Morphogenesis of the axolotl pronephric duct: a model system for the study of cell migration *in vivo*

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ABSTRACT Pronephric duct (PND) morphogenesis is a critical early event in the development of the vertebrate excretory system. This structure is the exit channel for both pronephric and mesonephric filtrate, forms the ureteric bud of the metanephros and gives rise to the *ductus deferens* of the testis. In addition, the PND and ureteric bud epithelia induce terminal differentiation of the mesonephric and metanephric mesenchyme, respectively. Elongation of the PND in all vertebrates involves active cell migration of the primordium. In urodele embryos — unlike in some anuran, avian and mammalian embryos — elongation of the PND occurs solely by cell migration. In the axolotl embryo, the PND primordium segregates as an ovoid tissue mass from the anterodorsal flank mesoderm directly beneath somites 3-7. The primordium then extends caudally along the ventral border of the developing somites until it reaches the cloaca. The ease with which these embryos can be manipulated microsurgically makes the PND system ideal for the study of the mechanisms controlling cell migration *in vivo*. This review summarizes the progress that has been made in characterizing the environmental cues and the cell surface recognition systems that drive this tightly regulated migration event.

KEY WORDS: *axolotl, pronephric duct, cell migration, urodeles, amphibian*

Introduction

Throughout development, the pronephric (also termed the Wolffian, mesonephric or nephric) duct (PND) constitutes the central component of the vertebrate excretory system. It is the functional waste conduit of the pronephros and mesonephros, gives rise to the *ductus deferens* of the male reproductive system and, in reptiles, birds and mammals, forms the ureteric bud of the metanephros. Moreover, the PND epithelium and its derivatives participate in the induction of the nephrons of both mesonephros and metanephros.

PND morphogenesis is very similar throughout the vertebrate lineage. The PND primordium is first observed in association with the incipient pronephros, arising from a mesodermal anlage ventral to the somites at the level of the cervical vertebrae. The duct then elongates caudally between the lateral and somitic mesoderm until it fuses with the cloaca. Studies of avian, amphibian and teleost embryos have revealed that duct elongation is accomplished via one or more of three possible mechanisms: 1) by *in situ* recruitment of mesoderm along the duct pathway, 2) by growth of the duct via cell division, or 3) by directed cell migration. For example, it has been shown that the avian PND is formed by growth via cell division as well as by active cell migration (Overton, 1959; Poole and Steinberg, 1984; Jacob *et al.*, 1991). Histological studies have led other workers to conclude that the mammalian mechanism is similar to that of birds (see Saxén *et al.*, 1986; Saxén, 1987). In *Xenopus laevis* and other anuran embryos, it was originally concluded that the PND formed solely by *in situ* recruitment of mesoderm along the duct pathway (Fox and Hamilton, 1964; Poole and Steinberg, 1984), but more recent evidence reveals that cell migration is also an important component of *Xenopus* PND morphogenesis (Lynch and Fraser, 1990; Cornish and Etkin, 1993). In urodeles as well as in some primitive teleosts, PND morphogenesis appears to be driven solely by active migration accompanied by cell rearrangement (Ballard and Ginsburg, 1980; Poole and Steinberg, 1981; Poole, 1988).

The urodele PND is particularly tractable for the study of directed cell migration *in vivo* because, unlike other such systems that are commonly studied in developing embryos (e.g., the neural crest and the involuting mesoderm of the amphibian gastrula), PND cells all have the same developmental fate and all migrate along a single, invariant route. In the axolotl, *Ambystoma mexicanum*, the incipient pronephros and PND first appear as a homogeneous bulge in the mesoderm directly ventral to somites 3-7, at approximately embryonic stage 20 (Bordzilovskaya and Dettlaff, 1979). Cells in the caudal region of this bulge, the presumptive PND, then move posteriorly as a cohesive stream along the ventral border of the somites until they reach and fuse with the cloaca at approximately stage 28-30 (Fig. 1). Early transplantation experiments showed that flank mesoderm ventral to the normal

Abbreviations used in this paper: PND, pronephric duct; GPI-linked, glycosylphosphatidylinositol-linked; PI-PLC, phosphatidylinositol-specific phospholipase C; ptk, protein tyrosine kinase.

