Interactions between Germ Cells and Extracellular Matrix Glycoproteins during Migration and Gonad Assembly in the Mouse Embryo

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Abstract. Cells are known to bind to individual extracellular matrix glycoproteins in a complex and poorly understood way. Overall strength of adhesion is thought to be mediated by a combinatorial mechanism, involving adhesion of a cell to a variety of binding sites on the target glycoproteins. During migration in embryos, cells must alter their overall adhesiveness to the substrate to allow locomotion. The mechanism by which this is accomplished is not well understood. During early development, the cells destined to form the gametes, the primordial germ cells (PGCs), migrate from the developing hind gut to the site where the gonad will form. We have used whole-mount immunocytochemistry to study the changing distribution of three extracellular matrix glycoproteins, collagen IV, fibronectin, and laminin, during PGC migration and correlated this with quantitative assays of adhesiveness of PGCs to each of these. We show that PGCs change their strength of adhesion to each glycoprotein differentially during these stages. Furthermore, we show that PGCs interact with a discrete tract of laminin at the end of migration. Closer analysis of the adhesion of PGCs to laminin revealed that PGCs adhere particularly strongly to the E3 domain of laminin, and blocking experiments in vitro suggest that they adhere to this domain using a cell surface proteoglycan.

Interactions between cell surface molecules and extracellular matrix (ECM)[1] glycoproteins are known to play critical roles in the survival, proliferation, differentiation, and migration of many cell types. This paper addresses the changing interactions between mouse primordial germ cells (PGCs) and purified ECM glycoproteins before, during, and after their migration. PGCs are a small population of cells, first found in the early mouse embryo during the gastrula stage (Ozdzenski, 1967; Ginsburg et al., 1990; cited by Tarkowski, 1975). Initially they are seen in the extraembryonic mesoderm, posterior to the primitive streak. Their expression of the enzyme alkaline phosphatase (AP; McKay et al., 1953; Chiquoine, 1954) allows their subsequent movements in the embryo to be followed. By embryonic age 8.5 d (E8.5), they are found embedded in the epithelium of the developing hind gut. At E9–9.5, they migrate out of the hind gut into its mesentery, and by E11.5 they are found mostly in the genital ridges, the sites of their future differentiation into gametes (for reviews see De Felici et al., 1992; Wylie and Heasman, 1993; Gomperts et al., 1994*a*).

Recently we showed that, during the E9.5 to E11.5 period, while PGCs emigrate from the gut, they interact with each other by the extension of long filopodial processes that link PGCs together. These appear to play an adhesive role (though there may be others) because 24 h later, the PGCs are all aggregated together into tightly apposed clusters in the genital ridges (Gomperts et al., 1994*b*). However, PGC:PGC interaction can only be a part of the migratory process, and here we present data on the adhesive interactions between PGCs and ECM glycoproteins.

ECM glycoproteins have often been implicated in PGC migration. The distribution of laminin (LM), fibronectin (FN), and collagen type IV (CIV) on the migratory route of avian PGCs suggests their involvement in migration (Urven et al., 1989). FN has been implicated in PGC migration in amphibians (Heasman et al., 1981), avians (En-

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1. *Abbreviations used in this paper*: AP, alkaline phosphatase; CIV, collagen type IV; E, embryonic day; ECM, extracellular matrix; FN, fibronectin; LM, laminin; PGC, primordial germ cell; SSEA-1, stage-specific embryonic antigen 1.

gland, 1983), and mammals (Fujimoto et al., 1985; Alvarez-Buylla and Merchant-Larios, 1986; Wylie et al., 1986; DeFelici and Dolci, 1989; ffrench-Constant et al., 1991). In addition, LM has been shown to support PGC adhesion in vitro (DeFelici and Dolci, 1989).

It is not clear from these studies how PGCs interact with individual ECM molecules or how these change at different stages of their migration. In mouse, for example, the distribution of FN has only been studied on part of the migratory route (Alvarez-Buylla and Merchant-Larios, 1986; Fujimoto et al., 1985), and studies of the interaction between FN and PGCs have had contradictory results (DeFelici and Dolci, 1989; ffrench-Constant et al., 1991). Here we have used a modified form of an adhesion assay developed by McClay and colleagues (1981) to measure the strength of initial adhesion (referred to here for simplicity as "adhesiveness") of PGCs taken from embryos before, during, and after migration. This method quantitates the number of cells detached from a substrate by a given centrifugal force, and we specifically scored PGCs by staining the cell preparations for alkaline phosphatase, either by histochemical staining and manual counting, or using a colorimetric reaction in solution. We show that PGC adhesiveness to three ECM glycoproteins alters in a differential manner during migration. We have also followed the distribution of the three ECM glycoproteins tested, using immunocytochemistry of whole-mount preparations. We find that PGCs assemble, at the end of migration, with a discrete region rich in laminin on each side of the embryo, whose position coincides with that of the future genital ridge. Given the changing adhesiveness of PGCs to laminin and its distribution, we investigated PGC:LM adhesiveness more closely in vitro. We show that PGCs adhere most strongly to the E3 domain of laminin and to a peptide within this domain containing a known heparin-binding site. Calcium dependency and blocking experiments with RGD peptides and heparin support the idea that PGCs attach to laminin in the early mouse embryo via both integrins and a cell surface heparan sulfate proteoglycan.

Materials and Methods

MF1 (OLAC) embryos from E8.5 to E12.5 (where noon on the day at which the vaginal plug was found is designated E0.5) were dissected from uteri in $Ca2^{+}/Mg^{2+}$-free PBS.

PGC Sample Enrichment by Percoll Gradient Purification

Mouse embryo regions containing PGCs were dissected as in Cooke et al. (1993) and incubated in PBS + EDTA (0.2%) for 15 min at room temperature. At E8.5, the entire allantois and posterior primitive streak were used. At E10.5, the dorsal mesentery, the aorta, and the genital ridges were used. At E12.5, the gonads were removed directly from the ventral wall without any other adherent tissue. Tissue fragments were triturated repeatedly in a small volume (150 μl) of the same solution. EDTA was removed by dilution and centrifugation (3 min at 270 *g*) of cells. The resulting pellet was resuspended in 260 μl of PB1 (Barton and Surani, 1993) and centrifuged in a premade Percoll (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) gradient to enrich for PGCs as described separately (García-Castro et al., 1997).

Adhesion Assay

Flat-bottom polyvinyl microtiter 5-mm-diam wells (Falcon 3912) were incubated for either 2 h or overnight (CO_2 25%, 37°C) with 50 μl of either collagen type IV (mouse; GIBCO BRL, Gaithersburg, MD), fibronectin (Bovine plasma; Sigma Chemical Co., St. Louis, MO), or laminin (mouse; Collaborative Research, Inc., Waltham, MA) at 20 μg/ml, bovine serum albumen (BSA, fraction V-SIGMA) at 20 μg/ml, or poly-D-lysine (PDL, to which all cells adhere) at 10 μg/ml. The coating solution was removed, and the surface was washed twice with PBS. Nonspecific binding to coated wells was prevented by incubating with a saturating solution of BSA 20 μg/ml for 1 h (CO_2 25%, 37°C). The solution was removed, and the wells were washed two times with PBS. Wells were freshly coated for each experiment. Details of coating for each experiment are given in the figure legends.

