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A novel retrovirally induced embryonic lethal mutation in the mouse: assessment of the developmental fate of embryonic stem cells homozygous for the 413.d proviral integration

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Summary

A genetic screen of transgenic mouse strains, carrying multiple copies of an MPSV*neo* retroviral vector, has led to the identification of a recessive embryonic lethal mutation, termed 413.d. This mutation is associated with a single proviral insertion and when homozygous, results in the failure of the early postimplantation embryo at the gastrulation stage of development. Embryonic stem cell lines (ES cells) were derived from 413.d intercross embryos. Genotyping, with respect to the 413.d integration site, identified wild-type, heterozygous and homozygous ES cell lines. The differentiation abilities and developmental potential of the ES cell lines were assessed using a number of *in vitro* and *in vivo* assays. Results indicate that the ES cell lines, regardless of genotype, are pluripotent and can give rise to tissue and cell types derived from all three germ layers. Furthermore, analysis of midgestation conceptuses (10.5 *p.c.*) and adult chimeras generated by injecting mutant ES cells into host blastocysts, provides strong evidence that the mutant cells can contribute to all extraembryonic tissues and somatic tissues, as well as to functional germ cells. These results indicate that the homozygous mutant cells can be effectively 'rescued' by the presence of wild-type cells in a carrier embryo.

Key words: mouse development, homozygous lethal, ES cells, chimeras.

Introduction

A large number of developmental mutations have been documented in the mouse (reviewed Lyon and Searle, 1989). The majority of these either occurred spontaneously or were obtained following exposure of animals to mutagens. With a few notable exceptions (for example Herrmann *et al.* 1990), this class of mutation has proven extremely difficult to characterize at the molecular level. More recently, the introduction of exogenous DNA into the mouse germ line has facilitated both the identification and molecular analysis of genes that play a role in embryogenesis. A number of routes have been used to generate insertional mutations including microinjection of DNA into fertilized eggs, and retroviral infection of embryos and embryonic stem (ES) cells (reviewed Gridley *et al.* 1987; Jaenisch, 1988). As introduced DNA sequences appear to integrate largely at random, this approach can potentially result in the isolation of a wide variety of novel genes required for completion of embryonic development. Indeed, a number of insertional mutations that perturb embryonic development have been identified in transgenic animals and, in some instances, complementary molecular studies have allowed the identification and characterization of the affected genes (Jaenisch *et al.* 1983; Schnieke *et al.* 1983; Woychik *et al.* 1985; Soriano *et al.* 1987; Maas *et al.* 1990; Woychik *et al.* 1990).

In a previous study, we used embryonic stem cells (ES cells) carrying multiple copies of a replication defective retroviral vector to generate germ line chimeras (Robertson *et al.* 1986). The rationale for this approach was twofold. First, as the mutational frequency of retroviruses is estimated to be approximately 5% (Soriano *et al.* 1987; Stoye *et al.* 1988; Spence *et al.* 1989), we wished to improve the efficiency of recovering these potentially rare events by screening multiple proviral integration sites within a small number of transgenic strains. Second, as previous reports have indicated that integration of a provirus has little consequence on the structure of the flanking DNA regions (reviewed Panganiban, 1985), insertional mutations generated in these animals would be relatively straightforward to characterize. Following genetic screening, a number of mutations have been identified in these animals (M. J. Evans, M. R. Kuehn, A.

Bradley, E.J.R. unpublished results). We have been studying one recessive lethal mutation, termed 413.d, which cosegregates with a single proviral insertion site. Here we show that this mutation results in embryonic failure shortly after implantation. The onset of developmental arrest appears to be coincident with the gastrulation stage of development.

Several possible explanations to account for the failure of early postimplantation stage embryos were considered. Lethality could result from the disruption of a gene required by all cells, or a gene essential for the embryo as a whole (e.g. perhaps reflecting metabolic changes at the time of implantation). Alternatively, developmental arrest could also reflect the inactivation of a gene necessary for the formation of specific tissues and/or correct positioning of subsets of cells. On the basis of morphological evidence alone, it is difficult to distinguish between these possibilities. The approach that we have taken to address these issues is to derive and analyze ES cells from homozygous embryos. ES cells are permanent tissue culture lines of pluripotential stem cells derived from blastocyst outgrowths (Evans and Kaufman, 1981; Martin, 1981). These cells can be induced to differentiate extensively in culture (Doetschman *et al.* 1985). In addition, our previous studies have shown that ES cells closely resemble early inner cell mass cells in their developmental potential as they routinely contribute to the extraembryonic, fetal and germ cell lineages (Bradley *et al.* 1984; Robertson and Bradley, 1986; Beddington and Robertson, 1989). Thus, an examination of the fates of ES cells carrying recessive lethal mutations may provide insight into the developmental role of the normal wild-type allele.

To characterize further the 413.d mutation, a panel of ES cell lines were obtained from embryos recovered from matings between heterozygous animals. When the lines were individually genotyped using DNA probes specific for the integration site, ES cell lines homozygous for the 413.d mutation were identified. This paper describes the differentiation abilities of these cell lines in a variety of *in vitro* and *in vivo* assays. Our analysis has shown that, while homozygous embryos are unable to complete embryogenesis, ES cells derived from them differentiate into a wide spectrum of cell and tissue types in multiple contexts. Indeed, their differentiation characteristics were found to be identical to those displayed by wild-type ES cells. The 413.d ES cell lines were shown to participate in the normal development of the embryo and, in chimeric adults, to contribute to all tissues and cell types including the functional germ line. These data imply that the mutation does not act in a cell autonomous fashion but rather that ES cells carrying the 413.d mutation can be rescued by the wild-type environment.

Materials and methods

Embryo histology

Embryos were dissected from the uterine horns of naturally mated females at 7.5 days or 8.5 days of development (the day of finding the vaginal plug was designated day 0.5). The embryos were retrieved within intact decidua, washed in phosphate-buffered saline (PBS) and fixed in Bouin's solution for 6–8 h. After this time, they were transferred to 70% alcohol, followed by dehydration and embedding in paraffin wax. 6 μm sections were collected and stained with hematoxylin and eosin.

Isolation and culture of ES cell lines

Embryos were recovered from matings between mice of the 129/Sv inbred strain that were heterozygous for the 413.d proviral insertion. ES cell lines were isolated from single, nondelayed or implantationally delayed, blastocysts according to previous protocols (Evans and Kaufman, 1981; Robertson, 1987). Briefly, normal or implantationally delayed blastocyst-stage embryos were explanted onto STO feeder layers. The resulting outgrowths were cultured for 4 to 5 days, and then dissociated onto fresh feeder layers. Subsequently, ES cell colonies were visually identified and expanded into individual cell lines. All cell lines were routinely maintained according to the protocol of Robertson (1987). Genotyping with respect to the 413.d locus and sex chromosome complement was accomplished by Southern blot analysis using appropriate probes (see below). Karyotypic analysis was undertaken between the 8th and 11th passage using the protocol described in Robertson (1987).

