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Expression of v-*src* induces aberrant development and twinning in chimaeric mice

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Summary

The role of the proto-oncogene c-*src* in mouse development has been investigated by studying the consequences of expressing its viral homologue, v-*src*. Embryonic stem (ES) cell lines with differing levels of v-*src* tyrosine kinase activity have been used to generate chimaeric mice. Whereas a low level of v-*src* expression is compatible with embryogenesis, chimaeras derived from ES clones with high levels of v-*src* activity develop abnormally and die on the 8th–9th day of gestation. These abnormalities are characterized by the formation of twin or multiple embryos within the same Reichert's membrane, and by the arrest of embryonic development at the late egg cylinder stage, accompanied by relative expansion of the visceral yolk sac (VYS) and hyperplasia of the VYS endoderm. These results demonstrate for the first time that deregulated expression of the *src* proto-oncogene product can induce developmental abnormalities during early embryogenesis.

Key words: v-*src*, twinning, embryogenesis, abnormal development.

Introduction

There is a growing body of evidence that proto-oncogenes play important roles in development. This has largely come from *Drosophila*, where a number of developmental genes have been shown to share considerable homology to proto-oncogenes (for example see Hafen *et al.* 1987; Rijsewijk *et al.* 1987). Recently, proto-oncogenes have also been implicated in vertebrate development; for instance ectopic expression of the *int*-1 gene in *Xenopus* leads to disruption of pattern formation (McMahon and Moon, 1989), while the c-*kit* gene maps to the *W* developmental locus in mouse (Geissler *et al.* 1988; Chabot *et al.* 1988). Indeed, a large number of proto-oncogenes show developmentally regulated patterns of expression, suggesting that they too may have roles in embryogenesis (Slamon and Cline, 1984; Adamson, 1987).

One such example is the c-*src* proto-oncogene. There is good circumstantial evidence for its involvement in embryonic development, since it is expressed in a specific spatial and temporal framework throughout embryogenesis in a large variety of species, including *Drosophila*, chick, mouse and human. In *Drosophila*, c-*src* transcripts are regionally expressed after germ band retraction, being particularly high in visceral mesoderm and in cells that will form the smooth muscle of the gut. In later embryos, expression is highest in dorsal and medial regions of the brain, in the ventral ganglia and in the eye (Simon *et al.* 1985). Expression in neural tissues has also been reported in the chicken, where high levels of c-*src* protein are found in the neural ectoderm until neural tube closure (Maness *et al.* 1986) and then again in developing cerebellum (Fults *et al.* 1985) and neural retina (Sorge *et al.* 1984). In fact, elevated c-*src* expression in neural tissues has been reported in a wide variety of species, (Cotton and Brugge, 1983; Maness *et al.* 1988; Levy *et al.* 1984). Taken together with the fact that an alternative form of the c-*src* protein is found in post-mitotic neurones (Brugge *et al.* 1985, 1987), these results have suggested that c-*src* may play an important role in neuronal

differentiation. However, it is also likely that c-*src* has a function in other cell types, since the gene is widely expressed in the embryo and adult.

Despite the accumulation of data on patterns of c-*src* expression in the embryo, little is known about its role in embryogenesis. The gene has however, been implicated in the growth and differentiation of a number of cell types in culture. Evidence for this is largely from studies on its closely related viral homologue, v-*src*. This is the transforming gene of Rous sarcoma virus (RSV), which encodes a tyrosine kinase, $pp60^{v\text{-}src}$, that has been shown to perturb the differentiation and self-renewal capacity of a wide variety of cell types both in culture and *in vivo*. In most cases, $pp60^{v\text{-}src}$ blocks terminal differentiation of cells, such as myoblasts (Holtzer *et al.* 1975; Fiszman and Fuchs, 1975), chondroblasts (Pacifici *et al.* 1977; Boettiger *et al.* 1983) and retinal melanoblasts (Boettiger *et al.* 1977). It can also induce the differentiation of the rat phaeochromocytoma cell line PC12 to neurones in the absence of nerve growth factor (Alema *et al.* 1985). In addition, it has been shown to affect a number of cell surface parameters, leading to reductions in cell adhesion (Warren and Nelson, 1987), gap junction communication (Azarnia and Loewenstein, 1984) and interaction with the extracellular matrix, for example *via* the fibronectin receptor complex (Hirst *et al.* 1986).

Analysis of temperature-sensitive mutations has shown that the effects caused by $pp60^{v\text{-}src}$ are due to its tyrosine kinase activity. In contrast to the viral protein, the *c-src* kinase is tightly regulated, largely by phosphorylation of a tyrosine residue at position 527 which greatly reduces its activity (Cooper *et al.* 1986). Replacement of this tyrosine by phenylalanine activates the $pp60^{c\text{-}src}$ kinase, which then exhibits some of the characteristics of its viral homologue (Piwnica-Worms *et al.* 1987; Cartwright *et al.* 1987; Kmiecik and Shalloway, 1987). $pp60^{v\text{-}src}$ lacks this tyrosine residue and consequently is constitutively active. Thus, the viral gene is an activated form of c-*src* and is probably exerting its effects through perturbing processes in which c-*src* is itself involved.