The adhesiveness of Percoll-purified PGCs (150–300 PGCs in 50 μl) from different stages was assayed using a modification of the McClay assay (1981). This is described in detail elsewhere (García-Castro et al., 1997). Briefly, PGC-enriched cell samples were added to "Adhesion" wells (coated with CIV, FN, LM, BSA, or PDL) and incubated for 0.5 or 1.5 h (CO_2 25%, 37°C). The adhesion wells were then inverted over "Recovery" wells (coated with PDL, 10 μg/ml) taped in place. The chamber so created was centrifuged upside down to create a quantitative detachment force. The PGCs displaced from the extracellular matrix glycoproteins were collected on the recovery wells. Cells in both wells were fixed with TCA 2% for 3 min and stained for the PGC marker AP. Alkaline phosphatase–positive cells, scored as PGCs, were counted in both wells. The strength of initial adhesion (adhesiveness) was defined as

$$\frac{\text{Number of PGCs in "adhesion well"} \times 100}{\text{Number of PGCs in "adhesion + recovery" wells}}\%.$$

Nonspecific binding of PGCs, measured as the percentage of PGCs adherent to BSA, was subtracted in each experiment. The specific conditions used here were arrived at after comparing different coating times and concentrations, adhesion times, g-force, and centrifugation time (García-Castro et al., 1997). Less than 10% of PGCs adhered to BSA under these conditions. All assays were performed in triplicate. Where different experiments used different conditions, these are given in the figure legends.

Quantitation of PGCs Using Alkaline Phosphatase Activity

Manual Counting. AP activity was detected in cell samples by adding 4 vol of AP staining solution (0.4 mg/ml Naphthol AS-MX phosphate [Sigma Chemical Co.], 1 mg/ml fast red or fast blue TR [Sigma Chemical Co.], 4 mM $MgCl_2$, Tris maleate, pH 9.0) and incubating for 10–15 min at room temperature. Detection of PGCs in the adhesion assay was carried out after fixing the cells with 2% TCA (Sigma Chemical Co.) for 3 min. Liquid was removed from the wells and 200 μl of AP staining solution was added. The wells were incubated at room temperature for 15 min, and the staining solution was substituted for PBS.

Using a Colorimetric Method. A rapid and quantitative method was established for quantitation of PGCs using a colorimetric substrate, p-nitrophenyl phosphate. Details are given separately (García-Castro et al., 1997). Briefly, samples after the adhesion assay were washed in 10 mM diethanolamine, pH 9.0 (DEA). This was then replaced by 50 μl of a colorimetric substrate for alkaline phosphatase (1 mg/ml p-nitrophenyl phosphate in DEA) for 230 min. The reaction was stopped by adding 50 μl 0.1 M EDTA, and the OD_{405} was measured in an ELISA plate reader.

Immunohistochemistry

Whole embryos were fixed for 2 h at room temperature in 2% TCA and processed immediately for whole-mount staining. Embryos were dissected to expose PGC-containing tissues. Antibody access to the tissues was promoted by incubating the samples for 15 min in PBTA (PBS containing 2 mg/ml BSA, 0.1% Triton X-100, and 0.02% sodium azide). Nonspecific antibody-binding sites were blocked by incubating samples for 1 h in PBTAS (PBTA containing 10% goat serum). The embryos were then incubated overnight at 4°C with the desired antibodies. PGCs were detected with an undiluted supernatant of the mouse hybridoma line TG1 (a gift from Peter Beverly, University College, London, England), which secretes a monoclonal antibody that reacts with the stage-specific embryonic antigen 1 (SSEA-1). LM was detected with a rabbit IgG antibody raised against mouse LM (Collaborative Research, Inc., Waltham, MA). A rabbit anti–mouse CIV antibody was a kind gift of Prof. K. von der Mark (University of Erlangen, Nürnberg, Germany). FN antibody was also a rabbit IgG raised against purified mouse plasma fibronectin (GIBCO BRL). All

three ECM antibodies were used at a 1:100 dilution. LM α1 chain was detected with rat monoclonal antibodies AL4 (a kind gift from Amy Skubitz, University of Minnesota) and mAb2000 (a kind gift from Peter Ekblom and Hubert Eng, University of Uppsala, Uppsala, Sweden), both raised against mouse LM α1. AL4 and mAb2000 were diluted 1:10. The samples were washed three times hourly in PBTA at room temperature before incubation overnight with the secondary antibody, either fluorescein or rhodamine-conjugated goat anti–mouse Ig or goat anti–rat Ig (diluted 1:50; Nordic Immunological Labs, Capistrano Beach, CA) or anti–rabbit biotinylated species-specific Ig from donkey (1:100; Amersham Corp., Arlington Heights, IL) in PBTA. After washing the samples three times (1 h each) in PBTA, streptavidin–Texas red (1:100; Amersham Corp.) was added and samples were incubated overnight at 4°C. After extensive washing, embryos were dehydrated through a methanol:PBS series to 100% methanol. All incubations were performed on a rotating platform. The embryos were cleared in benzoyl alcohol/benzoyl benzoate (1:2) and mounted in this mixture on cavity slides for viewing by confocal microscopy.

To stain embryo sections, TCA-fixed embryos were ethanol-dehydrated, embedded in PEDS wax, sectioned, and processed for antibody-staining. Alternatively, frozen sections were cut from unfixed embryos. Nonspecific antibody-binding sites were blocked by 30 min of incubation with blocking buffer (10% horse serum, 4% BSA in PBS). The buffer was substituted for the primary antibodies (diluted in PBS), and the sections were incubated overnight at 4°C. After three washes with PBS to remove all the antibody solution, the secondary antibody (fluorescent or biotinylated) was added and incubated for 2 h at room temperature. Texas red–streptavidin was added to biotinylated specimens and incubated for 2 h. After extensive washing, sections were embedded in mounting medium (90% glycerol, 100 mg/ml 1,4 diaza bicyclo [2,2,2] octane in PBS 0.1×). Stained sections were analyzed by fluorescent and confocal microscopy.

Results

PGC Adhesiveness to Purified ECM Glycoproteins Changes before, during, and after Migration

Adhesiveness values were obtained for PGCs on all three ECM glycoproteins at three stages of development; before (E8.5), during (E10.5), and after (E12.5) PGC migration. The conditions used (90 min of incubation of PGCs onto ECM glycoproteins coated at 20 μg/ml for 2 h on PVC) were as similar as possible to those reported by DeFelici and Dolci (1989) and ffrench-Constant et al. (1991) in order to compare our results with these studies. Centrifugation force and times were established empirically after several attempts in which the effects of these parameters were analyzed (García-Castro et al., 1997). The average results of one multiple experiment including all three stages and several experiments in which two developmental stages (E8.5 and E10.5, E8.5 and E12.5, or E10.5 and E12.5) were analyzed simultaneously are summarized in Table I and Fig. 1. Percoll gradient purification reveals that PGCs of two buoyant densities exist (low-density PGCs and high-density PGCs; García-Castro et al., 1997). In nearly all cases, changes in adhesiveness were the same for each population. The results show that PGCs change their adhesiveness to each of the three ECM glycoproteins in a differential manner during this period in development. Their adhesiveness to CIV changes very little; a large proportion of the PGCs (>90%) adhere to CIV at all three stages (Fig. 1 *A*). PGC adhesiveness to FN changes considerably (Fig. 1 *B*). This decreases during migration and continues to decrease after the PGCs colonize the genital ridges. PGC adhesiveness to laminin decreases during migration, but it either remains the same (high-density PGCs) or increases a little (low-density PGCs) when they colonize the genital ridges (Fig. 1 *C*). This last result was the only significant difference in behavior between high-density and low-density PGCs. The changes in adhesiveness were not due to differential losses of the less adhesive PGCs from the assays, since the total numbers (adhesive + nonadhesive PGCs) were not different in samples with high or low adhesiveness. This is shown in García-Castro et al. (1997).

ECM Distribution on the PGC Migratory Route

To correlate these adhesive changes with the distributions of the proteins concerned, we used double-label immunocytochemistry and confocal microscopy to study the spatial relationship between PGCs and the three ECM glycoproteins. PGCs were identified using antibodies against the carbohydrate antigen SSEA-1. LM, FN, and CIV were stained in the same specimens using specific antibodies.