Derivation of the pSS1/1 probe

A genomic library was constructed using DNA obtained from mice heterozygous for the 413.d MPSVmos^{-1}neo integration. DNA was partially digested with *Mbo*I, size selected and cloned into λGEM-11 (Promega). The library was then screened using a 400 bp *Nhe*I–*Sac*I restriction fragment derived from the retroviral LTR. Three independent clones were isolated, two of which contained the 3′ end of the retrovirus and about 15 kb of flanking genomic sequence (designated λKH 1/1 and λKH 4/1). The third clone contained the 5′ end of the retrovirus and approximately 14 kb of 'upstream' flanking genomic sequence (designated λKH 1/2). *Sac*I restriction fragments of the latter were subcloned into pBluescript KS(+). The clone containing sequences from the 5′ LTR and approximately 4.5 kb of the flanking genomic DNA (pAB106) was analyzed to identify non-repetitive regions. A 800 bp *Sph*I fragment found to contain only single copy sequences was subcloned into pGEM 3Zf(−). This probe, designated pSS1/1, hybridizes to a 7 kb *Bam*HI fragment representing the uninterrupted wild-type locus and a 9.5 kb *Bam*HI fragment representing the 413.d proviral integration (see Fig. 2).

Southern blot analysis

Genotyping of mice and cell lines was carried out using Southern Blot analysis. High molecular weight DNA was prepared from tail tissue biopsies or tissue culture cells using standard procedures. Briefly, DNA samples were digested to completion with *Bam*HI, fractionated on 0.8% agarose gels, blotted onto Gene Screen Plus (Dupont), and hybridized. The 800 bp *Sph*1 fragment was isolated from pSS1/1. The 900 bp *Pst*1 *neo* fragment was from pMCneo (Stratagene). The 720 bp *Eco*RI–*Sal*I fragment was recovered from the pY2 plasmid (Lamar and Palmer, 1984). Fragments were labeled with ^{32}P using a random priming kit (Multiprime, Amersham).

ES cell differentiation

In *vitro* differentiation of ES cells was induced using previously published protocols (Robertson, 1987). Briefly,

each cell line was grown for three days in the absence of a STO feeder layer. The monolayers were washed with phosphate-buffered saline (PBS), lightly trypsinized, and the resulting cell aggregates gently transferred, using a wide-bore pipette, into bacteriological Petri dishes. Simple embryoid bodies were formed by culturing the aggregates for 2–4 days in suspension. Cystic embryoid bodies were generated after an additional 6–10 days in suspension culture. Alternatively, the embryoid bodies were induced to form terminally differentiated cell types by replating onto gelatin-treated tissue culture plates and culturing for an additional 4–5 days. To identify nervous tissue, cultures were stained using a monoclonal antibody against neurofilament protein ($160\times10^3 M_r$ form, Boehringer Mannheim). The cultures were fixed in methanol, incubated in primary antibody, followed by HRP-conjugated goat anti-mouse IgG. HRP activity was visualized by DAB treatment.

To analyze differentiation *in vivo*, ES cells were injected into syngeneic 129/Sv male animals to produce teratocarcinomas. For each line approximately 4×10^6 cells in 0.2 ml PBS were injected subcutaneously into the flank region of two animals. The tumours were removed when they reached approximately 1–2 cm in diameter (generally 6–8 weeks postinjection), fixed in Bouin's solution, and processed for histology using standard procedures. Tumour sections (6 μm) were stained with hematoxylin and eosin.

Generation and analysis of chimeras

Host blastocysts were obtained from natural matings of either C57BL/6 (Charles River) or MF1 (Harlan Sprague-Dawley) animals. Expanded blastocysts were recovered 3.5 days *post coitum* (*p.c.*) and cultured in drops of Dulbecco's Modified Eagle's Medium (DMEM) plus 10% v/v fetal bovine serum, at 37°C in a 6% CO_2 humidified incubator. The cells used for microinjection were maintained in culture for less than 10 passages. Approximately 10–15 cells were injected into each host blastocyst according to the method of Bradley (1987). Following injection blastocysts were transferred, in groups of 5–7, into the uterine horns of 2.5 *p.c.* pseudopregnant (C57BL/6×CBA) F_1 females.

For the analysis of mid-gestation chimeras, conceptuses were recovered at 10.5 *p.c.* (i.e. 7 days post-transfer). Each conceptus was dissected and the following tissues isolated according to the protocol described in Beddington and Robertson (1989): fetus proper, amnion, visceral yolk sac, parietal endoderm and trophoblast giant cells. Tissue samples were rinsed extensively in PBS and stored at −20°C prior to GPI analysis.

To analyze the contribution to mature somatic tissues, selected adult chimeras were injected *i.p.* with 700 USP-K-1 units of heparin (Sigma), terminally anesthetized with a 2.5% solution of Avertin, and exsanguinated by opening the hepatic vein and introducing PBS into the aorta. Samples of individual organs and tissues were collected and stored at −20°C.

To test for germ line transmission of the 413.d provirus, chimeric males were mated to MF1 females. Progeny were screened for the presence of the dominant agouti coat colour. DNA samples obtained from tail biopsies of agouti progeny were analyzed by Southern blotting to confirm the transmission of the 413.d provirus.

Glucose phosphate isomerase assays

Tissue samples were frozen and thawed twice, diluted appropriately and run on Titan III electrophoresis plates (Helena Laboratories) according to the protocol described in Bradley (1987). The host blastocysts were from C57BL/6 matings (homozygous for the Gpi 1^b allele) or outbred MF1 strain mice (carrying various combinations of the Gpi 1^a and 1^b alleles). ES cell lines IMD-16 and IMD-11 were both Gpi 1^a1^c (data not shown). Therefore, ES cell derivatives were detected by the presence of a unique 1^c1^c band on gels and quantitated by comparing the intensity of this band to the other bands present in the same sample.

Results

Identification of a recessive lethal mutation in the 413.d transgenic strain

The generation of germ line chimeras from ES cells infected with the MPSVmos^{-1}neo retroviral vector has been described previously (Robertson *et al.* 1986). Progeny obtained by mating individual germ line chimeric males to females of the 129/Sv inbred strain were genotyped by Southern blot analysis using a *neo* probe specific for the retroviral sequence. Based on the restriction patterns of unique proviral junction fragments, we were able to determine the number of individual proviruses segregating in the germ lines of the founder chimeras. Male 413 proved to have a mosaic germ line composed of derivatives of two different germ cells. One of the germ cell progenitors (type 1) contained 4 proviral integrations while the other (type 2) had a large number (approximately 16). Type 1 derivatives predominated in the functional sperm so that approximately 70% of the progeny obtained from this animal carried a combination of 4 proviruses, designated bands a, b, c and d. F_1 animals, derived from the type 1 germ cells, were individually genotyped. Appropriate intercrosses were then set-up to test the 4 proviruses for homozygosity in the F_2 generation. A large number of offspring (approximately 100) were genotyped (Table 1). The predicted Mendelian inheritance patterns were obtained for viruses 413.a, b and c. By contrast, no live born individuals homozygous for the 413.d provirus were detected, although heterozygous and wild-type progeny were recorded at the expected frequency. Additionally, in many hundreds of progeny genotyped over successive generations, we failed to identify any animals homozygous for the 413.d locus. These data argue that

Table 1. *Identification of a homozygous lethal mutation in pedigrees derived from male 413*

Proviral band tested	Number of progeny genotyped	Wild type	Heterozygous	Homozygous
413.a	27	6 (22%)	12 (45%)	9 (33%)
413.b	42	12 (28.5%)	18 (43%)	12 (28.5%)
413.c	44	7 (16%)	21 (48%)	16 (36%)
413.d	79	26 (33%)	53 (67%)	-

A total of 106 F_2 progeny were genotyped. F_1 parents shared 1 or 2 bands.

the 413.d proviral integration site is associated with a recessive embryonic lethal mutation.

