Neonatal mouse gonocyte proliferation assayed by an *in vitro* clonogenic method

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Survival and proliferation of mouse gonocytes was studied using a single cell clonogenic assay *in vitro*. The effect of growth factors and extracellular matrix on clonogenic development was quantitated. Fundamental requirements for growth of neonatal gonocytes included addition of fetal calf serum and coating culture wells with collagen IV alone or with added fibronectin. After 4–5 days, colonies ranged in size from four to > 128 cells, and some contained very elongated cells indicating migratory behaviour. Soluble stem cell factor did not have any effect on clonogenicity, although STO (subline of SIM mouse fibroblasts) cells, which produce membrane-bound stem cell factor, reduced colony formation from $79 \pm 5.9\%$ to $20 \pm 3.3\%$ without added growth factor. The majority of gonocytes and type A spermatogonia express the *c-kit* receptor according to *in situ* hybridization studies. However, the results indicate that the receptor may not be functional in neonatal gonocytes and their immediate progeny. The current assay for gonocytes can be extended to test new growth factors or proliferation-inducing agents directly, as well as to study cell–cell interactions. This assay and long-term propagation of neonatal germ cells will provide the much needed resources to enable greater understanding of the early development of germ cells.

Introduction

Germ cells segregate from the somatic cell lineage during development and are derived from ectoderm and arise in the epiblast (McLaren, 1983). The first recognizable germ cell, the primordial germ cell, migrates to the developing gonad. In males, it subsequently undergoes mitotic arrest at about 14.5 days post coitum (McLaren, 1985) and does not re-enter mitosis until after birth. The quiescent primordial germ cell at birth is often referred to as a gonocyte or pro-spermatogonia. Mitosis resumes in the first few days after birth along with migration from the centre of the seminiferous cord to the periphery, thereby coming into close contact with the basement membrane and Sertoli cells (Clermont and Perey, 1957).

Although gonocytes and their immediate progeny have been studied extensively *in vivo*, development of a culture system for these germ cells is necessary for further progress. Observations from studies *in vivo* need to be applied to an *in vitro* environment to achieve this. *In vivo*, a proportion of gonocytes elongate and form pseudopod-like processes that allow active migration of the gonocyte; formation of these processes has also been observed *in vitro* (McGuinness and Orth, 1992a). Movement to the periphery of the cord and contact with the basement membrane is an interaction necessary for gonocyte differentiation to the type A

spermatogonial stage. The seminiferous tubule basement membrane is composed of laminin and collagen IV is located in the interstitial region (Sawada and Yazama, 1994). Other extracellular matrix (ECM) molecules found in the male gonad include fibronectin, proteoglycans and collagens I, II and IV (Pelliniemi *et al.*, 1984; Skinner *et al.*, 1985; Paranko, 1987; Gelly *et al.*, 1989). The effect of ECM on gonocyte development and survival has been studied *in vitro* using co-culture of Sertoli cells and gonocytes (Orth and McGuinness, 1991; Dissel-Emiliani *et al.*, 1993). An additional complex interaction in germ cell maturation involves gonocytes and cells of the testis microenvironment, in particular Sertoli cells. Co-culture experiments indicate that paracrine regulation of germ cell proliferation and differentiation occurs *in vivo* and *in vitro* (Orth and Boehm, 1990; Orth and McGuinness, 1991; Dissel-Emiliani *et al.*, 1993; De Miguel *et al.*, 1996).

Growth factors and their receptors are actively expressed in the embryonic and neonatal phases of testicular development (Kierszenbaum, 1994; Orth *et al.*, 1996; Loveland and Schlatt, 1997). However, there is some disparity in the detection of c-Kit at the gonocyte or undifferentiated type A spermatogonia stages, although there is agreement that differentiating spermatogonia express c-Kit uniformly. This controversy may be due in part to an innate difference in mice and rats, since Orth *et al.* (1996, 1997) and Munsie *et al.* (1997) reported c-Kit mRNA and protein expression in rat gonocytes. In mice, the c-Kit protein

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Table 1. *c-kit* *in situ* hybridization on mouse neonatal testis

Age (days)	Type of cell	Grains per cell antisense	Grains per cell sense control	Percentage of positive cells*
1	Gonocyte	11.7 ± 0.8	4.6 ± 0.2	77 ± 3
3	Gonocyte	16.6 ± 1.9	4.8 ± 0.1	88 ± 2
	Type A spermatogonia	20.8 ± 1.9	4.8 ± 0.3	92 ± 1
7	Type A spermatogonia	34.2 ± 5.3	3.8 ± 0.2	100 ± 0.4

Values are mean ± SEM, *n* = 5.