Fig. 2 shows an E9.5 embryo, when PGCs are leaving the hind gut. At this stage, the hind gut is part of the dorsal abdominal wall, before its mesentery has formed. All three glycoproteins were found to be concentrated in or around the basal laminae surrounding the neural tube, hind gut, and aorta. They were also found between cells of the connective tissue outside the basal laminae (interstitial expression). Thus, PGCs are surrounded by all three ECM glycoproteins as they emigrate from the hind gut.

By E10.5, the hind gut mesentery has formed, moving the hind gut away from the dorsal abdominal wall. PGCs were found concentrated in the dorsal part of the mesentery. Their morphology showed that they are actively motile at this stage (Merchant and Zamboni, 1973; Gomperts et al., 1994), and they are known to be actively motile in culture (Alvarez-Buylla and Merchant-Larios, 1986; Donovan et al., 1986). At this stage, the three ECM glycoproteins were found in highest concentration in and around

Table I. Adhesiveness of Low- and High-Density, Percoll-purified PGCs to BSA, Collagen IV, Fibronectin, and Laminin, before (E8.5), during (E10.5), and after (E12.5) Migration

Stage	Fraction	Percentage of PGCs remaining attached (± SEM) to:			
		BSA	Collagen IV	Fibronectin	Laminin
E8.5	LD	5.04 ± 1.26	98.10 ± 1.89	97.68 ± 0.72	71.41 ± 9.16
	HD	5.60 ± 0.46	97.16 ± 0.66	97.50 ± 0.90	80.10 ± 10.90
E10.5	LD	13.64 ± 5.60	99.27 ± 0.75	80.45 ± 3.78	50.67 ± 11.26
	HD	9.29 ± 4.60	94.45 ± 3.94	58.02 ± 10.00	48.75 ± 8.02
E12.5	LD	9.08 ± 3.90	99.87 ± 0.13	57.31 ± 2.19	63.74 ± 4.64
	HD	6.30 ± 2.64	97.93 ± 0.22	39.34 ± 8.47	41.35 ± 7.63

HD, high density; *LD*, low density.

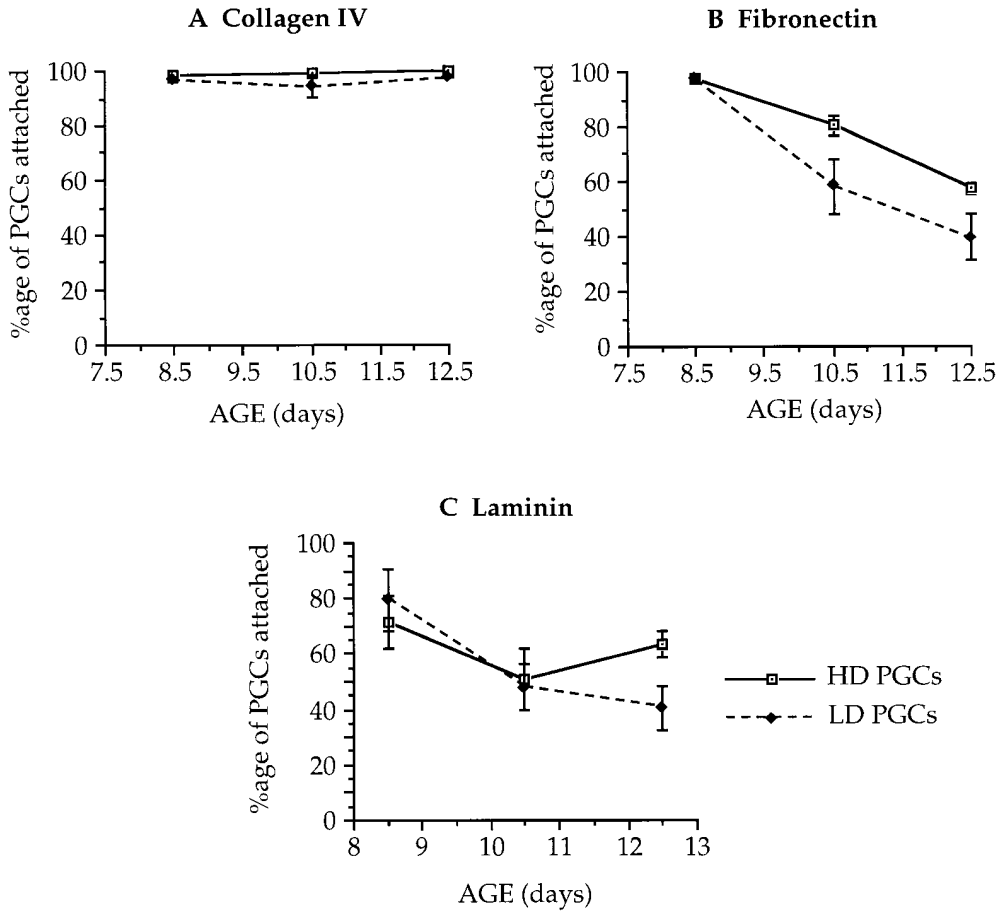

Figure 1. Graphs of the adhesiveness of low-density (*LD*) and high-density (*HD*) Percoll-purified PGCs to CIV (*A*), FN (*B*), and LM (*C*), before (E8.5), during (E10.5), and after (E12.5) migration. Values taken from Table I. Vertical bars show SEM from at least three experiments. Coating (20 μg/ml in each case) was for 2 h at room temperature. PGCs were incubated on substrata for 1.5 h at 37°C. Detachment was at 20 *g* for 3 min.

the basal laminae of the hind gut, neural tube, mesonephric ducts, epithelial lining of the abdominal wall, and the aorta. FN was distributed widely throughout the connective tissue (not shown; Fujimoto et al., 1985; Alvarez-Buylla and Merchant-Larios, 1986; Wylie et al., 1986). The distribution of laminin was particularly interesting (Fig. 3). In the hind gut mesentery, it is found interstitially (Fig. 3, *B* and *C*). However, during the 10th day, a basal lamina starts to form beneath the epithelial cells lining the coelom (Fig. 3 *B*). Immediately lateral to the root of the mesentery, this forms a ribbon of laminin-containing basal lamina, and the PGCs migrating laterally from the root of the mesentery accumulate on this ribbon. The earliest arrivals coincide with the first appearance of the laminin ribbon

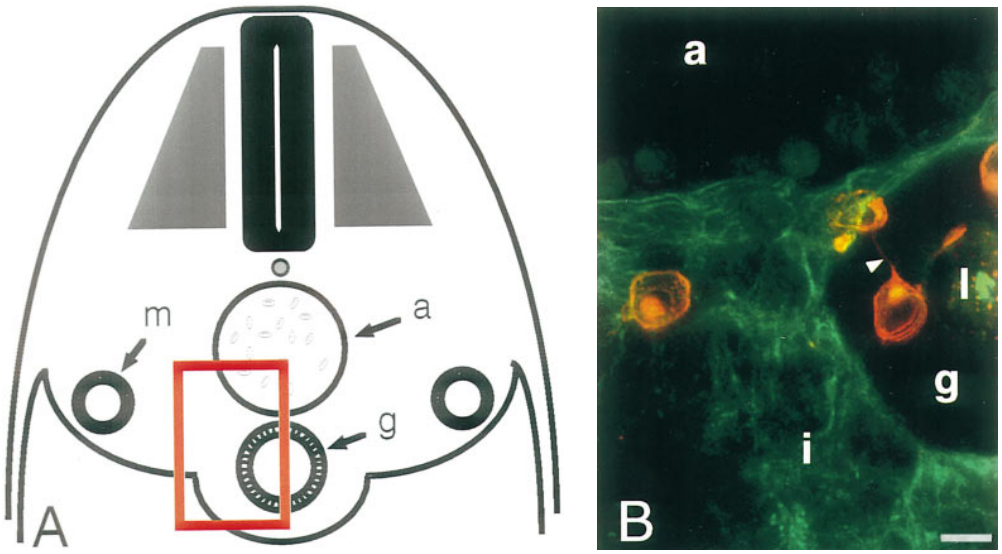

Figure 2. The relationship between PGCs and ECM glycoproteins as they leave the hind gut. The region of the hind gut and surrounding tissue is shown schematically in *A*. *m*, mesonephric duct; *a*, aorta; *g*, gut. (*B*) Profiles of four PGCs. Two are in the epithelial lining of the gut (*g*), one is entering the surrounding connective tissue, and one is migrating laterally away from the gut. All three ECM molecules (in this case, FN) are found interstitially around cells next to the gut (*i*). *a*, lumen of the aorta; *l*, lumen of hind gut; *arrowhead*, filopodial process of PGC; *red*, PGCs stained for anti-SSEA-1; *green*, FN. Bar, 10 μm.