The 413.d mutation is associated with embryonic failure of the early postimplantation stage embryo

To determine when homozygous embryos were dying, matings were set up between animals heterozygous for the 413.d provirus. In the first experiment, six females were killed at 9.5 days *post coitum* (*p.c.*). From a total of 40 decidua dissected, 31 contained morphologically normal somite-stage embryos. The remaining 9 decidua contained abnormal embryos, all of which exhibited similar morphological characteristics on gross inspection. The ectoplacental cone (EPC) region appeared to be present but reduced in size when compared to the presumed wild-type or heterozygous littermates. The remainder of the conceptus, while apparently composed of healthy cells, was retarded in size and highly disorganized. Careful visual inspection of these embryos failed to reveal any recognizable fetal structures. Moreover, a number of the abnormal embryos were already beginning to show signs of degeneration as evidenced by the presence of large numbers of maternal blood cells surrounding the conceptus.

Embryos were subsequently collected at 7.5 days and 8.5 days *p.c.*, fixed and sectioned within the intact decidual swellings. In the litters examined at 7.5 days *p.c.*, all the embryos appeared to be morphologically normal (24/24). However, 24 h later, at 8.5 days of development, we found that 25% of the implantation sites (5/21) contained grossly abnormal embryos (Fig. 1). The most striking feature of the presumed mutant embryos was the absence of a morphologically distinctive visceral yolk sac, allantois or amnion structures, or a definitive fetal portion. The region that would normally be occupied by the early-head-fold-stage embryo appeared to contain randomly disorganized, highly folded multicellular tissue layers inserted into the region of the ectoplacental cone. In contrast, some extraembryonic structures were readily identified. Primary and secondary trophoblast giant cells were present in normal quantities. In some embryos, large numbers of secondary giant cells were visible at the periphery of the EPC (see Fig. 1C). The parietal yolk sac (PYS) was a very distinctive structure in all the abnormal embryos examined (see Fig. 1D). However, in contrast to wild-type embryos, parietal endoderm cells appeared to be clustered on the Reichert's membrane matrix. Thus, it appears that the correct number of parietal endoderm cells form, but that the PYS fails to expand correctly in the absence of a normal, rapidly growing, fetal portion.

Thus, the histological analysis of embryos from heterozygous matings strongly suggests that the 413.d locus is associated with a recessive mutation. Although it was not possible to genotype the abnormal embryos, due to the small amount of tissue available, this unusual and highly distinctive phenotype was consistently recorded at the predicted frequencies in multiple litters examined at 8.5 days of development. The embryos become morphologically disorganized shortly after the eighth day of embryonic development. In comparison with the presumed wild-type and heterozygous embryos, we found no evidence for the formation of an embryonic axis or any recognizable fetal structures such as somites, head folds and beating heart.

Isolation and characterization of ES cell lines

To analyze further the developmental failure of homozygous 413.d embryos, we attempted to isolate ES cell lines derived from 413 intercross embryos. 22 independent stem cell lines were derived using previously described protocols (Evans and Kaufman, 1981; Robertson, 1987). ES cell lines were isolated from both delayed and non-delayed blastocysts with an overall efficiency of 23% (Table 2).

Genotyping of ES cell lines was performed by probing Southern blots with a *neo* probe specific for the MPSV mos^{-1}neo proviral sequence. To ensure accurate genotyping of the cell lines generated, blastocysts were collected from matings set up between animals heterozygous for 413.d (d/+) and animals which, in addition to being heterozygous for 413.d, were also homozygous for either proviral insertion 413.b or insertion 413.c. The previous genetic analysis (Table 1) had indicated that both the 413.b and the 413.c proviral integration sites were genetically silent (i.e. there appears to be no discernible phenotype associated with either integration site). Thus, the assignment of genotype could be determined by comparing the autoradiographic intensity of the 413.d proviral junction fragment to either the 413.b or 413.c proviral junction fragment as a single copy internal control.

Genomic DNA was digested with restriction enzymes and analyzed on Southern blots (see Fig. 2). Of the 19 lines genotyped, 3 were found to be wild type (+/+), 5 were heterozygous (d/+), and 11 lines were homozygous (d/d) with respect to the 413.d locus. We subsequently confirmed these results by hybridizing Southern blots with a single copy probe derived from genomic sequences flanking the insertion site (Fig. 2). This genomic probe, termed pSS1/1, distinguishes the wild-type and the 413.d mutant locus. When hybridized to *Bam*HI-digested DNA, the pSS1/1 probe detects a 7.0 kb fragment in wild-type DNA, whereas a 9.5 kb fragment is detected in the 413.d DNA. Interestingly, the genotype data obtained from the panel of ES cell lines did not conform to the predicted Mendelian ratios. Possible reasons for this are presented in the Discussion.

ES cell lines were also tested for the presence of a Y chromosome by hybridizing Southern blots with a Y-specific probe pY2 (Lamar and Palmar, 1984). Of the

Table 2. *Isolation of stem cell lines from 413.d intercrosses*

	Number of blastocysts	Number of ES cell lines	Efficiency of establishing cell lines
Nondelayed	48	13	27%
Delayed	47	9	19%
Total	95	22	23%