*Percentage of cells treated with antisense with a greater number of mean grains per cell than the sense control.

has not been detected in gonocytes (Yoshinaga *et al.*, 1991). In the present study, a sensitive *in situ* hybridization method was used to detect c-Kit mRNA in newborn gonocytes.

Co-culture experiments indicate that the neonatal phase of germ cell maturation is associated with highly specific and finely regulated controlling factors. However, the genes involved have yet to be clearly defined. An *in vitro* environment in which gonocytes can be manipulated directly is required to understand better the molecules that are involved. The aim of the present study was to develop a clonogenic culture system in which gonocytes are selected and the formation of germ cell colonies is assayed. Micromanipulation techniques were used to isolate gonocytes in a pure form for incubation and assay. In the long term, *in vitro* cultures of germ cells will enable access to sufficiently high numbers of cells to enable molecular analysis of the genes that regulate the early stages of germ cell differentiation.

Materials and Methods

Mice

Inbred HSD OLA:ICR Swiss mice were used throughout the study and the day of birth was denoted as day 1. In the experiments involving culture on STO cell underlays, the ROSA 26 mouse strain was used as a source of gonocytes (Friedrich and Soriano, 1991).

In situ hybridization

The SP71 vector carrying plasmid DNA consisting of a 900bp *c-kit* PvuII fragment was linearized with XhoI for antisense and with EcoRI for sense riboprobes. Riboprobes were generated and radiolabelled by incorporation of [³⁵S]cytidine 5'-triphosphate (CTP) (100 µCi at 1200 Ci mmol⁻¹) according to Russo *et al.* (1994) and Edmondson *et al.* (1995). A Ribocore kit (Promega, Sydney) was used to transcribe the riboprobes from linearized plasmid. Tissues were fixed in 10% (v/v) formalin and sections prepared on aminoalkylsilane-coated slides by conventional methods. Wax was removed from the sections and they were dehydrated, treated with the enzyme pronase E (125 µg ml⁻¹) for 10 min at 37°C, and fixed in 4% (w/v) paraformaldehyde in PBS for 10 min. Labelled riboprobes, at a concentration of 5 × 10⁵ c.p.m. probe in 25 µl hybridization buffer containing

50% (v/v) formamide per slide, were heated to 85°C and placed on slides. Coverslips were applied and the slides were incubated at 55°C overnight. After hybridization, slides were washed in 50% formamide with 2 × SSC (sodium tri-sodium citrate) at 55°C followed by RNase digestion for 1 h at 37°C, a wash in 2 × SSC, dehydration and air drying. Slides were dipped in LM-2 emulsion (Amersham, Castle Hill), and developed in D19 developer (Kodak, Melbourne) after 3 weeks exposure at 4°C. The slides were stained in Mayer's haematoxylin and mounted in DPX (BDH, Poole).

Grain counts were carried out on both antisense and sense controls. All grains overlying the nuclei and on the perimeter were counted. A total of 100 of each type of cell was analysed for the various time points and a mean value ± SE was calculated (*n* = 5 mice). The percentage of positive cells was determined by subtracting the mean sense value from the antisense value on corresponding testis sections. For each group, the mean value ± SE was calculated (Table 1).

