Axolotl pronephric duct migration requires an epidermally derived, laminin 1-containing extracellular matrix and the integrin receptor α6β1

Andrea R. Morris[1,*,†], Julie Drawbridge[2] and Malcolm S. Steinberg[1]

[1]Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA
[2]Department of Biology, Rider University, Lawrenceville, NJ 08648, USA
*Present address: Haverford College, Department of Biology, 370 Lancaster Avenue, Haverford, PA 19041, USA
[†]Author for correspondence (e-mail: armorris@haverford.edu)

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Summary

The epidermis overlying the migrating axolotl pronephric duct is known to participate in duct guidance. This epidermis deposits an extracellular matrix onto the migrating duct and its pathway that is a potential source of directional guidance cues. The role of this matrix in pronephric duct guidance was assayed by presenting matrix deposited on microcarriers directly to migrating pronephric ducts in situ. We found that reorientation of extracellular-matrix-bearing carriers prior to their presentation to migrating ducts caused a corresponding reorientation of pronephric duct migration. Subepidermal microinjection of function-blocking antibodies against α6 integrin, β1 integrin or the laminin-1/E8 domain recognized by α6β1 integrin, all of which were detected and localized here, inhibited pronephric duct migration. Moreover, pre-exposure to anti-laminin-1/E8 function-blocking antibody prevented reoriented carriers of epidermally deposited matrix from reorienting pronephric duct migration. These results are incorporated into an integrated model of pronephric duct guidance consistent with all present evidence, proposing roles for the previously implicated glial cell-line derived neurotrophic factor and its receptor as well as for laminin 1 and α6β1 integrin.

Key words: Pronephric duct, Cell migration, Axolotl, Extracellular matrix, Cell guidance, Laminin 1, α6β1 integrin

Introduction

Cell migration is essential for many morphogenetic events in multicellular organisms. Yet much remains unknown about the mechanisms that allow stationary cells to become motile, to follow the migratory pathways appropriate to them and ultimately to stop migration at the specified destination. We have investigated migration of the axolotl pronephric duct (PND), a model system for elucidating the molecular mechanisms that control directed cell migration during embryogenesis. The PND (also termed the Wolffian, mesonephric or nephric duct) is the central component in the development of the vertebrate kidney. In addition to forming the waste conduit of the pronephros and mesonephros, the amniote PND generates the ureteric bud of the metanephros, which will undergo further differentiation to form the ureter – the structure that transports urine to the bladder. The PND also gives rise to the ductus deferens – the major sperm conduit of the male reproductive system – and participates in the induction of both mesonephric and metanephric nephrons (for reviews, see Davies, 2001; Drawbridge and Steinberg, 1996; Saxén, 1987; Saxén et al., 1986).

The events of PND morphogenesis have been fairly well conserved during vertebrate evolution. The PND primordium is first observed in association with the incipient pronephros originating from lateral mesoderm located just ventral to the developing somites at the level of the cervical vertebrae. The PND then extends caudally along a precisely defined pathway between the lateral and somitic mesoderm. PND elongation continues until the PND fuses with the cloaca, the exit point of the excretory system. In urodeles, including the axolotl *Ambystoma mexicanum*, as well as some primitive teleosts, PND morphogenesis appears to occur entirely by active cell migration accompanied by cell rearrangements (Ballard and Ginsburg, 1980; Poole and Steinberg, 1981; Poole, 1988; Drawbridge et al., 2003). Therefore, the events of axolotl PND morphogenesis provide an excellent system for studying directed cell migration in vivo.

During their migration, PND cells come into direct contact with somitic mesoderm, lateral flank mesoderm and overlying epidermis, all of which are potential sources of local guidance information. By transplantation and rotation of epidermal grafts, Drawbridge et al. (Drawbridge et al., 1995) have shown that overlying epidermis provides directional cues to the migrating PND. The PND 'reads' this information along the anterior-posterior (AP) axis to direct migration from anterior to posterior (A→P) and along the dorsal-ventral (DV) axis to constrain migration to the PND pathway. This overlying epidermis has been shown to secrete an extracellular matrix (ECM) onto the migrating PND cells and their migration pathway (Poole and Steinberg, 1981; Gillespie and Armstrong, 1986). Because of evidence in other systems showing that ECM components can provide not only a permissive

substratum for cell migration but also a source of directional cues (reviewed in Boucaut et al., 1991; Winklbauer and Nagel, 1991; Johnson et al., 1992; Erickson and Perris, 1993; Perris, 1997; Perris and Perissinotto, 2000), we hypothesized that components of the epidermally secreted ECM might provide the PND with a migration substratum, directional information or both. Using a method described by Löfberg et al. (Löfberg et al., 1985) of "in vivo adsorption of embryonic extracellular matrix onto a 'microcarrier' of Nuclepore filter", we have examined the potential role of epidermally derived ECM in axolotl PND migration. The results presented here provide evidence that this ECM is a source of directional guidance cues for PND cells.

