Failure of mouse primordial germ cells to proliferate on fibroblasts from *Steel* mutant mice *in vitro*

MIA BUEHR[1], ALISON PEARCE-KELLY and ANNE MCLAREN[2*]

Medical Research Council Mammalian Development Unit,
Wolfson House, University College London, UK.

The Steel gene (Sl) encodes a growth factor (stem cell factor, SCF). Mice homozygous for Sl are deficient in germ cells: Addition of SCF to the culture medium of primordial germ cells grown in vitro improves survival and may also increase proliferation. We therefore compared the ability of fibroblasts taken from normal or homozygous Sl embryos to support the growth of primordial germ cells in vitro. Germ cells attached equally well to a normal or a homozygous Sl lung fibroblast monolayer, but after a further 24 hours in culture the number of germ cells on the normal fibroblasts had increased significantly, while the number on Sl/Sl fibroblasts had declined. Proliferation rate of germ cells, as judged by BUdR incorporation, was significantly greater on the monolayer grown from normal embryonic fibroblasts. We conclude that Sl/Sl fibroblasts, presumbly because or their genetic deficiency in SCF production, cannot adequately support the proliferation or the even survival of primordial germ cells in vitro.

Key words: *Embryonic fibroblasts, primordial germs cells, stem cell factor.*

INTRODUCTION

Mouse embryos homozygous for the *Steel* (*Sl*) gene die of anaemia before birth, because their haemopoietic stem cells are defective. Their gonads contain few any germ cells, because the primordial germ cells fail to reach their target. Homozygotes for weak alleles of *Sl* that survive beyond birth are therefore sterile. They are also white, owing to lack of pigment stem cells (melanoblasts).

SCF exists in both a soluble and a membrane-bound form. Extracellular matrix derived from *Sl/Sl* embryos differs from control matrix in failing to support melanoblast differentiation (11), but the membrane-bound product of the *Steel* gene appears to be required more for melanoblast survival than for differentiation (14). When mouse primordial germ cells are cultured *in vitro* with or without a feeder layer, soluble SCF has been shown to enhance survival, but it does not prolong survival nor does it appear to stimulate proliferation (2, 5). However, in combination with other growth factors, SCF (16), and in particular its membrane-bound form, has been reported to be required for proliferation (8).

In the present paper we report that cell monolayers derived from homozygous *Steel* embryos are inferior to control monolayers in supporting both survival and proliferation of primordial germ cells during their migratory phase.

[1] **Present address:** Institut for Molekylaer Biologi, Aarhus Universitet, C. F. Mollers Alle, DK.8000 Aarhus C, Denmark
[2] Present address: Wellcome / CRC Institute, Tennis Court Road, Cambridge, UK
* **Correspondence to:** Dr Anne McLaren, Wellcome / CRC Institute, Tennis Court Road, Cambridge CB2 1Q2 1QR, UK. Fax: (44-223) 334-089.

METHODS

Cell monolayers were grown in 3.5 cm plastic dishes, using STO cells, or lung cells from normal or *Sl/Sl* embryos 16-17 days *post coitum*. *Sl/Sl* embryos were distinguished from their litter mates by the pale colour and size of the liver. Litter mates could be either *Sl*/+ or +/+ in genetic constitution; these could not be distinguished phenotypically before birth, and were both classed as "normal" embryos.

When the cells had been grown to confluence, a ring of agar was poured into the dish, leaving a 2 cm diameter well in the middle into which cells from 2 dorsal mesenteries and associated tissues (see Fig 1), dissected from MF1 embryos 10.5 days *post coitum* and dissociated in EDTA, were seeded. For the BUdR incorporation experiment, only a single dorsal mesentery per well was used. The culture medium was Dulbecco's modification of Eagle's medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 0.1 mm mercaptoethanol, 100 units/ml penicillin and 0.05 mg/ml streptomycin. After 24 h the medium was drawn off and the cultures fixed in cold 80% ethanol. Staining for alkaline phosphatase activity (4) enabled germ cells to be distinguished from their accompanying somatic cells, as well as from cells of the underlying monolayer. After staining, the agar was removed and the stained cells counted. Confining the germ cells to a 2 cm diameter circle made scanning easier and avoided loss of cells at the edge of the dish. For the BUdR incorporation experiment, BUdR was added to the culture medium after 24 hours according to the instructions supplied with the Cell Proliferation Assay Kit (Amersham), and the cells cultured for a further 6 hours. The plates were fixed in cold 19:1 acetic acid-ethanol, stained for alkaline phosphatase activity, and then processed according to the procedure recommended by Amersham.

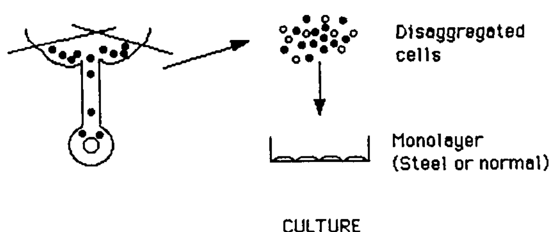

Fig 1: At 10.5 days *post coitum*, the primordial germ cells are migrating from the hind gut, up the dorsal mesentery and round the coelomic angles into the two nascent genital ridges. These regions of the embryo were dissected out, the tissues were dissociated and the resulting cell suspension, containing both primordial germ cells (black circles) and somatic cells (white), was seeded onto a monolayer derived from *Steel* or normal embryonic fibroblasts.