Immunofluorescence of ECM

Newborn testes were fixed in 10% (v/v) buffered formalin and processed for wax embedding. Fibronectin staining involved an unmasking step for 13 min at 37°C in 0.1% (w/v) trypsin in PBS followed by a 10 min blocking step using 1–5% sheep serum with 0.1% BSA. A rabbit anti-fibronectin antibody (DAKO Corporation, Sydney) was diluted 1:200, incubated with sections for 1 h at room temperature and then washed from slides with three changes of PBS. A fluorescent sheep anti-rabbit affinity purified Ig conjugated to fluoresceine isothiocyanate (FITC) diluted 1:200 was used to label tissues for 30 min at room temperature. The same procedure was carried out for laminin and collagen IV with omission of the unmasking step. The antibodies were rabbit anti-laminin (Sigma Chemical Co., St Louis, MO) diluted 1:30 and mouse anti-human collagen IV (DAKO) diluted 1:50. Sections were viewed on an Orthoplan microscope with fluorescent attachments. These experiments were carried out to confirm earlier reports (Sawada and Yazama, 1994; Skinner *et al.*, 1985; Pelliniemi *et al.*, 1984) and data are not shown here.

In vitro culture of gonocytes

Testes were dissected from 10 or more 1-day-old mice and stored in PBS. Enzymic digestion of testes involved a 20 min

Fig. 1. *In situ* hybridization using *c-kit* antisense and sense probes on mouse testis. (a) 1-day-old testis labelled with antisense *c-kit* showing autoradiographic grains localized over the gonocyte nucleus and (b) the corresponding sense probe with relatively little labelling associated with the gonocyte. (c) 3-day-old testis showing antisense labelling of a gonocyte and one in transition to a type A spermatogonia and (d) the corresponding sense probe. (e) 7-day-old testis showing labelling of type A spermatogonia adjacent to the base of the seminiferous tubule and (f) the corresponding sense probe. Scale bars represent 25 μm .

incubation at 37°C with a mixture containing hyaluronidase (100 $\mu\text{g ml}^{-1}$), trypsin (100 $\mu\text{g ml}^{-1}$), collagenase (100 $\mu\text{g ml}^{-1}$) and DNase 1 (5 $\mu\text{g ml}^{-1}$); all enzymes were purchased from Sigma Chemical Co. (St Louis, MO). After washing, the testes they were gently disrupted using a dounce homogenizer with Iscove's modified Dulbecco's medium (IMDM; Gibco BRL, Life Technologies Inc., Grand Island, NY) containing 1% (w/v) BSA (Fraction V, Sigma Chemical Co.) on ice. Gonocytes were collected individually using phase-contrast microscopy and a drawn out Pasteur pipette (tip diameter

approximately 250 μm). The cells were deposited into a second 60 cm diameter tissue culture treated Petri dish and single gonocytes were transferred to individual wells of a 96-well microtitre plate containing 150 μl culture medium with additives. Gonocyte cloning medium contained IMDM, nucleosides (Robertson, 1985), non-essential amino acids (Gibco BRL), 1×10^{-6} β -mercaptoethanol (Sigma Chemical Co.) and 20% fetal calf serum (FCS) with growth factors added as indicated below. Microtitre wells were precoated with collagen IV (Collaborative Biomedical

Fig. 2. A single cell suspension obtained by enzyme dissociation of 1-day-old mouse testis in which gonocytes appear as the largest cell type surrounded by somatic cells. Scale bar represents 62.5 μm .

Products, Becton Dickinson, Bedford, MA) for 1 h at room temperature, washed with IMDM, incubated with fibronectin ($5 \mu\text{g cm}^{-2}$; Boehringer-Mannheim, Melbourne), laminin ($5 \mu\text{g cm}^{-2}$; Boehringer-Mannheim) or collagen IV ($10 \mu\text{g cm}^{-2}$; Collaborative Research Products) for 1 h, and the plates were washed and used.