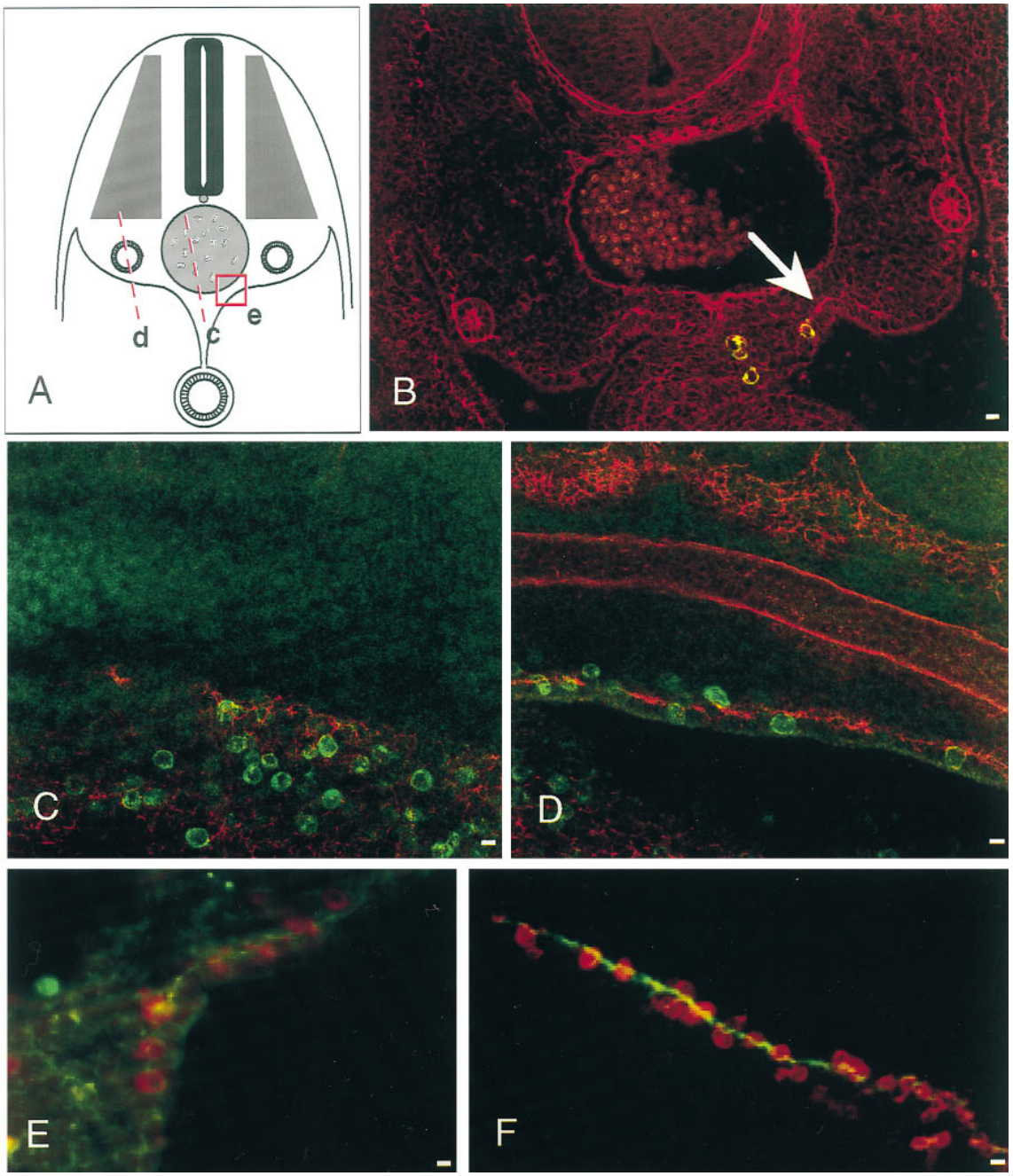

Figure 3. The relationship between PGCs and laminin at E10.5. (*A*) A schematic cross section. The mesentery of the hind gut has formed and now separates the gut from the aorta and other dorsal structures. *c* and *d* show the approximate positions of the longitudinal sections shown in *C* and *D*. *e* shows the region marked by the arrow in *B* and the region shown in *E*. (*B*) A low magnification transverse section, stained for laminin (*red*) and PGCs (*green*). LM is now becoming concentrated in basal laminae around the spinal cord, aorta, and mesonephric ducts. The forming basal lamina beneath the epithelial cells where the PGCs will accumulate is marked with an arrow. (*C* and *D*) Longitudinal sections through the positions indicated in *A*. Most of the PGCs (*green*) are in the mesentery, surrounded by interstitial laminin (*red*). The ones that have migrated most laterally (*D*) are found adjacent to the basal lamina shown in *B* (which is from the same specimen). (*E*) A double-stained specimen from an early E10.5 embryo, in transverse section, showing the close approximation of PGCs (*red*) and laminin (*green*) as the basal lamina first appears. (*F*) A later E10.5 embryo in longitudinal section (same orientation as in *D*). Many more PGCs have now accumulated on the ribbon of laminin. Bars, 10 μm.

(shown in transverse section in Fig. 3, *B*, *D*, and *E*). As more PGCs enter this region, increased numbers are found on the laminin ribbon (Fig. 3 *F*).

At E11.5, most PGCs have arrived in the genital ridges and are aggregated into closely apposed clusters, with few if any processes (Gomperts et al., 1994). They are also losing their ability to migrate in culture (Donovan et al., 1986). Laminin was found in high concentrations in the develop-