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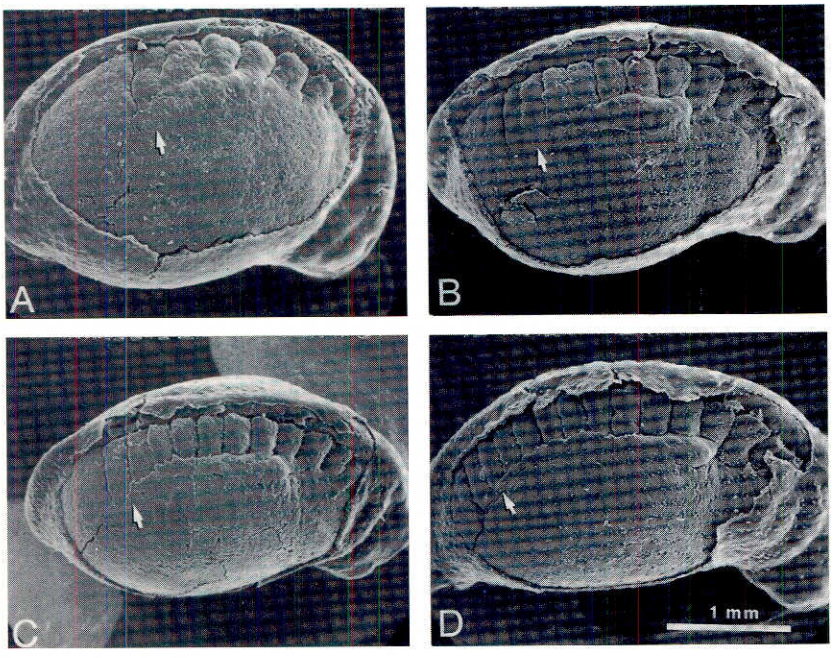

Fig. 1. Pronephric duct morphogenesis in the axolotl embryo. *Embryos were fixed, denuded of ectoderm on the right flank and visualized by scanning electron microscopy. The caudal tip of the pronephric duct is indicated by arrows. Anterior is to the right.* **(A)** *Stage 20 embryo;* **(B)** *stage 22 embryo;* **(C)** *stage 24 embryo;* **(D)** *stage 27 embryo.*

duct pathway can also support PND migration, highlighting the fact that some mechanism normally constrains these cells to their customary pathway (Maschkowzeff, 1936; Holtfreter, 1944; Nieuwkoop, 1947; Bijtel, 1948, 1968). This laboratory has taken advantage of the ease with which one can surgically manipulate the axolotl embryo to investigate the mechanisms governing PND migration. This review will discuss the progress that has been made in understanding the nature of both the environmental cues guiding the migrating cells and the recognition system displayed by the migrating cells that allows utilization of those cues.

What features characterize the PND guidance system?

Of the several strategies for guidance known to be employed by migrating cells, evidence for chemotaxis, contact guidance and haptotaxis has been sought experimentally in the axolotl PND system. Chemotaxis is defined as movement of cells toward a distant source of diffusible attractant; contact guidance and haptotaxis describe guidance systems that are local to the migration pathway. Contact guidance restricts cell movement to a permissive substratum, but the direction of movement along that substratum is not specified. In contrast, haptotaxis is defined as movement of cells up a gradient of adhesiveness to the substratum; thus haptotactic cell movement is *directional* along a permissive substratum.

Neither ectopic placement nor extirpation of the cloaca — the PND's target and therefore the presumptive source of potential chemoattractants — causes the PND to deviate from its normal pathway, eliminating chemotaxis as the strategy responsible for its guidance (Poole and Steinberg, 1982; Zackson and Steinberg, 1987). Rather, the migrating PND must navigate via cues local to the migration substratum. Transplantations of the PND ventral to its normal pathway in either normal or altered orientation reveal that PND migration is polarized such that, wherever the PND is able to migrate, it does so in a specific direction (Poole and Steinberg, 1982; Gillespie and Armstrong 1986; Zackson and Steinberg, 1988). Thus, contact guidance, a mechanism by which cells can move in either direction along a defined pathway, is also eliminated. These data have been interpreted to support haptotaxis as the most likely PND guidance mechanism (Poole and Steinberg, 1981; Zackson and Steinberg, 1986, 1987, 1988). However, until direct measurements of the adhesiveness of PND cells to their pathway are made, other mechanisms remain possible. For exam-