STO cell underlays (Ware and Axelrad, 1972) were made by treating cultures with mitomycin C ($10 \mu\text{g ml}^{-1}$; Sigma) for 3 h at 37°C and seeding 96-well plates with 2×10^5 cells per well in IMDM with 10% FCS. The medium was then aspirated and replaced with gonocyte cloning medium. All cultures were incubated at 37°C in a humidified 5% CO_2 :95% air atmosphere. A number of growth promoting molecules were tested for their effect on gonocyte colony formation, including rat stem cell factor (SCF, 100 ng ml^{-1} ; Amgen, Thousand Oaks, CA), platelet-derived growth factor (PDGF bb, 10 ng ml^{-1} ; Sigma), leukaemia inhibitory factor (LIF, 5 U ml^{-1} ; Amrad Biotech, Australia), oestradiol ($2 \mu\text{mol l}^{-1}$; Sigma) and a high affinity oestrogen receptor antagonist, ICI 182,780 (100 nmol l^{-1} , Tocris Cookson Ltd, Bristol).

An anti-SCF antibody was added to cultures to determine whether serum contained sufficient SCF to stimulate colony formation. Goat anti-mouse SCF was added to culture wells at $160 \mu\text{g ml}^{-1}$, which represented a 4- to 40-fold excess over the ND_{50} (antibody concentration giving half maximal inhibition of bioactivity of SCF) of $4\text{--}40 \text{ ng ml}^{-1}$ (Sigma). Normal marrow-derived mast cells were used as controls to assess the effectiveness of anti-SCF and these cells were cultured as described by Nocka *et al.* (1990). Mast cells were stained with Alcian blue and test cell aliquots contained $\geq 95\%$ Alcian blue-positive cells. Proliferating cells were labelled with $[6\text{-}^3\text{H}]\text{thymidine}$ that had a specific activity of $185 \text{ GBq mmol}^{-1}$ (Amersham Life Science, Castle Hill).

Germ cell nuclear antigen (GCNA-1) immunocytochemical staining

Paraffin wax sections of testes were prepared as controls and stained according to Enders and May (1994). Colony

cells at day 5 day of culture were pooled into a 5 ml tube (Falcon Plastics, Becton Dickinson), fixed in a 1% (w/v) para-formaldehyde solution for 10 min, washed, and resuspended in 0.1% (v/v) Triton-X100 in PBS. The GCNA-1 antibody (University of Kansas Medical Center, Kansas City, KS) hybridoma supernatant was not diluted and cells were incubated for 30 min on ice and washed with 0.1% (v/v) Triton-X100 in PBS. Anti-mouse F(ab')_2 -fluoresceine isothiocyanate conjugate (F(ab')_2 -FITC) was added for 30 min at 4°C and the cells were observed under a fluorescence microscope.

Results

In situ hybridization with c-kit riboprobes

In situ labelling of gonocytes was observed in 1- and 3-day-old testes with a *c-kit* antisense riboprobe (Fig. 1a,b), and was distinct from labelling in the sense control and in surrounding Sertoli cells (Fig. 1c,d). By day 7, differentiation into type A spermatogonia was complete and these cells also expressed *c-kit* mRNA (Fig. 1e,f). At day 14, type A spermatogonia continued to express *c-kit*, although there was a definite lack of expression in primary spermatocytes which are the predominant differentiated cell type (data not shown). Gonocytes and type A spermatogonia prepared for *in situ* hybridization were quantitated by grain counting for the various ages (Table 1). In all cases, there was a significant difference between antisense and sense controls (Fig. 1a,b). It was observed that 77% of newborn gonocytes expressed measurable *c-kit* receptors and had a mean of 11.7 ± 0.8 grains per cell.

Clonogenic assay of gonocytes

Gonocytes can be distinguished from all other somatic cells in the neonatal testis by size, thus micromanipulation was ideal for isolating gonocytes for culture *in vitro* (Fig. 2). Micromanipulation has the advantage of cell purity and the ability to isolate and transfer an individual cell into a well. The assay was based on depositing one gonocyte per well and detecting one colony per well, and using this to calculate the percentage of positive wells. The higher efficiency of cloning gonocytes was dependent largely upon the presence of an ECM coating (Fig. 3). The presence of collagen IV, fibronectin and laminin *in vivo* in the basement membrane of newborn mouse seminiferous tubules was confirmed (data not shown). Various ECM combinations were assayed *in vitro* and collagen IV alone or with added fibronectin was found to give large colony numbers reliably. In addition, the colony size peaked at $> 16\text{--}64$ cells per colony with collagen IV and $< 4\text{--}64$ cells for collagen IV with fibronectin (Fig. 3). With added laminin or no ECM coating the peak colony size was lower ($> 4\text{--}16$ cells per colony). In all subsequent experiments, the wells were coated with collagen IV and fibronectin, unless otherwise stated.