With this in mind, we have attempted to perturb c-*src* function in mouse development through expression of its viral homologue in transgenic mice. In order for this approach to be successful, the *v-src* gene must be introduced and expressed *in vivo* both efficiently and reproducibly, and consequently we have chosen to use embryonic stem (ES) cells (Evans and Kaufman, 1981; Martin, 1981) as a means of effecting ectopic expression in the embryo. ES cells provide a powerful tool for analyzing mouse development, since they are developmentally pluripotent, capable of contributing to many cell lineages, including the germ cells, on their introduction into the embryo (Bradley *et al.* 1984; Beddington and Robertson, 1989), and are amenable to genetic manipulation in culture (Robertson *et al.* 1986; Gossler *et al.* 1986). Here we describe the derivation of chimaeric mice from ES cell lines expressing varying levels of *v-src* tyrosine kinase activity, and the consequences of such expression on their development.

Materials and methods

Retroviral infection of ES cells

The ES cell line D3 (Gossler *et al.* 1986) was infected with the retroviral vectors SR1 and SR2 (Boulter and Wagner, 1988*a*). This cell line is derived from a 129/Sv×129/Sv embryo, having agouti coat colour and the GPI isoenzyme form 1A. Both retroviral vectors are based on Moloney Murine Leukaemia Virus and contain, in addition to the v-*src* gene, a selectable neomycin phosphotransferase (*neo*) gene, which confers resistance to G418. In SR1, the v-*src* gene is under the control of the Herpes Simplex virus thymidine kinase (TK) promoter, whereas in SR2 it is expressed from the human metallothionein (MT) promoter, inducible with dexamethasone and heavy metals such as zinc and cadmium. The viral promoter is located in the 5′ long terminal repeat (LTR) and drives expression of the *neo* gene in SR1, whereas in SR2 this gene is expressed from the TK promoter. Infections were performed as follows: 5×10^4 ES cells were plated in 60 mm dishes 1 day prior to infection with viral supernatants supplemented with $4\,\mu g\,ml^{-1}$ polybrene. Infection was performed with undiluted viruses for a period of 8 h, and selection in $750\,\mu g\,ml^{-1}$ G418 was started 24 h later. After 10 days of selection, the SR1 virus gave 1 or 2 G418-resistant ES cell colonies per plate, while the titre of the SR2 virus on ES cells was approximately 10-fold higher. ES cells were grown on feeder layers of mitomycin-treated primary embryo fibroblasts or STO cells as described by Robertson (1987), and were cultured in ES cell medium: Dulbeccos Modified Eagles Medium including glucose and glutamine, supplemented with 10% newborn calf serum, 5% foetal calf serum and 1×beta-mercaptoethanol (100× stock solution is $7\,\mu l$ in 10 ml PBS).

Determination of v-src *tyrosine kinase activity*

Levels of v-*src* tyrosine kinase activity were determined in SR1 and SR2 ES cell clones by performing assays on cell lysates, as described by Collett and Erikson (1978), after quantitation of total protein using Pierce reagent. A TBR polyclonal serum against v-*src* was used for the immunoprecipitation (obtained from S. Courtneidge, EMBL, Heidelberg).

In vitro *differentiation of ES cells*

ES cells were induced to differentiate *in vitro* by culturing in bacteriological dishes, as described by Robertson (1987). This results in the formation of embryoid bodies. Some of these were cultured further as aggregates, forming cystic embryoid bodies, while others were trypsinized and replated onto gelatinized tissue culture dishes. Extensive cell differentiation was observed in these cultures several days later.

Embryo manipulation and dissection

8-cell morulae and blastocysts were obtained from ICR×ICR, PO×PO (Pathology, Oxford) and MF1×MF1 matings, all three strains being albino and homozygous for the *Gpi-1b* allele. For blastocyst injection, ES cells were trypsinized and injected into the blastocoel cavity, as described by Bradley (1987). For embryo aggregation, a small clump of ES cells (4–6 cells) was aggregated with two morulae, according to a protocol described by Stewart (1982). Aggregated embryos were cultured overnight to the blastocyst stage in M16: ES cell medium 1:1. Manipulated embryos were transferred to the uterine horns of pseudopregnant (C57Bl6×C3H or PO×PO) recipient females (homozygous for the *Gpi-1b* allele).

Embryos isolated on the 8th and 9th days of gestation were dissected out of the decidua, photographed and fixed in

methanol:acetic acid 3:1. Alternatively, whole decidua were fixed in 4% buffered paraformaldehyde. Fixed tissue was dehydrated and embedded in paraffin wax according to standard procedures.

Analysis of glucose phosphate isomerase (GPI) activity

Samples of tissue for GPI analysis were freeze-thawed several times. GPI isoforms were resolved by electrophoresis using cellulose acetate plates and staining, as described by Bradley (1987).

