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The effect of retinoic acid pretreatment on the ability of murine embryonal carcinoma and inner cell mass cells to participate in chimaera development

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SUMMARY

Certain embryonal carcinoma (EC) cell lines can colonize the embryo following blastocyst injection or embryo aggregation, giving rise to EC–embryo chimaeras. However, such chimaeras often develop abnormally. For example, diploid P19 cells colonize the embryo readily but resulting chimaeras are usually abnormal, with persistence of tumour cells. Retinoic acid (RA) induces differentiation of EC cells to a variety of cell types *in vitro* but, in this study, it was shown that pretreatment of P19 cells with RA did not result in more normal development of P19–embryo chimaeras. The only significant effect of RA was to reduce the ability of P19 cells to participate in embryonic development at all after blastocyst injection. RA did not have a direct toxic or teratogenic effect on preimplantation mouse embryos and did not affect the ability of pluripotent embryo cells to colonize chimaeras. Therefore, RA may not be the normal inducer of differentiation in early embryogenesis.

INTRODUCTION

Murine embryonal carcinoma (EC) cells, the stem cells of teratocarcinomas derived from embryos or germ cells, can differentiate into a wide variety of cell types both *in vitro* and *in vivo* (Martin, 1980). *In vitro*, spontaneous differentiation usually gives rise to a fairly limited spectrum of cell types, but a broader range of differentiation can be induced by various agents. Retinoic acid (RA), a vitamin A derivative, is one such agent. Its effect on EC cells was first reported for the F9 murine EC cell line (Strickland & Mahdavi, 1978) which normally undergoes very limited differentiation *in vitro*, but will readily differentiate into extraembryonic endoderm in response to retinoic acid in concentrations as low as 10^{-9} M. P19, a euploid line of EC cells with a normal male karyotype (McBurney & Rogers, 1982), differentiates *in vitro* in a limited fashion spontaneously. It responds readily to retinoic acid induction by giving rise to a wider variety of cell types than does

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F9. These include muscle and neuronal cells, depending upon the conditions of induction (Jones-Villeneuve, Rudnicki, Harris & McBurney, 1983; Edwards & McBurney, 1983).

In vivo, EC cells differentiate in a disorganized fashion in teratomatous tumours. However, they can also participate in normal embryogenesis following injection into blastocysts, giving rise to genetic chimaeras with an EC cell-derived population (Mintz & Illmensee, 1975; Papaioannou, McBurney, Gardner & Evans, 1975). Not all EC cell lines demonstrate this ability to participate in chimaera development (reviewed by Papaioannou & Rossant, 1983) and the chimaeras that have been produced often exhibited sporadic and limited mosaicism. Furthermore, even diploid, karyotypically normal EC cell lines have given rise to abnormal chimaeras or animals with tumours. For example, P19 cells readily formed chimaeras after blastocyst injection but many of the chimaeras were abnormal at midgestation. At term live chimaeras contained P19 contributions to both normal tissue types and persisting tumours (Rossant & McBurney, 1982, 1983). This suggested that P19 cells were incompletely regulated by the embryo environment and retained their tumorigenicity. Some reduction in tumour growth and EC malignancy *in vivo* has been noted following retinoic acid (RA) treatment (Strickland & Sawey, 1980; Speers & Altman, 1984*a*,*b*). This was attributed to induction of EC differentiation *in vivo*. Therefore, it seemed possible that RA pretreatment might reduce the tumorigenicity of P19 cells and result in an increased percentage of normal chimaeric embryos. We tested this hypothesis and found that RA pretreatment did not improve development of P19 chimaeras but reduced the ability of P19 cells to generate chimaeras at all. In control experiments, we also tested the effect of RA exposure on the subsequent development of normal preimplantation embryos and inner cell masses (ICMs). No obvious developmental effect was observed after RA treatment of intact embryos nor was the ability of ICM cells to form chimaeras reduced or altered in any way.

