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Expression of the c-*kit* Gene Is Critical for Migration of Neonatal Rat Gonocytes In Vitro[1]

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ABSTRACT

Rat gonocytes migrate to the basement membrane during the first postnatal week, a change in position crucial for their survival. These cells express the c-*kit* gene from the day of birth through Day 5 in vivo and develop the ability to migrate in Sertoli cell-gonocyte cocultures. In this study, we asked whether c-*kit* expression and synthesis of Kit protein are required for pseudopod production by gonocytes in vitro. To determine whether gonocyte migration in vitro is invariably accompanied by c-*kit* expression, we quantified percentages of gonocytes expressing c-*kit* with increasing time in vitro and correlated these data with pseudopod development by individual cells. We also determined the effect of exposure to Kit antibodies on gonocyte migration in vitro, and, conversely, asked whether addition of exogenous stem cell factor (SCF), the Kit ligand, stimulates pseudopod development. We found that 1) increasing numbers of gonocytes express c-*kit* with increasing time in vitro; 2) once these cells begin migrating in vitro, the appearance of a pseudopod on a gonocyte is absolutely correlated with *kit* expression by that cell; 3) incubating cocultures with Kit antibodies significantly reduces the number of cells with pseudopods, without any detectable decrease in numbers of gonocytes; and 4) addition of exogenous SCF to cocultures prepared on Day 5 results in a transient but significant increase in the percentage of gonocytes with pseudopods even though we found that Sertoli cells in the cultures produce endogenous SCF. Thus, our findings provide evidence to support a role for c-*kit* expression by neonatal gonocytes and, presumably, SCF expression by neonatal Sertoli cells in stimulating migration of these germ cells in vitro.

INTRODUCTION

In the testis of the newborn rat, germ cells or gonocytes are uniformly separated from the basal lamina that surrounds the seminiferous cord by portions of Sertoli cells, to which they are metabolically coupled and to which they specifically adhere (for review, see [1]). However, on about the third postnatal day some gonocytes gain the ability to migrate actively, form pseudopods, and begin to move toward the periphery of the cord, where they come into contact with matrix factors in the basal lamina. This active relocation of gonocytes to the periphery of the cord anticipates the formation of the basal compartment at that location by about 2 wk [2] and is believed to be critical for further gonocyte maturation, since any cells that fail to move away from the central region of the seminiferous cord eventually degenerate [3]. Although until now little has been known about how migration of neonatal gonocytes is regulated, it has been suggested that movement of these cells depends at least in part on their interaction with adjacent Sertoli cells, an idea supported by a large body of evidence documenting the close relationship between these two cell types in testes of both adult and developing rats [1, 4]. Moreover, it seems especially likely that gonocyte migration in neonates depends on Sertoli cells since, unlike their progenitors the fetal germ cells [4], neonatal gonocytes are in intimate and initially exclusive contact with Sertoli cells from the time that movement of these cells to the basal lamina is initiated until they reach their destination [1].

In our laboratory we have been exploring the relationship between neonatal gonocytes and Sertoli cells with a variety of approaches both in vivo and in vitro and have characterized the development of both cell types in some detail. For example, we have defined the proliferative activity of both types of cells in postnatal rats and reported that there is an inverse correlation between their mitotic indices, with the rate of division among the Sertoli cell population diminishing just as that for gonocytes is increasing [4, 5]. Moreover, we now recognize that Sertoli cells and gonocytes are coupled by gap junctions [6] and that they recognize and adhere to each other via neural cell adhesion molecule [7], a non-cadherin, calcium-independent adhesion factor that disappears from the developing testis by about 2 wk of age (unpublished results). In addition, we recently reported that many neonatal gonocytes express the c-*kit* gene in vivo during the developmental period when they are actively moving toward the basement membrane [8]. This observation raises the possibility that expression of c-*kit* may be important for neonatal migration of these cells.

In previous work, we developed a neonatal Sertoli cell-gonocyte coculture system [6] and documented that in this system, gonocytes develop pseudopods and, via verified migratory mechanisms, actively move between Sertoli cells [8, 9]. Moreover, this behavior in vitro commences and continues with a temporal sequence essentially identical to that seen in vivo [9]. In the current study, we utilized the gonocyte-Sertoli cell coculture system developed in our laboratory [6] to explore the possible relationship between expression of the c-*kit* gene by neonatal gonocytes and development of migratory activity in these cells. We used in situ hybridization to detect expression of the gene, examined and quantified the temporal pattern of its expression by gonocytes during their maturation and acquisition of migratory ability in vitro, and verified that these cells synthesize Kit protein in coculture. In addition, we interfered with function of the Kit protein by incubating cocultures in Kit antiserum and then tested the ability of gonocytes to migrate under these conditions. Finally, we examined the effect of exogenously added ligand for the Kit receptor protein, stem cell factor (SCF), on the ability of gonocytes to migrate in vitro, and also determined whether cocultured neonatal Sertoli cells produce endogenous SCF. The out-

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come of these studies provides substantial new evidence supporting the concept that expression of c-*kit* by neonatal gonocytes, and presumably of the ligand SCF by Sertoli cells, has a critical role in supporting migration of these cells toward the basement membrane during the first week of postnatal life. The results also provide new information highlighting the critical nature of interaction between neonatal Sertoli and germ cells in ensuring normal development of the testis.