Results

Isolation and characterization of ES cell lines expressing pp60$^{v\text{-src}}$

The v-*src* gene was introduced into ES cells by infection with the selectable retroviral vectors SR1 and SR2 (Boulter and Wagner, 1988*a*; see Materials and methods). After selection several independent clones were isolated, each of which had a single intact copy of the vector from Southern blot analysis (data not shown). The level of v-*src* tyrosine kinase activity differed between clones, being highest in the SR1 ES cell lines (D3 SR1–1, SR1–2) and significantly lower in the uninduced SR2 clones (D3 SR2–1, SR2–2 and SR2–3) (see Fig. 1A,B). The low basal levels of v-*src* kinase activity in the SR2 clones were inducible 3- to 5-fold in the presence of 5×10^{-6} M cadmium chloride for a period of six hours (Fig. 1B).

Both SR1 and SR2 clones retained stem cell morphology, and continued to express the stem cell marker ECMA-7 (data not shown). We were interested to determine whether high levels of v-*src* kinase activity would interfere with their ability to differentiate in culture. This can be tested by growing the ES cells in bacteriological dishes, where they will form large aggregates in suspension called embryoid bodies. When, after a few days in culture, such aggregates are trypsinized and replated on tissue culture dishes, extensive cell differentiation is observed. We found that both SR1 and SR2 clones formed embryoid bodies (Fig. 2A), although the clones having higher levels of kinase activity aggregated less well, and formed fewer and smaller aggregates. Nevertheless, on trypsinization and replating of these embryoid bodies a wide variety of differentiated cell types was observed on the basis of cell morphology, including endoderm, neuronal cells and beating cardiac and skeletal muscle (Fig. 2B). Thus, expression of v-*src* tyrosine kinase activity in ES cells may reduce their ability to aggregate, but does not appear to compromise their ability to differentiate subsequently under these conditions. Indeed, as with uninfected ES cells, subcutaneous injection of SR1–1 and SR2–3 cells into syngenic mice results in the formation of teratocarcinomas, which are tumours containing many differentiated cell types, as well as proliferating stem cells (data not shown).

Derivation of chimaeric mice expressing a low level of pp60$^{v\text{-src}}$

Individual ES cell clones were initially introduced into ICR×ICR and PO×PO embryos by injection into the blastocoel cavity, and manipulated blastocysts transferred to recipient females for development to term. The extent of ES cell contribution was determined on the basis of coat colour and GPI activity (see Table 1). The two SR2 clones with barely detectable v-*src* kinase

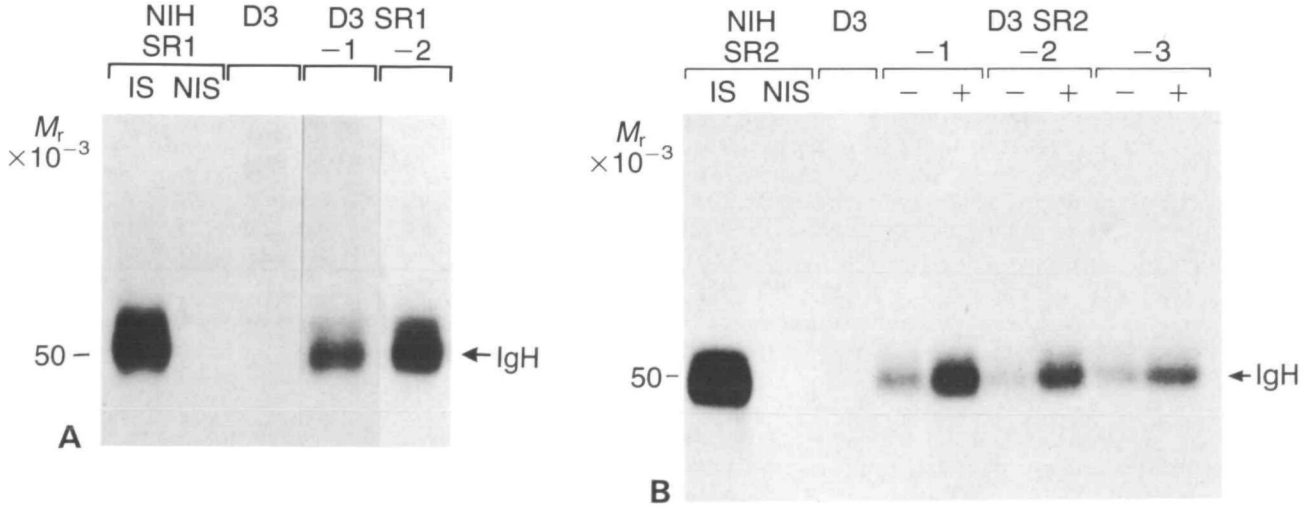

Fig. 1. (A,B) pp60$^{v\text{-}src}$ tyrosine kinase activity in ES SR1 clones (SR1–1 and SR1–2) and SR2 clones (SR2–1, SR2–2 and SR2–3), determined by *in vitro* IgH phosphorylation assays. The assay is based on pp60$^{v\text{-}src}$ catalysing the transfer of ^{32}P-labelled phosphate from ATP to the heavy chain of an antibody used to immunoprecipitate the v-*src* protein from cell lysates. After labelling, the heavy chain (IgH) is cleaved from the antigen–antibody complex under reducing conditions, and run on a denaturing 9% polyacrylamide gel, which is then autoradiographed. Assays were performed on the SR2 clones before (−) and after (+) induction with 5×10^{-6} M $CdCl_2$ for a period of 6 h. Also included are uninfected ES cells (D3) and NIH3T3 cells infected with the SR1 and SR2 retroviral vectors (Boulter and Wagner, 1988*a*), assayed with immune serum (IS) and non-immune serum (NIS). 100 mg total protein was included in each sample, with the exception of the NIH3T3 samples where 50 mg total protein was used.