We also wanted to identify specific ECM components that might serve as directional cues, as well as the corresponding receptors used by PND cells to recognize and respond to information from the ECM. Lallier et al. (Lallier et al., 1996) have reported that the expression pattern of the α6 integrin subunit during *Xenopus* development includes the elongating PND. Therefore, we have set out to determine whether axolotl embryos exhibit a similar α6 integrin expression pattern and, if so, whether α6 integrins play any role in PND extension. The anti-α6 integrin monoclonal antibody GoH3 blocks binding of α6-containing integrins to laminin proteins (Sonnenberg et al., 1986; Knapp et al., 1989; Aumailley et al., 1990). Using in vivo application of this and other function-blocking antibodies, we provide direct evidence that α6 and β1 integrins, and laminin 1 are required components of the PND migration system.

Materials and methods

Embryos and microsurgery

Wild-type and albino embryos were provided by the University of Indiana Axolotl Colony. Embryos were manually decapsulated with watchmaker's forceps and plastic transfer pipettes. Surgeries were performed with electrolytically sharpened tungsten needles, hairloops and watchmaker's forceps in Petri dishes lined with non-hardening modeling clay or 2% agarose. All surgical procedures and subsequent rearing of embryos were carried out in full strength Hepes-buffered Steinberg's solution (HBSt; Zackson and Steinberg, 1986) supplemented with antibiotics (Sigma; 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin). Embryos were staged according to Bordzilovskaya et al. (Bordzilovskaya et al., 1989).

ECM-bearing microcarriers: orientation and transplantation

Polycarbonate membranes (Poretics) of pore size 0.4 μm were cut with iris scissors into approximately 0.5 mm squares. Using sharpened tungsten needles, epidermal flaps of approximately four somite widths were made on one side of stage 22 donor embryos. Membrane fragments ('carriers') were then placed beneath the epidermal flaps and held there with glass bridges. Embryos were allowed to continue development overnight (to approximately stage 30) at room temperature, during which time ECM was deposited onto the carriers. The side of the carrier apposed to the epidermis is termed the 'E' face; the side apposed to the mesoderm is termed the 'M' face.

For transplantation of ECM-bearing carriers, epidermal flaps were cut over the PND pathway of stage 22 hosts. ECM-conditioned carriers were removed from donor embryos and placed under the host epidermal flap, presenting either the 'E' or the 'M' face of the ECM-bearing carrier to the migrating PND primordium. The flap was then closed and allowed to heal. Various rotations of the ECM-conditioned carriers (90° dorsal, 90° ventral, 180° or no rotation) were made prior to their introduction into hosts.

To present epidermally deposited ECM to the underlying mesoderm, a carrier must be turned over before re-implantation. This reverses either its AP or its DV axis. This axial reversal can be undone ('unrotated' carrier) by collecting ECM on one side of the embryo and presenting it to the mesoderm on the opposite side. For unrotated and 90° rotated 'E' carriers, ECM was collected on the left-hand side of donor embryos then transplanted to the right-hand side of hosts. Thus, both the AP and DV orientation of unrotated carriers relative to host embryos were maintained. '90° dorsal' and '90° ventral' rotations re-align both the AP and DV axes of the transplanted ECM. '90° dorsal' rotation aligns the ECM's original A→P axis with the host's V→D axis and its original D→V axis with the host's A→P axis. '90° ventral' rotation aligns the ECM's original A→P axis with the host's D→V axis and its original D→V axis with the host's P→A axis.

In the case of 180° rotations of 'E' carriers, ECM was collected on the left-hand side of donor embryos and then transplanted to the left-hand side of hosts to reverse only the AP axis of the transplanted ECM relative to that of the host PND pathway.

ECM for unrotated or 180° rotated 'M' carriers was collected on the left-hand side of donor embryos and transplanted to the left-hand side of hosts. Thus, unrotated 'M' ECM matched both the AP and DV axes of the underlying host PND pathway. 'M' carriers rotated 180° reversed both AP and DV axes of the transplanted ECM relative to host tissues.

Host embryos were cultured to stage 28 at room temperature and fixed in modified Karnovsky's fixative (1.5% paraformaldehyde, 1.5% glutaraldehyde and 0.1 M phosphate buffer, pH 7.4). Overlying epidermis was then peeled away and the carrier dissolved in chloroform. PND morphology was then observed and photographed. In all cases, the unoperated side of each host embryo was examined as an internal control for normal PND migration. Embryos in which PND migration was inhibited on the unoperated side were discarded, because they probably underwent abnormal development unrelated to our experimental manipulations. Fisher's exact test and likelihood ratio χ^2 statistical analysis were performed on this data.

Antibody treatment of embryos

Embryos were reared to stage 20-21 prior to injections. The Picospritzer II (General Valve) was used to inject bovine serum albumin (BSA), antibodies and Fab fragments under conditions previously described (Thibaudeau et al., 1993). Antibodies were obtained from the following sources: purified monoclonal anti-human α6 integrin (GoH3) (Pharmingen); monoclonal, function-blocking anti-β1 chicken integrin, (Sigma); monoclonal, non function-blocking anti-human β1 integrin (Chemicon International); and polyclonal, anti-mouse laminin-1/E8 fragment (gift of P. Yurchenco, UMDNJ). A polyclonal anti-laminin antibody against the basement membrane of an EHS mouse sarcoma (Sigma) was also used in whole mount immunocytochemistry (ICC). Embryos stained for laminin proteins were sectioned using standard techniques. All antibodies used in these experiments were characterized by western analysis and immunoprecipitation (IP) to insure antigen specificity. Fab fragments were made using papain digestion according to the manufacturer's recommended conditions (Pierce).

