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Genital ridges exert long-range effects on mouse primordial germ cell numbers and direction of migration in culture

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Summary

The functional gametes of all vertebrates first arise in the early embryo as a migratory population of cells, the primordial germ cells (PGCs). These migrate to, and colonise, the genital ridges (GR) during the early organogenesis period, giving rise to the complete differentiating gonad. PGCs first become visible by alkaline phosphatase staining in the root of the developing allantois at 8.5 days post coitum (dpc). At 9.5 dpc they are found in the wall of the hind-gut and, during the following three days, they migrate along the hind-gut mesentery to the dorsal body wall, and then to the genital ridges. By 12.5 dpc, the great majority of PGCs have colonised the genital ridges. During this period the number of PGCs increases from less than 100 to approximately 4000.

In a previous paper (Donovan *et al.* 1986), we showed that 10.5 dpc PGCs can be explanted from the hind-gut mesentery, and will spread and migrate on feeder cell layers. We showed also that the intrinsic ability of PGCs to spread and migrate changes as they colonise the genital ridges. In this paper, we examine extrinsic factors that control PGC behaviour *in vitro*. Using PGCs taken from 8.5 dpc embryos, at the beginning of their migratory phase, we show that culture medium conditioned by 10.5 dpc genital ridges causes an increase in the number of PGCs in these cultures. We also show that PGCs migrate towards 10.5 dpc genital ridges in preference to other explanted organs.

These experiments show that genital ridges exert long-range effects on the migrating population of PGCs. They also suggest that tropic factors released from the genital ridges play a role in PGC guidance.

Key words: mouse germ cells, migration, proliferation.

Introduction

In all vertebrates and many invertebrates, the gametes first arise in the early embryo as a small migratory population of cells, the primordial germ cells (PGCs). These migrate through the tissues of the embryo and colonise the genital ridges, thus forming the complete gonad (see Nieuwkoop and Sutasurya, 1979 for review). In mouse embryos, PGCs are first identifiable due to their content of alkaline phosphatase at 8.5 dpc, in the root of the developing allantois, posterior to the primitive streak. At 9.5 dpc, approximately 350 PGCs can be identified in the wall of the hind-gut. During the following three days, the PGCs migrate along the hind-gut mesentery to the dorsal body wall, and then laterally to the genital ridges, which provide the somatic tissue of the gonad. During this time, PGC numbers increase to 1000 at 10.5 dpc, 4000 at 12.5 dpc, and eventually to a maximum of 20 000–25 000 at 14.5 dpc, when the male PGCs enter mitotic arrest and the female PGCs enter meiosis (see Heath, 1978; Eddy *et al.* 1981; Wylie *et al.* 1985, for reviews).

The mechanisms that maintain the PGCs during their migration, and control their proliferation and guidance, are major unsolved questions in this field. There is little previous work on PGC proliferation, although studies on various cell types suggest that proliferation is controlled by a balance between different growth factors (see Sporn and Roberts, 1988; Mercola and Stiles, 1988 for reviews). The control of PGC guidance has been more extensively studied, although results are more fragmentary and have led to different proposals. There is evidence that PGCs interact with the extracellular matrix around them as they migrate. Fibronectin surrounds migrating PGCs in amphibians (Heasman *et al.* 1981), birds (England, 1983) and mice (Wylie *et al.* 1985), and plays a role in PGC adhesion in *Xenopus* (Heasman *et al.* 1981) and mouse (Alvarez-Buylla and Merchant-Larios, 1986; De Felici and Dolci, 1989). In *Xenopus* embryos, the substratum for migration *in vivo* is visibly aligned in the direction of travel (Wylie *et al.* 1979), although such alignment can only cause polarity, leaving open the question of direction.

Chemotropic agents are well known to play a role in the migration of several cell populations, of different lineages. In the developing nervous system, neurotropic agents are released by target tissues and influence the migration of outgrowing nerve processes (see Davies,

1987 for review). Diffusible agents also control the direction of movement of mammalian neutrophils and slime mould amoebocytes (both reviewed in Devreotes and Zigmund, 1988). There is some evidence for such tropic agents in PGC migration. Grafting experiments in birds (Dubois, 1968; Rogulska *et al.* 1971), and amphibians (Gipouloux, 1970; Giorgi, 1974) give indirect evidence for these. However, these experiments are difficult to interpret. The action of tropic factors can only be shown unambiguously *in vitro*, where the cells can be shown to respond directly to the target tissue in preference to other tissues.