MATERIALS AND METHODS

Gonocyte-Sertoli Cell Cocultures

Newborn male pups were obtained by mating Sprague-Dawley rats (Charles River Breeding Labs, Kingston, RI) that were maintained in an environmentally controlled facility and on water and lab chow ad libitum. For each experiment, 30–40 pups were killed on the day of birth by CO_2 asphyxiation, and Sertoli cells and gonocytes were isolated from their testes by sequential digestion with collagenase/hyaluronidase and collagenase and were plated as single cells on Matrigel (Collaborative Research, Waltham, MA) in either 8-chamber culture slides (Lab-Tek; Nunc, Naperville, IL) or 35-mm plastic petri dishes. These procedures have been described in detail previously [6]. Culture medium was hormone- and serum-free Eagle's D-val minimal essential medium (Gibco, Grand Island, NY) supplemented with sodium pyruvate (1 mM), nonessential amino acids (0.1 mM), sodium lactate (3 mM), transferrin (5 μg/ml), and retinol (50 ng/ml). Cocultures were maintained at 37°C in a water-saturated environment containing 5% CO_2 for up to 6 days.

RNA Isolation and Northern Analysis

Total RNA was isolated from gonocyte-Sertoli cell cocultures and Northern analysis was carried out as previously described [10]. Briefly, cultures prepared on the day of birth and maintained in vitro for 1–6 days were exposed to a denaturing solution containing 4 M guanidine thiocyanate and 0.8% 2-mercaptoethanol on ice for 2 min, followed by phenol:chloroform:isoamylalcohol extraction of the resulting cell lysate. After precipitation of the aqueous phase with isopropanol, the RNA preparations were treated with RNase-free DNase (2 U/ml, 37°C, 30 min; Promega, Madison, WI) and reextracted with phenol:chloroform. RNA content of the final samples was determined spectrophotometrically and Northern analysis carried out on equal amounts of RNA per sample with procedures previously described [10]. Samples were fractionated on a 1% agarose-formaldehyde gel and electrotransferred to positively charged nylon membranes (Biodyne B; GibcoBRL, Grand Island, NY). Blots were hybridized with a ^{32}P-labeled cDNA probe produced by random priming [11]. The probe was a *Bgl* II-*Hin*dIII fragment spanning nucleotides 2607 to 3710 of the murine c-*kit* gene, obtained from a subclone in a Bluescript KS vector provided by Dr. Alan Bernstein (Mt. Sinai Hospital, Toronto, ON, Canada). Hybridization was carried out overnight at 65°C in a buffer as detailed by Church and Gilbert [12]. Autoradiography was performed overnight at −80°C with Kodak MS film (Eastman Kodak, Rochester, NY).

In Situ Hybridization

Digoxigenin-labeled sense and antisense probes were synthesized with the Genius IV RNA labeling kit (Boehringer-Mannheim, Indianapolis, IN) from linearized plasmids containing the c-*kit* fragment described above, as recently reported [10]. After brief fixation in freshly prepared 4% paraformaldehyde, cultures were subjected to in situ hybridization with methods described in detail previously [10]. Hybridization was at 60°C overnight, and washes were at 60°C in gradually decreasing concentrations of formamide and saline-sodium citrate (SSC; single strength SSC is 0.15 M sodium chloride and 0.015 M sodium citrate), with final stringent washes in 0.2-strength SSC. In addition to exposure of some sections to sense probes, to ensure that positive signal indicated the presence of RNA-RNA hybrids, other controls included subjecting sections to RNase-free DNase prior to hybridization or RNase I treatment either before or after hybridization. After all sections were preblocked in 10% sheep serum-2% BSA, the Genius 3 Nucleic Acid Detection kit (Boehringer-Mannheim) was used to visualize hybridized probe in the cultures, with reagents supplied in the kit used at recommended concentrations. The color reaction was allowed to proceed for the same length of time for all slides in a single analysis, usually 20–30 min. After rinsing, culture slides were refixed in 4% paraformaldehyde, mounted in PBS:glycerol (1:1), and viewed and photographed with a Leitz Orthoplan 2 microscope equipped with differential interference contrast (DIC) optics and an Orthomat E camera (Leica, Malvern, PA).

Immunolocalizations

c-Kit Protein. To verify that Kit protein is produced by gonocytes in coculture with Sertoli cells, we carried out immunolocalization on some cocultures on each day in vitro, using approaches identical to those described previously [10]. For this, cultures were fixed briefly, preblocked in 10% normal goat serum in PBS, and incubated overnight at 4°C in a polyclonal primary antibody raised in rabbits (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:50). We recently used this primary antibody in a Western analysis and verified that it recognized the 160-kDa Kit protein [10]. In control incubations, either preimmune serum or "control peptide," as provided by the supplier, was substituted for primary antibody. Secondary antibody was goat anti-rabbit IgG conjugated to alkaline phosphatase (Jackson Immunoresearch, West Grove, PA; diluted 1:500). Signal was developed after 1-h incubation in secondary antibody followed by rinsing and reaction for alkaline phosphatase for 15 min.

SCF. Since our observations after exposure of cells to recombinant SCF suggested that Sertoli cells might supply endogenous SCF in cocultures (see *Results*), we also immunolocalized SCF in some cultures with a protocol similar to that used above for Kit protein except that primary antibody was rabbit polyclonal anti-mouse SCF (Genzyme, Cambridge, MA; 10 μg/ml) and secondary antibody was goat anti-rabbit IgG conjugated to rhodamine (Jackson Immunoresearch; diluted 1:1000). These cultures were viewed and photographed with a Leitz Orthoplan microscope equipped with epifluorescence and DIC optics.