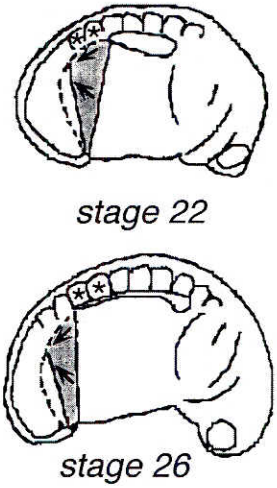

Fig. 2. A map of the embryonic region capable of supporting PND migration. *The regions of a stage 22 and a stage 26 embryo competent to support PND migration are indicated by wedge-shaped stippled areas. The fifth and sixth somites posterior to the anterior tip of the pronephros are marked by asterisks. Comparing the location of the competent region to the marked somites reveals that the competent area moves posteriorly along the flank. The dorsal border of this region lies at the ventral border of the two posterior-most somites; the anterior and posterior borders converge on the cloaca. In addition, the competent region is directional (indicated by arrows) such that migration within this region is restricted to caudad movement on the normal duct pathway or dorsocaudad movement ventral to the pathway. As the PND reaches the cloaca, this wedge-shaped region collapses on itself. By stage 30, no area of the flank can support PND migration. This "map" of guidance information actually represents the embryonic region within which at least two distinct but required sets of PND migration cues overlap. The epidermis overlying the duct pathway provides directional information; temporal restriction of duct migration is hypothesized to be a property of the flank mesoderm (Drawbridge et al., 1995). Anterior is to the right.*

ple, the PND could potentially migrate up a graded distribution of permissive, yet non-adhesive molecules, a strategy apparently used by ingressing mesodermal cells during gastrulation of amphibian embryos (Winklbauer and Nagel, 1991).

What are the spatio-temporal constraints on PND migration?

Transplantation of stage 20-21 PND to the lateral flank of older and younger embryos has allowed further characterization of the guidance information, allowing construction of a spatio-temporal map of the embryonic region capable of supporting PND migration (Fig. 2). These data show that PND guidance information is present not only on the normal duct pathway but also on the posterior lateral flank. Moreover, expression of PND guidance cues in the embryo is also transient, appearing at the onset of host PND migration and disappearing as host PND migration stops. Analysis of the spatial component of the guidance information reveals that substratum competence to support duct migration is lost anteriorly in synchrony with the caudad movement of the host duct (Poole and Steinberg, 1982). On the lateral flank, PND guidance information is never present in any region anterior to the host migrating duct tip (Poole and Steinberg 1982; Gillespie and Armstrong, 1986). The anterior border of this advancing wave can be visualized as rotated counter-clockwise to the dorsal-ventral axis such that the further ventrad a PND is grafted onto the flank, the further caudad it must be placed in order to migrate (Poole and Steinberg 1982; Gillespie and Armstrong, 1986). Gillespie and Armstrong calculated that the counter-clockwise rotation of the anterior border was 41° to the dorsal ventral-axis, but examination of the data of Poole and Steinberg (1982) suggests that the anterior border is more likely defined by a line from the cloaca to the migrating duct tip. Therefore, the angle between the dorsal-ventral axis and the anterior border appears to change as development continues (Fig. 2).

The work of Gillespie and Armstrong (1986) strongly indicates that the competent region also has a posterior border, approximately at the level of the posterior-most somite fissure. Analysis of the data presented in Poole and Steinberg (1982), Gillespie and Armstrong (1986) and Zackson and Steinberg (1986) suggests that this border is defined by a line drawn from the posterior-most somite fissure to the cloaca. Thus, at any given time during PND migration the "active" area within which the PND is allowed to migrate can be described as a wedge-shaped region on the lateral flank. The dorsal border of this region is represented by the duct pathway underlying the two posterior-most somites; both the anterior and posterior borders of this region converge on the cloaca. As development continues, the active region moves posteriorly along the flank in concert with the formation of new somites. In addition, the competent region is graded such that PND migration can only proceed caudad on the pathway and dorsocaudad on the flank.