In previous papers (Donovan *et al.* 1986; Stott and Wylie, 1986), we showed that mouse PGCs could be isolated, and that they would spread and migrate on feeder layers *in vitro*. In the experiments reported here, we wished to test whether PGCs on their migratory route are influenced in their behaviour by the tissues around them, using *in vitro* assays. We show that PGCs can be isolated at the beginning of their migratory phase, from 8.5 dpc embryos, and that isolated genital ridges release factors that influence both the numbers and the direction of migration of PGCs *in vitro*.

Materials and methods

Preparation of PGCs, genital ridges and feeder cell layers

Mature MF1 females were caged with breeding males and examined for vaginal plugs. The day on which a plug was identified was considered as 0 dpc. 8.5 and 10.5 dpc embryos were dissected from uteri in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS). The PGC-containing fragments of 8.5 dpc embryos (allantois and posterior primitive streak) were collected in PBS, washed several times and triturated to produce a single cell suspension. This was layered on top of a column of Dulbecco-modified Eagle's medium containing 15% fetal calf serum, 4 mM-glutamine and 1 mM-pyruvate (DMEM). After 5 min, the top two thirds of the column was removed. This contained a PGC suspension free of aggregates and debris.

To produce organ-conditioned media, 10.5 dpc genital ridges (GR), limb buds (LB), and hind-gut mesenteries (Mt) were isolated and washed several times in DMEM. They were then cultivated in 24-well plates (10 organs, or 10 pairs of GR per well and per ml of DMEM) for 36 h. The conditioned medium was centrifuged (3000 *g*, 15 min) to remove debris, and stored frozen. Before use, conditioned media were diluted 1:1 with DMEM. The amounts of tissue used to condition the media were compared by dissociating 10 organs of each type (10 pairs in the case of GRs) by trituration in PBS+0.2% EDTA. Cells were centrifuged at 100 *g* for 5 min, resuspended in 1 ml DMEM and counted (see Fig. 1D). Subsequently, 1 ml of DMEM was conditioned either by 12 Mt or 6 LB, in order to allow the medium to be conditioned by the same numbers of cells.

For feeder layers, STO cells were grown and irradiated as described previously (Donovan *et al.* 1986). Monolayers were produced by adding 150 μl of an irradiated STO cell suspension (2.5×10^5 cells ml^{-1}) in DMEM to the culture well, and leaving overnight at 37°C in 10% CO_2 to form a confluent monolayer.

Assay of PGC numbers

50 μl of an 8.5 dpc PGC suspension (representing between 0.3 and 1 embryo equivalent) was added to each well of a 96-well plate containing STO cell feeder layers in 100 μl of either DMEM or organ-conditioned DMEM. These cultures were kept at 37°C in 10% CO_2 for several days, and fed every day with fresh medium. After fixation and staining for alkaline phosphatase (Donovan *et al.* 1986), the PGCs in each well were counted, and the number in conditioned medium cultures expressed as the percentage of the number found in DMEM alone (considered as 100%). Results are given as the means (and standard errors) of 10 replicate cultures.

Assay for tropic factors

A diagram is shown in Fig. 2A. Three aligned wells were cut in Petri dishes with a gridded bottom (Nunclon) containing 10 ml of 7.5% agarose (dotted areas): the central well was seeded with STO cells and was kept overnight at 37°C in 10% CO_2, to obtain a confluent monolayer, and the lateral wells were then filled with DMEM. A suspension of 8.5 dpc PGCs (2 or 3 embryo equivalents per well) was seeded into the central well. Test tissues (GR, Mt and LB), explanted from 10.5 dpc embryos, were put in the lateral wells and the cultures left undisturbed at 37°C for 24 h. Each type of test was performed on a total of 12 replicate dishes. Cultures were then fixed in 4% paraformaldehyde and stained for alkaline phosphatase as described above. PGCs were counted separately in the three zones of the central well (see Fig. 2A), and given as a percentage of the total number of PGCs present in the entire central well. Each assay is presented as a group of 3 bars representing the percentage of total PGCs in end-zone A, middle zone and end-zone B, respectively. Each outer bar is shaded according to the content of the lateral well nearest to it. Error bars indicate the standard errors of the means of 12 replicate samples in each case.