MATERIALS AND METHODS

EC cells

P19 is an EC cell line with a normal male karyotype which was isolated from the primary tumour induced by grafting a 7·5 day C3H/He embryo to an adult testis (McBurney & Rogers, 1982). The P19S18 subclone (Jones-Villeneuve, McBurney, Rogers & Kalnins, 1982) and a naturally occurring variant, P19S1801A1, selected for ouabain and 6-thioguanine resistance (McBurney, Jones-Villeneuve, Edwards & Anderson, 1982), were gifts from M. W. McBurney and were used in these experiments. These clonal sublines behave like the parental line in culture in response to RA, and in chimaeras (Rossant & McBurney, 1982; Papaioannou & Rossant, 1983). The cells were maintained in culture as described previously (Rossant & McBurney, 1982).

Retinoic acid treatment of EC cells

Retinoic acid (all-*trans*, Eastman) was dissolved in DMSO at 10^{-2} M and stored as a stock solution at −70°C, for no longer than three months. Aliquots were diluted into culture medium immediately prior to each experiment. In 48 h RA treatments, RA was added to a subconfluent monolayer at 10^{-7} M for the first 24 h period. The cells were then harvested and diluted 1:4 into

a bacteriological grade Petri dish in fresh RA-containing medium and these suspension cultures were incubated for a further 24 h. In 24 h treatments, RA was added to a freshly established suspension culture.

Aggregation with 8-cell embryos

Small clumps of P19 cells resembling ICMs in size and morphology and containing 15 to 25 cells (Rossant & McBurney, 1982) were selected from treated and control suspension cultures for aggregation with 8-cell embryos. P19 cells are homozygous *Gpi-1^b/Gpi-1^b*, and 8-cell embryos were obtained from stock of homozygous *Gpi-1^a/Gpi-1^a* CD1 mice (Charles River, Quebec) maintained at Brock University. The 8-cell embryos were flushed in PB1 medium (Whittingham & Wales, 1969) containing 5 % foetal calf serum (FCS) and 5 % newborn calf serum (NBCS) from the oviducts on the afternoon of the third day after natural mating. Following zona removal by a brief incubation in acid Tyrode's solution, pH 2·5, the embryos were transferred to microdrops of α-modified MEM (GIBCO) with 5 % FCS and 5 % NBCS under oil. The P19 cell clumps were gently blown into contact with the embryos and aggregates were incubated overnight prior to transfer to the uterine horns of CD1 females on the third day of pseudopregnancy.

Blastocyst injections

Similar clumps of treated and control P19 cells were injected into *Gpi-1^a/Gpi-1^a* CD1 blastocysts and the injected blastocysts were transferred about one hour later to pseudopregnant recipients, as described previously (Rossant & McBurney, 1982).

Analysis of development of P19-containing embryos

Conceptuses were dissected from the uterus between day 9·5 and day 12·5 of development and embryo and extraembryonic yolk sac fractions were assayed for P19 contribution by glucose phosphate isomerase (GPI) electrophoretic analysis (Peterson, Frair & Wong, 1978; Rossant & Lis, 1979).

Immunofluorescence assays

Methanol-fixed outgrowths of P19 cells from suspension cultures were assayed for expression of the EC marker SSEA-1 (Solter & Knowles, 1978), utilizing anti-SSEA-1 (a gift from D. Solter) as the primary antibody and an FITC-conjugated rabbit anti-mouse IgM (Bionetics) as the secondary antibody, as described by McCue (McCue, Matthaei, Taketo & Sherman, 1983).

RA treatment of embryos

Embryos were collected at the 8-cell stage from random-bred CD1 mice and cultured in microdrops of RA-containing α-medium with 5 % FCS and 5 % NBCS for either 24 h or 48 h in bacteriological grade dishes. Some embryos were transferred to pseudopregnant recipients after 24 h culture. After 48 h culture, blastocysts were transferred to tissue culture dishes for outgrowth, or ICMs were isolated by immunosurgery (Solter & Knowles, 1975), utilizing a rabbit antiserum raised against mouse 12-day embryonic tissue and rabbit complement (CedarLane Laboratories). For these experiments, *Gpi-1^b/Gpi-1^b* embryos were cultured and their ICMs were injected into *Gpi-1^a/Gpi-1^a* host blastocysts from CD1 mice. Control ICMs were obtained from untreated blastocysts cultured for the same period of time.

