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The extended survival of t^{w5}/t^{w5} mouse embryo cells *in vitro*

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Summary

The t^{w5} haplotype is a recessive mutation which is lethal when homozygous in mouse embryos following implantation. This series of studies was undertaken to determine the effect of the t^{w5}/t^{w5} genotype on embryos developing *in vitro*. Blastocyst embryos from $+/t^{w5}$ *inter se* matings were compared with control blastocysts obtained from matings between $T/+$ and $+/+$ females and $+/t^{w5}$ males for their abilities to continue development *in vitro* in two culture media. The data show that there are no significant differences between the percentages of experimental and control blastocyst embryos which attach and outgrow or which contain inner cell masses on any day of culture up to equivalent gestation day 21 in either media. These findings show that the life span of cells from t^{w5}/t^{w5} embryos can be extended significantly by *in vitro* culture.

1. Introduction

Embryos homozygous for the t^{w5} haplotype die between gestation days 6 and 11 *in vivo* (Bennett & Dunn, 1958). Wudl & Sherman (1976) found that the t^{w5}/t^{w5} embryos are arrested in development on equivalent gestation days (e.g.d.) *in vitro*. The observed numbers of embryo outgrowths on e.g.d. 11 to 14 fit the numbers expected if the t^{w5}/t^{w5} genotype were lethal *in vitro* between e.g.d. 6 and 11. Hogan *et al.* (1980) reported between 30 and 50% of the inner cell mass cells (ICMs) immunosurgically removed from blastocyst embryos obtained from $+/t^{w5}$ *inter se* matings to be morphologically abnormal after seven days in culture. These abnormal ICMs were presumed to be homozygous for the t^{w5} haplotype. Although the stage(s) of death of the ICMs is not reported, the endodermal cells of some of these presumed t^{w5}/t^{w5} ICMs attached to the culture dish and outgrew into flat sheets of cells which ceased to grow after a few additional days in culture. Wudl & Sherman (1976) also found that t^{w5}/t^{w5} embryos could not be rescued when combined with wild-type embryos to produce chimeric blastocyst embryos which were allowed to continue development *in vitro*. All of the above studies suggest that cells from t^{w5}/t^{w5} blastocyst embryos do not survive *in vitro* beyond their normal lethal period *in vivo*.

Previous studies showed that embryos homozygous for two other lethal phenotypes, t^{12} and t^{6}, die *in vitro* at gestational stages comparable to their respective lethal stages *in vivo* (Hillman *et al.* 1970; Nadijcka & Hillman, 1975). These lethal phenotypic expressions have been used to determine the effects of various types of insemination on the transmission frequencies of the respective haplotypes (McGrath & Hillman, 1980*a*, *b*).

Recently, we attempted to utilize the reported *in vitro* lethality for t^{w5}/t^{w5} embryos as a means of determining the transmission frequencies of the t^{w5} haplotype following normal and delayed matings and *in vitro* fertilization. The data presented here show that the numbers of viable outgrowths with ICMs developing *in vitro* from the blastocyst embryos obtained from $+/t^{w5}$ *inter se* matings, and expected to include t^{w5}/t^{w5} embryos, do not differ from those found developing from control blastocysts up to e.g.d. 21. Therefore, *in vitro* embryonic death cannot be used to determine the transmission frequency of this haplotype. More importantly, however, the data show that the life span of cells from t^{w5}/t^{w5} embryos can be extended *in vitro* beyond their lethal period *in vivo*.

2. Materials and methods

BALB/c females and T/t^{w5} males were mated to obtain normal tailed $+/t^{w5}$ and short tailed $T/+$ offspring. The $+/t^{w5}$ animals were mated *inter se* to obtain the experimental embryos ($+/+$, $+/t^{w5}$, t^{w5}/t^{w5}), and the $+/t^{w5}$ males were mated with their

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$T/+$ siblings and with BALB/c ($+/+$) females to obtain the control embryos. The first control mating yields $+/+$, $+/t^{w5}$, T/t^{w5} and $T/+$ embryos and the second, $+/+$ and $+/t^{w5}$ embryos. Only the t^{w5}/t^{w5} genotype from the experimental matings is lethal to the embryo *in vivo* (Bennett & Dunn, 1958). In our colony, the transmission frequency of the t^{w5} haplotype from $+/t^{w5}$ males is 0·95 (1770 offspring). This transmission ratio distortion is not significanty different from that of the t^{w5} haplotype determined by karyotyping embryos developing from zygotes obtained from normal and delayed matings and from *in vitro* fertilization (Garside & Hillman, 1989).