Initiation of the development of a colony from a single gonocyte appeared to occur soon after incubation. After 48 h

Fig. 3. (a) The percentage of mouse gonocyte-derived colonies formed in 96-well plates coated with collagen IV (Col IV) and fibronectin (Fib), laminin (Lam) or without added growth factors, and cultured for 4–5 days. The mean $\pm$ SD was calculated from cultures over five experiments. (b) Size of colonies in (a) cultured with extracellular matrix components: $\square$, collagen IV; $\square$, collagen IV and fibronectin; $\blacksquare$, collagen IV and laminin; $\square$, no added factors.

there were up to 8 cells per colony, and colonies continued to enlarge up to day 5, at which time they were assayed (Fig. 4). Some of the colonies contained very elongated cells, which may represent a migratory subpopulation (Fig. 5). The growth characteristics of the gonocyte-derived colonies resulted in three basic colony types: compact, dispersed or matrix-associated (Fig. 6). Compact colonies were most numerous (70%) and were floating or not firmly attached to the extracellular matrix underlay. Dispersed colonies (10%) had a similar appearance, except individual clusters were

present that contained fewer cells. It is common for non-adherent types of cell to move about a vessel and establish a dispersed pattern. The matrix-associated colonies (20%) were more unusual: a crumpled clear acellular matrix (resembling cellophane) was associated with the cells, some of which were adhered to the outer rim. At this stage, it is not possible to explain this type of cellular growth pattern. Colony cells were routinely stained with GCNA antibody to confirm their germ cell nature (Fig. 7). Viability of cells within colonies was confirmed using 0.04% (v/v) trypan blue.

Fig. 4. Development of a single mouse gonocyte colony after (a) 24 h of culture compared with (b) 5 days of culture with stem cell factor and leukaemia inhibitory factor. Scale bars represent 50 μm .

Fig. 5. Development of 5-day-old mouse gonocyte-derived colonies cultured without added growth factors showing elongated cells protruding from the colony periphery. Scale bars represent 50 μm .

Effect of serum and growth factors on clonogenicity of gonocytes

Gonocyte-derived colony formation was reliant on fetal calf serum in a dose-dependent manner and colonies were detected in 45–75% of wells (Fig. 8). The effect of various growth factors, individually and in combination, was studied to determine their influence on both clonogenicity and proliferation (number of cells per colony). When soluble SCF was added to cultures, mean colony formation was 81% (three experiments, 84 wells per group) compared with 82% without SCF. An antibody to SCF did not lower clonogenicity, indicating the SCF had a negligible contribution (Table 2). Mouse mast cells were used as the control for the effectiveness of anti-SCF antibody to block SCF action (Nocka *et al.*, 1990). Addition of anti-SCF resulted in a decrease in proliferation and [^3H]thymidine was reduced from 4357 ± 110 c.p.m. to 1688 ± 121 or 2299 ± 398 c.p.m. with anti-SCF alone or anti-SCF and SCF, respectively. The membrane-bound form of SCF was also tested for promotion of gonocyte-derived colony formation using STO cells. STO feeder layers produced $20 \pm 3.3\%$ colony formation (Fig. 9) compared with $79 \pm 5.9\%$ for cells cultured on collagen IV-coated wells (mean of five experiments). STO cells are known to produce both SCF and LIF. Soluble SCF and LIF were added to cultures and compared with cultures in which no

factors were added to confirm that LIF did not have an inhibitory effect. Slightly higher numbers of colonies were obtained ($68 \pm 10.5\%$) compared with when no factors were added ($56 \pm 14.7\%$) (mean of four experiments).