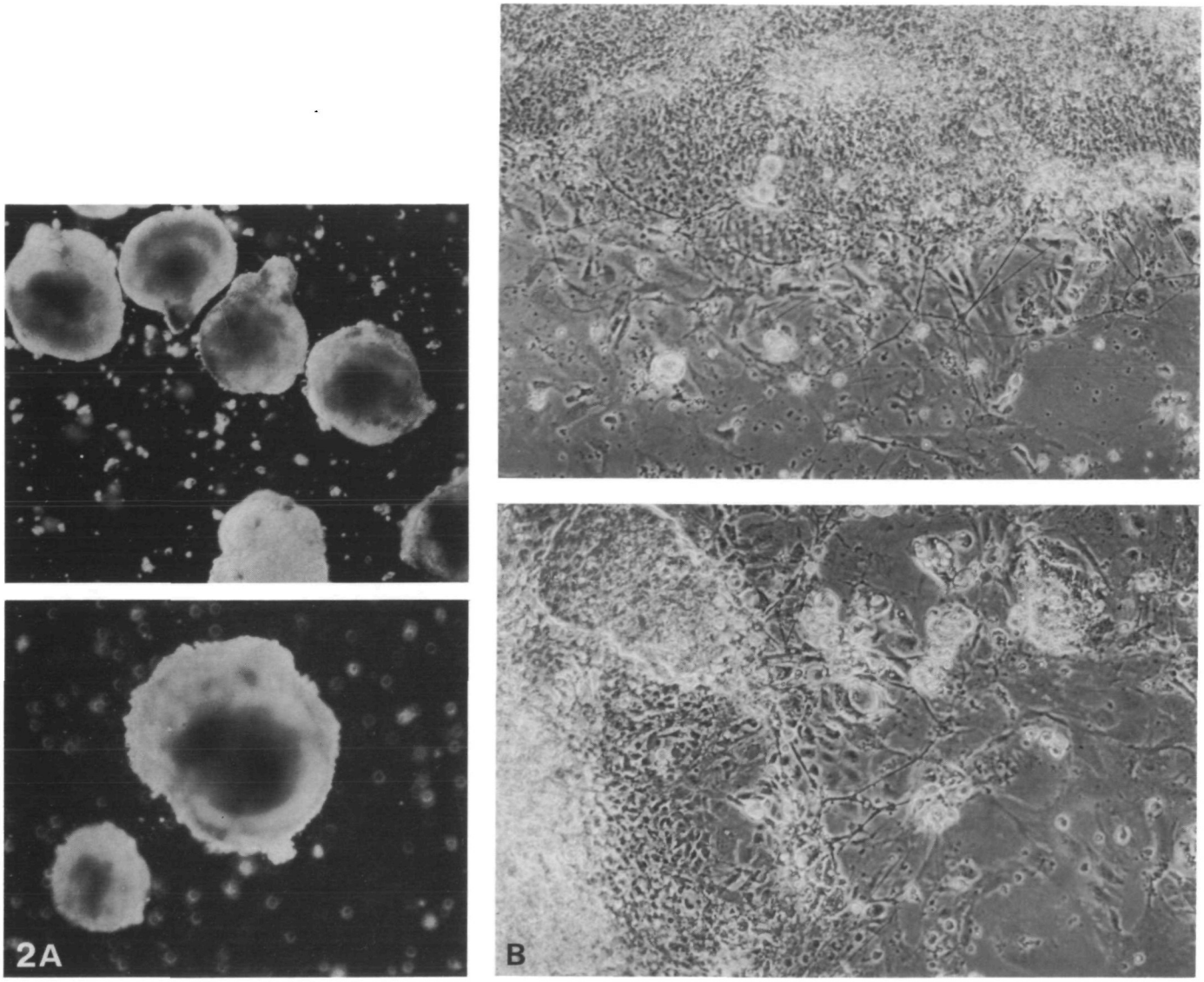

Fig. 2. Differentiation of SR1 and SR2 ES cell clones. (A) Formation of embryoid bodies derived from SR2–3 (upper panel) and from SR1–1 (lower panel), shown after 10 days in culture. A ridge of ectoderm is present in embryoid bodies derived from both clones. Magnification: upper panel, 10×; lower panel, 20×. (B) Extensive cell differentiation on trypsinizing and replating embryoid bodies derived from SR2–3 (upper panel) and SR1–1 (lower panel). Neural, muscle and endoderm cells are present in both cultures several days after replating. Magnification: 50×.

activity (SR2–2 and SR2–3) participated efficiently in embryogenesis, and of 65 mice born, 44 were chimaeric. Two of these mice were tested for v-*src* expression, and in both a low level of kinase activity was detected in a number of tissues, notably spleen, liver, lung and brain (data not shown), suggesting that a relatively low level of v-*src* expression can be tolerated during normal embryogenesis.