Quantitative analyses. To determine the effect on cocultures of incubation either with antisera against Kit protein or with exogenous SCF, we quantified and compared the percentages of gonocytes with pseudopods in treated and control cocultures, as in our previous studies. For this, we used a uniform, patterned scoring method to observe and count gonocytes in each sample, as described earlier

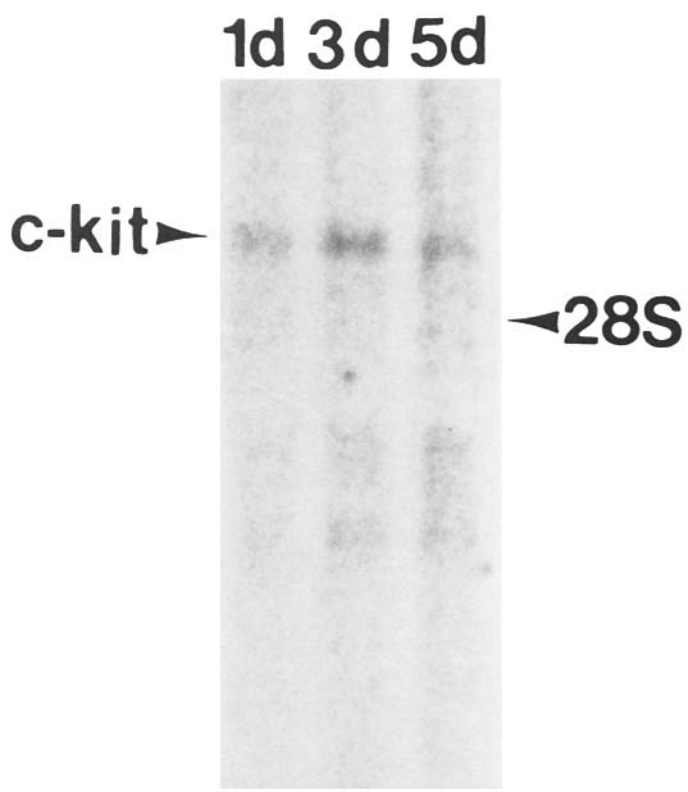

FIG. 1. Northern blot analysis of expression of c-*kit* mRNA in total RNA samples extracted from gonocyte-Sertoli cell cocultures prepared on the day of birth and maintained in vitro for 1 (1d), 3 (3d), or 5 (5d) days. All lanes were loaded with 10 μg of RNA. In each sample, the probe detected a transcript of approximately 5.5 kb, which is located just above the indicated position of the 28S ribosomal RNA.

[7], and employed criteria established in our laboratory [6] and utilized in previous studies [6, 7, 11] to identify cells with pseudopods. For each experiment, 200–300 gonocytes were scored in each chamber subjected to in situ hybridization or exposed to Kit antiserum or exogenous SCF for the presence or absence of signal and/or for the presence or absence of a pseudopod, as appropriate for that experiment. Three or more chambers were analyzed in this way for each treatment at each time point. For each experiment, the presence of significant differences among the groups was determined with ANOVA, and these differences were located by carrying out multiple comparisons with a Newman-Keuls test.

RESULTS

Migratory Gonocytes Preferentially Express the c-kit Gene

Northern analysis. Sertoli cell-gonocyte cocultures were prepared on the day of birth; total RNA was isolated from samples maintained for 1, 3, or 5 days in coculture; and RNA was subjected to Northern analysis with a ^{32}P-labeled probe specific for the c-*kit* message. A photograph of a typical autoradiogram is shown in Figure 1, with the position of the 28S ribosomal RNA, as seen in the corresponding ethidium bromide gel before transfer, indicated. In each of the lanes, a major signal was detected that migrated slightly above the 28S ribosomal RNA, indicating the presence of a transcript of approximately 5.5 kilobases (kb) that corresponds to the major c-*kit* message [10].

In situ hybridization. When cocultures were subjected to in situ hybridization with digoxigenin-labeled sense and antisense cRNA probes for the c-*kit* message, signal in all incubations with antisense probes was detected exclusively in gonocytes (Figs. 2 and 3). After 1 day of coculture, when all gonocytes were still round, some of these cells displayed strong signal for c-*kit* while others lacked signal entirely