Analysis of development of RA-treated embryos

Embryos treated with RA prior to transfer were recovered and examined on day 9·5 to day 16·5 of gestation. Embryos injected with RA-treated or control ICMs were recovered on day 13·5 and embryo and yolk sac samples were subjected to GPI analysis as already described. Additionally, yolk sac endoderm and mesoderm samples were enzymically separated (Gardner & Rossant, 1979) and assayed for GPI phenotype.

RESULTS

Effect of retinoic acid pretreatment on the ability of P19 cells to participate in chimaera development

The ability of RA-treated P19 cells to participate in chimaera development was tested (Table 1). In embryo aggregation experiments, P19 cells, whether treated or not, aggregated successfully with 8-cell embryos and formed morphologically normal blastocysts, but never contributed to viable chimaeras. The great majority of such aggregate embryos was resorbed prior to 9·5 days development in both control and aggregate groups. This is not a result of poor culture conditions since control embryos cultured 24 h *in vitro* prior to transfer as late morulae or early blastocysts are routinely recovered in high numbers in this laboratory with an average resorption rate of only 17% (Waters & Rossant, unpublished data). Thus, the presence of P19 cells in the aggregates is incompatible with embryonic development to 9·5 days. Histological examination revealed that embryonic death occurred soon after implantation. Decidua from 5·5 days and 6·5 days of development contained only trophoblast giant cells and a few small dispersed cells (not shown). RA-treated P19 embryo aggregates showed a slight, statistically insignificant ($\chi^2 = 2{\cdot}865$, $0{\cdot}05 < P > 0{\cdot}1$) improvement in embryo recovery over control aggregates. The few embryos that escaped early resorption following aggregation with treated or untreated P19 cells were developing normally at midgestation and no contribution by P19 cells was detected by GPI analysis of dissected conceptuses.

When treated or untreated P19 cell clumps were injected into blastocysts, the embryo recovery rate at midgestation (9 to 12 days pregnancy) was much greater than that seen following aggregation, and chimaeric embryos were recovered (Table 1). Increasing the RA pretreatment time appeared to reduce the chance of producing chimaeras following P19 injection. The difference in rate of chimaerism

Table 1. *The effect of retinoic acid pretreatment on the ability of P19 EC cells to participate in chimaera development following embryo aggregation or injection into blastocysts*

	Embryo aggregation		Blastocyst injection		
	untreated P19–embryo aggregates	24 h RA+ P19–embryo aggregates	untreated P19-injected blastocysts	24 h RA+ P19-injected blastocysts	48 h RA+ P19-injected blastocysts
Embryos transferred	48	29	34	35	36
Implants (% transferred)	33 (69)	25 (86)	30 (88)	23 (66)	32 (89)
Resorptions (% implants)	27 (82)	16 (64)	5 (17)	2 (9)	3 (9)
Embryos (% implants)	6 (18)	9 (36)	25 (83)	21 (91)	29 (91)
Chimaeras (% embryos)	0	0	8 (32)	4 (19)	0

Table 2. *GPI analysis of chimaeras recovered at 9·5 to 12·5 days gestation following injection of P19 cells into blastocysts*

Chimaera no.	Embryo	Yolk sac	Recovery day	Morphology
(A) *Chimaeras recovered after injection of untreated P19 cells*				
1			10·5	Normal
2			10·5	Normal
3			9·5	Misshapen head: tube open
4			10·5	Small, normal
5			9·5	Normal
6			12·5	Growth on head
7			10·5	Misshapen head: tube open
8			10·5	Misshapen head: tube open
(B) *Chimaeras recovered after injection of P19 cells treated for 24 h with 10^{-7} M-RA*				
1			10·5	Normal
2			9·5	Normal
3			10·5	Misshapen head: tube open
4			10·5	Small, open neural tube

The black portion of each diagram represents contribution by the P19 cells.

between 48 h pretreated and control injected blastocysts was statistically significant. No obvious differences were noted in the extent of mosaicism or the degree of abnormality in the two groups of chimaeras produced (Table 2). Therefore, RA pretreatment appears to reduce the rate of chimaerism but not the rate of abnormality.