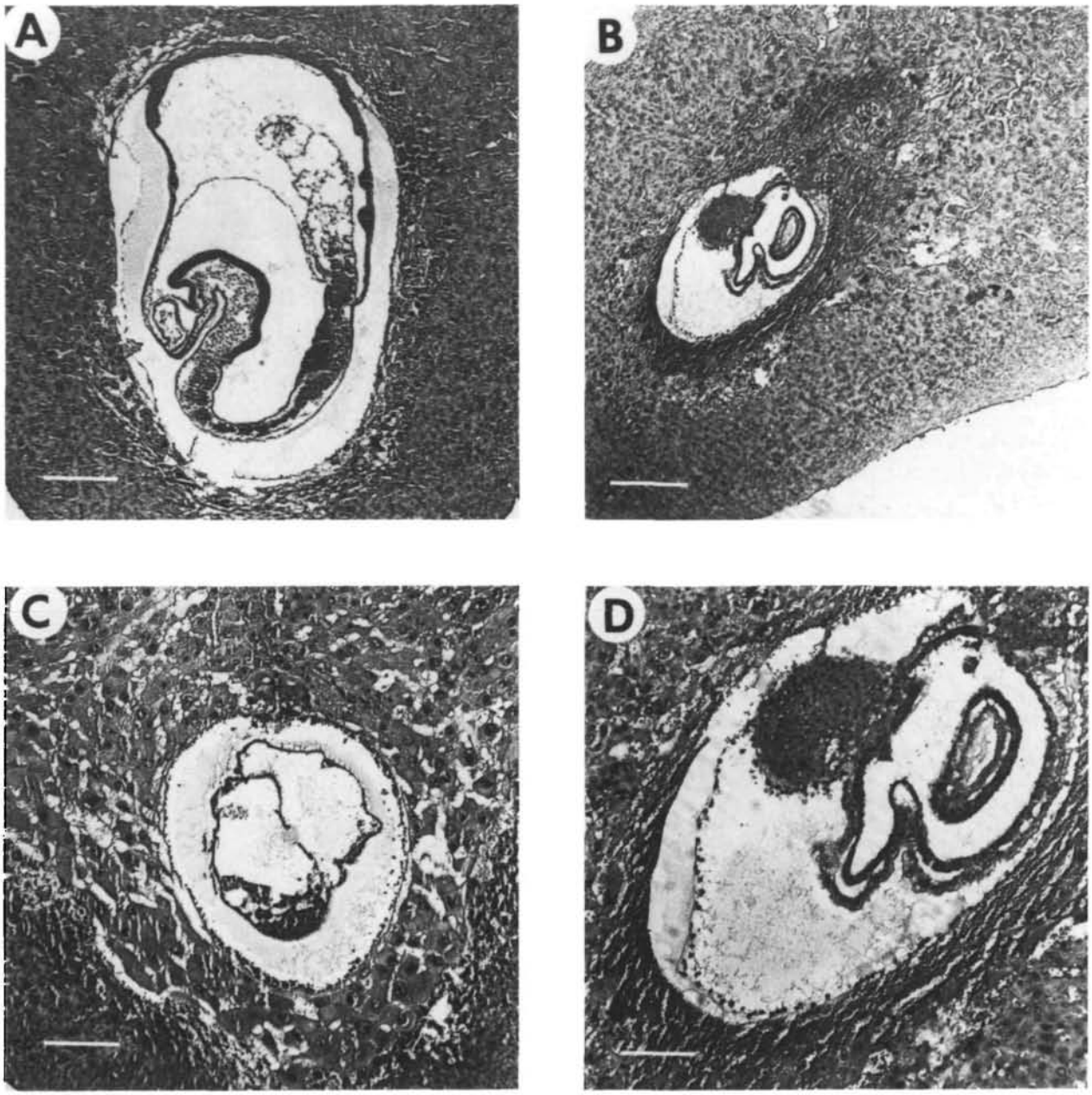


Fig. 1. Histological sections of hematoxylin- and eosin-stained 8.5 day *p.c.* embryos. (A) Sagittal sections through a wild-type 8.5 day *p.c.* embryo. (B,C) Sagittal sections through presumptive homozygous 413.d embryos showing the absence of a definitive fetal portion. (D) Higher magnification view of B showing the presence of several extra embryonic structures, such as parietal endoderm, Reichert's membrane, giant trophoblast giant cells and parietal yolk sac. For sections A, B and C scale bar 250 μm; for D scale bar 50 μm.

19 lines analyzed, 10 lines lacked complementary sequences and were presumably female (or X0). DNA from the remaining 9 ES cell lines hybridized to the pY2 probe (data not shown). For subsequent experiments, we selected one wild-type (IMD-13), one heterozygous (IMD-8), and two homozygous (IMD-11 and IMD-16) ES cell lines, of which three (IMD-8, IMD-11, and IMD-16) tested positive with the Y-specific probe. G-banding analysis of metaphase spreads showed all four cell lines to be diploid (data not shown).

None of the eleven 413.d homozygous ES cell lines were recognizably different, as judged by either cellular morphology or growth characteristics, from their wild-type or heterozygous counterparts. Since ES cell lines homozygous for the 413.d provirus were readily maintained *in vitro*, we conclude that embryonic failure cannot simply result from a general cell lethal mutation.

413.d homozygous ES cell lines differentiate in isolation to form derivatives of all primary germ layers

Previous studies have shown that alteration of growth

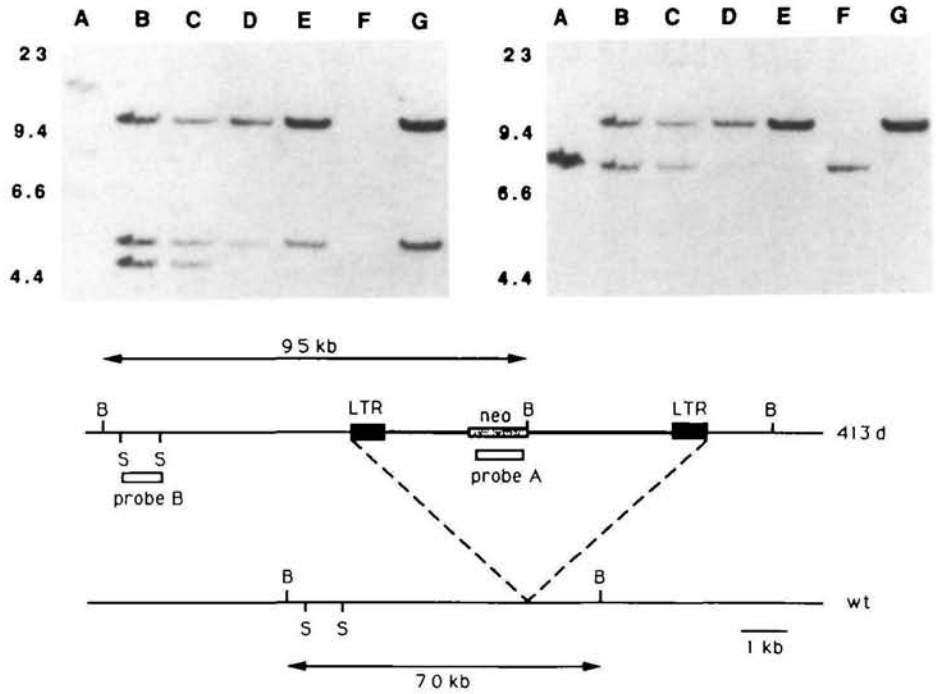


Fig. 2. Genotyping of ES cell lines derived from 413.d intercross embryos. DNA was isolated from cell lines and digested with *Bam*HI. DNA derived from the STO feeder layer is shown in lane A on both blots. *Upper left*, DNA was hybridized to a *neo* specific probe, (probe A on physical map) identifying a 9.4 kb fragment for proviral insertion 413.d, a 4.7 kb fragment for proviral insertion 413.c, and a 4.5 kb fragment for proviral insertion 413.b. *Upper right*, DNA was hybridized with a genomic junction fragment probe, pSS1/1 (probe B on physical map). The pSS1 detects a 7.0 kb fragment in wild-type DNA, lanes A and F, a 9.5 kb fragment in the homozygous 413.d DNA, lanes D, E, and G, and both a 7.0 kb and a 9.5 kb fragment in heterozygous DNA, lanes B and C. (C) Physical map of the 413.d locus. The MPSVneo provirus is oriented 5′ to 3′. The junction fragments identified by the pSS1/1 probe (probe B) in *Bam*HI digested genomic DNA are indicated. B: *Bam*HI, S: *Sph*I.

conditions are sufficient to induce the ES cells to differentiate *in vitro* (Doetschman *et al.* 1985; Robertson, 1987). So-termed embryoid bodies form as a consequence of culturing small aggregates of cells in suspension. The primary event, which occurs within 24–48 h, is the formation of an outer layer of primitive endoderm-like cells surrounding a core of undifferentiated ES cells. These structures are thought to resemble the embryonic ectoderm region of the 5 day *p.c.* conceptus (Martin *et al.* 1977). By culturing the embryoid bodies for an additional 6–10 days in suspension, differentiation progresses to give more complex cystic structures. These display some morphogenetic similarities to the 8.5 day *p.c.* conceptus, such as the formation of blood islands and beating muscle. Alternatively, if allowed to attach to a tissue culture substrate, embryoid bodies give rise to a chaotic array of terminally differentiated cell types.