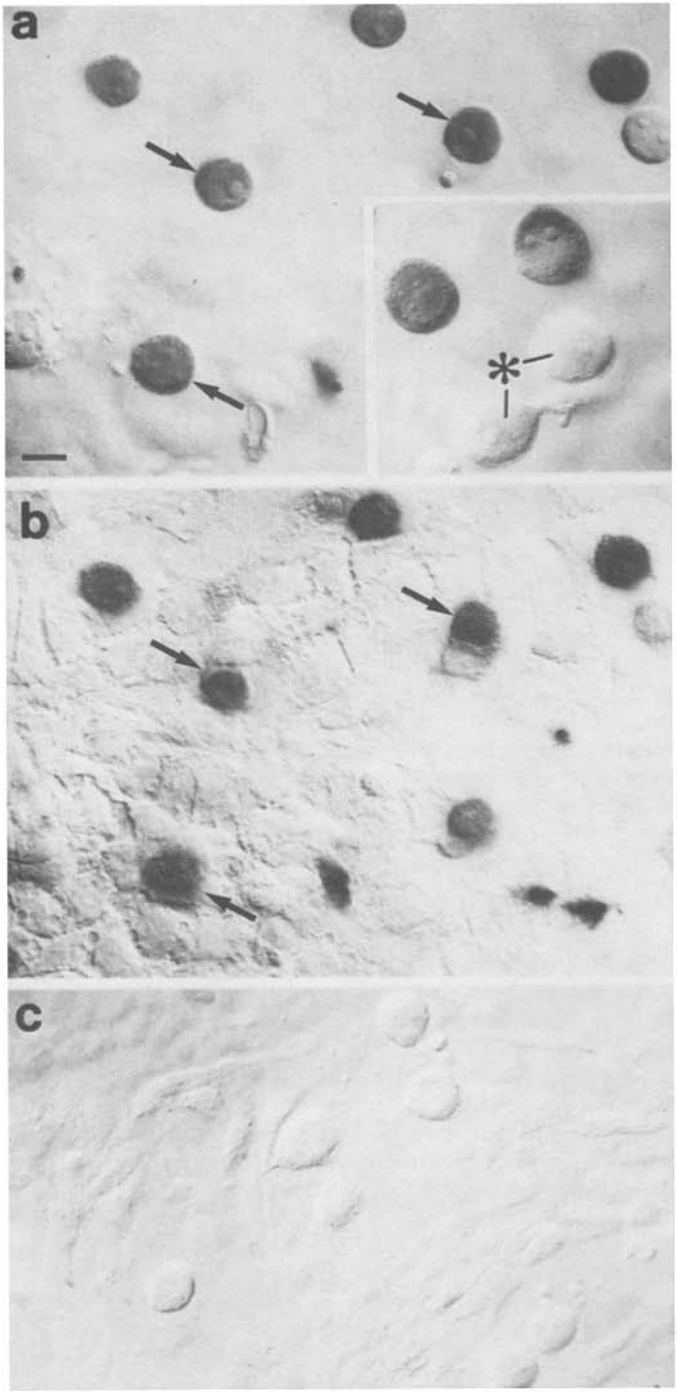

FIG. 2. Expression of c-*kit* mRNA in gonocyte-Sertoli cell cocultures after 1 day in vitro. In situ hybridization was carried out with digoxigenin-labeled cRNA antisense (**a, b**) or sense (**c**) probes and viewed with DIC optics. **a, b**) The same areas, focused in **a** on the gonocytes and in **b** on the underlying Sertoli cell monolayer, with arrows indicating the same gonocytes in both views. Reaction product indicating the presence of the c-*kit* message was restricted to gonocytes in antisense incubations, with no product in any Sertoli cells. After 1 day in vitro, some gonocytes displayed strong signal as in **a**, but other gonocytes lacking product were common (examples at asterisk in inset, **a**). No reaction product was detected in any cultures exposed to sense cRNA probes (**c**). Bar = 10 μm.

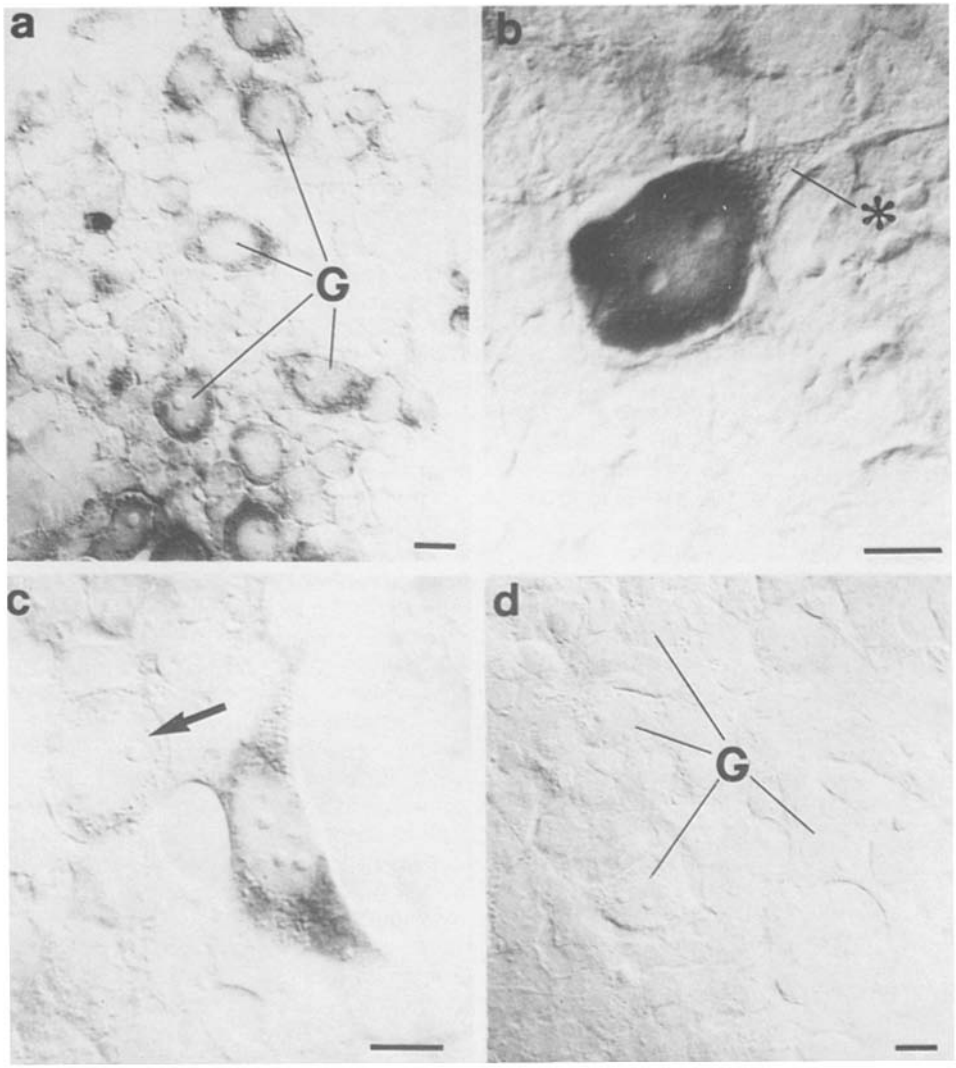

FIG. 3. Expression of c-*kit* mRNA in gonocyte-Sertoli cell cocultures after 5 days in vitro. Reaction product indicating the presence of hybridized digoxygenin-labeled antisense probes was restricted to gonocytes (G). Although it appeared that a large majority of these cells expressed c-*kit*, including most if not all cells with pseudopods (asterisk in **b**), round gonocytes lacking signal were also seen (arrow in **c**). In incubations with sense probes, gonocytes were uniformly without signal (G in **d**).