Fluorescent labelling of the somatic cells in PGC suspensions

These were stained with the carbocyanine dye, tetramethyl isothiocyanate coupled di-I (Molecular probe) at a concentration of 40 $\mu g\,ml^{-1}$ in DMEM. This dye, insoluble in water, incorporates into the lipid bilayer where it is freely mobile and can be seen in cultured living cells under the fluorescence microscope for up to three weeks (Honig and Hume, 1989).

Results

Genital ridges increase PGC numbers in culture

PGCs taken from 8.5 dpc embryos and cultured on STO feeder layers in DMEM adhere within 3 h. By that time, about 25% of the PGCs show a phenotype characteristic of motile cells (Fig. 1A). They are elongated and possess extended leading lamellae, uropods and retraction fibres. By 24 h, 50% of the PGCs have this appearance. During the following days, PGC number increases, reaching a maximum between 3 and 5 days. After this, PGC numbers decrease dramatically, and after the 7th day none can be found in these cultures (Fig. 1C).

We compared the time-courses of PGC numbers for 7 days, either in DMEM alone, or conditioned by one of three embryonic tissues: genital ridges (GR), limb buds (LB) or hind-gut mesentery (Mt). The results are shown in Fig. 1B and 1C. GR-conditioned medium had

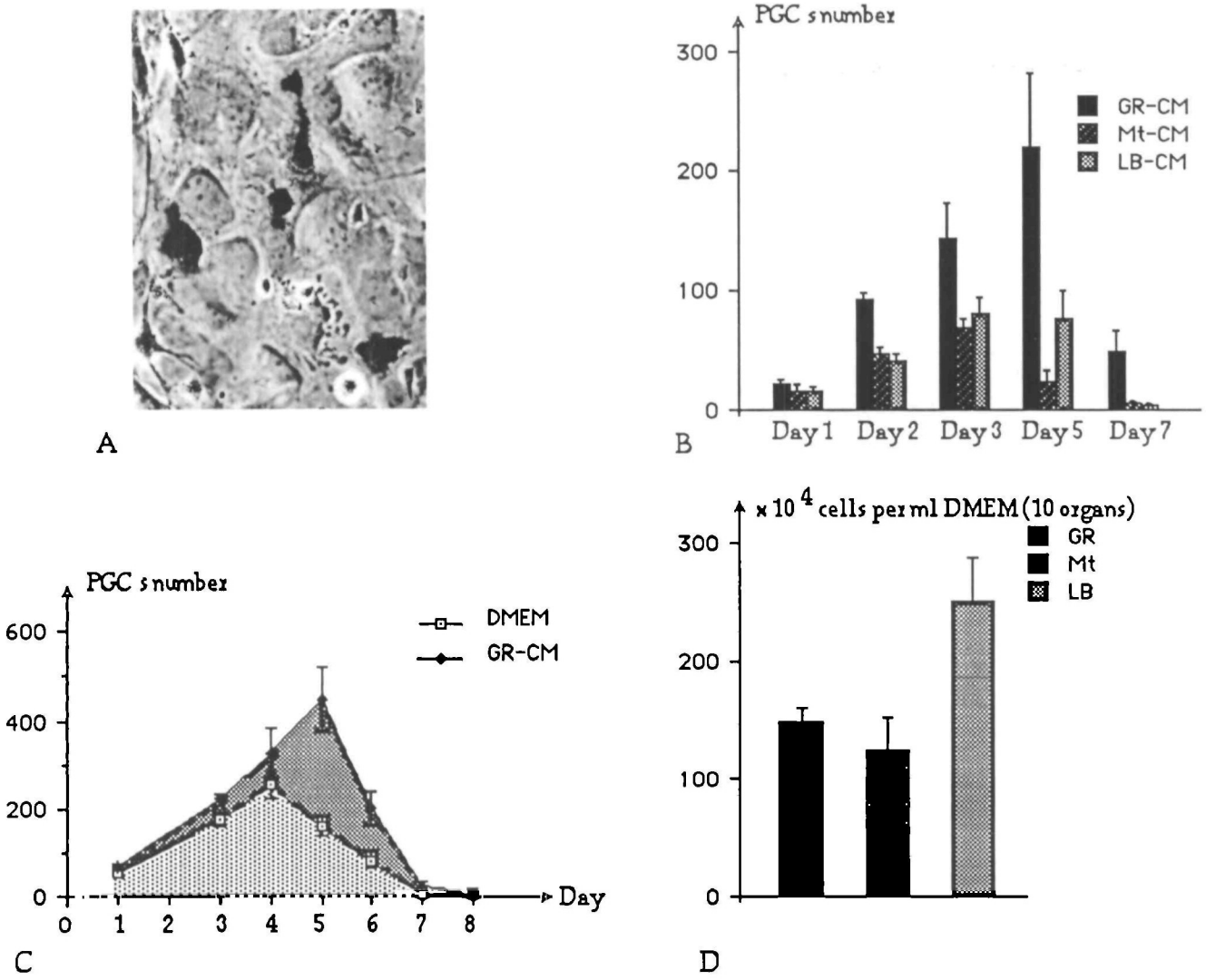
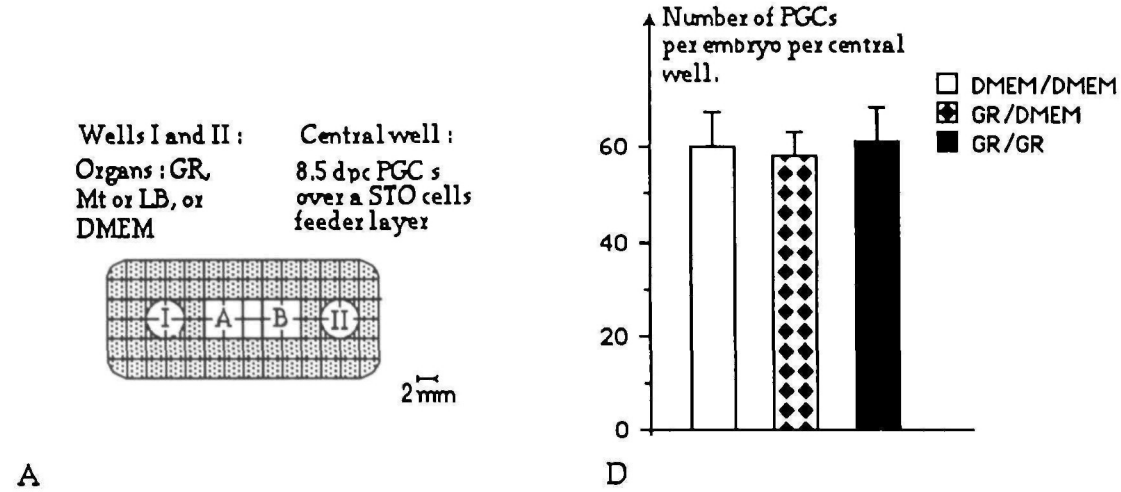