Effect of retinoic acid treatment on P19 cells in vitro

In each experiment where P19 cells were treated with retinoic acid prior to *in vivo* assessment of their developmental potential, the effect of the treatment was also monitored *in vitro*. Untreated P19 cell clumps plated out from suspension cultures formed colonies of EC cells. A few large flat cells resembling extra-embryonic endoderm were observed at the periphery of such colonies by the second day of culture. Following 24 h RA treatment, no change in morphology or SSEA-1 expression was detected in EC clumps, but differentiation was evident by the second day after plating and cells with various morphologies proliferated.

It was not possible to identify specific cell types at this stage, but cultures left for several more days contained neuronal cells as previously reported (Jones-Villeneuve *et al.* 1982). After 48 h treatment with 10^{-7} M-RA, clumps showed changes in surface properties as indicated by their inability to aggregate with 8-cell embryos and also by reduced expression of SSEA-1. However, some cells with an EC-like morphology were readily detected two days after plating out either 24 h or 48 h RA-treated P19 clumps. These cells were usually located in small groups at the centre of the outgrowths and made up approximately 5–10 % of all cells. An immunofluorescence assay employing anti-SSEA-1 as the primary antibody revealed persistence of this EC marker on these cells, while it was not detected on cells with a differentiated morphology (not shown). Thus EC-like cells persist for at least 48 h after RA treatment, although no such cells could be detected in long-term differentiated cultures derived from RA-treated cells (unpublished data; Jones-Villeneuve *et al.* 1982).

The effect of retinoic acid exposure on the preimplantation embryo

8-cell embryos were cultured for 48 h in the presence of various concentrations of RA. Embryos were able to form morphologically normal blastocysts even in high RA concentrations (Table 3). As a more stringent test for toxicity, 8-cell embryos were exposed for 24 h to 10^{-7} M-RA. These embryos were then transferred to recipient uteri and their subsequent development analysed (Table 4). A slightly lower embryo recovery rate was noted for RA-treated embryos, but the difference from controls was not significant. Furthermore, no abnormalities were noted in 11 RA-treated embryos recovered at 9·5 to 10·5 days development, or in the remaining foetuses recovered later in gestation (13·5 or 16·5 days). Therefore,

Table 3. *Effect of continuous retinoic acid exposure on the development of 8-cell embryos*

	RA concentration (M)						
	0	10^{-7}	5×10^{-7}	10^{-6}	5×10^{-6}	10^{-5}	10^{-4}
Number of embryos treated	55	69	37	18	5	5	5
Morphologically normal blastocysts within 48 h	48	66	33	16	4	5	3

Table 4. *The effect of a 24 h exposure to 10^{-7} M-retinoic acid beginning at the 8-cell stage on subsequent* in utero *development of transferred morulae*

	Embryos transferred	Implants (% transferred)	Resorptions (% implants)	Embryos (% implants)
Controls	39	23 (59)	4 (17)	19 (83)
RA-treated embryos	42	22 (52)	7 (32)	15 (68)

Table 5. *Embryo recovery following injection of RA-treated (48 h in 10^{-7} M-RA) or untreated ICM cells into blastocysts*

	Embryos transferred	Implants	Resorptions	Embryos	Chimaeric embryos	Chimaeric yolk sacs
Control blastocysts (untreated ICMs injected)	15	11	2	9	7	8
Treated blastocysts (RA-treated ICMs injected)	23	20	3	17	14	14

RA is not toxic to normal embryo cells and toxicity was unlikely to have been the reason for the decrease in chimaera recovery following RA treatment of EC cells.