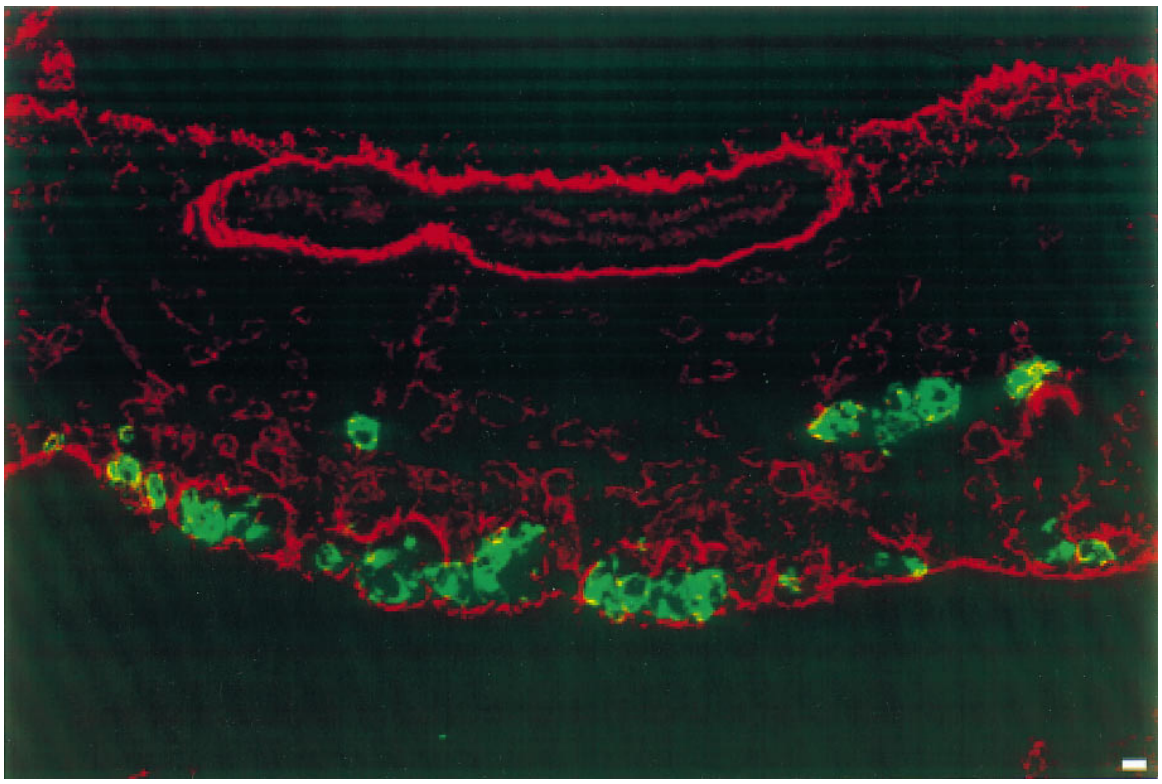

Figure 4. Longitudinal section at E11.5 stained for laminin (*red*) and PGCs (*green*). PGCs have now aggregated into clusters, each of which is now surrounded by a continuous layer of laminin. SSEA-1 antigen is disappearing from the PGCs, which causes the appearance of spaces between PGCs and laminin in some areas. Bar, 10 μm.

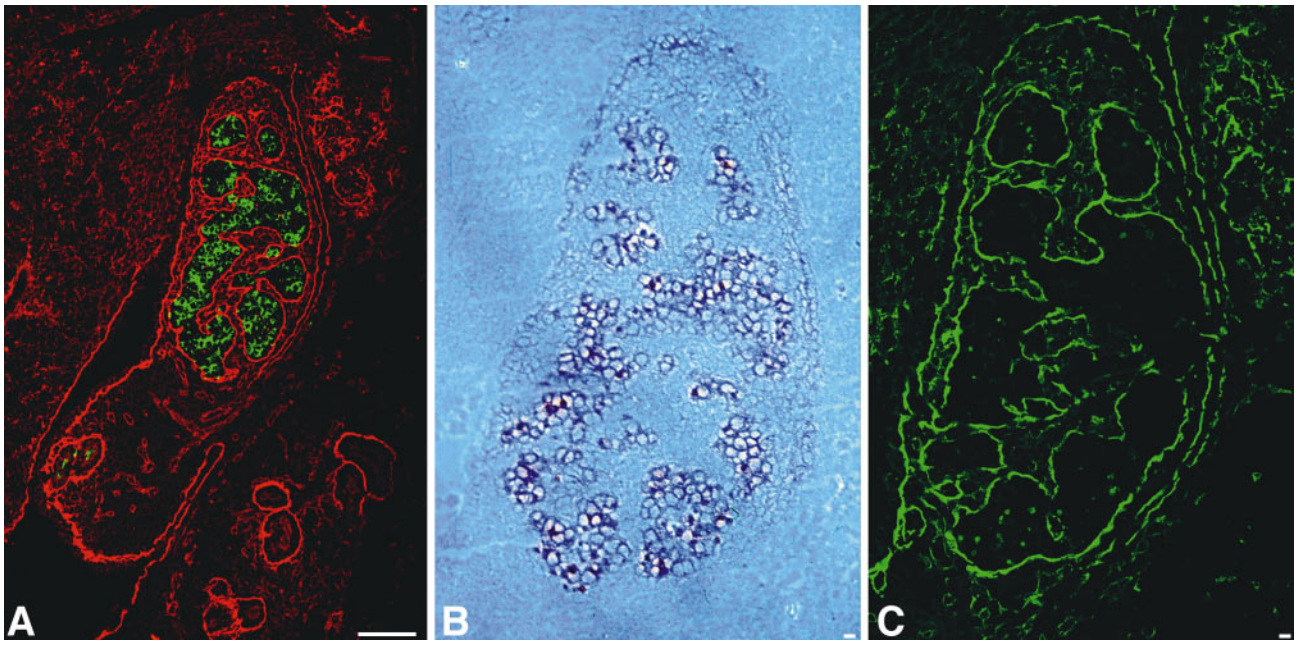

Figure 5. PGCs (*green* in *A*) arranged as sex cords, each of which is surrounded by a layer of laminin (*red*). (*B* and *C*) Adjacent sections stained for alkaline phosphatase (*B*) and with an antibody (AL4) against the α1 chain of laminin (*C*). The laminin surrounding the sex cords contains the α1 chain. mAb2000 gave the same result. SSEA-1 is being lost by the PGCs, giving the appearance of spaces in the sex cords in *A*. Bar: (*A*) 100 μm; (*B* and *C*) 10 μm.

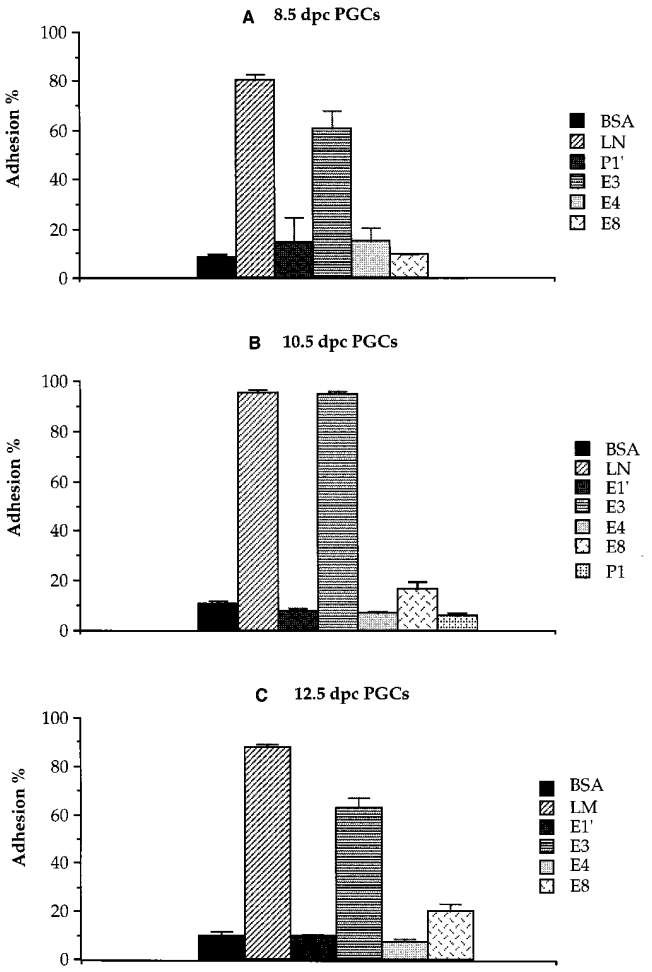

Figure 6. PGCs adhered most strongly to the E3 domain of laminin. Bars show the adhesiveness to whole laminin, and to different fragments of laminin, of PGCs isolated before (*A*), during (*B*), and after (*C*) migration. In each case, the g-force (20 *g*) and centrifugation time (1 min) detached PGCs from all fragments except E3. Coating concentration of whole laminin was 20 μg/ml and for each fragment was equivalent to 100 μg/ml of whole laminin. Coating was overnight at 4°C. PGCs incubated on substrata for 30 min at 37°C.

ing genital ridges, arranged as discrete layers surrounding each aggregate of PGCs (Fig. 4). At this stage, FN and CIV are expressed at low levels in the genital ridges, but at higher concentrations in the mesentery of the gut (not shown).

