series XJ: $(BALB/c \times CMA \leftrightarrow BF_2)$ Mean $Tg/- \leftrightarrow -/-$ $n = 15$	Mean $-/- \leftrightarrow -/-$ $n = 5$	P-value[a]
Primitive ectoderm lineage						
Eye (% albino)	47·86	47·69	0·873	37·70	44·00	0·965
Foetus	53·80	55·06	> 0·999	45·98	46·78	0·965
Amnion	51·97	59·84	0·459	44·86	46·92	0·896
YS mes	47·08	55·53	0·381	41·45	48·92	0·694
Primitive endoderm lineage						
YS end	48·82	51·42	0·973	47·24	50·30	0·827
P. end	57·56	57·93	0·920	43·51	68·14	0·193
Trophectoderm lineage						
Trophoblast	43·38	44·86	> 0·999	44·16	25·48	0·423
Placenta	45·89	47·61	0·866	55·07	34·34	0·206

[a] P-value from Mann–Whitney U-test.

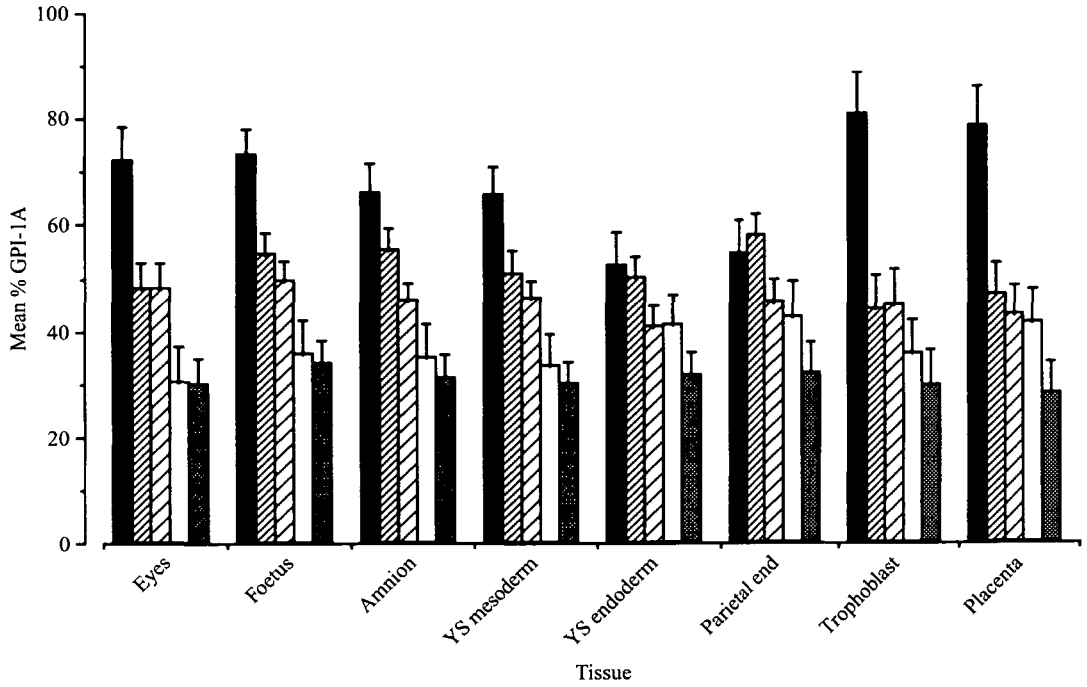

Fig. 4. Histogram comparing the mean % GPIA (or % albino in the eyes) in eight tissues of chimaeric conceptus from series XF, XL, XM, XJ and XN. ■, XF: $(AF_1 \times 83) \leftrightarrow BF_2$; ▨, XL: $(AF_1 \times CMA) \leftrightarrow BF_2$; ▨, XM: $(AF_1 \times AF_1) \leftrightarrow BF_2$; □, XJ: $(BALB/c \times CMA) \leftrightarrow BF_2$; ▨, XN: $(BALB/c \times AF_1) \leftrightarrow BF_2$.

the GPIA embryos are $Tg/-$. The composition of the $(AF_1 \times CMA) \leftrightarrow BF_2$ series did not differ significantly from the non-transgenic $(AF_1 \times AF_1) \leftrightarrow BF_2$ series. Similarly, $(BALB/c \times CMA) \leftrightarrow BF_2$ chimaeras did not differ significantly from $(BALB/c \times AF_1) \leftrightarrow BF_2$. These relationships show that the influence of strain combination, on the composition of the series of chimaeras, was unaffected by the presence of the $Tg/-$ genotype in the $(AF_1 \times CMA) \leftrightarrow BF_2$ and $(BALB/c \times CMA) \leftrightarrow BF_2$ chimaeras.