(Fig. 2). After 2 days in vitro, a few gonocytes began to produce pseudopods, as we reported previously [12]. In these cultures and in others maintained for up to 5 days, we detected c-*kit* signal in most if not all gonocytes with pseudopods as well as in some of the cells that remained round (Fig. 3). In all in situ hybridizations, no signal was detected in any incubations with sense probes (Figs. 2c and 3d), and all other methodology controls were negative; i.e., RNase I pretreatment prevented signal generation while DNase treatment did not, and posthybridization signal was insensitive to RNase I.

To determine 1) whether the percentage of gonocytes expressing c-*kit* changes with time in culture and 2) whether c-*kit* is preferentially expressed by gonocytes with pseudopods, as suggested by the qualitative observations described above, we carried out quantitative analyses of gonocytes cocultured on the day of birth and subjected to in situ hybridization on Days 1–5 after plating. First, we scored all gonocytes—regardless of morphology—for the presence or absence of hybridization signal and found that the numbers of cells expressing c-*kit* increased steadily and significantly ($p < 0.05$) with increasing time in coculture (Fig. 4). In addition, we located gonocytes with pseudopods on Days 3 through 5 of culture, when the percentage of cells displaying migratory activity is steadily increasing [12], and scored these cells for the presence or absence of c-*kit* signal. The result of the latter analysis, shown in Fig-

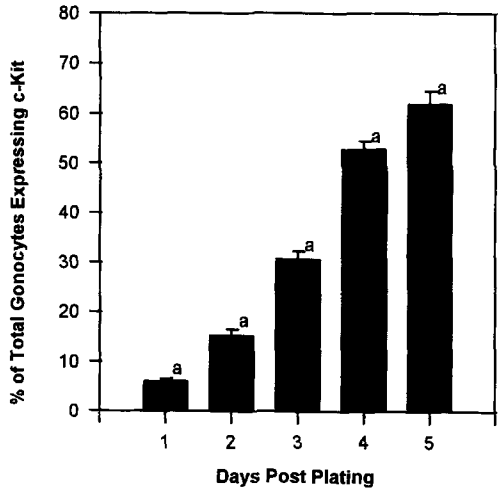

FIG. 4. The percentage of gonocytes expressing c-*kit* in cocultures prepared on the day of birth and subjected to in situ hybridization on Days 1–5 postplating. Three chambers were examined for each time point, and 300 gonocytes were scored for the presence or absence of signal in each chamber. a: $p < 0.05$.

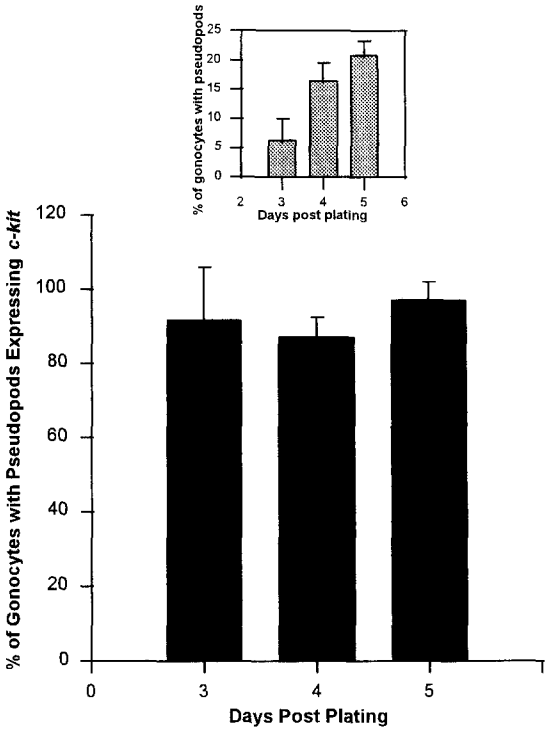

FIG. 5. The percentage of migratory gonocytes that express c-*kit* in cocultures fixed and subjected to in situ hybridization on Days 3, 4, and 5 in vitro. On each day, essentially 100% of these cells were positive for the c-*kit* transcript, with no significant differences among the three data points. Inset graph) The percentage of gonocytes that develop pseudopods with increasing time in vitro; virtually no cells with pseudopods were detected before the third day of culture.

ure 5, indicates that virtually all gonocytes with pseudopods expressed the c-*kit* message on each of the 3 days examined.

Anti-Kit Protein Blocks Gonocyte Migration In Vitro

Immunolocalization of Kit protein. To confirm that the Kit receptor protein is synthesized by gonocytes in coculture, we carried out immunolocalizations on Days 1–5 in vitro with a polyclonal primary antiserum and a secondary antiserum conjugated to alkaline phosphatase. An example of an immunolocalization performed on Day 5 in vitro is shown in Figure 6. Reaction product is clearly present in many gonocytes, including most or all of those with pseudopods, and careful inspection indicates that at least some of the signal in each cell is associated with the cellular surface (see inset, Fig. 6a). Control incubations in which either antigenic peptide or preimmune serum was substituted for the primary antibody were uniformly negative (Fig. 6c).

Blockade of the Kit receptor protein. To determine whether interfering with Kit protein might affect the ability of gonocytes to migrate in vitro, we incubated cocultured gonocytes in the presence of either polyclonal Kit antiserum or preimmune serum for up to 6 days in vitro and quantified the percentage of cells with pseudopods on Days 2 through 6 postplating (Fig. 7). On Day 2, only a very few cells with pseudopods could be located in any chamber, as expected from previous work [12]; and there was no difference between controls and antiserum-treated chambers, nor was any significant difference detected in the two groups by the next day, Day 3 after plating. However, on Day 4, essentially 1 day after onset of migration in vitro, the percentage of gonocytes with pseudopods was significantly less ($p < 0.01$) in chambers exposed to Kit antiserum compared to controls; and 2 days later, on Day 6 postplating, the difference between treated and untreated cultures was even greater, with about half as many migratory gonocytes present in antiserum-treated vs. control chambers. In addition, we also determined whether the presence of Kit antiserum affected the numbers of gonocytes in these cultures by quantifying and comparing total numbers of gonocytes in treated and untreated cocultures, and found (Fig. 8) that similar numbers of these cells were present in the two groups on each day.