In contrast, ES cell clones having higher levels of v-*src* kinase activity (SR1–1, SR1–2, SR2–1) failed to give rise to chimaeras; of 27 live-born offspring and 53 mid-gestation embryos examined, none were chimaeric. This result could not easily be explained by the death of chimaeric embryos early in development because the litter sizes were not significantly smaller than those obtained after blastocyst injection of SR2–2 and SR2–3 cells. An alternative explanation was that cells expressing higher levels of v-*src* kinase activity were failing to participate efficiently in embryogenesis after injection into the blastocyst. Given that the cells retained their ability to differentiate *in vitro*, this may have been due to failure of the cells to adhere to the inner cell mass (ICM) on their introduction into the blastocoel cavity. This would be consistent with the differences that we had observed in their ability to aggregate in culture. To overcome this problem, experiments were performed in which morulae and small clumps of ES cells were aggregated together, as described below.

Derivation of chimaeras by morula aggregation

Clumps of ES cells were aggregated with two

Table 1. *Generation of chimaeras by blastocyst injection*

Cell line	Time analysed	Number of blastocysts Transferred	Implanted	Number tested	Number chimaeric	Percentage chimaerism
SR1-1	mid-gest.	26	12+8R	12	0	–
	birth	26	17	17	0	–
	total	52	29+8R	29	0	–
SR1-2	mid-gest.	35	30	27	0	–
SR2-1	mid-gest.	15	14	14	0	–
	birth	34	12	12	0	–
	total	49	26	26	0	–
SR2-2	mid-gest.	29	25	21	6	10–40
	birth	21	12	12	6	10–50
	total	50	37	33	12	10–50
SR2-3	mid-gest.	11	11	11	5	30–70
	birth	97	54	53	38	20–95
	total	108	65	64	43	20–95

Chimaerism was determined on the basis of coat colour for live born mice and GPI activity in embryos analyzed mid-gestation (abbreviated to mid-gest.). R denotes resorption.

Table 2. *Correlation between embryo abnormalities and chimaerism with ES cells expressing high levels of* $pp60^{v\text{-src}}$

Cell line	Relative kinase activity	Embryos analysed	Normal embryos Number	Chimaeric	Abnormal embryos Number	Chimaeric
SR1-1	+++	17	11	0	6	6 (40–80%)
SR1-2	+++	20	12	2 (10–20%)	8	8 (40–80%)
SR2-1	++	4	1	0	3	3 (50%)
Total		41	24	2	17	17
SR2-3	+	10	10	5 (40–80%)	0	0

Chimaerism was determined by GPI assays on lysed embryonic cells. The range of percentage contribution of ES cells in the chimaeric embryos is given in parentheses. +, ++ and +++ indicate relative levels of v-*src* tyrosine kinase activity, see Fig. 1.

8-cell-stage embryos, after removal of the zona pellucida. This method differs from the protocol for blastocyst injection where cells are trypsinized to yield a single cell suspension, and instead involves gently treating the cells with EGTA to give sticky clumps of 4–6 cells. Both the SR1 and SR2 ES cell clones aggregated well with the embryos, and after overnight incubation formed composite blastocysts. These were transferred to the uterus of pseudopregnant recipients, and on the 9th day of gestation, embryos were isolated and assayed for ES cell contribution on the basis of GPI activity (Table 2). ES cell contribution was detected in half the embryos that had been aggregated with an SR2 ES cell line having a low level of v-*src* kinase activity (SR2-3), the extent of contribution ranging from 40 to 80%; all of these were morphologically normal, indicating that the aggregation procedure was compatible with normal development. In contrast, ES clones with higher levels of v-*src* expression (SR1-1, SR1-2 and SR2-1) gave a high frequency of abnormal embryos; all of the 17 abnormal embryos analyzed were chimaeric, containing 40 to 80% contribution from the introduced ES cells, whereas 22 of the 24 normal embryos produced had undetectable levels of chimaerism. Moreover, the ES cell contribution in the two remaining normal embryos was low (10–20%). Overall an excellent correlation was observed between morphological abnormality of the embryo and the extent of contribution from ES cells with relatively high v-*src* kinase activity, a result consistent with v-*src* expression above a threshold level leading to abnormal embryonic development.