(iv) *Correlations in tissue composition within a developmental lineage were unaffected by the presence of* Tg/− *cells*

Previous studies have shown that the tissue composition is always very significantly positively correlated among tissues within each of the three primary developmental lineages (West, Bücher, Linke & Dünnwald, 1984; James, Flockhart, Keighren & West, 1993; West & Flockhart, 1994; West *et al.*, 1995*c*). Table 8 shows that the same is true of the three series of chimaeras containing transgenic GPIA cells. This shows that the presence of the $Tg/-$ genotype did not disrupt these relationships. As in previous studies, any

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Table 7. *Statistical comparison of composition of 12½ d chimaeric conceptuses from series XM, XN, XL and XJ* (P-*values are shown*)

Sample	Statistical variation among four different series (Kruskal–Wallis tests)	Pairwise comparisons (Mann–Whitney *U*-tests)			
		XM *v.* XN $(AF_1 \times AF_1) \leftrightarrow BF_2$ versus $(BALB/c \times AF_1) \leftrightarrow BF_2$	XL *v.* XJ $(AF_1 \times CMA) \leftrightarrow BF_2$ versus $(BALB/c \times CMA) \leftrightarrow BF_2$	XM *v.* XL $(AF_1 \times AF_1) \leftrightarrow BF_2$ versus $(AF_1 \times CMA) \leftrightarrow BF_2$	XN *v.* XJ $(BALB/c \times AF_1) \leftrightarrow BF_2$ versus $(BALB/c \times CMA) \leftrightarrow BF_2$
Eyes (% albino)	0·005*	0·013*	0·008*	0·945	0·574
Foetus (%GPIA)	0·007*	0·012*	0·021*	0·708	0·826
Amnion	0·001*	0·013*	0·008*	0·175	0·879
Yolk sac mesoderm	0·002*	0·004*	0·013*	0·679	0·946
Yolk sac endoderm	0·033	0·087	0·122	0·154	0·238
Parietal endoderm	0·007*	0·038*	0·078	0·061	0·225
Trophoblast	0·271	0·073	0·661	0·798	0·185
Placenta	0·059	0·019*	0·560	0·932	0·076

* The % albino or % GPIA was considered to be significantly different when $P \leqslant 0{\cdot}05$.

Table 8. *Spearman rank correlation coefficients* (r_s) *and* P *values, for % GPIA (or mean % albino in the eyes) in different tissues of chimaeras*

	Series of chimaeras with transgene[a]								Series of chimaeras without transgene[a]			
	XF		XL (all)		XL (with transgene)		XJ (all)		XM		XN	
	r_s	*P*	r_s	*P*	r_s	*P*	r_s	*P*	r_s	*P*	r_s	*P*
Correlations within lineages												
Eye *v.* foetus	0·954	****	0·929	****	0·826	***	0·945	****	0·918	****	0·933	****
Eye *v.* amnion	0·864	***	0·798	****	0·608	**	0·917	****	0·789	****	0·923	****
Eye *v.* YS mes	0·919	***	0·831	****	0·684	**	0·920	****	0·873	****	0·919	****
Foetus *v.* Amnion	0·931	***	0·873	****	0·768	***	0·963	****	0·880	****	0·945	****
Foetus *v.* YS mes	0·946	****	0·904	****	0·854	***	0·953	****	0·934	****	0·943	****
Amnion *v.* YS mes	0·955	***	0·961	****	0·948	****	0·963	****	0·885	****	0·962	****
YS end *v.* P. end	0·761	**	0·760	****	0·644	**	0·798	****	0·517	**	0·777	****
Troph *v.* placenta	0·797	**	0·902	****	0·909	****	0·854	****	0·643	***	0·862	****
Correlations between primitive ectoderm and primitive endoderm lineages												
Eye *v.* YS end	0·480	ms	0·116	NS	−0·072	NS	0·605	****	0·162	NS	0·627	***
Eye *v.* P. end	0·476	ms	0·130	NS	−0·051	NS	0·509	****	0·206	NS	0·477	ms
Foetus *v.* YS end	0·447	NS	0·257	NS	0·187	NS	0·653	****	0·190	NS	0·632	***
Foetus *v.* P. end	0·434	NS	0·261	NS	0·125	NS	0·624	****	0·089	NS	0·615	**
Amnion *v.* YS end	0·317	NS	0·296	NS	0·301	NS	0·625	****	0·220	NS	0·628	***
Amnion *v.* P. end	0·234	NS	0·330	NS	0·317	NS	0·602	****	0·039	NS	0·561	**
YS mes *v.* YS end	0·371	NS	0·324	NS	0·300	NS	0·572	****	0·137	NS	0·547	**
YS mes *v.* P. end	0·340	NS	0·321	NS	0·284	NS	0·566	****	0·038	NS	0·534	**
Correlations between primitive ectoderm and trophectoderm lineages												
Eye *v.* troph	0·498	ms	0·343	NS	0·193	NS	0·394	ms	−0·023	NS	0·335	NS
Eye *v.* placenta	0·424	NS	0·346	ms	0·113	NS	0·499	ms	0·149	NS	0·394	ms
Foetus *v.* troph	0·408	NS	0·407	ms	0·413	NS	0·368	ms	0·067	NS	0·308	NS
Foetus *v.* placenta	0·446	NS	0·439	ms	0·384	NS	0·503	***	0·345	NS	0·396	ms
Amnion *v.* troph.	0·340	NS	0·464	ms	0·519	*	0·458	ms	0·047	NS	0·436	ms
Amnion *v.* placenta	0·362	NS	0·498	ms	0·488	ms	0·577	****	0·258	NS	0·490	ms
YS mes *v.* troph	0·363	NS	0·473	ms	0·528	*	0·473	ms	0·011	NS	0·453	ms
YS mes *v.* placenta	0·352	NS	0·516	*	0·532	*	0·582	****	0·324	NS	0·536	**
Correlations between primitive endoderm and trophectoderm lineages												
YS end *v.* troph	0·457	NS	0·447	ms	0·385	NS	0·222	NS	0·411	ms	0·200	NS
YS end *v.* placenta	0·400	NS	0·522	*	0·432	NS	0·266	NS	0·353	ms	0·155	NS
P end *v.* troph	0·542	*	0·330	NS	0·255	NS	0·156	NS	−0·0003	NS	0·110	NS
P end *v.* placenta	0·475	NS	0·522	*	0·160	NS	0·234	NS	−0·061	NS	0·180	NS