The experimental and control females were time ovulated (5 IU pregnant mare serum gonadotropin followed 48 h later with 5 IU human chorionic gonadotropin). Two-cell embryos were recovered from each female and each group placed separately into a 0·2 ml drop of Standard Egg Culture Medium (Biggers *et al.* 1971) under silicone oil contained in 10 × 35 mm tissue culture dishes (Falcon Plastics). The dishes were then placed into a 37 °C continuous gas flow incubator (5% CO_2 in air) and the embryos allowed to develop to the blastocyst stage. After hatching, the experimental and control blastocyst embryos were each divided into two groups. One group was transferred to culture dishes containing supplemented NCTC-109 (M.A. Bioproducts; Wudl & Sherman, 1976) and the other to modified Eagle's medium (Spindle & Pedersen, 1973). Following transfer and attachment, each embryo was scored for the presence of outgrowing trophoblast cells (hereafter referred to as outgrowths) and ICMs each subsequent day up to e.g.d. 21 using a Zeiss inverted microscope equipped with phase optics. An outgrowth was considered to be viable if it exhibited the normal series of morphological changes associated with development *in vitro* [e.g. attaching and spreading as a sheet of cells, continuing to enlarge through cell division, undergoing endoreduplication resulting in the formation of giant cells which reorganize to form a network (Sobel *et al.* 1980; Nadijcka *et al.* 1981)]. ICM cells were scored only for their presence or absence. An outgrowth or ICM was considered to be dead when, presumably through cell death and dispersal, it was reduced to only a few cells (Nadijcka & Hillman, 1975; Wudl & Sherman, 1976).

Significant differences between the percentages of outgrowths and the percentages of outgrowths with attached ICM cells from experimental and control crosses in each culture medium and between the two culture media were determined by Student's t test using arcsine transformed data. Significance was set at $P < 0{\cdot}05$.

3. Results

On the basis of the distorted transmission ratio of the t^{w5} haplotype, 0·95, the expected genotypic distribution

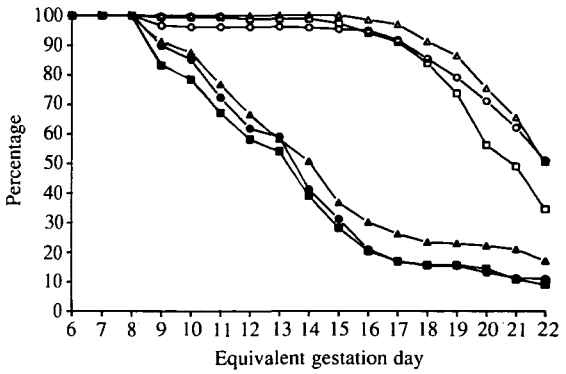

Fig. 1. Percentrage of viable outgrowths and of viable outgrowths with ICMs in NCTC-109. Outgrowths: ○, $+/t^{w5} \times +/t^{w5}$; △, $T/+ \times +/t^{w5}$; □, $+/+ \times +/t^{w5}$. Outgrowths with ICMs: ●, $+/t^{w5} \times +/t^{w5}$; ▲, $T/+ \times +/t^{w5}$; ■, $+/+ \times +/t^{w5}$.