Subepidermal injections of all agents to be tested were typically made along the lateral aspect of the embryo, just below the developing somites at points dorsal to the PND tip, immediately caudal to the tip and also along the migratory pathway, at 1 mg ml^{-1} concentrations. Successful injections resulted in raised 'blisters' at the injection site that flattened upon dispersal of the sample. After injections, embryos were transferred to fresh agarose-coated dishes containing HBSt with antibiotics and allowed to continue development overnight at room temperature, followed by fixation and manual removal of the overlying epidermis. For each set of injections, the location of the PND tip was determined for both the injected and uninjected sides, since diffusion of injected proteins across the embryonic midline does occur (Zackson and Steinberg, 1989; Thibaudeau et al., 1993).

Whole mount immunocytochemistry

Albino axolotl embryos were treated according to the methods described by Smith and Armstrong (Smith and Armstrong, 1990). Embryos were first fixed overnight at room temperature, in Dent's fixative (80% methanol, 20% dimethylsulfoxide, v/v) and then stored in absolute methanol at –20°C until use. Samples were rehydrated in TBS (0.05 M Tris-HCl, 0.85% NaCl, pH 7.6) then incubated in the appropriate primary antibody, diluted in TBS with 0.2% BSA as follows: α6, 1:1000; β1 (blocking and non-blocking), 1:500; laminin, 1:50; laminin 1/E8, 1:100. Embryos were washed in at least five changes of TBS/0.1% Tween over 24 hours. Whole mounts were then incubated in a biotinylated goat anti-mouse, goat anti-rat or goat anti-rabbit secondary antibody in TBS/0.2% BSA (1:1000 dilution), rinsed as before, then incubated in 1:1000 streptavidin/β-galactosidase in TBS/0.1% Tween. Antibody binding was detected by developing whole mounts in 0.42 mg ml^{-1} X-gal in 10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 3.0 mM potassium ferricyanide, 3.0 mM potassium ferrocyanide, 1.0 mM $MgCl_2$. For staining with α6 and β1 antibodies, epidermis was peeled from fixed embryos prior to initial rehydration. For laminin and LM-1, E8 fragment antibody staining, the epidermis was kept intact and the embryos were permeabilized with Dent's fixative prior to antibody introduction. Transverse sections of LM-stained were made using standard histological techniques.

Assessment of the extent of PND migration

In the axolotl, PND migration occurs in synchrony with the wave of somite segmentation (Gillespie et al., 1985; Gillespie and Armstrong, 1986). Gillespie and Armstrong (Gillespie and Armstrong, 1986) showed that the posterior tip of the axolotl PND is located approximately 2 somite widths anterior to the most posterior somite fissure throughout the entire period of PND migration. Thus, segmenting somites serve as anatomical landmarks to which the extent of PND migration can be compared. In agreement with Gillespie and Armstrong, we found that the posterior tips of PNDs in control embryos are found one, two and sometimes three somite widths anterior to the most posterior somite fissure; these were scored as 'last somite minus 1' (LS MINUS 1), LS MINUS 2 and LS MINUS 3, respectively. Thus, scores 'anterior to LS MINUS 3' indicate inhibition of PND migration. One-way ANOVA statistical analysis was also performed on this data.

Results

Directional guidance of PND migration by epidermally produced ECM

In order to investigate the role of epidermal ECM in PND migration, we first optimized our ability to isolate epidermally produced ECM. ECM deposited onto polycarbonate carriers by the overlying epidermis of stage 22 embryos was visualized by scanning electron microscopy. An extensive meshwork of proteins was apparent along the surface of the carrier apposed to the epidermis (the 'E' surface of the carrier) after overnight incubation. The observable extracellular material showed dense regions of crisscrossing fibrils, typical of such matrices (Löfberg et al., 1985).

To assay the ability of epidermally derived ECM to direct PND migration, the 'E' face of such conditioned polycarbonate carriers was presented to the PND and PND pathway of stage 22 host embryos in the following orientations: (1) unrotated, preserving both AP and DV axes; (2) rotated by 180°, reversing the AP axis while maintaining the DV axis; (3) rotated 90° dorsally; or (4) rotated 90° ventrally (Fig. 1). In the last two cases, the carrier's original A→P orientation was aligned

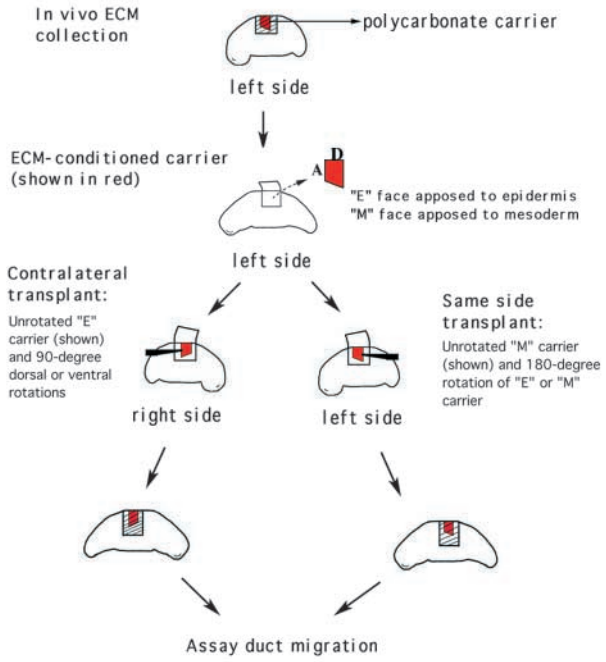

Fig. 1. ECM collection and carrier transplantation techniques. Polycarbonate carriers were cut and placed under donor epidermis, which was allowed to secrete ECM onto the carrier overnight. This 'ECM-conditioned' carrier was then placed, with or without rotation, under an epidermal flap along either the left or the right PND migration pathway of the host embryo. Carriers remained in embryos overnight, followed by fixation, removal of epidermis and analysis of PND location.