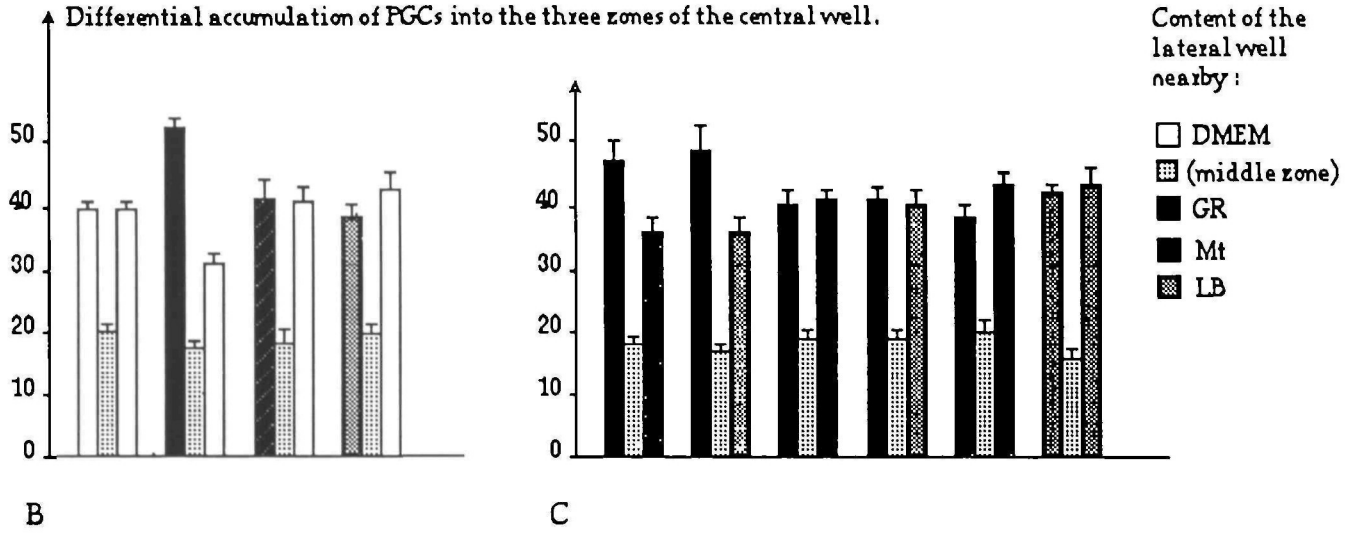
Fig. 1. (A) 8.5 dpc PGCs on a STO feeder layer, stained for alkaline phosphatase after 2 days in culture (×225). (B) Effect of organ-conditioned media on PGC numbers in culture. Media were conditioned with genital ridges (GR), limb buds (LB) or hind-gut mesentery (Mt). (C) Comparison of PGCs number in culture with GR- conditioned medium or DMEM over an 8-day period. (D) Comparison of the amount of tissue used to condition the media. After this assay, DMEM was conditioned with appropriate numbers of organs to allow conditioning by roughly equal numbers of cells.