[a] Series XF is $(AF_1 \times 83) \leftrightarrow BF_2$; XL = $(AF_1 \times CMA) \leftrightarrow BF_2$; XJ = $(BALB/c \times CMA) \leftrightarrow BF_2$; XM = $(AF_1 \times AF_1) \leftrightarrow BF_2$; XN = $(BALB/c \times AF_1) \leftrightarrow BF_2$.

* Spearman's rank correlation coefficient, * $r_s > 0{\cdot}5$, $P < 0{\cdot}05$; ** $r_s > 0{\cdot}5$, $P < 0{\cdot}01$; *** $r_s > 0{\cdot}5$, $P < 0{\cdot}001$; **** $r_s > 0{\cdot}5$, $P < 0{\cdot}0001$; ms, marginally significant ($P < 0{\cdot}05$ but $r_s < 0{\cdot}5$ and $> -0{\cdot}5$); NS, not significant ($P > 0{\cdot}05$).

other correlations (between the three different developmental lineages) were generally weaker. Both the $(BALB/c \times CMA) \leftrightarrow BF_2$ series (XJ) and the non-transgenic $(BALB/c \times AF_1) \leftrightarrow BF_2$ series (XN) also showed a significant correlation between primitive ectoderm and primitive endoderm-derived tissues. The biological significance of this remains unclear but both these series were also genotypically unbalanced (see Section iii). Although trophectoderm-derived tissues as well as primitive ectoderm and primitive endoderm derivatives were unbalanced (see Table 5 and West *et al.* 1995*c*), it is possible that a common factor caused both the correlation between primitive ectoderm and primitive endoderm compositions and the genotypic imbalance in favour of BF_2 cells. The possibility of non-random allocation of cells to different lineages at the blastocyst stage has been discussed elsewhere with respect to unbalanced strain combinations (West & Flockhart, 1994) and this could also underlie the unexpected correlations observed in series XJ and XN.

(v) *Chimaeric conceptuses were physically unaffected by the presence of* Tg/− *cells*

Comparisons of physical parameters (Table 9) revealed significant differences among the five series of chimaeras in conceptus weight, foetal weight, placental weight and foetal development (as assessed by the hind limb morphological index), which were mostly attributed to the larger conceptuses in series XF. More importantly, there were no significant differences

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Table 9. *Comparison of physical parameters*[a] *in different groups of 12½ d chimaeric conceptuses*