V→D or D→V with the host, its original DV axis also being rotated correspondingly. The results of these experiments were compared with those obtained with unconditioned, control carriers. In addition, mesodermally derived ECM (i.e. the 'M' face of conditioned carriers) was also presented to the migrating PND. The results are summarized in Table 1.

When 'E' carriers were introduced, with no rotation, into host embryos at the beginning stages of PND migration, we observed that the PND was able to migrate normally, beneath and beyond the carrier, in most cases (Fig. 2A). In a few cases, the migrating PND stopped beneath the carrier. Following 180° rotations of 'E' carriers, such that the host PND tip was presented with P→A oriented ECM, migration was arrested beneath the carrier in approximately half the cases observed. PND migration beneath and beyond the carrier along its normal migration pathway was also observed.

Only when 'E' carriers were introduced with 90° dorsal rotations were cases observed in which the PND was deflected dorsally from its normal migratory pathway just below the developing somites (Fig. 2B). Normal migration under and beyond the carrier and migration stalled under the carrier were also seen. Only following 90° ventral rotations of 'E' carriers were cases of ventral migration onto lateral flank mesoderm seen (Fig. 2C). In these experiments, some embryos also exhibited 'DV flaring'; that is, the PND tip spread both dorsally and ventrally, as previously described (Drawbridge et al., 1995). Unconditioned carriers restrained PND migration when placed over migrating host PNDs. Migration stopped beneath the carriers in most cases observed.

Table 1. The effect of ECM orientation on PND migration

Carrier rotation	PND migrates normally	PND migration stops under carrier	PND deflected ventrally	PND deflected dorsally	PND tip flares
Unconditioned carriers (n=16; P<0.001)	6	10	0	0	0
'E' carriers no rotation (n=11; P<0.001)	8	3	0	0	0
E carriers 180° rotation (n=11; P<0.001)	6	5	0	0	0
E carriers 90° dorsal (n=13; P=0.0116)	5	4	0	4	0
E carriers 90° ventral (n=15; P=0.0391)	3	5	5	0	2
Anti-LM- Ab-treated E carriers 90° ventral (n=14; P<0.001)	5	9	0	0	0
M carriers no rotation (n=12; P<0.001)	10	2	0	0	0
M carriers 180° rotation (n=13; P<0.001)	12	1	0	0	0

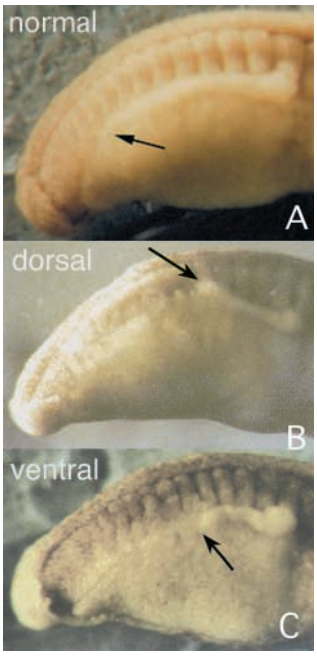

Fig. 2. Epidermal ECM provides directional cues to the PND. Epidermally secreted ECM was collected onto polycarbonate carriers, then presented in various orientations to the PND pathway of same-stage hosts (Fig. 1). (A) A control embryo undergoing normal PND migration. The PND migrates just below the somites and its tip is indicated by the arrow. (B) The PND has migrated onto somitic mesoderm – dorsal to its normal migratory pathway – following the introduction of a 90° dorsally rotated ECM-bearing carrier. The PND tip (arrow) has here traveled more than halfway up the sixth somite. (C) A 90° ventral rotation of an ECM-conditioned carrier prior to transplantation has redirected PND migration ventrally. The PND tip (arrow) is located ventral to the normal migration path, on lateral mesoderm. (A-C) Anterior is to the right and dorsal to the top.

Host PNDs were also presented with the side of the microcarrier that had been apposed to the mesoderm of the ECM donor (the 'M' side). Experiments in which the AP and DV axes of the implanted 'M' carrier were the same as those of the host embryo resulted in normal PND migration. When the AP axis of the 'M' carriers was rotated 180° relative to that of the host, PND migration was unaffected by this reorientation and these carriers also supported normal migration.

Inhibition of laminin binding to 'E' carriers prevents redirection of PND migration

Exposure of 'E' carriers to a polyclonal, anti-laminin antibody revealed the presence of laminin proteins on the carriers (data not shown). This raises the possibility that laminins might be involved in permitting and/or guiding PND migration. There is substantial evidence in other systems that extracellular components, particularly laminins, provide both a permissive substratum and also directional cues for cell migration (Lallier et al., 1992; Bronner-Fraser, 1993; Perris et al., 1996; Garcia-Castro et al., 1997; Luckenbill-Edds, 1997).