We initially used this *in vitro* system to characterize the differentiation abilities of homozygous ES cell lines. When grown in suspension culture, the IMD-13 (wild-type), IMD-8 (d/+), IMD-11 (d/d) and IMD-16 (d/d) ES cell lines demonstrated equal abilities to form simple embryoid bodies and endodermal derivatives. After an additional 6 days in culture, all four lines gave rise to cystic embryoid bodies. These structures were composed of fluid-filled cysts and Reichert's membrane-like material as well as two morphologically distinct types of endoderm. An example of a cystic embryoid body formed from the homozygous IMD-16 cell line is shown in Fig. 3A.

In subsequent experiments, simple embryoid bodies were replated onto tissue culture dishes. Several days after attachment, the cultures were scored visually. Cultures of homozygous cells contained a variety of morphologically recognizable cell types, such as beating muscle and fibroblast-like cells. In addition, immunohistochemical staining of differentiated embryoid bodies derived from the IMD-11 cell line, using a monoclonal antibody against neurofilament, revealed the presence of nerve tissue (Fig. 3B). There were no obvious qualitative or quantitative difference in the results obtained with 413.d homozygous cell lines in comparison with those obtained from wild-type or heterozygous ES cell lines.

While *in vitro* analysis is useful for assessing some of the differentiation capacities of ES cells, the spectrum of cell types that form in this assay is limited. To obtain a more extensive collection of terminally differentiated cell types, each of the four ES cell lines were injected subcutaneously into syngeneic 129/Sv hosts to generate teratocarcinoma tumours. All of the cell lines gave rise to solid tumours within 6 to 8 weeks postinjection. For each line, two independent tumours were examined

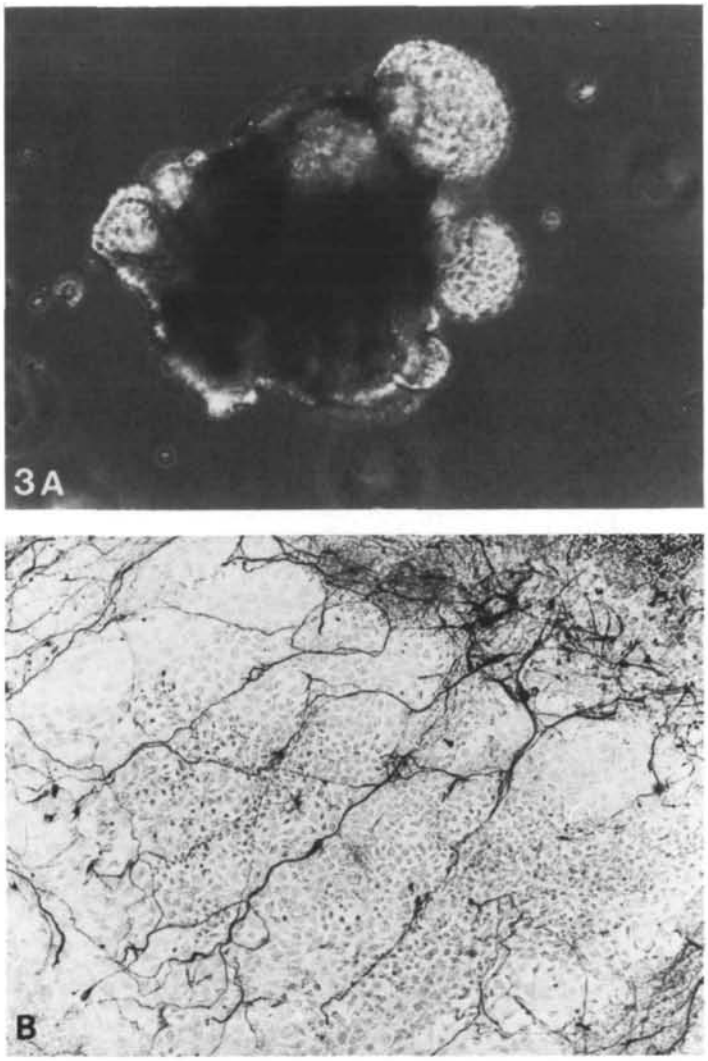


Fig. 3. *In vitro* differentiation of an ES cell line (IMD-16) homozygous for the 413.d proviral insertion. Upper panel shows an example of a cystic embryoid body in which the developing cysts are lined with a layer of endoderm-like cells; 100×. Lower panel shows the formation of differentiated nerve formed after the attachment of 413.d homozygous embryoid bodies; the cells were stained with anti-NF1; 100×.

histologically. A wide range of ectodermal and mesodermal derivatives were present in all tumours including large areas of keratinizing epithelium, secretory epithelium, pigmented melanocytes, adipose tissue, skeletal muscle, connective tissue, cartilage, bone, and red and white blood cells. An extensive analysis of multiple sections taken from each tumour did not reveal any obvious quantitative or qualitative differences in the differentiated derivatives formed by the various cell lines. Representative examples of cell and tissue types recorded in a homozygous ES cell derived tumour are shown in Fig. 4.

Homozygous ES cells can be 'rescued' by wild-type embryo cells

Although 413.d homozygous ES cell lines were judged to be pluripotent, the experiments described above did not rigorously demonstrate that these ES cells were capable of contributing to the entire repertoire of adult somatic tissues. It was therefore of interest to test whether 413.d homozygous cell lines, like normal ES cells, retain the ability to participate in development when returned to a wild-type embryonic environment.

IMD-11 and IMD-16, (both of which were karyotypically normal XY cell lines), were injected into wild-type host blastocysts. To distinguish donor and host derivatives, we made use of coat colour and glucose phosphate isomerase (GPI) isozyme variants. The 413.d mutation is maintained on the 129/Sv background, so both the IMD-11 and IMD-16 cell lines were homozygous for the agouti gene. Both IMD-11 and IMD-16 were Gpi type 1^a1^c. The 1^c allele was contributed by the original CCE ES cell line used to generate the 413 founder chimera. Host blastocysts were obtained from the MF1 outbred strain which, in addition to being albino, carries the Gpi 1^a and/or 1^b allele.

The manipulated embryos were allowed to proceed to term. The typical pattern and degree of mosaicism seen in adult chimeras obtained from the IMD-16 cell line is illustrated in Fig. 5. Previous studies have demonstrated that the extent of eye and coat hair pigmentation generally provide a good index of somatic contribution by ES cell derivatives. On this basis, it was immediately apparent that both of the homozygous 413.d cell lines contributed extensively to the tissues of adult animals. The contribution to live born animals by 413.d homozygous cells was as high as 50–70%. Moreover, we found no differences in rates of chimera formation using the 413.d homozygous cell lines in comparison with results reported previously for a number of ES cell lines of 129/Sv genotype in combination with MF1 host blastocysts (Bradley *et al.* 1984; Robertson *et al.* 1986). For example, in the first series of injections performed using the IMD-16 cell line, from 22 manipulated embryos transferred into pseudopregnant females, 16 live born progeny were obtained of which 11 were overtly chimeric. Thus, we conclude that homozygous ES cells can participate in the formation of a normal fetus, in conjunction with wild-type donor cells.

