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Review

Cellular and molecular aspects of mouse primordial germ cell migration and proliferation in culture

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Introduction

The development of primordial germ cells (PGCs), the ancestors of germ cells, is a complex coordinated process. So far we have had only a glimpse of the variety of interplay between germ cells and somatic cells performing and regulating the different steps involved. In the mouse embryo, using classical morphological approaches, the main events of PGC development have been described in detail (for reviews, see McLaren, 1981; De Felici and Dolci, 1987). However, our knowledge of the cellular and molecular mechanisms underlying these processes remains scant. The ability to isolate and purify mouse PGCs from various stages and to maintain them in culture (De Felici and McLaren, 1982, 1983; Donovan *et al.*, 1986; McCarrey *et al.*, 1987) now offers the opportunity to move from the realm of descriptive embryology to that of molecular biology in the study of PGC development.

In this review we shall follow mouse PGCs in their migration to the genital ridges (from 9.5 to 12.5 dpc) and focus on the recent findings, obtained mostly by *in vitro* culture studies, providing clues on how their migratory activity, survival and mitotic proliferation might be regulated.

PGC migration

The origin of germ cells from an extragonadal source and the ability of these cells to enter the gonadal ridges is no longer questionable, as has been demonstrated histologically and by genetic and experimental studies in all mammalian species examined (for references, see Eddy *et al.*, 1981). In the mouse, Chiquoine (1954), employing the Gomori histochemical method for alkaline phosphatase (ALP), was the first one to identify, in 8.5-day embryo, PGCs scattered among the cells of the caudal end of the primitive streak, the root of the allantois mesoderm and the underlying yolk sac splanchnopleure. More recently, Ginsburg *et al.* (1990) using a more sensitive method for detecting ALP activity, identified PGCs in early 7-day embryos as a cluster of about 8 cells lying in the extraembryonic mesoderm just posterior to the primitive streak. From here they move into the embryo proper, to the mesoderm of the primitive streak and then to the endoderm of the yolk sac forming part of the developing hindgut. Many observations indicate that PGC migration from the gut epithelium into the genital ridges occurs by active motility. Indeed, around day 10 mouse PGCs acquire the features of locomotor cells; they are seen to pass through the basal lamina of the gut epithelium into the dorsal mesentery by sending out a pseudopodium (Zamboni and Merchant, 1973; Clark and Eddy, 1975), and will migrate when placed in cell culture (see next paragraphs). About one day later they begin to enter the genital ridges and are enclosed into the gonadal tissues.

In analyzing PGC migration, at least three different steps have to be considered: (1) emigration from the gut epithelium; (2) migration itself, and (3) settlement into the genital ridges. As for all migrating cell types, interactions of PGCs with extracellular matrix (ECM) and the surrounding cells are likely to be involved in all three processes.

What role does fibronectin play?

Various lines of evidence suggest that fibronectin (Fn) might play an important role in PGC migration.

Fn might provide an adhesive substrate that PGCs need to move, thus constituting a pathway for PGC migration. Indeed, immunohistochemical studies demonstrate that large accumulations of Fn are present along the migration route of PGCs (Fujimoto *et al.*, 1985; Alvarez-Buylla and Merchant-Larios, 1986). Using an adhesion assay developed by us, we found that about 30% of PGCs isolated from the dorsal mesenteries of 10.5 dpc embryos, were able to adhere to tissue culture dishes coated with bovine plasma Fn (De Felici and Dolci, 1989). This relatively low adhesion level might be optimal for migratory cells that must be able to translocate within a dense fibrillar meshwork of Fn-containing fibrils. Moreover, we found that a few days after their arrival in the genital ridges, germ cells completely lost the ability to adhere to Fn. Thus, the adhesiveness of PGCs to Fn seems to be a developmentally regulated process.

It is well established that a family of cell surface receptors called integrins, heterodimers composed of different α and ß subunits, mediate cell attachment to different molecules of ECM. Cells often adhere to Fn via the integrin $\alpha_5ß_1$ that recognizes the fibronectin domain Arg-Gly-Asp-Ser (RGDS). We found that a polyclonal antibody (anti-gp140) raised against this integrin and two synthetic peptides containing the sequence RGDS were able to block PGC adhesion to Fn (De Felici and Dolci, 1989). Very recently, Dolci *et al.* (paper in preparation), using antibodies against different integrin subunits,

Abbreviations used in this paper: PGCs, Primordial germ cells; dpc, days post coitum; ALP, alkaline phosphatase; ECM, extracellular matrix; Fn, fibronectin; CAMs, cell adhesion molecules; LIF, leukemia inhibitory factor; MGF, mast-cell growth factor; AMH, anti-Mullerian hormone; dbcAMP, adenosine-3', 5'-cyclic monophosphate, N^6, O^2-dibutyryl; FRSK, forskolin; FCS, fetal calf serum; BrdU, bromodeoxyuridine.