To determine whether laminin 1, in particular, provides the migrating axolotl PND with a migration substratum, directional information or both, host embryos were presented with ECM-conditioned 'E' carriers that had been treated prior to transplantation with the function-blocking antibody against the laminin-1/E8 cell-binding domain (1 mg ml^{-1}). These antibody-treated carriers were rotated 90° ventrally before introduction into host embryos. In most cases, the PND stopped migrating under the carriers, as was seen when unconditioned carriers were presented to host PNDs (Table 1). In no case was the host PND deflected ventrally. By contrast, 90° ventral rotations of 'E' carriers that had not been treated with the anti-laminin-1/E8 antibody often caused ventral deflection. Thus, blocking the E8 cell-binding domain of laminin 1 in the epidermally derived ECM bound to rotated carriers prevents their re-orientation of PND migration.

Laminin 1 and its receptor ($\alpha6\beta1$ integrin) are expressed during PND migration

Whole mount ICC with either a polyclonal 'anti-laminin' antibody or an anti-laminin-1/E8 domain-specific antibody revealed the presence of laminin in the head and lateral flank of stage 24-28 embryos. Analysis of transverse sections of these embryos reveals that laminin 1 is present at the base of ectodermal epithelial cells, within the basal lamina overlying the head and along the lateral flank as well (Fig. 3C).

Because the $\alpha6\beta1$ integrin mediates binding to the ECM through the E8 domain of laminin 1 (Kramer et al., 1991; Aumailley and Gayraud, 1998), we looked at the expression of this integrin receptor during axolotl PND migration. IP and western blot analysis of stage 22-28 axolotl embryo proteins confirmed that the GoH3 antibody directed against the human $\alpha6$ integrin subunit recognizes an axolotl antigen of the predicted molecular weight of 130 kDa. Whole mount ICC of stage 23-26 axolotl embryos with the GoH3 antibody revealed expression on the migrating PND (Fig. 3A). GoH3 also recognizes antigen along the developing somites and the pronephros. IP and Western analysis with an anti-$\beta1$ integrin antibody revealed that it recognizes a peptide of the predicted size for the $\beta1$ subunit (120 kDa). Whole-mount ICC of stage 23-26 albino embryos showed $\beta1$ expression along the developing somites, the 'gill bud', the pronephros and the migrating PND (Fig. 3B).

Antibodies that block $\alpha6$ and $\beta1$ integrin function disrupt PND migration

Function-blocking antibodies against the $\alpha6$ and $\beta1$ integrin subunits were used to determine whether blocking active sites on the $\alpha6\beta1$ integrin receptor inhibits PND migration. Subepidermal injection of 1 mg ml^{-1} anti-$\alpha6$ integrin GoH3 Fab fragments significantly inhibited PND migration, such that

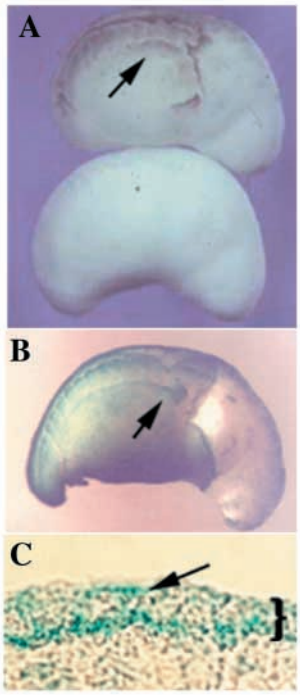

Fig. 3. Expression of α6 and β1 integrins and laminin 1 in axolotl embryos. (A) A stage 24 albino embryo was treated as described in Materials and Methods, with an anti-α6 integrin antibody (GoH3) followed by antibody detection (top). Antibody staining is evident along the somites, the pronephros (arrow) and the migrating PND. A control embryo to which no GoH3 antibody was added (bottom) did not show staining. (B) A stage 26 albino embryo was fixed and the epidermis overlying the lateral flank was removed. The embryo was then treated with an anti-β1 integrin primary antibody as described in Materials and Methods. Staining was evident throughout the lateral mesoderm, including the somites, the pronephros and the PND (arrow), indicating broad expression of β1-containing integrins. (C) The extracellular localization of laminin was observed following transverse sectioning through an axolotl embryo that had been previously treated with an anti-laminin-1 antibody. Strong staining was evident within the outermost epidermal layers (arrow and bracket) and highlights its expected localization to the basal lamina.