Effect of SCF on gonocyte migration in vitro. We also tested whether SCF, the ligand for the Kit receptor, stimulates gonocyte migration in vitro, as follows. Cocultures were prepared on postnatal Day 5, when gonocytes are actively migrating in vivo [12] and hence presumably capable of responding to SCF. Chambers were incubated in recombinant SCF or vehicle for 1, 2, 3, or 24 h, and the percentages of gonocytes with pseudopods were quantified and compared between the two groups at each time point (Fig. 9). By 2 h postplating, the proportion of gonocytes with pseudopods in the SCF-treated chambers was significantly elevated ($p < 0.01$) by about 40% over the control level, a difference that was essentially maintained through 3 h of SCF treatment. By 24 h postplating, the percentage of gonocytes with pseudopods in the treated group had fallen to control levels. In addition, analysis of variance indicated that the percentage of gonocytes migrating in vehicle-treated chambers was unchanged throughout the duration of the experiment. Thus, addition of SCF to cocultures prepared on Day 5 caused a transient but significant burst of migratory activity in the gonocyte population that was not seen in the absence of exogenous ligand.

Immunolocalization of SCF. The transient nature of the response to exogenous SCF detected in the analysis described above raised the possibility that endogenous SCF produced by Sertoli cells might prevent any further, more sustained effect of exogenous ligand. For this reason, we used immunolocalization to determine whether, by 24 h postplating, Sertoli cells synthesized SCF. Our findings (Fig. 10) indicate that SCF was present in most if not all Sertoli cells at this time and that, as expected, gonocytes uniformly lacked immunofluorescent signal.

DISCUSSION

Observations made during the course of the present study provide substantial evidence in support of a role for the Kit receptor and its ligand, SCF, in stimulating migration of neonatal gonocytes, at least in vitro. First, we verified with in situ hybridization that the c-*kit* gene is expressed exclusively by gonocytes in vitro, as we had previously shown for gonocytes in vivo [10]. In addition, since use of the Sertoli cell-gonocyte coculture system allowed us to view entire, nonsectioned cells, we were able to correlate the presence of pseudopods on individual gonocytes with their expression of c-*kit*. Using this approach, we evaluated random samples of the gonocyte population during its development in vitro and found that 1) only a very few gonocytes express the gene before the start of migration and 2) once migration begins, there is an absolute correlation between expression of c-*kit* and incidence of a pseudopod on a given gonocyte. These observations suggest a causal relationship between c-*kit* expression and gonocyte

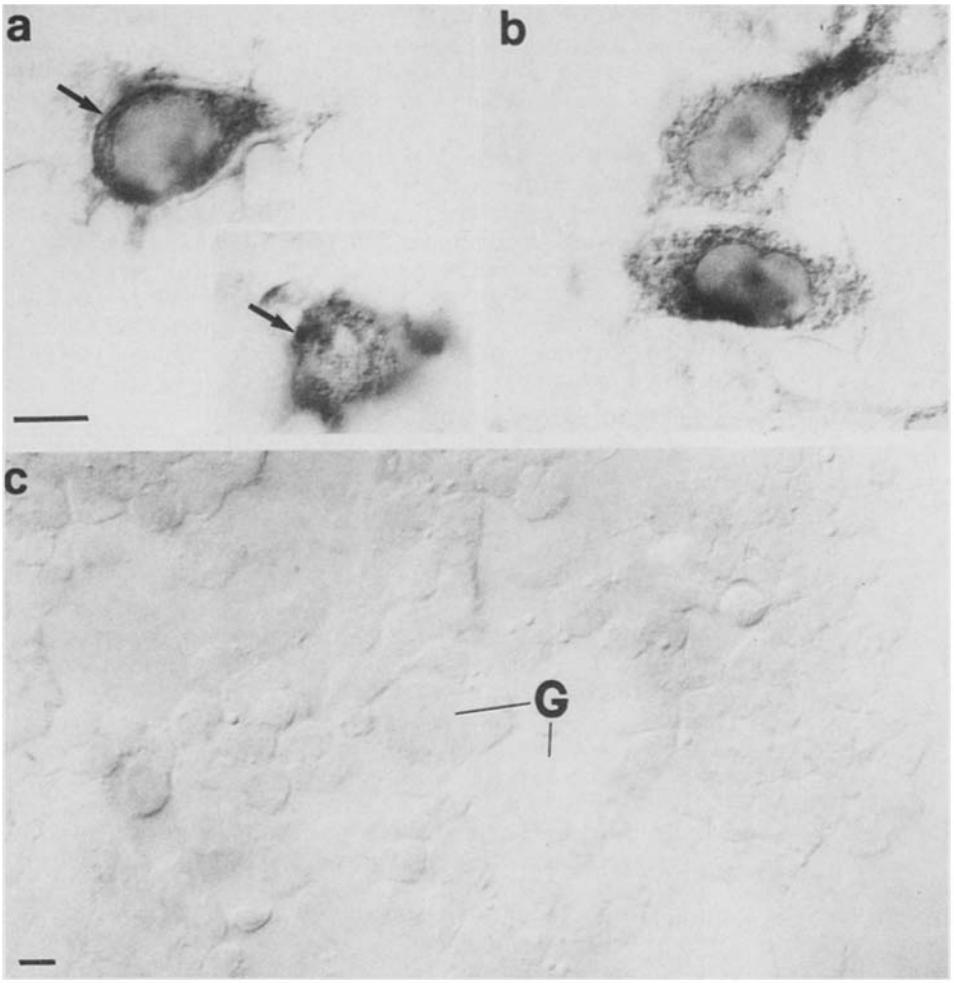

FIG. 6. Immunolocalization of the Kit receptor protein in gonocyte-Sertoli cell cocultures on Day 5 postplating. **a, b**) Incubation with Kit antiserum; **c**) incubation with preimmune serum. Many gonocytes, including most or all with pseudopods, were positive for the receptor protein. In many cases, some reaction product could be localized to the surface of a given cell, as in **a**, where arrows indicate the same gonocyte in both views and the plane of focus in the inset is on the upper surface of the cell. Bar = 10 μm.