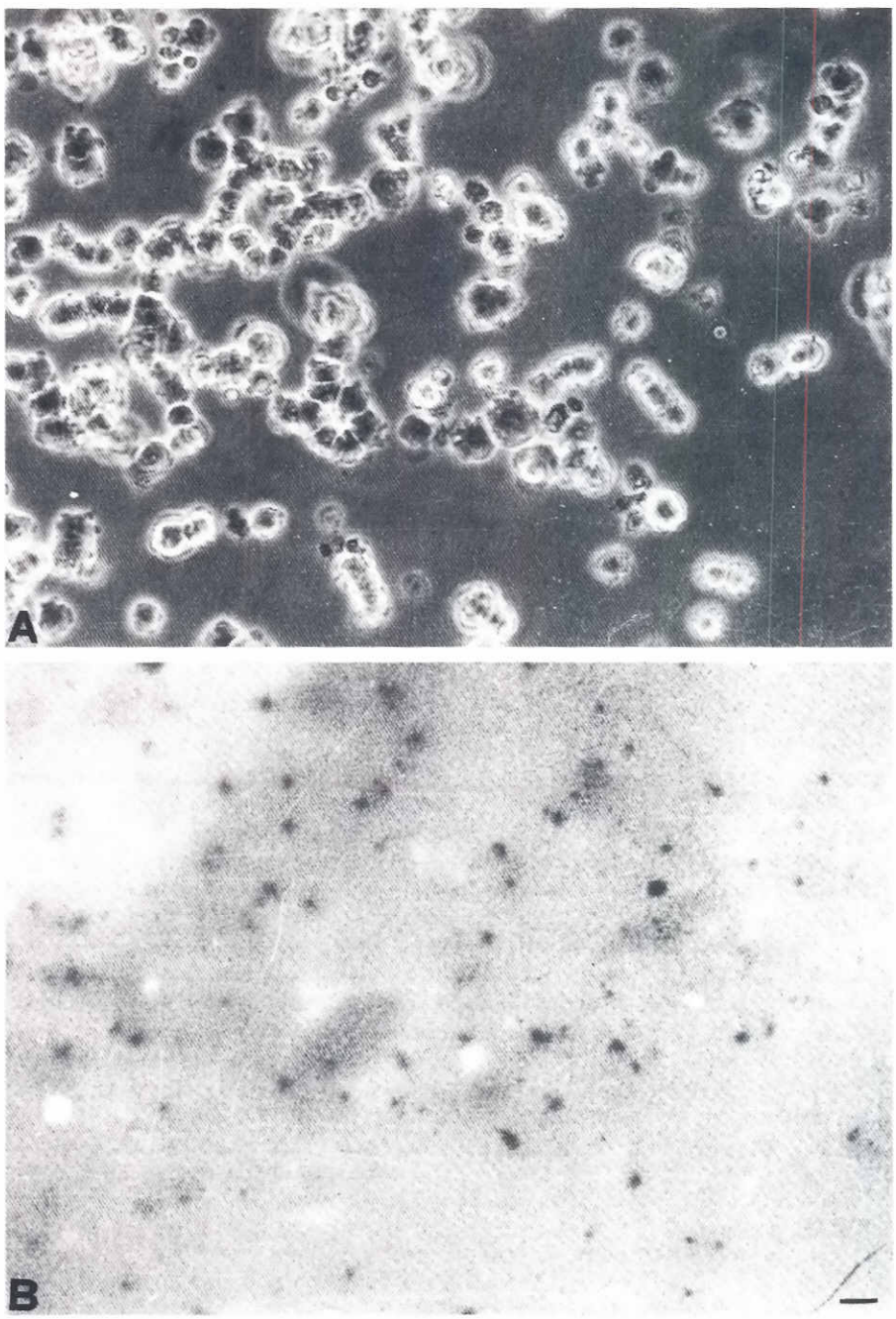

Fig. 1. Clusters of PGCs isolated from 11.5 genital ridges and attached to Fn. (A) *Bright field illumination.* **(B)** *Immunofluorescence visualization of the sites of the cell-substratum contact. Cells were fixed (2% paraformaldehyde) immediately after adhesion (40 min), stained with anti-Fn and then with second fluorescent antibodies. Notice black regions under small ventral area of many PGCs. Bar approximately 15 μm.*

have been able to immunoprecipitate three integrins ($\alpha_3\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$) from both 12.5 and 15.5 dpc germ cells. These last data represent the first direct evidence that germ cells express integrin receptors and establish the important point that the lack of adhesion to Fn of post-migratory germ cells (see above) is not due to the absence of the integrin receptor. It remains to be investigated why PGCs possess a multiplicity of integrins and what the function of each of them is.