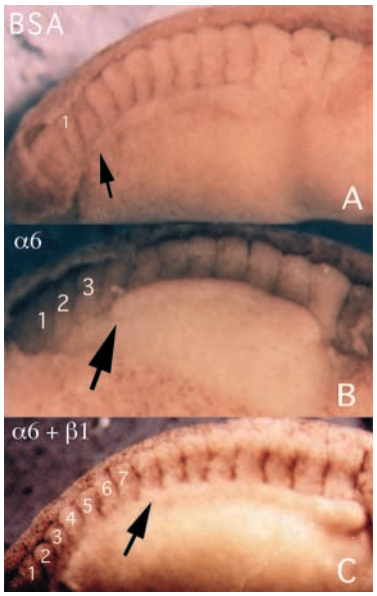

Fig. 4. Inhibition of PND migration following injection of function-blocking antibodies. Stage 21 embryos were injected with Fabs either against BSA or that specifically block integrin subunit function. (A) A BSA-injected control embryo in which the PND was found to have migrated normally. The PND tip (arrow) is at LS MINUS 1 in this embryo. (B) Following exposure to α6-integrin-function-blocking Fabs, PND migration has been inhibited, such that the PND tip (arrow) is at LS MINUS 3.5. (C) An embryo that has been co-injected with α6- and β1-integrin-function-blocking Fab fragments. These antibodies caused a significant inhibition of PND migration, such that the tip is located at LS MINUS 7 rather than its normal position of about LS MINUS 1 to LS MINUS 2.

the PND tip was located anterior to LS MINUS 3 in more than half the cases observed (P=0.005). In BSA-injected sibling controls, most cases showed the normal PND migration at LS MINUS 1 to LS MINUS 3 (Fig. 4A,B; Fig. 5A). Sub-epidermal injection of anti-β1 integrin Fabs resulted in inhibition of PND migration similar to that observed with the α6-blocking antibody (Fig. 5C; P=0.046).

When a mixture of both α6 and β1 blocking Fabs was injected into stage 21 embryos, we observed a more pronounced inhibition of PND migration than was found with either of these antibodies alone. In more than half the cases observed, the duct tip was anterior to LS MINUS 3 on the injected side of the embryos, including many cases in which the PND tip was located as far anterior as LS MINUS 7 (Fig. 4C, Fig. 5C; P<0.0001). In sibling BSA-injected control embryos, PND migration was anterior to LS MINUS 3 in only two embryos out of 21. The localization of the α6 and β1 integrin subunits to the migrating PND, together with the observed perturbation of migration upon introduction of function-blocking antibodies, indicates that α6β1 integrins are required for proper PND migration.

Antibody blocking the laminin-1/E8 domain inhibits PND migration

If the preceding results reflect more efficient blocking of the α6β1 integrin when antibodies to both subunits are injected together, blocking this integrin's ligand (the E8 domain of laminin 1) should be equivalent to blocking both α6 and β1 together. This would also provide a direct test of the involvement of laminin 1 in PND migration.

Injection of anti-laminin-1/E8 Fab fragments caused pronounced inhibition of PND migration. In these experiments, the tip of the duct was typically found anterior to LS MINUS 3 (Fig. 5D; P<0.0001). In sibling control embryos injected with BSA, this level of inhibition was never observed. These results are comparable to those observed with the combination of α6 and β1 antibody injections (Fig. 4C, Fig. 5D). We therefore conclude that inhibition of PND migration when antibodies to both α6 and β1 are co-injected reflects the specific blocking of α6β1 integrin.

In addition to antibodies, laminin-1/E8 peptide fragments were also injected into embryos. Such injections also caused inhibition of PND migration, presumably because of competition with endogenous laminin for binding to α6β1 integrin (Fig. 5E; P=0.008).

Injection of control proteins and antibodies does not inhibit PND migration

To examine the possibility that our Fab fragment injection results were due to non-specific effects of the injected proteins, control injections were also performed. Injection of a non-function-blocking anti-β1 antibody into embryos had little effect on PND migration, demonstrating that injected antibodies must bind to a functional epitope in order to inhibit PND migration in our assays (Fig. 5F; P=0.058). Likewise, when a 1:2 molar ratio of anti-laminin-1/E8 antibody:E8 fragments was injected, PND migration was essentially normal (Fig. 5G; P=0.0006), suggesting that the ability of the anti-E8 antibody to perturb PND migration depends on its E8-binding activity. These controls demonstrate that the observed inhibition of PND migration by microinjection of function-blocking antibodies and Fab fragments is due to the inhibition of specific molecular domains necessary for PND migration.