Addition of PDGF and SCF did not improve clonogenic capacity (Fig. 10), although with PDGF alone there was a shift in colony size to a modal value of ≥ 64 –128 cells compared with ≥ 16 –64 cells for SCF and PDGF or when no factors were added (Fig. 11). When LIF was added alone or in combination with SCF and PDGF, there was no effect on the incidence or size of colonies (data not shown).

Li *et al.* (1997) reported that 1 μmol oestradiol 1^{-1} stimulated purified rat gonocytes to synthesize DNA and that this could be blocked by the oestrogen antagonist ICI 163 384. In the present study, oestradiol caused a small reduction in the number of colonies, as did the antagonist ICI 182,780. Addition of both agents resulted in a number of colonies similar to that in control cultures (Fig. 12) and in all cases there was no shift in modal colony size.

Discussion

Newborn testis contains predominantly Sertoli cells, although 3–6% of the tissue comprises gonocytes (Orth and

Fig. 6. Phenotype of mouse gonocyte-derived colonies after 4–5 days of culture without added factors. Examples of (a) compact, (b) dispersed and (c) matrix-associated colonies are shown. Scale bars represent 50 µm.

Boehm, 1990), Leydig, peritubular and interstitial cells that are integral to the testis microenvironment which supports spermatogenesis. Cellular interactions are important for testicular function, but it is very difficult to study germ cells in this complex tissue. Therefore, control of germ cell proliferation and differentiation has been examined in isolated cells *in vitro*. Co-cultures have established functional coupling of Sertoli and gonocyte cells with regard to growth factor interactions and physical properties (Orth and Boehm, 1990; de Miguel *et al.*, 1996; van Dissel-Emiliani *et al.*, 1996).

Fig. 7. Photomicrograph of mouse gonocyte-derived colony cells stained with the anti-germ cell nuclear antigen antibody observed under a fluorescence microscope. The nuclei only are visible. Scale bar represents 25 µm.

In the present study, gonocytes were studied in isolation from all other cells to determine the direct effects of growth promoting agents, including extracellular matrix. At birth, type IV collagen is detected in the continuous epithelial basement membrane of seminiferous cords (Gelly *et al.*, 1989). Similarly, laminin lines the cords while fibronectin is more diffuse in the interstitial area. In the present study, collagen IV alone or with fibronectin enhanced the number and size (number of cells per colony) of gonocyte colonies *in vitro*, although the colonies were not firmly attached but were in contact with the extracellular matrix coating.

The growth characteristics and colony phenotype reflected some of the innate properties of gonocytes *in vivo*. A number of elongated cells were observed that were projecting away from the other cells in a migratory manner. The dispersed colony phenotype is presumably a result of such migratory gonocytes, which after initial proliferation move away and form new cell clusters. This property is apparent *in vivo* when gonocytes resume migration after birth to reach the periphery of the seminiferous tubule (McGuinness and Orth, 1992a). Re-initiation of cell division also occurs in neonatal rats from day 3 (McGuinness and Orth, 1992b) and mitotic gonocytes have been observed *in vivo* after birth in mice.

The critical events involved in resumption of germ cell proliferation after a period of mitotic arrest in developing testis are still unknown. However, proliferation is initiated within a few days of birth *in vivo* and is observed *in vitro* in co-culture experiments (van Dissel-Emiliani *et al.*, 1996). Colony development *in vitro* showed gonocyte-derived cell clusters after 48 h of culture. The gonocyte population in newborn testis showed some diversity in the degree of proliferation; some colonies were 4–16 cells and others contained > 128 cells. The modal colony size was influenced by some of the growth factors, but when no factors were added there was still a wide range in colony size, indicating a different potential of gonocytes and their progeny for proliferation. The size of the cells in the colonies also

Table 2. Effect of antibody to stem cell factor (SCF) on mouse gonocyte-derived colony growth and mast cell proliferation

Type of cell	SCF	anti-SCF antibody	SCF + anti-SCF antibody	None
Gonocyte-derived colony formation (%)*	92	90	94	88
Mast cells (c.p.m., [³ H]thymidine)	4357 ± 110	1688 ± 121	2299 ± 398	2712 ± 1078

*Duplicate experiments, 12–18 wells per group.
Duplicate experiments, mean ± SEM of five replicates per group.