A second role of Fn may be to stimulate PGC motility. Under the light microscope, however, we observed that migratory PGCs adhered to Fn-coated tissue culture dishes did not exhibit the characteristic features of locomotor cells (elongation, pseudopodia, filopodia) but remained mostly spherical. Moreover, immunofluorescent labeling for Fn appeared excluded from the central site of contact between PGCs and the Fn substratum (an area of about 0.7-0.8 μ^2) (Fig.1). (De Felici, unpublished observations). According to Grinnell (1980), this antibodies-excluding site should visualize focal contacts, adhesion structures characteristic of well-spread and stationary cells in which the distance cell-substrate is around 10-15 nm. Nevertheless, SEM reveals that

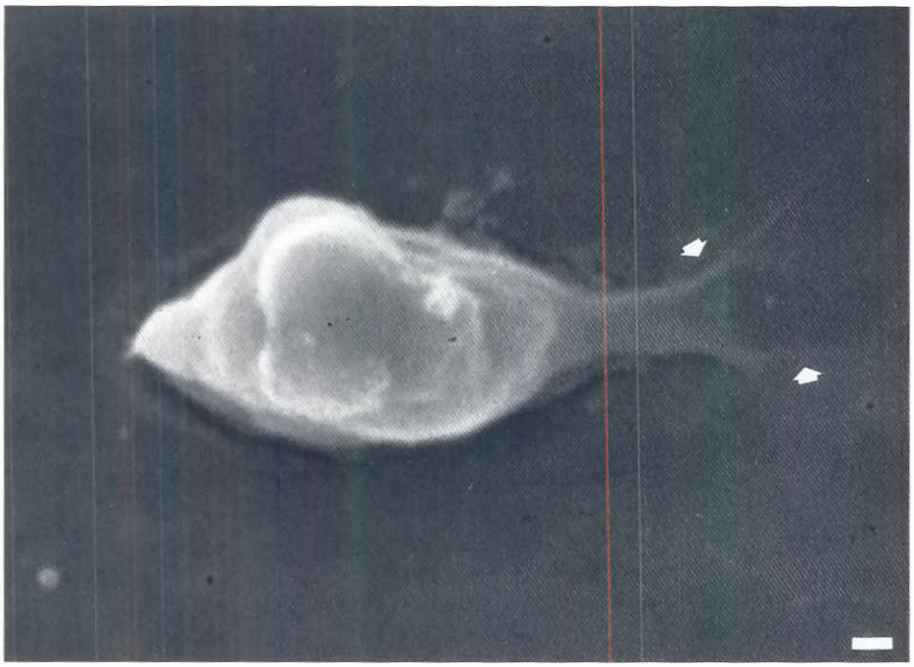

Fig. 2. SEM observations of a 11.5 dpc PGC cultured for 1-2 h on Fn substrate. *Notice a lobopodium with some filopodia (arrows). Bar approximately 1 μm.*

some PGCs adhered to Fn emit filopodial and/or lobopodial projections typical of motile cells that are absent in PGCs attached to an artificial substrate of poly-L-lysine (our unpublished observations) (Fig. 2). Taken together, these observations suggest that even if sometimes PGCs adhered to bidimensional Fn substrate show some features of motile cells, they mostly resemble stationary cells unable to displace themselves on Fn alone.

On the contrary, most of the PGCs appear to translocate when cultured within a tridimensional Fn-containing collagen gel (Alvarez-Buylla and Merchant-Larios, 1986) or on cellular monolayers (Donovan *et al.*, 1986, 1987; our unpublished observation). Ffrench-Constant *et al.* (1991), using an explant culture system, reported that PGCs will migrate from tissue fragments on a cell monolayer and that the addition of exogenous Fn stimulates this migration.

Interpreting these intriguing results we can hypothesize that multiple signals from the ECM molecules and/or from the surrounding cells are needed to stimulate PGC migration *in vivo* along a Fn pathway or that Fn must be presented to PGCs in a precise spatial conformation.

How PGCs move

As reported above, histological studies showed that *in vivo* PGCs emit pseudopodia during the time of active migration, suggesting that they move by typical ameboid motion (Chiquoine, 1954; Zamboni and Merchant, 1973; Clark and Eddy, 1975). In partial confirmation, using time-lapse cinematography, Blandau *et al.* (1963), Alvarez-Buylla and Merchant-Larios (1986) described pseudopodia and lobopodia in PGCs cultured *in vitro* on a glass substratum or on a collagen gel containing Fn, respectively. Moreover, these latter authors stressed that, when filmed within a tridimensional Fn-containing collagen gel, PGCs appear to elongate and translocate by cytoplasmic contraction. Recent *in vitro* studies revealed that PGCs placed on cellular monolayers exhibit a locomotory behavior in some way different from that of typical ameboid cells; they elongate with pseudopodia and filopodia and often a distinct leading lamella and uroid are present, indicating directional locomotion (Donovan *et al.*, 1986, 1987; Stott and Wylie, 1986; our unpublished observations) (Fig.3). Filming and interference reflection microscopy (Donovan *et al.*, 1986, 1987; Stott and Wylie, 1986) showed that PGCs on STO cell monolayers lacked focal contacts and possessed large areas of close contact; they exhibited relatively high rates of translocation (about 50 μm h^{-1}) and were able to penetrate beneath the cells of the monolayers.