of embryos from $+/t^{w5}$ *inter se* matings would be: 2·5% $+/+$; 50% $+/t^{w5}$; and 47·5% t^{w5}/t^{w5}. Based on the findings of Wudl and Sherman (1976), and on the transmission ratio of the t^{w5} haplotype, no more than 52·5% of the blastocyst embryos placed into NCTC-109 medium should be viable on e.g.d. 14. Moreover, the percentage of viable embryos might be even lower depending upon the level of background death. The data of the present studies show, however, that 95·8% (366/382 embryos) of the outgrowths from the experimental crosses are viable on e.g.d. 14 in this medium (Fig. 1). This percentage is the same as the percentage of viable outgrowths from $+/+ \times +/t^{w5}$ matings (98·8%; 165/167) and from $T/+ \times +/t^{w5}$ matings (100%; 257/257) on e.g.d. 14. Also, there are no significant differences among the percentages of outgrowths with attached ICMs from experimental and control blastocysts on e.g.d. 14 in this medium: experimental crosses (41·1%; 157/382 embryos); $+/+ \times +/t^{w5}$ control matings (38·9%; 65/167 embryos); and $T/+ \times +/t^{w5}$ control matings (50·6%; 130/257 embryos).

Similarly, there are no significant differences among the percentages of viable outgrowths from any of the matings on e.g.d. 14 in modified Eagle's medium (MEM) (Fig. 2). The data show that 93·1% (311/334) of the outgrowths originating from $+/t^{w5}$ *inter se* matings, 89·6% (172/192) of those from the $T/+ \times +/t^{w5}$ matings and 94·2% (114/121) of those from the $+/+ \times +/t^{w5}$ matings are viable on this day of culture. On e.g.d. 14 the percentage of outgrowths with ICM cells from $+/t^{w5}$ *inter se* matings is 56% (187/334 embryos), from $T/+ \times +/t^{w5}$ matings, 58·9% (113/192 embryos) and from $+/+ \times +/t^{w5}$ matings, 60·3% (73/121 embryos). There are no significant differences among these percentages.

The data show further that there are no significant differences between the percentages of experimental outgrowths with or without ICM cells when compared with their counterparts from either of the control crosses on any equivalent day of gestation in either

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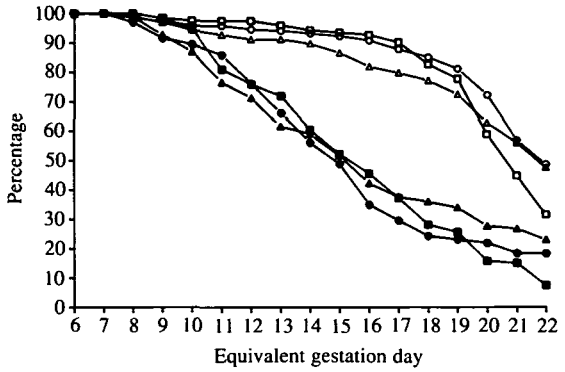

Fig. 2. Percentage of viable outgrowths and of viable outgrowths with ICMs in MEM. Outgrowths: ○, $+/t^{w5} \times +/t^{w5}$; △, $T/+ \times +/t^{w5}$; □, $+/+ \times +/t^{w5}$ Outgrowths with ICMs: ●, $+/t^{w5} \times +/t^{w5}$; ▲, $T/+ \times +/t^{w5}$; ■, $+/+ \times +/t^{w5}$.

culture medium (Figs 1, 2). A comparison of the two control groups shows that the only significant difference is on e.g.d. 16 when the percentage of viable outgrowths from $T/+ \times +/t^{w5}$ crosses (98·4%; 253/257) is significantly higher than that from $+/+ \times +/t^{w5}$ crosses (94·0%; 157/167) in NCTC-109 ($0{\cdot}01 > P > 0{\cdot}005$) (Fig. 1). In MEM, a significantly higher percentage of outgrowths are viable from $+/+ \times +/t^{w5}$ crosses (97·5%, 118/121) than from $T/+ \times +/t^{w5}$ crosses (91·1%, 175/192) on e.g.d. 12 ($0{\cdot}05 > P > 0{\cdot}02$) (Fig. 2). There are no differences between the percentages of the two groups of control outgrowths with ICMs developing in NCTC-109 or between those developing in MEM.