Characterization of embryo abnormalities

The morphological abnormalities observed fall into two classes, (see Fig. 3 and Table 3). The first, detected from the 7th day of gestation, is the formation of twin or multiple egg cylinders within a single Reichert's membrane. Of 65 embryos isolated after aggregation with SR1-1 and SR1-2, 32 were abnormal and more than 50% of these showed a twinning phenotype

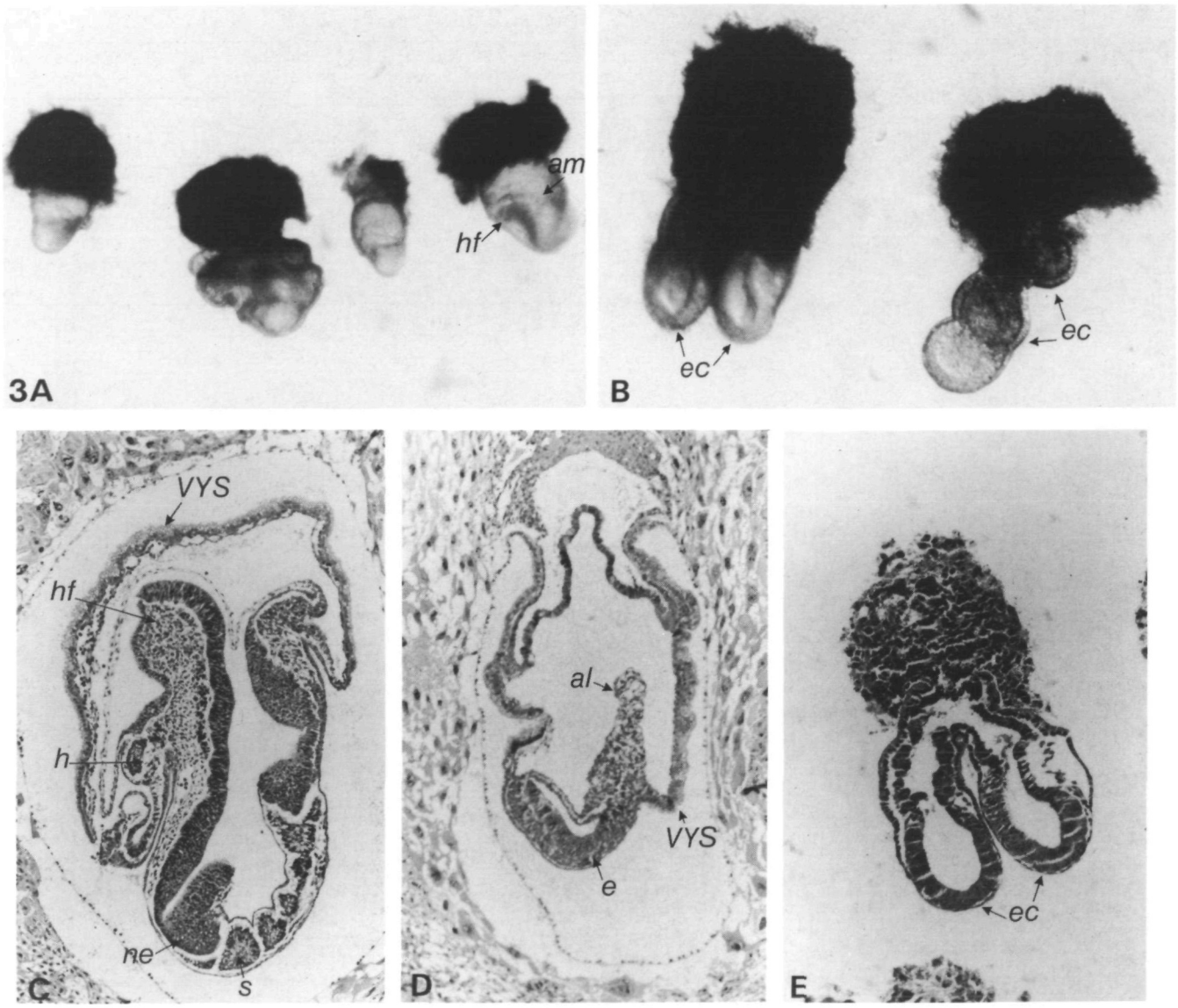

Fig. 3. (A) Morphology of embryos isolated on the 8th day of gestation, after morula aggregation with SR1–1. The embryo on the far right appears to be normal, with the head fold (hf) and amnion (am) discernable. Of the three remaining embryos, two have relatively expanded VYSs, while the third has multiple lobes. (B) Twin egg cylinders (ec) isolated on the 8th day of gestation, after aggregation with SR1–2. Each pair has been dissected out of a single Reichert's membrane. (C) Section of a normal 9th-day embryo, after aggregation with SR2–3, showing visceral yolk sac (VYS), headfold (hf), heart (h), neural ectoderm (ne) and somites (s). (D) Section of a 9th-day embryo, after aggregation with SR1–1, showing expanded VYS forming convoluted folds, and relatively retarded embryonic region (e). The allantois (al) is also present. (E) Section of one of the 8th-day embryos in panel B, showing twin egg cylinders (ec).

(Fig. 3). The morphology of the twinned embryos varies; some are apparently normal twin egg cylinders, whereas others form multiply-lobed structures. The relative size of the twin embryos also varies, in some cases one twin being retarded relative to the other, but in all cases the extraembryonic as well as the embryonic region appears to be duplicated. The division of the embryo is not a consequence of two morulae being used for the aggregation with ES cells, since we have observed the twinning phenotype when SR1–1 and SR1–2 cells have fortuitously associated with only one of the embryos.