Since the PND migrates between the epidermis and the lateral flank mesoderm, both tissues are potential sources of guidance cues. We have recently demonstrated that altering either the anterior-posterior (AP) or dorsal-ventral (DV) coordinates of the epidermis over the duct pathway perturbs PND migration in a manner consistent with the hypothesis that the epidermis is a source of directional information for the PND (Drawbridge *et al.*, 1995). Our data also indicate that the ability to direct PND migration is a stable property of flank epidermis throughout the period of PND migration. Thus, the map of guidance information presented in Figure 2 actually represents the embryonic region within which at least two distinct but required sets of PND migration cues overlap. The epidermis overlying the duct pathway provides directional

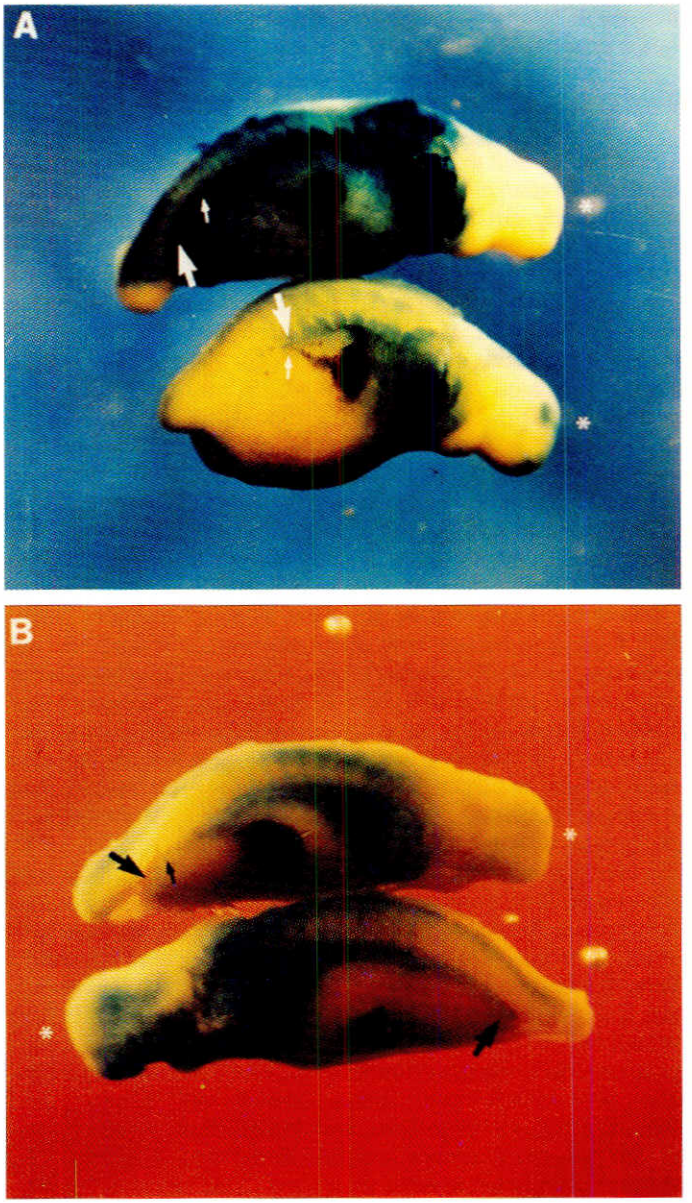

Fig. 3. GPI-linked proteins are required on the migrating PND not on the migration pathway. *Pigmented PNDs were transplanted to the flank of albino host embryos without PI-PLC treatment (top embryos in* **A** *and* **B***) or with prior PI-PLC treatment of either the host flank (bottom embryo in* **A***) or the pigmented graft (bottom embryo in* **B***). The embryos were fixed, denuded of ectoderm and stained for alkaline phosphatase (AP), a GPI-linked protein. The embryos in* **(A)** *were stained for 24 h to detect residual AP in the PI-PLC treated host; the embryos in* **(B)** *were stained for 1 h. The anterior of each embryo is indicated by asterisks. The caudal tips of host PNDs are indicated by large arrows; the caudal tips of graft PNDs are indicated by small arrows. These data show that removal of GPI-linked proteins from the PND pathway does not prevent migration, while removal of GPI-linked proteins from only the migrating cells is sufficient to prevent migration.*

information; temporal restriction of duct migration is hypothesized to be a property of the flank mesoderm.

What is the molecular basis of PND guidance?