A comparison of the viability of the outgrowths in the two media (cf. Figs. 1, 2) shows that a significantly higher percentage of experimental outgrowths have ICMs in NCTC-109 (100%; 382/382) than do their counterparts in MEM (97·0%; 324/334) on e.g.d. 8 ($0{\cdot}05 > P > 0{\cdot}02$). Otherwise, there are no differences between the experimental outgrowths in the two media. There are also some significant differences between the percentages of viable outgrowths and outgrowths with ICMs from the control crosses in the two media (cf. Figs. 1 and 2). Between e.g.d. 10 and 17, the percentages of viable outgrowths from $T/+ \times +/t^{w5}$ crosses are significantly higher in NCTC-109 than in MEM. From e.g.d. 10 to 15, the percentage of viable outgrowths in NCTC-109 remains at 100% (257/257), whereas in MEM the percentage decreases from 94·3% (181/192) on e.g.d. 10 ($0{\cdot}05 > P > 0{\cdot}02$) to 86·5% (166/192) on e.g.d. 15 ($P < 0{\cdot}001$). On e.g.d. 16, 98·4% (253/257) of the outgrowths from these crosses are viable in NCTC-109 whereas only 81·8% (157/192) are viable in MEM ($0{\cdot}002 > P > 0{\cdot}001$). Although the percentage of viable outgrowths in NCTC-109 from the $T/+ \times +/t^{w5}$ crosses drops to 96·1% (247/257) on e.g.d. 17, this percentage is still significantly higher than the percentage of their viable counterparts in MEM [79·7% (153/192); $0{\cdot}02 > P > 0{\cdot}01$]. The percentage of viable outgrowths from $+/+ \times +/t^{w5}$ crosses is significantly higher in NCTC-109 than in MEM on e.g.d. 13 (98.8%, 165/167 *vs.* 95·9%, 116/121; $0{\cdot}05 > P > 0{\cdot}02$) and on e.g.d. 14 (98·8%, 165/167 *vs.* 94·2%, 114/121; $0{\cdot}02 > P > 0{\cdot}01$). On e.g.d. 16, the percentage of viable outgrowths with ICMs is significantly higher ($0{\cdot}02 > P > 0{\cdot}01$) among the embryos developing in MEM (45·4%, 55/121) than among those developing in NCTC-109 (20·3%, 34/167). Otherwise there are no significant differences between the control outgrowths or outgrowths with ICMs in the two media.

4. Discussion

The current studies show that homozygosity for the t^{w5} haplotype which results in prenatal lethality between gestation days 6 to 11 is not lethal when embryos with this genotype are maintained *in vitro* from early developmental stages to e.g.d. 21. These observations contrast with those of Wudl & Sherman (1976) who reported that t^{w5}/t^{w5} embryos die *in vitro* before e.g.d. 14, i.e. a time equivalent to their *in vivo* lethal period. In their experiments, Wudl and Sherman noted that the observed percentage of dead embryos from $+/t^{w5}$ *inter se* matings was not significantly different from that expected on the basis of the transmission frequency of the t^{w5} haplotype. From these data they concluded the t^{w5} haplotype when homozygous to be a generalized cell lethal and that all cells of the embryo expressed the lethal phenotype at a specific stage or time; furthermore, neither embryos nor embryonic cells with this genotype could be rescued by *in vitro* culture. The discrepancy between the current results and those of Wudl & Sherman (1976) who also used NCTC-109 as the culture medium for embryos from $+/t^{w5}$ *inter se* matings is not easily explained. Differences in the genetic background of the t^{w5}-bearing animals used in the two studies might account for some of the observed differences. On the other hand, two separate studies, Magnuson *et al.* (1982) and Martin *et al.* (1987), succeeded in establishing pluripotent stem cell lines from the inner cell mass of blastocyst embryos that were, based on karyotypic and gene marker analyses, homozygous for the t^{w5} haplotype. They concluded that the t^{w5}/t^{w5} genotype does not cause generalized cell lethality and that cells from such embryos can be rescued. The data from the present study support the latter conclusion and clearly show that cells from homozygous t^{w5} embryos originating from *in vivo* matings survive *in vitro* beyond their *in vivo* lethal developmental period.