This decrease in chimaera recovery could also be a consequence of an altered potential of EC cells to colonize host conceptuses after RA treatment. To test whether RA treatment had a similar effect on embryo cells, we tested the ability of RA-treated ICMs to form chimaeras. Embryos developing from blastocysts injected with RA-treated ICM cells and from a control group of blastocysts injected with untreated cells were recovered from recipients at 13·5 days gestation and assessed for chimaerism (Table 5). All of the embryos were developing normally and most of the embryos recovered in each group were chimaeric. No differences were noted between injected RA-treated and control embryo cells in colonization of chimaeras. However, both types of ICM cells seemed to have been at some disadvantage in colonizing the foetus itself. Only two out of seven chimaeric foetuses in the control group and three out of fourteen chimaeric embryos in the RA-treated group were judged to be at least 50 % derived from the added ICM cells by GPI phenotype (Table 6). In contrast, five out of eight chimaeric yolk sacs in the control group and seven out of fourteen chimaeric yolk sacs in the treated group were judged to be at least 50 % derived from the added ICM cells. Analysis of separated yolk sac endoderm and mesoderm samples indicated that it was usually the extraembryonic endoderm fraction that contained the greater contribution from the added ICM cells. It seems probable that the preference for the extraembryonic lineage in both groups of chimaeras reflects a position effect. Injected ICM cells may be more likely to form the outer cell populations of the chimaeric ICM and hence contribute to primitive endoderm derivatives.

DISCUSSION

Although EC cells such as P19 respond very readily to retinoic acid by differentiating *in vitro*, this study has demonstrated that retinoic acid treatment does not improve development of P19 cells in embryos *in vivo*. No chimaeras were recovered following aggregation of clumps of untreated P19 cells with embryos. Indeed, very few viable embryos were recovered at all. Histological examination revealed that the majority of implanted conceptuses died soon after implantation.

Table 6A. *GPI analysis of foetuses recovered at 13·5 days gestation following injection of untreated ICMs into blastocysts*

Conceptus no.	Foetus	Yolk sac	Yolk sac endoderm	Yolk sac mesoderm
1				
2				
3			I.S.	
4			I.S.	
5				
6			I.S.	
7			I.S.	
8				
9				

The black portion of each diagram represents contribution by the added ICM cells.
I.S.: insufficient sample for GPI analysis.

The absence of any organized ICM derivatives in these implants is reminiscent of the results obtained when P19 cells were injected into trophectoderm vesicles and returned to the uterus (Rossant & Papaioannou, 1985). This suggests that aggregation of P19 cells with cleavage stage embryos produced too large a contribution of the EC cells to the ICM for regulation to occur. Aggregation of other EC cell lines to 8-cell embryos has resulted in higher rates of chimaerism but also in higher rates of abnormality than comparable blastocyst injections (Stewart, 1982; Fujii & Martin, 1983; Waters & Rossant, in preparation). Previous results have shown that P19 is very efficient at colonizing the host embryo (Rossant & McBurney, 1982) and so it is not surprising that the added advantage given to the EC cells by embryo aggregation results in complete disorganization of the embryo. Reduction in the proportion of EC cells to embryo cells might improve the development of P19–embryo aggregates (Stewart, 1982). Pretreatment of the P19 EC cells with RA for 24 h did not improve the development of the aggregates. Again most conceptuses were completely disorganized soon after implantation. Undifferentiated EC cells were shown to persist in *in vitro* outgrowths of RA-treated EC clumps for at least two days. In later cultures, EC cells are probably overgrown by differentiated derivatives but their proliferation in the embryonic environment early in development may have resulted in developmental failure.