Group	*n*	Conceptus wt (mg)	Foetus wt (mg)	Placenta wt (mg)	Foetus/placenta wt ratio	Crown–rump length (mm)	Hind limb morphology score[a]
Chimaeras grouped by series							
XF	18	365·5 ± 13·4	119·6 ± 7·1	109·0 ± 3·8	1·11 ± 0·07	9·22 ± 0·15	7·75 ± 0·25
XL	35	310·6 ± 9·0	96·4 ± 3·7	85·5 ± 2·6	1·13 ± 0·04	9·18 ± 0·16	7·00 ± 0·50
XM	33	330·2 ± 9·5	107·9 ± 3·5	93·0 ± 2·6	1·16 ± 0·03	9·17 ± 0·11	7·50 ± 0·50
XJ	26	319·9 ± 7·1	104·2 ± 5·1	92·5 ± 3·3	0·98 ± 0·06	9·09 ± 0·14	7·50 ± 0·00
XN	29	327·3 ± 10·5	98·1 ± 4·4	89·7 ± 2·3	1·09 ± 0·04	9·18 ± 0·18	7·50 ± 0·50
P-values from statistical tests for variation among five series of chimaeras							
ANOVA		0·0098	0·0106	< 0·0001	0·0770	0·9889	—
Kruskal–Wallis		—	—	—	—	—	0·0029
P-values excluding series XF							
ANOVA		0·4086	0·1606	0·1597	0·0316	0·9745	—
Kruskal–Wallis		—	—	—	—	—	0·0266
Chimaeras in series XL, XJ[b], XM and XN grouped by presence of transgene (Tg)							
Tg absent	81	322·2 ± 6·3	101·3 ± 2·5	90·2 ± 1·6	1·12 ± 0·02	9·14 ± 0·10	7·50 ± 0·50
Tg present	36	320·6 ± 6·7	98·8 ± 2·9	91·0 ± 2·5	1·11 ± 0·04	9·29 ± 0·09	7·25 ± 0·25
P-values from statistical tests for variation between chimaeras with and without the transgene							
t-test		0·8745	0·5567	0·7775	0·6590	0·3290	—
U-test		—	—	—	—	—	0·2843

[a] The physical parameters are summarized as mean ± S.E. except for hind limb morphology which is given as median ± median absolute deviation.
[b] For comparisons between the chimaeras with and without the transgene, the six XJ chimaeras of unknown transgenic status were excluded.
ANOVA = one-way factorial analysis of variance; Kruskal–Wallis = non-parametric Kruskal–Wallis test; *t*-test = unpaired Student's *t*-test; *U*-test = non-parametric Mann–Whitney *U*-test.

Table 10. *Viability of different series of chimaeras at 12½ d*

	Number (%)			
	Series with the transgene		Series without the transgene	
	XL	XJ	XM	XN
Aggregated embryos transferred[a]	128	97	138	140
Females with implantations	17	13	14	31
Total number of implantations	67 (52%)	44 (45%)	63 (46%)	71 (51%)
Resorbing moles	23	9	22	30
Normal foetuses	44 (66%)*	35 (80%)*	41 (65%)*	41 (58%)*

[a] Excluding those transferred to females with no implantation sites.
* Expressed as a percentage of total implantations.

in any of the physical characteristics between 81 $-/-\leftrightarrow-/-$ and 36 $Tg/-\leftrightarrow-/-$ chimaeras in the other four series. This shows that the presence of $Tg/-$ cells had no effect on the size or gross morphology of the chimaeric conceptus.

(vi) *The survival of chimaeric conceptuses was unaffected by the presence of* Tg/− *cells*

Comparisons of preimplantation and post-implantation survival of chimaeras in four different series (Table 10) revealed no significant differences between series with $Tg/-$ cells and those without the transgene.

4. Discussion

Analysis of three series of $Tg/-\leftrightarrow-/-$ foetal chimaeras and comparison with two previous series of $-/-\leftrightarrow-/-$ chimaeras revealed no significant effect of the presence of hemizygous ($Tg/-$) transgenic cells on the overall composition, size or gross morphology of the chimaeric foetuses, placentas or extraembryonic membranes. Also, a previously described maternal genetic effect, of the strain combination, on the composition of chimaeric tissues occurred in the presence or absence of transgene. These tests have demonstrated that hemizygous $Tg/-$ cells are not at a selective disadvantage, when incorporated into

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mouse aggregation chimaeras with non-transgenic (−/−) cells. However, they do not rule out the possibility that hemizygous *Tg*/− cells may influence other developmental processes, such as cell mixing, that were not tested. Thus, our quantitative analyses imply that transgene TgN(Hbb-b1)83Clo is selectively neutral (Oster-Granite *et al.* 1981) in the hemizygous state. It provides a good lineage marker for quantitative studies because it is cell-localized, cell-autonomous, stable, present in all nucleated cells, easy to detect (although not usually detected in 100% of cells in histological sections), genetically polymorphic, and selectively neutral but it is not yet known whether it is completely developmentally neutral (West, 1984). Further studies are also needed to test whether homozygous *Tg*/*Tg* cells are selectively neutral like the hemizygous *Tg*/− cells studied here.

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