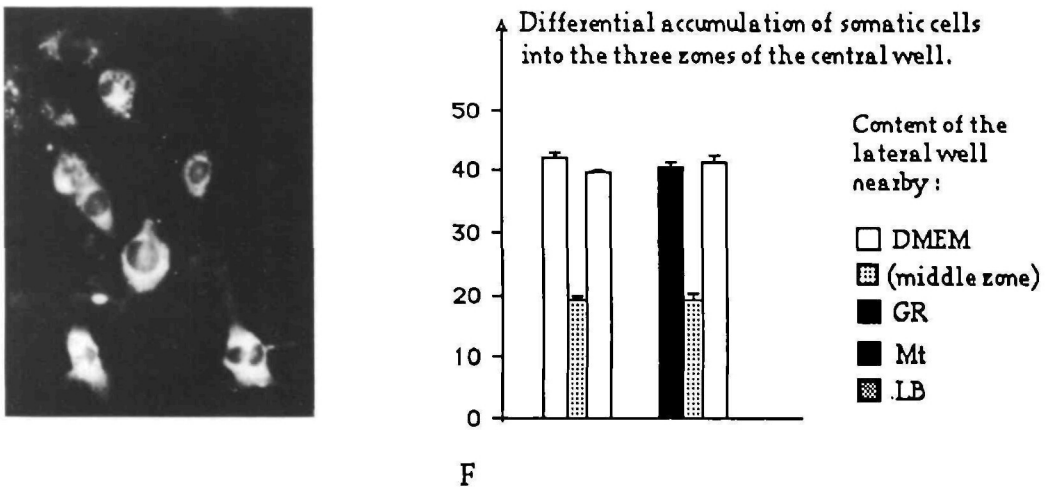
two effects; firstly, it produced a one-day prolongation of the PGC growth period, and, secondly, it caused increased PGC numbers in the cultures. However, it did not significantly delay the time of disappearance of PGCs from the cultures. This makes it unlikely that the effect is due to prolongation of PGC survival. In Fig. 1B, the media were conditioned by 10 explanted organs in each case. In order to exclude the possibility that the effect of GR is due simply to more tissue in these explants, we disaggregated and counted the cells in the explanted tissues. The counts are shown in Fig. 1D and show that GRs used to condition the medium contain equal or smaller numbers of cells than the other organs used.

Genital ridges exert a chemotropic effect on 8.5 dpc PGCs

In order to test whether the direction of PGC migration is influenced either by the mesentery through which PGCs pass, or by their target tissue, 8.5 dpc PGCs were seeded onto STO cell feeder layers and confronted by the same three tissue explants as used previously, in a new chemotactic assay derived from the 'under-agarose' method. Three wells were cut in an agarose layer in a gridded culture dish, a central rectangular well, seeded with feeder cells, and a lateral well at each end, separated by 1 mm from the central well (Fig. 2A). The central well was divided into three zones, a middle zone, and two 'end zones', A and B, which are twice as big as the middle zone. Their surface areas are each 40 % of the total surface area of the well floor, and that of the middle zone is 20 %. PGCs were seeded over the middle zone, but settled randomly over the whole of the central well. If they then migrated randomly, after 24 h, their distribution in the three zones would be in proportion to the surface areas (40 % of the total PGC







number in each end zone, 20% in the middle zone). The percentage of the total PGCs in end zone A (%A) would be the same as that in B (%B) and therefore the value %A/%B would be approximately 1. When the lateral wells were filled with culture medium only (Fig. 2B), after 24 h, the PGCs had migrated apparently randomly (%A/%B=1.02±0.05). However, when one lateral well was filled with culture medium and other