Some egg cylinder stage embryos show a second phenotype, characterized by the embryonic region being retarded and the VYS relatively expanded (Fig. 3; Table 3). In addition, the VYS endoderm appears to be hyperplastic, thrown into convoluted folds which can be seen on sectioning (Fig. 3D). By the 10th day of gestation there is a marked increase in resorption frequency, as witnessed by the number of haemorrhagic decidua.

Neither of these abnormal phenotypes have been observed in chimaeras made with control ES cell lines which do not express v-*src* (uninfected ES cells) or which express at a very low level (SR2–3) (Table 3). This confirms that the aggregation of ES cells with

Table 3. *Occurrence of morphological abnormalities in embryos aggregated with SR1–1, SR1–2 and SR2–1*

Cell line	Day of gestation	Number transferred	Number implanted	Number of embryos Normal	Abnormal	Number of twinned embryos	Number with expanded VYS
SR1–1	8th	17	13	7	6	3*	3
	9th	24	13	6	7	2	5
	Total	41	26	13	13	5	8
SR1–2	7th	20	12 (+1R)	5	7	5	2
	8th	7	6 (+1R)	2	4	2	2
	9th	34	21 (+8R)	13	8	6	2
	10th	6	0 (+4R)	0	0	–	–
	Total	67	39	20	19	13	6
SR2–1	9th	8	4 (+1R)	1	3	1	2
SR2–3	7th	7	2	2	0	–	–
	9th	23	13	13	0	–	–
	Total	30	15	15	0	–	–
ES	7th	10	7 (+1R)	7	0	–	–
	9th	8	4	4	0	–	–
	Total	18	11	11	0	–	–

Embryos aggregated with uninfected ES cells and with SR2–3 were morphologically normal. Embryos were isolated on the 7th–10th day of gestation, dissected from their decidua and viewed. The total of abnormal embryos is broken down into those showing the twinning phenotype and those with a relatively expanded VYS. R denotes resorption, and ES uninfected ES cells. * One of the twin embryos was dissected into two separate egg cylinders, and ES cell contribution determined in each. Whereas one of the egg cylinders was normal and had an undetectable level of ES cell contribution, the other, which was morphologically abnormal, contained 25% ES cell contribution.

embryos does not result in embryo abnormalities *per se*, and that these are only observed with ES cell lines expressing relatively high levels of v-*src* tyrosine kinase activity.

Discussion

The experimental approach

The aim of this work was to elucidate the role of the c-*src* proto-oncogene in mammalian development, through expression of its viral homologue. The approach we have taken is essentially a genetic one, namely to disrupt the expression pattern of the gene and, by studying the consequences of doing this, to infer its normal function. The c-*src* gene encodes a tyrosine kinase that is tightly regulated, its activity in the cell normally being suppressed by phosphorylation of a negative regulatory site at tyr^{527} (Cooper *et al.* 1986). We have therefore introduced and ectopically expressed its viral homologue, $pp60^{v\text{-}src}$, a mutant form of the protein that has constitutive tyrosine kinase activity. The viral protein is closely related to its cellular counterpart. Indeed, replacement of a single amino acid at position tyr^{527} in $pp60^{c\text{-}src}$ results in activation of the kinase domain and confers on the cellular protein some of the properties of $pp60^{v\text{-}src}$. It is therefore a reasonable assumption that expression of the v-*src* gene would simulate ectopic expression of active $pp60^{c\text{-}src}$ and would consequently interfere with normal c-*src* function.

We chose to use ES cells as a means of introducing the v-*src* gene into mouse embryos because this route has a number of important advantages over other methods of generating transgenic mice. The most pertinent of these is that the manipulated ES cells can be characterized before being introduced *in vivo*, and so chimaeric mice can be derived from clonal populations of ES cells having defined levels of expression of the introduced gene. Furthermore the derivation of chimaeric mice is highly efficient, and so results can be easily reproduced, an important consideration in cases where expression of the gene may have a dominant disruptive effect on embryogenesis, precluding the generation of transgenic lines.

ES cells with high v-src kinase activity retain stem cell morphology

In this paper, we describe the derivation of ES cell lines with a range of v-*src* tyrosine kinase levels. Despite having relatively high levels of kinase activity, the SR1 ES cell clones retain stem cell morphology and continue to express the stem cell marker ECMA-7. In fact, they appear to be very stable and, unlike uninfected ES cells, do not spontaneously differentiate when grown without feeder cells or the growth factor DIA/ LIF (Smith *et al.* 1988; Williams *et al.* 1988*b*; R.L.W. and C.A.B., in preparation). Nevertheless, they will differentiate *in vitro* under appropriate conditions, giving rise to embryoid bodies when aggregated in bacteriological dishes, and a wide variety of differentiated cell types when these are trypsinized and replated. We did, however, note differences in the efficiency with which