Mutant ES cells contribute extensively to the somatic tissues and germ cells of adult chimeras

Based on these results, it was clear that 413.d homozygous ES cells give rise to pigmented melanocytes derived from the neural crest cells. However, it was possible that the mutant ES cells might be specifically excluded from some adult somatic tissues. Thus, studies using aggregation chimeras have shown that parthenogenic cells, while colonizing tissues derived from all three embryonic lineages, are systematically eliminated from adult skeletal muscle and liver tissue (Fundele *et al.* 1989; Nagy *et al.* 1989). To examine the distribution of homozygous 413.d cells in adult tissues and organs, we performed a rigorous analysis of two adult chimeras (one male and one

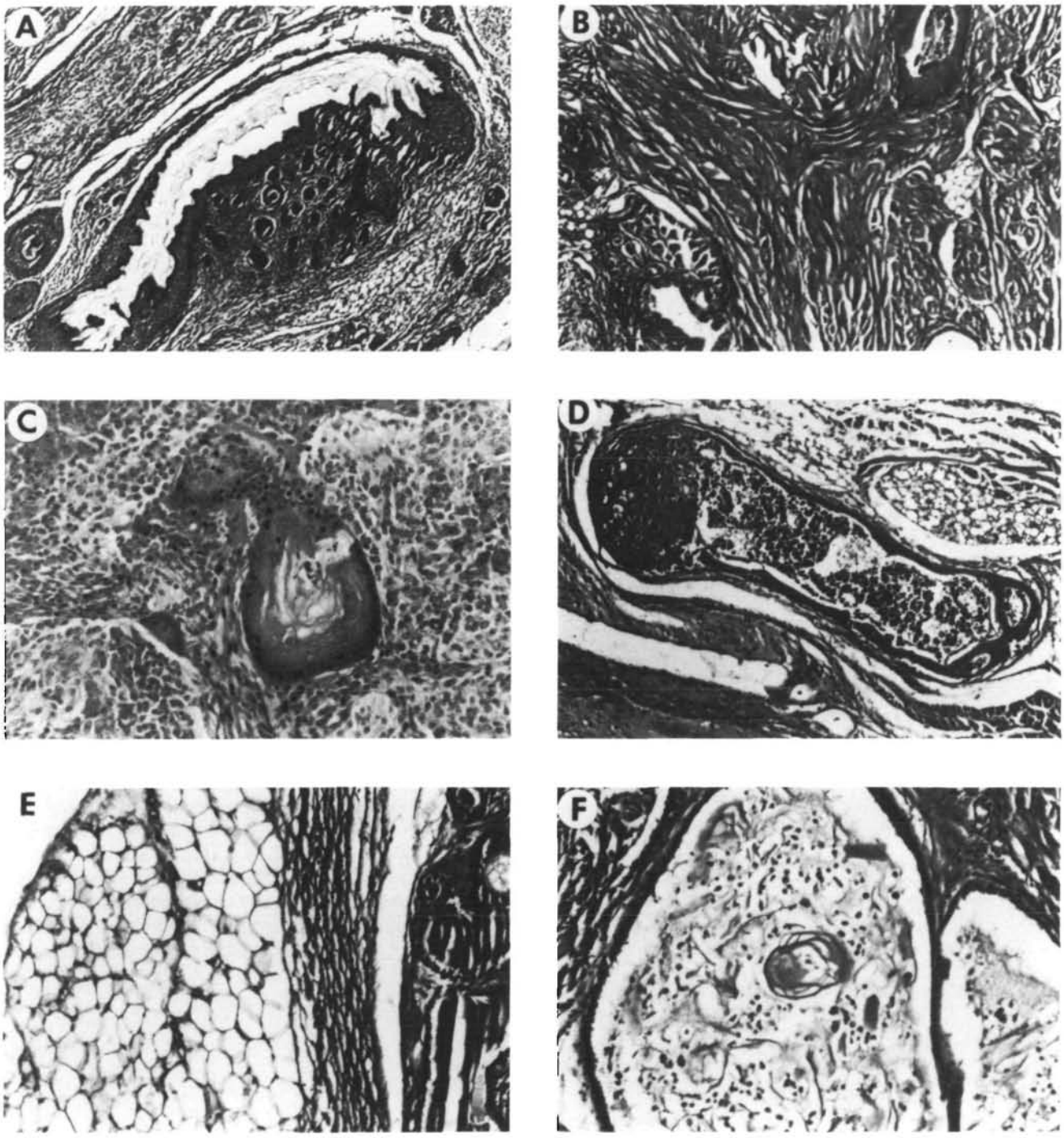


Fig. 4. Histological sections of a teratocarcinoma derived from the IMD-16 ES cell line homozygous for the 413.d proviral insertion. Sections (6 μm) were stained with hematoxylin and eosin. (A) Keratinizing epithelium, (B) skeletal muscle, (C) pigmented melanocytes, (D) small bone, (E) fat and muscle, (F) keratin swirls. A, B, D, E and F magnification 40×; C magnification 100×.

female). The animals were perfused to remove blood contamination and tissue samples collected for GPI analysis. As shown in Table 3, 413.d/d ES cell derivatives were found to contribute to all tissues tested in this assay. Moreover, in some instances the ES cell derivatives constituted the predominant somatic cell population.

We next assessed the ability of homozygous 413.d ES cells to contribute to the functional germ line. Matings of IMD-16 chimeric males to wild-type MF1 females were initially screened for progeny carrying the dominant agouti coat colour gene. Of the 10 chimeric males tested, 4 separate males sired litters of mixed albino and agouti pups. As predicted, Southern blot

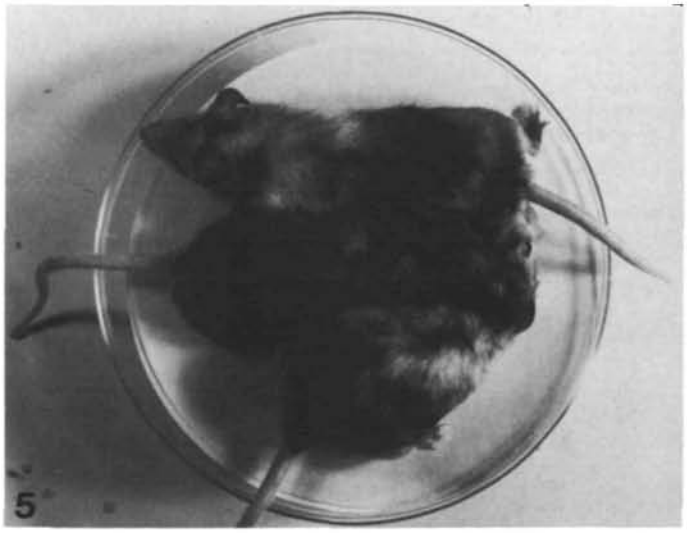


Fig. 5. Litter of chimeric mice generated by the injection of homozygous 413.d cells into MF1 albino host blastocysts. The agouti coat color is contributed by the 413.d homozygous ES cells.

Table 3. *Percentage distribution to adult tissues*

	Male	Female
Bladder	25–50	0
Blood*	25–50	0
Brain	50–75	10–25
Colon	25–50	10–25
Diaphragm	50–75	50–75
Duodenum	50–75	10–25
Eye	50–75	<10
Heart	75–100	25–50
Kidney	25–50	10–25
Liver	50–75	50–75
Lung	10–25	0
Muscle (Skeletal)	75–100	75–100
Pancreas	50–75	0
Salivary Gland	50–75	10–25
Spleen	25–50	0
Stomach	<10	10–25
Tongue	<10	75–100
Thymus	25–50	<10
Testies	10–25	–
Uterus	–	10–25
Ovaries	–	10–25

The female chimera was only 5–10% ES-cell derived as judged by coat color. All of the phenotypic females obtained in this study showed a similar level of contribution. This is because the majority of XX host blastocysts develop as phenotypic males as a result of the high level of incorporation of the XY IMD-16 cells.