Fig. 2. Chemotropic assays. (A) Plan of agar plates used. (B) and (C) Assays in which PGCs were confronted with either explanted tissue in one lateral well and DMEM in the other (B), or explanted tissues in both lateral wells (C). In both cases, PGCs always accumulated preferentially in the end zone nearest the GRs. Each assay is presented as a group of three bars. These represent the percentage of total PGCs in end zone A, middle zone, and end zone B, respectively. Each outer bar is shaded according to the contents of the lateral well nearest to it. Error bars indicate the S.E.M. of 12 replicate assays. (D) The total number of PGCs per central well was recorded for each assay and divided by the number of embryo equivalents for accurate comparison between different sets of experiments. The accumulation of PGCs near GR is not due to a local effect on proliferation. (E) Di-I labelling of somatic cells. The suspension of 8.5 dpc PGCs was exposed to Di-I fluorescent dye (Molecular probe; 40 $\mu g\,ml^{-1}$) for 1 h at 37°C, plated on STO cells and alkaline phosphatase stained. Almost no PGCs were found after such a treatment, whereas somatic cells are highly fluorescent (×233). (F) Di-I labelled somatic cells were seeded into the central wells and treated as before. They are evenly distributed after 24 h and thus are not responsible for the preferential accumulation of PGCs near GR.

with either GR, Mt or LB explants, GR caused PGCs to accumulate in the end zone nearest them, at the expense of accumulation in the end zone furthest away from them (%A/%B=1.73±0.14). Neither Mt nor LB explants had this effect (%A/%B=1.09±0.14, and 0.99±0.11, respectively) (Fig. 2B).

The fact that Mt and LB do not preferentially attract PGCs makes it unlikely that GR effect is due solely to the presence of embryonic tissue. However, to exclude this possibility, the next series of experiments included explanted tissues in both lateral wells (Fig. 2C). All combinations of the three tissues were used. The results show that when there is a choice between GR and one of the other tissues, PGCs always accumulate preferentially on the side of the culture towards GR. However, PGCs accumulate equally in the two end zones when GR are in both lateral wells, or when any combination of Mt and LB is used. These results cannot be explained simply on the basis of the amounts of target tissue used, since approximately the same number of cells were used in each case (see Fig. 1D). They also cannot be explained on the basis of differential PGC proliferation, since the proliferation results above show that GR do not exert an effect at 24 h. Furthermore, when the total numbers of PGCs in the central well (both end zones and the middle section) are counted after 24 h, they are approximately the same in experiments in which either GR or medium only was placed in both lateral wells (Fig. 2D). Lastly, the results cannot be explained by differential initial adhesion of PGCs to different areas of the central well, because the total number of PGCs in the entire central well after 24 h is the same whether GRs or DMEM is present in the two lateral wells (Fig. 2D).

In all these experiments, the PGCs never moved out of the central well, though they often accumulated at its edges. The feeder cell front did sometimes spread under the agarose, but did so to the same extent in all directions, and never as far as the lateral wells. During the period of the assay, 24 h, the target tissues adhered to the dish at the bottoms of the lateral wells and started to spread. These outgrowths did not underlap the agarose at the edges of the wells. When GR or Mt were used, their own population of PGCs could be seen by the alkaline phosphatase staining. These PGCs did not leave the wells, but remained attached to the outgrowths.

8.5 dpc PGC suspensions are heavily contaminated with somatic cells, derived from the allantoic rudiment and surrounding tissue. In order to exclude the possibility that the chemotropic effect is exerted on the somatic cells, which would then carry the PGCs, we carried out the chemotropic assay with somatic cells alone, labelled with Di-I (see Fig. 2). GRs had no effect on the distribution of the somatic cells after 24 h.