At E12.5, there was no longer any interstitial staining of LM; it was restricted to developing basal laminae surrounding the large aggregates of PGCs. Only the outermost PGCs in these clumps are attached to the layers of laminin seen (Fig. 5); the rest of the PGCs are attached to each other. Little FN, and no detectable CIV, was found in the E12.5 gonad in this study (not shown).

PGC Adhesiveness to Laminin Subunits

The association of PGCs with laminin at the end of their migration and assembly into sex cords led us to examine this interaction more carefully in vitro. First, we carried out adhesion assays to see which fragments of laminin PGCs adhere to most strongly and whether this changed during migration and gonad assembly. At first, we used the adhesion assay conditions reported above (coating time and with equivalent molar fragment concentrations, as well as adhesion incubation time and centrifugation time and force). However, we found very low or no adhesion to laminin fragments. Therefore, we modified the assay conditions in several ways: We used higher molar fragment concentrations (equivalent to 100 μg/ml of LM) and overnight coating; we reduced the adhesion incubation time as much as possible (to 30 min) to avoid interference from endogenous ECM deposits; and we reduced the centrifugation time (to 1 min). Fig. 6 shows the result of McClay assays, using different fragments of laminin. These were kindly donated by Peter Yurchenco (Robert Wood Johnson School of Medicine) and Roberto Perris (Reference Center for Oncology, Aviano, Italy), and their positions on the whole laminin molecule are given in Yurchenco and Cheng (1993). PGCs from before (E8.5), during (E10.5), and after (E12.5) migration were used. PGCs adhered most strongly at all three stages to E3, the COOH-terminal region of the α1 chain. In these experiments, adhesion assays from different stages were done separately so that stage to stage differences cannot be compared, as was the case in Fig. 1, where we compared PGCs from different stages in the same experiment.

Second, we tested PGC adhesiveness to individual peptide regions within COOH terminus of the α1 chain with known cell binding sites. These peptides were donated by Amy Skubitz, and their positions on the laminin molecule are given in Skubitz et al. (1991). Fig. 7 shows the adhesiveness of E12.5 PGCs to each peptide at different coating concentrations. PGCs adhered most strongly to the peptide GD2, measured here by the fact that they remained attached to this peptide at the lowest coating concentration.

The peptide GD2 contains a site known to bind heparin (Skubitz et al., 1991). We therefore tested the effect of heparin on binding of PGCs to whole laminin, E3, and the peptide GD2. Fig. 8 shows that heparin completely blocked attachment to E3 and GD2 at the g-force and time used and partially blocked attachment to whole laminin.

Since the adhesiveness of PGCs was greatest to a region of the α1 chain of laminin, we used two antibodies specific for the α1 chain (AL4 and mAb2000) to stain embryos at different stages of migration. We found that the α1 chain was present at all the locations, and at all stages, shown above (Fig. 5).

Effect of RGD Peptide, and EDTA, on PGC Adhesion to ECM Glycoproteins

Fig. 9 shows the effect of adding 100 μg/ml of either the peptide G**RGD**SP or G**RGE**SP on the initial adhesion of E12.5 PGCs to all three ECM glycoproteins. Adhesion of PGCs to both FN and LM was reduced, by 58% and 32.7%, respectively, by -RGD- but not -RGE- containing peptide. Adhesion to CIV was unaffected. This experiment was repeated with identical results. The fact that divalent cations are required for adhesiveness to whole laminin and the other glycoproteins used is shown in Fig. 10. 5 and 10 mM EDTA reduce the number of PGCs left in the adhesion wells, the effect on laminin being the most dramatic.

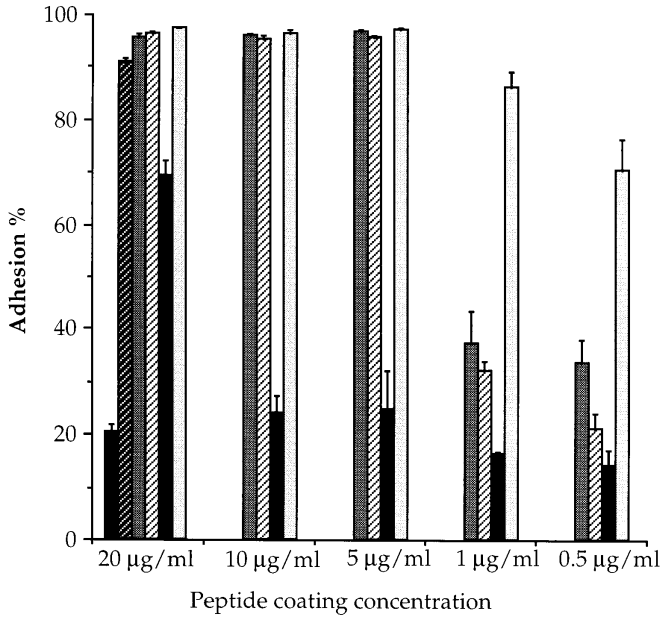

Figure 7. PGCs adhered best to the peptide GD2. Progressively reducing the coating concentration shows that PGCs were detached from all peptides except GD2 at coating concentrations of 0.5 μg/ml. PGCs were incubated on substrata for 30 min at 37°C, followed by centrifugation at 20 *g* for 1 min.

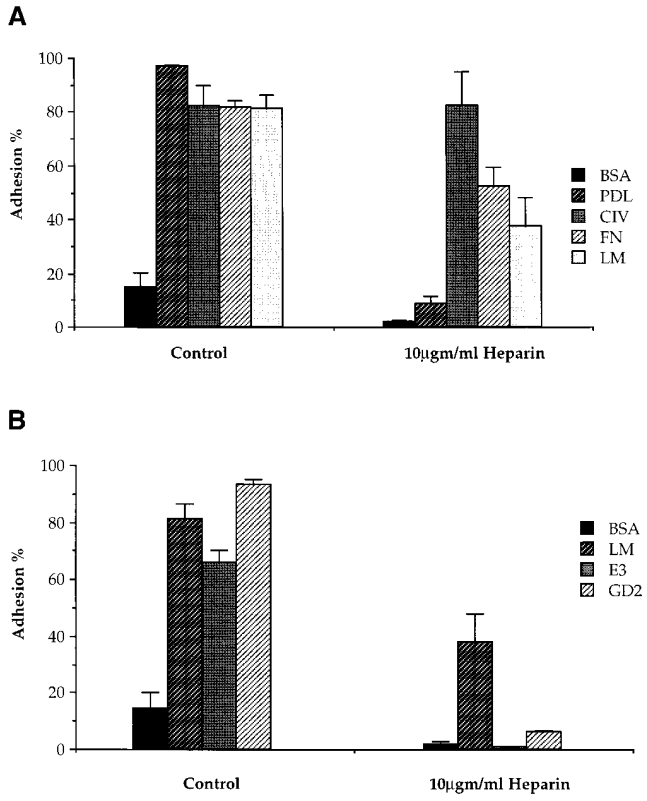

Figure 8. Heparin partially blocked adhesion to whole laminin (*A*) and completely abolished adhesion to E3 fragment and to GD2 peptide (*B*). (*A*) Effect of heparin on adhesion to whole glycoproteins. Coating was overnight at 4°C at a concentration 20 μg/ml in each case. 30 min contact of PGCs with substrata, centrifugation at 20 *g* for 1 min. (*B*) Effect of heparin on adhesion to E3 and GD2. Coating concentrations of E3 and GD2 equivalent to 100 μg/ml of whole laminin. Other conditions are as in *A*.