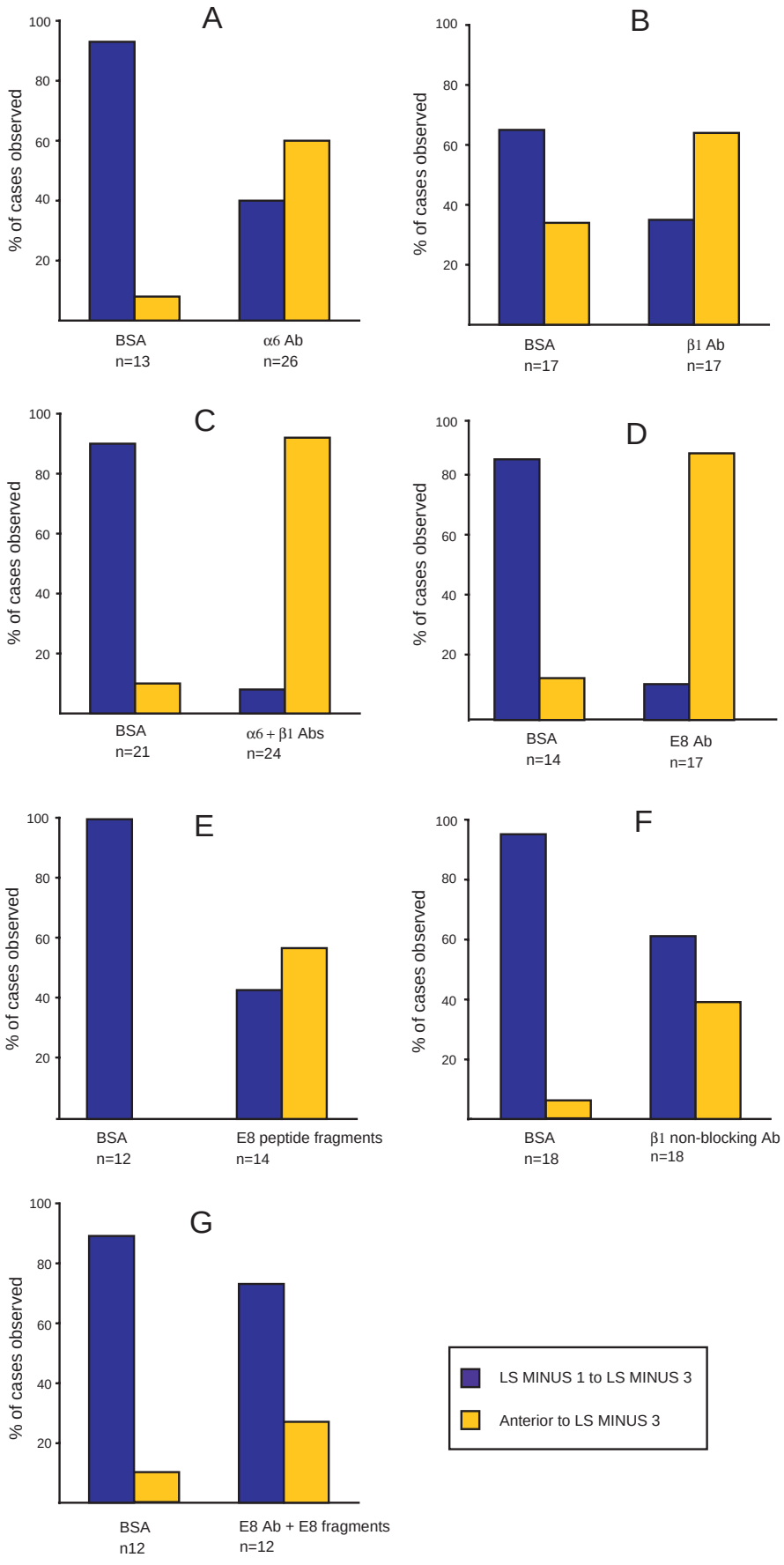

Fig. 5. Summary of PND migratory inhibition. The effects of injecting function-blocking versus non-blocking agents are summarized here. BSA-injected embryos appear to undergo normal PND migration (LS MINUS 1 to LS MINUS 3) and are shown in blue for each graph. Blocking α6β1 integrins and laminin 1 causes inhibition of PND migration (anterior to LS MINUS 3), as indicated in yellow.

Discussion

Epidermal ECM provides directional information to the migrating PND

In studies of the effects of epidermal grafts upon axolotl PND migration, Drawbridge et al. (Drawbridge et al., 1995) first reported on the role of the overlying epidermis in providing directional cues to the migrating PND. These results showed that the direction of PND migration could be altered by reorienting the overlying epidermis, indicating that the overlying epidermis is a source of directional PND guidance information. These directional cues were proposed to be a property of the ECM secreted by the epidermis. We show here that the direction of PND migration can be similarly altered by reorientation of epidermally derived ECM collected on polycarbonate carriers. Furthermore, rotated 'E' carriers are as efficient at reorienting PND migration as rotated epidermal grafts [compare results presented here with those in Drawbridge et al. (Drawbridge et al., 1995)]. From these observations, we conclude that the directional information previously shown to be a property of the epidermis overlying the migrating PND resides in the epidermal ECM.

Our carrier implantation experiments also allowed us to determine whether ECM deposited on polycarbonate carriers by lateral mesoderm ('M' ECM) underlying the PND can influence PND migration. The data in Table 1 reveal that both mesodermal ECM and unrotated epidermal ECM are much more effective than polycarbonate carriers alone at supporting PND migration. Thus, both epidermal and mesodermal ECM provide a permissive substratum for PND migration. However, 'M' ECM supports PND migration equally well when aligned with or opposed to the AP and DV axes of host ECM, whereas 'E' ECM only supports normal PND migration when its AP and DV axes are aligned with those of the host. Thus, we conclude that 'M' ECM permits, but does not guide PND, migration, whereas 'E' ECM both permits and guides it.

Although changes in the direction of PND migration with carrier rotations were observed in significant numbers of cases, both normal and arrested PND migration were also frequently observed. These results might be explained by the fact that removal of epidermis does not completely remove already-secreted epidermal ECM from the PND surface (J.D., unpublished). In our ECM-conditioned carrier transplantation experiments, ECM is likely to be left on the PND following epidermal removal, leaving some host-derived A→P guidance information available to the PND. We speculate that this information competes with information from the transplanted, donor ECM bound to the carrier, presenting the migrating PND with conflicting directions when the carrier is rotated. The PND responds to such conflicting information by ceasing migration altogether or by following the more abundant of the two competing sets of guidance cues.