*GPI analysis of blood from two separate chimeric mice demonstrated that the homozygous 413.d ES cells can contribute to both the red and white blood cells.

analysis of genomic DNA from a sample of 6 agouti progeny confirmed that each had inherited a single copy of the 413.d provirus (data not shown).

Homozygous mutant ES cells colonize all the extraembryonic lineages of the embryo

Our previous studies have shown that normal ES cells resemble early inner cell mass cells in their developmental potential and can therefore contribute to the derivatives of all three primary lineages of the developing embryo (Beddington and Robertson, 1989). It was possible that the developmental arrest occurring in the 413.d mutation resulted from an inability of homozygous embryos to form extraembryonic tissues correctly. To examine this point, we analyzed the contribution of 413.d homozygous cells to these cell lineages.

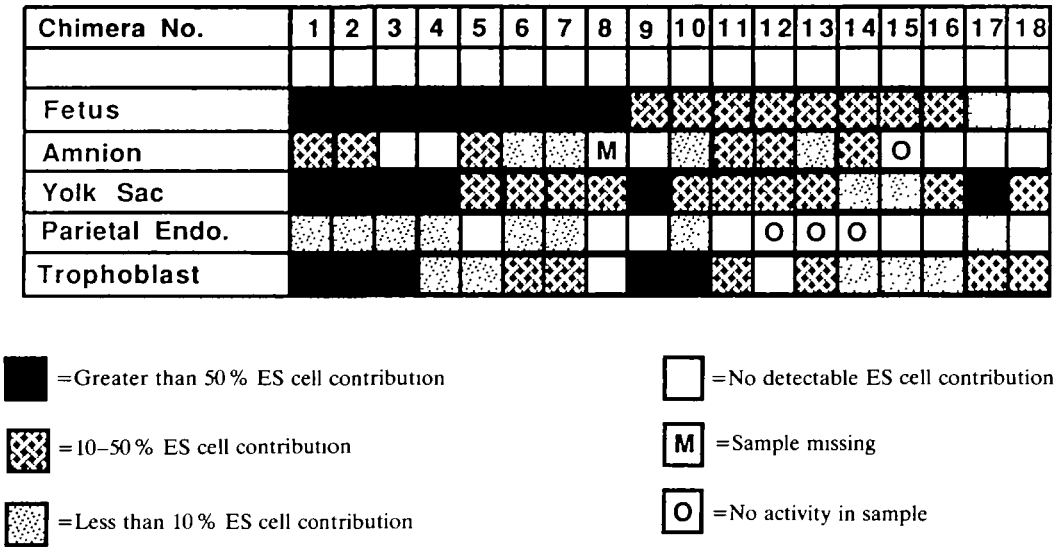
Chimeric conceptuses, generated by injecting ES cells into C57BL/6 blastocysts, were recovered at 10.5 days of gestation (i.e. 7 days post-transfer), dissected and various tissues isolated as described by Beddington and Robertson (1989). In total, 18 midgestation conceptuses, all of which proved to be chimeric, were analyzed. The results are presented in Table 4. Derivatives of the IMD-16 cell line were shown to contribute extensively to the embryo proper and were consistently present in the visceral yolk sac. Additionally, IMD-16 cells were shown to contribute routinely to the amnion and secondary trophoblast giant cells. IMD-16 ES stem cells contributed less frequently to the parietal endoderm (8/18 samples unequivocally showed a low level contribution). In comparison with the behavior of wild-type ES cells analyzed in similar experiments (Beddington and Robertson, 1989; F.L.C. unpublished observations), the only noticeable difference was an increased propensity of 413.d/d cells to colonize the trophoblast giant cells. We observed 5 instances where a contribution exceeding 50% was detected, whereas wild-type cells rarely give a contribution in excess of 10–20%. We have not yet determined whether this property is specific to the IMD-16 cell line or is attributable to the 413.d mutation.

In summary, ES cell derivatives were present in all the tissues derived from the trophectoderm, primitive endoderm and embryonic epiblast. Thus, we conclude that the ES cells homozygous for the 413.d provirus are capable of contributing to the three primary tissue lineages of the embryo.

Discussion

Here we describe a recessive embryonic lethal mutation that segregates with a specific proviral integration site in the mouse germ line. Based on previous reports analyzing insertional mutations (Jaenisch *et al.* 1983; Soriano *et al.* 1987; Weiher *et al.* 1990), we assume that viral integration interferes with the function of endogenous gene(s) required for normal embryogenesis. Previous studies on viral transduction have demonstrated that proviral insertions can lead to alterations in gene expression through a number of different mechanisms including disruption of normal patterns of transcription, alternative splicing, and/or stimulation of transcription from viral enhancers or promoters (reviewed Varmus, 1988). Our preliminary analysis of the 413.d locus has supported the prediction that no gross rearrangements of the genomic DNA sequences

Table 4. *Distribution of homozygous ES cells at 10.5 p.c.*



have occurred as a consequence of the viral integration (authors, unpublished results). We are presently analyzing transcripts derived from sequences flanking the proviral insertion site, to identify candidate gene(s) whose expression is affected by the 413.d provirus.

Histological experiments analyzing the morphology of abnormal conceptuses indicated that development proceeds beyond implantation. At 8.5 days *p.c.*, the homozygous embryos were morphologically grossly abnormal but the constituent cells, and in particular those of a subset of extraembryonic lineages, appeared to be viable. A more detailed analysis will be required to determine whether homozygous embryos proceed normally through the primitive streak stage of development and can generate any normal embryonic or extraembryonic mesodermal cell types.

We used the ability to isolate ES cells from the 413.d recessive lethal mutation to address the question as to whether developmental failure results from a general cell lethal effect. Previous studies analyzing mutations mapping to the t-complex have shown that ES cell lines can be derived from embryos homozygous for the t^{w5} haplotype (Magnuson *et al.* 1982) and t^{w18} haplotype (Martin *et al.* 1987) but not from homozygous embryos of the t° haplotype (Martin *et al.* 1987). Similarly, an analysis of mutations at the albino deletion complex has demonstrated the feasibility of isolating homozygous ES cells from C^{3H} deletion but not from C^{6H} deletion embryos (Niswander *et al.* 1988). Experiments reported here show that karyotypically normal, homozygous ES cell lines can be isolated from the 413.d mutation.

Genotypes of the cell lines were initially assigned based on comparisons of relative intensities of the proviral junction fragments hybridized with a *neo* specific probe. These results were confirmed using a single-copy probe which readily distinguishes the wild-type and 413.d locus. The ability to isolate homozygous ES cell lines demonstrates that the 413.d insertion does not disrupt a gene required for cell growth. When the genotype data, obtained from a sample of 19 individual cell lines, was subjected to a Chi squared analysis, a statistically significant deviation from the predicted Mendelian ratios was obtained. Thus, we recovered more homozygous and fewer wild-type and heterozygous cell lines. The factors that determine the ability to derive ES cells from cultured embryos remain largely obscure. The 413.d mutation may act to enhance the efficiency with which pluripotential cells can be rescued from blastocyst outgrowths, by suppressing cellular differentiation. However, the overall frequency of isolating lines (23%) from 413.d intercross embryos is in the same range as that found previously using wild-type 129/Sv strain embryos. The reasons underlying these biases thus remain unclear.