To increase the likelihood of obtaining chimaeras, blastocyst injection experiments were also performed. These experiments also allowed longer pretreatment of the EC cells with RA. EC cells pretreated for 48 h with RA showed altered surface properties which prevented their aggregation with cleavage stage embryos,

Table 6B. *GPI analysis of foetuses recovered at 13·5 days gestation following injection of ICMs from embryos cultured 48 h in 10^{-7} M-RA into blastocysts*

Conceptus no.	Foetus	Yolk sac	Yolk sac endoderm	Yolk sac mesoderm
1				
2				
3				
4				
5				
6				
7			I.S.	I.S.
8				
9				
10				
11				I.S.
12			I.S.	
13				
14				
15				
16				
17				

The black portion of each diagram represents contribution by the RA-treated ICM cells.

but the same cells could be successfully injected into blastocysts. As expected from a previous study (Rossant & McBurney, 1982), some chimaeras were recovered after injection of untreated P19 cells into blastocysts. However, again as reported previously, some of these chimaeras exhibited abnormalities. The rate of chimaera production was lower in the control group than previously reported, but was within the range of variation in the rate of chimaera production noted originally. RA treatment of P19 cells before injection into blastocysts did not improve either the extent of their contribution to or the normality of development of the resulting chimaeras. The only significant effect of RA pretreatment was a reduction in the ability of the P19 cells to contribute in any detectable manner to embryonic development after blastocyst injection. This decrease in the rate of chimaera recovery following RA treatment could have been due to toxicity of RA treatment

to the P19 cells, a decreased capacity for integration of the P19 cells with the host ICM, or induced differentiation of the P19 stem cells to cell types inappropriate to an early stage of development. These cell types would probably not persist and hence not contribute to embryonic development. The effect of RA treatment on normal embryo cells was compared with its effect on EC cells to try to assess which of these explanations was most likely.

A retinoic acid exposure comparable to that effective in inducing differentiation of EC cells did not affect the subsequent development of preimplantation embryos in any obvious manner. Limited data showed that 8-cell embryos were capable of developing into morphologically normal blastocysts *in vitro* even in very high RA concentrations known to be toxic for some EC cells (Matthaei, Andrews & Bronson, 1983). A more extensive series of experiments was performed on embryos treated with RA at a concentration of 10^{-7} M, as used for the EC cells. 8-cell embryos treated for 24 h at this concentration were capable of normal development *in utero* as well as *in vitro*. Retinoic acid is known to be a potent teratogen that induces a wide variety of defects in rodents and other laboratory animals, including limb defects (Kwasigroch, Skalko & Church, 1984) and neural tube defects (Dempsey & Trasler, 1983) following administration to the mother during pregnancy. While the sample examined in this study was quite small, no abnormalities were noted in the embryos and foetuses treated with RA prior to implantation. When embryos were cultured 48 h in 10^{-7} M-RA, a highly effective dose for inducing differentiation of EC cells, no change was seen in the percentage of chimaeras or in the pattern of colonization of chimaeras following injection of the RA-treated ICMs into blastocysts compared with a control set of chimaeras produced by injection of untreated ICMs. RA pretreatment of ICM cells thus affected neither their ability to integrate into the host ICM nor their ability to differentiate into normal embryonic tissues at the correct stage of development.

Thus, it appears that RA does not affect pluripotent embryonic cells in a manner similar to its effects on EC cells. These results, therefore, shed little light on why RA-treated EC cells fail to form chimaeras, other than to suggest that generalized toxicity is not the reason for failure. It seems most likely that the differentiated cell types that obviously develop from RA-treated EC cells *in vitro* are simply not capable of either integration or further development in the embryonic environment. This could only be tested by following the fate of RA-treated P19 cells shortly after implantation using *in situ* marker systems. If treated cells could be introduced experimentally into the embryo later in development either *in vitro* (Beddington, 1982) or *in vivo* (Jaensich, 1985), they might be able to colonize normal tissues.