All these findings demonstrate that environmental cues can influence how PGCs move. Furthermore, they also suggest that PGCs are potentially able to adapt their locomotory behavior to the different substrata they encounter during migration (basal lamina, ECM molecules, mesenchymal and epithelial cells) and under certain circumstances can even develop an invasive phenotype.

PGC homing

How do PGCs find their way and why do they cease migration and settle in the genital ridges?

The specificity of the timing and the route of migration suggest that either PGCs are guided to the genital ridges by surrounding tissues (contact guidance), or they are lured by attractants coming from the genital ridges (chemotaxis). The experimental evidence available suggests that both mechanisms might contribute to the homing of PGCs to their final site. Early studies by Rogulska *et al.* (1971) showed that when pieces of mouse hindgut containing PGCs were transplanted into the coelomic cavity of chick embryos, some of the mouse germ cells settled in or near the host gonads, suggesting that a non-species-specific attractant might be involved in PGC migration. Recently, Godin *et al.* (1990) reported that PGCs cultured on STO feeder layers (a condition necessary for PGC survival and migration in culture) migrated towards 10.5 dpc genital

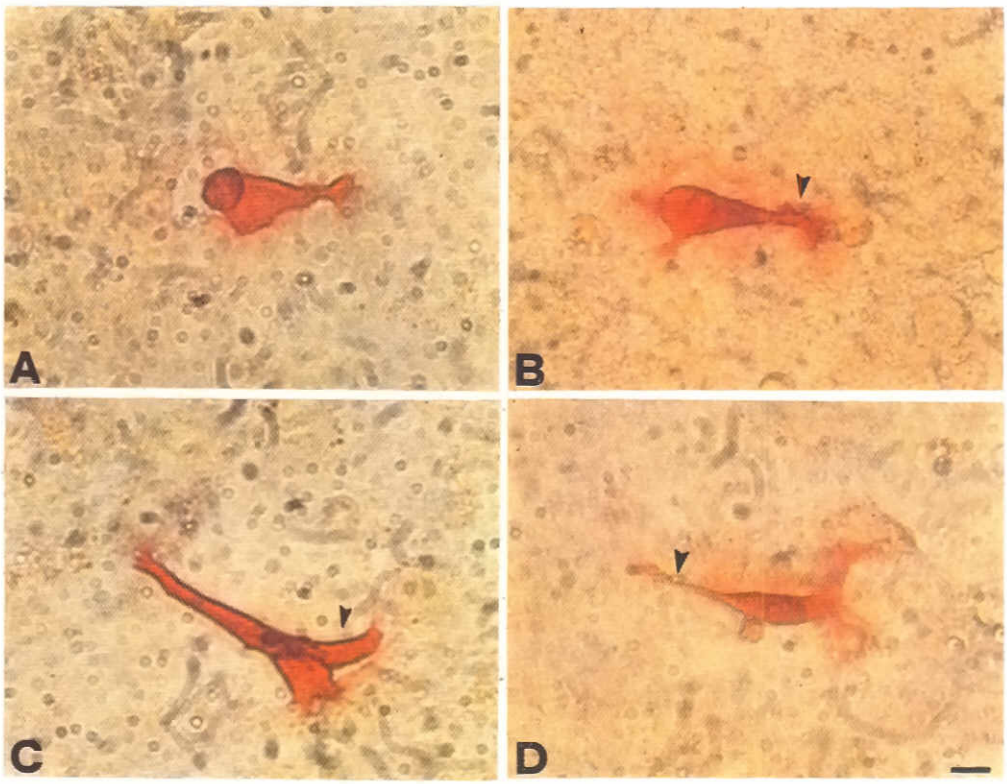

Fig. 3. Locomotory morphologies of 10.5 dpc PGCs cultured on TM_4 cell feeder layers. *Arrows indicate:* **(A)** *lobopodium,* **(B)** *leading lamella and* **(C)** *uroid. Bar approximately 4 μm.*

ridges in preference to other explanted organs. In this culture system, the PGC attractant released by the genital ridges seems to be $TGF\beta_1$ or a closely related molecule (Godin and Wylie, 1991).