high- and low-expressing *src* clones form embryoid bodies. In contrast to uninfected ES cells and low *src*-expressing clones, the high expressing clones (SR1–1, SR1–2) aggregated poorly, giving fewer and smaller aggregates which were more ragged in appearance. The failure of these cells to aggregate efficiently might be explained by cell surface differences between uninfected cells and cells expressing relatively high levels of v-*src* kinase activity. Indeed, this has already been reported in fibroblasts, where phosphorylation of the fibronectin receptor complex by v-*src* results in reduced association with the extracellular matrix (Hirst *et al.* 1986), and where v-*src* expression induces decreased numbers of adhesion plaques on the cell surface (Warren and Nelson, 1987). It will be of interest to determine whether adhesion molecules, such as uvomorulin, which is known to be present on the surface of early embryonic cells (Hyafil *et al.* 1980), are affected by v-*src* expression.

High levels of v-src *kinase activity lead to developmental abnormalities*

The aggregation of morulae with ES cells expressing relatively high levels of v-*src* kinase activity frequently gives rise to twin embryos. The phenotype of twinning is very unusual, since the normal incidence of monozygotic twins in the mouse, as in most other mammals, is extremely low (Gluecksohn-Schoenheimer, 1949; Tarkowsky, 1965; Kaufman and O'Shea, 1978). The twins we describe have multiple egg cylinders within the same Reichert's membrane, which indicates that the splitting of the embryo must take place after the formation of the ICM. They resemble the monozygotic twins described by Hsu and Gonda (1980), which developed at a very low frequency from blastocysts cultured *in vitro*. These authors proposed that twinning was the result of subdivision of the ICM due to physical constraints imposed on a small subset of blastocysts in culture. A similar mechanism might account for twinning in our case, in that the ICM may be subdivided: surface differences between ES cells with high v-*src* activity and the host embryo could be responsible for this fission, perhaps by disrupting communication between cells. Indeed, it has been reported in fibroblasts that expression of v-*src* or the activated c-src^{527} mutant induces a reduction in gap junction communication (Azarnia and Loewenstein, 1984; Azarnia *et al.* 1988). Although this has not been demonstrated in early embryonic cells, gap junctions have been detected in the preimplantation mouse embryo and are thought to play an important role in early development (Lo and Gilula, 1979; Lee *et al.* 1987). It will be of interest to determine whether ES cells expressing high levels of v-*src* activity have fewer gap junctions than uninfected ES cells or cells of the ICM and, if so, whether this effect can be induced in the SR2 clones by culturing the cells in the presence of heavy metals.

Chimaeric embryos derived from ES cell lines with relatively high *src* kinase activity are arrested in development at the late egg cylinder stage. Although the tissues usually found at this stage appear to be present, these embryos are morphologically abnormal, having a retarded embryonic region and a relatively expanded VYS, with hyperplasia of the VYS endoderm. It is unknown whether expression of v-*src* is directly disrupting the growth and differentiation of cell types present at this stage or whether it is perturbing their interaction with each other, both of which might result in arrest of further development.

We have previously investigated the effects of v-*src* expression on cell types of the early mouse embryo *in vitro*, using embryonal carcinoma (EC) cells. These cells serve as a model system for embryogenesis since they can be induced to differentiate *in vitro* into a variety of cell types found in the early embryo (Martin, 1980). Although c-*src* transcripts are found at a low level in parietal and visceral endoderm derived from the EC cell lines F9 and PC13 (Boulter and Wagner, 1988*b*), expression of v-*src* does not appear to induce immortalization of these cells in culture (Boulter and Wagner, 1988*a*). This would suggest that the effect of v-*src* expression on VYS endoderm *in vivo* may not be a cell-autonomous effect, but might be influenced by other cell types. In order to address this question it will be important to localise v-*src* transcripts in these chimaeric embryos, as well as the position of ES-derived cells. To this end, we are introducing a beta-galactosidase gene as a marker into the SR1 and SR2 ES cell clones, which will enable us to determine their location in chimaeric embryos.

In this paper, we demonstrate that deregulated expression of the *src* gene product during embryogenesis can reproducibly disrupt early development. Disruption of embryogenesis has also been reported in chimaeric mice expressing polyoma middle T antigen which can complex with $pp60^{c\text{-}src}$, thereby increasing its activity (Williams *et al.* 1988*a*). In this case however, the embryos appear to develop normally until after the tenth day of gestation, when they die as a result of multiple haemangiomas disrupting blood vessel formation.

The mechanisms by which $pp60^{v\text{-}src}$ disrupts early embryogenesis are unclear, but may well involve perturbation of normal patterns of cell–cell interactions, cell differentiation or cell division. Certainly $pp60^{c\text{-}src}$ has been implicated in all of these processes. A mosaic analysis of chimaeric embryos using marked ES cells should allow us to localize the effect of v-*src* expression in developmentally compromised embryos, and thereby establish the cellular basis of the disruption. This will be a prerequisite for relating molecular dysfunction to the gross abnormalities seen. This use of ES cells expressing a mutant molecular function, combined with chimaeric analysis, may serve as a general strategy for determining the function of specific genes during early mammalian development.

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