While the mechanism of RA-induced differentiation of EC cells is largely unknown at present, a cytoplasmic RA-binding protein has been implicated (Jetten & Jetten, 1979; Schindler, Matthaei & Sherman, 1981). This binding protein has been detected in the day 10 mouse embryonic limb bud (Kwarta, Kimmel, Kimmel & Slikker, 1984) but whether it is present in the pluripotent cells of the very early

embryo is unknown. The results from this study, which show no effect of exogenous RA on normal embryonic development, suggest that the binding protein may not be present and/or that retinoic acid may not be the signal for differentiation in the early stages of normal embryonic development. In EC cells, RA might trigger differentiation events normally signalled by other means in the early embryo.

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REFERENCES

BEDDINGTON, R. S. P. (1982). An autoradiographic analysis of tissue potency in different regions of the embryonic ectoderm during gastrulation in the mouse. *J. Embryol. exp. Morph.* **69**, 265–291.

DEMPSEY, E. & TRASLER, D. (1983). Early morphological abnormalities in splotch mouse embryos and predisposition to gene- and retinoic acid-induced neural tube defects. *Teratology* **28**, 461–472.

GARDNER, R. L. & ROSSANT, J. (1979). Investigation of the fate of 4.5d post coitum mouse inner cell mass cells by blastocyst injection. *J. Embryol. exp. Morph.* **52**, 141–152.

EDWARDS, M. K. S. & MCBURNEY, M. W. (1983). The concentration of RA determines the differentiated cell types formed by a teratocarcinoma cell line. *Devl Biol.* **98**, 187–191.

FUJII, J. J. & MARTIN, G. R. (1983). Developmental potential of teratocarcinoma stem cells *in utero* following aggregation with cleavage stage mouse embryos. *J. Embryol. exp. Morph.* **74**, 79–96.

JAENISCH, R. (1985). Mammalian neural crest cells participate in normal development when microinjected into postimplantation mouse embryos. *Nature, Lond.* **318**, 181–183.

JETTEN, A. M. & JETTEN, M. A. R. (1979). Possible role of RA binding protein in retinoid stimulation of embryonal carcinoma cell differentiation. *Nature, Lond.* **278**, 180–182.

JONES-VILLENEUVE, E. M. W., MCBURNEY, M. W., ROGERS, K. A. & KALNINS, V. I. (1982). Retinoic acid induces EC cells to differentiate into neurons and glial cells. *J. Cell Biol.* **94**, 253–262.

JONES-VILLENEUVE, E. M. W., RUDNICKI, M., HARRIS, J. & MCBURNEY, M. W. (1983). Retinoic acid-induced neural differentiation of EC cells. *Mol. cell. Biol.* **3**, 2271–2279.

KWARTA, R. JR, KIMMEL, C., KIMMEL, G. & SLIKKER, W. JR (1984). Identification of the cellular retinoic acid binding protein (cRABP) within the embryonic mouse (CD-1) limb bud. *Teratology* **29**, A43.

KWASIGROCH, T., SKALKO, R. & CHURCH, J. (1984). Mouse limb bud development in merged culture: quantitative assessment of the effects of *in vivo* exposure to retinoic acid. *Teratogenesis, Carcinogenesis, and Mutagenesis* **4**, 311–326.

MATTHAEI, K., ANDREWS, P. W. & BRONSON, D. L. (1983). Retinoic acid fails to induce differentiation in human teratocarcinoma cell lines that express high levels of a cellular receptor protein. *Expl Cell Res.* **143**, 471–474.

MARTIN, G. R. (1980). Teratocarcinomas and mammalian embryogenesis. *Science* **209**, 768–776.

MCBURNEY, M. W., JONES-VILLENEUVE, E. M. V., EDWARDS, M. K. S. & ANDERSON, P. J. (1982). Control of muscle and neuronal differentiation in a cultured EC cell line. *Nature, Lond.* **299**, 165–167.

MCBURNEY, M. W. & ROGERS, B. J. (1982). Isolation of male embryonal carcinoma cells and their chromosomal replication patterns. *Devl Biol.* **89**, 503–508.