It is not clear why PGCs cease migration once they have arrived in the genital ridges. Donovan *et al.* (1986) favor the view that PGCs undergo an intrinsic loss of motility. Environmental factors and contact with the somatic cells of the gonads might be involved as well. At variance with results obtained by Donovan *et al.* (1986), we found that under certain conditions of culture, germ cells isolated from 12.5-13.5 dpc gonads can resume their locomotory and invasive phenotype (De Felici and Dolci, 1989; our unpublished observations). In addition, whereas the ability of PGCs to adhere *in vitro* to ECM molecules declines as they settle in the gonads, their ability to adhere to gonad somatic cells increases (De Felici and Siracusa, 1985; De Felici and Dolci, 1989) (Fig.4). Our knowledge about cell-to-cell adhesion molecules expressed by PGCs and/or somatic cells of the fetal gonads is limited (see De Felici and Siracusa, 1985; De Felici and Dolci, 1987). The possible role of peculiar or known cell adhesion molecules (CAMs), integrins and selectins remains to be investigated.

The control of PGC number

Control of cell number is the result of the dynamic balance between cell proliferation and cell death.

During the migratory period, and for 2-3 days after their arrival in the genital ridges, germ cells proliferate actively. In about eight replicative cycles, their number increases from approximately 50 at the beginning of migration to about 25,000 around 13.5 dpc (Mintz and Russell, 1957; Tam and Snow, 1981). Distinct waves of degeneration drastically reduce the number of germ cells before birth. As far as we know, no detailed studies on the kinetics of germ cell degeneration in the mouse embryo have been performed. By analogy with other species, we can assume that there is usually little or no degeneration in migratory PGCs whereas extensive cell death happens later at gonia and meiotic stages (for a review, see Siracusa *et al.*, 1985).

LIF and MGF as survival and/or proliferation factors

Many attempts have been made to culture populations of proliferating PGCs *in vitro* (for reviews, see De Felici and Dolci, 1987; De Felici *et al.*, 1991). Recent studies have shown that mouse PGCs from 8.5-10.5 dpc can be grown in culture for 3-5 days provided suitable cell feeder layers are employed (Donovan *et al.*, 1986; Godin *et al.*, 1990; De Felici and Dolci, 1991). Evidence is emerging that the process by which feeders support PGC survival and proliferation is probably multifunctional, involving both contact-dependent mechanisms and diffusible factors. Screening the effects of a variety of growth factors on the survival and proliferation of PGCs cultured on TM_4 cell feeder layers, we have recently reported that leukemia inhibitory factor (LIF) causes a significant increase of the survival of 10.5 dpc PGCs (De Felici and Dolci, 1991) (Fig.5). It is interesting to note that LIF, in addition to its primary ability to influence the differentiation of hemopoietic cells, exerts a number of effects on a variety of cells in culture (for a review, see Metcalf, 1991); notably, it is able to maintain proliferating and totipotent embryonic stem cells, thus suggesting that LIF may have a role in early embryogenesis. No information is so far available on

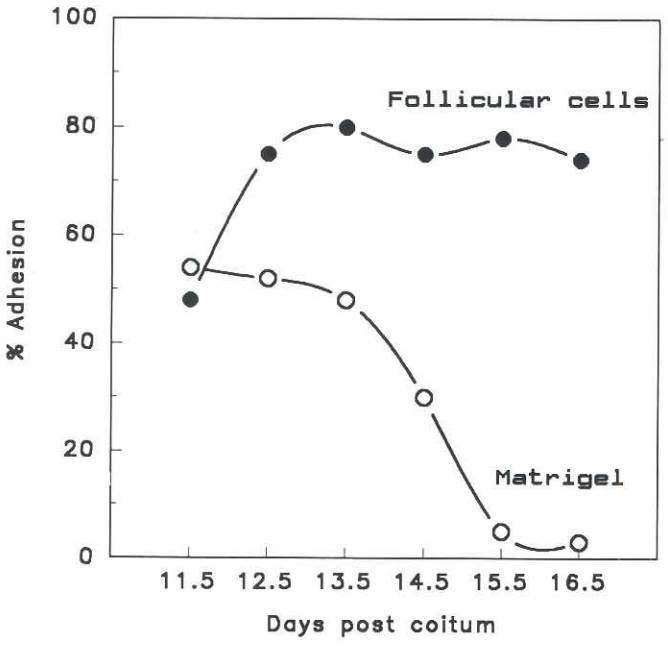

Fig. 4. *In vitro* adhesion of germ cells obtained from female embryos of various ages to follicular cell monolayers (De Felici and Siracusa, 1985) and to natural basement membrane (Matrigel, Collaborative Research) (our unpublished data).

the molecular structure and function of the LIF receptor. Is LIF produced by somatic cells surrounding PGCs *in vivo?* Is it able to act directly on PGCs? These and other questions are being investigated in various laboratories.