The rescue of cells from embryos homozygous for other lethal *t* haplotypes by explanting them to a different environment before developmental arrest has been reported by several laboratories. Artzt & Bennett (1972) found that t^{w18}/t^{w18} embryos, which normally die around gestation day nine, could be rescued by

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transplanting them into the testes of mice where they develop into tumours. The observations of Wudl *et al.* (1977) support these results. The latter investigators also reported that t^{w18}/t^{w18} embryos remained viable under *in vitro* conditions. On e.g.d. 22, the percentage of viable outgrowths originating from the experimental embryos ($+/t^{w18} \times +/t^{w18}$) was the same as that originating from embryos from control matings ($+/+ \times +/t^{w18}$). Axelrod *et al.* (1981) found that explanted t^{w12}/t^{w12} embryos, which have a protracted lethal period ranging between gestation days nine and 20, can also be rescued if they are explanted to mouse testes. Moreover, cells obtained from the tumours developing from these explanted embryos have been successfully maintained *in vitro*.

Collectively, the results from the studies by Magnuson *et al.* (1982), Martin *et al.* (1987), Artzt & Bennett (1972), Wudl *et al.* (1977), Axelrod *et al.* (1981) and those from the present studies show that individual cells from embryos homozygous for some *t* haplotypes can survive past their *in vivo* lethal period if they are placed into specific culture media or into appropriate ectopic sites. The fact that cells from homozygous lethal embryos can be rescued is not surprising since the results of light and electron microscopic studies of t^{12}/t^{12}, t^{w32}/t^{w32} and t^6/t^6 embryos show that embryos which are developmentally arrested as a result of their lethal genotype contain both pycnotic and healthy cells, many of which are undergoing mitoses (Hillman *et al.* 1970; Hillman & Hillman, 1975; Nadijcka & Hillman, 1975). Thus not all cells of the embryo die simultaneously. Initial cell death of t/t embryos occurs either at random in cleavage staged embryos (Hillman *et al.* 1970; Hillman, 1972; Hillman & Hillman, 1975) or in specific cell types in later staged embryos following implantation and cellular differentiation (Artzt & Bennett, 1972; Bennett, 1975; Nadijcka & Hillman, 1975; Axelrod *et al.* 1981). The exact stage of *in vivo* embryonic death can be related to the time of onset of cell death, to the proportion and relative position of necrotic cells to healthy cells (Hillman, 1975) and to the type(s) of cells which express the mutant phenotype (e.g. overgrowth of the primitive streak; Artzt & Bennett, 1972). *In utero*, those embryos which contain necrotic cells or are phenotypically aberrant stop development and ultimately all cells become necrotic. Thus, *in utero*, the t/t genotype interferes with the developmental sequence, ultimately producing a cascade of effects resulting in embryo death.

In spite of the fact that those t/t embryos (t^{12}; t^{w32}) which die during the preimplanation stages and those that die at implantation (e.g. t^6/t^6) contain both normal and necrotic cells at the time of embryo developmental arrest, there have been no reports showing that the embryos or cells from these embryos can be rescued either by allowing them to develop *in vitro*, by combining them with wild-type embryos to produce chimeric embryos, or by transplanting them to ectopic sites (Mintz, 1964; Hillman, 1975; Wudl *et al.* 1977). Only cells from t/t embryos which die at later stages of gestation have been rescued. However, since the *t*-lethal mutations are not allelic (Artzt *et al.* 1982; Shin *et al.* 1983) it is highly unlikely that the cause of lethality is the same for all t/t embryos and, consequently, it is not reasonable to expect that cells from all t/t embryos can be rescued.

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References

Artzt, K. & Bennett, D. (1972). A genetically caused embryonal ectodermal tumor in the mouse. *Journal of the National Cancer Institute* **48**, 141–158.

Artzt, K., McCormick, P. & Bennett, D. (1982). Gene mapping within the *T/t*-complex of the mouse. I. *t*-lethal genes are nonallelic. *Cell* **28**, 463–470.

Axelrod, H. R., Artzt, K. & Bennett, D. (1981). Rescue of embryonic cells homozygous for a lethal haplotype of the *T/t* complex: t^{w12}. *Developmental Biology* **86**, 419–425.

Bennett, D. (1975). The *T*-locus of the mouse. *Cell* **6**, 441–445.