MCCUE, P. A., MATTHAEI, K. I., TAKETO, M. & SHERMAN, M. I. (1983). Differentiation defective mutants of mouse embryonal carcinoma cell: response to hexamethylene bisacetamide and RA. *Devl Biol.* **96**, 416–426.

MINTZ, B. & ILLMENSEE, K. (1975). Normal genetically mosaic mice produced from malignant teratocarcinoma cells. *Proc. natn. Acad. Sci. U.S.A.* **72**, 3585–3589.

PAPAIOANNOU, V. E. & ROSSANT, J. (1983). Effects of the embryonic environment on proliferation and differentiation of embryonal carcinoma cells. *Cancer Surveys* **2**, 165–183.

PAPAIOANNOU, V. E., MCBURNEY, M. W., GARDNER, R. L. & EVANS, M. J. (1975). Fate of teratocarcinoma cells injected into early mouse embryos. *Nature, Lond.* **258**, 70–73.

PETERSON, A. C., FRAIR, P. M. & WONG, G. G. (1978). A technique for detection and relative quantitative analysis of glucosephosphate isomerase isozymes from nanogram tissue samples. *Biochem. Genet.* **16**, 681–690.

ROSSANT, J. & LIS, W. T. (1979). The possible dual origin of the ectoderm of the chorion in the mouse embryo. *Devl Biol.* **70**, 249–254.

ROSSANT, J. & MCBURNEY, M. W. (1982). The developmental potential of a euploid male teratocarcinoma cell line after blastocyst injection. *J. Embryol. exp. Morph.* **70**, 99–112.

ROSSANT, J. & MCBURNEY, M. W. (1983). Diploid teratocarcinoma cell lines differ in their ability to differentiate normally after blastocyst injection. In *Teratocarcinoma Stem Cells, Cold Spring Harbor Conferences on Cell Proliferation*, vol. 10, pp. 625–633.

ROSSANT, J. & PAPAIOANNOU, V. E. (1985). Outgrowth of embryonal carcinoma cells from injected blastocysts *in vitro* correlates with abnormal chimaera development *in vivo*. *Expl Cell Res.* **156**, 213–220.

SCHINDLER, J., MATTHAEI, K. I. & SHERMAN, M. I. (1981). Isolation and characterization of mouse mutant embryonal carcinoma cells which fail to differentiate in response to retinoic acid. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1077–1080.

SOLTER, D. & KNOWLES, B. B. (1978). Monoclonal antibody defining a stage-specific mouse embryonic antigen. *Proc. natn. Acad. Sci. U.S.A.* **75**, 5565–5569.

SOLTER, D. & KNOWLES, B. B. (1975). Immunosurgery of mouse blastocyst. *Proc. natn. Acad. Sci. U.S.A.* **72**, 5099–5101.

SPEERS, W. & ALTMANN, M. (1984*a*). Chemically induced differentiation of murine embryonal carcinoma *in vivo*: Transplantation of differentiated tumors. *Cancer Res.* **44**, 2129–2135.

SPEERS, W. & ALTMANN, M. (1984*b*). Malignant neoplasms of differentiated cells occurring after retinoic acid treatment of murine embryonal carcinomas *in vivo*. *Cancer Res.* **44**, 2136–2143.

STEWART, C. L. (1982). Formation of viable chimaeras by aggregation between teratocarcinomas and preimplantation mouse embryos. *J. Embryol. exp. Morph.* **67**, 167–179.

STRICKLAND, S. & SAWEY, M. J. (1980). Studies on the effects of retinoids on the differentiation of teratocarcinoma stem cells *in vitro* and *in vivo*. *Devl Biol.* **78**, 76–85.

STRICKLAND, S. & MAHDAVI, V. (1978). The induction of differentiation in teratocarcinoma stem cells by retinoic acid. *Cell* **15**, 393–403.

WHITTINGHAM, D. G. & WALES, R. G. (1969). Storage of two-cell mouse embryos *in vitro*. *Aust. J. biol. Sci.* **22**, 1065–1068.

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