Discussion

There is much evidence that the migration of embryonic founder cell populations is dependent on extracellular matrix components surrounding them. Interaction with individual matrix glycoproteins and proteoglycans, and indeed with domains within some of these molecules, has been established by many elegant experiments using neural crest (Lallier et al., 1991), neurons (Tomaselli et al., 1990), and myoblasts (Goodman et al., 1991), as well as other cell types. However, only fragmentary and, in some cases, contradictory data exist for the interactions between PGCs and ECM during migration. This is largely because PGCs have not been collected in sufficient numbers for quantitative analysis. At the migratory stage, they consist of a population of only a few hundred cells, with no easy way to distinguish them in the living state from surrounding somatic cells.

In this work, we have started a systematic study of PGC:ECM interactions in the mouse embryo, using a combination of techniques that make this feasible. These include partial purification of PGCs, before, during, and after migration, a quantitative detachment assay, and a simple colorimetric assay for the PGC marker alkaline phosphatase (García-Castro et al., 1997). The reason for using a centrifugal detachment assay is that changes in the strength of adhesion might be expected to accompany the various phases of migration of PGCs (exit from the gut, migration in the mesentery, and cessation of migration at the sites of genital ridge formation). If so, this should be revealed by the sort of quantitative assay used here.

These assays proved highly reproducible, with small standard errors. They showed that the adhesiveness of PGCs to different purified ECM glycoproteins is different. Furthermore, their adhesiveness to each glycoprotein changes over the migratory period with a different profile. The change in adhesiveness to fibronectin confirms an earlier study, using a nonquantitative adhesion assay (ffrench-Con-

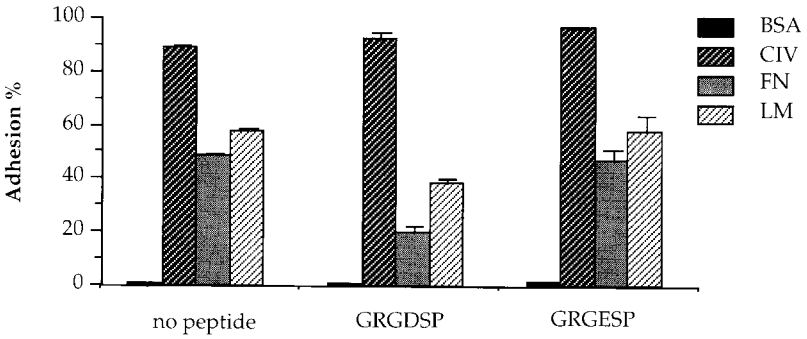

Figure 9. Effect of -RGD- containing peptide on adhesion of PGCs to FN, LM, and CIV. RGD reduced adhesiveness to LM and FN, but not to CIV. Control -RGE- containing peptide had no effect. PGCs were incubated in 100 μg/ml of each peptide for 1 h, followed by 30 min of incubation on substrata, and then were centrifuged at 50 *g* for 1 min. All substrata coating concentrations were 20 μg/ml for 2 h at room temperature.

stant et al., 1991). The reductions in adhesiveness of PGCs to both FN and LM fit well with similar data from cultured cells. For example, the migration speed of human myoblasts depends on their attachment strength, with maximum migration speed at an intermediate level of cell:substratum adhesiveness (DiMilla et al., 1993). The experiments reported here show that such changes occur during the normal migration of an embryonic founder cell type, and thus confirm that migratory cells in vivo do modulate their strength of adhesiveness in a manner predicted by studies on cells in culture. Of course these adhesive assays in culture do not tell us how these molecules are made available in vivo.

Adhesion assays, using different regions of laminin, showed that the adhesiveness of PGCs is highest for the E3 domain, before, during, and after migration. This suggests that this is the major site on laminin necessary for strength of adhesion of PGCs at all stages. The adhesion assay used here does not preclude the possibility that PGCs also adhere to other regions of laminin, only that this adhesiveness was not sufficient to prevent them from being removed by the centrifugal force used. Within the E3 domain, one region of PGC adhesiveness was shown to be the peptide GD2. This has been shown previously to be a heparin-binding site (Skubitz et al., 1991). This was supported by the fact that adhesion of PGCs to this peptide, and to the E3 domain, is abolished by heparin. The fact that RGD-containing peptide, and EDTA, both partially block PGC adhesiveness to whole laminin suggests that proteoglycan-mediated adhesion is not the only mechanism used by PGCs.

The distribution of ECM glycoproteins around PGCs during this same period shows significant differences with time. As they emigrate from the epithelial lining of the gut, PGCs encounter an ECM-rich region that surrounds the dorsal aorta and hind gut epithelium. Here all three glycoproteins are present in high concentration, and apparently in intimate contact with PGCs. Without electron microscopy, we cannot eliminate the possibility that somatic cell processes extend between PGCs and this sur-

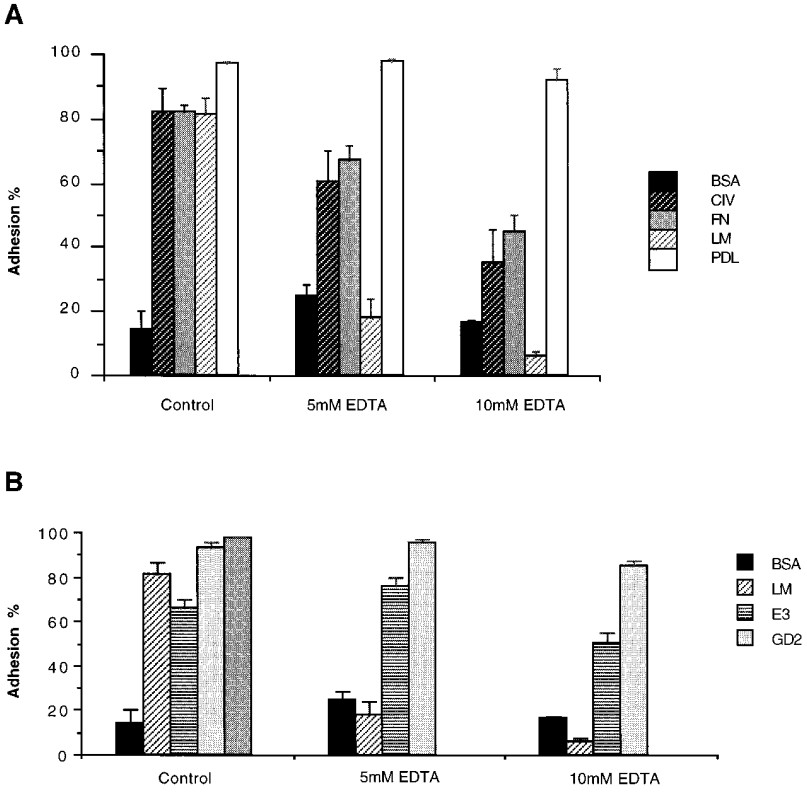

Figure 10. The effect of EDTA on PGC adhesiveness to whole glycoproteins (*A*) and fragments/peptides (*B*). Removal of calcium dramatically reduced adhesion to whole laminin (*A*), but not to fragment E3 or peptide GD2 (*B*). Coating concentrations were 20 μg/ml for whole proteins, 100 μg/ml equivalents for E3 and GD2. PGCs were incubated in solutions for 1 h, then on substrata for 30 min, followed by centrifugation at 20 *g* for 1 min.

rounding matrix. However, such processes would have to be extremely fine since there is no measurable distance between PGCs and surrounding ECM in the confocal images shown here. At E10.5, while in the mesentery and at the coelomic angles, PGCs are also surrounded by an interstitial-type matrix of ECM. However, a discrete laminin-rich layer appears in the region of the coelomic angles where the genital ridges form. In double-labeled images, the PGCs nearest to this area can be seen to align precisely with this layer. From its position, we assume this layer to be the forming basal lamina of the coelomic epithelial cells that are destined to become somatic cells of the gonad. In the following 24 h, this laminin-rich layer becomes organized in discrete layers around aggregates of PGCs as they accumulate in the gonad, a process that continues during the following 2 d as the primary sex cords form. The most attractive interpretation of the adhesion assays and the confocal images is that the first PGCs to encounter the epithelial cells that are going to form the somatic tissue of the gonad (the "pioneer PGCs") contact and adhere to a specialized laminin-rich ECM produced by these cells. This occurs in the E10–11 period. Later-emerging PGCs contact these pioneers, as well as each other, by means of long filopodial processes (Gomperts et al., 1994*a*,*b*). PGC aggregation will then cause PGCs to accumulate in the genital ridges. Here, PGCs assemble, in cooperation with laminin (we cannot distinguish cause and effect here), to form the primary sex cords. Further experiments will be required to prove whether this view of PGC migration is correct.