To obtain a better understanding of the role of the 413.d locus, we examined the spectrum of cell types formed when homozygous 413.d ES cells were induced to differentiate in isolation. We initially examined the growth and differentiation of 413.d ES cells *in vitro*. Homozygous 413.d/d ES cells were indistinguishable from wild-type and heterozygous ES cell lines, in terms of their cellular morphology and their potential to form primitive endoderm-like cells and terminally differentiated cell types of both mesodermal and ectodermal origin, such as contractile muscle and nerve. When homozygous ES cells were induced to differentiate in the context of a solid tumour mass, a wide variety of mature somatic tissues were formed including, for example, epithelia, hair follicles and small bones. This suggests that the 413.d mutation does not compromise the cellular interactions involved in the formation of at least a subset of normal mature tissues.

One informative approach for studying embryonic lethal mutations is to study the fate of mutant cells in chimeric embryos generated by the aggregation of mutant and wild-type embryos. In the case of two early acting postimplantation recessive lethals, namely lethal yellow (A^y) (Papaioannou and Gardner, 1979; Barsh *et al.* 1990) and the t^{w5} haplotype of the t-Complex (Magnuson *et al.* 1983), it did not prove possible to

rescue cells carrying the homozygous genotype. In contrast, aggregation chimeras have been particularly valuable in analyzing the fates of androgenetic and parthenogenetic embryonic cells (Surani *et al.* 1975; Stevens *et al.* 1977; Nagy *et al.* 1989; Clarke *et al.* 1988; Thompson and Solter, 1988; Fundele *et al.* 1989). Similarly, chimera analyses have demonstrated that the dominant white spotting (*W*) mutation acts in a cell autonomous fashion (Mintz, 1970). The recent demonstration that the *c-kit* gene, which encodes a cell surface tyrosine kinase receptor, maps to the *W* locus, has provided a genetic basis for the observed cell autonomy of this particular mutation (Chabot *et al.* 1988; Geissler *et al.* 1988).

In this report, we chose to utilize ES cells, identified as homozygous for the 413.d mutation, to test the developmental potential of mutant cells following their transfer into blastocysts. We used GPI isozyme markers to examine the contribution by 413.d homozygous ES cell derivatives to the tissues of adult chimeras. Rigorous analysis of widely representative tissues established that homozygous cells can extensively colonize adult somatic tissues. These experiments unambiguously demonstrated that 413.d homozygous cells contribute to derivatives of the embryonic ectoderm, including the germ cells. Indeed our analysis revealed a number of instances where homozygous cells formed the predominant cell population in specific adult organs. We therefore conclude that the 413.d mutation does not act in a cell autonomous fashion. However, formal proof of this will require a more sophisticated *in situ* analysis using cell marking, to establish that all of the cell populations of heterogeneous tissues contain descendants of the homozygous cells.

We also considered the possibility that insertion of the 413.d provirus might have affected a gene required for differentiation of extraembryonic tissues. To test this, we assessed the ability of homozygous ES cells to contribute to derivatives of all three primary tissue lineages in mid-gestation chimeric conceptuses. We found multiple instances where descendants of homozygous ES cells had colonized all tissues, including the trophectoderm. In comparison with wild-type cells, we found no significant differences in the pattern of colonization, with the exception that 413.d cells frequently made substantial contributions to the trophectodermal lineages. Additional experiments will be required to determine whether this is due to the particular ES cell line used or to the mutation.

One possible explanation to reconcile the observation that 413.d conceptuses fail to develop beyond early postimplantation stages, while homozygous 413.d ES cells behave identically to wild-type ES cells and participate in the formation of all of the primary embryonic cell lineages, is that some tissues may be especially affected by the absence of the gene product encoded at the 413.d locus. For example, it is known that ES cells do not make substantial contributions to the primitive endoderm derivatives following injection into the blastocyst. The 413.d gene product may be predominantly involved in the development of this particular lineage of cells. It is possible that, in chimeric conceptuses, the predominant population of wild-type cells is able to compensate fully for the presence of small numbers of 413.d/d-derived cells. In principle, this hypothesis could be tested by transferring wild-type ES cells into 413.d/d host blastocysts. This would result in the formation of embryos with 413.d/d cells predominating in the extraembryonic lineages. These experiments await the ability to genotype accurately individual preimplantation stage embryos.

Alternatively, the 413.d provirus may have disrupted a gene that has a specialized developmental function, perhaps being necessary to specify positional information. This is consistent with the finding that homozygous 413.d cells can proliferate and differentiate normally in concert with wild-type cells. We found no evidence suggesting that the incorporation of a high proportion of mutant cells (>50%) compromises chimeric conceptuses. The insertion at the 413.d locus may block the production of a secreted and/or diffusible molecule required for cell–cell interactions. The ability of cells carrying such a mutation to respond and behave normally when provided with a wild-type environment is not without precedent in the mammalian embryo. For example, a variety of tissue grafting experiments have shown that the *Steel* mutation acts in an environment-specific fashion allowing Sl/Sl cells to be fully rescued by the presence of wild-type cells (reviewed Silvers, 1979). It is interesting to contrast our results with those of Magnuson and coworkers. They reported that ES cells homozygous for the t^{w5} mutation were capable of extensive differentiation *in vitro* or as solid tumours, including the formation of mesodermal derivatives (Magnuson *et al.* 1982, 1983), whereas the inclusion of t^{w5}/t^{w5} embryos in aggregation chimeras resulted in abnormal embryonic development. These observations suggest that the t^{w5} mutation does not interfere with differentiation *per se*, but that, in contrast to 413.d mutant cells, t^{w5}/t^{w5} cells are unable to respond in an appropriate manner in response to developmental cues provided by the wild-type cells.

To distinguish these various models, it will be necessary to examine the exact spatial distribution of 413.d homozygous derivatives in chimeric embryos. Analysis of mosaics of marked wild-type and mutant cells in *Drosophila* have demonstrated that *arm* and *notch* gene products act in a cell autonomous manner during embryogenesis to control cell fate (Wieschaus and Riggleman, 1987; Hoppe and Greenspan, 1986, 1990). Conversely, gynandromorph studies of the wingless mutation (*wg*) have demonstrated that mutant *wg* clones can be rescued when juxtaposed to wild-type cells. This was taken as evidence that the *wg* product may be involved in cell–cell communication (Morata and Lawrence, 1977; Baker, 1988). Therefore, the 'rescue' phenomenon that we have documented for the 413.d mutation may occur only when mutant cells are positioned adjacent to wild-type cells. We plan to develop a nuclear or cytoplasmic marker to trace the

fate of single homozygous 413.d cells or their clonal descendants.

In summary, our preliminary analysis of the 413.d mutation demonstrates that the product of this gene is required for normal postimplantation development. Moreover, the mutation does not act in a cell autonomous fashion, as cells homozygous for the 413.d mutation can be rescued in all embryonic lineages in chimeras. The unravelling of the underlying causes of the cellular disturbances that characterize the 413.d mutant phenotype will ultimately rely on complementary molecular analysis of this genetic locus to identify and characterize candidate gene products. However, we believe that this general approach utilizing ES cells should be generally applicable to the study of further mutations that perturb differentiation and morphogenesis of the mouse embryo.

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