RESULTS AND DISCUSSION

Embryonic lung fibroblasts were selected to form the monolayer, because *Sl* is known to be strongly expressed in the lung of normal embryos (1). Disaggregated dorsal mesenteries and their associated tissues taken from mouse embryos 10.5 days *post coitum* contain migrating primordial germ cells as well as somatic cells. The germ cells grow well if seeded onto a STO cell monolayer (3), and on a monolayer of lung fibroblasts taken from normal embryos the number of germ cells remaining after 24 hours proved to be not signicantly less than on a STO cell monolayer (Table I). When the monolayer was derived from fibroblasts taken from *Sl/Sl* embryos, however, significantly fewer primordial germ cells were detected after 24 hours than in either of the other two groups (Table I).

Table I

Primordial germ cells derived from dissociated dorsal mesenteries of mouse embryos 10.5 days *post coitum*, cultured for 24 h on cellular monolayers and counted after histochemical staining for alkaline phosphatase activity

	No. of cultures	No. of primordial germ cells identified after 24 h (mean ±s.e.)	Significance of difference
STO cells	5	307 ±49	
			P > 0.05
Normal fibroblasts	15	228 ±33	
			P < 0.001
S1/S1 fibroblasts	18	93 ±13	

Since initial cell counts indicated that we were seeding our culture wells with up to 1000 primordial germ cells, there was clearly a large initial loss of cells, attributable to cell death and/or failure to attach to the feeder layer. To determine whether the reduced number of germ cells present after 24 h on *Sl/Sl* monolayers resulted from failure of attachment or failure of subsequent survival or proliferation, we allowed our cultures 5 h for cell attachment, washed off the unattached cells (many of these were fragmented and degenerating), and counted germ cells in half the cultures at 5 h and the remainder at 24 h, as before. The results (Table II) show that there is no significant difference in the number of primordial germ cells attaching to Sl/Sl and normal fibroblasts.

Since on normal fibroblasts the number of germ cells almost doubled between 5 and 24 h (Table II; $P<0.05$), cell proliferation must have occurred *in vitro*. The significantly reduced number of germ cells remaining on the Sl/Sl monolayer suggests a deficiency of some factor (presumably SCF) required for germ cell survival. Whether the proliferation rate of the germ cells was also reduced in these conditions was not clear.

To clarify the above point, we carried out a further experiment, in which primordial germ cells and their associated somatic cells were seeded onto fibroblast monolayers derived from either normal or *Sl/Sl* embryos, and cultured for 24 h as before. BUdR was then added to the culture medium and the cells were cultured for a further 6 h. At the end of the culture period, they were stained for alkaline phosphatase activity and processed for BUdR incorporation, as a measure of cell proliferation. The results (Table III) show that the rate of germ cell proliferation was significantly lower on the *Sl/Sl* than on the normal fibroblasts monolayer, but that some proliferation was still continuing. This

Table II

Primordial germ cells attached to the feeder layer after 5 h, and after a further 24 h culture period

Source of monolayer	No. of primordial germ cells (mean ± s.e.) identified after: 5 h	29 h	Difference	Significance of difference
Normal fibroblasts	184 ± 23 (6)	329 ± 46 (10)	+145 ± 51	P < 0.02
Sl/Sl fibroblasts	166 ± 28 (10)	99 ± 14 (8)	–67 ± 31.3	P < 0.05

Number of cultures in parenthesis.

Table III

The proportion of primordial germ cells labelled with BUdR as an index of proliferation rate, following culture for 24 h on a monolayer of lung fibroblasts taken from either normal or homozygous *Steel* embryos

Source of monolayer	No. of cultures	Total no. of primordial germ cells	Mean % labelled (± se)	Significance of difference
Normal fibroblasts	18	1264	39.6 ± 2.17	P<0.05
Sl/Sl fibroblasts	6	110	25.6 ± 6.04	

proliferation may have been supported by SCF produced by the somatic cells from dissociated mesenteries, seeded onto the monolayer along with the primordial germ cells (see Fig 1).

Our results suggest that the *Steel* fibroblast monolayer cannot adequately support either survival or proliferation of primordial germ cells during their migratory phase. Since the *Steel* and normal monolayers are genetically similar except at the *Steel* locus, SCF appears to be required both for proliferation and for survival. Other growth factors are required also: in our system these will be provided by both *Steel* and normal monolayers, but under other conditions they may be lacking. This may explain why some studies conclude that SCF is required only for survival of germ cells (2,5), while others regard it as necessary for their proliferation (8). For long-term survival and proliferation of germ cells *in vitro* ("immortalization"), a combination of SCF, leukaemia inhibitory factor (LIF), and basic fibroblast growth factor (bFCF) is effective (10, 13).

It could be that germ cell proliferation cannot occur without the activation of the SCR/c-kit signal tranduction pathway (6, 15, 17). c-kit expression has been reported at 7.5 days (7); SCF expression has been detected along the germ cell migratory pathway by 9 days but not earlier (9). In an earlier study (4), we saw evidence of mitosis in mouse primordial germ cells *in vivo* at 8 days *post coitum*, but not earlier. The clump of tissue staining diffusely for alkaline phosphatase activity remained at approximately the same size between 7 and 8 days; on the other hand, within that clump the number of putative primordial germ cells showing a spot of intense alkaline phosphatase activity increased from about 10 to about 100 in the same period. We did not know whether to interpret this increase as evidence of proliferation or cell differentiation, and we were therefore unable to come to any conclusion as to the likely size of the initial germ cell pool. It may be that our failure to detect mitosis before 8 days represents a real absence of germ cell proliferation prior to this time, owing to absence of SLF. This would imply that the initial germ cell pool consists of about 100 cells, the number present at 8 days, and would bring the germ cell lineage into line with somatic cell lineages, for which an initial pool size of 100-200 cells has been postulated (12).

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