The concentrations of CIV and FN around PGCs decrease from E10.5 onwards, so that by E14.5 there is no detectable CIV and very little FN in the gonads. We assume from this that PGC adhesiveness to these two molecules is not used in the construction of the gonad, although this does not rule out the roles of small concentrations of these molecules.

In conclusion, this work has shown the changing distribution of three ECM glycoproteins around PGCs during migration and gonad construction, and that PGCs alter their adhesiveness to each of these during the same period. These changes suggest a possible mechanism of PGC accumulation in the genital ridges and sex cord formation. Further experiments will be required to fully analyze PGC adhesion of laminin and how this changes during migration and gonad assembly.

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References

Alvarez-Buylla, A., and H. Merchant-Larios. 1986. Mouse primordial germ cells use fibronectin as a substrate for migration. *Exp. Cell Res.* 165:362–368.

Barton, P., and A. Surani. 1993. Manipulation of genetic constitution by nuclear transplantation. *In* A Guide to Techniques in Mouse Development. P.M. Wassarman and M.L. DePamphilis, editors. Academic Press, New York. 732–746.

Chiquoine, A.D. 1954. The identification, origin and migration of the primordial germ cells of the mouse embryo. *Anat. Rec.* 118:135–146.

Cooke, J., I. Godin, C. ffrench-Constant, J. Heasman, and C.C. Wylie. 1993. Culture and manipulation of primordial germ cells. *Methods Enzymol.* 225:37–58.

DeFelici, M., and S. Dolci. 1989. *In vitro* adhesion of mouse fetal germ cells to extracellular matrix components. *Cell Diff. Dev.* 26:87–96.

De Felici, M., S. Dolci, and M. Pesce. 1992. Cellular and molecular aspects of mouse primordial germ cell migration and proliferation in culture. *Int. J. Dev. Biol.* 36:205–213.

DiMilla, P., J. Stone, J. Quin, S. Albeda, and D. Lauffenberger. 1993. Maximal migration of Human Smooth muscle cells on fibronectin and type IV collagen occurs at an intermediate attachment strength. *J. Cell Biol.* 122:729–737.

Donovan, P., D. Stott, L. Cairns, J. Heasman, and C.C. Wylie. 1986. Migratory and non-migratory mouse primordial germ cells behave differently in culture. *Cell.* 44:831–838.

England, M. 1983. The migration of primordial germ cells in avian embryos. *In* Current Problems in Germ Cell Differentiation, A. McClaren and C. Wylie, editors. Cambridge University Press, Cambridge. 91–114.

ffrench-Constant, C., A. Hollingsworth, J. Heasman, and C.C. Wylie. 1991. Response to fibronectin of mouse primordial germ cells before, during and after migration. *Development (Camb.).* 113:1365–1373.

Fujimoto, T., K. Yoshinaga, and I. Kono. 1985. Distribution of fibronectin on the migratory pathway of primordial germ cells in mice. *Anat. Rec.* 211:271–278.

García-Castro, M., J. Heasman, and C.C. Wylie. 1997. A rapid and quantitative assay of adhesiveness of mouse primordial germ cells that can be applied to many types of early embryonic cell. *Methods Cell Sci.* 19:1–9.

Ginsburg, M., M.H.L. Snow, and A. McLaren. 1990. Primordial germ cells in the mouse embryo during gastrulation. *Development (Camb.).* 110:521–528.

Gomperts, M., M. García-Castro, C. Wylie, and J. Heasman. 1994*a.* Interactions between primordial germ cells play a role in their migration in mouse embryos. *Development (Camb.).* 120:135–141.

Gomperts, M., C. Wylie, and J. Heasman. 1994*b.* Primordial germ cell migration. *In* Germline Development. Ciba Foundation Symposium Vol. 182. J.M.a.J. Goode, editor. John Wiley and Sons Ltd., London. 121–134.

Goodman, S.L., M. Aumailley, and H. von der Mark. 1991. Multiple cell surface receptors for the short arms of laminin: α1β1 integrin and RGD-dependent proteins mediate cell attachment only to domains III in murine tumor laminin. *J. Cell Biol.* 113:931–941.

Heasman, J., R.O. Hynes, A.P. Swan, V. Thomas, and C.C. Wylie. 1981. Primordial germ cells of *Xenopus* embryos: the role of firbonectin in their adhesion during migration. *Cell.* 27:437–447.

Lallier, T., R. Deutzmann, R. Perris, and M. Bronner-Fraser. 1991. Avian neural crest cell attachment to laminin: involvement of divalent cation dependent and independent integrins. *Dev. Biol.* 113:1069–1084.

McClay, D., G. Wessell, and R. Marchase. 1981. Intercellular recognition: quantitation of initial binding events. *Proc. Natl. Acad. Sci. USA.* 78:4975–4979.

McKay, R.G., A.T. Herting, E.C. Adams, and S. Danziger. 1953. Histochemical observations on the germ cells of human embryos. *Anat. Rec.* 117:201–219.

Merchant, H., and L. Zamboni. 1973. Fine morphology of extragonadal germ cells of the mouse. *In* The Development and Manipulation of the Ovary and Its Functions. H. Peters, editor. Experta Medica, Amsterdam. 95–100.

Ozdzenski, W. 1967. Observations on the origin of the primordial germ cells in the mouse. *Zool. Polon.* 17:367–379.

Skubitz, A.P.N., P.C. Letourneau, E. Wayner, and L.T. Furcht. 1991. Synthetic peptides from the carboxy-terminal globular domain of the A chain of laminin: their ability to promote cell adhesion and neurite outgrowth, and interact with heparin and the β1 integrin subunit. *J. Cell Biol.* 115:1137–1148.

Tarkowski. 1975. Discussion on germ cells origin. *In* Embryogenesis in Mammals. Vol. 40. K. Elliot, M. O'Connor, editors. Elsevier/Excerpta Medica, London. 110.

Tomaselli, K.J., D.E. Hall, L.A. Flier, K.R. Gehlsen, D.C. Turner, S. Carbonetto, and L.F. Reichardt. 1990. A neuronal cell line (PC12) expresses two β1-class integrins—α1 β1 and α3 β1—that recognize different neurite outgrowth-promoting domains in laminin. *Neuron.* 5:651–662.

Urven, L., U. Abbot, and C.A. Erickson. 1989. Distribution of extracellular matrix in the migratory pathway of avian primordial germ cells. *Anat. Rec.* 224: 14–21.

Wylie, C.C., and J. Heasman. 1993. Migration, proliferation, and potency of primordial germ cells. *Semin. Dev. Biol.* 4:161–170.

Wylie, C.C., D. Stott, and P.J. Donovan. 1986. Primordial germ cell migration. *In* The Cellular Basis of Morphogenesis, 2nd ed. L. Browder, editor. Plenum Press, New York. 433–450.

Yurchenco, P., and Y.-S. Cheng. 1993. Self-assembly and calcium-binding sites in laminin. *J. Biol. Chem.* 268:17286–17299.