migration, a possibility we tested directly by incubating cocultures prepared from newborns in the continuous presence of Kit antiserum. In this analysis, we detected a significant drop in the percentage of treated cells that were migrating compared to controls, a difference that became more pronounced with time of exposure to the antiserum. Finally, we also cocultured gonocytes from pups 5 days old, an age when these cells are already actively migrating in vivo, and exposed some of these cultures to exogenous Kit ligand, SCF, from plating onward. With this approach, we detected a rapid increase in the percentage of gonocytes with pseudopods in SCF-treated cultures compared to con-

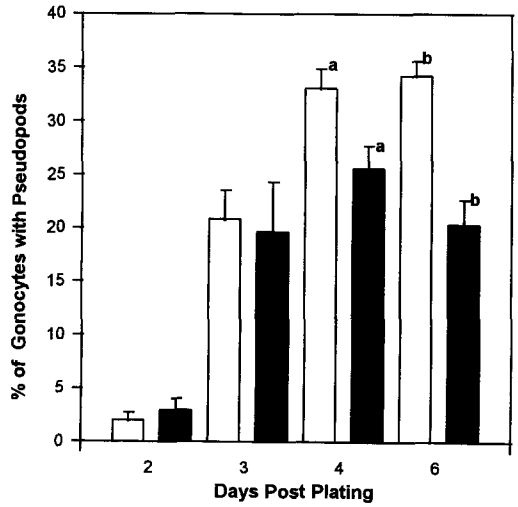

FIG. 7. The percentage of gonocytes with pseudopods in cultures prepared on the day of birth and exposed to either control peptide (open bars) or Kit antiserum (solid bars) from the time of plating on the day of birth through Day 6 in vitro. a, b: $p < 0.05$.

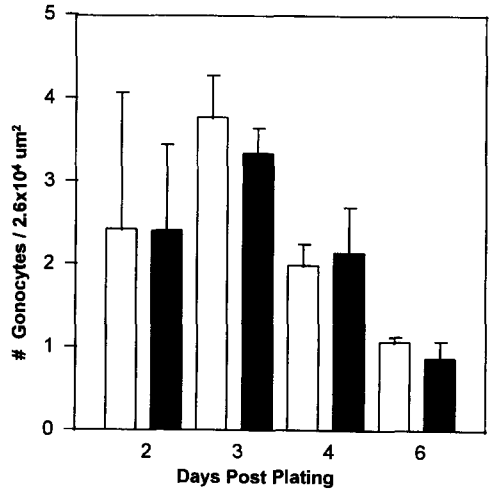

FIG. 8. The numbers of gonocytes per unit area culture in chambers exposed to either control peptide (open bars) or Kit antiserum (solid bars) from the time of plating on the day of birth through Day 6 in vitro. No significant differences were detected between treated and control chambers at any time point.

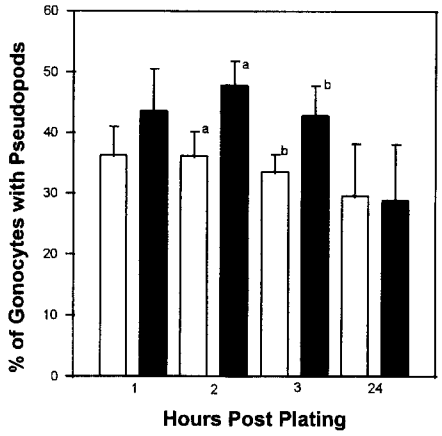

FIG. 9. The percentage of gonocytes with pseudopods in cocultures prepared on Day 5 after birth and exposed to either vehicle (open bars) or exogenous SCF (solid bars) from 1 through 24 h postplating. a: $p < 0.01$; b: $p < 0.05$.

trols. Taken together, these findings identify the Kit-SCF receptor-ligand system as critical for migration of neonatal gonocytes in vitro.

These observations confirm our earlier report [10] that at least some gonocytes express the c-*kit* gene in vivo on the day of birth and our suggestion that more cells might express this gene, at least as detected by in situ hybridization on sectioned material, as neonatal development proceeds and gonocytes begin migrating to the basement membrane. Moreover, the current findings provide the first clear-cut indication of a role for this gene and its encoded protein in the neonatal testis. Earlier reports from other laboratories raised the possibility that the Kit receptor might act as a survival factor, at least for primordial germ cells of fetuses [4] and some spermatogonia of adults [13]. For example, Yoshinaga et al. [14] found that injection of anti-Kit into adult mice resulted in depletion of differentiating spermatogonia, leaving behind a subset of surviving spermatogonia. These surviving spermatogonia were presumed to be stem cells, and this has led to the suggestion that, at least in adults, stem spermatogonia might not depend on Kit for their survival and hence might be c-*kit* independent [13]. However, it is not yet clear whether there is indeed a subpopulation of spermatogonia in adults that fail to express this gene. In our recent study of c-*kit* expression in vivo [10], we observed strong signal for the c-*kit* message in many spermatogonia of adults, but we did not address the question of whether any spermatogonia lacked signal. However, since some neonatal gonocytes are likely to represent either early spermatogonial stem cells or the precursors of these cells, it would be interesting to ask whether gonocytes of newborns cease expressing c-*kit* once their migration to the basal lamina in vivo or their burst of migratory behavior in vitro is completed. If so, this would lend support to the idea that spermatogonial stem cells are c-*kit* independent. Our current data are consistent with this possibility, since in samples encompassing the time of gonocyte migration in vitro, many nonmigrating gonocytes failed to express the gene and some or all of these may represent a c-*kit*-independent subpopulation of developing germ cells. This interesting question will be the subject of future investigations into the relationship between c-*kit* expression and gonocyte maturation.