A second factor that happens to be essential to PGC survival and proliferation is mastocyte growth factor (MGF), also known as stem cell factor (SCF) or Steel factor (SF). Using *in vitro* culture systems, a number of groups have proved MGF is essential for PGC survival, and that the PGC life-supporting effect of STO cell feeder layers is partly attributable to the production of such growth factor (Dolci *et al.*, 1991; Godin *et al.*, 1991, Matsui *et al.*, 1991). A proliferative response of PGCs to MGF has also been reported (Matsui *et al.*, 1991). MGF is encoded by the murine *steel* (Sl) locus and is a ligand for the tyrosine kinase receptor protein encoded by the proto-oncogene c-kit at the murine *white spotting* (W) locus. Mutations at each of these loci result in deficiencies of hemopoietic stem cells, melanocytes and PGCs (for a review, see Besmer, 1991). The findings that c-kit is expressed in PGCs (Orr-Urtreger *et al.*, 1990), while MGF is expressed in cells present in the PGC migratory pathway and in the genital ridges (Matsui *et al.*, 1990), together with the functional studies on PGCs in culture reported above, are consistent with a crucial role for the c-kit receptor system in supporting survival and/or proliferation of PGCs. Interestingly, LIF

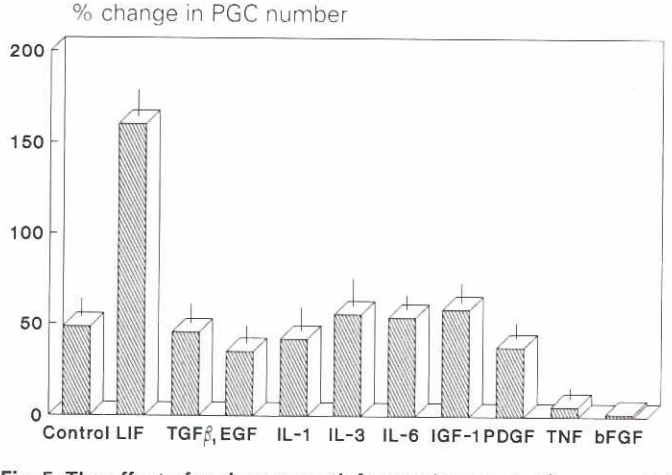

Fig. 5. The effect of various growth factors (concentration range 0.1-50 ng/ml) on the number of 10.5 dpc PGCs cultured for three days on TM_4 feeder layers: *leukemia inhibitory factor (LIF) only caused a marked increase of the PGC number. Bar, Standard error.*

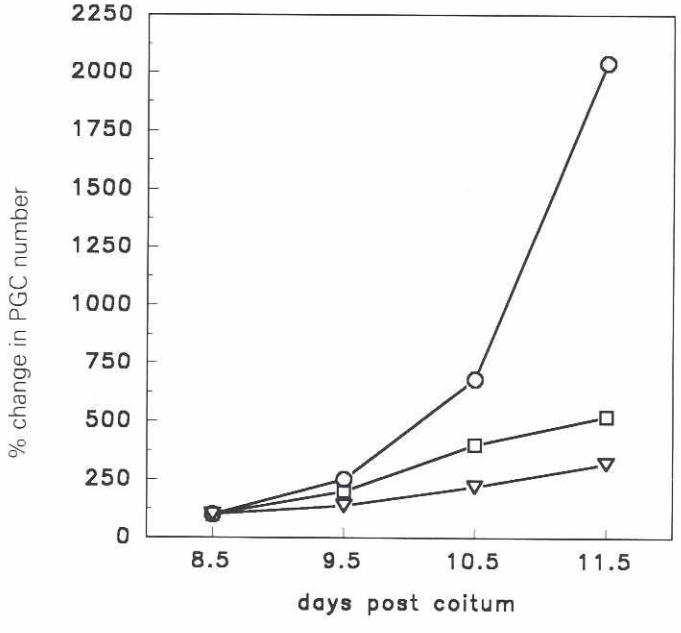

Fig. 6. Increase of the PGC number *in the mouse embryo (○) (from Tam and Snow, 1982) and in culture on STO (□) (from Matsui* et al., *1991; Dolci* et al., *1991) or TM_4 (▽) (from De Felici and Dolci, 1991) cell feeder layers.*

was found to act in synergy with MGF on PGCs in culture (Matsui *et al.*, 1991). It is to be stressed, however, that conditioned media from feeder layers or LIF and MGF alone and in combination cannot substitute for the feeder support (De Felici and Dolci, 1991; Godin *et al.*, 1991; our unpublished observations).

Other factors controlling PGC multiplication

By comparing *in vivo* and *in vitro* PGC proliferation between 8.5 and 11.5 dpc (Fig. 6), it is evident that the latter is still only a fraction of that *in vivo*. What is lacking in the *in vitro* culture conditions? In partial answer to this question we have recently found (De Felici *et al.*, paper in preparation) that N^6,O^2-dibutyryl cyclic AMP (dbcAMP) and compounds that are able to increase the level of intracellular

cAMP, such as forskolin and cholera toxin, are mitogenic on 8.5-11.5 dpc PGCs, as they promote incorporation of bromodeoxyuridine (BrdU) in PGCs and cause a drastic increase of their number in culture on TM_4 feeder layers (Fig.7). Whether PGC proliferation in the embryo is supported by mitogenic factors that active cAMP-dependent mechanisms remains to be elucidated, but the existence of such a mitogenic response in cultured cells suggests that it may be a key event in controlling germ cell proliferation *in vivo*.