Specific cell surface and substratum molecules that govern the directed migration of the PND primordium have yet to be characterized in any detail. However, studies of chick, mouse and amphibian embryos are beginning to yield some valuable clues as to the possible identity of some of the key players. At present, there is evidence for only one candidate ECM component involved in PND guidance. Misdirection of the chick PND by the YIGSR peptide — a putative inhibitory agent for cell-binding to laminin — and the presence of α6 integrin subunits on both chick and amphibian PND cells (Jacob *et al.,* 1991; Bronner-Fraser *et al.,* 1992; Lallier *et al.,* 1993) provide evidence that laminin or laminin-like ECM components might play a role in PND morphogenesis.

Genetic studies of organisms such as *Drosophila melanogaster* are beginning to provide evidence that protein tyrosine kinases (ptks) are required to support cell migration (see Montell, 1994 for review). Recent work by Pachnis *et al.* (1993) and Schuchardt *et al.* (1994) has implicated the ptk, *c-ret,* in migration of the mouse PND. *C-ret* is expressed throughout the nervous system and the developing pronephric duct as well as in the forming tubules of the metanephros. Mice lacking *c-ret* die shortly after birth as a result of kidney agenesis and megacolon. Although these data do not conclusively demonstrate that the *c-ret* tyrosine kinase is directly involved in cell migration, the distribution of *c-ret* on the migrating neural crest and PND and the phenotype of the mutant embryos are consistent with this idea.

In the axolotl, two classes of molecules important for cell migration have been identified. Gillespie *et al.* (1985) showed that brief trypsinization of the flank mesodermal surface disrupts both somitogenesis and PND migration. However, when Ca^{++} is present during trypsinization, PND migration proceeds normally. Resistance to trypsinization in the presence of Ca^{++} is characteristic of the cadherins — a group of homotypic adhesion molecules that are developmentally regulated and believed to direct many morphogenetic events during embryogenesis (Takeichi, 1991; Steinberg and Takeichi, 1994). Although these experiments do not tell us whether the Ca^{++}-resistant system resides on the migrating PND, the migration substratum or both, we have initiated functional studies of axolotl cadherins to address such issues.

Glycosylphosphatidylinositol (GPI)-linked proteins have also been demonstrated to be important for PND migration in the axolotl embryo. *In vivo* application of phosphatidylinositol-specific phospholipase C (PI-PLC) — an enzyme that releases GPI-linked proteins from cell surfaces (see Ferguson and Williams, 1991 for review) — results in defective pharyngeal pouch formation, failed trunk elongation and failed PND migration (Zackson and Steinberg, 1989; Thibaudeau *et al.,* 1993; Drawbridge *et al.,* 1994). Thibaudeau *et al.* (1993) took advantage of the ease with which the PND can be manipulated *in vivo* to ask whether the migrating PND, its migratory pathway or both require GPI-linked proteins. PI-PLC treatment of either the migration substratum or the migrating PND showed that the PI-PLC-sensitive component of this system resides on the migrating cells; substratum denuded of GPI-linked proteins supports PND migration (Fig. 3).

The recent work of Bellairs *et al.* (1995) has shown that digestion of the polysialic acid component of NCAM with the enzyme endo-N blocks PND extension in the chick embryo. This finding may indicate a difference between avian and mammalian PND extension; elimination of the NCAM gene in mice has no obvious effect on development of the urogenital system (Cremer *et al.,* 1994). The GPI-linked form of NCAM has been found to be polysialylated in several systems (e.g. Lipinski *et al.,* 1987; Roth *et al.,* 1989; Fredette *et al.,* 1993), but it is not known which form(s) of NCAM the urodele PND displays. We are testing the possibility that PI-PLC's blockage of urodele PND migration is due to removal of a polysialylated, GPI-linked form of NCAM.

Conclusion

Pronephric duct elongation is the seminal event in the establishment of the vertebrate urogenital system. Failure of this structure to form or migrate properly results in agenesis of both the genital ducts and the kidneys. Because of the accessibility of the axolotl PND to *in vivo* manipulation, this system has contributed more than any other to our understanding of this critical developmental event. As biochemical, genetic and cellular studies continue to identify potential molecular components of the PND migration system and as function-blocking probes for these components become available, the axolotl embryo will continue to offer significant advantages as an experimental system in which the function of these molecules can be elucidated.

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