Since it has been recognized for some time that relocation of neonatal gonocytes to the basement membrane is essential for their survival [3], our conclusion that c-*kit* expression is critical for gonocyte migration is consistent with the characterization by others of the Kit receptor as a survival factor, as discussed above (see [4] for review). However, Packer et al. [15] suggested that expression of c-*kit* ensures survival of germ cells by preventing apoptosis, as implied by their detection of an unusually high rate of apoptosis among spermatogonia in anti-Kit-treated adult mice. We addressed this issue indirectly in the present study by quantifying total numbers of gonocytes in cocultures with and without sustained exposure to c-*kit* antiserum. With this analysis we found that, under the same conditions that interfered significantly with gonocyte migration, there was no change in the number of gonocytes (or Sertoli cells; data not shown) in the treated cultures. This observation argues against the possibility that c-*kit* expression ensures survival of gonocytes by directly protecting them from programmed cell death. In addition, in preliminary investigations into regulation of apoptosis in the neonatal gonocyte population, we found no change in the incidence of apoptotic gonocytes, as measured by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling reaction, in cocultures exposed for several days to Kit antiserum (unpublished results). Thus, our findings in neonates suggest that the apparent increase in apoptosis among anti-Kit-treated spermatogonia detected by Packer et al. [15] in adults may reflect interference by the antiserum with an essential, as yet unidentified, cell function, and that apoptosis resulting from the antibody treatment may be secondary to that event.

As an additional test of the hypothesis that the Kit receptor is involved in gonocyte migration, we also asked

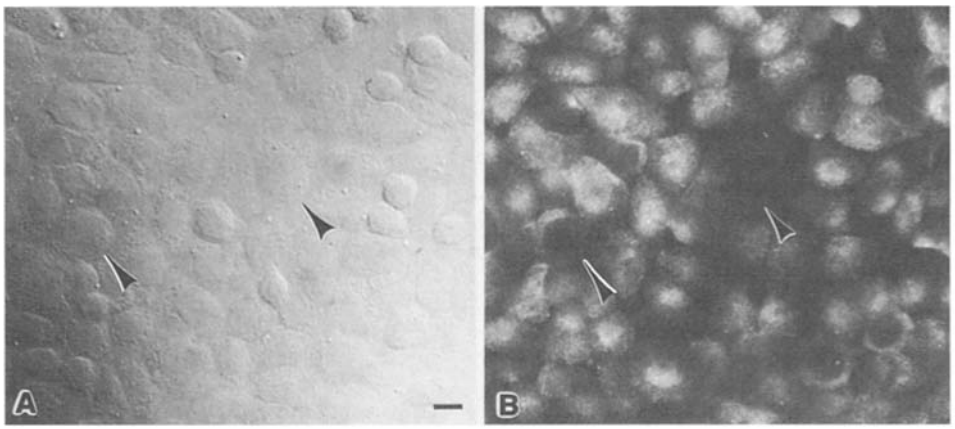

FIG. 10. Immunolocalization of SCF in cocultures prepared on Day 5 and subjected to analysis 24 h postplating. **A**) DIC optics; **B**) epifluorescence. Fluorescent signal is restricted to Sertoli cells, with gonocytes (examples at arrowheads) uniformly lacking fluorescence. Bar = 10 μm.

whether addition of exogenous Kit ligand to cocultured gonocytes would stimulate their migration in vitro. Our observation that this treatment caused a rapid but only transient migratory response among these cells led us to ask whether some or all neonatal Sertoli cells synthesize endogenous SCF in cocultures, thus providing a likely explanation for the short duration of the response to exogenous peptide. With immunolocalization, we found that SCF immunofluorescence was restricted to Sertoli cells, as expected, and that many if not all of these cells produce the peptide in vitro. Thus, the transient nature of the response of gonocytes to exogenous SCF that we detected in our study is perhaps attributable to the fact that—perhaps after some period of recovery following the cell isolation—Sertoli cells continue or resume producing SCF in both treated and control cultures, thus masking any further effect of exogenous peptide. In addition, it is also possible that exogenously added, soluble SCF is a poor substitute for endogenous, membrane-bound peptide on the Sertoli cell plasma membrane, and thus is capable of eliciting a response only during the initial stages of culture, before close contact between gonocytes and Sertoli cells is reestablished and gonocytes have access to available endogenous SCF on Sertoli cell surfaces. Although the precise nature of the interaction between SCF and Kit receptor protein in the neonatal testis remains to be explored, our observations constitute preliminary evidence that normal migratory activity of gonocytes from newborns may be at least transiently stimulated by soluble SCF.

In summary, we have used a variety of approaches to identify expression of the c-*kit* gene by neonatal gonocytes and, presumably, activation of the Kit receptor protein upon binding of SCF as crucial for successful reestablishment of migratory behavior among these cells. It will be important to identify the cellular mechanism(s) specifically targeted by activation of the Kit tyrosine kinase receptor that leads to onset of migration, as well as to determine the subsequent fate of those neonatal germ cells that successfully reach the future site of the basal compartment, two important goals of our future studies.

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