Bennett, D. & Dunn, L. C. (1958). Effects on embryonic development of a group of genetically similar lethal alleles derived from different populations of wild house mice. *Journal of Morphology* **103**, 135–157.

Biggers, J. D., Whitten, W. K. & Whittingham, D. G. (1971). The culture of mouse embryos *in vitro*. In *Methods in Mammalian Embryology* (ed. J. E. Daniel, Jr.), pp. 86–116. San Francisco: W. H. Freeman.

Garside, W. & Hillman, N. (1989). The *in vivo* and *in vitro* transmission frequencies of the t^{w5} haplotype in mice. *Genetical Research* **53**, 21–24.

Hillman, N. (1972). Autoradiographic studies of t^{12}/t^{12} mouse embryos. *The American Journal of Anatomy* **134**, 411–424.

Hillman, N. (1975). Studies of the *T*-locus. In *The Early Development of Mammals* (ed. M. Balls and A. E. Wild), pp. 189–206. London: Cambridge University Press.

Hillman, N. & Hillman, R. (1975). Ultrastructural studies of t^{w32}/t^{w32} mouse embryos. *Journal of Embryology and Experimental Morphology* **33**, 685–695.

Hillman, N., Hillman, R. & Wileman, G. (1970). Ultrastructural studies of cleavage stage t^{12}/t^{12} mouse embryos. *The American Journal of Anatomy* **128**, 311–339.

Hogan, B., Spiegelman, M. & Bennett, D. (1980). *In vitro* development of inner cell masses isolated from t^0/t^0 and t^{w5}/t^{w5} mouse embryos. *Journal of Embryology and Experimental Morphology* **60**, 419–428.

McGrath, J. & Hillman, N. (1980*a*). The *in vitro* transmission frequency of the t^6 allele. *Nature* (*London*) **283**, 479–481.

McGrath, J. & Hillman, N. (1980*b*). The *in vitro* transmission frequency of the t^{12} mutation in the mouse. *Journal of Embryology and Experimental Morphology* **60**, 141–151.

Magnuson, T., Epstein, C. J., Silver, L. M. & Martin, G. R. (1982). Pluripotent embryonic stem cell lines can be derived from t^{w5}/t^{w5} blastocysts. *Nature* (*London*) **298**, 750–753.

Martin, G. R., Silver, L. M., Fox, H. S. & Joyner, A. L. (1987). Establishment of embryonic stem cell line from preimplantation mouse embryos homozygous for lethal mutations in the *t*-complex. *Developmental Biology* **121**, 20–28.

Mintz, B. (1964). Formation of genetically mosaic mouse embryos, and early development of 'lethal (t^{12}/t^{12})-normal' mosaics. *Journal of Experimental Zoology* **157**, 273–292.

Nadijcka, M. & Hillman, N. (1975). Studies of t^6/t^6 mouse embryos. *Journal of Embryology and Experimental Morphology* **33**, 697–713.

Nadijcka, M., Morris, M. & Hillman, N. (1981). The effect of delay on the expression of the t^6/t^6 genotype. *Journal of Embryology and Experimental Morphology* **63**, 267–283.

Shin, H.-S., Bennett, D. & Artzt, K. (1983). Gene mapping within the *T*/*t*-complex of the mouse. IV. The inverted MHC is intermingled with several *t*-lethal genes. *Cell* **39**, 573–578.

Sobel, J. S., Cooke, R. & Pedersen, R. A. (1980). Distribution of actin and myosin in mouse trophoblast: correlation with changes in invasiveness during development *in vitro*. *Developmental Biology* **78**, 365–379.

Spindle, A. I. & Pedersen, R. A. (1973). Hatching, attachment and outgrowth of mouse blastocysts *in vitro*: fixed nitrogen requirements. *Journal of Experimental Zoology* **186**, 305–318.

Wudl, L. R. & Sherman, M. I. (1976). *In vitro* studies of mouse embryos bearing mutations at the *T* locus: t^{w5} and t^{12}. *Cell* **9**, 523–531.

Wudl, L. R., Sherman, M. I. & Hillman, N. (1977). Nature of lethality of *t*-mutations in embryos. *Nature* (*London*) **270**, 137–140.