Discussion

We report here that 10.5 dpc genital ridges of mouse embryos release factors that change the behaviour of migrating PGCs in culture, and that two other test tissues do not have these effects.

The effect on PGC number could be due to an increase either in cell proliferation or cell survival. These have not been distinguished experimentally here. It is unlikely that the genital ridges significantly affect PGC survival in culture, since the PGCs disappear at the same time as in control cultures. However, we are currently testing this directly.

The assay for chemotropic factors is complicated by two factors; the necessity for a feeder layer, and the fact that the PGC suspension also contains somatic cells. The feeder cells, although irradiated, are still capable of movement. This is unlikely to be affecting the outcome of the migration studies, since we found in previous studies that PGC movement is random with respect to the feeder cells (Donovan *et al.* 1986; Stott and Wylie; 1986). Furthermore, in these experiments, the position of the edge of the feeder monolayer was carefully monitored, and migrated out symmetrically in all experiments. We intend to repeat these experiments on cell-free substrata. However, we have not so far been able to show that PGCs from early migratory stages will spread and migrate reproducibly on purified extracellular matrix components. Second, there are a large number of contaminating somatic cells in the PGCs population used. These come from the allantoic rudiments which were dissected from the 8.5 dpc embryos. We therefore carried out the same chemotaxis assays with somatic cells only, after labelling with the fluorescent marker Di-I (Fig. 2E and F). GR explants had no effect on the distribution of these cells after 24 h, showing that they are not physically carrying the PGCs towards GR explants. However, we cannot exclude the possibility that somatic cells may act as signalling intermediates. GR too are a mixed population of cells, consisting of the somatic tissues of the ridges them-

selves, as well as the PGCs that have already colonised them (which, at 10.5 dpc, represents approximately 8 % of the total PGCs population *in vivo* (Tam and Snow, 1981) and 60 % *in vitro*, since GR explants include the most dorsal part of Mt). Since Mt, which also contain migrating PGCs, has no effect, it is unlikely that the effects seen are due to the PGC population of the 10.5 dpc ridges, unless the gonadal and mesenteric PGCs are already different from each other at this early stage.

There are several mechanisms whereby a diffusible factor released by GRs could cause asymmetric migration of PGCs *in vitro*, and we have not attempted to discriminate between these experimentally here. First, the rate of PGCs movement could be increased (chemokinesis). Second, the substratum could be conditioned to improve adhesion of the leading lamellae of PGCs (haptotaxis). Third, a factor could act on the motile machinery of the PGCs *via* a surface receptor, which would result in migration of the PGCs towards the source of the signal (chemotaxis). This mechanism has been shown unequivocally for the migration of neutrophils (Zigmund, 1978) and slime mould amoebocytes (Newell, 1981). The advantage of this experimental system is that it will allow us to distinguish between the various possibilities, by using specific agonists (e.g. growth factors) and antagonists (e.g. antibodies or competing peptides).

It is possible that both effects of GR are mediated by the same molecule. There is now considerable evidence that individual growth factors have both proliferative and chemotactic effects on their target tissues: PDGF acts as a chemoattractant for vascular smooth muscle cells (Antoniades *et al.* 1987; Deuel *et al.* 1987; Zerwes and Risau, 1987), fibroblasts, neutrophils and monocytes (Antoniades *et al.* 1987), and as so is implicated in wound-healing (Harvey *et al.* 1987), morphogenesis of blood vessels and pathological conditions such as atherosclerosis (Raghow *et al.* 1987) or atherogenesis (Deuel *et al.* 1987). TGF-β also possesses chemotactic properties (Raghow *et al.* 1987; Wahl *et al.* 1987) and, as it is present at high levels in platelets, it is thought to play a role together with PDGF in wound repair (Derynck, 1987). FGF (Connolly *et al.* 1987) and IGF-I (Grant *et al.* 1987) stimulate chemotaxis in endothelial cells and could be involved in neovascularization processes.

In conclusion, we have now demonstrated two factors that control PGC migration in mouse embryos; firstly, the intrinsic capacity of PGCs to spread and move, which is progressively lost between 10.5 and 13.5 dpc (Donovan *et al.* 1986), and secondly, extrinsic factors released from the target tissues that control the direction of migration and support the PGC population. The results presented here will allow the study, at the molecular level, of the way GR exert long-range control over the PGCs that are migrating towards them.

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