Entry into proliferative arrest is another crucial point of the PGC cycle. Interestingly, testicular teratomas seem to originate from PGCs that escape mitotic arrest (Stevens, 1967; Regenass *et al.*, 1982). When cultured *in vitro*, however, PGCs appear to have a finite proliferative capacity (De Felici and Dolci, 1991; Dolci *et al.*, 1991; Donovan *et al.*, 1986; Godin *et al.*, 1991; Matsui *et al.*, 1991), which correlates with the timing of their mitotic arrest (male) or the beginning of meiosis (female) *in vivo*. It has been suggested that this might result from down-regulation of the c-kit receptor and/or by the uncoupling of the receptor from the intracellular signaling (Manova and Bachvarova, 1991; Matsui *et al.*, 1991). The existence of mitotic inhibitors produced by the somatic cells of the gonads has been also postulated (Clermont and Mauger, 1976) and several investigations have provided experimental evidence that the fetal testis produces molecules able to inhibit PGC proliferation (Regenass *et al.*, 1982; Prépin *et al.*, 1985; Vigier *et al.*, 1987). Some clues concerning the identity of this putative inhibitor have been obtained. Godin and Wylie (1991) reported that $TGF\beta_1$ inhibits *in vitro* proliferation of 8.5 dpc PGCs, and in the rat Vigier *et al.* (1987), using organ culture, found that the anti-Mullerian hormone (AMH), another member of the TGFß family, was able to induce a drastic reduction of the number of oogonia.

How do PGCs in culture die?

As reported above, PGC degeneration *in vivo*, during migration and soon after their arrival in the genital ridges, is probably a rare event, while, when cultured *in vitro* in the absence of feeder layers and certain growth factors, PGC numbers rapidly decline.

A recent view is that cell death can be classified into two different categories: necrosis, which occurs as a result of tissue damage, and apoptosis (programmed cell death), which is a process of active cellular self-destruction. The latter may occur either in response to a stimulus (i.e., glucocorticoid hormones for immature thymocytes) or to the disappearance of a stimulus (i.e., removal of specific growth factors from hemopoietic cells). Preliminary results obtained in our laboratory indicate that PGC death in culture has the hallmark of being apoptotic in nature. We found that most 11.5-12.5 dpc *in vitro* isolated PGCs rapidly assume a nuclear morphology and produce membrane-bound cell fragments (apoptotic bodies) that closely resemble the classic description of cells undergoing apoptosis (reviewed by Wyllie *et al.*, 1980) (Fig. 8). These data, although interesting, need to be confirmed by further studies, and the importance of apoptosis, in the context of germ cell development, should be clarified.

Perspectives

The ability to culture mouse PGCs on feeder layers of defined cell lines will, we hope, permit a detailed investigation of factors regulating PGC interactions with ECM and somatic cells. In particular, the identification of molecules modulating PGC adhesion to the somatic cells of the fetal gonads will make it possible to understand their role in homing and, possibly, in other PGC differentiation events. In this regard, the c-kit receptor system provides an excellent model for studying the mechanisms underlying synergism between different signalling systems (i.e., cAMP, LIF) in regulating germ cell migration, proliferation and survival. Undoubtedly, we are still in the early stages of understanding the complexities of PGC development, but can now look forward to the study of this subject with more perspectives than in the past.