Fig. 8. Relationship between percentage of mouse gonocyte-derived colonies formed (mean ± SD) and concentration of fetal calf serum.

Fig. 10. Percentage of mouse gonocyte-derived colonies formed (mean ± SD) in the presence of stem cell factor (SCF) and platelet-derived growth factor (PDGF) or PDGF alone, or without added growth factors.

differed, which may be a reflection of cell cycle phase (G1 smaller than G2 cells) or differentiation of germ cells as colony formation takes place. Dispersed colonies usually comprised numerous clusters of a small number of cells, whereas matrix-associated colonies were typically very large. The matrix appeared to be synthesized by the germ

Fig. 9. Mouse gonocyte-derived colonies cultured without growth factors on STO feeder layers for 5 days. Scale bars represent 50 µm.

cells since it formed even when gonocytes were cloned on STO cell feeder layers or ECM-coated wells. The composition of this cell matrix is unknown, but may have some role in cell communication or may be an environmental factor.

SCF is a vital factor for the survival and maintenance of primordial germ cells in culture (Dolci *et al.*, 1991) and acts synergistically with LIF to stimulate proliferation of primordial germ cells (Matsui *et al.*, 1991). The entry of primordial germ cells into mitotic arrest may be correlated with a decrease in the expression of c-Kit (Manova *et al.*, 1990) or an uncoupling of the receptor and intracellular

Fig. 11. Distribution of colony size of mouse gonocytes cultured with (a) stem cell factor (SCF) and platelet-derived growth factor (PDGF), (b) PDGF alone, or (c) without added growth factors.

signalling (Rottapel *et al.*, 1991). The observations from the present study support the latter since the majority of newborn gonocytes expressed c-Kit, but optimal colony formation by gonocytes occurred without added soluble or membrane-bound SCF. Growth of colonies on STO feeder cells, which produce both SCF and LIF, was 22% compared with 79% when no factors were added. This inhibition may be due to LIF, but when LIF and SCF were added, colony formation was comparable with controls ($68 \pm 10.5\%$ and $56 \pm 14.7\%$, respectively). From these experiments, it is concluded that optimal colony formation occurred with FCS alone, that STO cell underlays gave suboptimal stimulation and soluble SCF did not increase colony formation. The PDGF receptor is expressed in rat gonocytes during the first 5 days after birth and purified gonocytes undergo a four-fold increase in the incorporation of bromodeoxyuridine when treated with PDGF *in vitro* (Li *et al.*, 1997). In the present study, PDGF had little effect on the incidence of colonies, but was observed to increase the modal size of colonies and may act to enhance proliferation rather than initiate it in resting gonocytes.

The *in vitro* culture method described in this paper enabled growth factors and proliferation-inducing agents that affect gonocytes to be assayed directly. Isolation from secondary pathways that stimulate germ cell proliferation, such as paracrine effects from Sertoli and Leydig cells, is

Fig. 12. Percentage of mouse gonocyte-derived colonies formed (mean $\pm$ SD) in the presence of oestradiol ($2 \mu\text{mol l}^{-1}$), oestradiol antagonist ICI 182780 (100 nmol l^{-1} ; ICI), oestradiol and ICI 182780, or without added growth factors.

crucial for establishing long-term culture of germ cells. The results of the present study indicate that certain properties of germ cells are expressed innately, even when isolated from the microenvironment of the testis. This was apparent from the elongation of colony cells and characteristic migratory behaviour displayed during colony development. Although this method promotes growth of germ cells, it raises the question of the nature of the growth regulatory molecules, since marked effects on proliferation were not observed with factors that are used routinely for propagation of primordial germ cells. ECM was essential for colony formation and indicates that this may be a crucial mechanism for signalling germ cell proliferation. Cell-cell interactions can also be studied in this assay by cell mixing experiments, as has been shown with STO cell underlays. In the future, this approach has the potential to unravel the differentiation and regulation of early germ cell development that occurs as the spermatogenic stem cell population is formed in the testis.

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