Role of α6β1 integrins and laminin 1 in PND migration

Can the PND-guiding property of 'E' ECM be traced to specific cell receptors and ECM components? Our results demonstrate the presence of both α6 and β1 integrin subunits on the PND itself and of laminin 1 on the flank that serves as the PND pathway. We also show that blocking either laminin 1's integrin-binding sites or the α6β1 integrin receptor that binds to them inhibits PND migration. Moreover, a function-blocking anti-laminin-1 antibody neutralizes the ability of ECM bound to a rotated carrier implant to redirect PND migration. Thus, laminin 1 is a required component of the PND migratory pathway and α6β1 integrins are a required component of the PND pathfinding apparatus.

Laminin proteins have been reported to provide both permissive and directive substrata for migrating cells (Lallier and Bronner-Fraser, 1991; Yao et al., 1996; Bendel-Stenzel et al., 1998; Jones et al., 2000). Furthermore, laminins have also been shown to be required components of the PND (Wolffian duct) migration substratum in both chick and mouse embryos (Bellairs et al., 1995; Willem et al., 2002). Two possibilities present themselves here based upon our findings – either laminin 1 itself provides both permissive and directive PND guidance information or laminin 1 provides only a permissive substratum, directional cues being provided by a different component of the ECM.

Of the ducts confronted with 'E' carriers rotated by 90°, 32% were deflected in the corresponding direction and another 32% were blocked beneath the carrier. By contrast, of the ducts confronted with anti-laminin-1-treated 'E' carriers rotated by 90°, 0% were deflected in the corresponding direction, whereas 64% were blocked beneath the carrier. These data suggest that application of anti-laminin-1 to 'E' carriers prevents the ECM-mediated duct diversion by blocking the further migration of those ducts that would otherwise have been diverted. Those ducts consequently end up in the 'stops under carrier' category. There is no significant effect of antibody treatment of the carriers on the fraction of ducts that migrate normally. These are the results to be expected if, in the absence of antibody, a proportion of the ducts beneath the rotated carrier adhere primarily to residual host ECM and are thus unaffected by the ECM on the carrier, whereas the remaining ducts adhere primarily to the laminin 1 on the carrier and are diverted. Thus, anti-laminin-1 treatment of the ECM-bearing carrier would only prevent ducts adhering primarily to the carrier's ECM from migrating further.

Possible models for axolotl PND guidance

Although the studies reported here indicate that an interaction between α6β1 integrins on PND cells and laminin 1 on their migration substratum is required for PND migration, they do not tell us whether laminin 1 provides permissive cues, directional cues or both to the migrating PND. Recently, Drawbridge et al. (Drawbridge et al., 2000) identified the ligand-binding component of the Ret receptor, glial-cell-line-derived neurotrophic family receptor α-1 (GFRa-1), and its ligand, glial-cell-line-derived neurotrophic factor (GDNF), as components of the PND directional pathfinding machinery. They proposed a model in which PND cells expressing GFRa-1 migrate up a gradient of GDNF to the cloaca. However, GDNF is secreted not by epidermal cells but by cells of the lateral mesoderm (Moore et al., 1996; Sanchez et al., 1996; Homma et al., 2000). Thus, the data presented here and that of Drawbridge et al. (Drawbridge et al., 2000) suggest at least two possible models for PND guidance.

First, it is known that growth factors can be deposited in ECM from which they can subsequently be released (Taipale and Keski-Oja, 1997). Therefore, our present results and those of Drawbridge et al. (Drawbridge et al., 2000), support a model of normal PND guidance in which the lateral flank epidermis secretes a laminin-1-containing ECM overlying the migrating PND. In this model, lateral mesoderm posterior to the migrating duct secretes GDNF, which binds to epidermal ECM in an A→P gradient. The PND cells migrate by attachment to the laminin-1/E8 cell-binding domain via their α6β1 integrins and chose their direction by migrating up the gradient of ECM-bound GDNF.

Alternatively, epidermally deposited laminin 1 and mesodermally provided GDNF might provide the migrating PND with overlapping but independent sets of guidance cues. Direct evidence from this study and that of Drawbridge et al. (Drawbridge et al., 1995) shows that laminin-1-containing epidermal ECM provides directional information to the migrating duct. However, Drawbridge et al. (Drawbridge et al., 1995) also showed that epidermal guidance cues are present throughout the tailbud stages of development and are therefore not responsible for the observed temporal restriction on PND migration (Poole and Steinberg, 1982; Gillespie and Armstrong, 1986). They proposed that two sets of guidance cues govern the timing and direction of PND migration: cues from the epidermis are required for directional information; and cues derived from lateral mesoderm are required to restrict migration temporally (Drawbridge et al., 1995; Drawbridge et al., 2003; Drawbridge and Steinberg, 1996). Therefore, a second model of PND guidance is also possible, in which laminin-requiring epidermal cues and mesodermally derived GDNF provide overlapping but independent sets of guidance information.

In summary, the present study identifies the laminin-1/α6β1-integrin ligand/receptor system as essential for PND guidance in axolotl embryos. Furthermore, these data, integrated with previous studies, suggest two possible ways in which PND guidance might be accomplished in these embryos. These two alternative models suggest direct tests that will help future workers to elucidate the molecular and cellular basis of this guidance system in further detail.

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