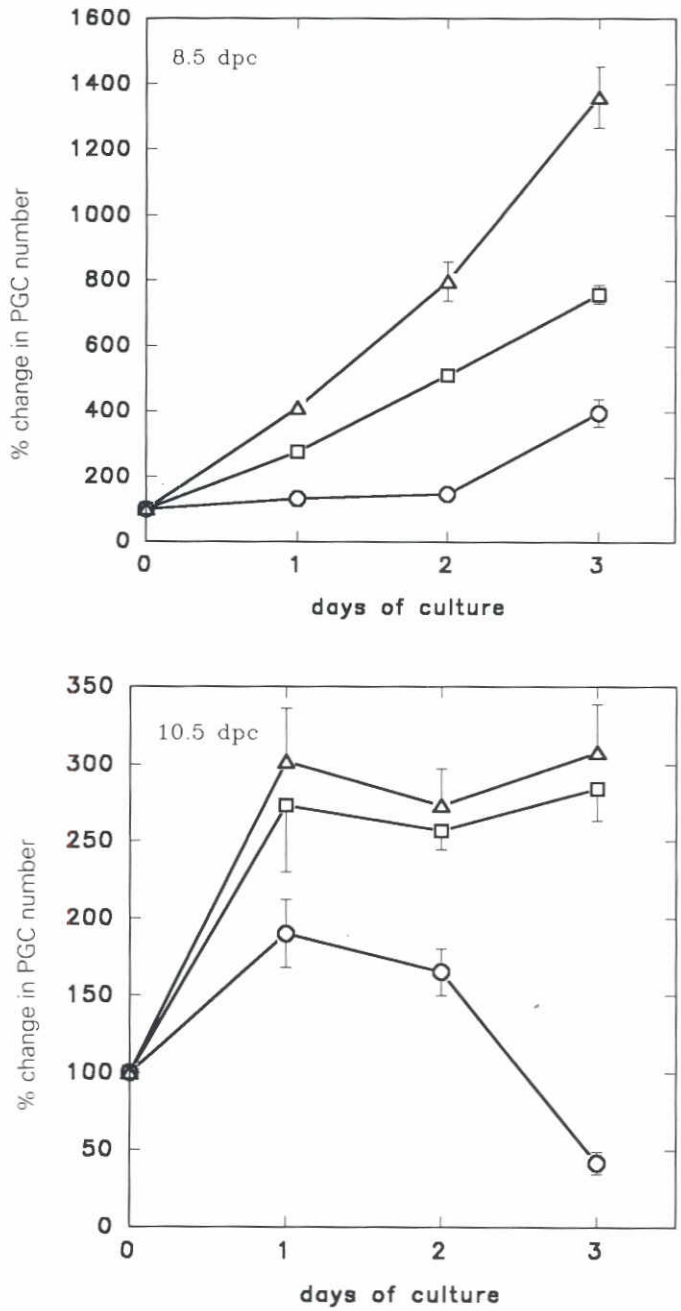

Fig. 7. The effect of 20 μM forskolin (△) and 1 mM dbcAMP (□) on the number of PGCs cultured on TM_4 feeder layers; (○) control.

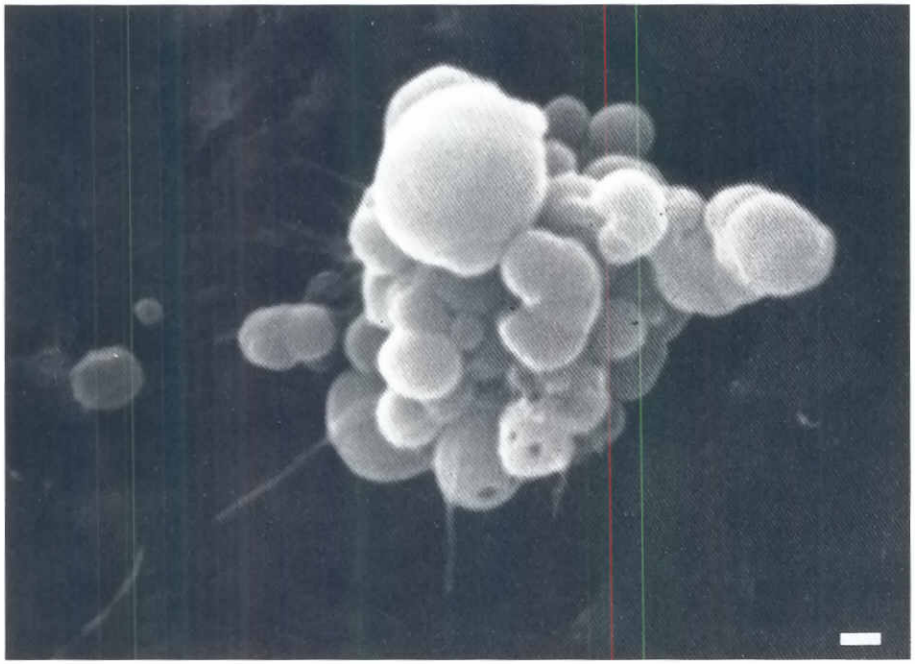

Fig. 8. SEM observation of a 11.5 dpc PGC cultured for 5 h in MEM + 10% FCS. *Cell fragmentation closely resembles the formation of apoptotic bodies typical of cells undergoing apoptosis. Bar approximately 1 μm.*

Summary

The development of mouse primordial germ cells is followed from their first appearance in the extraembryonic mesoderm of the posterior amniotic fold (7 dpc embryo) to their settlement in the genital ridges (12.5 dpc embryo). The role of fibronectin as adhesive substrate and/or in stimulating cell motility during PGC migration is discussed. Recent papers showing how PGCs migrate when cultured *in vitro* on cellular monolayers are reviewed. The process of PGC homing is proposed to be controlled by chemotaxis as well by developmentally regulated cell-to-cell interactions. Finally, evidence that survival and proliferation of PGCs is strictly dependent on growth factors such as LIF and MGF, and possibly on a cAMP-dependent mechanism is reported.

KEY WORDS: *primordial germ cells, cell migration, fibronectin, germ cell homing, chemotaxis, leukemia inhibitory factor, mastocyte growth factor, dbcAMP, cell degeneration.*

Acknowledgments

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